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THE METABOLOMICS OF HOST-PARASITOID
INTERACTIONS

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degree of Doctor of Philosophy

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Abstract

This thesis examines the relationship between insect life history and behavioural decisions and underlying cellular biochemistry, with particular focus on bethylid parasitoid wasps in the genus *Goniozus*. This comprises the first major body of work attempting to draw links between the underlying metabolome of an organism and its behaviour. This thesis further optimised the first known example of a combined LC-MS and NMR metabolomic approach capable of analysing extremely low biomass samples (<1 mg), a vital requirement when studying the behaviour of individual organisms. Part 1 of this thesis details the optimisation and validation of this metabolomic approach, whilst also examining the effects of aging on the metabolome of adult *Goniozus* wasps. Part 2 applies this approach to examine the effects of diet, host species and host aging on *Goniozus* wasp behaviour and biochemistry. Comparisons of the metabolomes of starved and honey fed wasps indicate that *G. legneri* is capable of utilising a carbohydrate rich diet as an energy source. Aged honey fed wasps possessed higher levels of large storage lipids, such as tri- and diacylglycerides, than starved wasps of the same age. Metabolomic analysis also detected a legacy effect on the metabolome of *G. legneri* associated with differences in the species of host each wasp was reared on. A similar legacy effect was confirmed when examining the metabolomes of wasps reared on artificially aged hosts. Whilst *Goniozus* wasp oviposition behaviour was altered by the species of host presented, no links between changes in a wasp's metabolome and its resulting contest behaviour were found. Part 3 of this thesis examines the morphological, behavioural and chemical mimicry of another wasp, the hyperparasitoid *Gelis agilis*. *G. agilis* demonstrated an enhanced predation avoidance rate compared with control species, similar to that of the black garden ant *Lasius niger*. Agitation of *G. agilis* also resulted in the chemical emission of a known ant alarm pheromone.

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Table of Contents

Abstract	ii
Acknowledgements	iv
Table of contents	v
List of figures	xiii
List of tables	xvi
Chapter 1: General introduction	1
1.1 Parasitoids	2
1.1.1 Taxonomy and classification	3
1.2 Host quality	5
1.2.1 Host quality and oviposition decisions	8
1.2.1.1 Clutch size	9
1.2.1.2 Sex ratio	9
1.3 Contest behaviour	9
1.4 Bethyid wasps	13
1.4.1 Biology of <i>Goniozus legneri</i>	14
1.4.2 Biology of <i>Goniozus nephantidis</i>	15
1.4.3 Bethyid contest behaviour	15
1.5 The metabolomic approach	18
1.5.1 The terminology problem	21
1.5.2 Eco- and "Ento"-metabolomics	21
1.5.3 Nuclear magnetic resonance spectroscopy (NMR)	25
1.5.4 Mass spectrometry (MS)	27
1.5.5 Establishing a metabolomic workflow	29
1.5.5.1 Sample preparation	30
1.5.5.2 Experimental analysis	31
1.5.5.3 Data pre-processing and metabolite identification	33
1.5.5.4 Metabolomics and multivariate data analysis	36

1.5.6 Current limitations of ento-metabolomic studies	39
1.6 Summary of thesis structure.....	40
1.6.1 Part 1: Employing an optimised metabolomic protocol: the effects of aging and diet on adult parasitoid contest behaviour	40
1.6.2 Part 2: Host parasitoid interactions: the effects of aging and host species	40
1.6.3 Part 3: Behavioural and biochemical mimicry by a hyperparasitoid wasp	41
1.6.4 Thesis format	41
Chapter 2: Addressing the challenge of low biomass in insect metabolomics - Part 1: Optimisation of procedures	43
2.1 Abstract	43
2.2 Introduction.....	44
2.2.1 Solvent extraction protocol optimisation	47
2.2.2 Aims and objectives	47
2.3 Materials and methods	48
2.3.1 Parasitoid origin and cultures	48
2.3.2 Analytical strategy	48
2.3.3 Single insect extraction methodology optimisation	49
2.3.3.1 Methanol-chloroform-water (MCW) extraction.....	49
2.3.3.2 Hexane-isopropanol-water (HIW) extraction.....	49
2.3.3.3 Ethanol-heptane-water (EHW) extraction	50
2.3.4 Optimisation of metabolomic analysis by NMR spectroscopy	51
2.3.4.1 Optimisation of NMR spectral resolution.....	51
2.3.4.2 Optimisation of NMR analytical stability	52
2.3.4.3 2D NMR spectral identification	53
2.3.5 Optimisation of non-polar metabolomic analysis by LC-MS	53
2.3.5.1 Optimisation of non-polar extract LC-MS pre-processing	54
2.3.5.2 Optimisation of LC-MS column choice	54

2.3.5.3 Validation of LC-MS analytical precision and stability	55
2.3.5.4 Optimisation of non-polar metabolite identification by LC-MS/MS.....	56
2.4 Results.....	57
2.4.1 Single insect extraction methodology NMR optimisation.....	57
2.4.2 Optimisation of NMR spectral resolution.....	57
2.4.3 Optimisation of NMR analytical stability	58
2.4.4 2D NMR spectral identification	61
2.4.5 Optimisation of non-polar extract pre-processing.....	61
2.4.6 Optimisation of LC-MS column choice	70
2.4.7 Validation of LC-MS analytical precision and stability	70
2.4.8 Optimisation of non-polar metabolite identification by LC-MS/MS.....	75
2.5 Discussion	76
2.5.1 Single insect extraction methodology optimisation	76
2.5.2 Optimisation of NMR spectral resolution.....	77
2.5.3 Optimisation of non-polar extract pre-processing.....	77
2.5.4 Validation of LC-MS analytical precision and stability	78
2.5.5 Validation of NMR and LC-MS spectral identifications.....	78
2.6 Conclusions	80
Chapter 3: Addressing the challenge of low biomass in insect metabolomics - Part 2: Validation of procedures	81
3.1 Abstract	81
3.2 Introduction.....	82
3.2.1 Biology of <i>Goniozus legneri</i>	83
3.2.2 Aims and objectives	84
3.3 Materials and methods.....	85
3.3.1 Parasitoid origin and cultures	85

3.3.2 Experimental design and wasp metabolite extraction.....	85
3.3.2.1 NMR spectroscopy	86
3.3.2.2 LC-MS analysis	86
3.3.2.3 LC-MS stability validation	87
3.3.3 Data analysis	87
3.4 Results.....	89
3.4.1 NMR spectroscopy	89
3.4.2 LC-MS analysis.....	89
3.5 Discussion	102
3.6 Conclusions	105
Chapter 4: The effects of nutritional status on bethylid wasp longevity, biochemistry and contest behaviour.....	107
4.1 Abstract	107
4.2 Introduction.....	108
4.2.1 Aims and objectives	111
4.3 Materials and methods.....	112
4.3.1 Host and parasitoid rearing	112
4.3.2 Part 1: The effects of a carbohydrate diet on parasitoid longevity	112
4.3.3 Part 2: The effects of a carbohydrate diet on parasitoid contest behaviour	112
4.3.4 Part 3: The effects of dietary treatment on the metabolomic profile of parasitoid wasps	113
4.3.4.1 NMR spectroscopy	114
4.3.4.2 LC-MS analysis	114
4.3.4.3 LC-MS stability validation	115
4.3.5 Data analysis	115
4.4 Results.....	118

4.4.1 Part 1: The effects of a carbohydrate diet on parasitoid longevity	118
4.4.2 Part 2: The effects of a carbohydrate diet on parasitoid contest behaviour	118
4.4.3 Part 3: The effects of dietary treatment on the metabolomic profile of parasitoid wasps	119
4.4.3.1 NMR spectroscopy	119
4.4.3.2 LC-MS	124
4.5 Discussion	137
4.6 Conclusions	142

Chapter 5: The assessment and basis of host quality - life history, behavioural and metabolomic assays using bethylid wasps..... 143

5.1 Abstract	143
5.2 Introduction.....	144
5.2.1 <i>G. legneri</i> biology and host range	147
5.2.2 <i>G. nephantidis</i> biology and host range.....	147
5.2.3 Aims and objectives	148
5.3 Materials and methods.....	150
5.3.1 Parasitoid origin and cultures	150
5.3.2 Part 1: Adult parasitoid host acceptance and offspring utilisation.....	150
5.3.3 Part 2: Effects of host species on <i>G. legneri</i> development	150
5.3.4 Part 3: The effects of host species on parasitoid contest behaviour	151
5.3.5 Part 4: Metabolomic analysis of larval hosts and adult	152
5.3.5.1 Host extraction protocol.....	152
5.3.5.2 Parasitoid extraction protocol	153
5.3.5.3 NMR spectroscopy	153
5.3.5.4 LC-MS analysis	154

5.3.5.5 LC-MS stability validation	154
5.3.6 Data analysis	155
5.4 Results.....	157
5.4.1 Part 1: Adult parasitoid host acceptance and offspring utilisation.....	157
5.4.2 Part 2: Effects of host species on <i>G. legneri</i> development	158
5.4.3 Part 3: The effects of host species on <i>G. legneri</i> contest behaviour	164
5.4.4 Part 4: Metabolomic analysis of larval and parasitoid tissue extracts	164
5.4.4.1 Host NMR spectral identification.....	164
5.4.4.2 NMR spectroscopy.....	167
5.4.4.3 Host species LC-MS.....	167
5.4.4.4 Parasitoid LC-MS.....	169
5.5 Discussion	178
5.6 Conclusions	183
Chapter 6: Host age legacy does not influence parasitoid contest performance	184
6.1 Abstract	184
6.2 Introduction.....	185
6.2.1 Aims and Objectives	187
6.3 Material and methods	188
6.3.1 Parasitoid origin and cultures	188
6.3.2 Part 1: The influence of nutritional legacy on <i>G. legneri</i> contest outcome	188
6.3.3 Part 2: Metabolomic analysis of larval and parasitoid tissue extracts	190
6.3.3.1 Host extraction protocol.....	191
6.3.3.2 Parasitoid extraction protocol	193

6.3.3.3 NMR spectroscopy	193
6.3.3.4 LC-MS analysis	193
6.3.3.5 LC-MS stability validation	194
6.3.3.6 LC-MS/MS Analysis	194
6.3.4 Data analysis	194
6.4 Results.....	196
6.4.1 Part 1: The influence of nutritional legacy on <i>G. legneri</i> contest outcome	196
6.4.2 Part 2: Metabolomic analysis of larval and parasitoid tissue extracts	196
6.4.2.1 Host aging NMR spectroscopy	196
6.4.2.2 Parasitoid NMR spectroscopy	200
6.4.2.3 Host aging LC-MS	201
6.4.2.4 Parasitoid LC-MS.....	205
6.5 Discussion	210
6.6 Conclusions	215
Chapter 7: Multi-trait mimicry of ants by a parasitoid wasp.....	217
7.1 Abstract	217
7.2 Introduction.....	218
7.2.1 Aims and objectives	220
7.3 Materials and methods.....	221
7.3.1 Insect culturing	221
7.3.2 Predation bioassays	221
7.3.3 Statistical analyses	222
7.3.4 Chemical analyses	222
7.4 Results.....	225
7.4.1 Predation bioassays	225
7.4.2 Chemical analyses	225

7.5 Discussion	227
7.6 Conclusions	230
Chapter 8: General discussion and conclusions.....	231
8.1 Summary of results	231
8.2 The applicability of a combined LC-MS and NMR low biomass insect approach	233
8.3 The influence of the host species on the fitness of <i>Goniozus</i> wasps ..	234
8.4 The effects of adult wasp diet on the lifespan and contest behaviour of <i>G. legneri</i>	237
8.5 The metabolomic legacy of post-paralysis host aging on <i>G. legneri</i> offspring	238
8.6 The prevalence of chemical mimicry within parasitoids.....	239
8.7 The association of metabolomic changes with parasitoid contest behaviour	240
8.8 Final conclusions	241
References.....	242
Appendices	278

List of figures:

Figure 1.1 The five major Bethyloid subfamilies as described by Carr et al. (2010).....	16
Figure 1.2 Contest Behaviour in <i>Goniozus</i> wasps.....	17
Figure 2.1 Physiological comparison of <i>G. legneri</i> on host with brood and <i>D. melanogaster</i>	46
Figure 2.2 Comparisons of 600 MHz polar NMR spectra.....	58
Figure 2.3 Comparisons of 800 MHz and 600 MHz NMR spectra generated from single wasp polar extracts	59
Figure 2.4 PCA validation of NMR spectral stability	60
Figure 2.5 Representative ¹ H NMR and 2D spectrum of <i>Goniozus legneri</i> methanol extracts.....	62
Figure 2.6 Solvent extract pre-processing comparison	64
Figure 2.7 Representative key extracted LC-MS ion chromatograms of Palmitic acid, Linolenic acid and Stearic acid.....	65
Figure 2.8 Representative total ion chromatograph of whole wasp extract in ESI- and ESI+ mode	67
Figure 2.9 Representative mass spectra labelled for fatty acyl phospholipid, diacylglyceride and triacylglyceride key ions.....	68
Figure 2.10 Principle components analysis of wasp non-polar extract analytical precision and stability replicates	71
Figure 2.11 Representative LC-MS/MS fragmentation spectra of wasp non-polar key ions.....	74
Figure 3.1 Principle components analysis of aged wasp NMR spectra	90
Figure 3.2 Principle components analysis of aged wasp LC-MS chromatograms	91
Figure 3.3 Metabolite differences between 0 day, 3 day and 7 day old <i>G. legneri</i>	92
Figure 3.4 Representative LC-MS/MS spectra of individual lipid classes ...	99
Figure 4.1 Cohort survival of starved and honey-fed <i>G. legneri</i>	119
Figure 4.2 Comparisons of wasp NMR spectral region 4.5 - 3.0 ppm.....	121

Figure 4.3 Principle components analysis of 3 day old dietary wasp NMR spectra	122
Figure 4.4 Principle components analysis of 7 day old dietary wasp NMR spectra	123
Figure 4.5 Polar metabolite differences between starved and honey-fed <i>G. legneri</i>	127
Figure 4.6 Principle components analysis of 3 day old dietary wasp LC-MS chromatograms	128
Figure 4.7 Non-polar metabolite differences between 3 day old starved and 3 day old host-fed <i>G. legneri</i>	131
Figure 4.8 Principle components analysis of 7 day old dietary wasp LC-MS chromatograms	133
Figure 4.9 Non-polar metabolite differences between 7 day old starved and 7 day old host-fed <i>G. legneri</i>	136
Figure 5.1 Comparisons of <i>G. legneri</i> and <i>G. nephantidis</i> clutch size and offspring mean dry weight when parasitizing <i>C. cephalonica</i>	158
Figure 5.2 <i>G. legneri</i> reproduction in response to host weight and host species	160
Figure 5.3 <i>G. legneri</i> reproduction in response to host weight and host species (restricted weight range)	161
Figure 5.4 Profile of behaviour interactions during <i>G. legneri</i> aggression contests	163
Figure 5.5 Principle components analysis of <i>G. legneri</i> and host NMR spectra	166
Figure 5.6 Principle components analysis of host LC-MS data	168
Figure 5.7 Significantly differing lipids between host species	170
Figure 5.8 Principle components analysis of parasitoid LC-MS data	173
Figure 5.9 Biomarker lipids between parasitoids reared across four lepidopteran hosts	177
Figure 6.1 Schematic representation of the host-aging protocol prior to owner-owner contests	189
Figure 6.2 A schematic diagram outlining host-aging treatments prior to liquid nitrogen snap freezing and metabolomic analysis	191

Figure 6.3 A schematic diagram outlining host-aging protocol for offspring parasitoid liquid nitrogen snap freezing and metabolomic analysis	192
Figure 6.4 The effect of host legacy on contest outcome in owner-owner contests.....	197
Figure 6.5 Principle components analysis of parasitoid LC-MS data.....	198
Figure 6.6 Polar metabolite abundances across young, middle-aged and old hosts.....	200
Figure 6.7 PCA of aged wasp NMR data	201
Figure 6.8 Comparison of wasp NMR spectra with control spectra	202
Figure 6.9 Principle components analysis of aged host LC-MS data.....	203
Figure 6.10 Lipid abundances across young, middle-aged and old host extracts.....	205
Figure 6.11 Principle components analysis of aged wasp LC-MS data.....	206
Figure 6.12 Lipid abundances for young, middle-aged and old host reared wasps.....	208
Figure 7.1 Morphological comparisons of <i>Gelis agilis</i> Fabricius and <i>Lasius niger</i> Linnaeus.....	219
Figure 7.2 Mean percentage of prey consumed by wolf spiders in paired choice tests.....	223
Figure 7.3 Representative APCI-MS spectrum of <i>G. agilis</i> emissions	226
Figure 7.4 GC-MS chromatogram of <i>G. agilis</i> volatile emissions	226
Figure 7.5 GC-MS spectrum of <i>G. agilis</i> volatile emissions.....	228

List of tables

Table 1.1 Glossary of metabolomic definitions and techniques	22
Table 1.2 Insect metabolomics studies	24
Table 2.1 Relative standard deviations of key spectral bins for NMR stability replicates.....	61
Table 2.2 ¹ H and ¹³ C chemical shift assignments of <i>G. legneri</i> NMR spectra	63
Table 2.3 Key ion peak area RSDs for technical replicates and quality control sample	72
Table 2.4 Key ion retention time RSDs for technical replicates and quality control samples	73
Table 3.1 Summary of polar biomarkers with tentative identities that significantly differ between 0 day old and 7 day old wasp extracts	93
Table 3.2 Summary of non-polar biomarkers with tentative identities that significantly differ between 0 day old and 7 day old wasp extracts	94
Table 4.1 The effects of dietary treatment on <i>G. legneri</i> contest outcome	120
Table 4.2 Summary of polar biomarkers with tentative identities that significantly differ between starved wasps and host-fed wasp extracts at 3 days old and 7 days old	125
Table 4.3 Summary of non-polar biomarkers with tentative identities that significantly differ between 3 day old starved wasps and 3 day old host-fed wasp extracts.....	129
Table 4.4 Summary of non-polar biomarkers with tentative identities that significantly differ between 7 day old starved wasps and 7 day old honey-fed wasp extracts	134
Table 5.1 Performance comparisons of two species of <i>Goniozus</i> wasps reared on four lepidoteran host species	159
Table 5.2 Average values for <i>G. legneri</i> oviposition probability, clutch size and female offspring weight	162
Table 5.3 Variation in aggression contest behaviour profile according to host species	163

Table 5.4 ^1H and ^{13}C chemical shift assignments of <i>C. cephalonica</i> NMR spectra	165
Table 5.5 Summary of non-polar biomarkers with tentative identities that significantly differ between host species extracts	171
Table 5.6 Summary of non-polar biomarkers with tentative identities that significantly differ between wasps reared on different host species.....	175
Table 6.1 Summary of polar biomarkers with tentative identities that significantly differ between young and old host extracts	199
Table 6.2 Summary of polar biomarkers with tentative identities that significantly differ between young and old host extracts	204
Table 6.3 Summary of non-polar biomarkers with tentative identities that significantly differ between wasps reared from young hosts and wasps reared from old host extract.....	207

Chapter 1: General introduction

Competition for resources is a struggle common to all organisms, regardless of kingdom or phyla. This struggle has often resulted in arms races to gain control of an essential resource (Darwin 1859; Dawkins and Krebs 1979). These arms races can be intraspecific, such as those between trees of the same species competing for light, and interspecific, such as those between individuals of different species competing for access to a watering hole. Furthermore the selection pressures on the parties may be asymmetrical, particularly in evolutionary arm races between predators and prey (Slobodkin 1974; Dawkins and Krebs 1979). Whilst the failure of a predator to catch its prey may result in an incremental reduction in reproductive opportunities due to starvation, prey that fails to escape a predator will lose all further reproductive opportunities; this is also known as the 'life-dinner principle' (Dawkins 1979). Other arms race scenarios also exist, such as those between a parasite and its host (Edmunds 1974; Davies et al. 1989; Langmore et al. 2003) or a parasitoid and its host (Kraaijeveld and Godfray 1997) and even within mating pairs and between parents and their offspring (Trivers and Hare 1976; Dawkins and Krebs 1979; Parker 2006).

Competition often takes the form of contests between individuals or groups of individuals, with the aim of excluding other contestants from access to a resource. In many cases the contested resource is access to mating opportunities, though other resources, such as food, shelter and territory can similarly be competed for (Hardy and Briffa 2013). Animal contests consist of a diverse range of behaviours and conflicts between individuals. Some of these behaviours include aggressive, escalated physical encounters, which may pose a serious risk of injury to contestants. Since the 1970's, the understanding of factors that underlie animal contests has been revolutionised by the application of game-theoretical concepts, significantly altering, and enhancing, the understanding of animal behaviour (Maynard Smith and Price 1973; Maynard Smith 1974; Kokko 2013).

This thesis has its conceptual basis in the field of competitive interactions, particularly parasitoid contest interactions and the effects of interactions between hosts and parasitoids on the contest and reproductive performance of the parasitoids. The methodological basis of the thesis is in the

metabolomic approach to evaluating the underlying biochemical states of hosts and parasitoids. The application of metabolomics to parasitoid, and indeed any, evolutionary ecology is still in its infancy and as such this thesis is also concerned with methodological aspects, particularly the development of a reliable metabolomic approach catered for low biomass samples. This thesis further focuses on applying chemical analytical techniques to parasitoid evolutionary ecology.

1.1 Parasitoids

Insect parasitoids are excellent organisms with which to study such varied areas as predation (Strassman 1981; Schmidt et al. 2003; Borer et al. 2007), resource acquisition (Sequeira and Mackauer 1992; Heimpel et al. 1996; Häckermann et al. 2007), sex ratio (King 1987; Fox 1990; Ode and Heinz 2002; Ode and Hardy 2008), fecundity (Ellers et al. 1998; Heimpel et al. 2003; Jervis et al. 2012) and contest behaviour (Taylor 1986; Petersen and Hardy 1996; Stokkebo and Hardy 2000; Humphries et al. 2006; Hardy and Briffa 2013).

Recognised now for over a century as a category separate from true parasites (Reuter 1913), parasitoids in many ways present an intermediate between the aggressive resource acquisition strategies of predators and the host exploitation strategies of true parasites. Whilst some adult parasitoids do paralyse hosts during resource acquisition, most adult parasitoids do not consume their prey (Godfray 1994). The majority of parasitoids are also distinguished from most parasites by the lack of a relatively benign relationship with their host. Whilst the loss of host nutrients to a parasite may be enough to damage its overall reproductive performance, it does not normally prevent host reproduction occurring, whereas parasitoids kill or chemically castrate their host during development. Parasitoids are further distinguished from parasites by their nutritional background during development. Immature parasitoids feed directly on the tissues of a single host during development, before emerging from the host as a free-living adult. Organisms that are recognisably parasitoid in nature occur among arthropods, plants, fungi and bacteria (Kuris 1974; Eggleton and Gaston 1990; Blaxter 2003; Male and Roberts 2005), though Price (1975) restricted the use of the term to insects. In the case of insect parasitoids, hosts

commonly include the eggs, larvae, pupae and even adults of a majority of insect orders, along with other arthropods such as spiders (Eggleton and Belshaw 1992; Godfray 1994).

1.1.1 Taxonomy and classification:

Whilst parasitoids have been reported in several insect orders, including the Diptera, Lepidoptera, Coleoptera, Neuroptera, Strepsiptera and the Trichoptera, approximately 80 % of all described species belong to the Hymenoptera (Clausen 1940; Gordh and Móczár 1990). Parasitoids have been estimated to comprise 8 - 25 % of all insect species (Crosskey 1980; Gordh and Móczár 1990; Gaston 1991; Godfray 1994). Approximately 65,000 species of hymenopteran parasitoid have been described and the total number of parasitoid species has been estimated at 225,000 (Godfray 1994; Gordh et al. 1999). These species have been grouped into a number of superfamilies within the parasitic Apocrita and Orussoidea (Whitfield 1998).

Parasitoids can be categorised according to oviposition behaviour. The females of many parasitoid species possess modified ovipositors with which they paralyse suitable hosts by injecting venom. Koinobionts only paralyse hosts temporarily prior to depositing their eggs in or rarely on the host. This allows the host to continue to feed and grow, thus increasing resources for the developing parasitoid (Godfray 1994). By comparison, idiobionts permanently immobilise hosts prior to oviposition, limiting development to the finite amount of resources possessed by the host at the moment of oviposition (Askew and Shaw 1986). These approaches are employed by both solitary parasitoids, where only a single parasitoid develops on each host, and gregarious parasitoids, where multiple parasitoids can develop on a single host.

Egg placement during oviposition also differs. Parasitoids that place their eggs inside hosts are referred to as endoparasitoids. Endoparasitoids develop and pupate entirely inside the host, only emerging as adults. Ectoparasitoids directly lay eggs on the surface of paralysed hosts. Ectoparasitoid larvae develop external to the host, remaining attached to host tissues by the mouth parts. Endoparasitoid and ectoparasitoid lifestyles

have associated advantages and disadvantages; the internal nature of endoparasitoid development avoids external predation hazards and behavioural defences of the host, whereas ectoparasitoids are less vulnerable to immune responses by the host (Schmidt et al. 2001). Ectoparasitoids are also more vulnerable to ovicide by competing parasitoids, or hyperparasitism. Broadly speaking, most endoparasitoids are koinobionts, whereas most ectoparasitoids are idiobionts, though Quicke (1997) reviewed some of the more notable exceptions.

Two major categories of egg maturation strategies also exist within parasitoids. Pro-ovigenic parasitoids mature eggs during development, emerging from pupation with a fixed level of ripe eggs. Synovigenic parasitoids emerge with a limited egg load, then mature eggs throughout the adult life. The fixed level of mature eggs present in pro-ovigenic parasitoids has been demonstrated to be affected by a range of factors, including the density of host species within the local environment (Chan et al. 1993; Segoli and Rosenheim 2013), the quality of available host resources relative to egg resource allocation (Iwasa et al. 1984; Harvey et al. 2001; Carbone and Rivera 2003), temperature change (Lysyk 1998; Ris et al. 2004; Denis et al. 2012), and travel costs during initial dispersion and host location (Ellers et al. 2000; Ellers and Jervis 2004). Parasitoids exhibit a continuum between the two extremes, with relatively few species exhibiting strict pro-ovigeny or synogeny (Jervis et al. 2001; Strand and Casas 2008).

Alternative resource acquisition strategies exist outside of primary parasitism. One such strategy is superparasitism, in which a second parasitoid of the same species will oviposit on a host upon which another brood is already developing (Godfray 1994). This strategy can carry greater risks than primary parasitism, due to the often lower quality resource available, competitiveness between the developing broods, and the possibility of overlap with sibling broods and even accidental self-superparasitism. Whilst the possibility of self-superparasitism has previously been attributed to parasitoid error, this could potentially be an adaptive strategy for parasitoids when hosts are scarce (van Alphen and Visser 1990). Despite these risks, non-sib superparasitism can result in higher yields, as the initial brood may have exhausted the defences of the host prior to

competition with the second laid brood. A further strategy is hyperparasitism, in which the second brood, usually of a different species, instead feeds on the developing parasitoid brood instead of the host (Godfray 1994). Facultative hyperparasitoids are capable of both primary and secondary parasitism, whilst obligate hyperparasitoids remain dependent on other parasitoids for development (Sullivan and Völkl 1999).

1.2 Host quality

Resource limitations result in fundamental trade-offs when considering reproductive decisions concerning parasitoid nutrient allocation (Strand and Casas 2008). Parasitoid wasps feed on a single host throughout immature development (Godfray 1994). This host thus represents the only source of nutrients (capital resources) available to the developing parasite, and its quality can have a significant influence on parasitoid life histories (Mackauer et al. 1996). As a result, the ability of a parasitoid to assess the quality of a given host can be a major limiting factor for parental fitness (Steiner et al. 2007). This assessment is complicated by the wide range of factors that have been demonstrated to affect host quality. These factors include the host species, diet, size, age and developmental instar (Häckermann et al. 2007; Hegazi et al. 2007; Hegazi and Khafagi 2008; Sampaio et al. 2008; Khidr et al. 2012). Most behavioural and developmental studies have examined the effects of variation in host size and larval instar (Humphries et al. 2006; Li and Sun 2011; Liu et al. 2011, Khidr et al. 2012). There has also been a recent increase in the use of metabolic studies to examine the composition and allocation of major host resources, such as lipid dry weight, during parasitism (Giron et al. 2002; Casas et al. 2005; Haspel et al. 2005; Visser et al. 2010). Despite these advances, only three studies have attempted to assess a wide range of host metabolites simultaneously (Thompson et al. 2001; Khidr et al. 2012; Mrinalini et al. 2014). As the metabolic state of the host is subject to a wide range of factors at any given time, it is clear that simultaneous assessment of different metabolite categories is likely to generate a greater understanding of the nutritional components of host value.

The underlying metabolic and immune states of the resources available to a developing parasitoid are a factor when considering a metabolomic

approach. The nutritional reserves of the emerging adult parasitoid consist largely of storage carbohydrates and lipids, the most common of which are glycogen and glycerolipids (Dean et al 1985; Stanley-Samuelson et al. 1988). These nutrients are regulated by the insect fat body, a tissue unique to insects that surrounds the gut and reproductive organs (Dean et al. 1985; Law and Wells 1989). The basic cell of the fat body, the adipocyte, acts as a storage repository for glycogen and fat droplets consisting mainly of triglycerides (Dean et al. 1985; Arrese and Soulages 2010). The majority of all circulating carbohydrates are also generated from glycogen mobilisation within the adipocytes, producing major hemolymph sugars such as trehalose and glucose (Candy et al. 1997; Meyer-Fernandes et al. 2000, 2001; Marciano et al. 2009).

The fat body also plays an essential role in embryogenesis, through the mobilisation of energy stores and the increased expression in apolipoprotein genes (Becker et al. 2001; Lorenz and Anand 2004). The key lipoprotein in insect egg yolk, vitellogenin, is produced in the fat body (Wahli et al. 1981; Sappington et al. 2002). Insect hemolymph also acts as a reservoir for storage proteins, amino acids and circulatory sugars (Sutcliffe 1963; Depledge and Bjerregaard 1989; Phalaraksh et al. 2008). A parasitoid relies on these capital reserves in order to survive initial metamorphosis, reproduction and diapause during any prolonged periods of limited host availability (Hagedorn and Kunkel 1979; Aluja et al. 1998; Ringel et al. 1998; Lalonde 2004). Regulation of glycerol and proline, notable insect cryoprotectants, by the fat body is a documented aspect of overwintering behaviour (Bennett and Lee 1997; Košťál et al. 2011a, 2011b; Storey and Storey 2012).

As the available host represents the only form of nutrients available to a parasitoid throughout development, the parasitoid is reliant on its quality in order to survive pupation, metamorphosis and eclosion. The initial suitability of an available host depends on its ability to provide the minimal level of nutrients these processes require (Sampaigo et al. 2008). Whilst the term host quality is largely used in the context of individual hosts within a species, the term host suitability refers to an entire species or population of potential hosts (Mackauer et al. 1996). The suitability of a given host species is further dependent on the ability of the larvae to overcome either behavioural or

immune defences employed by host in responses to parasitism, an ability that significantly affects host acceptance decisions (Allen 1925; Götz 1985; McAllister et al. 1990; Kraaijeveld and Godfray 1997). As parasitoids are a significant source of host mortality, many of these host species are naturally selected for counter defences, decreasing their suitability as hosts (Kraaijeveld and Godfray 1997). It follows from this that parasitoids also face evolutionary trade-offs in developing mechanisms to overcome these counter-defences (Strand and Pech 1995). Whilst some hosts exhibit external defences prior to oviposition i.e. aggressive behaviour, internal defences present a challenge to endoparasitoid development (Rotheray et al. 1984; Strand and Pech 1995).

Koinobionts are particularly vulnerable to immune responses by the host in the time period between paralysis termination and the resumption of host activity (Schmidt et al. 2001). Host immune responses are two-fold, comprising both humoral and cellular elements. The cellular response has received the most attention, particularly in regard to haemocyte aggregation (Vinson 1971; Lackie 1988; Ratcliffe 1993; Strand and Pech 1995). Upon the detection of a foreign tissue, i.e. the developing parasitoid, haemocytes circulating within the insect body cavity adhere to this tissue. Through the formation of a multi-layered capsule, known as 'encapsulation', the parasitoid is isolated from the hemocoel (Nappi et al. 1971; Kraaijeveld and Godfray 2006). The parasitoid is then killed either through asphyxiation or through the direct effects of compounds released from the capsule (Nappi et al. 1995). Parasitoids possess both direct and indirect methods of avoiding encapsulation. Some parasitoids only oviposit in tissues removed from direct circulatory access, such as the brain and fat body (Klomp and Teerink 1978; Grbić et al. 1992; Kraaijeveld and van Alphen 1994; Strand and Pech 1995). Egg parasitoids similarly avoid an immune response, as most insect eggs lack an immune system until more advanced stages of embryogenesis (Strand et al. 1986). Many adapted parasitoids are capable of mimicking host tissues through the use of surface chemistry characteristics (Salt 1965, 1968; Vinson 1974, 1990). Larger parasitoids are capable of physically disrupting cuticle formation through vigorous movement. Other developing endoparasitoids may be capable of directly manipulating the host's immune system through hormonal releases (Harvey et al. 1999; Beckage and Gelman 2004; Fors et al. 2014). Injection by the adult parasitoid of poly-

DNA-viruses or other virus-like agents during initial paralysis can further manipulate host immune signal transduction (Fleming 1992; Dahlman and Vinson 1993; Jones and Coudron 1993).

Having eclosed as adults, some parasitoids are capable of utilising local resources, such as nectar and honey, as way of providing extra proteins and carbohydrates (Olson et al. 2000). Some adult idiobionts are also capable of gaining nutrients through feeding on the hemolymph of a host (host feeding) (Godfray 1994). However, these nutrients are rarely translated to gains in storage lipids; indeed, many parasitoid species are incapable of *de novo* lipogenesis (Ellers 1996; Olson et al. 2000; Casas et al. 2003; Giron and Casas 2003; Visser and Ellers 2008; Visser et al. 2012). Lipogenesis loss likely resulted from exposure of developing parasitoids to an abundance of environmental lipids (Visser et al. 2008). With a high level of lipids available throughout larval development, this evolutionary redundancy of *de novo* lipogenesis may have resulted in its status as a non-selective trait (Visser et al. 2010).

1.2.1 Host quality and oviposition decisions

Two major considerations govern parasitoid oviposition behaviour: the fitness of the parasitoid and the availability of acceptable hosts (Godfray 1987; West et al. 2001). In the case of gregarious parasitoids, a large number of studies have further demonstrated that host quality, usually considered in terms of size, has a significant effect on clutch size (Sequeira and Mackauer 1992; Hardy et al. 1992; Heimpel et al. 1996; West et al. 2001; Bezemer and Mills 2003; Häckermann et al. 2007). It has been proposed that an optimal clutch size based on host quality will be produced when a parasitoid is attempting to maximise its reproductive fitness (Lack 1947; Charnov and Skinner 1984; Hardy et al. 1992).

1.2.1.1 Clutch size

Maternal clutch size decisions largely determine the level of competition between siblings for resources, with larger clutch sizes resulting in lower resource availability per parasitoid. This lower availability has potentially negative effects for adult parasitoid fitness, including smaller body size,

lower host finding ability, and decreased lifetime fecundity (Hardy et al. 1992; Stearns 1992; Mangel et al. 1994; Vet et al. 1994; Visser 1994, Ellers et al. 1998; Zaviezo and Mills 2000; Jervis et al. 2008). As a result it is expected that female parasitoids will control egg allocation in response to host quality in order to maximise maternal fitness (Godfray 1994; Bezemer and Mills 2003; Häckermann et al. 2007).

1.2.1.2 Sex ratio

A number of studies of parasitoid sex ratio allocation have demonstrated that aspects of host quality play a significant role in female parasitoid sex allocation decisions (Ode and Hardy 2008). Hymenopteran parasitoids have haplo-diploid sex determination, in which males emerge from unfertilised eggs and are haploid, whereas females emerge from fertilised eggs and are diploid. Unmated females are constrained to only produce haploid male offspring. Brood sex ratio is controlled by adult females, which are capable of selectively fertilising individual eggs (Charnov et al. 1981). Aspects of host quality affecting sex allocation decisions include dietary status, age, and size (Charnov et al. 1981; Kraaijeveld and van Alphen 1986; King 1990; Gutierrez et al. 1993; King 1993; Ode and Strand 1995). Size in particular has frequently been selected as an indicator of host quality, (King 1993; Godfray 1994; Napoleon and King 1999; West 2009) though some studies have indicated that the correlation is imperfect (Häckermann et al. 2007). In the case of species exhibiting sexually separate returns upon host resource investment, sex allocation theory indicates that mothers are more capable of influencing the fitness of one sex (Charnov et al. 1981). For many parasitoids, both solitary and gregarious, females show greater responses to variation in host quality than males (King et al. 1995; Heinz 1998; Heimpel and Lundgren 2000; Ode and Heinz 2002). Ovipositing females tend to lay sons in or on lower quality hosts and daughters on higher quality hosts; where host quality has been considered in terms of host age, a higher proportion of females are typically laid on younger hosts (King 1993; Ode and Hardy 2008).

1.3 Contest behaviour

Within the field of behavioural ecology, particular focus has been placed on aggressive intraspecific behaviour related to resource acquisition. In

situations where two or more individuals contest a resource, attention has been placed on context specific contest behaviours, and the factors underlying contest outcome. Historically research in this area has focused on interference competition by males (referred to as male-male) over limited environmental resources, including food, water access and reproductive opportunities (Stevens 1988; Birkhead and Parker 1997; Chamorro-Florescano et al. 2011; Jordan et al. 2014). Agonistic contests over territorial ownership or hierarchical status are often directly related to resource acquisition, as males possessing a larger territory or higher status within a community may benefit from enhanced reproductive opportunities or greater resource availability (Huntingford and Turner 1987; Andersson 1994). In this direct competition, contestants aim to completely exclude rivals from a resource that is critical to the survival/fecundity of each (Schoener 1977, 1983). Female-female resource contests form the majority of contest studies in parasitoids (Stokkebo and Hardy 2000; Goubault et al. 2006; Bentley et al. 2009; Stockermans and Hardy 2013; Hardy and Briffa 2013; Pusey and Schroeffer-Walker 2013). Violent contests over reproductive opportunities have been documented, including infanticide of the offspring of competing females (Williams et al. 2002). More indirect forms of competition are also observed, such as exploitation competition, where two individuals share a resource and presence of the one individual negatively affects the ability of the other to acquire a vital resource.

If the resource has a high enough value to both competitors, direct contests for the resource may occur. Within these contests, it is expected that individuals will attempt to maximise their performance. The application of economic game theory to agonistic contests generated the concept of an Evolutionarily Stable Strategy (ESS) (Maynard Smith and Price 1973; Maynard Smith 1974; Taylor et al. 1978). An ESS is a refinement of the Nash equilibrium that refers to a strategy that, if adopted by enough individuals within a population, becomes evolutionarily advantageous against an alternative or 'mutant' strategy (Maynard Smith and Price 1973; Eschel 1983). When this strategy is adopted, natural selection alone is enough to prevent successful invasion by the mutant strategy. Whilst further research has attempted to refine the theory, primarily to take into account dynamic asymmetries within a biological context (van Rhijn and Vodegel 1980; Maynard Smith 1982; Foster and Young 1990; Mesterton-Gibbons

1992), this theory has been emphasised when determining how individuals react in a contest environment.

A number of parameters underlie animal decisions in direct contests, with specific behaviours relying on a combination of factors such as relatedness, resource value, prior ownership, fighting ability, perceptions of the opponent and a range of costs and benefits (Leimar and Enquist 1984; Enquist and Leimar 1987; West et al. 2001). This range of dynamics includes the possibility of non-agonistic contests, which may consist of display behaviour, often accompanied by visual or acoustic behaviour aimed at resolving the contest without the risk of injury to either contestant (Maynard Smith 1982; Huntingford and Turner 1987). However, an escalation of these displays to aggressive fighting behaviour may occur when individuals contest a resource that is highly valuable, are nearly equally capable of acquiring the resource, or where aggressive interactions involve little chance of injury (Maynard Smith and Price 1973; Enquist and Leimar 1990). Performance within these aggressive contests is governed by a combination of two major asymmetries; the ability of an individual to aggressively obtain and retain a resource, and the value of the resource contested (Maynard Smith and Parker 1976; Arnott and Elwood 2008; Hardy and Briffa 2013). The first of these is referred to as the Resource Holding Potential (RHP) of a competitor (Parker 1974; Maynard Smith and Parker 1976; Hsu et al. 2006). The second of these is the value of a contested resource (resource value) (Maynard Smith and Parker 1976; Arnott and Elwood 2008; Hardy and Briffa 2013).

If it assumed that the resource in contest is of equal value to both competitors, then the outcome of the contest will be determined by their RHP (Parker and Rubenstein 1981). Components of the RHP can include prior resource ownership, contestant size and age, energy reserves, prior contest experience, signalling and weaponry (Maynard Smith 1982; Emlen 2014). Furthermore the difference between the RHP of competitors can govern the length and aggressiveness of a contest, a large asymmetry in RHP may result in shorter and less aggressive contests than in the case of a lower asymmetry (Taylor et al. 2001; Taylor and Elwood 2003; Arnott and Elwood 2009). Inference of the RHP of an opponent may result in the settlement of contests by set conventions, rather than aggressive encounters, an ability

that has been incorporated into some 'war of attrition' (WOA) contest models (Mesterton-Gibbons et al. 1996; Payne and Pagel 1996).

Other factors may influence contests when the second of the two major contest parameters, the resource Value (RV), is not equal to each competitor. If one competitor places a higher value on a resource than does the other, it may be willing to incur greater costs in order to obtain it, thus displaying a higher level of aggression. This difference in RV may be enough to override the effect of a higher RHP in the other competitor. A multitude of studies on the assessment of resource value has resulted in conflicting information as to how variations in resource value effect contest behaviour (Taylor and Elwood 2003). Assessment of a resource is indicated by the costs a contestant is willing to occur in order to gain it, which have previously been inferred by examining contest duration (Thornhill 1984; Arnott and Elwood 2009), energy expenditure (Briffa et al. 1998), and the rate of contest occurrence (possibly including disruptive stimuli) (Elwood et al. 1998). However, these measurements only provide an indirect estimate of resource value assessment. Certain factors, such as contest duration and energy expenditure, also have the disadvantage of only providing information on resource assessment by the loser, no measurements of the costs that the winner would have been prepared to incur are possible (Taylor and Elwood 2003). Some of these previous studies have also varied the ability of contestants to gather and integrate information regarding RV; in some studies both contestants are capable of resource assessment, whilst in others only one or neither (Taylor and Elwood 2003; Briffa and Elwood 2009). Some study systems have indicated that a lack of resource assessment is likely due to limits in cognitive or sensory ability (Taylor et al. 2001; Taylor and Elwood 2003). In the case of active resource assessment, owner-intruder contests have focused on the advantage conferred to the owner of a resource (Hammerstein 1981; Goubault et al. 2006; Bentley et al. 2009; Small et al. 2009). As the first contestant to encounter a resource has a greater exposure period it possesses a better assessment of resource value and thus of the costs it is willing to occur in defence of a resource. By comparison, the intruder has no such assessment period and might be expected to fight at an assumed average resource value. However, in the case of more artificial owner-owner contests, or if the required assessment period is extremely small, no such owner advantage may be present.

There is also debate as to the degree that contestants assess their opponent's RHP, and whether the assessment of the asymmetries contributes to contest behaviour (Taylor and Elwood 2003). The claim has been made that in some cases an apparent negative relationship between RHP asymmetry and contest duration is due to other underlying mechanisms (Taylor and Elwood 2003). For example, contestants may persist in aggression based solely on their own RHP. In this scenario costs are incurred with no consideration of the opponent's attributes. There are several theories that address RHP assessment within contests, including the cumulative assessment model (CAM) (Payne and Pagel 1997; Payne 1998) the sequential assessment model (SAM) (Enquist and Leimar 1983), and the 'best-so-far rule' (Payne and Pagel 1996). In the CAM, a contest begins with the contestants repeating a given signal, the intensity of which may change as the contest proceeds. Signal intensity is considered in a cumulative manner, regardless of the strength or weakness of an individual signal. This accumulation represents a war of attrition (WOA) scenario where decisions by a contestant are dependent on the amount of cost they are willing incur. When physical contact is incorporated into this model, competitors are not capable of assessing the damage taken by an opponent during the contest. By comparison the SAM rule allows for mutual assessment of the opponent's RHP by repetition of a signal between competitors without alteration in the intensity of the signal. As this signal is repeated, the competitor generates a more precise estimate of its opponents RHP. The last of these models, the best-so-far rule, allows the contestants to resolve a contest with the minimum loss of future fitness (Payne and Pagel 1996). The contest begins with a low cost signal by both contestants. This signal is then amplified until one contestant concedes.

1.4 Bethylid wasps

It has been previously mentioned that hymenopteran parasitoid wasps are currently classified within a group of superfamilies (Whitfield 1998). Within one of these superfamilies, the Chrysoidea, is a family of ectoparasitoid wasps consisting of approximately 2000 described (and possibly an equal number of undescribed) species (Gordh and Móczár 1990) known as the Bethylidae. These aculeate wasps mainly utilise larval or pupal Coleopteran or Lepidopteran species as hosts for larval development (Evans 1978; Hawkins and Gordh 1986; Gauld and Bolton 1988; Gordh and Móczár 1990).

Due to their life history, bethylid wasps have been widely utilised to examine the mechanisms underlying clutch size decisions, developmental mortality, sex ratio decisions, and dyadic contests (Green et al. 1982; Hardy et al. 1992; Hardy and Cook 1995; Bachelor et al. 2005). Some of the most intensely studied Bethylid wasps belong to the genus *Goniozus*, specifically the two species; *Goniozus legneri* Gordh (Hymenoptera: Bethylidae), and *Goniozus nephantidis* (Muesebeck) (Hardy et al. 1991, 1992, 1999; Hardy and Cook 1995; Pérez-Lachaud et al. 2002; Goubault et al. 2006, 2007a; Humphries et al. 2006; Bentley et al. 2009; Lizé et al. 2012; Khidr et al. 2013a, 2013b). Like all Bethylid wasps, *Goniozus* species are ectoparasitoid idiobionts, producing broods of offspring that develop on the surface of a single host (Gordh and Moćzár 1990). Furthermore, *G. legneri* and *G. nephantidis* exhibit similar host paralysis, oviposition, and host defence behaviour. Each wasp utilises its stinger to inject venom into hosts in order to induce paralysis. Oviposition takes place between 1-3 days post-paralysis, with the clutch size being correlated to host size (Hardy et al. 1992). Single broods tend to consist of 3-18 eggs in the case of *G. nephantidis* (Hardy et al. 1992) and 1-20 for *G. legneri* (Hardy and Mayhew 1998); both are capable of producing over 100 eggs if presented with a series of potential hosts (Gordh et al. 1983; Hardy et al. 1992, 1998). Females guard developing broods until larval pupation, at which stage they may disperse to seek other hosts (Hardy et al. 1999, 2000; Stokkebo and Hardy 2000; Goubault et al. 2007; Bentley et al. 2009). Developing broods are characterised by a very heavy bias towards females, with male offspring comprising between 9-19% of the brood (Gordh et al. 1983; Hardy and Cook 1995; Hardy et al. 1999, 2000; Khidr et al. 2013; Stockermans and Hardy 2013).

1.4.1 Biology of *Goniozus legneri*

Many aspects of the life-history and behaviour of the ectoparasitoid wasp *G. legneri* have been well documented (Gordh et al. 1983; Butler and Schmidt 1985; Hardy et al. 1998, 2000; Bentley et al. 2009). It is native to South America but has been utilised in North America and the Middle East as an agent of biological pests control (Gothilf and Mazor 1987; Legner et al. 1992), and is also a useful laboratory study organism for behavioural research (Hardy et al. 2000; Lizé et al. 2012). Although its 'natural' host or host range is not currently known, it has been observed to attack the larvae

of several non-native lepidopteran species in South American agroecosystems (Legner and Warkentin 1988) and can be reared on further (facitious) host species. These species include snout moth larvae (Lepidoptera: Pyralidae) such as the navel orangeworm *Amyelois transitella* Walker (Gordh and Moćzár 1990), the locust bean moth *Ectomyelois ceratoniae* Zeller (Legner and Silveiraguido 1983) and the rice moth *Corcyra cephalonica* Stainton (Cook 1993). Informal laboratory observations have also confirmed its capability to parasitize the mill moth *Ephestia kuehniella* Zeller, the almond moth *Ephestia cautella* Walker, the Indian meal moth *Plodia interpunctella* Hübner (ICW Hardy, personal communication) and the greater wax moth *Galleria mellonella* (Linnaeus) (personal observation).

1.4.2 Biology of *Goniozus nephantidis*

The gregarious ectoparasitoid *G. nephantidis* exhibits both a more limited geographical and host species range than *G. legneri*. *G. nephantidis* is the major parasitoid of *Opisina arenosella* Walker, a lepidopteran pest species that feeds on various life stages of coconut palm throughout southern India, Bangladesh, Myanmar (Burma) and Sri Lanka (Perera et al. 1989). *G. nephantidis* is also capable of parasitizing several other host species, including the greater wax moth *Galleria mellonella* Linnaeus and *Corcyra cephalonica* (Hardy and Blackburn 1991; Cook 1993). *Corcyra cephalonica* in particular has been utilised in laboratory culturing and mass rearing systems to maintain *G. nephantidis* for over two decades (Kapadia and Mittal 1986; Hardy and Blackburn 1991; Hardy et al. 1992; Humphries et al. 2006; Venkatesan et al. 2007; Khidr et al. 2013).

1.4.3 Bethyloid contest behaviour

In both *G. legneri* and *G. nephantidis*, adult females compete directly for the possession of hosts (Figure 1.2). These contests can escalate into aggressive encounters, involving chasing, biting, wrestling and stinging, but only rarely result in either the death of a contestant or lasting physical injury (Petersen and Hardy 1996; Humphries et al. 2006). Prior investigations into *Goniozus* contest behaviour provide a comprehensive background to the factors that underlie animal aggression, as well as the major aspects of *Goniozus* parasitoid life history and ecology (Batchelor et al. 2005; Goubault et al. 2006, 2007, 2008; Goubault and Hardy 2007; Lizé et al. 2012; Stockermans

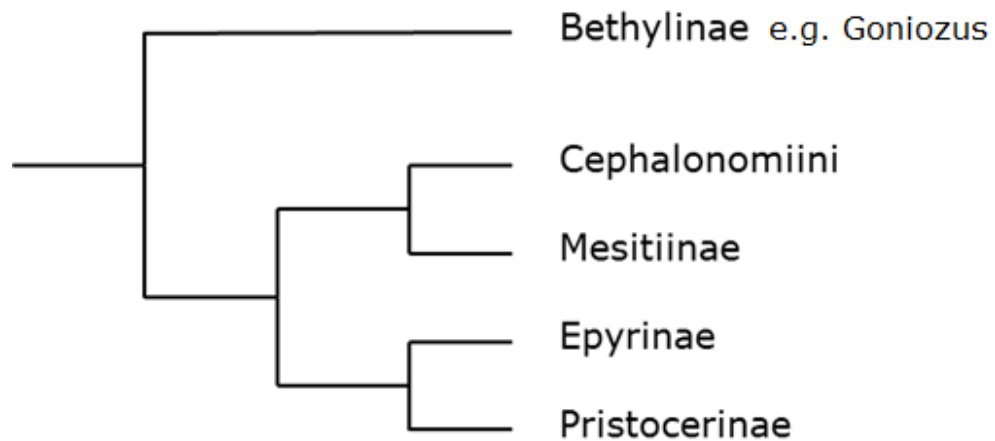


Figure 1.1. The five major Bethyloid subfamilies as described by Carr et al. (2010).

and Hardy 2013; Hardy and Briffa 2013). A number of factors influence the contest behaviour of *Goniozus* wasps. The three most common contest scenarios in *Goniozus* contest studies have been intruder-intruder contests, in which neither contestant is the prior owner of a host, owner-intruder contests, in which only one contestant is in prior possession of a host, and owner-owner contests, in which both contestants own hosts at the start of the dyadic interaction.

Contestant size and fighting ability have been linked, with larger contestants exhibiting an increased probability of winning (Stokkebo and Hardy 2000). In intruder-intruder contests, asymmetry in prior contest experience has been demonstrated to affect contest outcome, with more experienced wasps possessing greater fighting ability (Goubault et al. 2006; Bentley et al. 2009). In the case of owner-intruder contests, the prior owner of a host exhibits an advantage in agonistic contests (Goubault et al. 2006; Bentley et al. 2009). This advantage, likely due to the owner possessing greater information as to the quality of the host, is large enough to overcome that conferred by smaller size asymmetries in the intruders favour (Peterson and Hardy 1996; Bentley et al. 2009). Stokkebo and Hardy (2000) found that ownership of a host affects the owner's egg load, and that egg load also affects contest outcome, probably due to a host possessing a higher RV to a

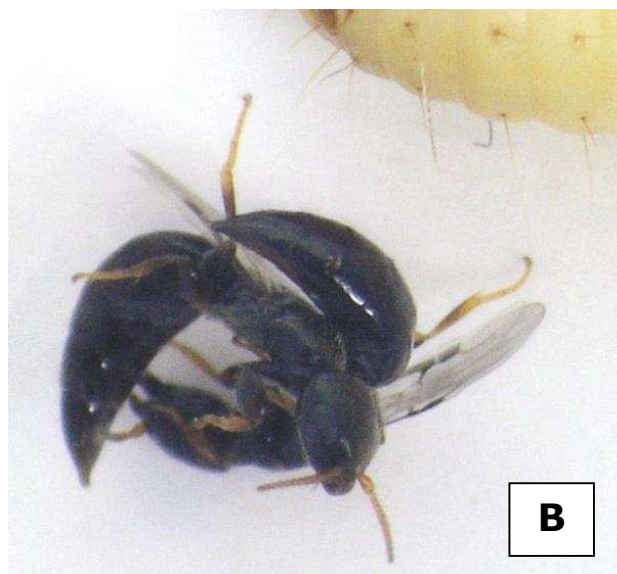
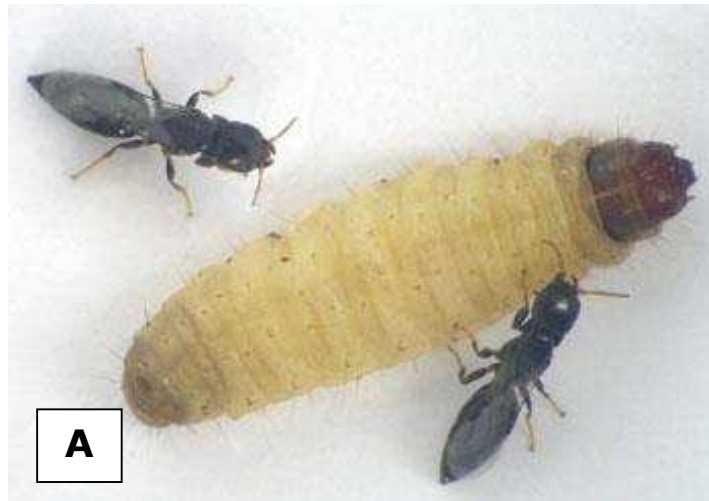


Figure 1.2. Contest Behaviour in *Goniozus* wasps. A. Owner female encounters an intruder B. Fully escalated fighting behaviour (from Goubault et al. 2006). Aggressive behaviour between females includes chasing, biting, wrestling, and occasionally fatal stinging (Humphries et al. 2006) Photo credit: Sonia Dourlot.

contestant with a higher amount of mature eggs. However, the probability of an intruder displacing an owner increased with the size and age of the intruder (Humphries et al. 2006). Finally, in owner-owner contests, females possessing a larger host are generally more successful (Humphries et al. 2006). When no asymmetries were present within owner-owner contests, contests involving larger hosts were more aggressive (Stockermans and Hardy 2013).

The most commonly employed indicator of host quality by parasitoid behavioural ecologists, host size, has been found to significantly affect bethylid contest behaviour (Humphries et al. 2006; Stockermans and Hardy 2013). In owner-owner contests with a size asymmetry, wasps defending a larger host had a greater probability of winning (Humphries et al. 2006). When no asymmetry was present between owners, contests involving larger hosts exhibited a greater proportion of aggressive interactions (Stockermans and Hardy 2013). The host background of contestants, particularly whether their development occurred on the same host, also significantly affects the possibility of contest success, with contests between less related wasps exhibiting a higher proportion of aggressive interactions (Lizé et al. 2012). Whether or not a contestant has already oviposited can further affect contest outcome. If an owner has already laid eggs, the size and age of the brood can affect contest outcomes, with older larvae receiving a lower level of maternal defence (Bentley et al. 2009). This is likely due to their reduced vulnerability, and the lower value of the mostly consumed host. The nutritional status of a host can also significantly alter contest behaviour. Wasps defending older hosts, i.e. those with a substantial time lag between initial paralysis and parasitism, have a lower probability of contest success (Khidr et al. 2012). Through the use of a metabolomic approach, this reduced probability of contest success was found to be correlated with declines in metabolites associated with energy production.

1.5 The metabolomic approach

Rapid improvements in technology since the 1980's has resulted in the adoption of analytical approaches that attempt to move beyond single molecule assays or pathway analyses, instead aiming to profile the biochemical mechanisms underlying complex biological systems. This "-omics revolution" has resulted in the development of the separate disciplines of genomics, transcriptomics and proteomics. The emergence of the newest of these -omics disciplines, metabolomics, provides a valuable tool to assess the effects of a particular condition of interest on the phenotype (Rochfort 2005; Kristal et al. 2007; Kaddurah-Daouk et al. 2008; Snart et al. 2015; Maag et al. 2015). Metabolomics (also referred to as metabonomics or metabolic profiling) accomplishes this by quantifying the global profile of small molecules that mediate complex biochemical systems (Fiehn 2002). This focus on the overall picture is key to the understanding of how individual

metabolites can form an integrated system outside of their simple role as the end-products of gene translation. By characterising the metabolic fingerprint underlying a particular environmental perturbation, it is possible to ascertain the identities of potential diagnostic biomarkers, metabolites associated with a disruptive metabolic condition e.g. a specific drug treatment or disease state. Initially applied in the related fields of pharmacology and toxicology, the utility of the metabolomic approach as a diagnostic tool has led to its widespread application to the study of metabolic disease states (Kaddurah-Daouk et al. 2008).

Current applications include the reporting of biomarkers for numerous cancers (Blekherman et al. 2011; Armitage and Barbas 2014), neurodegenerative disorders such as amyotrophic lateral sclerosis (Blasco et al. 2013), Alzheimer's disease (Han et al. 2011) and Huntington's disease (Verwaest et al. 2011), cardiovascular diseases (Shah et al. 2009, Rhee et al. 2012; Senn et al. 2012), diabetes (Wang-Sattler et al. 2012) and preeclampsia (Austdal et al. 2014). The ability of metabolomics to assay the metabolic effect of environmental influences has seen its further utilisation in such fields as systems biology (Weckworth 2003; McLean 2013), plant biotechnology (Saito et al. 2010; Okazaki et al. 2012), microbial biotechnology (Mashego et al. 2007), nutriogenomics (Davis and Milner 2004; Zeisel 2007), and ecology (Sardans et al. 2011; Rivas-Ubach et al. 2013). As the most 'downstream' of the -omics technologies, metabolomics can be used as a complement to genomic, transcriptomic and proteomic work, moving through the biological questions of 'what can occur' (genomics), 'what can we see occurring' (transcriptomics), 'what is causing it to occur' (proteomics) and 'what is actually occurring' (metabolomics).

Developments in the fields of both mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy have made it possible to simultaneously measure hundreds of metabolites within a given biofluid or tissue sample in a high throughput manner. More specific instrumentation such as infrared spectroscopy (Harrigan et al. 2004), Raman spectroscopy (Cherney et al. 2007), matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Want et al. 2005) and imaging mass spectrometry (McDonnell and Heeren 2007) has further refined the current utility of metabolomic analysis. Though the selection of a specific technique relies

heavily on the parameters of the experiment in question, it is now possible to generate reliable datasets at a comparatively low cost (although start-up costs remain high). The sheer size and complexity of the metabolome has also presented unique challenges for this field, requiring rapid advances in the development of analytical technology capable of simultaneously detecting and quantifying high numbers of metabolites (Smith et al. 2006; Patti et al. 2012). Limitations regarding the 'curse of dimensionality', the problem of drawing conclusions from the very large datasets generated by metabolomic analysis, have been increasingly removed by the development of specialist bioinformatic tools and software capable of producing an unbiased analysis of metabolites (Chi and Muller 2010). Improvements in metabolite cataloguing, along with the availability of comprehensive and widely available metabolite databases, have facilitated rapid metabolite identification. Examples of these include the Human Metabolome Database (HMDB), the LIPID Metabolites and Pathways Strategy (LIPID MAPS) and the Scripps Centre for Metabolomics (METLIN Scripps). Finally, a historical lack of consistent reporting within the field has led to calls for a unified reporting procedure, emphasising the documentation of instrument parameters, data analysis approaches, and the full disclosure of supporting analytical metadata (Fiehn et al. 2006; Goodacre et al. 2007; Sumner et al. 2007; Dunn et al. 2011; Zhang et al. 2012).

Another significant factor behind the increasing adoption of this approach is the growing understanding that genome sequencing and mapping does not fully explain the nature of complex metabolic systems (ter Kuile and Westerhoff 2001; Hirai et al. 2005). It is becoming increasingly apparent that the control of metabolic fluxes, the rate of transfer of molecules through a given metabolic network, is rarely explained by gene expression alone (Fell et al. 1992; ter Kuile and Westerhoff 2001). The metabolomic approach also benefits from a number of advantages when compared with the other -omics disciplines, perhaps the most exquisite of which is its comparative simplicity (Fuhrer and Zamboni 2015). The lack of extensive sample preparation required for the detection of small metabolites in complex biofluids (Keun and Athersuch 2011; Zhang et al. 2012), along with the existence of long established solvent extraction protocols applicable to tissue metabolite extraction (Folch et al. 1956; Hara and Radin 1976; Wu et al. 2008), provides a major advantage in terms of sample preparation time, an

attribute that can be crucial in the case of large clinical studies. The less invasive sample collection of a metabolomic investigation is especially advantageous within a clinical setting, where common biofluids such as urine and blood are regularly used in a diagnostic fashion (Zhang et al. 2012). Despite these inherent advantages, metabolomics retains a number of disadvantages. Though sample preparation is simple and comprehensive for biofluids, there is currently no single protocol capable of extracting the full range of metabolites from a given tissue or organism. Similarly, no instrument is currently capable of analysing the full range of metabolites simultaneously. As a result each investigation requires a tailored experimental design that is highly dependent on sample type, analytical platform and the metabolites of interest.

1.5.1 The terminology problem

Despite rapid investigative progress within the field, there has been a lingering controversy within the metabolomic community as to the exact definition of some key terminology. This extends to the name itself, the overlapping nature of the terms metabolomics and metabonomics has led to the two being used in a somewhat interchangeable manner. Within a more specific context, metabonomics has come to be regarded as “quantitative measurement of time-related multiparametric metabolic responses of multicellular systems to pathophysiological stimuli or genetic modification”, whereas metabolomics has been applied within a broader context (Oliver et al. 1998; Nicholson et al. 1999; Rochfort 2005). To simplify the structure of this thesis, the more frequently used term, metabolomics, is employed throughout. A summary of metabolomics related definitions and techniques is provided in Table 1.1.

1.5.2 Eco- and “ento”-metabolomics

The last decade has seen an increasing number of applications of the metabolomic approach in environmental studies. The attractive combination of the global nature of metabolomic analysis and the rapid nature of metabolic fingerprinting has fuelled the development of a new sub-discipline, that of environmental- or ecological-metabolomics (Bundy et al. 2009; Sardans et al. 2011; Lankadurai et al. 2013). A large number of studies now exist that investigate the response of metabolic systems to the presence of

Table 1.1. Glossary of metabolomic definitions and techniques (adapted from Goodacre et al. 2004; Dettmer et al. 2007).

Term	Definition
Metabolite	Small molecules that mediate metabolic systems and that are required for the maintenance, growth and normal function of a cell.
Metabolome	The complete set of metabolites in an organism or tissue.
Metabolomics	Global detection and quantification of the whole metabolome of a biological system under a given set of conditions.
Metabonomics	Analysis of metabolomic perturbations in response to disease, drugs or toxins. This definition was prevalent in older NMR-based studies.
Metabolic profiling	Focused quantitative analysis of a specific class of metabolites or those associated with a specific biochemical pathway. In the case of a limited number of metabolites, this may be referred to as a targeted analysis. Also known as metabolomic profiling.
Metabolic fingerprinting	Unbiased, global classification of samples based in response to a given set of conditions. This approach aims to characterise the underlying pattern or “fingerprint” of the response to environmental or genetic perturbations with the aim of identifying biomarkers. Also known as metabolomic fingerprinting.
Metabolic footprinting	The global analysis of extra-cellular metabolites within a cell culture medium. This approach aims to characterise cell uptake or excretion. Also known as metabolomic footprinting.

an external environmental stressor (Sardans et al. 2011). These stressors can be abiotic, such as temperature and climate stressors, or biotic interactions such as predation, defence and disease (Seki et al. 2007; Guy et al. 2008; Hoover et al. 2008; Marti et al. 2013; Meyer et al. 2013). Furthermore, optimised protocols for general eco-metabolomic studies are now available (Rivas-Ubach et al. 2013). Despite these advances, there remain distinct challenges for the conducting of an eco-metabolomic approach. The majority of prior approaches have largely focused on single factor approaches, when the metabolome can be influenced by numerous environmental factors at any one time. These factors include but are not limited to feeding status, organism age, circadian rhythm and health status (Sardans et al. 2011). Common metabolomic issues, such as the inability pesticides (Derecka et al. 2013), and temperature dependent stresses (Michaud and Denlinger 2007, 2008; Košťál et al. 2011a, 2011b). This wide range of applications has also resulted in a large variation in to characterise every metabolite within an organism or tissue, can also be a limiting factor within environmental studies.

Within eco-metabolomics there are an increasing number of studies investigating the effects of external stressors on insects. Although the current body of this "ento-metabolomic" literature remains small (<50 publications, Table 1.2, Snart et al. 2015), it has already contributed in understanding to a wide range of topics, including behavioural ecology (Lenz et al. 2001), parasitism (Thompson et al. 1990), development (Phalaraksh et al. 2008; Wu et al. 2012), hypoxia (Coquin et al. 2008; Feala et al. 2008), insect- bacterial symbiosis (Wang et al. 2010), experimental approaches, with some studies lacking rigorous data analysis and reporting (Snart et al. 2015). In addition to the common issues that beset environmental metabolomic approaches, ento-metabolomic investigations have lacked rigorous reporting of technical factors and experimental validation. Further experimental optimisation is also required for approaches utilising extremely low biomass samples, as current protocols have either focused on large quantities of insect biofluids (most notably hemolymph), or high volume solvent tissue extracts. Mass spectrometry has proven a useful analytical tool to generate single insect metabolomic analyses (Kamleh et al. 2008, 2009; Hammad et al. 2011, Košťál et al. 2011a, 2011b; Bratty et al. 2012; Colinet and Renault 2012), however, no low biomass equivalent currently exists for NMR methodologies. This absence is unsurprising, as the

Table 1.2 Insect metabolomics studies. Updated from Snart et al. 2015.

Insect order	Species	Research topic	Sample type	Techniques utilised	Conclusions	
Diptera	<i>Aedes aegypti</i>	Juvenile hormone regulation	Solvent extract	HPLC-FD*	Mevalonate and juvenile hormone pathways are highly dynamic and linked to reproductive physiology ¹ .	
	<i>Anopheles gambiae</i>	Temperature stress response	Solvent extract	GC-MS	Seasonal variations in climate resulted in metabolic and reproductive depression in older females ² .	
	<i>Belgica antarctica</i>	Temperature stress response	Solvent extract	GC-MS	Freezing and desiccation are associated with increases in metabolites associated with carbohydrate metabolism and a decrease in free amino acids ³ . Shifts in metabolite pools are associated with changes in gene regulation related to dehydration ⁴ .	
	<i>Chymomyza costata</i>	Cryopreservation	Solvent extract, biofluid	GC-MS, LC-MS	Survival of cryopreservation is associated with increased proline levels in larval tissues ⁵ .	
	<i>Drosophila melanogaster</i>	Metabolomic profiling	Solvent extract, biofluid	GC-MS, LC-MS	Cold shock disturbs short- and long-term cellular homeostasis ^{6,7,8,9} . Inbreeding, both in the absence and the presence of temperature stress, alters metabolic processes ¹⁰ . Lower rates of glycolysis occur in adapted flies undergoing hypoxia ^{11,12,13} . Age-related decline of hypoxia tolerance is linked to reduced recovery of mitochondrial respiration ¹⁴ . > 230 metabolites profiled across four <i>Drosophila</i> subspecies ^{15,16} . Bowman-Birk inhibitor disrupts energy metabolism ¹⁷ . Long-term cold acclimation modifies the larval metabolome ¹³ . Absolute quantification of 28 phospholipids ¹⁸ . Larvae with the y mutation have altered lysine metabolism ¹⁹ . CO ₂ exposure causes metabolic changes during short term recovery ²⁰ .	
		Temperature stress responses				
		CO ₂ anaesthesia				
		Bacterial infection Hypoxia			Infection by <i>Listeria monocytogenes</i> results in loss of energy store regulation ²¹ . Developmental and adult cold acclimation strongly promoted cold tolerance and restored metabolic homeostasis ² .	
	<i>Drosophila montana</i>	Temperature stress responses	Solvent extract	GC-MS, LC-MS	Seasonal variations in thermoperiod are correlated with differential expression of myo-inositol, proline and trehalose ²³ .	
<i>Sarcophaga crassipalpis</i>	Temperature stress response	Solvent extract	GC-MS, 1D NMR	Rapid cold-hardening elevates glycolysis associated metabolites whilst reducing levels of aerobic metabolic intermediates ²⁴ .		
Hemiptera	Aphids (multiple species)	Trehalose analysis	Solvent extract, biofluid	1D NMR	High concentrations of trehalose are present in aphid hemolymph ²⁵ . Removal of bacterial-insect symbiosis reduced expression of dietary metabolites, including essential amino acids ²⁶ .	
		Insect-bacterial symbiosis				
Hymenoptera	<i>Apis mellifera</i>	<i>Nosema ceranae</i> infection	Solvent extract, biofluid	GC-MS, LC-MS	Exposure to infectious pathogens and neonicotinoid pesticides results in altered larval and adult metabolism ^{27,28} .	
		Pesticide exposure				
	<i>Praon volucre</i>	Diapause induction	Solvent extract	GC-MS	Cold acclimation eliminated cryo-stress associated homeostatic perturbations ²⁹ .	
	<i>Venturia canescens</i>	Temperature stress responses	Solvent extract	GC-MS	Increases in cold tolerance are associated with the accumulation of cryoprotective metabolites ³⁰ .	
Lepidoptera	<i>Helicoverpa armigera</i>	Diapause induction	Solvent extract	GC-MS, MALDI-TOF	Diapause induces metabolic alterations associated with photoperiodic information and energy storage ³¹ .	
	<i>Manduca sexta</i>	Host parasitism	Biofluid	1D NMR	Insect parasitism enhances glucogenesis induction and halts lipogenesis ^{32,33} . Concentrations of small molecule metabolites change alongside larval development ³⁴ .	
	<i>Spodoptera frugiperda</i>	Metabolomic profiling	Solvent extract	LC-MS	Identification of major pathways associated with cellular protein productivity ³⁵ .	
	<i>Trichoplusia ni</i>	Metabolomic profiling	Solvent extract	LC-MS	Major pathways associated with cellular protein productivity identified ³⁵ .	
	Orthoptera	<i>Chorthippus</i> (multiple species)	Metabolomic profiling	Solvent extract	GC-MS	Determination of water soluble and lipid components of abdominal secretions of grasshoppers ^{36,37} .
<i>Locusta migratoria</i>			Developmental phase transition	Solvent extract	1D NMR	Onset of solitary-group behavioural phase transitions are regulated by carnitine expression ³⁸ .
<i>Schistocerca gregaria</i>			Social behaviour	Biofluid	1D NMR	Concentrations of trehalose and lipids were lower in the haemolymph of crowd- than in solitary-reared nymphs ³⁹ .
Phasmatodea	<i>Anisomorpha buprestoides</i>	Venom analysis	Biofluid	1D, 2D NMR	Stick insect defence secretions contain high levels of glucose, lysine, histidine, serotonin and sorbitol ⁴⁰ .	
	<i>Peruphasma schultei</i>	Venom analysis	Biofluid	1D NMR	Individual insects produce different stereoisomeric mixtures ⁴¹ .	
Plecoptera	<i>Dinocras cephalotes</i>	Hypoxia	Solvent extract	1D NMR/DI-MS	Metabolic shifts associated with heat stress are more pronounced under hypoxia ⁴² .	

* High-Performance Liquid Chromatography with Fluorescence Detection

¹ Rivera-Perez et al., 2014; ² Mamai et al., 2014; ³ Michaud et al., 2008; ⁴ Teets et al., 2012; ⁵ Košťál et al., 2011b; ⁶ Malmendal et al., 2006; ⁷ Overgaard et al., 2007; ⁸ Malmendal et al., 2013; ⁹ Williams et al., 2014; ¹⁰ Pedersen et al., 2008; ¹¹ Feala et al., 2008; ¹² Feala et al., 2009; ¹³ Košťál et al., 2011a; ¹⁴ Coquin et al., 2008; ¹⁵ Kamleh et al., 2008; ¹⁶ Kamleh et al., 2009; ¹⁷ Li et al., 2010; ¹⁸ Hammad et al., 2011; ¹⁹ Bratty et al., 2012; ²⁰ Colinet and Renault, 2012; ²¹ Chambers et al., 2012; ²² Colinet et al., 2012a; ²³ Vesala et al., 2012; ²⁴ Michaud and Denlinger, 2007; ²⁵ Moriwaki et al., 2003; ²⁶ Wang et al., 2009; ²⁷ Aliferis et al., 2012; ²⁸ Derecka et al., 2013; ²⁹ Colinet et al., 2012b; ³⁰ Foray et al., 2013; ³¹ Zhang et al., 2012; ³² Thompson et al., 1990; ³³ Thompson et al., 2001; ³⁴ Phalaraksh et al., 2008; ³⁵ Monteiro et al., 2013; ³⁶ Buszewska-Forajta et al., 2014b; ³⁷ Buszewska-Forajta et al., 2014a; ³⁸ Wu et al., 2012; ³⁹ Lenz et al., 2001; ⁴⁰ Zhang et al., 2007; ⁴¹ Dossey et al., 2006; ⁴² Verberk et al., 2013.

comparatively low sensitivity of NMR spectroscopy would require expensive high field instrumentation and could potentially result in impractically long sample analysis times.

As a result some studies have instead employed sample pooling methodologies (Michaud and Denlinger 2007; Pedersen et al. 2008). This also has the bonus of reducing the complexity of the dataset prior to data processing. However, if sample pooling is performed at the expense of sample size, this can result in a decrease in the number of degrees of freedom available during statistical analysis. This reduced number of samples results in a loss of analytical power during parametric validation of significantly different metabolite intensities. In the case of extremely small changes, such as those associated with low biomass samples, the resulting loss in analytical power during statistical validation may result in an increase in type II error i.e. a false positive (Banerjee et al. 2009). However the increase in the number of institutions capable of providing high-field analysis has increased the viability of the approach, especially when the general attractiveness of the structural elucidation capacities of NMR are factored in (Agrawal et al. 1992; Kwan and Huang et al. 2008). For this approach to be reliably implemented, it is clear that a degree of modification to any of the widely available solvent extraction protocols (e.g. Folch et al. 1956; Hara and Radin 1976; Wu et al. 2008) would be required, as these ratio-based approaches presume a large sample volume.

1.5.3 Nuclear magnetic resonance spectroscopy (NMR)

Though NMR spectroscopy has been employed in single metabolite insect studies since the early 80s (Ivie et al. 1983; Heinstra et al. 1990; Thompson 1990), the last decade has seen a significant rise in the number of insect studies utilising NMR spectroscopy in a recognisably metabolomic approach (Table 1.2; Snart et al. 2015). NMR has been extensively applied in characterising the main insect biofluid, hemolymph (Lenz et al. 2001; Moriwaki et al. 2003; Phalaraksh et al. 2008), resulting in a large number of robust, highly conserved peak assignments represented by multiple model spectra. NMR has also been employed to evaluate metabolomic changes during larval development and parasitism in Lepidoptera (Thompson et al. 2001; Phalaraksh et al. 2008), behaviour (Lenz et al. 2001; Wu et al. 2012) and heat stress (Verberk et al. 2013).

NMR presents a sophisticated tool that relies on the properties of certain atomic nuclei, including ^1H , ^{13}C , ^{15}N and ^{31}P (Abraham et al. 1988; Ikura et al. 1990). Of these, ^1H or proton NMR spectroscopy has been employed the most extensively within insect studies, with ^{13}C NMR being largely employed to further validate initial ^1H assignment (Keeler 2010). The usage of any type of NMR relies on the atomic property of spin (I), a property possessed by nuclei with odd numbers of protons and neutrons. Nuclei possessing an odd number of both protons and neutrons experience integer spin, those with only an odd total number of protons and neutrons exhibit half integer spin. In the presence of an electromagnetic field these nuclei transition between spin estates through a process of absorption and relaxation, given an external energy source. At a particular radio frequency a specific nuclei rapidly oscillates between these two energy states, producing an effect known as nuclear magnetic resonance. An NMR spectrum is the resulting plot of the intensity of the resonance at a given frequency. The intensity at a given peak is proportional to the number of nuclei undergoing resonance at that particular frequency. As the frequency of a given peak is directly proportional to the field strength of a particular instrument, the chemical shift scale (δ) is used, resulting in peak positions expressed in parts per million (ppm) rather than frequency (Hz) or wavelength. On this scale, peak positions are independent of the field strength, making it possible to compare spectra utilising different field strengths. In order to set up this scale, a standardised reference compound is used to generate a reference proton/peak (V_{ref}), which is then set at 0.00 ppm (Miller 2005). For proton NMR either dimethyl-silapentane-sulfonate (DSS) or tetramethylsilane (TMS) are used, due to their low reactivity with the sample itself.

Nuclear magnetic resonance spectroscopy possesses significant advantages from a metabolomic standpoint, being highly reproducible, non-destructive to the initial sample, moderately high-throughput, and in the case of biofluids, requiring very little sample preparation. However, it does have disadvantages associated with detecting hundreds of metabolite peaks simultaneously (Fiehn 2002). Highly complex metabolite mixtures often display overlap between spectral peaks, this can result in difficulty generating accurate metabolite assignments (Widarto et al. 2006). Furthermore, NMR has greater limitations in concentration sensitivity when compared to mass spectrometry (roughly 5nM for liquid chromatography-mass spectrometry vs 10 μM for NMR), resulting in a less stable analysis

time (Brown et al. 2005; Pan and Raftery 2007). This uncertainty in 1D assignment can be overcome by the generation of supporting 2D NMR spectra (Izrayelit et al. 2013). These spectra plot intensity vs two frequency axes, the exact axes depending on the type of experiment. Homonuclear spectra correlate shifts between matching nuclei, whereas heteronuclear spectra correlate shifts between different nuclei. For example, heteronuclear single-quantum correlation (HSQC) experiments are widely used to record correlations between ^{13}C and proton nuclei, with the proton being the observed nucleus. Other common 2D NMR experiments include correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and heteronuclear multiple-bond correlation (HMBC). These 2D spectra are highly reliable and are directly comparable to available spectra standard databases, such as the Biological Magnetic Resonance Data Bank (University of Wisconsin–Madison, USA) and the Human Metabolome Database (Human Metabolome Project, Canada).

1.5.4 Mass spectrometry (MS)

The majority of MS-based insect metabolome studies have utilised the model organism *Drosophila melanogaster* Meigen (Kamleh et al. 2008, 2009; Hammad et al. 2011; Košťál et al. 2011a, 2011b; Bratty et al. 2012; Colinet and Renault 2012). The combination of a large well-characterised stock of genetic mutants, genetic tractability, and a known organismal complexity makes *D. melanogaster* an ideal choice for generating insight into the composition and organisation of metabolic networks (Kamleh et al. 2008, 2009). Outside of metabolite profiling, studies have focused on such aspects of insect physiology as diapause (Colinet et al. 2012a; Zhang et al. 2012), infection and symbiosis (Chambers et al. 2012; Aliferis et al. 2012), hypoxia (Feala et al. 2008; Coquin et al. 2008; Feala et al. 2009, Košťál et al. 2011a, 2011b; Verberk et al. 2013), pesticide exposure (Derecka et al. 2013) and temperature stress responses (Michaud and Denlinger 2007; Michaud et al. 2008; Pedersen et al. 2008; Košťál et al. 2011a, 2011b; Colinet et al. 2012; Teets et al. 2012; Vesala et al. 2012; Foray et al. 2013).

Mass spectrometry ionises the molecules within a sample and separates them according to their mass to charge ratio (m/z), along with measuring their relative abundance within a sample (Hoffmann and Stroobant 2007; Hiraoka et al. 2013). A number of different ionisation techniques are utilised

for MS, the most common of which are electron impact (EI), electrospray ionisation (ESI), matrix-assisted laser desorption ionisation (MALDI) and atmospheric pressure chemical ionization (APCI). Ionised atoms are detected according to their negative or positive charge, generated by the addition or loss of a charged ion respectively. Charged ions are then sorted by a mass analyser according to their m/z , which is determined by their behaviour in electromagnetic fields. Commonly employed mass analysers include variations of time of flight (TOF) MS, quadrupole MS, ion-trap MS, orbital-trap MS and fourier transform-ion cyclotron resonance MS (FT-MS) (Hoffmann and Stroobant 2007; Hiraoka et al. 2013). Finally these sorted ions are recorded by a detector, usually consisting of a surface that records the induced AC current produced by the striking or passing of an ion. Mass Spectrometry is also regularly coupled with either liquid (LC) or gas (GC) chromatography prior to ionisation. As a result the output from MS differs from that of NMR spectroscopy, producing a time-based chromatogram with individual spectra observable at each time point. The main separation methods used in metabolomics are GC-MS and LC-MS, and their use depends on the nature of the metabolites of interest and the study in question (Marquet 2012).

Combined with recent developments in automated processing, MS based approaches are capable of analysing a large number of complex biofluid samples within a single day. The comparatively lower detection limits for MS, roughly 5 nM vs. 10 μ M for NMR, allow a more stable time of analysis (Pan and Raftery 2007; Brown et al. 2005). This enhanced sensitivity does lead to other processing problems, the most significant being difficulties in simplifying the enlarged data set, along with other ion suppression effects (the presence of less volatile compounds affecting the amount of charged ions that are detected). However, NMR is also subject to a degree of suppression: ^1H NMR in particular encounters line broadening problems due to interactions between individual molecular nuclei, resulting in a reduction of spectral resolution (Romanova et al. 2008).

As with all metabolomic techniques, MS requires extensive validation to accurately assign metabolite identities to individual ions (Dettmer et al. 2007). Elucidation of an ion's structure is enhanced through the use of LC-MS/MS, with individual metabolite classes possessing a specific

fragmentation structure. During LC-MS/MS analysis, ions are typically fragmented by an inert gas, such as argon or helium. The resulting m/z and fragmentation data can then be compared with either a similarly generated standard fragmentation pattern, or publicly available mass spectral databases (Taylor and Johnson 1997; Dettmer et al. 2007). A range of these databases are now freely available, including Lipidmaps (National Institute of General Medical Sciences, USA), Metlin Scripps (Scripps center for metabolomics, USA), HMDB (Human metabolome project, Canada) and Massbank (Massbank project, Japan) and Lipidblast (Fiehn lab, USA).

NMR spectroscopy and mass spectrometry both possess disadvantages when analysing particular classes of metabolites. However, using the two techniques together can yield enhanced information about the metabolic changes being investigated. For example, NMR is capable of rapidly generating complex polar single spectra with a rich amount of supporting structural information. LC-MS requires more specialised techniques in order to accurately separate and quantify polar metabolites, and metabolite identification elucidation is often reliant on the availability of standards. However NMR suffers from higher sensitivity thresholds compared to mass spectrometry and also displays a large degree of signal overlap when analysing lipid samples. GC-MS is also limited to volatile compounds. By utilising a combination of these methods, information can be generated for a wide range of metabolites, regardless of if they are volatile, non-volatile, polar, non-polar, high abundance or low-abundance.

1.5.5 Establishing a metabolomic workflow

A number of reviews and methodology publications already exist outlining the major analytical and statistical steps involved in the establishment of an appropriate workflow for conducting a metabolomics, or indeed any '-omics', investigation (Broadhurst and Kell 2006; Tiziani et al. 2011; Nikolskiy et al. 2013; Ibáñez et al. 2013). From the perspective of entomological research, it is likely that any investigation will utilise either a complex biofluid (Phalaraksh et al. 2008) or, especially for smaller insect species, solvent extraction of a targeted tissue or whole organism (Wu et al. 2008). Individual tissue types may be targeted (Coquin et al. 2008), thus requiring a degree of dissection, or the organism as a whole may undergo a direct solvent extraction (Košťál et al. 2011a, 2011b).

1.5.5.1 Sample preparation

Liquid-liquid sample extraction methodologies are commonly employed for sample preparation using a wide range of solvents such as chloroform-methanol, acetonitrile, isopropanol, hexane, heptane, ethanol and water (Folch et al. 1957; Hara and Radin 1978; Wu et al. 2008; Lin et al. 2006). A similar basic extraction approach is conserved across the majority of these methodologies: treatment/homogenisation of experimental tissues in cooled solvents, centrifugation of the homogenate to isolate the supernatant and often a sample concentration stage generating an extract in a relevant analytical solvent (e.g. D₂O for NMR spectroscopy) (Lin et al. 2006; Wu et al. 2008). The choice of extraction solvent can be based on the chemical nature of the metabolites of interest where these are relevant to the goals of the investigation. For example, an experimental approach focusing on polar (water soluble) metabolites would utilise a polar solvent such as water, methanol or ethanol. Alternatively, a study focusing on non-polar or lipid metabolites would utilise a non-polar solvent such as chloroform or heptane. An efficient 'one-step' separation of polar and non-polar metabolite fractions can be achieved using the widely-applied Folch method (Folch et al. 1957).

Using a combination of solvents is effective for focusing on both polar and non-polar metabolites and also for focusing on only one (Lin et al. 2006). The improved separation between metabolite classes has been shown to decrease the possibility of polar/non-polar phase contamination (Le Belle et al. 2002; Wu et al. 2008) which is important in reducing unwanted analytical interference, such as the effects of non-polar molecules on ¹H NMR spectroscopy. The study of certain non-polar metabolites also comprises a sub-category of metabolomic research, termed 'lipidomics' (Wenk 2005, 2010). As previously outlined, the insect fat body is an organ of great biosynthetic activity, and accounts for most intermediary metabolism, including, but not limited to, lipid and carbohydrate metabolism (Arrese and Soulages 2010; Roma et al. 2010). Its importance in insect physiology may require adoption of recent lipid-centric approaches, several of which have been previously described (Guan and Wenk 2006; Watson 2006; Shevchenko and Simons 2010; Wenk 2010). Despite the existence of standardised sample preparation methodologies, problems may arise with specific organisms, in the case of insect studies this is often specifically

related to low biomass (Lorenz et al. 2011; Marcinowska et al. 2011; Kim et al. 2013).

It has been previously discussed that the majority of extraction methodologies are tailored for larger biomass samples, the volumes and ratios associated with these approaches require adaptation if adopted for low biomass investigations, such as in Wu et al. (2008). If biomass is relatively constant among samples the inclusion of a fixed solvent volume, rather than ratio based mL/mg approach, may be more appropriate to avoid unworkably low solvent volumes. Whilst the metabolomic approach requires case-by-case optimisation for individual sample types, there are several general steps that all investigations must consider. Experimental tissues must be snap frozen (usually using liquid nitrogen) prior to analysis in order to halt all metabolic activity, a process often referred to as 'quenching'. In order to prevent cross contamination, it is optimal to clean the organism using high-purity water, or another appropriate solvent, prior to snap freezing. This is a major consideration for entomological investigations, due to many laboratory insect populations being reared in conditions where the surface of the specimen may be exposed to culture media and/or faecal matter that could affect the outcome of analysis if detected. Several common culturing diet components, including glucose, glycerol and ethanol are readily detectable in metabolomic analysis, particularly in the case of ^1H NMR spectroscopy (Phalaraksh et al. 2008; Chapter 4). Diet should also be considered, particularly as highly sensitive analytical approaches may also detect differences in gut composition. A possible approach to eliminating this issue would be to perform similar extractions and analytical profiling of the insect diet: dietary spectral data could then be compared with experimental samples, and used to rule out any observed background resonances or ions.

1.5.5.2 Experimental analysis

Selection of an analytical method will be influenced by the type of sample, and the goals of the investigation. As previously discussed, the two major forms of analysis, NMR and MS, have associated advantages and disadvantages, requiring different considerations before their utilisation (Pan and Raftery 2007). Whilst NMR spectroscopy is not capable of the sensitivity MS-based approaches can provide, it is a stable and well-established technique requiring comparatively little sample preparation. However, less

metabolite rich samples, such as those generated from low biomass organisms, often require higher sensitivity approaches. This may involve the use of a cryoprobe (a helium cooled probe that reduces noise detection, resulting in significant signal-to-noise enhancement) or high field instrumentation (Sobolev et al. 2005). These approaches may also extend the time of analysis for individual samples.

A consistent problem encountered by both approaches is spectral/chromatographic alignment. In order to compare individual samples, it is required for generated metabolomic data to be correctly aligned to avoid the potential for peak misidentification (Hoffman et al. 2012). In the case of NMR, fluctuations in sample pH can result in noticeable spectral shifts which are capable of disrupting alignment (Choy et al. 2001; Miyataka et al. 2007). Choosing an appropriate buffer pH (typically close to neutral, pH 6.5 - 8) is also important in the case of MS mobile phases in order to improve the reproducibility of analyte ionisation and chromatographic retention times. NMR samples are capable of using common salt-based buffers without interfering with spectral analysis, examples of which include sodium phosphate, HEPES and PIPES (Sterner et al. 2000). By comparison, salt based buffers cannot be used in MS mobile phases (Sterner et al. 2000), requiring the use of volatile buffers such as ammonium acetate or formic acid (Cappiello et al. 1997).

A major goal in most metabolomic investigations is the generation of a reliable and consistent data set relating to changes in metabolite concentration. Differentiation between metabolites is usually established by comparison of the spectral or chromatographic peak areas, validated by parametric statistical analysis (see below). Quantification in NMR approaches is most commonly achieved by the inclusion of a known chemical substance with the analytical sample, referred to as an internal standard (Lanza et al. 2010). Quantification for individual metabolites is then achieved by normalisation of the peak area to the area of the standard peak (Wishart et al. 1995). Two different approaches can be employed when selecting an internal standard. When the analyte of interest is known, a deuterated form of the analyte can be utilised to provide targeted quantitative information. If the approach is global or untargeted, a common standard can be applied. However, care should be taken to ensure that reference compounds used in

this manner do not overlap with resonances associated with potential analytes of interest (Dettmer et al. 2007).

The most common NMR standard for organic analysis is tetramethylsilane (TMS) (Wishart et al. 1995). As TMS is not soluble in aqueous samples, sodium salts, such as 3-trimethylsilyl-2,2,3,3-d₄-propionate (TSP) and 3-trimethylsilyl-propane-1-sulfonic acid (DSS) are employed instead (Shimizu et al. 1994; Schicho et al. 2010). By comparison, high-resolution MS instrumentation, though capable of highly accurate mass determinations, require calibration standards for each individual metabolite to achieve absolute quantitative analysis, which adds an extra level of analytical complexity. In most cases, MS-based metabolomics relies on relative quantitation, in which changes in the metabolite signals are compared with the analysis of the sample set. Normalisation procedures are often employed to minimise small variations in the weight of samples or to account for technical variations during the analysis. The most common of these procedures in MS-based methods is normalizing to the sum of the intensities of the ions within the spectrum, also referred to as the total ion count (TIC). However, there are associated disadvantages for some forms of data normalisation, such as the potential loss of biological information (Cairns et al. 2007).

1.5.5.3 Data pre-processing and metabolite identification

Different approaches are required for NMR and MS data in order to accomplish these steps. Spectral alignment in NMR is achieved through the addition of an internal standard during sample preparation, similar to quantification. As the positioning of spectral resonances in NMR (referred to as chemical shift) is relative to the instrument's magnetic field, the use of an internal standard with a known position (e.g. DSS/TSP) provides a reference point for alignment. Peak shifting can also be affected by environmental fluctuations, such as pH, temperature, and the concentration of ions (ionic strength) within a solution (Beneduci et al. 2011). Correct sample preparation should compensate for these potential sources of spectral variance. Alignment techniques for MS are also subject to shifting, particularly in terms of chromatogram retention time. These shifts can be partially compensated for by expanding the retention time window for peak integration (Hoffmann et al. 2012). Utilisation of internal standards for MS

quantitation can also aid in retention time correction, providing useful chromatogram landmarks.

As spectral/chromatographic data generated by metabolomic approaches is highly complex, data pre-processing procedures must be applied to the aligned data prior to any form of statistical analysis. Both NMR and MS approaches utilise specific techniques to reduce data complexity, these are respectively spectral binning (NMR) and peak framing (MS). Spectral binning (also known as bucketing) involves splitting a raw NMR spectrum into several hundred regions (Anderson et al. 2010), each represented by a numerical value corresponding to the intensity of the peak in that region. Binning does result in a reduction in data resolution, a drawback that is not shared by newer techniques focusing on full-resolution analysis (Stoyanova et al. 2004; Schoonen et al. 2007). However, binning remains a popular technique for metabolomic analysis, due to its high throughput nature. Peak framing of MS data consists of generating a unique 'frame' for each group of peaks with a certain mass and retention time. This approach relies heavily on having high resolution data (Cai et al. 2013) and requires further validation in order to accurately distinguish background noise from genuine chromatogram peaks. Both these processes reduce the dimensionality of the data, resulting in easier application of multivariate analyses.

Whilst such pre-processing aids in gas chromatography-, liquid chromatography-MS and NMR spectral processing, it can still result in difficulties in the chemical identification of metabolites, as there can be dozens of potential compound identities for each ion size/spectra peak position (Dunn et al. 2011). In the case of NMR, it is possible to directly compare spectral information generated from common metabolites with the raw spectra itself; this can eliminate some of the uncertainties associated with database identification. Several metabolite databases, including the previously mentioned HMDB, provide these spectra free of charge. A drawback to this approach is that identifications based on region overlap rely on these spectra being correctly aligned with the experimental spectra (Marion et al. 1989). Furthermore, the instrument parameters under which these model spectra have been generated have a relatively high probability of differing from the instrument parameters under which experimental data were acquired, decreasing the accuracy of direct comparisons of spectra.

Overlap between model metabolite spectra is also possible, particularly in the case of high intensity spectral regions.

Similar identification problems also apply to high resolution mass spectrometry which focuses on generating the mass to charge ratio (m/z) of a candidate ion. Whilst often capable of very high mass accuracy determination (<1 ppm) (Balogh 2004), this approach still encounters the problem of higher mass ions having multiple complex 'hits' in the metabolite databases, making it difficult to obtain a definitive identity of a metabolite from the database alone. Accurate differentiation and identification of these compounds requires correct experience-based filtering in order to eliminate potential ambiguities surrounding the identification. Such an approach could first focus on identifying molecules within NMR and mass spectra based on metabolomic database validation, before progressing to further validation of these putative identities utilising simultaneously generated structural information (Kind and Fiehn 2007). Pre-existing knowledge can also be used to reduce the number of candidate identities and to reliably label spectral compounds. There are defined workflows that facilitate this process, including some that utilise databases of known metabolite identities (Weingart et al. 2012; Tautenhahn et al. 2012; Nikolskiy et al. 2013).

Extended areas of metabolomic identification, such as structure elucidation, can also greatly aid in accurate ion identification (Ceglarek et al. 2009; Tian et al. 2013). Tandem mass spectrometry (MS/MS) fragmentation and two dimensional (2D) NMR have been increasingly adopted in order to overcome limitations in standard metabolomic approaches. The primary obstacle to correct identification in MS is the existence of many candidate identities for a specific m/z . Whilst prior knowledge of a particular biological system can reduce the number of candidate identities, the achievement of unambiguous identification requires further structural information. MS/MS techniques use gas based (usually argon) collision fragmentation in order to generate a specific set of product ions (Huddleston et al. 1993, Grebe and Singh 2011). This product ion set can be traced to a particular ion of interest. Alternative experimental setups can narrow the set of precursor ions exposed to fragmentation, resulting in the generation of product ions only associated with these ions (Grebe and Singh 2011). After correct precursor-product identification, the fragmentation pattern can then be utilised to narrow the

range of candidate identities. Similarly to standard MS identification, this is accomplished through comparisons with spectral databases or chemical standards (Dettmer et al. 2007). By comparison, overlapping spectra resonances remain the most challenging obstacle to unambiguous identification for NMR based approaches. 2D NMR works by spreading the signals across an extra dimension, providing further separation and relieving congestion within heavily populated peak areas. 2D NMR is also equally capable of generating global quantitative data with only a single internal standard (Lewis et al. 2007). However, the acquisition of high resolution 2D NMR spectra can take many times as long as the acquisition of similarly resolved 1D spectra (Viant 2003).

1.5.5.4 Metabolomics and multivariate data analysis

The fusion of metabolite profiling technologies with modern statistical analyses has facilitated the recent expansion of metabolomic research. The primary goal of combining these techniques has been to simplify large metabolite data sets into the principal sub-sets of metabolites that account for significant change as a result of biochemical perturbations (Kell et al. 2000; Beckonert et al. 2007). This necessitates the use of a combination of chemometric and mathematical modelling methods in order to generate useful information about potential biomarkers (Diamandis 2004; Crockford et al. 2006). Spectra generated through metabolomic approaches display high complexity, a large number of unique peaks are often detected from complex biological samples. Specialist data analysis is needed to prevent potentially important compounds from being overshadowed by larger peaks (Zhang et al. 2011). These approaches include long-established methods, such as principal components analysis (PCA) and associated discriminant analyses such as partial least squares-discriminant analysis (PLS-DA) and its orthogonal counterpart (OPLS-DA) (Quinn and Keough 2002). Techniques such as artificial neural networks (Ala-Korpela et al. 1997) and Bayesian spectral decomposition (Ochs et al. 1999) have also been employed to detect possible biomarkers.

PCA has been applied most frequently for initial spectral classification, and can be readily used for identifying potential separation between experimental classes (Stoyanova and Brown 2001; Nyamundanda et al. 2010). PCA operates by summarising large data variations generated by the underlying

metabolite profile into dimensional co-ordinates, known as principle components. By plotting these co-ordinates in a scores plot, a visual representation of differences between metabolite profiles associated with a physiological state or treatment can be generated (Stoyanova and Brown 2001). A similar plot, known as a loadings plot, is generated from individual metabolites within each sample. Metabolites associated with a particular treatment occupy similar co-ordinates in the loading plot as samples do within the scores plot, facilitating quick identification of potential biomarkers. This method does not differentiate between any predetermined classes during the data analysis (e.g. wild type vs. experimental, non-diseased vs. diseased) and is consequently ideal for establishing initial subset/class separation and providing an overview of complex data sets (Pan and Raftery, 2007).

Whilst PCA is important for preliminary data assessment and biomarker identification, it does not constitute statistical separation of the experimental classes (Eriksson et al. 2005). Significance of potential separation between treatment classes can be assessed by supervised multivariate analyses, such as PLS-DA/OPLS-DA and factor analysis (FA) (Rozett and Peterson 1975). Of these, PLS-DA/OPLS-DA is the most commonly utilised in metabolomic data analysis. These approaches include sample class information, attempting to construct a statistical model that can predict sample class membership from the data (Want and Masson 2011). Similarly to PCA, PLS-DA/OPLS-DA analysis produces scores and loadings plots. Unlike PCA, this is achieved by modelling the association between the data table, referred to as X, and a matrix of responses (age, diet, growth, pH etc.), referred to as Y. A PLS-DA model attempts to separate classes based on their X values (bins/ions). If certain parameters of the PLS-DA, such as the 'goodness of fit' (R^2X) and the 'goodness of prediction' (Q^2X), exceed accepted separation thresholds, a list of statistically significant bins (NMR) or ions (MS) can be generated (Eriksson et al. 2005). Using the scores plot in combination with the loadings plot, a list of bins/ions associated with experimental treatments can be generated and ranked in terms of their correlation to different classes (i.e. the Y matrix), a process also known as weighting. These modelling approaches can be applied either through the use of specialist software, such as SIMCA (Umetrics Ltd), or through the generation of specific processing algorithms. However, these multivariate approaches do require a degree of cross-

validation in order to assess the stability of the fitted model and to avoid overfitting of the model to the data set.

Whilst multivariate modelling is an integral tool for establishing separation between experimental groups, and for explaining the variation that accounts for this separation, parametric statistical modelling is required to assess these potential biomarker bins/ions (Quinn and Keough 2002). Significant separation in intensity between these bins/ions across experimental treatments can be assessed utilising general linear models (GLMs), such as an analysis of variance (ANOVA). This validation is an important step, as it is possible for multivariate models to generate noise instead of variations amongst real peaks. This noise, along with the multivariate nature of the dataset, is of particular interest when considering false discovery rates (Broadhurst and Kell 2006), i.e. the rejection of a correct null hypothesis (Type I error). One cause of Type I error is low sample size, multivariate data compounds this problem as the number of response variables vastly outnumbers the sample size in most metabolomic datasets (Ioannidis 2005; Broadhurst and Kell 2006). This high number of variables should also be considered from the perspective of multiple hypothesis testing. Classical statistical approaches largely focus on univariate analyses, with a single variable being tested independently. However, the probability of a variable generating a p-value below a specific threshold (e.g. <0.05) increases proportionally to the number of independent tests performed (Broadhurst and Kell 2006). As a result, a level of stringency is required when assessing the significance of a generated p-value. A number of potential corrections for this have been utilised; the most conservative, as well as the easiest to implement, is the Bonferroni correction (Quinn and Keough 2002). Further scrutiny of raw spectral/chromatogram data can also aid validation, confirming the presence of distinct spectral peaks in both NMR and MS spectra, along with a distinct time of elution for MS data. It is possible for multivariate analysis to flag baseline fluctuations (for NMR) or noisy background ions as contributing to treatment separation. As a result, the subtraction of the spectral baseline (NMR) and genuine peak identification (MS) prior to data analysis is essential to eliminating the possibility of generating a false positive. This approach can also produce a more accurate estimate of the true number of variables within the dataset, aiding multiple hypothesis correction.

1.5.6 Current limitations of ento-metabolomic studies:

Whilst ento-metabolomic studies have covered a range of issues, most issues have been addressed only one or two studies (Table 1.2). Furthermore a lack of consistent rigour is present in the existing literature, particularly when considering sample preparation, analytical methodology, and statistical analysis and reporting (Snart et al. 2015). The success or failure of a metabolomic investigation can depend on the rigour of the planning and developmental process preceding the investigation. As a result a central aim of this thesis is to rigorously apply the metabolomic approach, with particular care taken to consider experimental design, analytical validation and data analysis.

Employment of the metabolomic approach to the study of insect behaviour is particularly limited, consisting of two published studies (Lenz et al. 2001; Wu et al. 2012). These two studies attempted to generate biomarkers for solitary and gregarious behavioural phases in the locusts *Schistocerca gregaria* and *Locusta migratoria* respectively, with Wu et al. (2012) identifying carnitines as the key regulatory metabolites behind these behavioural states. Despite these studies much of the biochemistry underlying insect life history variation remains unexplored. In particular changes in underlying metabolic processes associated with predation, contest aggression and outcome, aging, oviposition and brood development are currently unknown. This thesis aims to apply an optimised, rigorous metabolomic approach to assess each of these areas.

1.6 Summary of thesis aims

This thesis investigates the behavioural, molecular and chemical properties of the bethylid parasitoids *G. legneri* and *G. nephantidis*. In all, this thesis provides a rigorously validated application of the metabolomic approach to advance understanding of how the underlying biochemistry of a parasitoid or its host can affect parasitoid life history choices. It comprises three parts, which are structured as follows:

1.6.1 Part 1. Employing an optimised metabolomic protocol: the effects of aging and diet on adult parasitoid contest behaviour

Chapters 2 and 3 detail the development and validation of an optimised method for conducting metabolomic investigations using a low-biomass insect sample, in this case *G. legneri*. The aim is to present an accurate and reproducible protocol that can be applied in future low-biomass investigations.

Chapter 4 investigates the effects of a high carbohydrate diet on *G. legneri* contest behaviour. The protocol outlined in chapter 2 and 3 was simultaneously employed to examine dietary effects on the adult parasitoid metabolome. The aim is to examine the effects of a carbohydrate diet on circulating hemolymph sugars and fat body reserves in an insect lacking *de novo* lipogenesis.

1.6.2 Part 2. Host parasitoid interactions: the effects of aging and host species

Chapter 5 explores the effects of host aging on the development and contest behaviour of *G. legneri*. Correlations between the larval and adult parasitoid metabolome were generated using an optimised metabolomic approach. The aim is to assess the effects of differing resource value on contest behaviour.

Chapter 6 examines the development and contest behaviour of *G. legneri* and *G. nephantidis* on four different lepidopteran host species. An optimised metabolomic approach is utilised to examine correlations between metabolome of host species and adult parasitoids. The aim is to investigate which host species result in better parasitoid performance, and which species are of the highest value.

1.6.3 Part 3. Behavioural and biochemical mimicry by a hyperparasitoid wasp

Chapter 7 examines the multi-trait mimicry of the garden ant *Lasius niger* by the hyperparasitoid wasp *Gelis agilis* using a combined behavioural and metabolite profiling approach. The aim was to assess the predation defences of *G. agilis* from common spiders.

1.6.4 Thesis format

The five experimental chapters of this Thesis, along with the manuscript in the appendix, are presented in the format of standard journal papers, except that references and acknowledgements for all chapters are formatted as a single list. Chapter 7 has been previously published (Malcicka et al. 2015).

Part 1: Employing an optimised metabolomic protocol: the effects of aging and diet on adult parasitoid contest behaviour

Chapter 2: Addressing the challenge of low biomass in insect metabolomics - Part 1: Optimisation of procedures

2.1 Abstract

Non-targeted metabolite profiling has been increasingly employed in insect study systems over the last two decades, revolutionizing approaches for testing biochemical and nutritional hypotheses within insect biology. However, the majority of small organism studies have been limited to pooling insect samples in order to employ traditional solvent extraction and analytical approaches. The generation of metabolite profiles of single small insects subjected to different experimental treatments is required if metabolomics approaches are to be used in conjunction with behavioural and ecological studies. This chapter outlines the first part of the optimisation process required to generate a low biomass solvent extraction approach compatible with a rigorous metabolomic approach. Optimisation focused on developing a reliable solvent extraction methodology capable of generating metabolite rich phases for NMR and LC-MS analysis. An optimal methanol-chloroform-water solvent extraction procedure was developed that successfully addressed the challenges associated with low biomass in metabolomic analysis. This approach was further demonstrated to be highly stable and precise, as well as capable of generating readily identifiable spectra and supporting structural information. Further validation of this approach, in the form of a full metabolomic workflow investigating the effects of aging on the metabolome of *G. legneri*, is detailed in Chapter 3.

2.2 Introduction

Metabolomics is a relatively newly established '-omics' platform that, together with genomics and proteomics, aims to advance systems biology approaches in biochemistry and cellular biology (Patti et al. 2012). Metabolites are small molecules that represent the end product of cellular biochemical processes and thus metabolite profiles correlate better with phenotypes than genomic, transcriptomic and proteomic profiles (Patti et al. 2012). Global metabolite profiling, often referred to as untargeted metabolomics, operates in a non-selective manner to uncover patterns and relationships between stimulus and response (metabolome) that generate further hypotheses; this is particularly useful in exploring a range of environmental and ecological (e.g., diet, circadian rhythm, social behaviour) influences on organisms (Lenz et al. 2001; Gattolin et al. 2008, Bundy et al. 2009).

The use of the metabolomic approach in insect study systems has rapidly increased over the last decade (Maag et al. 2015; Snart et al. 2015), with individual studies focusing on a wide range of ecological, behavioural and physiological applications. Metabolite profiling in insects has been used repeatedly to associate metabolites with responses to abiotic factors such as oxygen limitation, desiccation, heat and cold stress, cold tolerance, acclimation and hardening (Overgaard et al. 2007; Coquin et al. 2008; Malmendal et al. 2006; Michaud et al. 2007, 2008; Hawes et al. 2008; Pedersen et al. 2008; Feala et al. 2009; Waagner et al. 2010; Vesala et al. 2012; Košťál et al. 2011, 2012; Teets et al. 2012a, 2012b; Colinet et al. 2012a, 2012b, 2013; Verberk et al. 2013), and biotic factors such as density dependent phase transition, symbiosis and disease (Wang et al. 2010; Aliferis et al. 2012; Wu et al. 2012). *Drosophila* flies are one of the most targeted organisms for metabolomics studies (Malmendal et al. 2006; Overgaard et al. 2007; Coquin et al. 2008; Pedersen et al. 2008; Feala et al. 2009; Li et al. 2010; Košťál et al. 2011, 2012; Vesala et al. 2012; Sarup et al. 2012; Colinet et al. 2012, 2013).

In the majority of studies sample size has consisted of a pooled group of individual insects (Overgaard et al. 2007; Pedersen et al. 2008; Coquin et al. 2008; Feala et al. 2009; Vesala et al. 2012; Košťál et al. 2011a, 2012;

Sarup et al. 2012; Colinet et al. 2013) as dictated by the size of the focal organism (many insects are small) and the analytical power of methods used. Sample biomass remains a limiting factor for nuclear magnetic resonance spectroscopy (NMR), as important compounds present at lower concentrations are subject to sensitivity limits and possible overshadowing by larger peaks (Pan and Raftery 2006). By comparison mass spectrometry (MS) exhibits higher sensitivity (Dettmer et al. 2007) but is dependent on the size and type of molecule, with polar macromolecules being more difficult to analyse, although lately the use of hydrophilic interaction chromatography (HILIC) has offered new approaches in analysing polar metabolites (Want et al. 2007). Moreover, sample biomass constraints may limit the application of established, low variability solvent extraction protocols (Folch et al. 1957; Bligh and Dyer 1959; Lin et al. 2006; Wu et al. 2008), many of which assume solvent tissue ratios that are infeasible for a low biomass (e.g. <1 mg). A few studies have applied modified metabolite extraction methodologies to individual insects, with a particular focus on larger organisms (Teets et al. 2012; Verberk et al. 2013). However, such studies have often been limited to targeting specific groups of metabolites (e.g., Iwahata et al. 2011), and may also encounter metabolite fluctuations associated with whole organism analysis. Of particular concern is the unpredictable content of the gut, whilst this can be controlled for in many investigations, it may become a serious limitation in the case of nutritional and field-based investigations (Snart et al. 2015).

Insect and other arthropod study systems have played a considerable role in the development of behavioural and evolutionary theory, and empirical studies rely on the observation of individuals (Davies et al. 2012). Insect parasitoids represent an important group of insects that have been used for biological control and also for probing more fundamental questions in both ecology and evolution (Godfray 1994; Kapranas and Hardy 2014). The metabolomes of insect parasitoids remain under-explored, currently comprising of only two studies examining diapause (Foray et al. 2013) and the effects of developmental temperature (Colinet et al. 2012). However, owing to their low biomass a generation of an optimised metabolomic approach is needed.

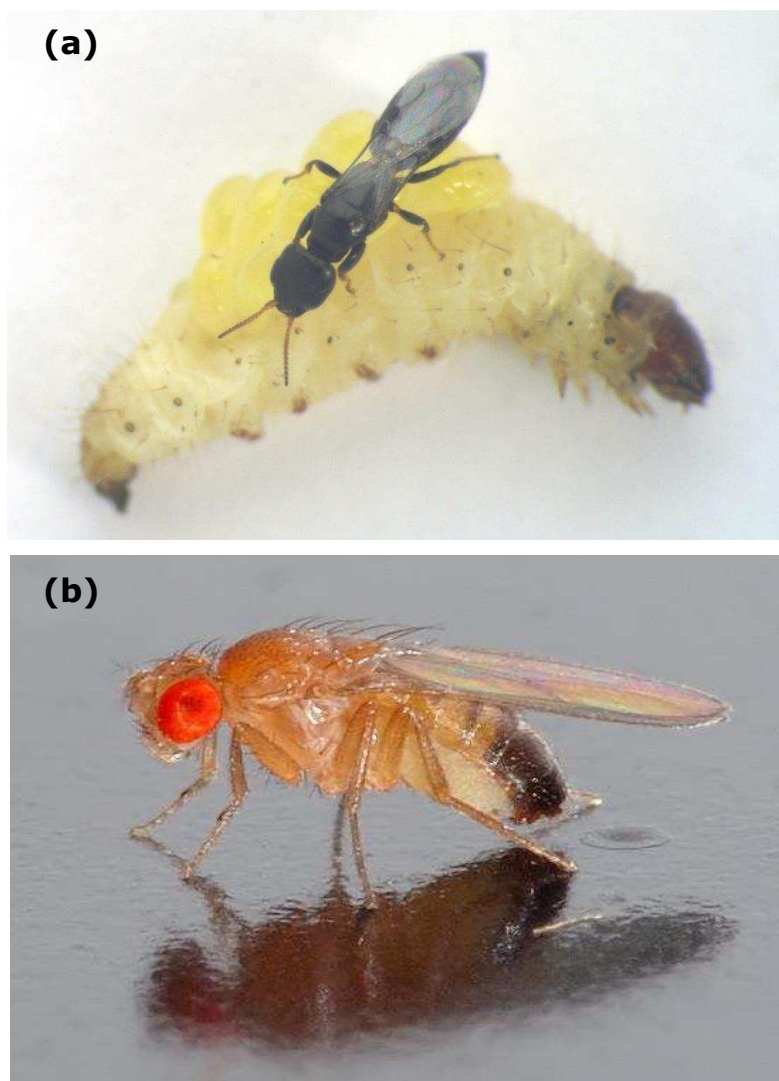


Figure 2.1. Physiological comparison of (a) *G. legneri* on host with brood and (b) *D. melanogaster*. Whilst *G. legneri* is of a similar biomass to that of *D. melanogaster* ($\sim 1\text{mg}$), it possesses a thick sclerotized cuticle that poses a technical challenge for sample extraction. The successful generation of complex 1D NMR spectra from extracts generated under this methodology confirms that this extraction protocol is equally applicable to hard, disruption resistant low biomass samples, a sample type that remains underexplored in NMR studies. Photos: (a) Sonia Dourlot (b) André Karwath, Wikimedia Commons. Photos are reproduced under the Creative Commons Attribution-Share Alike 2.5 Generic license.

2.2.1 Solvent extraction protocol optimisation

This chapter is presented as the first in two complementary parts detailing the optimisation and validation of a low biomass metabolomic workflow. Part 1 details the optimisation process of a specialised solvent extraction approach capable of assessing the metabolite profiles of individual low biomass organisms. Unlike previous low biomass studies, this approach attempts to simultaneously generate both polar and non-polar phases by employing a modified Bligh and Dyer (1959) methodology. Previous low biomass NMR extraction approaches have utilised soft bodied organisms, with little consideration of hard bodied organisms or samples. To test the applicability of this extraction protocol for such samples, this study deliberately employs a non-model organism, the bethylid parasitoid wasp *Goniozus legneri* (Hymenoptera: Bethyridae) as a study organism (typical female mass approx. 1 mg). *G. legneri* was selected for this optimisation due to its status as a useful laboratory study organism for behavioural research (Hardy et al. 2000, Lizé et al. 2012). *G. legneri* possesses a thick outer exoskeleton consisting of multiple levels of cuticle that are resistant to physical disruption (Hillerton et al. 1982) (Figure 2.1).

2.2.2 Aims and objectives

This chapter aimed to generate a low-biomass solvent extraction approach capable of producing solvent phases that were compatible with polar and non-polar metabolomic analysis by NMR spectroscopy and LC-MS respectively. Specific objectives required to fulfil this aim are:

- To optimise a solvent extraction approach that produces easily separable solvent phases with a reliable total volume and minimal contamination.
- To optimise an NMR approach capable of producing complex and highly reproducible spectra from single wasp polar extracts.
- To optimise a stable LC-MS approach capable of reliably producing complex LC-MS chromatograms and mass spectra from single wasp non-polar extracts with a minimum of sample pre-processing.

2.3 Materials and methods

2.3.1 Parasitoid origin and cultures

The host of *G. legneri*, the rice moth *Corcyra cephalonica* (Stainton), (Lepidoptera: Pyralidae) was reared on a diet of glycerol, honey, corn meal, wheat bran and yeast (Stockermans and Hardy 2013). The parasitoid strain was originally obtained from a commercial insectary in the USA, and both host and parasitoid strains were the same as utilised in a number of previous behavioural and biochemical studies (Hardy et al. 2000; Goubault and Hardy 2007; Bentley et al. 2009; Lizé et al. 2012; Stockermans and Hardy 2013; Khidr et al. 2013a, 2013b, 2014). *Goniozus legneri* were reared on larvae of *C. cephalonica* by introducing an adult female wasp and a caterpillar in a glass vial (2.5 x 7.5 cm) plugged by gauze and cotton. Parasitoid and moth culturing was carried out in a climate room (~27 °C, 16:8 hour light:dark cycle, 60 - 70 % relative humidity).

2.3.2 Analytical strategy

The analytical strategy adopted during the development of this metabolomic approach is centred upon the challenge that a small sample size presents to NMR analysis. To compensate for the lower sensitivity of NMR spectroscopy compared to LC-MS, a degree of optimisation was required, particularly when considering the sample extraction methodology and the instrumentation employed. As a result initial optimisation of the wasp extraction methodology was focused on confirming if NMR is capable of reliably generating complex high resolution spectra from single insect solvent extracts generated using established-solvent protocols. Another priority was to generate an approach capable of high throughput analysis with available instrumentation, as increasing spectral resolution without changing to higher resolution instrumentation also increases the analysis time. Due to this the optimisation of the extraction methodology for LC-MS focused on the processing of the generated lipid phase prior to LC-MS analysis to improve mass spectral signal intensity. Further optimisation of LC-MS analysis focused on the technical replicability of the approach, along with its stability across and extended experimental timeframe.

2.3.3 Single insect extraction methodology optimisation

A total of 18 3 day old adult female *G. legneri* were snap frozen in liquid nitrogen and stored at -80 °C until solvent extraction. Three different extraction solvent combinations were investigated and compared throughout wasp extraction optimisation, methanol-chloroform-water (MCW), hexane-isopropanol-water (HIW) and ethanol-heptane-water (EHW) (Folch et al. 1957; Bligh and Dyer 1959; Hara and Radin 1978; Lin et al. 2006; Wu et al. 2008). In order to produce a consistent final extract volume, these approaches were modified to include a fixed solvent quantity as outlined below. A total of six replicates were performed for each extraction methodology. All solvents were of a high LC-MS grade purity (Chromasolv Sigma-Aldrich) and all samples were randomised throughout initial sample extraction and analysis.

2.3.3.1 Methanol-chloroform-water (MCW) extraction

Individual wasps were placed in a 2.0 mL lysing matrix Z MP-BIO® vial containing 320 µL of methanol and 128 µL of water and homogenised using an MP-BIO Fast Prep® homogenizer. The homogeniser programme consisted of 3 x 30s of homogenisation with a 30 second cool down period between each homogenisation period. The resulting homogenate was transferred to a 1.5 mL solvent resistant Eppendorf® Biopur® tube by Pasteur pipette. 320 µL of chloroform and 160 µL of water were then added and extracts were vortexed for 30 seconds (s). Extracts were then centrifuged for 10 minutes (min) at 10,000 g and left for 5 min at room temperature before phase separation. Solvent phases were separated using a Pasteur pipette and transferred to either a 1.5 mL solvent resistant Eppendorf® Biopur® tube (polar phase) or a 2 mL borosilicate glass vial (non-polar phase). Both phases were stored at -80 °C prior to analysis.

2.3.3.2 Hexane-isopropanol-water (HIW) extraction

Individual wasps were placed in lysing matrix vial containing 240 µL of hexane, 160 µL of isopropanol and 200 µL of sodium dodecyl sulfate (SDS) and homogenised as outlined in the MCW extraction protocol. The generated homogenate was transferred to a solvent resistant eppendorf and vortexed, centrifuged as outlined in the MCW extraction protocol. As HIW extraction results in upper non-polar and lower polar phases as opposed to the upper

polar and lower non-polar phases generated in MCW analysis, phase separation was facilitated by freezing the sample at -20 °C. The non-frozen upper fragment was removed by Pasteur pipette, whilst the lower fragment was removed after thawing. Both phases were stored as outlined in the MCW extraction protocol until sample analysis.

2.3.3.3 Ethanol-heptane-water (EHW) extraction

The EHW extraction protocol was similar in outline to that of MCW extraction, with the following exceptions. Individual wasps were homogenised in a lysing matrix vial containing 160 µL of SDS and 160 µL of water. The resulting homogenate was transferred to a solvent resistant eppendorf, to which 320 µL of ethanol and 320 µL of heptane were added. Samples were vortexed, centrifuged, separated and stored as outlined in the MCW extraction protocol prior to NMR analysis.

All polar samples were dried by rotary evaporation and reconstituted in 0.6 mL of D₂O before analysis. 1D proton NMR spectroscopy was used to obtain spectra of polar wasp extracts. Acquisition was performed using a Bruker Avance 600 spectrometer, equipped with a 5mm quadruple nuclei probe (QNP) and a Bruker Sample Jet autosampler. The spectrometer operated at resonance frequency of 600.13 MHz and measured samples with an internal probe temperature of 298.1 K. A pre-saturation solvent suppression pulse sequence (NOESYPRESAT) was employed during spectral acquisition to minimise the large water signal. 128 scans were collected from each sample within a spectral width of 13 ppm. Exponential line broadening of 0.3 Hz was applied to free induction decays (FIDs), which were zero-filled by a factor of 2 prior to Fourier Transform. Spectra acquisition was automated using IconNMR, followed by manual spectra phasing and baseline correction using TopSpin version 3 (Bruker, GmbH). The generated spectra were then assessed in TopSpin for spectral complexity i.e. the number of visible spectral peaks, and spectra peak intensity. In addition, sample phase separation and sample volume consistency were considered when selecting the optimal extraction approach. The results of this comparison are displayed in Figure 2.2.

2.3.4 Optimisation of polar metabolomic analysis by NMR spectroscopy

The optimum extraction methodology identified from the previous section, methanol-chloroform-water, was further optimised to improve spectral resolution and to assess sample stability throughout the course of a model experimental run. Whilst MCW spectra were the most complex and reliable of the three methodologies, spectral resolution remained poor. To attempt to improve this resolution, a new set of polar extracts were generated under the same methodology and analysed using a high field 800 MHz spectrometer equipped with a cryoprobe. As analysis of large NMR datasets requires an extended experimental time period, a stability test was also performed to test for potential sample degradation. A number of stability samples were repeatedly analysed throughout a model NMR experiment, constituting sample analysis from three separate treatment categories. In order to identify any generated NMR resonances a 2D NMR experiment was performed using a pooled wasp extract to provide structural information.

2.3.4.1 Optimisation of NMR spectral resolution

To optimise spectral resolution, a total of 6 individual wasp polar extracts were generated using the MCW extraction protocol outlined in chapter 2.3.3.1. Polar extracts were dried in a rotary evaporator and reconstituted in 600 μ l of D₂O with 0.5 mM of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). The addition of a DSS signal served to provide an internal reference (0.000 ppm) for spectral alignment and quantification. All NMR experiments were acquired on a Bruker Avance 800 MHz III spectrometer using a 5mm QCI Cryoprobe. Data were collected with a spectral width of 13 ppm and signal averaged over 512 scans using a noesy 1D pre-saturation experiment to achieve water suppression. Total recycling delay was 4.7 s. Pre-saturation power and frequency were determined in advance on representative samples and fixed for all acquisitions. Samples were referenced and locked using the internal D₂O signal with a secondary reference of DSS (0.5 mM) present in all samples. Data were processed by application of an exponential window function set to give a 0.3 Hz line broadening prior to zero filling and Fourier transformation. Spectral acquisition was automated by IconNMR, manual spectral phasing, baseline correction and alignment was performed in TopSpin version 3 (Bruker, GmbH). The resulting spectra were compared with those generated by 600 MHz spectrometry to assess spectral complexity

i.e. the number of visible spectral peaks, and spectra peak intensity. Comparisons of 600 MHz and 800 MHz spectra are provided in Figure 2.3.

2.3.4.2 Optimisation of NMR analytical stability

To assess sample stability throughout an extended experimental time period, a model NMR experiment was performed. Individual *G. legneri* females were presented with a *C. cephalonica* larva and allowed to rear broods. Upon eclosion, female offspring were placed separately into 0.5 mL Eppendorf tubes. Individual wasps were either (a) provided with a honey diet (Honey-fed) (b) provided with a pre-paralysed host on which to feed for day one then starved until freezing (Host-fed) or (c) provided with no diet (Starved). Wasps were snap frozen in liquid nitrogen 7 days after initial isolation and stored at -80 °C prior to analysis. Polar and non-polar extracts were generated from individual wasps using the MCW extraction protocol detailed in chapter 2.3.3.1. 20 replicates were performed for each experimental treatment. A number of control extracts, consisting of an extraction replicate with the wasp removed, were also generated to act as quality control samples. Polar extracts were then dried, reconstituted and analysed as outlined in chapter 2.3.4.1. A total of 4 wasps from the starved treatment and 3 wasps from the host fed treatment died prior to sample freezing, these were considered censors for subsequent analysis. Three honey fed wasp spectra were also removed during initial PCA, as spectral consideration indicated that these wasps had not ingested the available diet (this is explored further in Chapter 4).

Throughout NMR analysis, spectra were acquired from a randomly selected sample from each treatment class after every ten spectral acquisitions. The generated spectra were phased, baseline corrected and referenced to the internal DSS signal using TopSpin version 3 (Bruker, GmbH). A user generated TopSpin script was used to perform non-discriminant data alignment and binning. The data set was simplified by dividing spectra into a total 931 non-overlapping bins with a spectral width of 0.01 ppm. Integrated spectral intensities were assigned to each of these bins. Microsoft excel was used to exclude the DSS and water signal regions (5.2 - 4.5 ppm) and to normalise spectral bins to the total spectral intensity. Normalised bins were imported into Simca 13.0.3 (Umetrics) where samples were visualised using principle component analysis (PCA). Autoscaling and mean centring

was applied prior to PCA model generation. Experimental classes were assessed for initial clustering and separation. Stability sample replicates were assessed for clustering and drift over time. Ten metabolite associated bins were selected within each set of replicates and the relative standard deviations of their intensity were assessed. In line with acceptability thresholds for bioanalytical method validation suggested by the Food and Drug administration (FDA USA) and by Dunn et al. (2011), a tolerance of 30 % for a minimum of 70 % of bins was judged to be acceptable. PCA comparisons of dietary treatment spectra are provided in Figure 2.4. Relative standard deviations of the selected key bins across stability replicates are provided in Table 2.1.

2.3.4.3 2D NMR spectral identification

To aid confirmation of metabolite identity, two-dimensional NMR spectra were generated from a pooled extract of 20 starved wasps generated utilising the same MCW methodology. A heteronuclear single-quantum correlation (HSQC) experiment was employed. HSQC spectra were acquired using 48 transients per increment for 160 scans collected for 4096 complex points, covering a ^1H spectral width of 8484.163 Hz and a ^{13}C spectral width of 33339.457 Hz. Putative NMR resonances were validated through direct comparisons between generated 2D NMR spectra and 2D spectral standards from the Biological Magnetic Resonance Data Bank (BMRB). Individual standards were aligned to the D_2O signal in topspin then validated by overlaying them directly with the 2D wasp spectra using CCPNMR (CCPN project). The generated HSQC spectra and spectral identifications are displayed in Figure 2.5 and Table 2.2.

2.3.5 Optimisation of non-polar metabolomic analysis by LC-MS

Pre-processing of the selected non-polar fragment extraction methodology was optimised to improve spectral yield. Comparisons between two available LC columns were made to assess the peak shape and separation of generated LC-MS chromatograms. To assess technical replication within an extended experimental time frame, a number of non-polar samples were injected in triplicate and assessed for the relative standard deviations of the peak area and retention times of a number of key ions. Repeated injections of a pooled QC sample were made throughout the experimental timeframe

to assess LC-MS stability. Finally a tandem mass spectrometry (LC-MS/MS) approach was optimised to validate tentative sample identifications.

2.3.5.1 Optimisation of non-polar extract LC-MS pre-processing

To assess the applicability of the selected solvent extraction approach for metabolomic profiling using LC-MS, a number of approaches were considered for processing generated non-polar chloroform extracts prior to analysis. A total of 30 non-polar extracts were generated from individual female *G. legneri* as outlined in section 2.3.3. These extracts were subject to either (a) no modification or (b) rotary evaporation and reconstitution in 70 μL of isopropanol. A total of ten replicates were generated for each treatment class.

Accurate mass LC-MS was performed on non-polar extracts (10 μL injection volume) using an Accela LC coupled with an Exactive mass spectrometer (Thermo Fisher Scientific, USA) in negative and positive electrospray ionisation modes (ESI). An Hypersil gold C18 (1.9 μm , 2.1 x 50 mm) column was maintained at 40 $^{\circ}\text{C}$. The flow rate was set at 300 $\mu\text{L}/\text{min}$ initially then increased linearly with a variable flow rate of 300-400 $\mu\text{L}/\text{min}$ depending on gradient time. Mobile phases utilised for the gradient program consisted of (A) 80:10:10 water/isopropanol/acetonitrile, the phase was modified with 0.01 % ammonium acetate and (B) 50:50 IPA/acetonitrile modified with 0.01 % ammonium acetate. All solvents used were of high LC-MS grade purity (Chromasolv, Sigma-Aldrich). The LC gradient program had an initial starting proportion of 62.5 % B with a flow rate of 300 $\mu\text{L}/\text{min}$ followed by a linear increase to 99 % B with a flow rate of 400 $\mu\text{L}/\text{min}$ over 3 min. This proportion was maintained for a further 5 min followed by a linear decrease to the starting proportion of 62.5 % B and flow rate of 300 $\mu\text{L}/\text{min}$ over 2 min. Ions were monitored within the range of m/z 100 to 1500 (ESI voltage: 3500, capillary temperature: 350 $^{\circ}\text{C}$, scan rate: 250 ms). The generated spectra were then contrasted for their average number of real chromatographic peaks. Representative chromatograms are displayed in Figure 2.6.

2.3.5.2 Optimisation of LC-MS column choice

As a large number of ions detected using the Hypersil gold C18 column previously did not exhibit sharp chromatographic peaks, 10 non-polar wasp

extracts prepared using the optimal pre-processing methodology (reconstitution in 50 μ L of isopropanol) were re-analysed using an Ace Excel 2 Super C18 (2 μ m, 2.1 x 50mm) column equipped with an appropriate guard column. Column conditions and gradient were maintained from the previous experiment. A total of 35 readily available peak ions were compared between column chromatograms and assessed for their peak shape. Representative comparisons are provided in Figure 2.7. Fully annotated LC-MS chromatogram and mass spectra with the retention times and mass to charge ratios (m/z) of visible key ions is provided in Figure 2.8 and 2.9.

2.3.5.3 Validation of LC-MS analytical precision and stability

To assess the analytical precision and stability of the LC-MS approach throughout an extended analytical timeframe, 20 non-polar wasp extracts were prepared using the previous determined optimal pre-processing methodology (reconstituted in 50 μ L of isopropanol) and the highest performing column (Ace Excel 2 Super C18). A pooled quality control (QC) sample was also generated from 10 μ L of each sample. Technical replicability was established through triplicate injections of 10 μ L of each sample throughout the analytical timeframe. 10 μ L of the QC sample was injected five times in succession before every 20 technical replicates, for a total of 20 QC injections. An extra five injections of the QC sample were made prior to experimental analysis in order pre-equilibrate the column to the sample conditions.

The generated dataset was aligned and framed without discrimination using the propriety software Sieve 2.0 (Thermo Fisher Scientific, USA). A frame width of 0.5 min was employed during alignment to remove variation caused by minor chromatographic shifts. Samples were framed with an arbitrary intensity threshold of 50,000. The first 0.5 min of spectral acquisition was excluded from framing to remove variation from potential polar metabolite contamination. Two samples exhibited abnormal alignment and were treated as censors for further analysis. The framed data set was exported to Microsoft excel where individual frames were normalised to total peak area. Normalised frame tables were imported into Simca 13.0 (Umetrics, Sweden), where samples were visualised by principle component analysis (PCA). Individual variables were mean-centred and pareto-scaled prior to PCA visualisation. The generated PCA plot was scrutinised to assess the tightness

of clustering within each sample triplicate. QC replicates were expected to form a tight cluster with the centre of the PCA plot. PCA plots of technical and QC replicates are displayed in Figure 2.10. The relative standard deviations (RSD) (%) of the peak area and retention time were also assessed within each triplicate and across all 20 QC samples for each of the 35 key ions (Table 2.3 and 2.4). An acceptability threshold of 30% for a minimum of 70% of key ions was applied when assessing analytical stability (FDA USA; Dunn et al. 2011).

2.3.5.4 Optimisation of non-polar metabolite identification by LC-MS/MS

To aid in the validation of tentative ion identities, a tandem mass spectrometry (LC-MS/MS) approach was developed. Remaining extracts from the previous LC-MS optimisation were pooled to generate a larger sample volume size for LC-MS/MS optimisation. 10 μ L of this pooled sample was injected into the LC-MS/MS with a collision energy of either (a) 10 V (b) 25 V or (c) 40 V to assess fragmentation quality. The LC column was equilibrated prior to sample analysis through five injections of 10 μ L of the pooled sample. The LC column, mobile phases and gradient program were replicated from LC-MS optimisation. Analysis was performed utilising an Accela LC coupled with an LTQ Velos Pro Dual-Pressure linear ion trap mass spectrometer (Thermo Fisher Scientific, USA). Ions were monitored within the range of m/z 100 to 1500 (ESI voltage: 3000, capillary temperature: 275 °C, scan rate: 50 ms). MS² fragmentations were performed with a maximum ion isolation window of 5 Da. Key ion identifications were validated through direct comparisons of the comparisons of generated LC-MS/MS fragmentation spectra with the 'Lipidblast fragmentation database' (Lipidblast project, Kind et al. 2013). Further validation was provided by direct comparisons to external lipid standard databases (Lipidmaps, HMDB, database). Generated MS-MS spectra for each collision energy level were scrutinised and selected for optimal fragmentation. A number of representative spectra from each collision energy are displayed in Figure 2.11.

2.4 Results

2.4.1 Single insect extraction methodology NMR optimisation

Comparisons of NMR spectra generated from each extraction approach indicated that the methanol-chloroform-water (MCW) extraction resulted in the highest number of visible non-lipid peaks (Figure 2.1). Hexane-isopropanol-water (HIW) spectra displayed a low number of total peaks. MCW spectra also displayed a higher signal to noise ratio (S/N) than HIW spectra. However both approaches resulted in a peak yield significantly below that required for reliable spectral analysis, and spectra displayed an extremely low signal to noise ratio (MCW S/N = 7.04, HIW S/N = 1.64). As a result of this poor peak count and intensity the HIW extraction protocol was excluded from further consideration as a solvent extraction approach. Ethanol-heptane-water (EHW) spectra exhibited easily the highest peak intensity (S/N = 43316.33); however these peaks were identified as lipid contamination from the non-polar extract. EHW peaks separated poorly during sample extraction, this may be the cause of the observed lipid contamination. Due to these factors, the EHW extraction protocol was excluded from further consideration. The MCW extraction protocol was selected for further optimisation, with the aim of increasing sample peak yield and the signal to noise ratio.

2.4.2 Optimisation of NMR spectral resolution

Comparisons of generated 800 MHz NMR spectra with the previously generated MCW extract spectra indicated a vast improvement in signal to noise ratio for both the aromatic (800 MHz S/N = 885.48, 600 MHz S/N = 8.49) and the aliphatic spectral regions (800 MHz S/N = 6248, 600 MHz S/N = 7.04) (Figure 2.2). The internal DSS signal was clearly visible at 0.001ppm. Preliminary analysis of the generated 800 MHz spectra assigned a total of 30 putative identities to 66 spectral bins. A degree of overlap between spectral resonances in the region δ 4 - 3 prevented precise identification of individual peaks, as a result all spectral resonances in this region were considered approximate until further analysis. Due to this improvement in spectral resolution, along with the increased presence of identifiable spectral peaks, 800 MHz NMR spectroscopy was selected for all further wasp extract analysis.

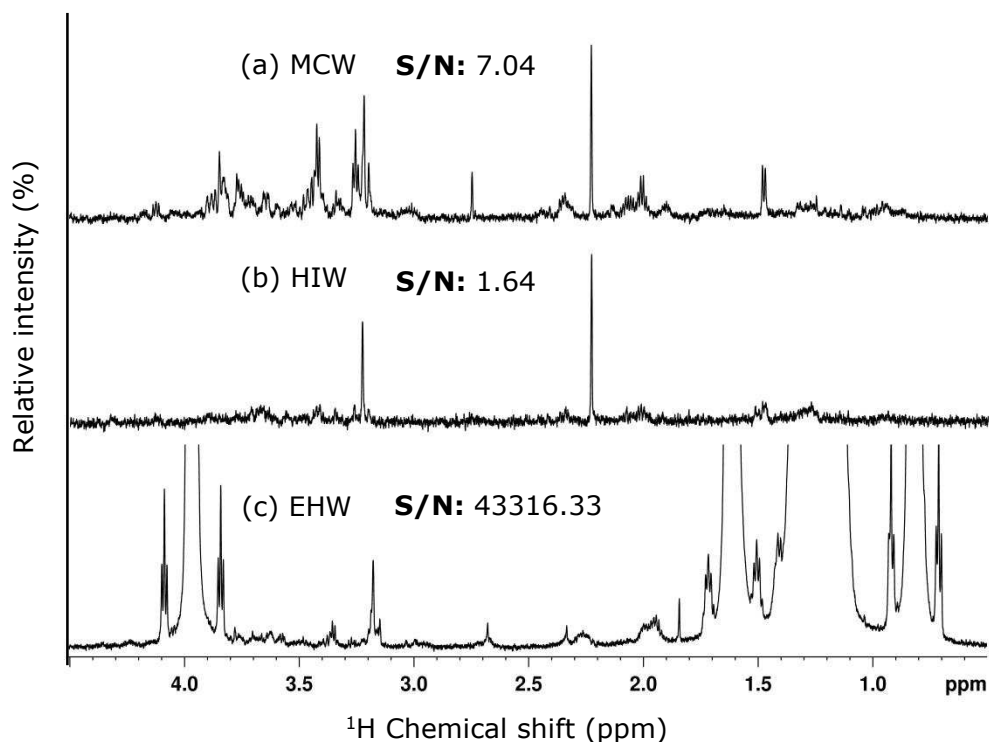


Figure 2.2. Comparisons of 600 MHz polar NMR spectra generated from (a) methanol-chloroform-water (b) hexane-isopropanol-water and (c) ethanol-heptane-water extracts. Spectral comparisons are limited to the aliphatic spectral region (4.5 - 0.5 ppm) as no clear peaks were detected in the aromatic spectral region (9.0 - 5.2 ppm).

2.4.3 Optimisation of NMR analytical stability

PCA comparisons of the generated dietary extracts displayed clear separation between honey fed wasps and starved/host fed wasps. Control spectra clustered separately from other treatment classes. Separation between experimental classes was a result of the elevated intensities of spectra peaks in the high density δ 4 - 3 region in honey fed wasp spectra. Further exploration of the NMR separation between wasp diets, and their effects on *G. legneri* physiology and behaviour, is detailed in Chapter 4. With separation between diets established, the clustering of stability replicates was assessed. Individual replicates clustered loosely together, remaining within experimental treatments. Whilst some variation in sample coordinates was visible, this variation did not trend with time, samples were positioned randomly within their specific clusters. With the exception

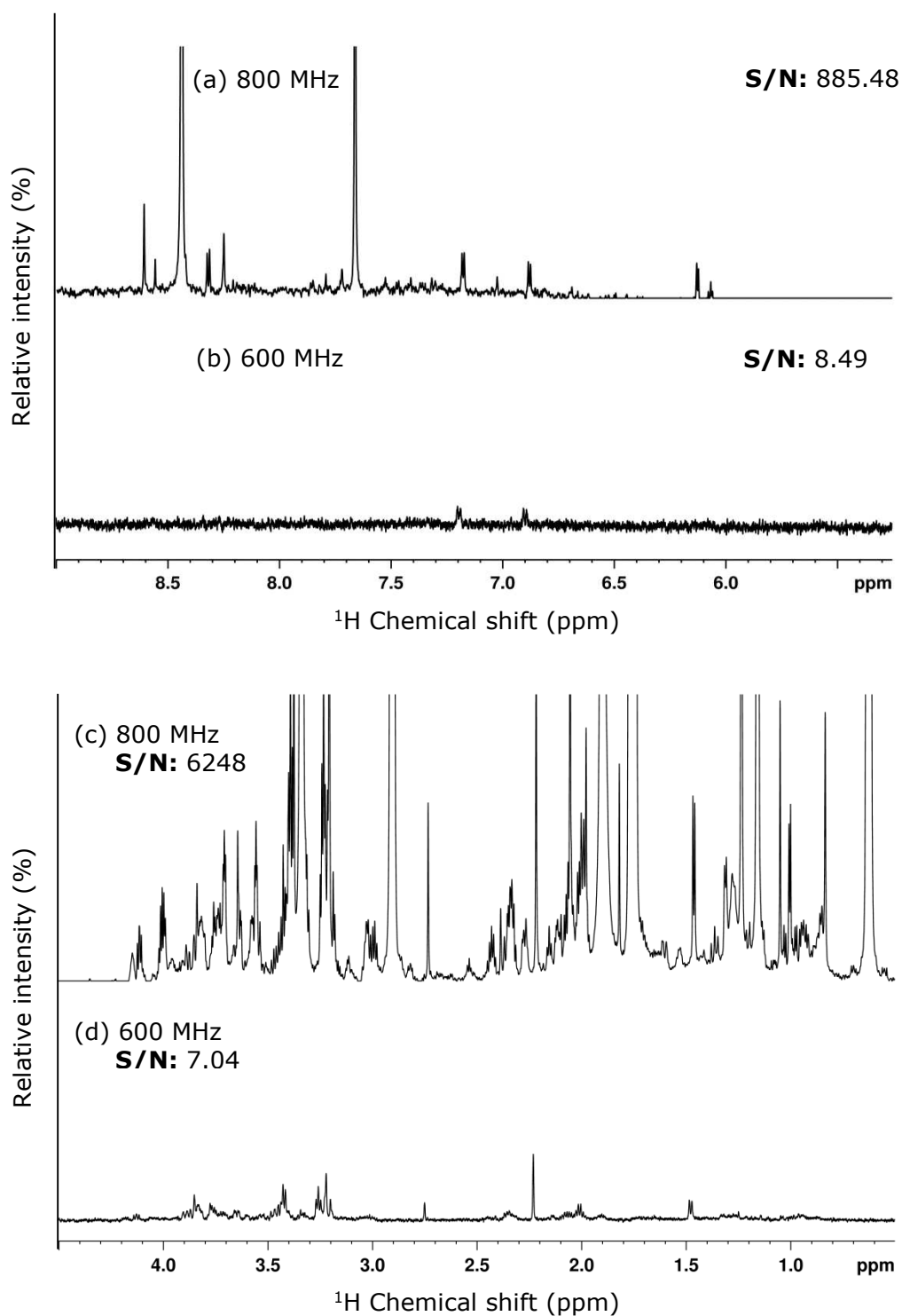


Figure 2.3. Comparisons of 800 MHz and 600 MHz NMR spectra generated from single wasp polar extracts (a) Wasp aromatic 800 MHz polar spectra (b) Wasp aromatic 600 MHz polar spectra (c) Wasp aliphatic 800 MHz polar spectra (d) Wasp aliphatic polar spectra generated by 600 MHz NMR spectroscopy. The scale of improvement of 800 MHz spectra over 600 MHz spectra is attributable to the use of a cryoprobe throughout analysis.

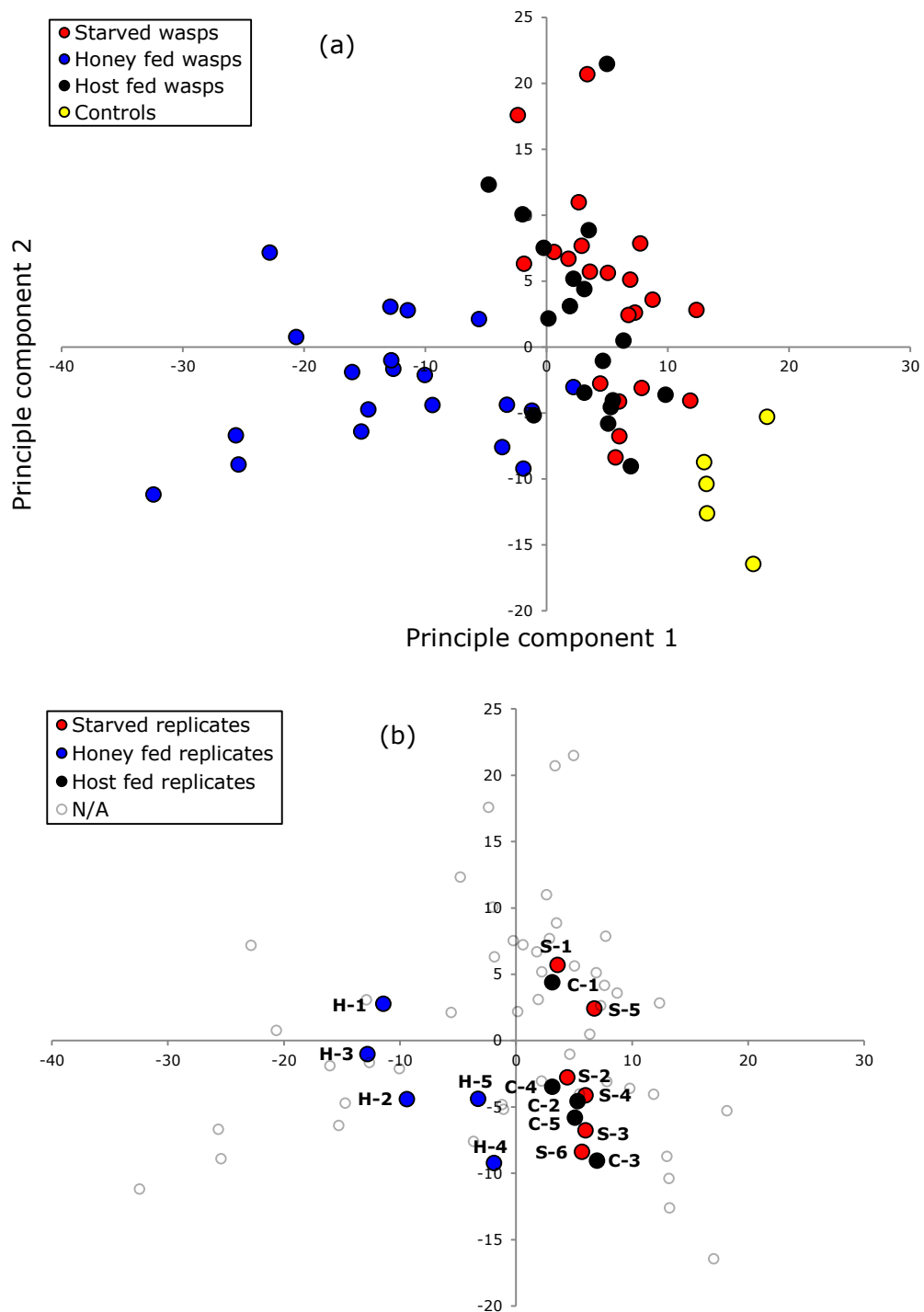


Figure 2.4. PCA validation of NMR spectral stability (a) PCA comparisons of dietary treatment polar NMR spectra of *G. legneri* (PC1 = 13.5%, PC2 = 7.12%, $R^2X = 0.513$) (b) PCA comparisons of NMR stability replicates within dietary treatment classes (H = honey-fed replicate, S = starved wasp replicate, C = host-fed replicate).

Table 2.1. Relative standard deviations of key spectral bins for NMR stability replicates (H = honey-fed replicate, S = starved wasp replicate, C = host-fed replicate).

Bin Identity (ppm)	Relative Standard Deviation (%)		
	H	S	C
3.70 (Glucose)	3.71	22.62	7.86
8.53 (ATP)	27.83	41.58	27.15
3.82 (Trehalose)	6.90	23.61	14.77
2.01 (Arginine)	11.67	13.76	9.17
3.55 (Glycerol)	10.54	5.74	2.66
2.34 (Glutamate)	16.62	7.30	19.79
0.99 (Valine)	15.85	14.82	22.89
2.01 (Proline)	11.67	13.76	9.17
3.59 (Threonine)	11.78	5.01	4.85
0.95 (Leucine)	12.44	8.05	10.48

of bin 8.53 (ATP) for starved wasps, the peak area RSDs of all key bins were within the acceptable threshold limits (Table 2.1).

2.4.4 2D NMR spectral identification

The generated HSQC spectra displayed a large number of both major and minor spectral resonances. Comparisons between the generated spectra and NMR standard spectra verified a total of 19 unique compounds; these included organic acids, free amino acids and assorted sugars (Figure 2.4a). As with most biological samples characterisation of some spectral resonances was not possible. A degree of overlap from major resonances in 1D spectra had presented difficulties for initial assignment; this was a particular issue with the highly overlapping sugar signals in the region δ 4-3 (Figure 2.4a). These overlapping spectra were clearly assignable through analysis of 2D HSQC spectra (Figure 2.4b). 2D NMR peak assignments are further outlined in Table 2.2.

2.4.5 Optimisation of non-polar extract pre-processing

Comparisons of concentrated and non-concentrated indicated significantly higher chromatographic yield in samples concentrated in isopropanol

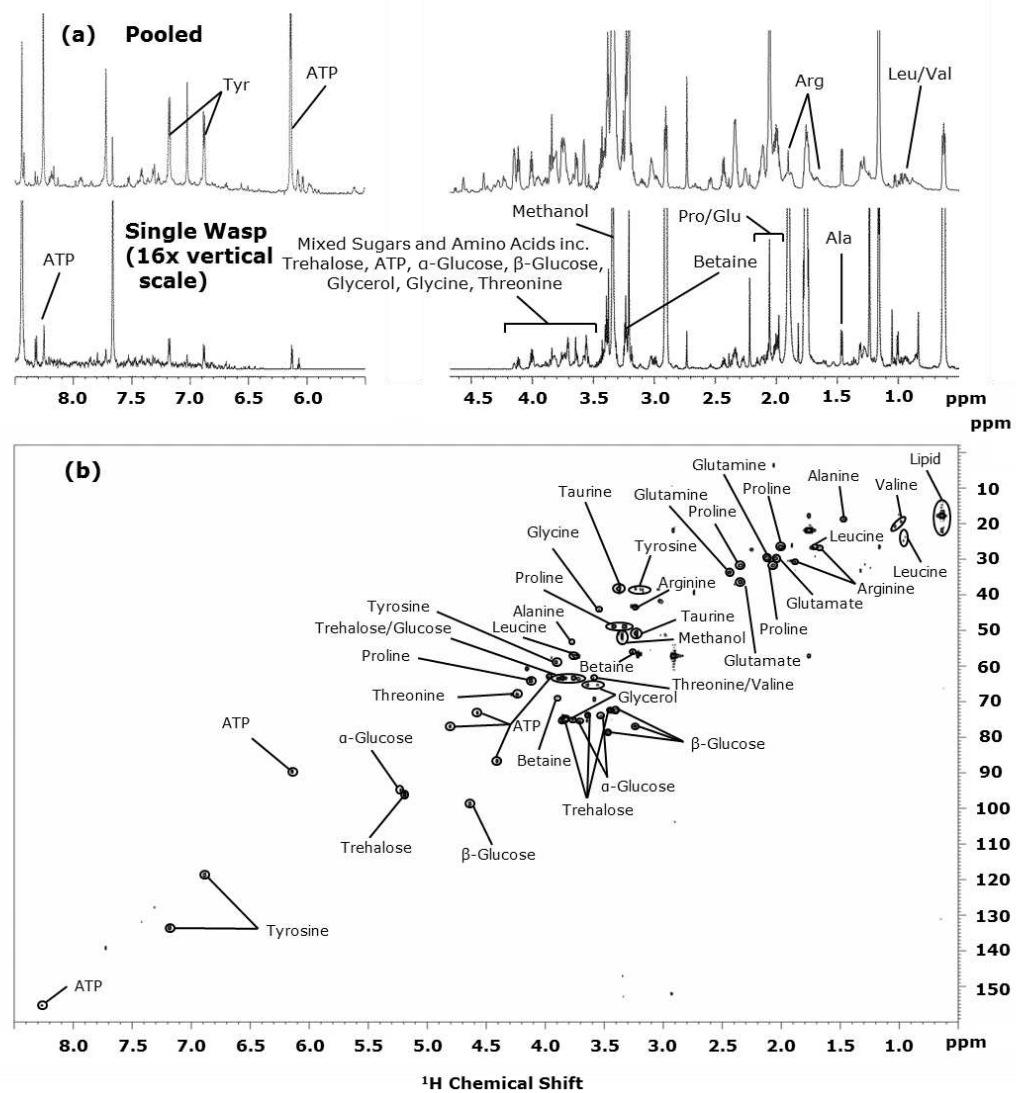


Figure 2.5. Representative ^1H NMR and 2D spectrum of *Goniozus legneri* methanol extracts. (a) A spectral comparison between ^1H NMR spectra of 20 wasps (pooled) and a single wasp amplified vertically x16 with 1D spectral resonance assignments (b) 2D HSQC spectra of pooled wasp sample including NMR resonance assignments. Complete ^1H and ^{13}C chemical shift assignments are listed in Table 2.1.

Table 2.2. ¹H and ¹³C chemical shift assignments of *G. legneri* NMR spectra.

Metabolite	¹H Chemical Shift (Multiplicity*)	¹³C Chemical Shift
ATP	4.24 (m), 4.30 (m), 4.41 (m), 4.58 (m), 4.77 (t), 6.15 (d), 8.26 (s), 8.53 (s)	67.78, 67.78, 86.68, 72.96, 72.96, 89.57, 155.42, 142.76
Formate	8.45 (s)	-
Tyrosine	3.04 (dd), 3.18 (dd), 3.92 (dd), 6.89 (d), 7.19 (d)	38.58, 38.51, 58.94, 118.68, 133.52
α-Glucose	3.45 (t), 3.52 (dd), 3.70 (t), 3.76 (m), 3.82 (ddd), 3.83 (m), 5.22 (d)	72.42, 74.2, 75.53, 63.34, 74.15, 63.34, 94.86
Trehalose	3.44 (t), 3.64 (dd), 3.75 (m), 3.82 (m), 3.84 (m), 3.86 (m), 5.19 (d)	72.47, 73.82, 63.32, 74.93, 75.30, 63.33, 96.02
β-Glucose	3.24 (dd), 3.4 (m), 3.47 (m), 3.74 (m), 3.88 (dd), 4.64 (d)	76.8, 72.4, 78.65, 63.28, 63.28, 98.67
Threonine	1.32 (d), 3.59 (d), 4.24 (m)	63.21, 67.98
Proline	2.01 (m), 2.08 (m), 2.35 (m), 3.33 (dt), 3.42 (dt), 4.12 (dd)	26.5, 31.66, 31.73, 48.84, 48.84, 64.06
Glycine- Betaine	3.26 (s), 3.90 (s)	56.16, 68.93
Arginine	1.67 (m), 1.91 (m), 3.23 (t), 3.76 (t)	26.72, 30.42, 43.33, 57.05
Alanine	1.47 (d), 3.77 (q)	18.99
Glycerol	3.55 (m), 3.64 (m), 3.77 (tt)	65.24, 65.24, 74.94
Glutamine	2.12 (m), 2.44 (m), 3.76 (t)	29.57, 33.61, 56.96
Glutamate	2.05 (m) 2.12 (m), 2.34 (m), 3.74 (d)	29.72, 29.72, 36.35, 57.37
Valine	0.99 (d), 1.04 (d), 2.34 (m), 3.6 (d)	19.41, 20.73, 31.66, 63.04
Glycine	3.54 (s)	44.23
Taurine	3.23 (t), 3.39 (t)	50.78, 38.24
Methanol	3.35 (s)	51.66
Leucine	0.95 (t), 1.75 (m), 3.74 (m)	23.6, 26.62, 56.86

*Letters m, t, d, s & q denote peak multiplicity, where m = multiplet, t = triplet, d = doublet, s = singlet & q = quartet

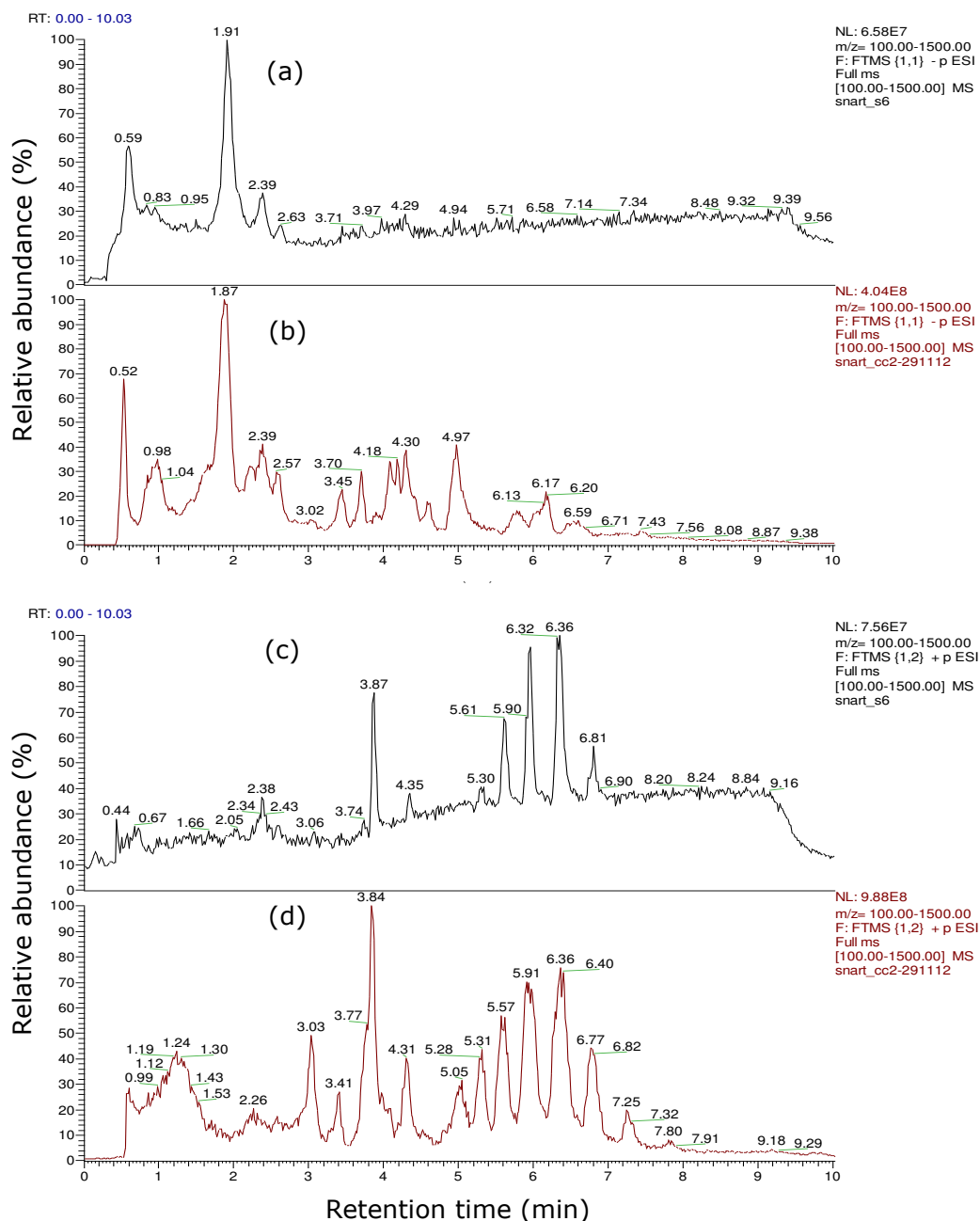


Figure 2.6. Solvent extract pre-processing comparison (a) Non-concentrated wasp extract negative ESI chromatogram (b) Concentrated wasp extract negative ESI chromatogram (c) Non-concentrated wasp extract positive ESI chromatogram (d) Concentrated wasp extract positive ESI chromatogram.

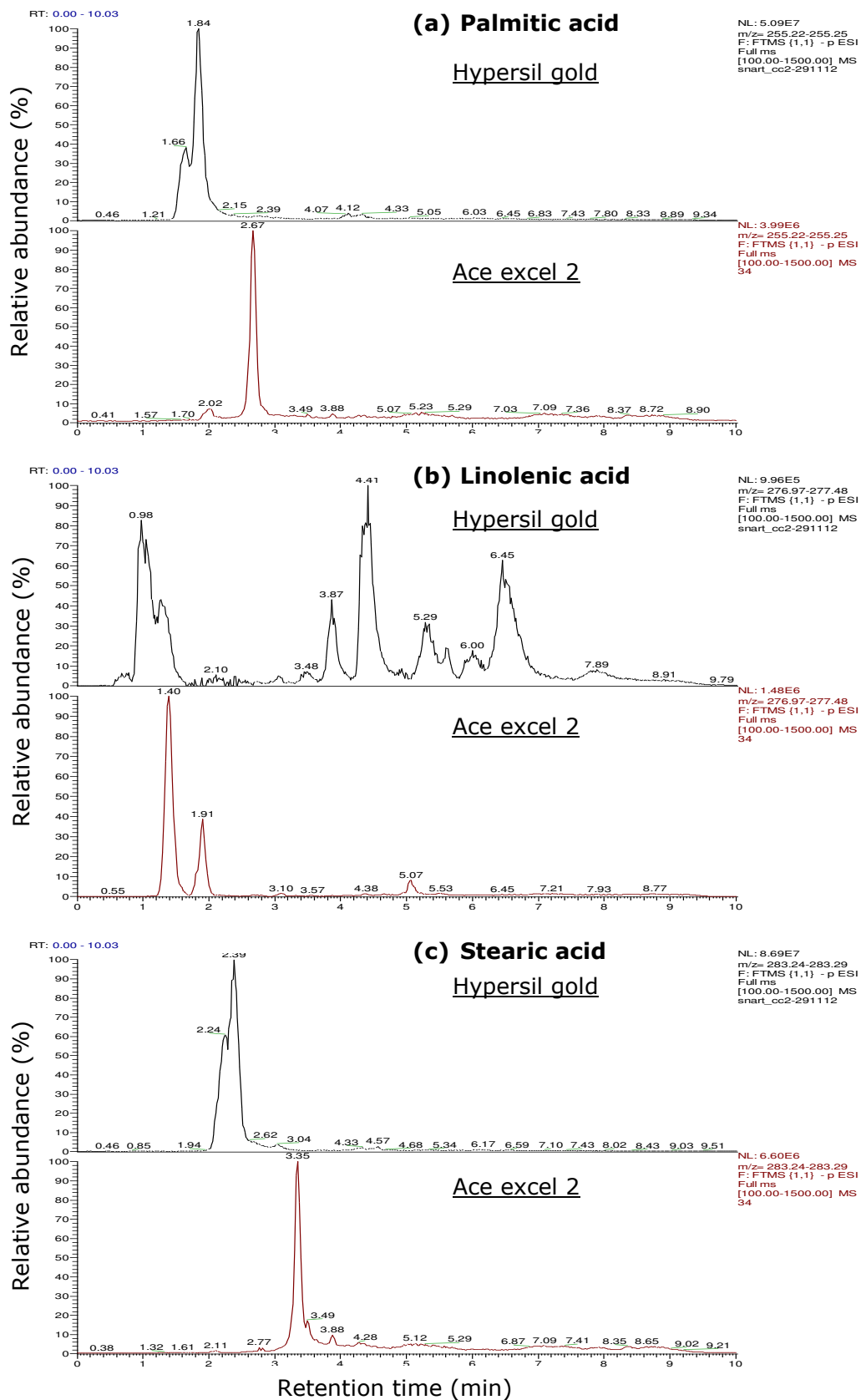


Figure 2.7. Representative key extracted LC-MS ion chromatograms of (a) Palmitic acid (b) Linolenic acid (c) Stearic acid.

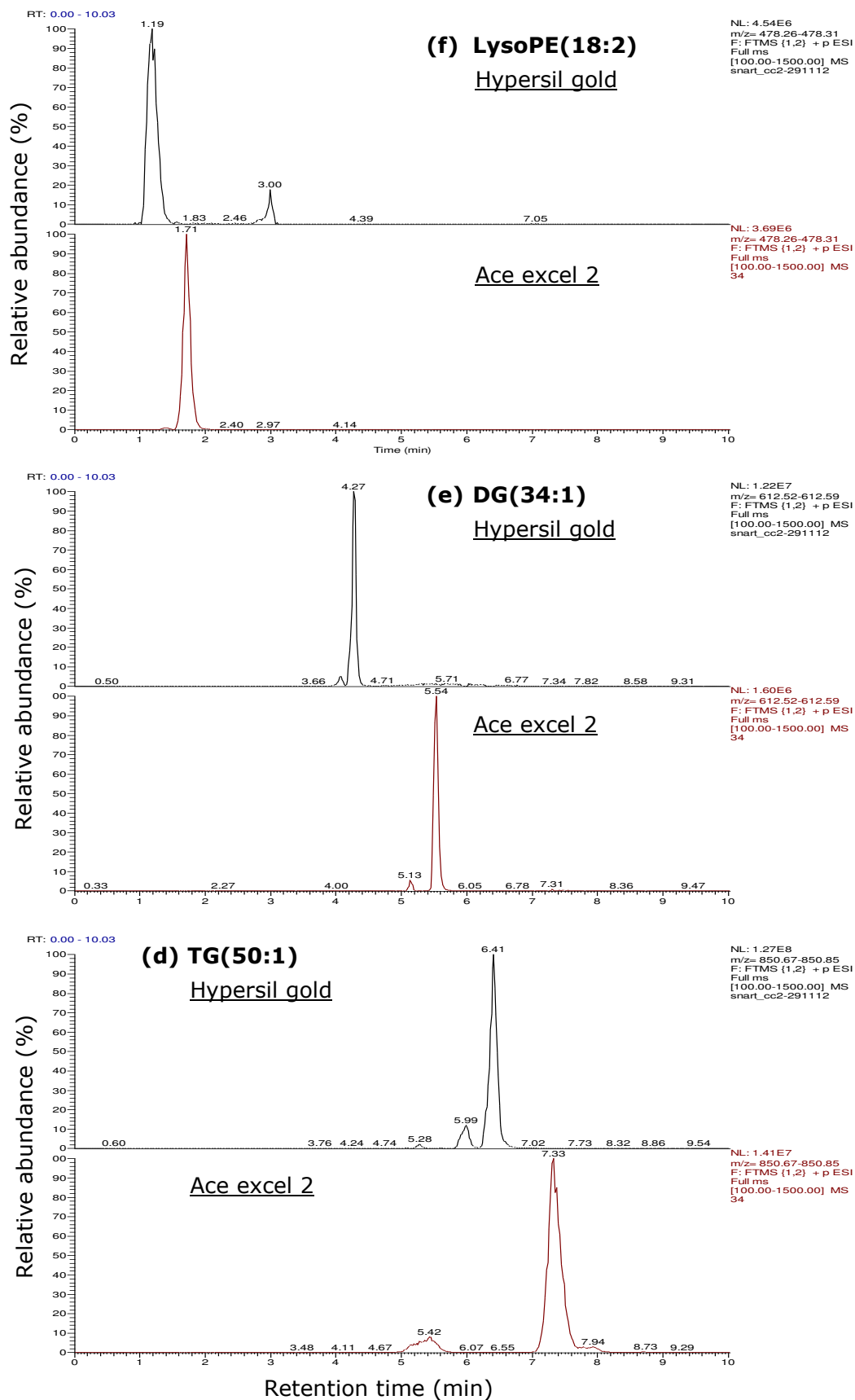


Figure 2.7. cont. Representative key extracted LC-MS ion chromatograms of (a) Palmitic acid (b) Linolenic acid (c) Stearic acid.

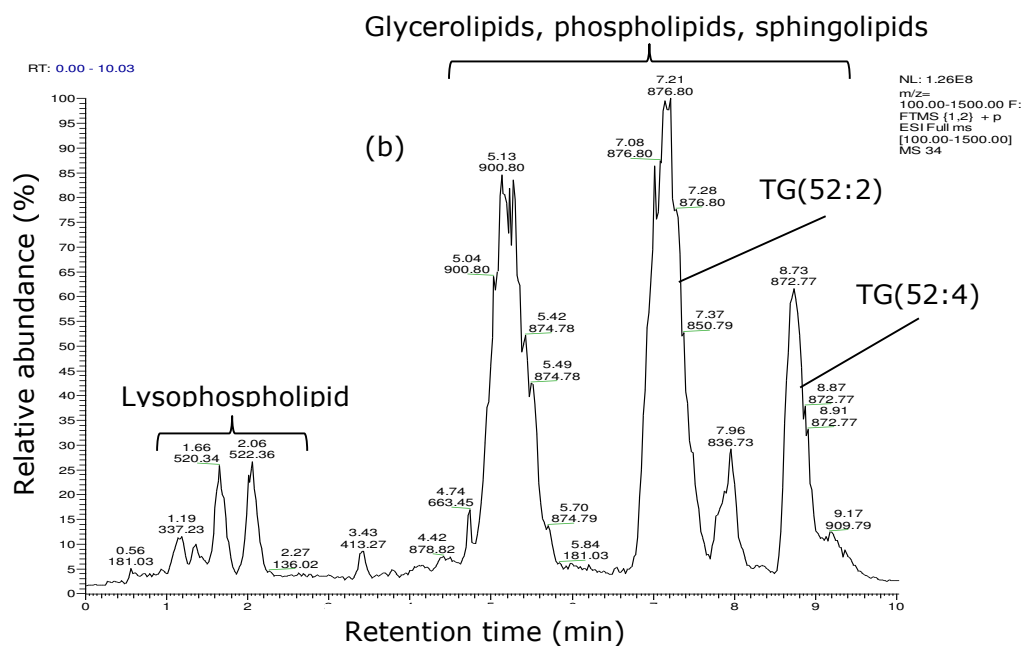
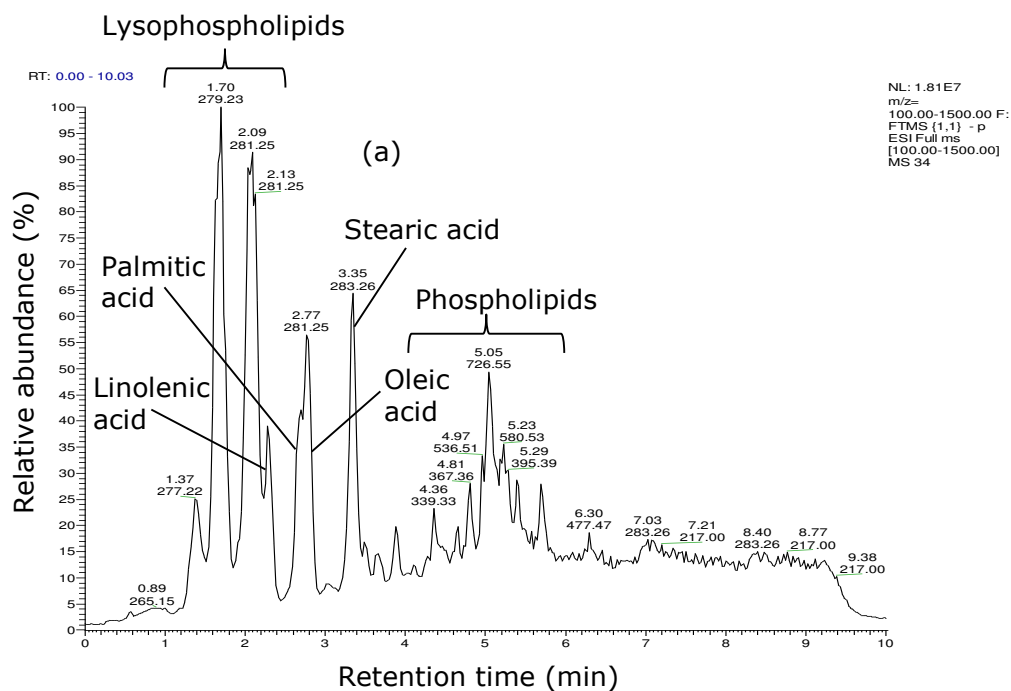


Figure 2.8. Representative total ion chromatograph of whole wasp extract in (a) ESI- and (b) ESI+ mode. Labels represent visible key ions and major lipid categories. TG = triacylglyceride. The unusual appearance of lysophospholipids alongside fatty acyls in ESI- mode was found to be due to in source fragmentation of ESI+ lysophospholipids. These peaks matched the retention time and corresponded to major LC-MS/MS fragments of the corresponding lysophospholipid seen in ESI+. These ions were absent from chromatograms acquired during Velos LC-MS/MS analysis.

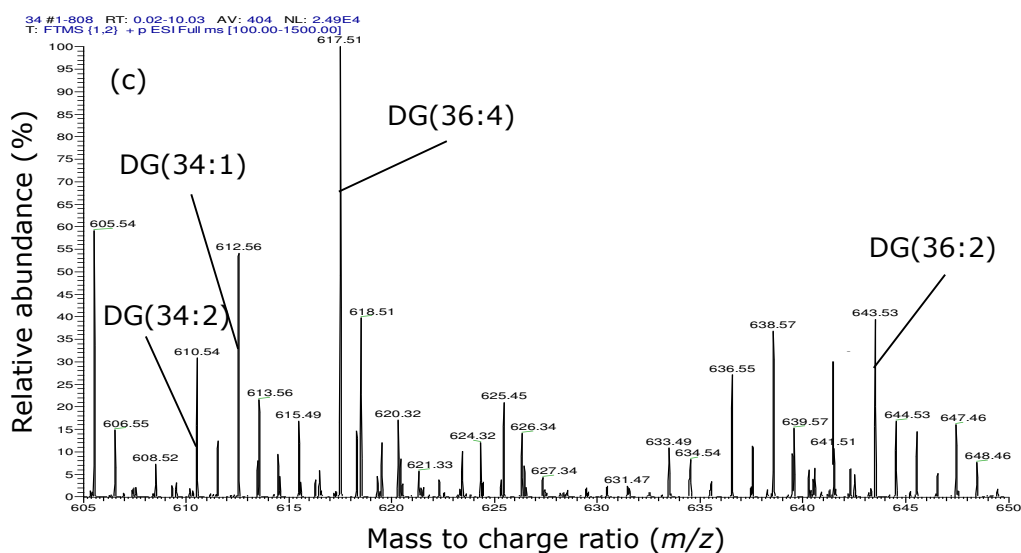
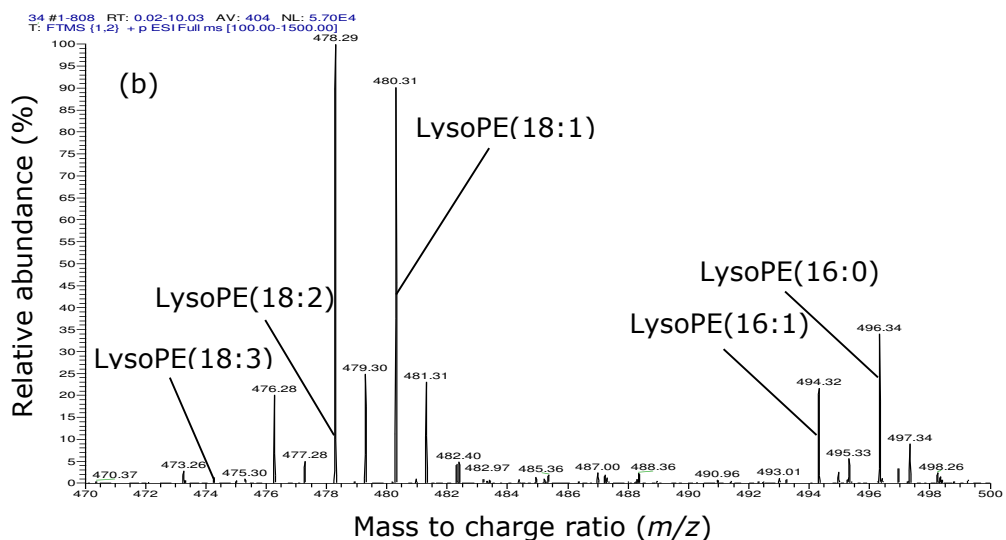
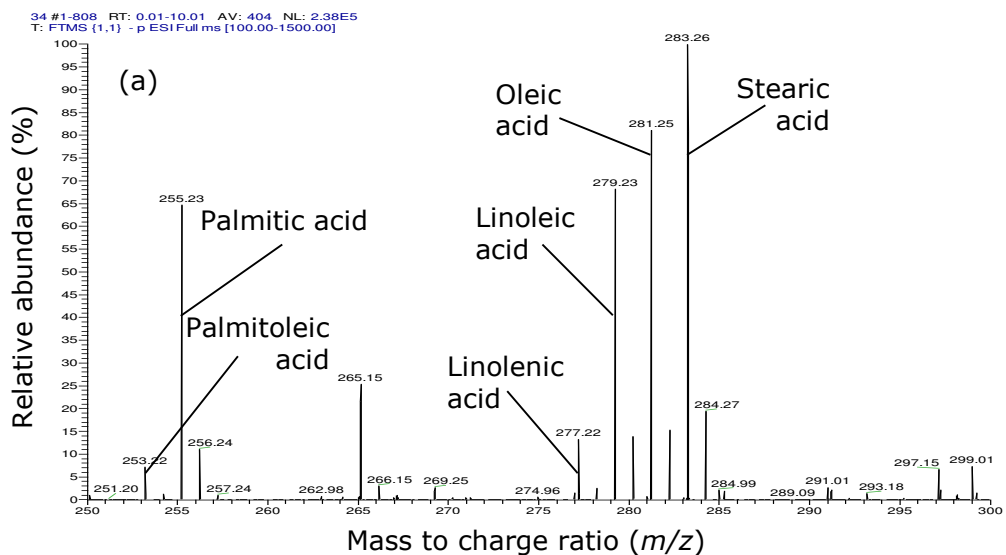


Figure 2.9. Representative mass spectra labelled for (a) fatty acyl (b) lysophospholipid and (c) diacylglyceride key ions. PE = phosphoethanolamine, DG = diacylglyceride.

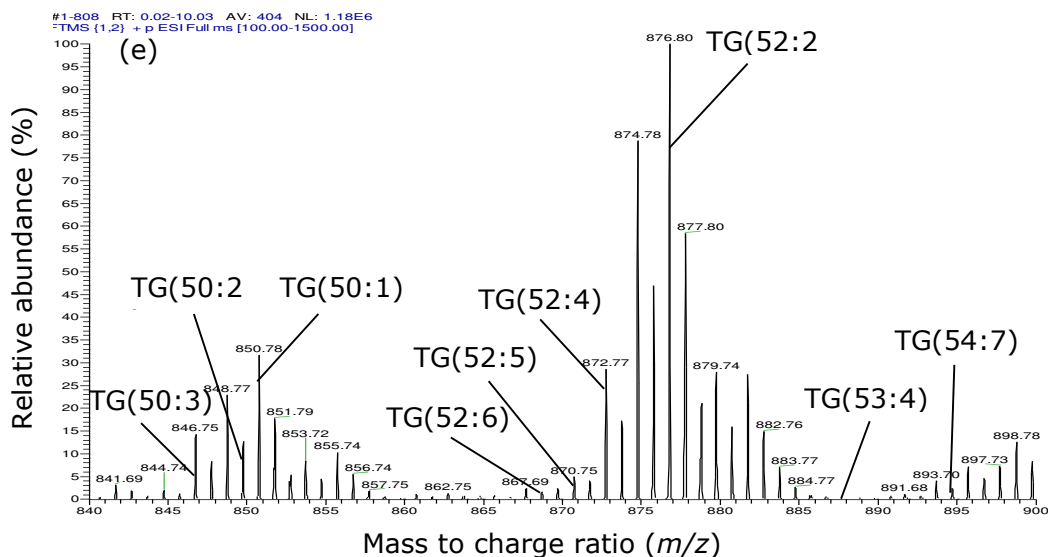
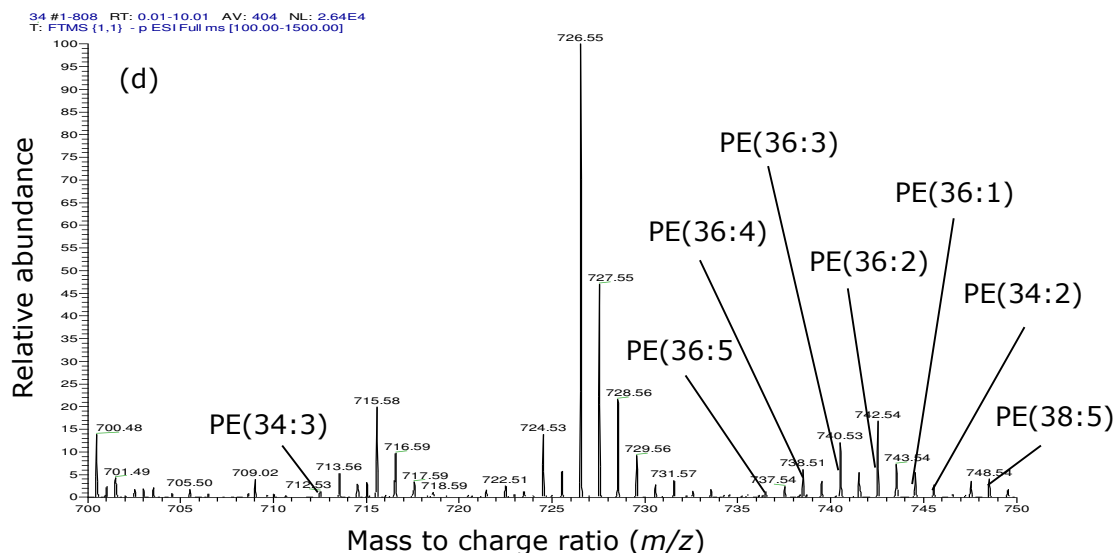


Figure 2.9. *cont.* Representative mass spectra labelled for (d) fatty acyl (b) phospholipid and (c) triacylglyceride key ions. PE = phosphoethanolamine, TG = triacylglyceride.

(Figure 2.5). Analysis of concentrated extracts found a total of 1109 unique ions compared to 176 for non-concentrated extract. Inspections of sample extracts after experimental analysis furthermore displayed a lower level of evaporation in concentrated extracts, likely due to the lower volatility of the solvent. Due to these factors, all further generated sample extracts underwent sample concentration prior to LC-MS and LC-MS/MS analysis.

2.4.6 Optimisation of LC-MS column choice

Initial scrutiny of the LC-MS chromatograms and mass spectra generated using either a Hypersil Gold C18 or Ace Excel 2 Super C18 column concluded that greater separation was apparent between individual lipid categories for the Ace Excel 2 column (Figure 2.7 and 2.8). Examination of the extracted chromatograms for individual key ions indicated an improvement in peak sharpness and a lower incidence of peak tailing for those generated using the Ace Excel 2 column (Figure 2.7). As a result of these factors, the Ace Excel 2 column was selected for all future LC-MS and LC-MS/MS analysis of wasp non-polar extracts. Representative key ion extracted chromatograms are displayed in Figure 2.7, chromatogram and mass spectral annotations displaying key ions are presented in Figure 2.8 and 2.9 respectively.

2.4.7 Validation of LC-MS analytical precision and stability

Preliminary assessment of the generated PCA scores plot for wasp non-polar extract triplicate injections (Figure 2.10a) indicated tight clustering within each technical replicate. Validation of analytical precision within triplicates indicated that 100 % of key ions displayed average peak area relative standard deviations (RSD) under the acceptability threshold of 30 %. A total of 94 % of key ions had RSD ranges under the acceptability threshold. Assessment of the retention times of key ions within triplicates indicated that 100 % of retention time RSDs were under the acceptability threshold. The average peak area RSD across all key ions was 5.54%, with a total range of 0.02 - 42.60 %. The average retention time RSD across all key ions was 0.88 %, with a total range of 0.03 - 6.43 %. Individual key ion RSD averages and ranges for peak area and retention are outlined in Table 2.3 and Table 2.4.

Assessment of QC sample clustering within the generated PCA scores plot for wasp non-polar extract triplicate injections (Figure 2.10b) indicated tight central QC clustering. With the exception of initial equilibration samples (Eq), no time related shifts in QC position were noted between QC category. Validation of analytical stability within QC samples indicated that the peak area RSDs of 100 % of key ions were under the acceptability threshold. Assessment of key ion retention times within QC samples also indicated that 100 % of retention time RSDs were under the acceptability threshold. The average peak area RSD across key ions within QC samples

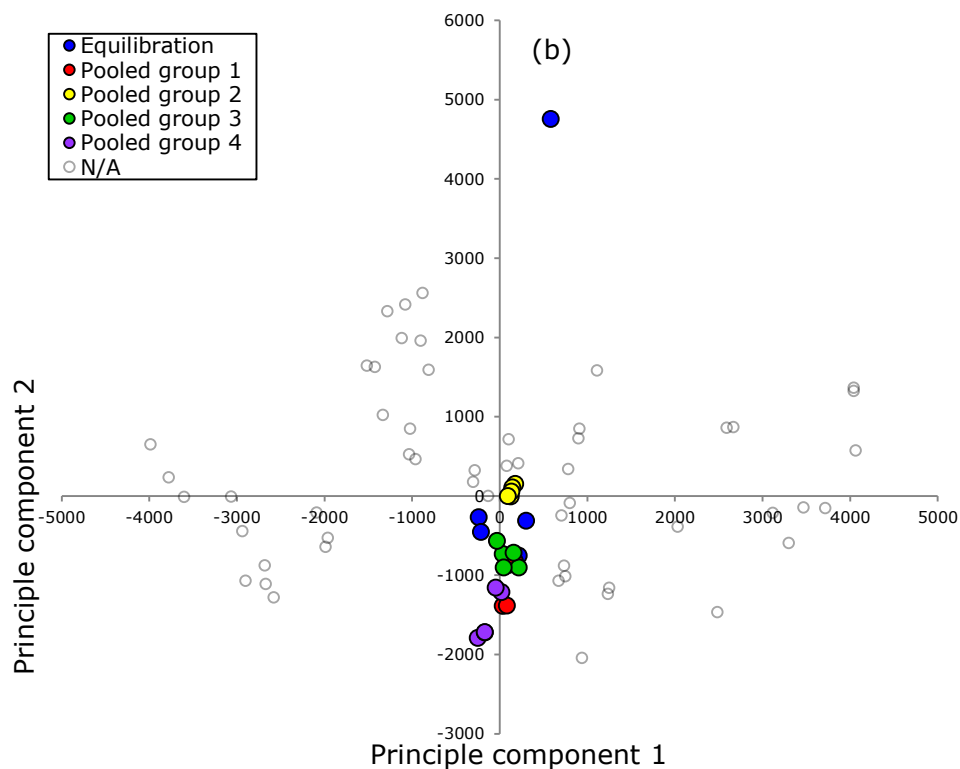
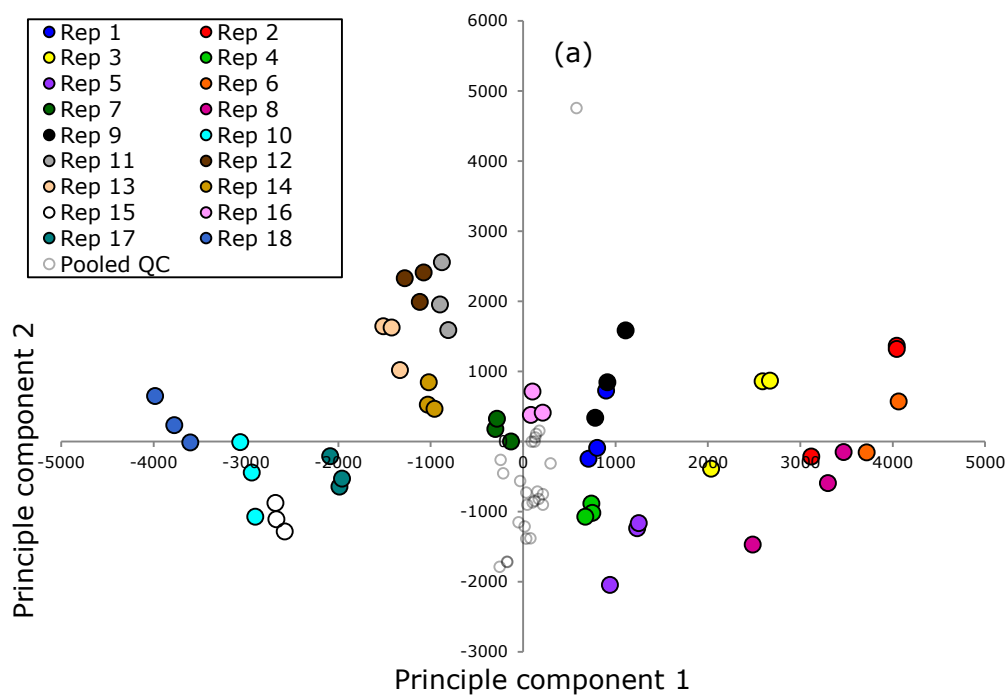


Figure 2.10. Principle components analysis of wasp non-polar extract analytical precision and stability replicates (a) PCA of wasp non-polar technical replicates and (b) PCA of wasp non-polar QC samples.

Table 2.3 Key ion peak area RSDs (%) for technical replicates and quality control (QC) samples. RT = retention time, RSD = relative standard deviation. PE = phosphoethanolamine, PC = phosphocholine, PG = phosphoglycerol, DG = diacylglyceride, TG = triacylglyceride.

M/Z	Average RT (min)	Tentative Identification	Phase	Adduct	Formula	Mass error (ppm)	Peak area RSD (%)	Peak area RSD range (%)	QC peak area RSD (%)
253.22	2.13	Palmitoleic acid	Negative	M-H	C ₁₆ H ₃₀ O ₂	0.073	5.32	0.58 - 13.73	11.02
255.23	2.67	Palmitic acid	Negative	M-H	C ₁₆ H ₃₂ O ₂	0.03	3.88	1.34 - 12.12	6.99
277.22	1.91	Linolenic Acid	Negative	M-H	C ₁₈ H ₃₀ O ₂	0.120	4.11	0.38 - 16.31	9.67
281.25	2.79	Oleic Acid	Negative	M-H	C ₁₈ H ₃₄ O ₂	0.180	4.11	0.21 - 25.33	9.37
283.26	3.35	Stearic Acid	Negative	M-H	C ₁₈ H ₃₆ O ₂	0.030	9.05	0.75 - 8.87	8.76
452.28	1.57	LysoPE(16:0)	Positive	M+H	C ₂₁ H ₄₄ NO ₇ P	0.189	5.16	0.04 - 9.76	8.74
474.27	0.95	LysoPE(18:3)	Positive	M+H	C ₂₃ H ₄₂ NO ₇ P	5.8	4.13	0.50 - 13.58	6.86
478.29	1.72	LysoPE(18:2)	Positive	M+H	C ₂₃ H ₄₄ NO ₇ P	0.2	3.33	0.17 - 9.51	8.61
480.31	2.13	LysoPE(18:1)	Positive	M+H	C ₂₃ H ₄₆ NO ₇ P	0.5	3.25	0.35 - 8.31	8.31
494.32	1.49	LysoPC(16.1)	Positive	M+H	C ₂₄ H ₄₈ NO ₇ P	0.139	9.88	0.23 - 8.24	8.24
496.34	1.95	LysoPC(16.0)	Positive	M+H	C ₂₄ H ₅₀ NO ₇ P	0.1	2.87	1.57 - 19.08	17.19
610.54	5.15	DG(34:2)	Positive	M+NH ₄	C ₃₇ H ₆₈ O ₅	0.298	4.53	0.56 - 11.84	9.32
612.56	5.52	DG(34:1)	Positive	M+NH ₄	C ₃₇ H ₇₀ O ₅	0.448	4.41	0.96 - 10.12	8.63
617.51	5.51	DG(36:4)	Positive	M+H	C ₃₉ H ₆₈ O ₅	2.975	4.21	1.50 - 8.20	8.14
641.51	5.13	DG(36:3)	Positive	M+Na	C ₃₉ H ₇₀ O ₅	0.743	4.35	0.55 - 9.17	9.17
643.53	5.52	DG(36:2)	Positive	M+Na	C ₃₉ H ₇₂ O ₅	0.993	4.92	1.32 - 8.79	8.10
712.49	4.41	PE(34:3)	Negative	M-H	C ₃₉ H ₇₂ NO ₈ P	0.645	6.36	1.30 - 22.37	16.40
736.49	4.13	PE(36:5)	Negative	M-H	C ₄₁ H ₇₂ NO ₇ P	0.845	6.47	2.07 - 12.78	9.69
738.51	4.39	PE(36:4)	Negative	M-H	C ₄₁ H ₇₄ NO ₈ P	0.695	5.14	1.19 - 12.74	11.05
740.52	4.65	PE(36:3)	Negative	M-H	C ₄₁ H ₇₆ NO ₈ P	0.245	7.36	0.02 - 14.53	14.53
742.53	5.02	PE(36:2)	Negative	M-H	C ₄₁ H ₇₈ NO ₇ P	0.980	14.41	0.32 - 18.55	16.05
744.56	5.45	PE(36:1)	Negative	M-H	C ₄₁ H ₈₀ NO ₇ P	0.345	5.01	3.51 - 25.37	23.77
745.50	5.45	PG(34:2)	Negative	M-H	C ₄₀ H ₇₅ O ₁₀ P	0.715	8.45	0.33 - 11.36	10.87
748.53	4.39	PE(38:5)	Negative	M-H	C ₄₃ H ₇₆ NO ₇ P	0.260	17.73	1.15 - 20.09	12.51
794.50	5.08	PE(36:6)	Negative	M+Hac-H	C ₄₁ H ₇₀ NO ₈ P	1.756	5.07	2.12 - 34.44	23.61
846.75	8.71	TG(50:3)	Positive	M+NH ₄	C ₅₄ H ₉₈ O ₆	1.214	2.04	0.21 - 6.18	6.18
849.77	5.29	TG(50:2)	Positive	M+NH ₄	C ₅₃ H ₉₈ O ₆	2.236	3.49	0.81 - 4.62	4.00
850.78	7.29	TG(50:1)	Positive	M+NH ₄	C ₅₃ H ₁₀₂ O ₅	1.614	4.35	1.12 - 7.78	6.41
868.74	8.71	TG(52:6)	Positive	M+NH ₄	C ₅₅ H ₉₄ O ₆	1.364	3.78	0.32 - 12.09	11.86
870.75	7.95	TG(52:5)	Positive	M+NH ₄	C ₅₅ H ₉₈ O ₅	1.814	3.21	0.38 - 8.47	7.55
872.77	8.73	TG(52:4)	Positive	M+NH ₄	C ₅₅ H ₉₈ O ₆	1.464	3.89	0.23 - 7.20	6.41
876.78	7.09	TG(52:2)	Positive	M+NH ₄	C ₅₅ H ₁₀₂ O ₆	0.564	4.53	0.07 - 42.60	14.53
887.78	7.48	TG(53:4)	Positive	M+NH ₄	C ₅₆ H ₁₀₀ O ₆	7.735	4.85	0.76 - 7.76	5.94
894.75	8.73	TG(54:7)	Positive	M+NH ₄	C ₅₇ H ₉₈ O ₅	0.114	4.61	0.77 - 12.20	12.20

Table 2.4 Key ion retention time RSDs (%) for technical replicates and quality control (QC) samples. RT = retention time, RSD = relative standard deviation. PE = phosphoethanolamine, PC = phosphocholine, PG = phosphoglycerol, DG = diacylglyceride, TG = triacylglyceride.

M/Z	Average RT (min)	Tentative identification	Phase	Adduct	Formula	Mass error (ppm)	RT RSD (%)	RT RSD range (%)	QC peak area RSD (%)
253.22	2.13	Palmitoleic acid	Negative	M-H	C ₁₆ H ₃₀ O ₂	0.073	2.43	0.44 - 6.43	1.89
255.23	2.67	Palmitic acid	Negative	M-H	C ₁₆ H ₃₂ O ₂	0.03	0.75	0.26 - 1.18	0.87
277.22	1.91	Linolenic Acid	Negative	M-H	C ₁₈ H ₃₀ O ₂	0.120	1.03	0.15 - 2.26	1.66
281.25	2.79	Oleic Acid	Negative	M-H	C ₁₈ H ₃₄ O ₂	0.180	1.26	0.04 - 6.04	1.09
283.26	3.35	Stearic Acid	Negative	M-H	C ₁₈ H ₃₆ O ₂	0.030	0.60	0.17 - 1.08	0.70
452.28	1.57	LysoPE(16:0)	Positive	M+H	C ₂₁ H ₄₄ NO ₇ P	0.189	1.17	0.09 - 1.81	1.64
474.27	0.95	LysoPE(18:3)	Positive	M+H	C ₂₃ H ₄₂ NO ₇ P	5.8	2.01	0.14 - 3.75	2.96
478.29	1.72	LysoPE(18:2)	Positive	M+H	C ₂₃ H ₄₄ NO ₇ P	0.2	1.02	0.10 - 1.74	1.38
480.31	2.13	LysoPE(18:1)	Positive	M+H	C ₂₃ H ₄₆ NO ₇ P	0.5	1.04	0.39 - 1.64	1.24
494.32	1.49	LysoPC(16.1)	Positive	M+H	C ₂₄ H ₄₈ NO ₇ P	0.139	1.24	0.42 - 1.81	1.67
496.34	1.95	LysoPC(16.0)	Positive	M+H	C ₂₄ H ₅₀ NO ₇ P	0.1	1.98	0.60 - 3.53	2.71
610.54	5.15	DG(34:2)	Positive	M+NH ₄	C ₃₇ H ₆₈ O ₅	0.298	0.43	0.04 - 0.72	0.56
612.56	5.52	DG(34:1)	Positive	M+NH ₄	C ₃₇ H ₇₀ O ₅	0.448	0.44	0.06 - 0.76	0.50
617.51	5.51	DG(36:4)	Positive	M+H	C ₃₉ H ₆₈ O ₅	2.975	0.43	0.06 - 0.74	0.46
641.51	5.13	DG(36:3)	Positive	M+Na	C ₃₉ H ₇₀ O ₅	0.743	0.40	0.04 - 0.78	0.56
643.53	5.52	DG(36:2)	Positive	M+Na	C ₃₉ H ₇₂ O ₅	0.993	0.47	0.06 - 0.95	0.50
712.49	4.41	PE(34:3)	Negative	M-H	C ₃₉ H ₇₂ NO ₈ P	0.645	1.46	0.02 - 2.35	2.76
736.49	4.13	PE(36:5)	Negative	M-H	C ₄₁ H ₇₂ NO ₇ P	0.845	0.58	0.18 - 0.99	0.96
738.51	4.39	PE(36:4)	Negative	M-H	C ₄₁ H ₇₄ NO ₈ P	0.695	0.95	0.26 - 1.64	1.64
740.52	4.65	PE(36:3)	Negative	M-H	C ₄₁ H ₇₆ NO ₈ P	0.245	0.65	0.03 - 2.37	2.41
742.53	5.02	PE(36:2)	Negative	M-H	C ₄₁ H ₇₈ NO ₇ P	0.980	1.15	0.13 - 3.98	4.52
744.56	5.45	PE(36:1)	Negative	M-H	C ₄₁ H ₈₀ NO ₇ P	0.345	1.14	0.20 - 2.05	2.04
745.50	5.45	PG(34:2)	Negative	M-H	C ₄₀ H ₇₅ O ₁₀ P	0.715	0.40	0.18 - 0.85	0.39
748.53	4.39	PE(38:5)	Negative	M-H	C ₄₃ H ₇₆ NO ₇ P	0.260	1.38	0.12 - 2.09	1.65
794.50	5.08	PE(36:6)	Negative	M+Hac-H	C ₄₁ H ₇₀ NO ₈ P	1.756	1.04	0.10 - 2.17	1.87
846.75	8.71	TG(50:3)	Positive	M+NH ₄	C ₅₄ H ₉₈ O ₆	1.214	0.49	0.18 - 0.97	1.00
848.77	5.29	TG(50:2)	Positive	M+NH ₄	C ₅₃ H ₉₈ O ₆	2.236	0.61	0.10 - 1.12	0.93
850.78	7.29	TG(50:1)	Positive	M+NH ₄	C ₅₃ H ₁₀₂ O ₅	1.614	0.49	0.06 - 0.72	1.08
868.74	8.71	TG(52:6)	Positive	M+NH ₄	C ₅₅ H ₉₄ O ₆	1.364	0.52	0.10 - 0.83	0.76
870.75	7.95	TG(52:5)	Positive	M+NH ₄	C ₅₅ H ₉₈ O ₅	1.814	0.57	0.14 - 0.99	0.87
872.77	8.73	TG(52:4)	Positive	M+NH ₄	C ₅₅ H ₉₈ O ₆	1.464	0.43	0.18 - 0.85	0.98
876.80	7.09	TG(52:2)	Positive	M+NH ₄	C ₅₅ H ₁₀₂ O ₆	0.564	0.55	0.13 - 1.08	0.96
887.78	7.48	TG(52:0)	Positive	M+NH ₄	C ₅₆ H ₁₀₀ O ₆	7.735	0.46	0.15 - 0.82	0.82
894.75	8.73	TG(54:7)	Positive	M+NH ₄	C ₅₇ H ₉₈ O ₅	0.114	0.46	0.05 - 0.75	0.75

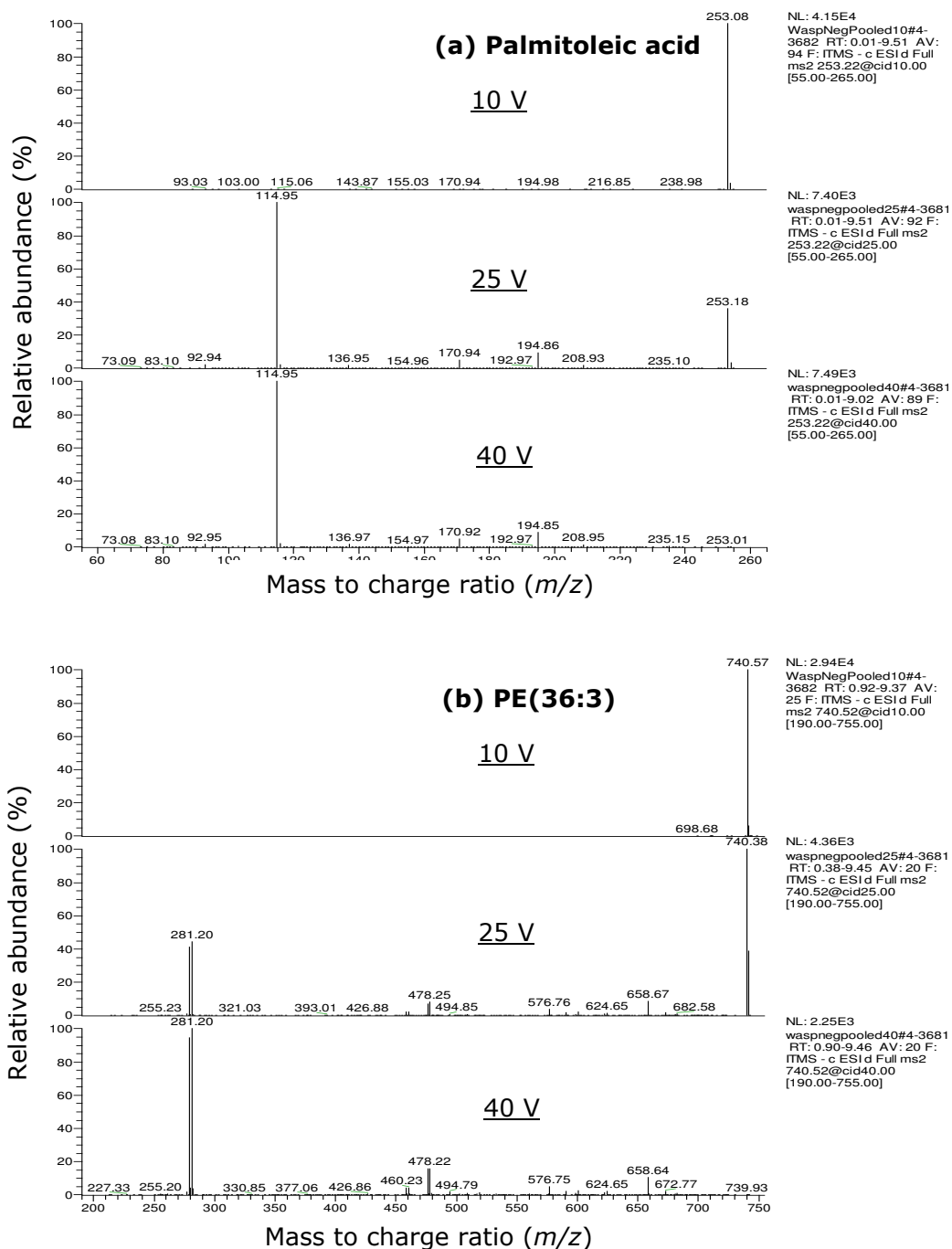


Figure 2.11. Representative LC-MS/MS fragmentation spectra of wasp non-polar key ions (a) Palmitoleic acid and (b) PE(36:3) for collision energies of 10, 25 and 40 V. PE = phosphoethanolamine.

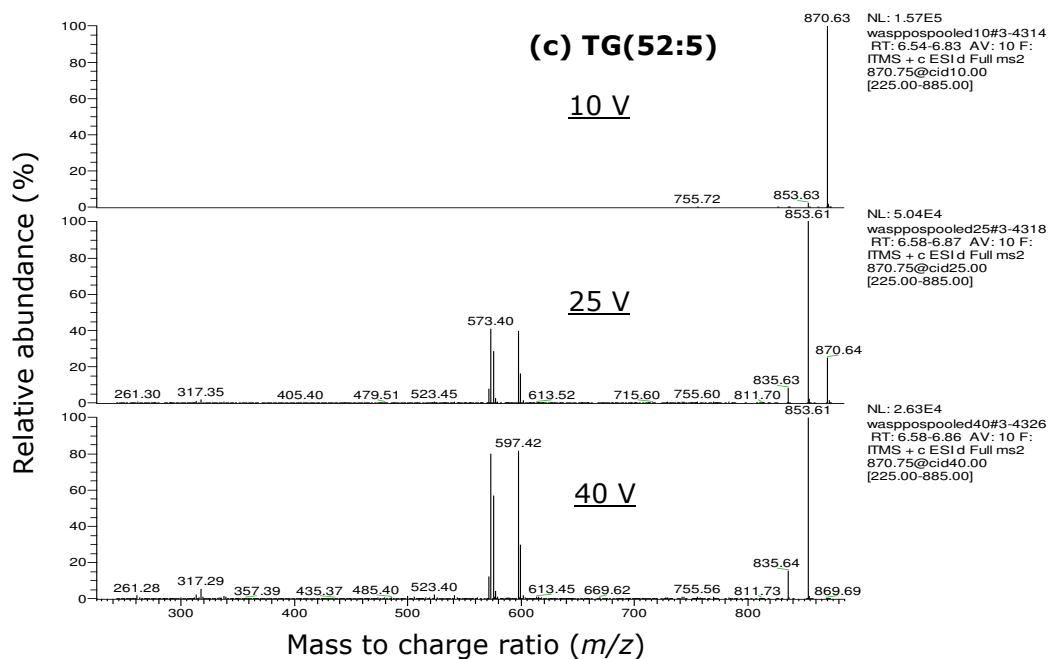


Figure 2.11. *cont.* Representative LC-MS/MS fragmentation spectra of wasp non-polar key ion (c) TG(52:5) for collision energies of 10, 25 and 40 V. TG = triacylglyceride.

was 10.61 %, with a range of 4.00 - 23.61 %. The average retention time RSD across key ions within QC samples was 1.38 %, with a range of 0.39 - 4.52 %.

2.4.8 Optimisation of non-polar metabolite identification by LC-MS/MS

Assessment of fragmentation data generated LC-MS/MS indicated that no recognisable fragment ions were present in spectra generated under a collision energy of 10 V, with only precursor peaks being readily identifiable. By comparison fragmentation data generated using a collision energy of 40 V were readily assignable when compared to both library standards and when matched with the Lipidblast database. Fragmentation data generated with a collision energy of 25 V generally appeared as an intermediate in fragmentation pattern between 10 V and 40 V MS/MS spectra.

2.5 Discussion

Part 1 of this chapter aimed to address the challenges associated with employing a rigorous metabolomic approach to assess changes in the biochemistry of low biomass insects. Such an approach is required to be efficient, precise and reproducible when being applied for the potential elucidation of experimental biomarkers (Dunn et al. 2011; Want et al. 2011). This requires a sample extraction methodology capable of producing clear, uncontaminated polar and non-polar solvent phases of a reliable volume. Pre-processing of these solvent phases should be minimal in order to produce highly complex, readily identifiable NMR and mass spectra. Furthermore the generation of these spectra should be stable and reproducible throughout an extended analytical timeframe (Dunn et al. 2011; Want et al. 2011). From the results outlined in this chapter it can be concluded that this optimisation process has generated an approach for metabolomic analysis of single *G. legneri* samples that meets these requirements.

2.5.1 Single insect extraction methodology optimisation

The thick outer cuticle of *G. legneri* was considered to present a possible technical challenge for generating metabolite rich solvent extraction, due to its disruption resistant nature. However, no major difficulties were noted during sample homogenisation, likely due to the highly disruptive nature of homogenisation method employed. The successful generation of complex NMR and mass spectra from a fixed solvent extraction volume suggests that this is an effective way of overcoming applicability problems associated with a ratio based solvent extraction approach. In contrast to the unworkably low sample volumes that a ratio based approach would generate for a sub milligram organism dry weight (e.g. 10.5 μL for low biomass polar and 2.5 μL for non-polar tissue extracts generated by Wu et al. 2008), the MCW approach applied here was capable of consistently generating solvent extracts with an approximate volume of 250 μL and 200 μL respectively for polar and non-polar phases. However, the solvent ratio of methanol/water when homogenising the parasitoids is 2:1 vs 2:0.625. The total ratio of solvent volume to mass is more than 40 times higher than employed by Wu et al. (2008) as this was necessary to handle such low biomass samples. Whilst similar solvent extract volumes were generated for both HIW and EHW extraction protocols, the lower spectral yield (HIW) and the higher levels of contamination (EHW) observed precluded these from further consideration.

2.5.2 Optimisation of NMR spectral resolution

Whilst analysis using a 600 MHz spectrometer did not result in an acceptable signal-to-noise ratio to separate a large number of metabolite associated from the surrounding baselines, a cryoprobe based 800 MHz NMR spectroscopy approach was capable of generating complex spectral resonances that clearly separated from the surrounding baseline. This is significant as a major consideration for NMR analysis of low biomass organisms is the relative time of analysis for the selected instrumentation. As the signal to noise ratio for is proportional to the number of scans performed throughout analysis, spectral signal to noise ratios can be increased by acquiring a larger number of scans and thus increasing the time of analysis per sample (Keeler 2010). However the signal to noise ratio only doubles with the square root of the number of scans, as a result replicating a higher signal to noise ratio on an instrument with lower field strength will require significantly longer acquisition times. Due to this principle, replicating the same signal noise ratio (S/N: 6248) observed by 800 MHz cryoprobe spectroscopy using 600 MHz NMR spectroscopy would require an acquisition time per sample of almost 30 years with over 100 million scans, compared to approximately 50 minutes and 512 scans per sample for 800 MHz. The possibility of such absurd acquisition times remains a significant limiting factor for low-biomass metabolomics using NMR, and as a result it must be concluded that the approach outlined in this chapter relies upon the availability of high field spectroscopic equipment and the use of a cryoprobe to remain relatively high throughput in nature.

2.5.3 Optimisation of non-polar extract pre-processing

Concentrated wasp extracts reconstituted in isopropanol displayed a greater chromatographic yield when analysed by LC-MS than non-concentrated chloroform samples. This is unsurprising, as a higher proportion of the concentrated sample was injected into the LC than the non-concentrated sample (10 μ L out of 60 μ L total sample volume compared to 10 μ L out of approximately 250 μ L). The lower relative evaporation exhibited by concentrated wasp extracts is likely due to isopropanol being a less volatile solvent than chloroform. From an analytical standpoint a minimal level of sample volume evaporation throughout the analytical timeframe is most attractive. In the case of highly volatile solvents, this evaporation can change the relative concentrations of individual samples, with the sample volume

injected for samples later in the analytical timeframe representing a higher proportion of the overall sample and thus a higher concentration. To attempt to minimise sample concentration variations throughout experimental analysis, isopropanol was selected as the reconstitution solvent for all future LC-MS and LC-MS/MS analysis.

2.5.4 Validation of LC-MS analytical precision and stability

Analytical stability for both NMR and LC-MS indicated the vast majority of peak area and retention time RSDs were well within the RSD acceptability threshold of 30% for analytical precision (FDA USA; Dunn et al. 2011). Whilst PCA clustering for NMR stability samples was looser than ideal, some variation is to be expected when the analytical approach is close to the limits of instrument sensitivity. Most significantly no correlation between PCA position and time was observed for any of the three stability samples, indicating that instrument sensitivity is a larger factor in sample variability than any time related sample metabolite degradation. As a result of these factors, both the NMR and LC-MS approaches utilised were considered to demonstrate a high degree of analytical stability throughout extended periods of sample analysis.

2.5.5 Validation of NMR and LC-MS spectral identifications

Validation of NMR and MS identities indicated that generated 2D NMR spectra and LC-MS/MS fragmentation spectra were readily assignable when compared to online spectral databases (BMRB, Lipidmaps, HMDB, Massbank databases), and in the case of 2D NMR resonances, model metabolite spectra. However both approaches required multiple extracts to be pooled prior to analysis in order to remain feasible. Whilst this limitation was not critical for LC-MS/MS samples, 2D NMR acquisition was only possible with the use of pooled samples. As a result, whilst it can be stated that this protocol is capable of single low biomass sample analysis, the validation required for such an approach still relies on some degree of sample pooling.

Although there have been studies that apply a metabolomic approach to examining insect biology, in the majority of cases the analytical sample consisted of a group of individuals (pooled biological samples), owing to the small biomass of insects (Snart et al. 2015). In the few cases that the

analytical sample equated a biological sample, the study targeted a limited range of metabolites, ranging from the broadly water soluble metabolites to specific class sugars and amino acids (Iwahata et al. 2011; Teets et al. 2012; Verberk et al. 2013). To the best of knowledge, this chapter presents the first low biomass solvent extraction approach capable of assessing multiple classes of both polar and non-polar metabolites. However, this chapter has been limited to optimising the individual aspects of a metabolomic workflow, rather than validating an entire metabolomic experiment. As such a fully validated metabolomic workflow examining the effects of aging on the metabolome of adult female *G. legneri* is presented in Chapter 3. Chapter 3 also outlines the finalised protocol in full, along with its associated data analysis and internal quality control aspects. This validated workflow is further applied throughout chapters 4, 5 and 6, examining the effects of wasp diet, host aging and wasp developmental background on the metabolomes of *G. legneri* and host larvae.

2.6 Conclusions

This chapter successfully optimised a modified methanol-chloroform-water solvent extraction procedure for low biomass insect samples. The generated extraction protocol was capable of producing consistent sample volumes with minimal contamination between solvent phases. This chapter also successfully validated a combined low biomass NMR and LC-MS metabolomic approach for stability and technical precision. The selected NMR approach used an 800 MHz NMR spectrometer equipped with a cryoprobe. Due to improved chromatogram peak shape, an Excel ace 2 column was employed as part of an Exactive LC-MS approach. The selected LC-MS approach utilised a concentration step during which non-polar extracts were concentrated in isopropanol prior to experimental analysis. Technical and stability replicates for LC-MS and NMR indicated acceptable key ion variations in both peak areas and retention times. NMR and LC-MS analysis was capable of generating complex readily identifiable spectra from individual polar and non-polar *G. legneri* extracts.

Chapter 3: Addressing the challenge of low biomass in insect metabolomics - Part 2: Validation of procedures

3.1 Abstract

This chapter aims to validate the low biomass metabolomic approach previously optimised in Chapter 2. To accomplish this, the previously optimised NMR and LC-MS approach was employed to assess the effects of aging on the metabolome of adult female *Goniozus legneri*. It has previously been confirmed that a majority of parasitoids do not possess *de novo* lipogenesis, an evolutionary trait likely lost due to the high abundance of lipids available from a parasitoid's host throughout larval development. As a result, parasitoid lipid reserves are highest upon initial eclosion, declining monotonically throughout the insect's remaining lifespan. This known decline provides an ideal model for the validation of a low biomass metabolomic approach. This chapter demonstrates that a combined NMR and LC-MS based metabolomics method can differentiate between the metabolite profiles of individual parasitoids of different ages. The major classes of metabolites that vary between newly emerged and older wasps were large glycerolipids, amino acids and circulatory sugars. This successfully validated metabolomic approach is further employed throughout this thesis to assess the effects of dietary status and developmental background on the behaviour and metabolome of *G. legneri*.

3.2 Introduction

First observed nearly 20 years ago, the lack of *de novo* lipogenesis is now known to be a widespread trait amongst hymenopteran parasitoids (Ellers et al. 1996). In the years since the majority of studied parasitoids have been found to lack the ability to accumulate new lipid reserves throughout adult life (Visser and Ellers 2008; Visser 2010). Due to this lack of lipogenesis, adult parasitoids emerging from their pupae retain a fixed amount of lipid reserves that decline throughout their remaining lifespan (Ellers et al. 1996; Eijs et al. 1998; Giron and Casas 2003; Casas et al. 2005; Jervis et al. 2008; Visser and Ellers 2008; Visser et al. 2010). This decline remains evident even in the presence of a carbohydrate-rich diet; though the rate of decline is significantly lower than in starved individuals (Ellers et al. 1996). Whilst carbohydrate sources are available in a typical parasitoid environment in the form of plant nectar, honey dew and host haemolymph, it is unlikely for a parasitoid to encounter new lipid sources throughout adult-life (Eijs et al. 1998; Giron et al. 2002; Giron and Casas 2003; Visser and Ellers 2008).

The loss of such an important process does not however result in lethal consequences for the organism, due to the abundance of lipids available from a parasitoid's host throughout larval development. As noted by Visser et al. (2008), this loss of lipogenesis may have resulted from relaxed selection due to its low consequences for the organism's phenotype. Traits tend to have a higher chance of being lost when selection is relaxed (Lahti et al. 2009; Ellers et al. 2012), the evolutionary redundancy of *de novo* lipogenesis for parasitoids may have resulted in it being neutrally selected (Visser et al. 2008; Ellers et al. 2012). However the physiological mechanisms that underlie the inability of parasitoids to convert dietary carbohydrates into large storage lipids have yet to be fully elucidated, in particular whether synthesis of fatty acid synthase (FAS) remains functional in adult parasitoids (Visser and Ellers 2008). Similarly it is yet to be conclusively determined whether lipogenesis is widespread amongst larval parasitoids (Yagan 1972; Thompson 1979; Visser and Ellers 2008).

Previous work by Visser et al. (2010) has confirmed that adult *Goniozus* wasps are incapable of *de novo* lipogenesis, even in the presence of a sugar rich diet. As it has been confirmed that the total lipid reserves of *Goniozus* wasps will decline over time after initial eclosion, this provides an excellent

opportunity to validate the low-biomass approach previously optimised by this thesis. Furthermore the short lifecycle of laboratory cultured *G. legneri* (approximately 7 - 9 days) allows this decline in lipid reserves to be readily assessed across the organism's entire lifespan. Alongside decreases in large storage lipids, starvation related declines in polar metabolites, including amino acids and circulatory sugars, should be observable by a combined NMR and LC-MS approach.

This chapter aims to validate the low-biomass metabolomic approach previously optimised in Chapter 2. This chapter outlines the final protocol for solvent extraction and metabolomic analysis applied throughout this thesis. To validate the feasibility of a low biomass metabolomic approach, it further applies this method to assess the effects of post eclosion aging on the metabolite profiles of individual adult female *G. legneri*. This approach utilises a combined NMR spectroscopy and mass spectrometry workflow that aims to generate untargeted metabolite profiling of individual adults of the parasitoid wasp *Goniozus legneri* (Hymenoptera: Bethyridae). This chapter further discusses the implications of the availability of a low-biomass metabolomic approach for the study of insect physiology, behaviour and ecology.

3.2.1 Biology of *Goniozus legneri*

Goniozus legneri Gordh (Hymenoptera: Bethyridae) is a gregarious parasitoid wasp, the life history and behaviour of which has been extensively studied (Gordh et al. 1983; Butler and Schmidt 1985; Hardy et al. 1998, 2000; Goubault et al. 2006, 2007; Goubault and Hardy 2007; Bentley et al. 2009; Lizé et al. 2012; Stockermans and Hardy 2013; Hardy and Briffa 2013). Individual *G. legneri* females paralyse the larvae of a range of lepidopteran host species before laying a batch of 1 - 20 eggs onto the host surface over the next 24 hours (Hardy et al. 1998). Upon hatching 24 hours or so later the developing larvae develop by feeding on the host externally through punctures in the integument. After initial oviposition has occurred females remain in close proximity to host, and will aggressively defend hosts from any intruding conspecific females (Petersen and Hardy 1996; Humphries et al. 2006). Whilst *G. legneri* is known to parasitize a wide range of lepidopteran larvae, its 'natural' host or host range remains unknown.

3.2.2 Aims and Objectives

This chapter aims to validate the low-biomass metabolomic approach previously optimised in Chapter 2. Specific objectives required to fulfil this aim are:

- To detect separation between the NMR spectra of polar extracts generated from 0 day, 3 day and 7 day old adult female *G. legneri*.
- To detect separation between the LC-MS spectra of non-polar extracts generated from 0 day, 3 day and 7 day old adult female *G. legneri*.
- To identify potential polar and non-polar biomarkers associated with wasp aging.

3.3 Materials and methods

3.3.1 Parasitoid origin and cultures

Laboratory cultures of both *G. legneri* and its host, *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae) were reared under the same diet as outlined in Chapter 2 (Stockermans and Hardy 2013). The parasitoid strain was obtained from a commercial insectary in the USA, both host and parasitoid strains have been used in prior behavioural and biochemical studies (Hardy et al. 2000; Goubault and Hardy 2007; Bentley et al. 2009; Lizé et al. 2012; Stockermans and Hardy 2013; Khidr et al. 2013a, 2013b, 2014). All insect culturing was carried out in a culture room with a fixed 16:8 hour light:dark cycle, at a relative humidity of 60 - 70 % maintained by a water bath.

3.3.2 Experimental design and wasp metabolite extraction

Individual *G. legneri* females were presented with a *C. cephalonica* larva and the resulting parasitoid broods were allowed to develop. Upon emergence, female offspring were weighted to an accuracy of 0.01 mg. All wasps utilised were within the weight range 0.94 - 1.63 mg. To assess the effects of wasp age on metabolomic state, wasps were either sampled upon adult emergence (0 days) or placed in a 0.5 mL Eppendorf tube and subsequently sampled at three or seven days post-emergence.

Individual parasitoids were sampled by snap freezing in liquid N₂ and stored at -80 °C until extraction. A modified Bligh and Dyer (1959) biphasic lipid extraction protocol was employed using a final ratio of solvents in a methanol/chloroform/water solution of 2:2:1.8. A two-step solvent addition process as described by Wu et al. (2008) was employed. All solvents used were of high LC-MS grade purity (Chromasolv Sigma-Aldrich) and were kept chilled during sample extraction. Wasps were placed in a 2.0 mL lysing matrix Z MP-BIO® vial and homogenised in 320 µL methanol and 128 µL water in a MP-BIO Fast Prep® homogenizer. 320 µL of chloroform and 160 µL of water were then added and extracts were vortexed for 30 seconds (s). Extracts were then centrifuged for 10 minutes (min) at 10,000 g and left for 5 min at room temperature before phase separation. For each extract, lipid phase (lower layer) was transferred to a 2 mL borosilicate glass vial. The upper phase (polar compounds, ~ 400 µL) was transferred to a 1.5 mL

solvent resistant Eppendorf ®Biopur® tube. Both phases were stored at -80 °C prior to analysis. All samples were randomly allocated to the treatment groups during sample extraction and prior to analysis to allow for the greatest reliability and validity of statistical estimates of treatment effects.

3.3.2.1 NMR spectroscopy

Individual polar extracts were dried in a rotary evaporator and reconstituted in 600 µL of D₂O with 0.5 mM of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) added as internal reference prior to analysis. All NMR experiments were acquired on a Bruker Avance 800 MHz III spectrometer using a 5mm QCI Cryoprobe. Data were collected with a spectral width of 13 ppm and signal averaged over 512 transients using a noesy 1D pre-saturation experiment to achieve water suppression. Total recycling delay was 4.7 s. Pre-saturation power and frequency were determined in advance on representative samples and fixed for all acquisitions. Samples were referenced and locked using the internal D₂O signal with a secondary reference of DSS (0.5 mM) present in all samples. Data were processed by application of an exponential window function set to give a 0.3 Hz line broadening prior to zero filling and Fourier transformation. To aid confirmation of metabolite identity, two-dimensional NMR spectra were generated from a pooled extract of 20 wasps generated utilising the same methodology. Total correlation spectroscopy (TOCSY) and heteronuclear single-quantum correlation (HSQC) experiments were employed.

3.3.2.2 LC-MS analysis

Extracts of single wasps were assessed using a lipidomic LC-MS method. The full non-polar fraction (approximately 250 - 300 µL) was dried by rotary evaporation and reconstituted in 50 µL of isopropanol. A pooled extract (comprising 10 µL from each experimental sample) was generated and injected after every 15 samples for quality control (QC) purposes. Accurate mass LC-MS was performed on non-polar extracts (10 µL injection volume) of individual wasps using an Accela LC coupled with an Exactive mass spectrometer (Thermo Fisher Scientific, USA) in negative and positive electrospray ionisation modes (ESI). An Ace Excel 2 Super C18 (2 µm, 2.1 x 50mm) column equipped with an appropriate guard column was maintained at 40 °C with a variable flow rate of 300 - 400 µL/min depending on gradient

time. Mobile phases utilised for the gradient program consisted of (A) 80:10:10 water/isopropanol/acetonitrile, the phase was modified with 0.01 % ammonium acetate and (B) 50:50 isopropanol/acetonitrile modified with 0.01 % ammonium acetate. All solvents used were of high LC-MS grade purity (Chromasolv, Sigma-Aldrich). The LC gradient program had an initial starting proportion of 62.5 % B followed by a linear increase to 99 % B over 3 min. This proportion was maintained for a further 5 min followed by a linear decrease to the starting proportion of 62.5% B over 2 min. Ions were monitored in full scan mode within the range of m/z 100 to 1500 (ESI voltage: 3500, capillary temperature: 350 °C, scan rate: 250 ms).

A tandem mass spectrometry approach was employed to validate putative identifications of each ion. Remaining non-polar phases were pooled prior to analysis to improve yield. Analysis was performed utilising an Accela LC coupled with an LTQ Velos Pro Dual-Pressure linear ion trap mass spectrometer (Thermo Fisher Scientific, USA). The column, mobile phases and gradient programs were replicated from the previous LC-MS analysis. Ions were monitored within the range of m/z 100 to 1500 (ESI voltage: 3000, capillary temperature: 275 °C, scan rate: 50 ms). MS2 fragmentations were performed with a collision energy of 40 V with a maximum ion isolation window of 5 Da.

3.3.3 LC-MS stability validation

To assess the stability of the LC-MS approach, the relative standard deviations of peak areas and retention times of key ions outlined previously examined to establish instrument precision and analytical stability in Chapter 2 were assessed within QC samples. An RSD threshold of <30 % for 70 % of key ions was considered acceptable to establish LC-MS stability. Central clustering of QC samples within the generated PCA was further considered as a requirement to confirm LC-MS stability.

3.3.4 Data analysis

All NMR spectra were referenced to the internal DSS signal at 0.001 ppm. Individual spectra were manually phased and baseline corrected in Topspin (Bruker Scientific Instruments, Bruker) prior to non-discriminant data alignment and binning utilising a user generated script. Data simplification

was achieved by dividing spectra into a total of 931 non-overlapping regions (bins) with a width of 0.01 ppm, to which integrated spectral intensities were assigned. The water signal region (5.2 - 4.5) was excluded prior to multivariate analysis. LC-MS data were aligned and framed without discrimination using the propriety software Sieve 2.0 (Thermo Fisher Scientific, USA). A frame width of 0.5 min was employed during alignment to remove variation caused by minor chromatographic shifts. Samples were framed with an arbitrary intensity threshold of 50,000. The first 0.5 min of spectral acquisition was excluded from framing to remove variation from potential polar metabolite contamination.

The generated frame/bin tables for both NMR and LC-MS were exported to a spread sheet package (Microsoft Excel) for baseline correction and normalised to total intensity to remove variation in sample concentration. Normalised tables were separately imported into the Simca P+ (Umetrics) package, where preliminary sample visualisation was accomplished using principle component analysis (PCA). Individual variables were mean-centred and scaled prior to PCA visualisation. Prior to model generation, NMR data were autoscaled, whereas LC-MS data underwent pareto scaling. Supervised multivariate analysis of parasitoid profiles was conducted utilising OPLS-DA to establish separation between treatment classes. The generated OPLS-DA model was cross validated by a combination of cross-validation ANOVA and through the generated of a series of predictive models. These models were generated by dividing NMR and LC-MS samples into a randomly selected training set (comprising 20 % of total samples) and a test set (remaining 80 % of total samples). A total of five predictive models were generated and assessed for correct prediction rate and Fisher's exact p-value. Comparisons of the generated loading and scores plots were utilised to aid in the selection of potential biomarkers, along with the generation of class weightings for each variate. Ions were validated using a one-way binomial ANOVA, utilising total sample intensity as the binomial total. Generated p-values were adjusted using the Bonferroni correction to account for multiple hypothesis testing (Quinn and Keogh 2002). Metabolites with an adjusted p-value <0.05 were considered to display significant separation between treatments. A minimum fold change of 1.5 was required for a metabolite to be considered as a potential biomarker. ANOVA's were performed using the statistical package Genstat v.15 (VSN International).

3.4 Results

3.4.1 NMR spectroscopy

Using NMR, the generated PCA model exhibited a goodness of fit (R^2X) of 73.1% for wasp age treatments (0 days, 3 days and 7 days). Cross validation of the model resulted in an average correct prediction rate of 92.5% ($P < 0.001$). Associated PCA scores plots indicated a degree of separation between 0 days and 7 days treatment, with 3 days samples indicating a loose degree of intermediate clustering (Figure 3.1a). Whilst individual treatments did not exhibit tight clustering, significant separation between 0 days and 7 days treatment was established by OPLS-DA analysis (Figure 3.1b). Further parametric validation of weighted scores plots by logistic ANOVA (d.f. = 2, 57, $P < 0.05$) generated a total of 63 significant bins between 0 day and 7 day old wasps. Of these bins, 59 were elevated in the 0 days treatment, compared to four in the 7 day treatment. Spectral assignments of bins associated with 0 days treatment identified a number of metabolites that were elevated in 0 days wasps, including common insect sugars and amino acids. Bins associated with the 7 days treatment between 6.62 - 6.40 ppm were identified as baseline fluctuations rather than real spectral peaks. Individual metabolite identities, F values and p -values are outlined in Table 3.1. Individual metabolite levels are displayed in Figure 3.4.

3.4.2 LC-MS analysis

This LC-MS approach detected 1076 unique ions. These comprised 360 ions in negative mode ESI and 749 in positive mode ESI. Lipid categories tentatively identified included fatty acyls, phospholipids, sphingolipids, glycerolipids and lysophospholipids. ESI- mode spectra were dominated by common fatty acyls and phospholipids. Spectra of ESI+ mode were dominated by large storage lipids and mobilisation lipids including diacyl- and triacylglycerides. Cross validation of the model using Fisher's exact test resulted in an average correct prediction rate of 98.8 % ($P < 0.001$). QC samples exhibited very little drift over time, clustering centrally between experimental groups. Assessment of peak areas of quality control ions within QC samples found that 88.24 % of ions displayed RSDs with the acceptability threshold for experimental stability. QC ions had an average peak area RSD of 14.05 % with a range of 2.62 - 51.39 % and an average

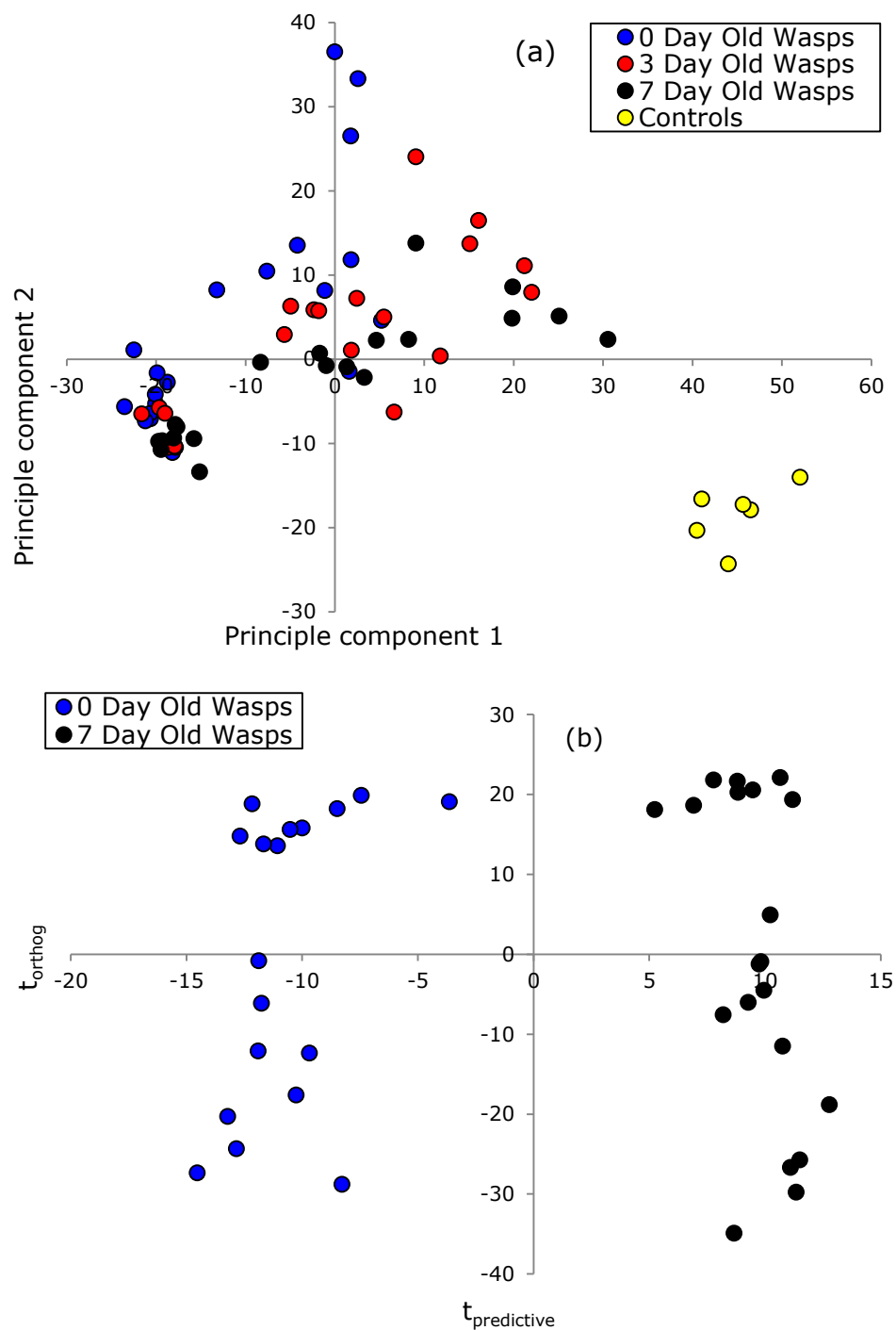


Figure 3.1. Principle components analysis of aged wasp NMR spectra (a) PCA of NMR samples of wasps aged 0 days, 3 days and 7 days (PC1 = 44.3 %, PC2 = 14.1 %, PC3 = 8.28 %, $R^2X = 0.731$), including control samples (b) OPLS-DA analysis of NMR samples of wasps aged 0 days and 7 days ($R^2X = 0.873$, $Q^2 = 0.688$).

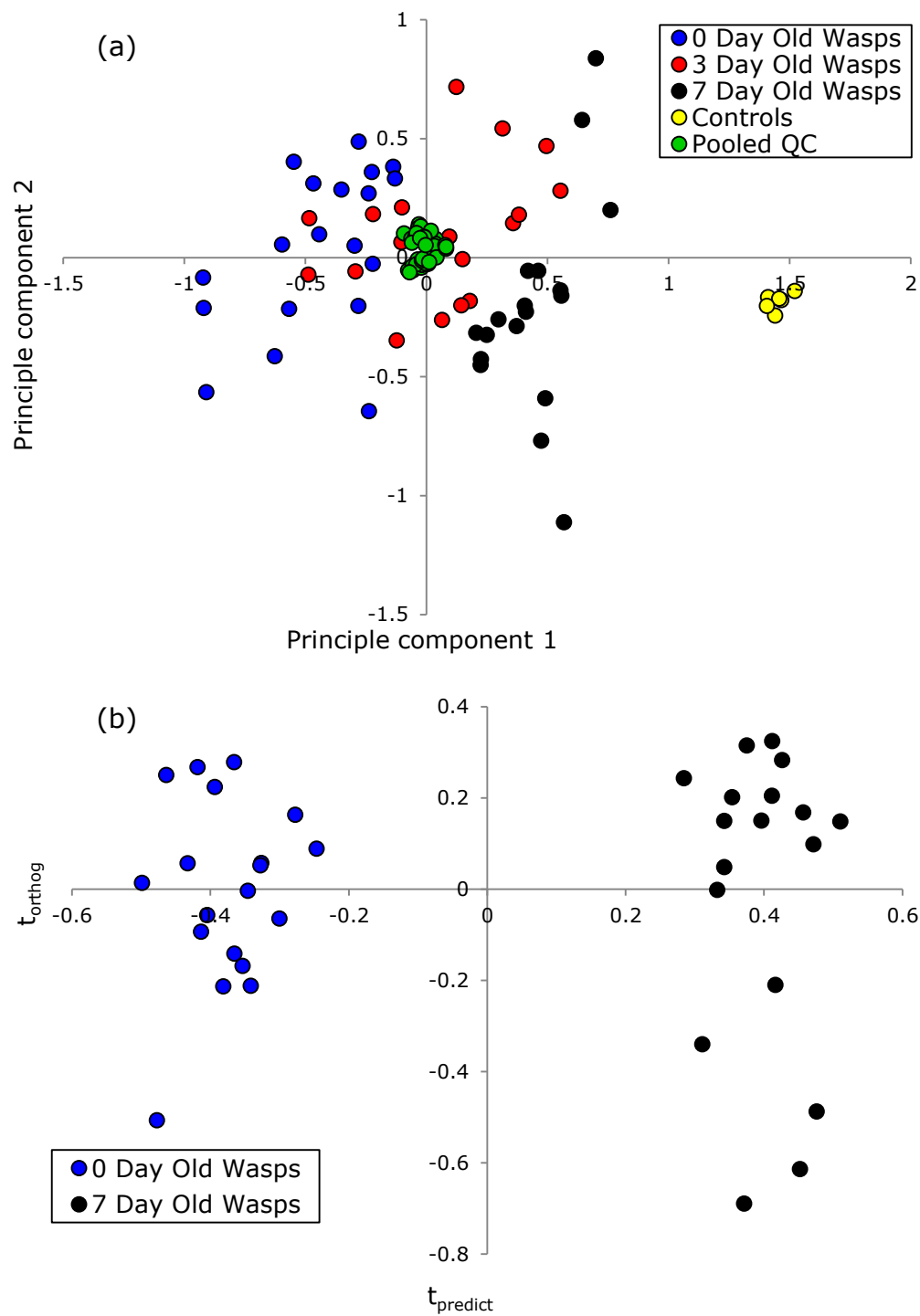


Figure 3.2. (a) PCA of LC-MS samples of wasps aged 0 days, 3 days and 7 days (PC1 = 27.1 %, PC2 = 18.7 %, PC 3 = 9.51 %, $R^2X = 0.875$) (b) OPLS-DA of LC-MS samples of wasps aged 0 days and 7 days ($R^2X = 0.726$, $Q^2 = 0.818$).

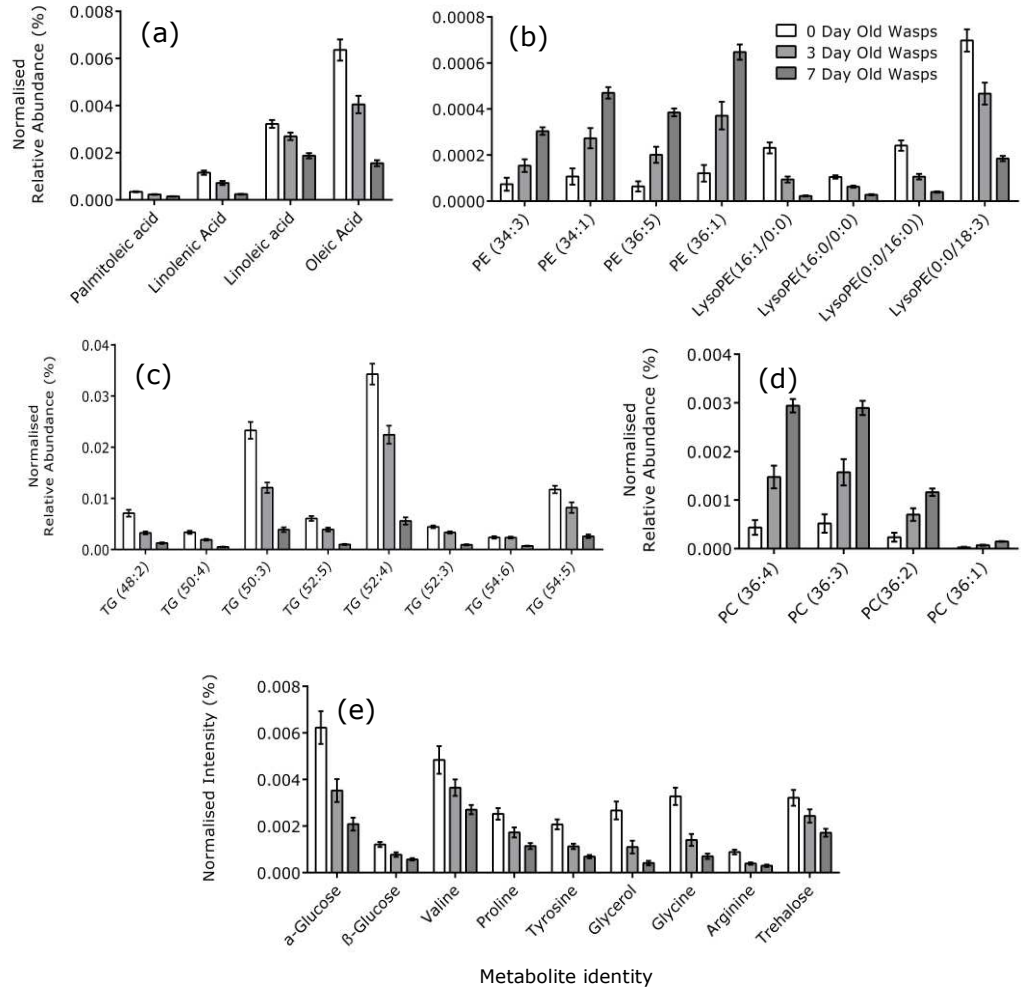


Figure 3.3. Metabolite differences between 0 day, 3 day and 7 day old *G. legneri*. Biomarker metabolites are separated by class and scale, comprising of (a) Fatty acyls (b) Phosphoethanolamines (PE) and LysoPE's (c) Triacylglycerides (d) Phosphocholines (e) Sugars and amino acids. The displayed values consist of the mean normalised metabolite area; error bars show standard error.

retention time RSD of 0.9 % with a range of 0.14 - 4.30 %. Assessment of retention time RSDs within QC samples found that 100 % of ions displayed RSDs within the acceptability threshold for experimental stability. Due to these results the stability of the LC-MS analysis was considered to be appropriately validated. Analysis of generated scores plots according to wasp age treatments indicated separation between 0 days and 7 days treatment in across the second principle component, whereas the 3 day treatment exhibited partial separation from the 0 day and the 7 day

Table 3.1. Summary of polar biomarkers with tentative identities that significantly differ between 0 day old and 7 day old wasp extracts.

Common Name	Chemical shift (ppm)	d.f.	Formula	F ratio	P-value (B)	Fold change ¹
α -Glucose	3.45 (t), 3.52 (dd), 3.70 (t), 3.76 (m), 3.82 (ddd), 3.83 (m), 5.22 (d)	2, 57	C ₆ H ₁₂ O ₆	28.36	<0.01	-2.99
Arginine	1.67 (m), 1.91 (m), 3.23 (t), 3.76 (t)	2, 57	C ₆ H ₁₄ N ₄ O ₂	21.92	<0.01	-2.98
Glycerol	3.55 (m), 3.64 (m), 3.77 (tt)	2, 57	C ₃ H ₈ O ₃	27.13	<0.01	-6.46
Glycine	3.54 (s)	2, 57	C ₂ H ₅ NO ₂	36.71	<0.01	-4.65
Proline	2.01 (m), 2.08 (m), 2.35 (m), 3.33 (dt), 3.42 (dt), 4.12 (dd)	2, 57	C ₅ H ₉ NO ₂	15.35	<0.01	-2.21
β -Glucose	3.24 (dd), 3.4 (m), 3.47 (m), 3.74 (m), 3.88 (dd), 4.64 (d)	2, 57	C ₆ H ₁₂ O ₆	11.12	<0.01	-2.12
Trehalose	3.44 (t), 3.64 (dd), 3.75 (m), 3.82 (m), 3.84 (m), 3.86 (m), 5.19 (d)	2, 57	C ₁₂ H ₂₂ O ₁₁	11.46	<0.01	-1.87
Tyrosine	3.04 (dd), 3.18 (dd), 3.92 (dd), 6.89 (d), 7.19 (d)	2, 57	C ₉ H ₁₁ NO ₃	33.38	<0.01	-3.01
Valine	0.99 (d), 1.04 (d), 2.34 (m), 3.6 (d)	2, 57	C ₅ H ₁₁ NO ₂	11.53	<0.01	-1.79

¹ Negative fold changes indicate metabolites that declined in abundance between 0 day old and 7 day old wasps, positive fold changes indicate metabolites that increased in abundance between 0 day old and 7 day old wasps.

treatment, clustering intermediately between the two (Figure 3.3a). OPLS-DA analysis confirmed the separation between 0 days and 7 days aged wasp samples ($R^2X = 0.726$, $Q^2 = 0.818$) (Figure 3.3b). Comparisons between 3 day wasp samples and each of the other treatments found no clear separation.

Parametric validation of the generated weighted comparisons scores by logistic ANOVA (d.f. = 2, 57, $P < 0.05$) found a total of 247 ion intensities that significantly differed between 0 day and 7 day wasps after Bonferroni correction. Of these ions, a total of 138 were assigned tentative identifications, 71 of which were successfully validated by LC-MS/MS. Due to the presence of multiple sodium adducts, this corresponded to a total of 61 unique lipids. These identifications comprised of 4 free fatty acyls, 17

Table 3.2. Summary of non-polar biomarkers with tentative identities that significantly differ between 0 day old and 7 day old wasp extracts. PE = phosphoethanolamine, PC = phosphocholine.

M/Z	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Fold change¹	Mass error (ppm)
253.217	2.14	M-H	Negative	Palmitoleic acid	C ₁₆ H ₃₀ O ₂	2, 57	44.19	<0.01	-2.32	0.304
277.218	1.41	M-H	Negative	Linolenic Acid	C ₁₈ H ₃₀ O ₂	2, 57	36.11	<0.01	-4.77	0.696
279.233	2.36	M-H	Negative	Linoleic acid	C ₁₈ H ₃₂ O ₂	2, 57	28.16	<0.01	-1.72	0.046
281.249	2.13	M-H	Negative	Oleic Acid	C ₁₈ H ₃₄ O ₂	2, 57	42.4	<0.01	-4.09	0.396
450.263	1.62	M-H	Negative	LysoPE(16:1/0:0)	C ₂₁ H ₄₂ NO ₇ P	2, 57	39.4	<0.01	-10.13	0.387
452.277	1.58	M+H	Positive	LysoPE(0:0/16:0)	C ₂₁ H ₄₄ NO ₇ P	2, 57	54.43	<0.01	-6.08	0.737
452.279	2.05	M-H	Negative	LysoPE(16:0/0:0)	C ₂₁ H ₄₄ NO ₇ P	2, 57	60.06	<0.01	-3.76	0.737
474.263	1.44	M-H	Negative	LysoPE(18:3/0:0)	C ₂₃ H ₄₂ NO ₇ P	2, 57	29.94	<0.01	-4.69	0.387
476.277	1.43	M+H, M+NA	Positive	LysoPE(0:0/18:3)	C ₂₃ H ₄₂ NO ₇ P	2, 57	60.91	<0.01	-3.77	1.263
476.279	1.74	M-H	Negative	LysoPE(18:2(9Z,12Z)/0:0)	C ₂₃ H ₄₄ NO ₇ P	2, 57	37.26	<0.01	-4.44	0.737
478.293	1.75	M+H, M+NA	Positive	LysoPE(0:0/18:2)	C ₂₃ H ₄₄ NO ₇ P	2, 57	46.77	<0.01	-3.25	0.087
478.294	2.13	M-H	Negative	LysoPE(18:1(9Z)/0:0)	C ₂₃ H ₄₆ NO ₇ P	2, 57	36.68	<0.01	-4.05	0.087
480.308	2.17	M+H, M+NA	Positive	LysoPE(0:0/18:1)	C ₂₃ H ₄₆ NO ₇ P	2, 57	37.15	<0.01	-3.10	0.465
494.324	1.50	M+H, M+NA	Positive	LysoPC(0.0/16.1)	C ₂₄ H ₄₈ NO ₇ P	2, 57	57.3	<0.01	-5.98	0.115
496.34	1.96	M+H, M+NA	Positive	LysoPC(0.0/16.0)	C ₂₄ H ₅₀ NO ₇ P	2, 57	38.07	<0.01	-2.99	0.235
506.325	2.05	M-H	Negative	LysoPE(20:1(11Z)/0:0)	C ₂₅ H ₅₀ NO ₇ P	2, 57	43.74	<0.01	-3.72	0.213
520.339	1.35	M+H, M+NA	Positive	LysoPC(0:0/18:2)	C ₂₆ H ₅₀ NO ₇ P	2, 57	50.57	<0.01	-3.67	0.765
522.355	2.09	M+H, M+NA	Positive	LysoPC(0:0/18:1)	C ₂₆ H ₅₂ NO ₇ P	2, 57	45.08	<0.01	-3.73	0.416
540.305	1.35	M+Na	Positive	LysoPC(0:0/18:3)	C ₂₆ H ₄₈ NO ₇ P	2, 57	55.96	<0.01	-4.26	1.057

¹ Negative fold changes indicate metabolites that declined in abundance between 0 day old and 7 day old wasps, positive fold changes indicate metabolites that increased in abundance between 0 day old and 7 day old wasps.

Table 3.2. *Cont.* Summary of non-polar biomarkers with tentative identities that significantly differ between 0 day old and 7 day old wasp extracts. PE = phosphoethanolamine, PC = phosphocholine, TG = triacylglyceride.

<i>M/Z</i>	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	<i>F</i> ratio	<i>P</i> value (B)	Fold change ¹	Mass error (ppm)
546.352	2.64	M+Na	Positive	LysoPC(0:0/18:0)	C ₂₆ H ₅₄ NO ₇ P	2, 57	31.6	<0.01	-1.79	1.008
580.363	2.04	M-Hac-H	Negative	LysoPC(18:1)	C ₂₆ H ₅₂ NO ₇ P	2, 57	40.54	<0.01	-3.88	1.009
682.448	6.16	M+Na	Positive	PE(30:2)	C ₃₅ H ₆₆ NO ₈ P	2, 57	54.4	<0.01	6.78	0.8
712.494	4.38	M-H	Negative	PE(34:3)	C ₃₉ H ₇₂ NO ₈ P	2, 57	13	<0.05	4.12	1.721
716.525	5.03	M-H	Negative	PE(34:1)	C ₃₉ H ₇₆ NO ₈ P	2, 57	15.38	<0.01	4.39	1.421
736.493	4.13	M-H	Negative	PE(36:5)	C ₄₁ H ₇₂ NO ₇ P	2, 57	24.87	<0.01	6.01	0.721
738.509	4.38	M-H	Negative	PE(36:4)	C ₄₁ H ₇₄ NO ₈ P	2, 57	30.88	<0.01	6.74	1.071
740.521	4.32	M+H	Positive	PE(36:4)	C ₄₁ H ₇₄ NO ₈ P	2, 57	22.19	<0.01	6.59	0.171
740.525	4.68	M-H	Negative	PE(36:3)	C ₄₁ H ₇₆ NO ₈ P	2, 57	26.52	<0.01	5.58	1.421
742.526	4.38	M-H	Negative	PE(36:2)	C ₄₁ H ₇₈ NO ₇ P	2, 57	21.96	<0.01	4.92	3.3
744.555	5.15	M-H	Negative	PE(36:1)	C ₄₁ H ₈₀ NO ₇ P	2, 57	12.94	<0.05	5.58	0.121
762.503	4.32	M+Na	Positive	PE(36:4)	C ₄₁ H ₇₄ NO ₈ P	2, 57	22.66	<0.01	5.12	0.985
762.509	4.31	M-Hac-H	Negative	PC(30:1)	C ₃₈ H ₇₄ NO ₈ P	2, 57	28.32	<0.01	6.85	3.479
766.535	4.88	M+Na	Positive	PE(36:2)	C ₄₁ H ₇₈ NO ₇ P	2, 57	26.93	<0.01	5.50	0.723
782.568	4.32	M+H	Positive	PC(34:1)	C ₄₂ H ₈₂ NO ₈ P	2, 57	26.63	<0.01	6.44	0.977
792.707	7.83	M+NH ₄	Positive	TG(42:2(12:0/18.1/16.1))	C ₄₅ H ₈₂ O ₆	2, 57	81.84	<0.01	-5.73	0.564
794.722	8.76	M+H, M+NA	Positive	TG(46:1(14:0/16.0/16.1))	C ₄₉ H ₉₄ O ₅	2, 57	100.79	<0.01	-5.13	1.214
800.494	5.01	M-HCOO	Negative	PC(35:5)	C ₄₃ H ₇₆ NO ₈ P	2, 57	24.15	<0.01	5.45	2
806.566	4.66	M+Na	Positive	PC(36:3)	C ₄₄ H ₈₂ NO ₈ P	2, 57	23.8	<0.01	5.29	1.023

¹ Negative fold changes indicate metabolites that declined in abundance between 0 day old and 7 day old wasps, positive fold changes indicate metabolites that increased in abundance between 0 day old and 7 day old wasps.

Table 3.2. *Cont.* Summary of non-polar biomarkers with tentative identities that significantly differ between 0 day old and 7 day old wasp extracts. PC = phosphocholine, TG = triacylglyceride.

M/Z	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Fold change¹	Mass error (ppm)
816.578	4.85	M-Hac-H	Negative	PC(34:2)	C ₄₂ H ₈₀ NO ₈ P	2, 57	29.24	<0.01	6.31	1.994
818.496	4.13	M-Hac-H	Negative	PC(34:1)	C ₄₂ H ₈₂ NO ₈ P	2, 57	25.3	<0.01	6.34	1.756
818.721	7.68	M+NH ₄	Positive	TG(48:3(14:1/16.1/18.1))	C ₅₁ H ₉₂ O ₆	2, 57	70.86	<0.01	-5.51	2.214
820.738	8.90	M+H, M+NA	Positive	TG(48:2(16:0/16.1/16.1))	C ₅₁ H ₉₄ O ₆	2, 57	53.43	<0.01	-5.63	0.864
836.547	3.86	M-Hac-H	Negative	PC(36:6)	C ₄₄ H ₇₆ NO ₈ P	2, 57	29.75	<0.01	5.52	2.294
838.561	4.09	M-Hac-H	Negative	PC(36:5)	C ₄₄ H ₇₈ NO ₇ P	2, 57	29.26	<0.01	6.35	0.644
840.576	4.38	M-Hac-H	Negative	PC(36:4)	C ₄₄ H ₈₀ NO ₇ P	2, 57	30.35	<0.01	6.74	0.006
842.592	4.73	M-Hac-H	Negative	PC(36:3)	C ₄₄ H ₈₂ NO ₈ P	2, 57	23.45	<0.01	5.58	0.344
844.608	5.12	M-Hac-H	Negative	PC(36:2)	C ₄₄ H ₈₄ NO ₇ P	2, 57	13.23	<0.05	4.92	0.694
844.738	7.90	M+NH ₄	Positive	TG(50:4(16:0/16.1/18.3))	C ₅₃ H ₉₆ O ₅	2, 57	97.16	<0.01	-6.56	0.864
846.624	5.99	M-Hac-H	Negative	PC(36:1)	C ₄₄ H ₈₆ NO ₈ P	2, 57	20.25	<0.01	5.59	1.044
846.753	8.71	M+H, M+NA	Positive	TG(50:3(16:0/16.1/18.2))	C ₅₄ H ₉₈ O ₆	2, 57	150.54	<0.01	-5.99	1.514
849.694	7.90	M+NH ₄	Positive	TG(50:2(16:0/16.1/18.1))	C ₅₃ H ₉₈ O ₆	2, 57	98.39	<0.01	-4.47	0.259
868.738	7.22	M+NH ₄	Positive	TG(52:6(16:1/18.2/18.3))	C ₅₅ H ₉₄ O ₆	2, 57	55.53	<0.01	-18.77	0.864
870.753	7.90	M+NH ₄	Positive	TG(52:5(16:0/18.2/18.3))	C ₅₅ H ₉₈ O ₅	2, 57	111.22	<0.01	-6.05	0.894
872.769	8.74	M+H, M+NA	Positive	TG(52:4(16:0/18.2/18.2))	C ₅₅ H ₉₈ O ₆	2, 57	191.68	<0.01	-6.13	1.164
875.709	7.93	M+NH ₄	Positive	TG(52:3(16:0/18.1/18.2))	C ₅₅ H ₁₀₀ O ₆	2, 57	116.78	<0.01	-4.76	0.909
876.799	8.67	M+NH ₄	Positive	TG(52:2(16:0/18.1/18.1))	C ₅₅ H ₁₀₂ O ₆	2, 57	167.36	<0.01	-7.43	2.464
887.779	8.73	M+NH ₄	Positive	TG(53:4(17:1/18.1/18.2))	C ₅₆ H ₁₀₀ O ₆	2, 57	56.35	<0.01	-2.41	2.061

¹ Negative fold changes indicate metabolites that declined in abundance between 0 day old and 7 day old wasps, positive fold changes indicate metabolites that increased in abundance between 0 day old and 7 day old wasps.

Table 3.2. *Cont.* Summary of non-polar biomarkers with tentative identities that significantly differ between 0 day old and 7 day old wasp extracts. TG = triacylglyceride.

M/Z	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Fold change¹	Mass error (ppm)
894.754	7.15	M+NH4	Positive	TG(54:7(18:2/18.2/18.3))	C57H98O5	2, 57	32.42	<0.01	-5.19	0.514
896.775	8.09	M+NH4	Positive	TG(54:6(18:1/18.2/18.3))	C57H100O5	2, 57	37.64	<0.01	-3.33	4.836
898.784	8.63	M+H, M+NA	Positive	TG(54:5(18:1/18.2/18.2))	C58H104O5	2, 57	101.31	<0.01	-4.52	0.594
901.725	7.78	M+NH4	Positive	TG(54:4(18:1/18.1/18.2))	C57H102O6	2, 57	66.6	<0.01	-3.98	0.559

¹ Negative fold changes indicate metabolites that declined in abundance between 0 day old and 7 day old wasps, positive fold changes indicate metabolites that increased in abundance between 0 day old and 7 day old wasps.

glycerolipids, and 40 phospholipids. All 17 glycerolipids were triacylglycerides, the primary form of storage in the insect fat body. All of these lipids were elevated in 0 day wasps compared to 7 day wasps, with 3 day old wasps exhibiting peak area levels intermediate between the two. Phospholipids comprised of glycerophosphocholines (13), glycerophosphoethanolamines (11), lysophosphatidylcholines (7) and lysophosphatidylethanolamines (10). Whilst all 17 Lysophospholipids were elevated in 0 day old wasps, the majority of glycerophospholipids were elevated in 7 day old wasps (11 phosphoethanolamines and 12 phosphocholines elevated vs. 1 phosphocholine reduced). Individual mass to charge ratios, identities, elution times, adducts, F values and fold changes of significant metabolites are outlined in Table 3.2. Metabolite levels for each lipid category are displayed in Figure 3.4. Representative fragmentation spectra of lipid classes are provided in Figure 3.5.

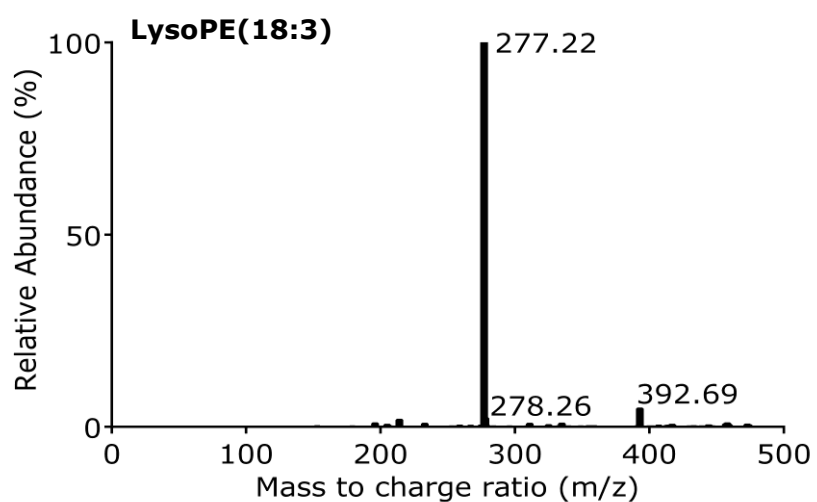
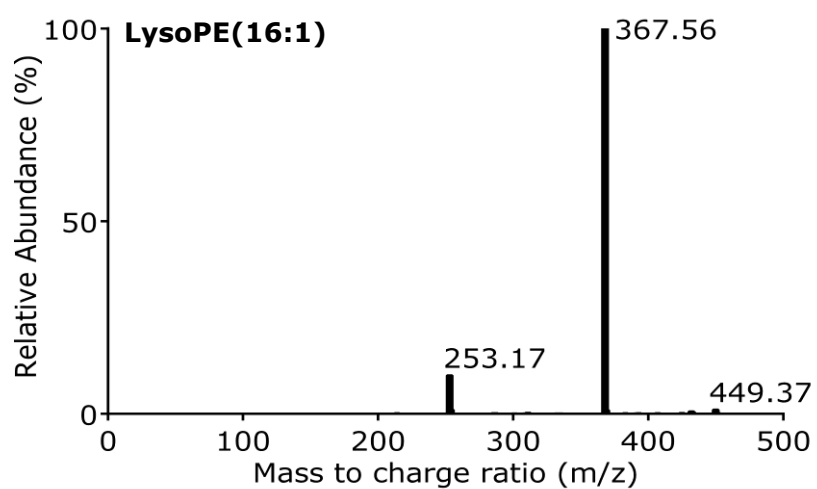
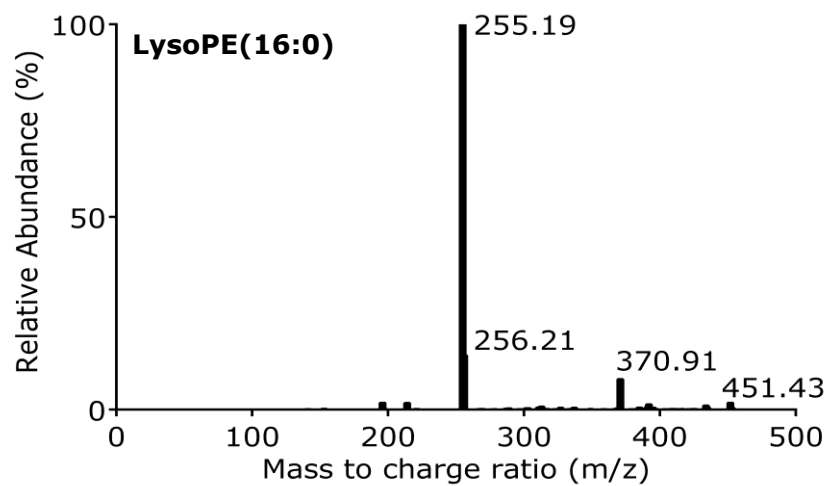


Figure 3.4. Representative LC-MS/MS spectra of individual lipid classes. LysoPE = lysophosphoethanolamine.

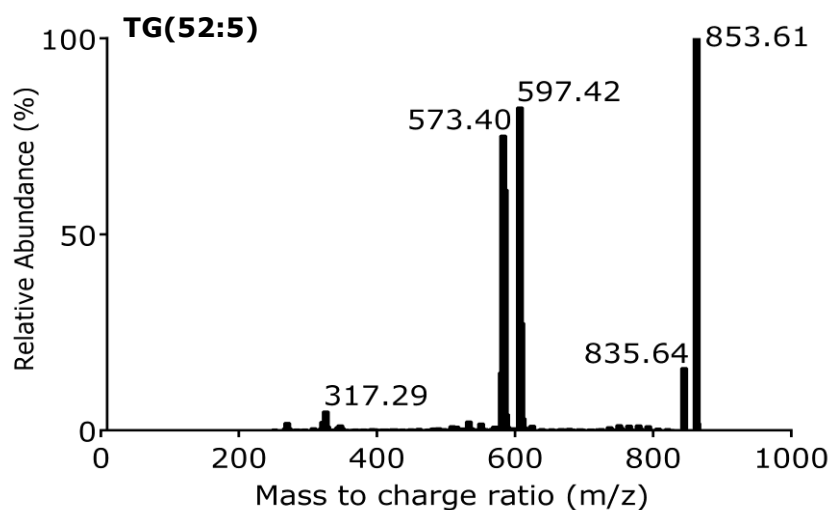
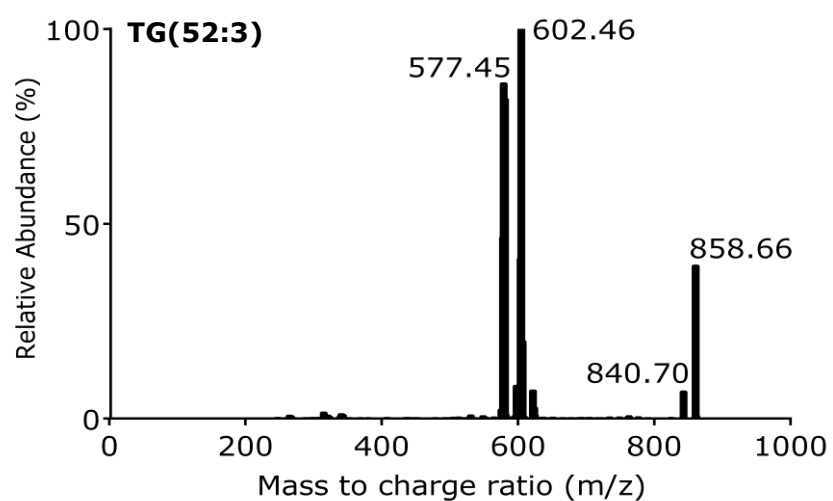
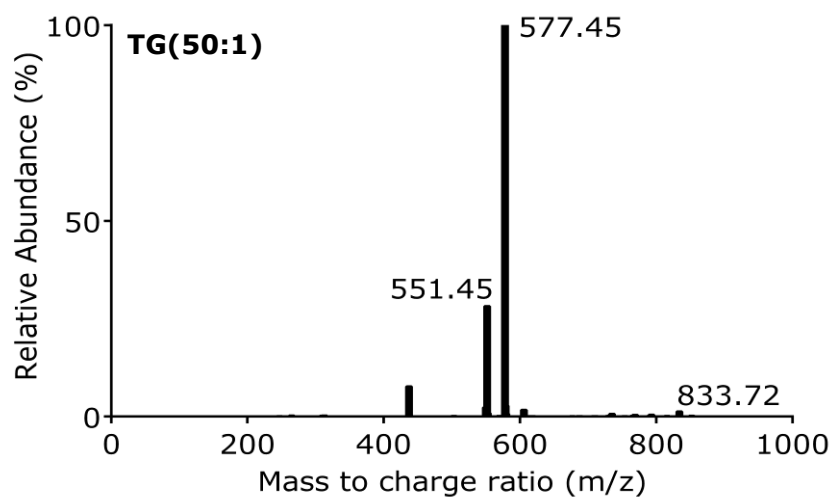


Figure 3.4. *cont.* Representative LC-MS/MS spectra of individual lipid classes. TG = Triacylglycerides.

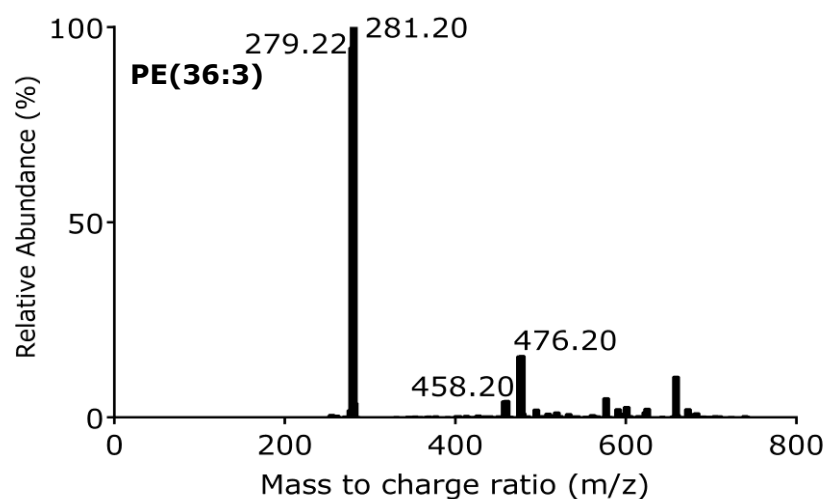
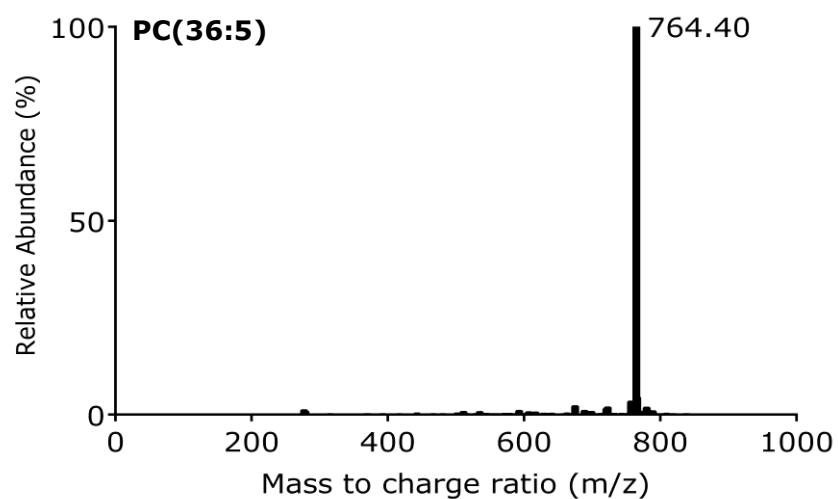
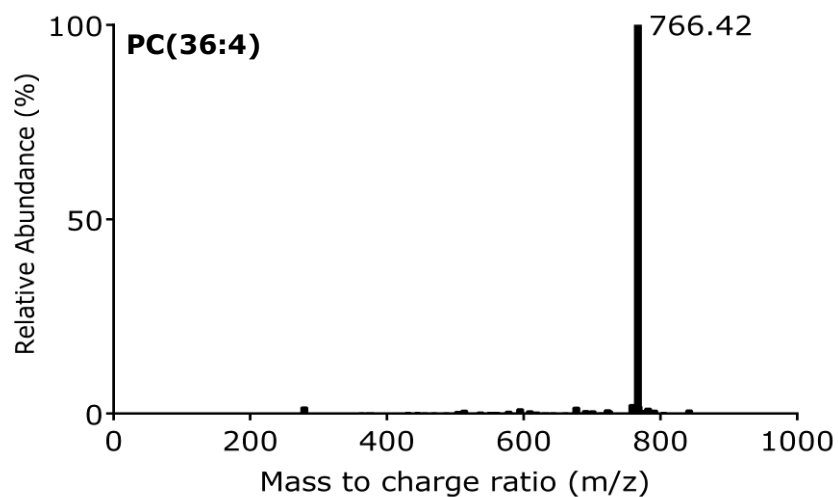


Figure 3.4. *cont.* Representative LC-MS/MS spectra of individual lipid classes. PC = phosphocholine, PE = phosphoethanolamine.

3.5 Discussion

The protocol successfully generated metabolite profiles of individual adult female *G. legneri* using both NMR and LC-MS approaches and demonstrated that these vary according to wasp age. This study also confirms that the two-step methanol/chloroform/water extraction protocol optimised in Chapter 2 is suitable for obtaining metabolome profiles of small (circa. 1 mg) individual insects (Lin et al. 2006; Wu et al. 2008). Thus this study presents for the first time the feasibility of a validated and untargeted metabolomics method suitable for both polar and non-polar metabolites of individual organisms with ultra-low biomass.

Most significantly, NMR based metabolite profiling was able to differentiate polar metabolite profiles from extracts from single individual wasps of different ages despite their low biomass. Whilst both Wong et al. (2014) and Nagato et al. (2015) considered the applicability of low biomass NMR, I know of no prior study that has established separation between NMR spectra of single low biomass organisms in response to experimental treatment without the use of a solid state NMR approach. NMR spectral features exhibited a high degree of conservation with those of previously published insect spectra (Lenz et al. 2001; Moriwaki et al. 2003; Overgaard et al. 2007; Phalaraksh et al. 2008). The general decline of carbohydrates such as glucose, glycerol and trehalose (the latter being the most abundant carbohydrate of insect hemolymph) with age indicates the loss of energy reserves. Furthermore the decline of several amino acids after seven days can be viewed as the result of starvation rather than of a temporary immediate decline due to cuticle sclerotization as shown in other insects (e.g. in mosquitoes, Mamai et al. 2014).

The LC-MS based profiling demonstrated that the main classes of metabolites that significantly varied among different age treatments were triacylglycerides and phospholipids. Triacylglycerides, along with glycogen, are considered the main energy reserves in animal cells. Triacylglycerides are the main constituent of the insect fat body (90%) and are generally synthesized from dietary carbohydrates via lipogenesis (Arrese and Soulages 2010). Lipids, and their allocation to eggs and metabolic maintenance, have been studied holistically in insect parasitoids (Jervis et al. 2008). As

previously outlined parasitoids lack the ability to synthesize lipids from dietary intake (Visser et al. 2010). These results demonstrate a gradual decline in triacylglyceride levels, consistent with reported patterns of fat reserve decline in starved parasitoids (Casas et al. 2005; Jervis et al. 2008; Visser and Ellers 2008). Diacylglyceride intensities did not demonstrate any significant changes in response to aging. Diacylglycerides are the immediate breakdown products of triacylglycerides during lipid mobilisation, thus it could be expected that temporary starvation would induce an elevation in their levels, although prolonged starvation would lead to their ultimate depletion (Arrese and Soulages 2010). Nonetheless, their levels were largely similar among age treatments. Lysophospholipids are important signalling molecules (Rivera and Chun 2006) whereas glycerophospholipids are structural components in cell membranes (van Meer et al. 2008). Whilst the former declined in abundance in older parasitoids, the latter were elevated in older parasitoids, probably as result of cellular membrane breakdown within the fat body.

Of particular concern in generating metabolite profiles of whole organisms is the unpredictable composition of the gut (i.e., its contents), which is also a considerable limitation in the case of dietary investigations. Thus another aspect that might become important in working with small organisms is whether whole body extracts pose a limitation for the integrity of the study as dietary gut contents introduce unpredictable sources of variation whenever the influence of nutrition on the insect metabolome is explored. In such cases a biofluid extraction approach could assess the effect of experimental treatments without directly analysing current gut components, though this does not completely eliminate the possibility of dietary metabolite fluctuations. *Drosophilid* fly hemolymph has previously been utilised for metabolomic analysis, however difficulties in yielding a large sample volume for single flies has required sample pooling for experimental analysis (Košťál et al. 2011a, 2012). Whilst this chapter concerned starved individuals, Chapter 4 further explores how different diets can affect the practicability of this methodology.

There has also been tremendous interest in developing methodology for determining the age of individual insects, particularly for species of medical importance, such as mosquitoes (Hayes and Wall 1999). Considerable effort

has gone into molecular approaches, particularly transcriptomics and proteomics (Cook et al. 2006, 2007; Caragata et al. 2011; Wang et al. 2013; Hugo et al. 2013). The later approach, however, relies on the existence of sequenced genomes, a pre-requisite that is not required for an equivalent metabolomic analysis. Metabolomics has been used for developing biomarkers of aging in a variety of model organisms, such as *C. elegans* and *Drosophila* (reviewed in Mishur and Rea 2011). However, studies on these organisms have relied on pooled samples rather than screening the metabolome profiles of individuals; this methodology has demonstrated that low biomass samples do not necessarily need to be pooled. A prerequisite for age biomarker development is the reliable discrimination of a suite of metabolite levels across young and old individuals. In the case of parasitoid wasps it seems that specific lipids could be targeted as biomarkers, particularly because they decline with age and because parasitoids lack the ability to synthesize them *de novo* (Visser et al. 2010).

3.6 Conclusions

The solvent extraction approach optimised in Chapter 2 was confirmed as capable of yielding reproducible metabolite-rich polar and non-polar samples from adult female *G. legneri* which have a typical biomass of approximately 1 mg. This approach successfully detected age related changes in the *G. legneri* metabolome, confirming that this tailored approach is capable of accurately detecting biological changes in low-biomass organisms. Compounds associated with energy metabolism were found to deteriorate with age, including hemolymph rich sugars and large storage glycerolipids. This validated workflow is further applied throughout this thesis to examine the effects of dietary status (Chapter 4), wasp developmental background (Chapter 5) and post-paralysis host aging (Chapter 6).

**Part 2: Host parasitoid interactions: the effects of diet,
aging and host species**

Chapter 4: The effects of nutritional status on bethylid wasp longevity, biochemistry and contest behaviour

4.1 Abstract

It is now well established that the majority of studied parasitoid wasps lack *de novo* lipogenesis, with adult lipid reserves being limited to those acquired throughout larval development. Some parasitoid species have also been shown to be capable of using external energy sources, such as simple sugars or host haemolymph, throughout adult life. A number of studies have demonstrated that carbohydrate and lipid rich diets increase parasitoid lifespan and fecundity, potentially by acting as an energy source to maintain vital metabolic processes. However, the effects of diet on the exact composition of parasitoid fat reserves, as well as parasitoid contest behaviour, remain unknown. This chapter assesses the effects of a simple carbohydrate diet on the longevity, metabolomic state and contest performance of the bethylid parasitoid wasp *Goniozus legneri*. The longevity of adult *G. legneri* provided with a honey diet was significantly increased compared to starved wasps, with honey-fed individuals displaying on average more than twice the lifespan of starved wasps. A combined NMR and LC-MS approach detected increased concentrations of common insect haemolymph sugars and amino acids in 3 day old and 7 day old honey-fed wasps, along with increased storage lipid concentrations in 7 day old honey-fed wasps. 3 day old host-fed wasps also displayed higher concentrations of fatty acyls and lysophospholipids when compared to starved wasps of the same age. However, dietary status did not significantly affect the probability of contest victory in either 3 day old or 7 day old *G. legneri*. This may be due to the enhanced RV of a contested host to a starved wasp equalising any enhancements in RHP resulting from the honey wasp diet. Alternatively, the changes in lipid composition detected between 7 day old honey-fed and starved wasps may be sizeable enough to maintain vital metabolic processes in later life, but not large enough to significantly enhance the RHP of honey-fed wasps over that of starved wasps.

4.2 Introduction

Dyadic (or pair-wise) contests for resources are a subject of longstanding interest within behavioural ecology (Maynard-Smith and Price 1973; Maynard Smith and Parker 1976; Hardy and Briffa 2013). Resolution of these contests relies on a combination of factors, including fighting ability, relatedness, the resource quality and the competitor's perceptions of their opponent (Leimar and Enquist 1984; Enquist and Leimar 1987; West et al. 2001). Bethyloid parasitoids are known to compete for the possession of hosts which are essential to their reproduction; these contests can escalate in aggressive agonistic encounters (Batchelor et al. 2005; Goubault et al. 2006, 2007, 2008; Goubault and Hardy 2007; Lizé et al. 2012; Stockermans and Hardy 2013; Hardy and Briffa 2013). The success of individual parasitoids within these agonistic contests is influenced by two major asymmetries, the respective resource holding potentials (RHP) of the contestants and the value that each contestant places on possession of the contested resource; 'resource quality' (RV) (Parker 1974; Maynard Smith and Parker 1976; Hsu et al. 2006; Kokko 2013). There may be several components contributing to RHP and RV. Components of RHP can include a contestants size, age, resource ownership and prior contest experience (Petersen and Hardy 1996; Stokkebo and Hardy 2000; Goubault et al. 2006; Humphries et al. 2006; Bentley et al. 2009). Conversely the RV of a contested host can be influenced by its size, species, larval instar, disease state and age post-paralysis (Humphries et al. 2006, Stockermans and Hardy 2013). RV can also be influenced by the physiological status of the contestants: for example, an older wasp may value a given host more highly than might a younger contestant, due to its more limited future reproductive opportunities (Humphries et al. 2006).

The nutritional status of animal contestants has previously been viewed as a major influence of contest behaviour and contest resolution (Maynard Smith and Parker 1976; Crowley et al. 1988; Marden and Rollins 1994; Riechert 1998). For example, individuals that have experienced a greater quality of juvenile or adult nutrition than others may be capable of maintaining contests for longer periods, due to their enhanced energy reserves (Poole 1989; Kemp and Alcock 2003; Martínez-Lendeck et al. 2007). Moreover an organism's physiological or metabolic state can influence the value it places on a particular resource. Individuals with depleted bodily

reserves may, within their physiological limits, be likely to fight more aggressively for a contested resource (Riechert 1998; Humphries et al. 2006). The nutritional quality of available resources has been demonstrated to influence physiological status, along with ecological interactions and individual behaviour (Davidson 1997, 1998; Raubenheimer et al. 2009, 2012). Differences in availability of macronutrients (protein, fats and carbohydrates) have been demonstrated to promote dominance-related behaviours in ants (Davidson 1997, 1998; Grover et al. 2007; Raubenheimer et al. 2009, 2012). Other metabolites are known to physiologically constrain contest behaviour. In hermit crabs, metabolites related to energy reserves (glycogen), aerobic capacity (lactic acid) and various hormones (biogenic amines) are known to influence contestant motivation and contest duration (Briffa and Elwood 2001, 2005, 2007; Briffa and Sneddon 2007). However the effects of adult nutrition on bethylid wasp contest behaviour remain unexplored.

As explored in Chapter 3, many adult parasitoids lack *de novo* lipogenesis; as a result, adults are limited to the fat reserves accumulated throughout larval development (Ellers et al. 1996; Eijs et al. 1998; Giron and Casas 2003; Casas et al. 2005; Jervis et al. 2008; Visser and Ellers 2008; Visser et al. 2010). Despite this, a number of parasitoids have been observed to acquire environmental nutrients, such as plant nectar, honey dew and host haemolymph, throughout adult life (Jervis et al. 1993; Eijs et al. 1998; Giron et al. 2002; Giron and Casas 2003; Visser and Ellers 2008). Specific strategies for the acquisition of these nutrients are known to vary depending on parasitoid life-history (Strand and Casas 2008). Many synovigenic species are capable of acquiring nutrients by feeding on host haemolymph, acquired nutrients can then be allocated to further egg production by adults (Strand and Casas 2008; Jervis et al. 2008). However, host feeding comes at a cost to the parasitoid, as the consumption of host tissues may sacrifice an immediate reproductive opportunity in favour of future opportunities (Rivero and West 2005). Previous studies have indicated that the rate of lipid reserve decline is significantly lower in some parasitoid species when provided with carbohydrate and lipid-rich diets (Ellers et al. 1996, 2011; Heimpel et al. 1997; Lee et al. 2004; Winkler et al. 2006; Wäckers et al. 2008; Desouhant et al. 2010; Gómez et al. 2012; Harvey et al. 2012). Provision of a carbohydrate diet can also result in a significantly longer lifespan (Heimpel et al. 1997; Pexton and Mayhew 2002; Lee et al. 2004; Wäckers et al. 2008;

Gómez et al. 2012), though its effects on fecundity are slightly more varied across parasitoid species (Heimpel et al. 1997; Winkler et al. 2006; Harvey et al. 2012).

As with many parasitoids, the bethylid ectoparasitoid wasp *Goniozus legneri* Gordh has previously been reported to ingest rich carbohydrate diets (Visser et al. 2010, supplementary figures). This work found that providing *G. legneri* with a carbohydrate rich diet did not translate to increased lipid reserves, indicating its lack of lipogenesis. However, Visser et al. (2010) did not present data comparing the rate of lipid loss to that of starved individuals. Unpublished work by Visser et al. (pers. comm.) and Kapranas et al. (pers. comm.) has further indicated that *G. legneri* fed with a honey diet displayed a significantly increased longevity when compared with starved wasps. This increased lifespan suggests that *G. legneri* is capable of using dietary carbohydrates as a maintenance energy supply. As a result it is hypothesised that the higher reserves of honey-fed wasps may constitute a 'de-aging' factor when compared to starved wasps. Humphries et al. (2006) previously reported that contestant success increased with age, likely due to the enhanced value of the contested host to older wasps (Stockermans and Hardy 2013). As a result it could be expected that starved contestants may possess an advantage, due to their limited reproductive opportunities providing a greater contest motivation. Conversely contestants with carbohydrate rich diets may have higher RHP due to possessing higher energy reserves.

This chapter explores how honey, a rich carbohydrate dietary source, affects metabolic energy reserves and longevity in adult female *G. legneri*. This chapter used staged intruder-intruder contests between 3 day old (approximate time of parasitoid dispersal) and 7 day old (average maximum lifespan) starved and host-fed adult female *G. legneri*. Intruder-intruder contests are employed in order to avoid potential compounding effects associated with host ownership, particularly the possibility of variable degrees of host feeding between contestants. This chapter also employs the combined NMR and LC-MS metabolomic approach optimised and validated in chapters 2 and 3. This approach was previously validated using starved wasps to avoid any potential compounding effects from variable levels of dietary intake between insects. This chapter assesses the appropriateness of

this technique for distinguishing between dietary treatments whilst also generating accurate metabolomic data. With the exception of Foray et al. (2013) and Visser et al. (2013), most studies assessing lipid levels in parasitoids utilised established assay-based approaches which do not provide extensive information as to specific lipid compositions. By employing this metabolomic approach, it is possible to detect specific changes in individual lipids between wasp dietary treatments across multiple lipid classes. This approach also has the advantage of simultaneously producing complementary polar metabolite information.

4.2.1 Aims and objectives

This chapter aims to explore whether dietary carbohydrates influence the longevity, biochemistry and contest behaviour of the Bethyloid parasitoid *G. legneri* Gordh. Specific objectives required to fulfil these aims are:

- To conduct a longevity study directly comparing the lifespans of honey-fed and starved adult female *G. legneri*.
- To stage intruder-intruder contests between 3 day and 7 day old starved and honey-fed adult female *G. legneri*.
- To perform combined NMR and LC-MS analysis of 3 day old and 7 day old starved, honey and host-fed adult female *G. legneri*.

4.3 Materials and Methods

4.3.1 Host and parasitoid rearing

Goniozus legneri were reared on larvae of *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae) by introducing an adult female wasp and a caterpillar in a glass vial (2.5 × 7.5 cm) plugged by gauze and cotton. Culturing and contest experiments were carried out in a climate room at 27 °C with a relative humidity of 60 - 70 % maintained by water bath evaporation. A light:dark cycle of 16:8 hours was maintained throughout culturing. The parasitoid strain was obtained from a commercial insectary in the USA, both host and parasitoid strains were the same as those utilised in several previous studies (Hardy et al. 2000, Goubault and Hardy 2007, Bentley et al. 2009, Lizé et al. 2012, Stockermans and Hardy 2013, Khidr et al. 2013a; 2013b; 2014). All wasps utilised were newly emerged (0 - 12 hours) from cocoons at the onset of experimentation.

4.3.2 Part 1: The effects of a carbohydrate diet on parasitoid longevity

To explore the effects of a carbohydrate diet on the longevity of an adult female parasitoid wasp, a total of 100 newly emerged wasps were isolated in 0.5 mL eppendorf vials. The inner lid of half of these vials had previously been randomly streaked with a droplet of carbohydrate rich honey. This resulted in a total of 50 honey-fed wasps and 50 starved wasps. Wasps were then maintained in isolation until death. The occupant of each vial was inspected daily for signs of parasitoid life (movement of the body in response to gentle vial shaking, movement of the mandibles or legs) until wasp death.

4.3.3 Part 2: The effects of a carbohydrate diet on parasitoid contest behaviour

To assess whether a carbohydrate rich diet (honey) affected parasitoid contest behaviour and outcome, a series of intruder-intruder contests were performed. All contests were staged under a similar experimental setup to that utilised by Lizé et al. (2012). An intruder-intruder contest design was utilised, rather than an owner-owner design, to remove the possibility of variable host feeding affecting parasitoid behaviour immediately prior to contests. A one-way design was selected; contests were staged between

honey-fed vs. starved wasps. Newly emerged female wasps were weighed to an accuracy of 0.01 mg then isolated in 0.5 mL Eppendorf vials. The inner wall of half these vials was randomly streaked in advance with a honey droplet as a source of carbohydrates. Wasps were maintained in isolation until the onset of the contest period, at either 3 days or 7 days post isolation. Two separate series of contests were staged between both 3 day old and 7 day old non-siblings (wasps emerging from different hosts), all contestants were the same age within contests. Individual wasps were closely matched by weight, with the largest difference between contests being 0.04 mg (approx. 5 % of wasp body weight). Individual females were distinguished within contest replicates by a dot of red or yellow acrylic paint on the dorsal surface of their thorax (Driessen and Hemerik 1992; Petersen and Hardy 1996).

Contests were staged in an opaque light grey plastic contest block consisting of 3 chambers connected by a slot filled with movable barriers and covered with a transparent Plexiglas lid (Petersen and Hardy 1996; Goubault et al. 2006). A pre-paralysed host of known weight, within the range 30.11 to 38.95 mg, was placed into the central chamber; contestants were placed separately in the lateral chambers. A 60 minute (min) acclimatisation period was employed, after which the barriers were withdrawn sufficiently to connect the three chambers. Contest behaviour was recorded from above for 60 minutes using a SONY HDR-CX 190 digital camcorder, from the time that the barriers were withdrawn. Wasp interactions, classified as non-aggressive, chasing, biting, stinging and full contact fighting behaviour, were recorded and the identity of any winner was noted. The identity of the contest victor was defined as the wasp that remained within the immediate vicinity of host at the end of the contest period (Petersen and Hardy 1996).

4.3.4 Part 3: The effects of dietary treatment on the metabolomic profile of parasitoid wasps

The protocol utilised during wasp contests was replicated to produce honey-fed and starved wasps. In addition a third dietary treatment, host-fed wasps, was generated by exposing adult female *G. legneri* to a pre-paralysed host (*C. cephalonica*) for 24 hours immediately after eclosion. Immediately after this, host-fed wasps were isolated in 0.5 mL eppendorf vial until either 3 or 7 days post eclosion. At either 3 or 7 days of age, starved, honey-fed or

host-fed wasps were snap frozen in liquid nitrogen then stored at -80 °C prior to solvent extraction. In 7 day old treatments a total of four starved wasps and 4 host-fed wasps died prior to snap freezing, as a result these replicates were excluded from further analysis.

The modified Bligh and Dyer (1959) biphasic extraction approach outlined in Chapter 2 was employed to generate polar and non-polar extracts of individual wasps. The generated solvent phases were separated by Pasteur pipette after extraction; polar phases were transferred to a sterile 1.5 mL solvent resistant Eppendorf (Biopur), non-polar phases were transferred to a sterile 2 mL borosilicate glass vile. Sample order was randomised prior to extraction and again before sample analysis. Solvent phases were dried and reconstituted for experimental analysis, as outlined in Chapter 2. A pooled quality control sample was generated using 10 µL of each reconstituted sample. All solvents used were of high LC-MS grade purity (CHROMASOLV Sigma-Aldrich) and were kept chilled during sample extraction. Reconstituted polar phases were analysed using NMR spectroscopy, whilst reconstituted non-polar phases were analysed by LC-MS.

4.3.4.1 NMR spectroscopy

1D NMR spectra were generated from individual parasitoid polar phases using a Bruker Avance 800 MHz III spectrometer equipped with a 5 mm QCI cryoprobe. A spectral width of 13 ppm was utilised for spectral acquisition; the spectral signal was averaged over a total 512 transients. A nosey pre-saturation experiment selectively suppressed any water resonances throughout spectral acquisition; frequency and power of the pre-saturation experiment was determined prior to experimental analysis using a set of representative wasp samples generated under the same extraction protocol. The internal D₂O signal was utilised for sample locking, with the DSS signal referenced to 0.0 ppm. Total recycling delay was 4.7 s. An exponential window function was applied to give line broadening of 0.3 Hz before zero filling and Fourier transformation.

4.3.4.2 LC-MS analysis

The lipidomic Accela LC-Exactive MS approach outlined in Chapter 2 was employed for analysis of non-polar wasp extracts. The generated pooled QC

sample was repeatedly injected into the LC-MS to pre-equilibrate the column. This sample was further injected throughout the experimental timeframe to assess column stability. As with NMR, a set of representative samples were injected in triplicate prior to experimental analysis to establish the technical stability between sample replicates. MS parameters were replicated from those employed in Chapter 3 (Ion range: 100 - 1500 m/z, ESI voltage: 3500, capillary temperature: 350 °C, scan rate: 250 ms).

Ion identities were validated by LC-MS/MS fragmentation. Remaining parasitoid non-polar extracts were pooled prior to LC-MS/MS analysis to improve ion yield. LC-MS/MS analysis replicated the analytical and instrument setup outlined in Chapter 3. Analysis was performed utilising an Accela LC coupled with an LTQ Velos Pro Dual-Pressure linear ion trap mass spectrometer (Thermo Fisher Scientific, USA). The column, mobile phases and gradient programs were replicated from the previous LC-MS analysis. An ion range of 100 - 1500 m/z was monitored (ESI voltage: 3000, capillary temperature: 275 °C, scan rate: 50 ms) and a collision energy of 40 V was employed during fragmentation.

4.3.4.3 LC-MS stability validation

To validate the stability of the LC-MS approach, the relative standard deviations of the peak areas and retention times of the key ions outlined in Chapter 2 were assessed within the pooled QC samples of both 3 day and 7 day analyses. An RSD acceptability threshold of <30 % for a minimum of 70 % of key ions was considered acceptable to establish LC-MS stability. QC samples were further required to cluster centrally within the generated PCA plots to confirm LC-MS stability. As LC-MS analysis of 3 day old *G. legneri* dietary treatments was performed alongside the wasp aging data used to validate this LC-MS approach, QC key ion peak area and retention time RSDs are identical to those previously outlined in Chapter 3.

4.3.5 Data analysis

Data generated in Parts 1 and 2 were analysed using generalized linear models with the GenStat statistical package (Version 15, VSN International, Hemel Hempstead, UK). The effects of diet on the longevity of adult parasitoids were assessed using parametric cohort survival analysis

(Crawley, 1993). This specifically assessed survival time, age-dependency and the effects of initial parasitoid weight on parasitoid longevity. Contest data from part 2 was analysed using a logistic analysis of variance (ANOVA) to determine whether dietary treatment affected the probability of a given wasp winning a contest. Size differences have been consistently found to influence *G. legneri* contest outcomes in previous studies (Goubault et al. 2006; Bentley et al. 2009). As a result the weight difference between contestants in each replicate was fitted as a continuous variable alongside the categorical factor of diet. Model simplification to the minimal adequate model was achieved by backward stepwise procedures (Crawley 1993; Hardy and Field 1998; Briffa et al., 2013).

Data from Part 3 were analysed using a combination of multivariate data analysis and generalised linear modelling. NMR/LC-MS data was aligned and binned/framed using the propriety software Sieve 2.0 (Thermo Fisher Scientific) as outlined in Chapter 2. Microsoft excel was used to baseline correct (NMR), normalise and add treatment labels to the generated top level tables. Frames/bins were normalised to either wasp/host dry weight (NMR) or total chromatogram ion count (LC-MS) before multivariate data analysis. The generated table was imported into Simca P+ (Umetrics), where preliminary sample classification was visualised through a principle components analysis (PCA). Automatic mean-centring and scaling was applied as outlined in Chapter 3. Differentiation between dietary classes was established through orthogonal partial least squares-discriminant analysis (OPLS-DA). Generated OPLS-DA models were validated through a combination of cross-validation ANOVA and through the generation of a series of predictive models as outlined in Chapter 3.

Ions were identified through weighted comparisons between experimental classes and through scrutiny of the underlying PCA loading plots across the first three principle components. Selected ions were validated by one-way binomial ANOVAs using GenStat v.15. Metabolite intensity/area was utilised as numerator and total spectral intensity/chromatogram area was utilised as denominator. As with prior parametric analysis empirically estimated scale parameters were employed where possible to account for potential over-dispersion. The Bonferroni correction was utilised to account for potential multiple-comparisons (Quinn and Keough 2002), an adjusted p-value of 0.05

was used to confirm significant differentiation in metabolite levels between treatments. A combination of the Lipidmaps, Metlin and Human Metabolome databases (HMDB) were consulted in assigning identities to significantly differing lipids, these identities were further validated through comparisons of generated LC-MS/MS data with the Lipid blast fragmentation database (Lipidblast project; Kind et al., 2013). NMR bins were identified through comparisons with 2D NMR data generated in Chapter 2.

4.4 Results

4.4.1 Part 1: The effects of a carbohydrate diet on parasitoid longevity

The majority of starved wasps died early into the second week post-eclosion, no starved wasps remained alive after 10 days had elapsed (Figure 4.1). By comparison the majority of honey-fed wasps survived into the third week, with some individuals surviving for more than 30 days post-eclosion (Figure 4.1). Survival data was initially assessed by fitting two different models of survival time: an exponential model (estimating the rate of mortality and assuming this rate remains constant over time) and a Weibull model (estimating the rate of mortality and additionally estimating variations in this rate over time). The Weibull model resulted in a significantly better fit for both starved ($G = 149.5$, d.f. = 1, $P < 0.001$) and honey-fed ($G = 117.3$, d.f. = 1, $P < 0.001$) survival times, indicating that wasp mortality increases with age (Figure 4.1). Adding individual wasp weights to this model also significantly improved the model fit for both starved ($G = 23.73$, d.f. = 2, $P < 0.001$) and honey-fed wasps ($G = 39.68$, d.f. = 2, $P < 0.001$), indicating that there is a relationship between wasp size and longevity. When a Weibull model is applied across both datasets, the addition of wasp diet to this model produces a significantly better fit ($G = 533.72$, d.f. = 4, $P < 0.001$), indicating that there is a relationship between wasp diet and longevity (Figure 4.1).

4.4.2 Part 2: The effects of a carbohydrate diet on parasitoid contest behaviour

Out of a total of 80 contests, 54 resulted in a clear contest outcome for 3 day old wasps. Out of a total of 60 contests, 31 resulted in a clear outcome for 7 day old contestants. Within these contests, the probability of *G. legneri* winning a contest was not significantly affected by diet for either 3 day ($G_1 = 2.53$, $P = 0.112$) or 7 day old wasps ($G_1 = 0.16$, $P = 0.686$). Contestant weight difference, host weight and their interaction term had no significant effect on the probability of 3 day or 7 day wasp contest success. The interactions between wasp diet and contestant weight difference and between wasp diet and host weight had no significant effect on the probability of wasp contest success for either 3 day old or 7 day old wasps. The interaction term between wasp diet, contestant weight difference and

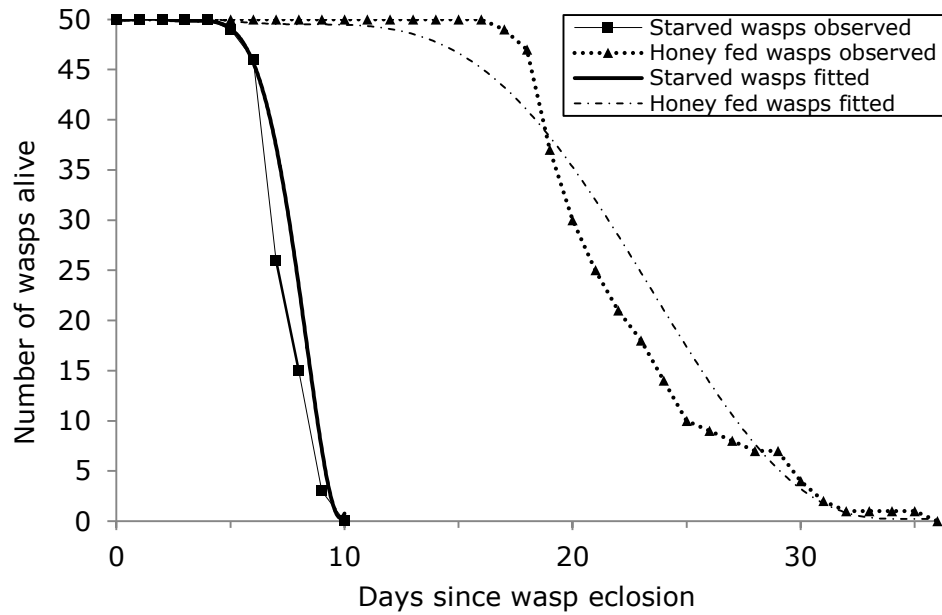


Figure 4.1. Cohort survival of starved and honey-fed *G. legneri*. By the beginning of week 2 over 50 % of starved individuals had died and by the end of week 3, 50 % of honey-fed individuals had died.

host weight had no significant effect on wasp contest success for 3 day or 7 day old wasps. Individual deviance ratios, p-values and degrees of freedom are given in Table 4.1.

4.4.3 Part 3: The effects of dietary treatment on the metabolomic profile of parasitoid wasps

4.4.3.1 NMR spectroscopy

Raw spectral comparisons of different treatment groups identified a consistent sugar signature between 4.15 - 3.2 ppm across the majority of both 3 day old and 7 day old honey-fed wasps. Comparisons of this signature with NMR spectra collected from raw honey samples found a high degree of overlap with the equivalent region in honey-fed wasps (Figure 4.2). It was concluded that this resonance overlap was most likely due to detection of honey within the intestinal tract of honey-fed wasps. A number of wasps (2 in 3 day old and 3 in 7 day old wasps) did not display these resonances, moreover these samples clustered alongside starved and host-

Table 4.1. The effects of dietary treatment on *G. legneri* contest outcome. Results are from backwards stepwise logistic ANOVA.

Source	Degrees of Freedom (3 days)	Degrees of Freedom (7 days)	G (3 days)	G (7 days)	P (3 days)	P (7 days)
Red wasp diet	1	1	2.53	0.16	0.112	0.686
Contestant weight diff	1	1	0.25	1.42	0.62	0.234
Host weight	1	1	0.04	0.34	0.844	0.562
Contestant weight diff x Red wasp diet	1	1	0.68	0.23	0.408	0.635
Host weight x Red wasp diet	1	1	0.75	0.32	0.385	0.569
Contestant weight diff x Host weight	1	1	3.79	1.21	0.052	0.272
Contestant weight diff x Host weight x Red wasp diet	1	1	0.59	1.84	0.443	0.175
Residual	46	23				
Total	53	30				

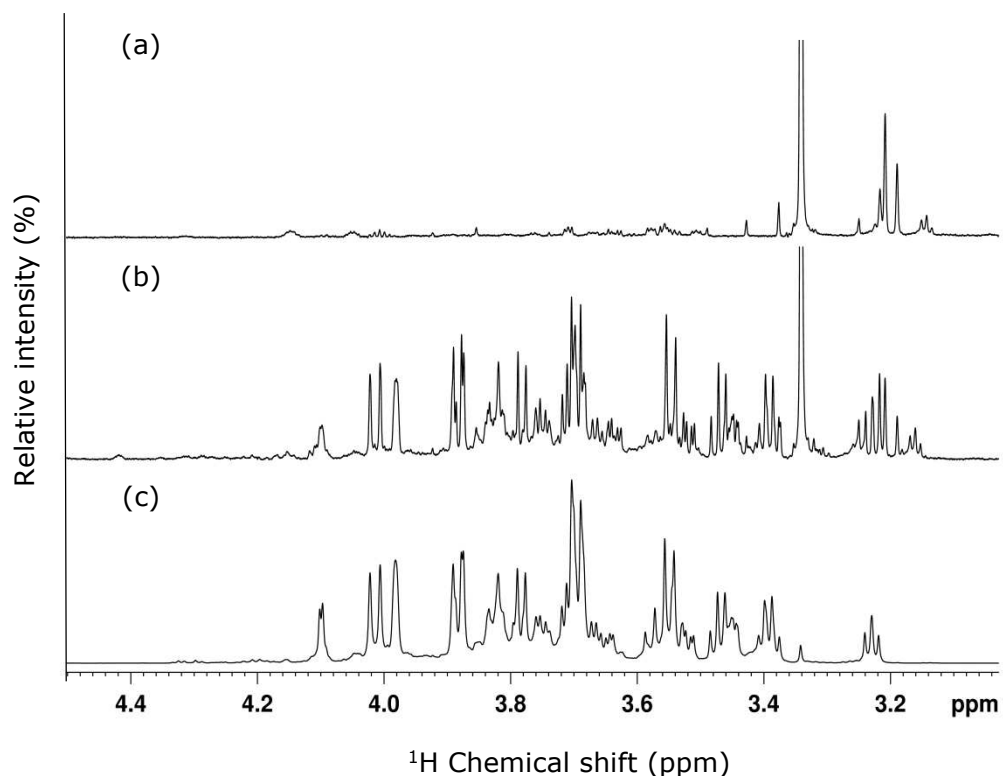


Figure 4.2. Comparisons of wasp NMR spectral region 4.5 - 3.0 ppm (a) Representative 7 day old starved wasp spectra (b) Representative 7 day old honey-fed wasp spectra (c) raw honey spectra. The presence of highly conserved honey resonances was considered to validate ingestion of the honey diet.

fed wasps in PCA score plots. It was concluded that this lack of signal was due to the lack of ingestion of the honey-fed diet by these wasps. Due to their unrepresentative nature of the honey-fed treatment group, these samples were removed from further NMR and LC-MS data analysis.

Analysis of the generated binned NMR PCA scores plots indicated clear separation between honey-fed and starved/host-fed treatments for both 3 day old and 7 day old wasps (Figure 4.3a and 4.4a). This separation was successfully validated by OPLS-DA (Figure 4.3b and 4.4b). Validation of the 3 day old wasp OPLS-DA model by cross validation (CV) ANOVA resulted in a p-value of 1.45×10^{-11} after Bonferroni correction. The generated predictive models gave an average correct prediction rate of 96% and an average Fisher's exact probability of <0.000001 . Cross validation of the 7 day old wasp model by CV ANOVA resulted in a p-value of 6.14×10^{-5} after

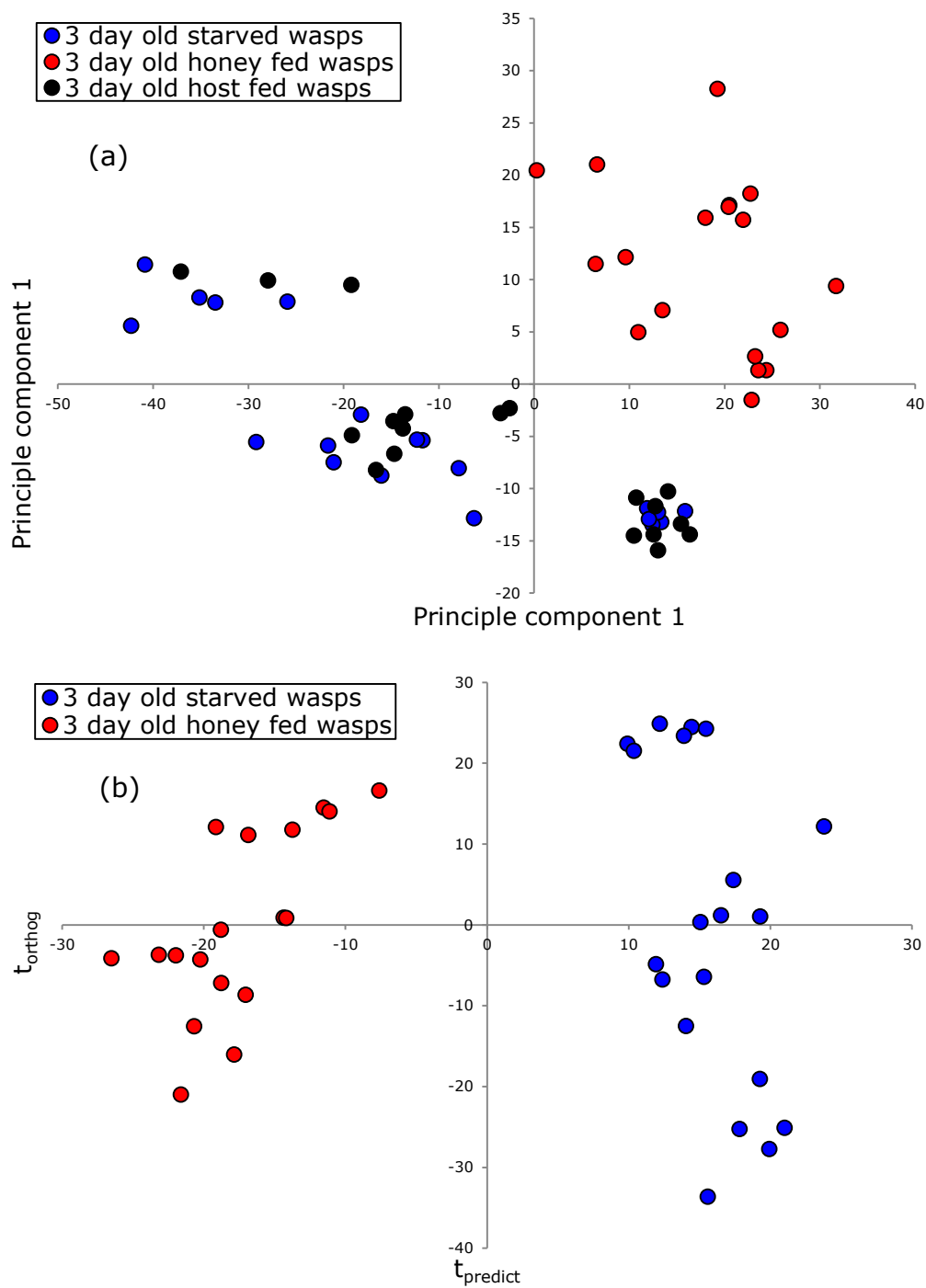


Figure 4.3. Principle components analysis of 3 day old dietary wasp NMR spectra (a) PCA of NMR samples of 3 day old starved, honey-fed and host-fed (PC1 = 53.3 %, PC2 = 14.3 %, PC3 = 6.07 %, $R^2X = 0.803$) (b) OPLS-DA analysis of NMR samples of 3 day old starved and honey-fed wasps ($R^2X = 0.718$, $Q^2 = 0.906$).

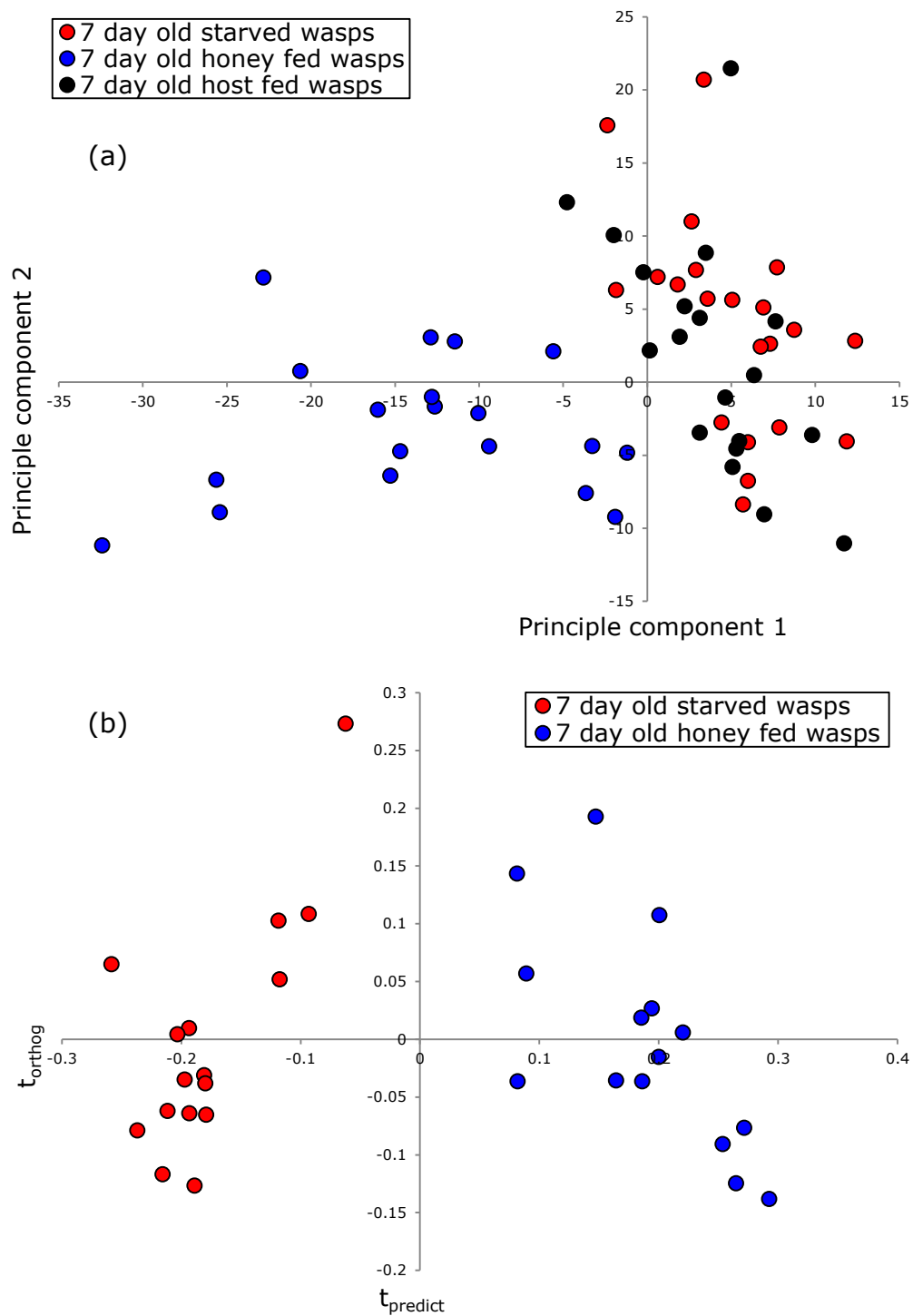


Figure 4.4. Principle components analysis of 7 day old dietary wasp NMR spectra (a) PCA of NMR samples of 7 day old starved, honey-fed and host-fed wasps (PC1 = 19.1 %, PC2 = 7.7 %, PC3 = 7.3 %, $R^2X = 0.582$), including control samples (b) OPLS-DA analysis of NMR samples of 7 day old starved and honey-fed wasps ($R^2X = 0.613$, $Q^2 = 0.803$).

Bonferroni correction. Generated predictive models had a total correct prediction rate of 92.5 %, along with a Fisher's exact probability of <0.00001. No separation was apparent between host-fed and starved wasps for either 3 day old wasps or 7 day old wasps (Figure 4.3 and 4.4). A total of 74 bins were associated with honey-fed wasps, compared to 6 associated with starved wasps, when comparing 3 day old wasp treatments. All 74 bins associated with honey-fed wasps indicated significantly higher intensities than the equivalent bins in starved wasps; similarly all 6 bins associated with starved wasps indicated higher intensities than honey-fed wasps ($p < 0.01$). Spectral identities were assignable to a total 29 honey-fed associated bins, remaining bins consisted of 40 unknown spectral peaks and 4 unknown lipid peaks; 7 bins were further identified as baseline fluctuations. When considering 7 day treatments, 72 bins were associated with honey-fed wasps and 7 were associated with starved wasps. Of these, 60 bins were significantly higher in honey wasps, whilst 2 bins were significantly higher in starved wasps. Spectral identities were assigned to 30 honey-fed associated bins, with remaining bins consisting of 28 unknown peaks and 1 unknown lipid. Honey-fed associated bins included common insect hemolymph sugars, along with a number of amino acids that varied between 3 day and 7 day treatments. All bins associated with starved wasp treatments were identified as baseline fluctuations. Significantly elevated metabolite levels for 3 day old and 7 day old NMR spectra are displayed in Figure 4.5. Bin identities, chemical shift assignments, chemical formulae, F ratio, p -value and fold changes are given in Table 4.2.

4.4.3.2 LC-MS

PCA visualisations of 3 day old wasp LC-MS data resulted in a generated model with a goodness of fit (R^2X) of 85.6 % for wasp dietary treatments. Comparisons of the associated PCA scores plots indicated poor separation between 3 day old honey-fed and starved treatments (Figure 4.6a). OPLS-DA comparisons between treatments resulted in a generated model with a poor R^2X (0.465) and Q^2 (0.475). Predictive modelling resulted in a low correct prediction rate (71.5 %) and a low Fisher's probability of 0.20. From this combination of poor separation and lack of predictive accuracy it was concluded that no significant separation was present between 3 day starved and honey-fed wasps. A degree of separation was apparent

Table 4.2. Summary of polar biomarkers with tentative identities that significantly differ between starved wasps and host-fed wasp extracts at 3 days old and 7 days old. NS = not significant for this treatment.

Common Name	1D Chemical shift (ppm)	Formula	d.f. (3 days)	d.f. (7 days)	F ratio (3 days)	F ratio (7 days)	P-value (B) (3 days)	P-value (B) (7 days)	Fold change ¹ (3 days)	Fold Change ¹ (7 days)
α-Glucose	3.45 (t), 3.52 (dd), 3.70 (t), 3.76 (m), 3.82 (ddd), 3.83 (m), 5.22 (d)	C ₆ H ₁₂ O ₆	2, 57	2, 47	117.08	40.34	<0.01	<0.01	-6.00	-113.89
Arginine	1.67 (m), 1.91 (m), 3.23 (t), 3.76 (t)	C ₆ H ₁₄ N ₄ O ₂	2, 57	2, 47	NS	22.02	NS	<0.01	NS	-3.21
Glycerol	3.55 (m), 3.64 (m), 3.77 (tt)	C ₃ H ₈ O ₃	2, 57	2, 47	126.57	37.79	<0.01	<0.01	-7.32	-46.52
Glycine-Betaine	3.54 (s)	C ₂ H ₅ NO ₂	2, 57	2, 47	93.31	32.48	<0.01	<0.01	-6.64	-24.38
Proline	2.01 (m), 2.08 (m), 2.35 (m), 3.33 (dt), 3.42 (dt), 4.12 (dd)	C ₅ H ₉ NO ₂	2, 57	2, 47	46.06	21.89	<0.01	<0.01	-4.34	-15.45
β-Glucose	3.24 (dd), 3.4 (m), 3.47 (m), 3.74 (m), 3.88 (dd), 4.64 (d)	C ₆ H ₁₂ O ₆	2, 57	2, 47	114.46	36.25	<0.01	<0.01	-8.29	-103.04
Taurine	3.23 (t), 3.39 (t)	C ₂ H ₇ NO ₃ S	2, 57	2, 47	NS	31.22	NS	<0.01	NS	-11.96
Trehalose	3.44 (t), 3.64 (dd), 3.75 (m), 3.82 (m), 3.84 (m), 3.86 (m), 5.19 (d)	C ₁₂ H ₂₂ O ₁₁	2, 57	2, 47	109.45	42.63	<0.01	<0.01	-6.00	-1109.08
Tyrosine	3.04 (dd), 3.18 (dd), 3.92 (dd), 6.89 (d), 7.19 (d)	C ₉ H ₁₁ NO ₃	2, 57	2, 47	66.55	NS	<0.01	NS	-4.84	NS
Valine	0.99 (d), 1.04 (d), 2.34 (m), 3.6 (d)	C ₅ H ₁₁ NO ₂	2, 57	2, 47	73.06	26.96	<0.01	<0.01	-3.16	-10.62

¹ Negative fold changes indicate metabolites that were more abundant in honey-fed wasps; positive fold changes indicate metabolites that were more abundant in starved wasps.

between host-fed and starved 3 day old wasps. Significant separation between these treatments was established by OPLS-DA analysis (Figure 4.6b). Predictive modelling resulted in an acceptable correct prediction rate of 88 % with a Fisher's exact probability <0.001 . Further parametric validation of treatment associated weighted scores plots by logistic ANOVA (d.f. = 1, 57 $P < 0.05$) resulted in a total of 76 significant bins between 3 day old starved and host-fed wasps. Of these bins, 45 were successfully identified and validated by LC-MS/MS. Due to the presence of multiple adducts this corresponded to a total of 28 unique lipids. Lipid identities comprised of 3 fatty acyls, 12 lysophospholipids and 13 phospholipids. Lysophospholipids comprised of 7 lysophosphatidylethanolamines and 5 lysophosphatidylcholines. Phospholipids comprised of 4 phosphoethanolamines, 6 phosphocholines and 3 phosphatidylglycerols. Specific lipid categories separated by treatment; fatty acyls and lysophospholipids were elevated in host-fed wasps, whereas phospholipids were elevated in starved wasps. Individual mass to charge ratios, identities, elution times, adducts, F values and fold changes of significant metabolites are given in Table 4.3. Metabolite levels for individual lipids are outlined in Figure 4.7.

PCA visualisations of 7 day old wasp LC-MS data indicated a degree of separation between honey-fed and starved wasp extracts (Figure 4.8a). Host-fed wasp extracts clustered with, and did not significantly separate from, starved wasp extracts (Figure 4.8a). Further analysis by OPLS-DA established significant separation between 7 day old starved and honey-fed wasps (Figure 4.8b). Cross validation of the generated OPLS-DA model resulted in an average correct prediction rate of 92.5% ($p < 0.001$). Further validation of weighted PCA scores plots by logistic ANOVA (d.f. = 1, 47 $p < 0.05$) generated a total of 67 ions that significantly differed between 7 day old starved and honey-fed wasps. Of these, 40 ions were assigned tentative identities through metabolite database comparisons. 37 ion identities were successfully validated by LC-MS/MS, due to the presence of multiple adducts this corresponded to 21 unique lipids. Lipid identities consisted of 5 lysophospholipids, 3 diacylglycerides, 1 phospholipid and 12 triacylglycerides. Within classes, lysophospholipids consisted of 2 lysophosphatidylethanolamines, 3 lysophosphatidylcholines, and the single treatment associated phospholipid was a phosphocholine. All 21 lipids were

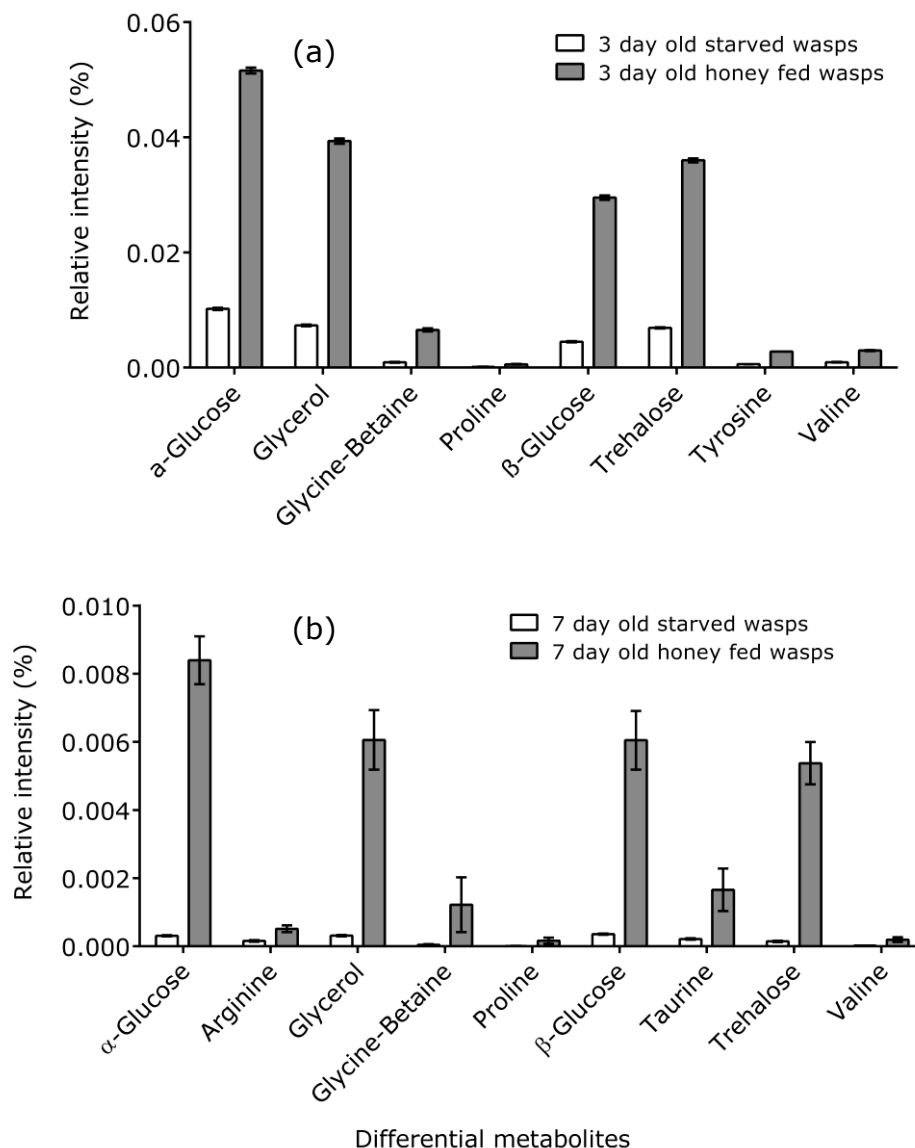


Figure 4.5. Polar metabolite differences between starved and honey-fed *G. legneri* (a) metabolite differences between 3 day old starved and 3 day old honey-fed *G. legneri* (b) metabolite differences between 7 day old starved and 7 day old honey-fed *G. legneri*. The displayed values consist of the mean normalised metabolite area, error bars show standard error.

elevated in honey-fed wasps. Individual mass to charge ratios, identities, elution times, adducts, *F* values and fold changes of significant metabolites are outlined in Table 4.4. Metabolite levels for each lipid category are displayed in Figure 4.9.

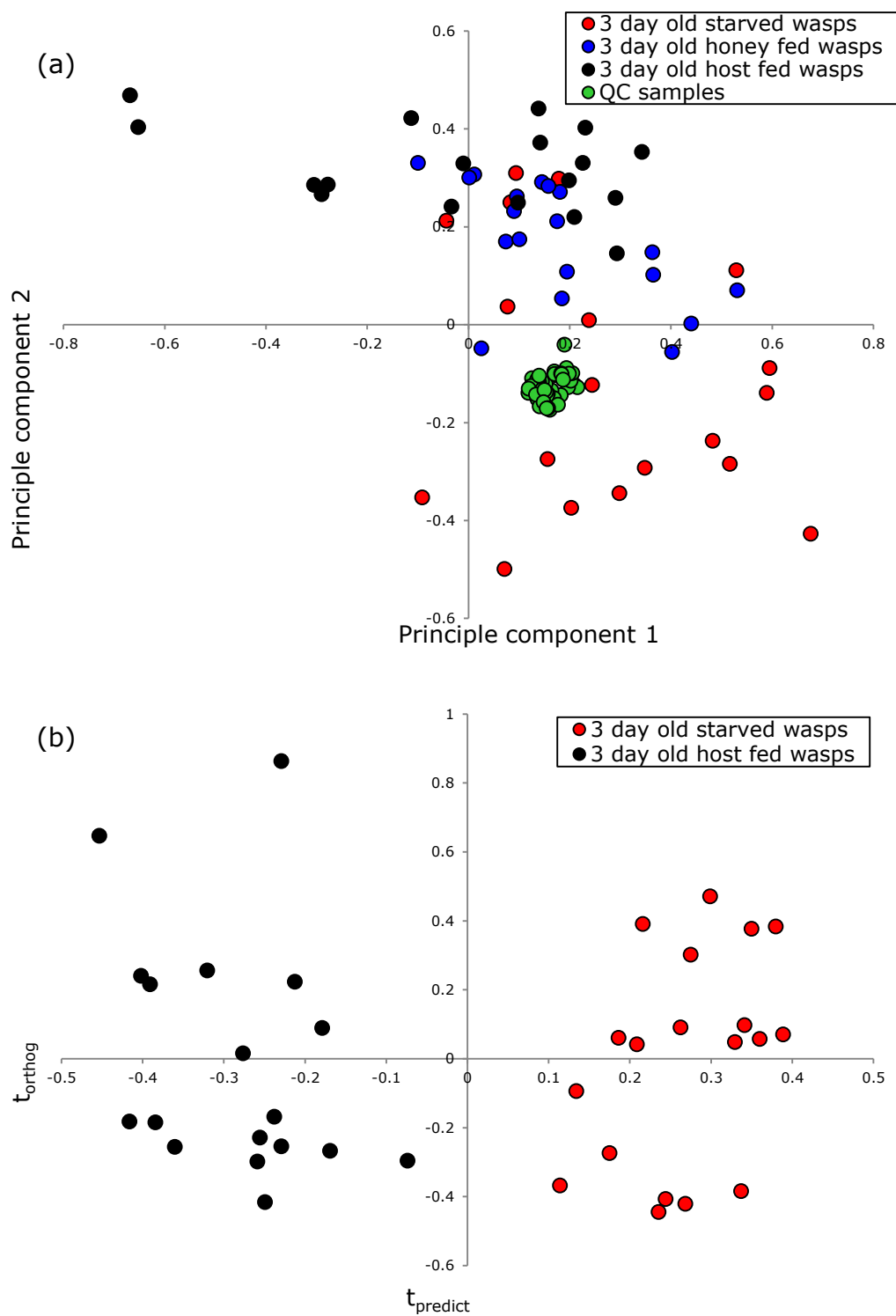


Figure 4.6. Principle components analysis of 3 day old dietary wasp LC-MS chromatograms (a) PCA of LC-MS samples of 3 day old starved, honey-fed and host-fed wasps (PC1 = 25 %, PC2 = 13.8 %, PC 3 = 11.3 %, $R^2X = 0.856$) (b) OPLS-DA of LC-MS samples of 3 day old starved and host-fed wasps ($R^2X = 0.578$, $Q^2 = 0.686$).

Table 4.3. Summary of non-polar biomarkers with tentative identities that significantly differ between 3 day old starved wasps and 3 day old host-fed wasp extracts. PE = phosphoethanolamine, PC = phosphocholine.

<i>M/Z</i>	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Fold change ¹	Mass error (ppm)
253.217	2.14	M-H	Negative	Palmitoleic acid	C ₁₆ H ₃₀ O ₂	2, 55	18.59	<0.01	-2.34	0.304
277.218	1.41	M-H	Negative	Linolenic Acid	C ₁₈ H ₃₀ O ₂	2, 55	19.25	<0.01	-2.09	0.696
281.249	2.82	M-H	Negative	Oleic Acid	C ₁₈ H ₃₄ O ₂	2, 55	13.95	<0.05	-1.79	0.396
474.263	1.44	M+H	Positive	LysoPE(18:3/0:0)	C ₂₃ H ₄₂ NO ₇ P	2, 55	17.04	<0.01	-1.98	0.387
476.279	1.74	M+H	Positive	LysoPE(18:2/0:0)	C ₂₃ H ₄₄ NO ₇ P	2, 55	14.3	<0.05	-1.76	1.263
478.294	2.13	M+H, M-H	Positive	LysoPE(18:1/0:0)	C ₂₁ H ₄₆ NO ₇ P	2, 55	14.99	<0.01	-1.77	0.087
494.324	1.50	M+H, M+Na	Positive	LysoPC(0.0/16.1)	C ₂₄ H ₄₈ NO ₇ P	2, 55	24.39	<0.01	-2.48	0.115
496.34	1.96	M+H, M+Na	Positive	LysoPC(0.0/16.0)	C ₂₄ H ₅₀ NO ₇ P	2, 55	15.83	<0.01	-1.76	0.235
502.294	1.34	M-H	Negative	LysoPE(20:3/0:0)	C ₂₅ H ₄₆ NO ₇ P	2, 55	19.65	<0.01	-1.82	0.087
504.31	1.68	M-H	Negative	LysoPE(0:0/20:2)	C ₂₅ H ₄₈ NO ₇ P	2, 55	16.74	<0.01	-1.76	0.437
506.325	2.05	M-H	Negative	LysoPE(20:1/0:0)	C ₂₅ H ₅₀ NO ₇ P	2, 55	18.19	<0.01	-1.80	0.213
518.324	1.35	M+H, M+Na, M+K	Positive	LysoPC(18:3)	C ₂₆ H ₄₈ NO ₇ P	2, 55	22.3	<0.01	-2.10	0.115
520.34	1.68	M+H, M+Na, 2M+Na	Positive	LysoPC(18:2)	C ₂₆ H ₅₀ NO ₇ P	2, 55	16.8	<0.01	-1.80	0.235
522.355	2.09	M+H, M+Na	Positive	LysoPC(18:1)	C ₂₆ H ₅₂ NO ₇ P	2, 55	18.84	<0.01	-1.82	0.416
545.34	2.05	M+NH ₄	Positive	LysoPE(0:0/22:5)	C ₂₇ H ₄₆ NO ₇ P	2, 55	16.01	<0.01	-2.37	4.988
740.525	4.68	M-H	Negative	PE(36:3)	C ₄₁ H ₇₆ NO ₈ P	2, 55	14.23	<0.05	33.15	1.421
742.54	5.01	M-H	Negative	PE(36:2)	C ₄₁ H ₇₈ NO ₇ P	2, 55	17.88	<0.01	14.13	0.771
744.553	4.88	M-H	Negative	PE(36:1)	C ₄₁ H ₈₀ NO ₈ P	2, 55	18.91	<0.01	11.93	1.879
756.553	4.37	M-H, M+H, M+Na	Negative	PC(36:2)	C ₄₂ H ₈₀ NO ₈ P	2, 55	20.35	<0.01	28.19	1.879

¹ Negative fold changes indicate metabolites that were more abundant in host-fed wasps; positive fold changes indicate metabolites that were more abundant in starved wasps.

Table 4.3. *Cont.* Summary of non-polar biomarkers with tentative identities that significantly differ between 3 day old starved wasps and 3 day old host-fed wasp extracts. PE = phosphoethanolamine, PC = phosphocholine.

M/Z	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Fold change¹	Mass error (ppm)
760.584	5.27	M+H	Positive	PE(37:1)	C ₄₂ H ₈₂ NO ₈ P	2, 55	29.07	<0.01	42.61	1.081
766.54	4.38	M+Na	Positive	PC(33:2)	C ₄₁ H ₇₈ NO ₈ P	2, 55	21.43	<0.05	42.16	1.51
780.551	4.67	M+Na	Positive	PC(34:2)	C ₄₂ H ₈₀ NO ₈ P	2, 55	23.53	<0.01	38.70	0.378
782.568	4.32	M+Na	Positive	PC(34:1)	C ₄₂ H ₈₂ NO ₈ P	2, 55	18.44	<0.01	37.18	0.977
784.583	4.82	M+Na	Positive	PC(34:0)	C ₄₂ H ₈₄ NO ₈ P	2, 55	21.9	<0.01	37.44	0.327
804.55	4.32	M+Na	Positive	PC(36:4)	C ₄₄ H ₈₀ NO ₈ P	2, 55	21.55	<0.01	37.55	1.035
816.577	4.72	M+NH ₄	Positive	PG(38:4)	C ₄₄ H ₇₉ O ₁₀ P	2, 55	23.24	<0.01	47.79	2.092
840.576	4.38	M+NH ₄	Positive	PG(40:6)	C ₄₆ H ₇₉ O ₁₀ P	2, 55	23.26	<0.01	2.05	1.092
842.592	4.72	M+NH ₄	Positive	PG(40:5)	C ₄₆ H ₈₁ O ₁₀ P	2, 55	21.04	<0.01	58.38	1.442
1062.66	1.64	2M+3H ₂ O+2H	Positive	LysoPC(18:3)	C ₂₃ H ₄₂ NO ₇ P	2, 55	16.59	<0.01	1.91	3.201

¹ Negative fold changes indicate metabolites that were more abundant in host-fed wasps; positive fold changes indicate metabolites that were more abundant in starved wasps

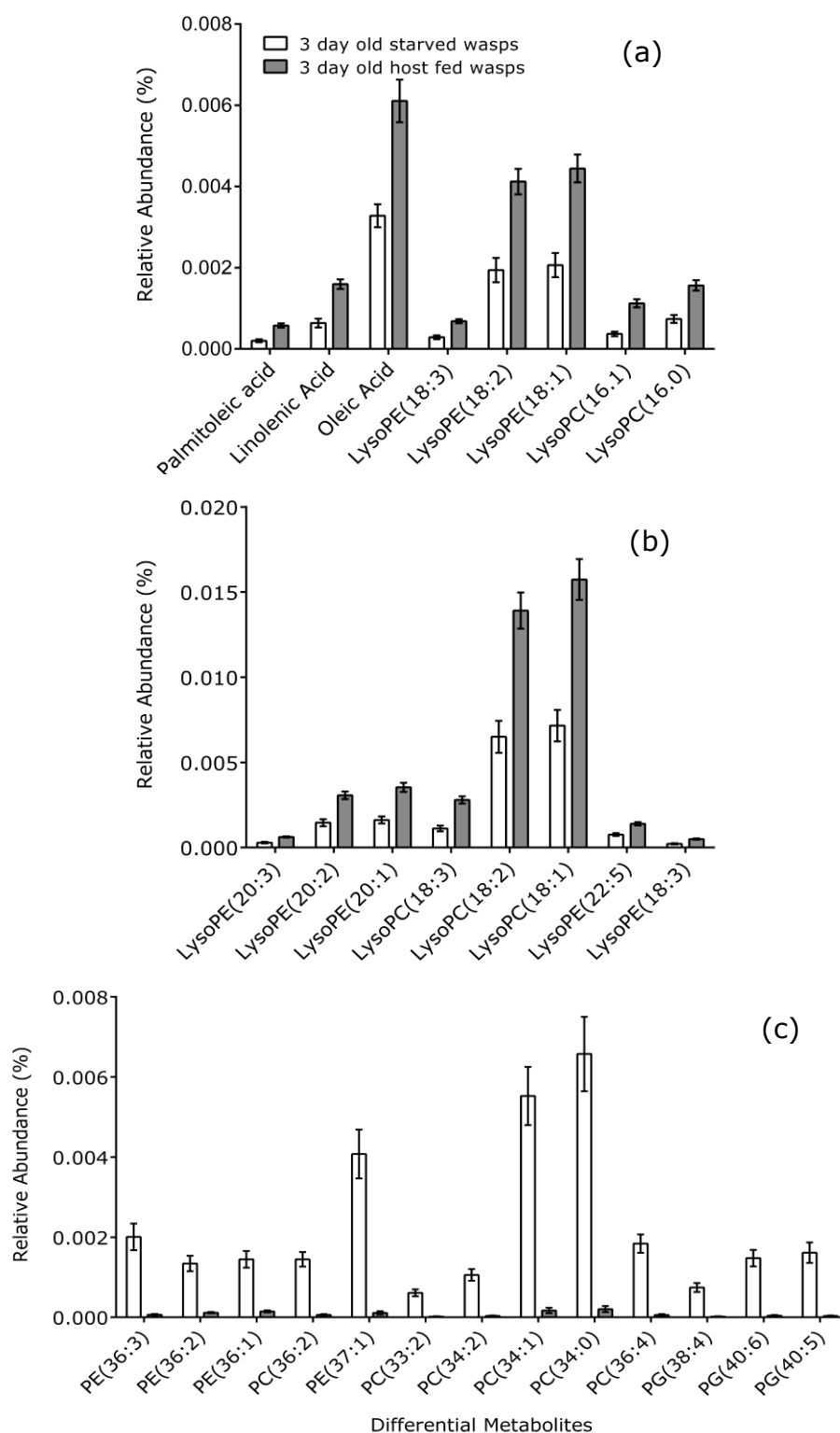


Figure 4.7. Non-polar metabolite differences between 3 day old starved and 3 day old host-fed *G. legneri* (a) Fatty acyls and lysophospholipids (b) lysophospholipids (c) Phospholipids. PE = phosphoethanolamine, PC = phosphocholine, PG = phosphatidylglycerol. The displayed values consist of the mean normalised metabolite area, error bars show standard error.

QC samples for both 3 day old and 7 day old LC-MS samples clustered centrally between treatment groups and exhibited very little drift over time (Figure 4.5a and 4.7a). Assessment of the peak areas of key ions within 3 day old wasp QC samples found that 88.24 % of ions displayed RSDs within the acceptability threshold for analytical stability. QC ions displayed an average peak area RSD of 14.05 % with a range of 2.62 – 51.39 % and an average retention time RSD of 0.9 % with a range of 0.14 – 4.3 %. Assessment of retention time RSDs within QC samples found that 100 % of ions displayed RSDs within the acceptability threshold for experimental stability. 7 day old wasp QC samples demonstrated an average key ion peak area RSD of 15.28 %, with a range of 3.02 – 29.00 %. Retention time RSDs averaged 1.43 % with a range of 0.32 – 7.38 %. In total 90.9 % of key ions RSDs and 100 % of retention time RSDs were within the acceptability threshold.

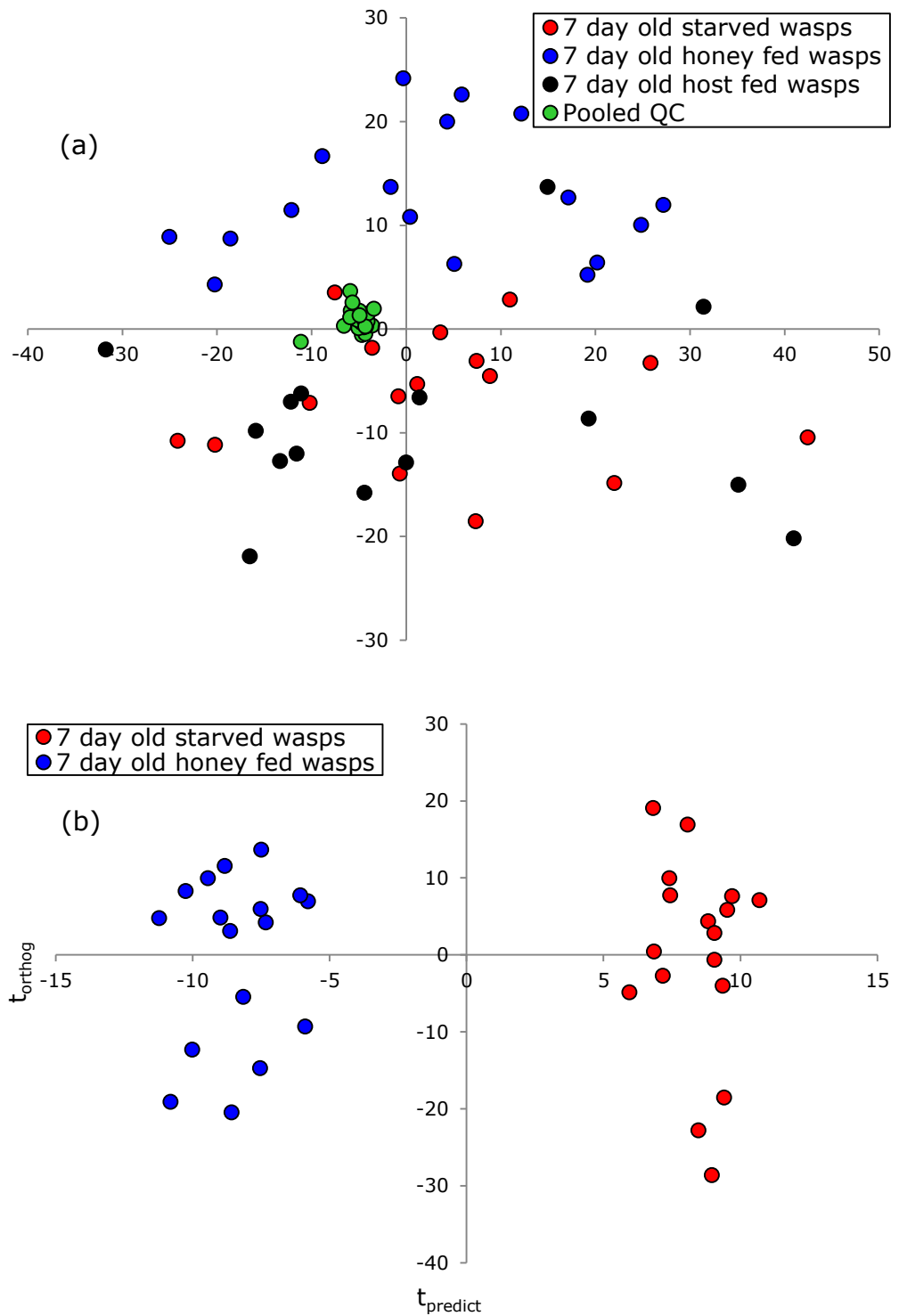


Figure 4.8. Principle components analysis of 7 day old dietary wasp LC-MS chromatograms (a) PCA of LC-MS samples of 7 day old starved, honey-fed and host-fed wasps (PC1 = 37.1 %, PC2 = 16.2 %, PC 3 = 5.86 %, $R^2X = 0.718$) (b) OPLS-DA of LC-MS samples of 7 day old starved and honey-fed wasps ($R^2X = 0.588$, $Q^2 = 0.653$).

Table 4.4. Summary of non-polar biomarkers with tentative identities that significantly differ between 7 day old starved wasps and 7 day old honey-fed wasp extracts. PE = phosphoethanolamine, PC = phosphocholine, DG = diacylglyceride, TG = triacylglyceride.

<i>M/Z</i>	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Fold change ¹	Mass error (ppm)
480.308	2.01	M+H	Positive	LysoPE(0:0/18:1)	C ₂₃ H ₄₆ NO ₇ P	2, 47	16.81	<0.01	-1.80	0.425
494.324	1.44	M+H	Positive	LysoPC(0.0/16.1)	C ₂₄ H ₄₈ NO ₇ P	2, 47	19.8	<0.01	-2.73	0.115
502.29	2.04	M+H	Positive	LysoPE(18:1/0:0)	C ₂₃ H ₄₆ NO ₇ P	2, 47	14.9	<0.05	-1.62	0.407
522.355	1.98	M+H, M+Na	Positive	LysoPC(0:0/18:1)	C ₂₆ H ₅₂ NO ₇ P	2, 47	16.94	<0.01	-2.08	0.416
548.37	2.09	M+H	Positive	LysoPC(20:2/0:0)	C ₂₈ H ₅₄ NO ₇ P	2, 47	36.82	<0.01	-3.79	1.066
659.522	5.76	M+CH ₃ OH+H	Positive	DG(37:6(22:6/15:0/0:0))	C ₄₀ H ₆₆ O ₅	2, 47	20.34	<0.01	-7.78	2.514
675.496	5.76	M+Na	Positive	DG(40:7(20:2/0:0/20:5))	C ₄₂ H ₆₈ O ₅	2, 47	30.9	<0.01	-19.04	0.107
687.553	6.33	M+CH ₃ OH+H	Positive	DG(38:6(18:2/0:0/20:4))	C ₄₂ H ₇₀ O ₅	2, 47	13.37	<0.05	-4.35	2.814
743.616	7.37	M+Na	Positive	TG(42:1(14:0/14:1/14:0))	C ₄₅ H ₈₄ O ₆	2, 47	14.35	<0.05	-3.41	0.008
766.691	7.88	M+NH ₄ , M+Na, M+K	Positive	TG(44:1(15:0/14:1/15:0))	C ₄₇ H ₈₈ O ₆	2, 47	25.1	<0.01	-7.02	0.914
769.631	7.21	M+Na	Positive	TG(44:2(14:0/14:1/16:1))	C ₄₇ H ₈₆ O ₆	2, 47	16.67	<0.01	-6.12	0.658
770.606	5.89	M+H	Positive	PC(36:2)	C ₄₄ H ₈₄ NO ₇ P	2, 47	14.6	<0.05	-1.65	0.183
792.707	7.87	M+NH ₄	Positive	TG(42:2(12:0/18.1/16.1))	C ₄₉ H ₉₀ O ₆	2, 47	27.73	<0.01	-5.92	0.564
794.722	8.98	M+NH ₄ , M+Na, M+K	Positive	TG(46:1(14:0/16.0/16.1))	C ₄₉ H ₉₂ O ₆	2, 47	32.38	<0.01	-14.30	1.214
797.663	7.97	M+Na	Positive	TG(46:2(14:0/14:1/18:1))	C ₄₉ H ₉₀ O ₆	2, 47	28.29	<0.01	-5.86	0.041
818.723	7.86	M+NH ₄ , M+Na	Positive	TG(48:3(14:1/16.1/18.1))	C ₅₁ H ₉₂ O ₆	2, 47	21.92	<0.01	-3.87	0.214
820.739	8.94	M+NH ₄ , M+Na, M+K	Positive	TG(48:2(16:0/16.1/16.1))	C ₅₁ H ₉₄ O ₆	2, 47	20.86	<0.01	-6.59	0.136
844.739	8.19	M+NH ₄ , M+Na, M+K	Positive	TG(50:4(16:0/16.1/18.3))	C ₅₃ H ₉₄ O ₆	2, 47	15.35	<0.05	-3.90	0.136

¹ Negative fold changes indicate metabolites that were more abundant in honey-fed wasps; positive fold changes indicate metabolites that were more abundant in starved wasps.

Table 4.4. *Cont.* Summary of non-polar biomarkers with tentative identities that significantly differ between 7 day old starved wasps and 7 day old honey-fed wasp extracts. TG = triacylglyceride.

M/Z	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Fold change¹	Mass error (ppm)
846.753	8.92	M+NH ₄ , M+K	Positive	TG(50:3(16:0/16.1/18.2))	C ₅₃ H ₉₆ O ₆	2, 47	13.76	<0.05	-3.93	1.514
863.653	7.27	M+K	Positive	TG(50:5(14:1/18:3/18:1))	C ₅₃ H ₉₂ O ₆	2, 47	14.52	<0.05	-3.17	0.451
887.781	8.92	M+K	Positive	TG(50:0(17:0/18:3/18:3))	C ₅₅ H ₁₀₈ O ₅	2, 47	13.85	<0.05	-2.61	1.835

¹ Negative fold changes indicate metabolites that were more abundant in honey-fed wasps; positive fold changes indicate metabolites that were more abundant in starved wasps.

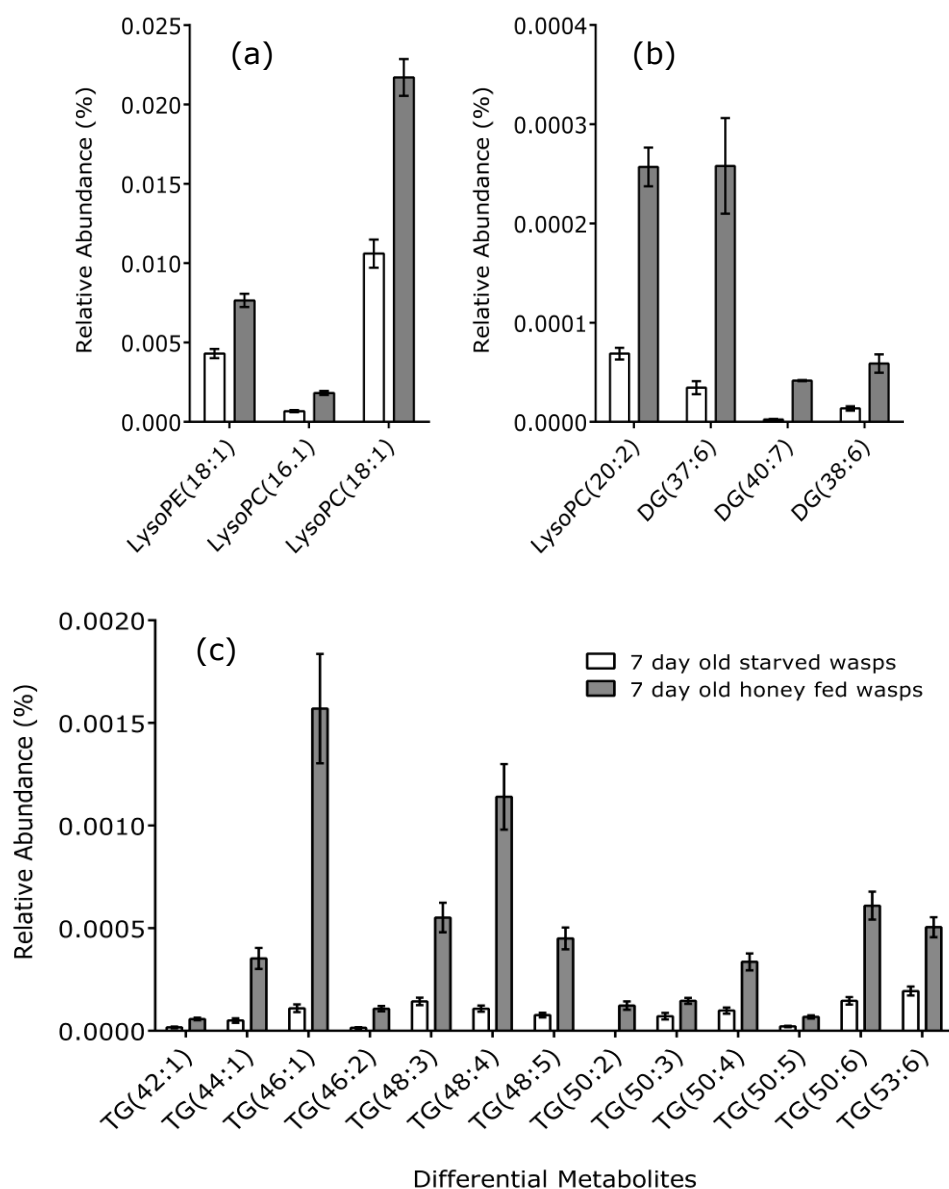


Figure 4.9. Non-polar metabolite differences between 7 day old starved and 7 day old host-fed *G. legneri* (a) Fatty acyls and lysophospholipids (b) lysophospholipids (c) Phospholipids. PE = phosphoethanolamine, PC = phosphocholine, PG = phosphatidylglycerol. The displayed values consist of the mean normalised metabolite area, error bars show standard errors.

4.5 Discussion

The results reported in this chapter suggest that whilst dietary status does affect a number of important life history parameters in bethylid wasps, it does not affect contestant performance. However a significant number of polar and non-polar metabolites associated with energy storage and metabolism were elevated in honey-fed wasps; a number of non-polar metabolites associated with lipid signalling were also elevated in 3 day old host-fed wasps. No clear separation was detected between the lipid profiles of 3 day old honey-fed and 3 day old starved wasps, with significant separation only becoming apparent in 7 day old wasps. These results also indicate that any advantages conferred by the availability of honey do not appear to be a major physiological factor until close to the maximum lifespan of starved *G. legneri*. Honey-fed wasps also displayed a significantly greater longevity than starved wasps, suggesting that honey-fed wasps are capable of preserving long term lipid stores by using dietary carbohydrates as a maintenance source for energy metabolism. From these results it can be concluded that the fitness of adult female *G. legneri* is significantly enhanced by the availability of a carbohydrate diet, via increased longevity. Whilst this study did not evaluate parasitoid fecundity, it is also likely that the use of dietary carbohydrates to maintain vital body processes would allow greater allocation of lipid reserves towards egg production and maturation, as demonstrated by a number of previous studies (Jervis and Kidd 1986; Heimpel et al. 1997; Winkler et al. 2006; Harvey et al. 2012).

The results of this study indicate that the applied metabolomic approach is capable of detecting both polar and non-polar changes in the metabolome of low biomass insects in response to dietary intake. Whilst many metabolomic studies have previously considered the metabolomic effects of dietary intake (German et al. 2002, 2003; Davis and Milner 2004; Zeisel et al. 2005; Wang et al. 2009; Ametaj et al. 2010), the current study appears to be the first to consider dietary fluctuations in the metabolome within the context of animal resource competition. This methodology was further capable of reliably producing identifiable NMR spectra associated with both starved and honey-fed wasps. The detection of the highly conserved honey signal in fed wasp NMR spectra proved instrumental in identifying experimental outliers, in this case wasps that had not ingested the provided diet. However, it should be stated that as this methodology employs whole insect analysis, it does not

distinguish between resonances generated from insect hemolymph and those generated from diet within an insect's gastrointestinal tract. As a result further experimentation, such as the longevity study outlined in this chapter, is required to confirm that an insect is capable of utilising a particular dietary resource other than merely ingesting it.

The lack of significant effects of a honey diet on *G. legneri* contest performance contrasts the results of both the longevity study and the metabolomic study. However a number of factors could account for the lack of significant differences in performance between starved and honey-fed wasps. The higher levels of storage lipids of 7 day old honey-fed wasps suggest that these contestants would have a higher RHP than starved wasps. However, when the results of this study are considered from the perspective of RV, it could be expected that starved wasps would place a greater value on the contested host than honey-fed wasps, due to their diminished reserves (Humphries et al. 2006). As a result any gains in RHP resulting from dietary treatment may have been effectively equalised by an increase in perceived RV by starved wasps. If this is the case any potential effects on contest behaviour related to dietary treatment would be limited to differences in size between contestants. However this factor was also found to be of no significance in determining contest outcome, likely due to efforts to minimise size differences.

An alternative possibility arises when the results of dietary metabolomic analysis are compared with those of the wasp aging study outlined in Chapter 3. Whilst LC-MS analysis of 7 day old wasps found increased levels of storage glycerolipids in honey-fed wasps, this number was significantly reduced from those which were elevated in 0 day old starved wasps compared to 7 day old starved wasps. Elevation of other biomarkers associated with wasp aging detected in Chapter 3, such as increased levels of phospholipids, was not detected when comparing 7 day old starved wasps to 7 day old honey-fed wasps. This suggests that honey-fed wasps are still subject to a degree of lipid reserve deterioration throughout the experimental time frame. As a result it is possible that the separation between starved and honey-fed wasp lipid stores is too small to translate into an increased RHP within wasp contests. This finding is further supported by elevations in diacylglyceride concentration in honey-fed wasps. Release of diacylglycerides into the

hemolymph constitutes the major form of fat mobilisation within insects (Arrese et al. 2000). Diacylglycerides are also the primary source of fuel for insect flight (Vogt et al. 2000; Harrison and Fewell 2002). Whilst 7 day old honey-fed wasps displayed higher levels of diacylglycerides than starved wasps, their presence may indicate that lipid store mobilisation is already occurring in 7 day old honey-fed *G. legneri*.

Further understanding of this result may be generated through the study of individual contest behaviours, rather than contest outcome. The study of aggression within *G. legneri* contests has previously indicated that older individuals display higher levels of aggression in attempting to acquire hosts (Stockermans and Hardy 2013). By staging contests within honey-fed and starved fed wasps (i.e. honey-fed vs. honey and starved vs. starved), further information on the role of RHP and RV in influencing dietary contest outcome could be generated. Under such a setup it would be hypothesised that increased aggression in starved wasps would indicate that starved wasps assign a higher RV than honey-fed wasps. This would also confirm that the enhanced lipid stores displayed by honey-fed wasps do have a significant effect on wasp fitness.

The longevity of host-fed wasps was not examined in this study, as this treatment group in metabolomic studies was initially included in metabolomic analysis to assess its potential role as a confounding variable in owner-owner contests. However, the similar proportion of host-fed wasp deaths during 7 day old wasp metabolomic analysis (20 % of wasps in either treatment) may suggest that host-fed wasps have a similarly restricted lifespan to starved wasps. However further analysis would be required to confirm this: due to time limitations it was not possible to include such a study in this chapter. Whilst common fatty acyls, such as palmitic acid and oleic acid, and a number of lysophospholipids were elevated in 3 day old host-fed wasps, no elevations in long term storage molecules, or polar metabolites, were detected. This result is consistent with previous reports that host feeding does not increase the lipid reserves of parasitoids incapable of *de novo* lipogenesis (Olson et al. 2000; Giron et al. 2002; Rivero and West 2005). Due to the high sugar content of host hemolymph it could be expected that host feeding would increase wasp sugar levels (Thompson et al. 1990; Phalaraksh et al. 2008), however no elevation was observed. This suggests that the quantity of extra

sugars gained from host feeding is too low to significantly alter the metabolome of *G. legneri*. Alternatively any increases in wasp haemolymph sugar levels are exhausted prior to the 3 day mark and as a result are no longer apparent in wasp NMR spectra. Casas et al. (2005) previously reported that carbohydrates are rapidly exhausted in place of storage lipids for maintenance, such a rapid utilisation of host acquired carbohydrates may account for this absence.

The energetic costs of contest behaviour have previously been examined in a range of species, including chlidid fish (Neat et al. 1998a, 1998b), hermit crabs (Briffa and Elwood 2001, 2002, 2004, 2007), pigs (Camerlink et al. 2015), red deer (Clutton-Brock and Albon 1979), shore crabs (Sneddon et al. 1999), swimming crabs (Thorpe et al. 1994) and spiders (Prestwich 1983a, 1983b, 1988a, 1988b; Decarvalho et al. 2004; Elwood and Prenter 2013). As it is difficult to track energy expenditure in the field, most studies of contest energetics have been limited to laboratory studies (Briffa and Sneddon 2007), with some exceptions (Dearborn et al. 2005). These studies have predominantly focused on the depletion of immediately available energy reserves, such as glycogen, and the accumulation of muscle waste products, such as lactic acid (Briffa and Sneddon 2007). The investigation presented in this chapter focused on long term energy stores and did not consider the role of these compounds. This was largely due to the inability of the employed NMR approach to reliably detect glycogen or lactic acid (Chapter 2). Lactic acid was tentatively identified in 1D NMR spectra, however further analysis by 2D HSQC experiments was incapable of validating this assignment. Due to the brevity (usually under 10 seconds of escalated fighting) of individual *Goniozus* contests (Petersen and Hardy 1996), it is possible that these immediately available energy stores play a greater role in determining *G. legneri* RHP than long term lipid stores. The potential for rapid aerobic metabolism of these energy stores and the inability to quickly mobilise lipid reserves may also force contestants to rely on anaerobic energy metabolism in order to continue costly contest behaviours (Briffa and Elwood 2000; Briffa and Sneddon 2007). If this is the case, more targeted metabolite profiling may be appropriate for assessing energy expenditure within contests of a short duration. Conversely, energy expenditure within contests of extended duration may be ideal candidates

for metabolomic assessment, due the increased possibility of lipid store mobilisation throughout the contest period.

4.6 Conclusions

The availability of a honey diet significantly affected the longevity of adult female *G. legneri*: Honey-fed individuals typically had over twice the lifespan of starved individuals. Significant differences in polar metabolite concentrations were present between treatments for both 3 day old and 7 day old wasps. Higher concentrations of common hemolymph sugars and amino acids were detected in honey-fed wasps than either starved or host-fed wasps. Whilst no significant separation in lipid profile was detected between 3 day old honey-fed and starved wasps, 7 day old honey-fed wasps displayed elevated levels of storage glycerolipids and lysophospholipids over starved and host-fed wasps. 3 day old host-fed wasps also displayed elevated levels of common fatty acyls and lysophospholipids. Despite these detected metabolomic differences, dietary treatment had no significant effect on wasp contest performance. This lack of significance may be due to a number of factors, whilst honey-fed wasps displayed levels of energy rich metabolites, these increased levels may not be high enough to translate into a raised RHP. Alternatively an increase in the perceived value of a host by starved competitors may have neutralised any advantage in RHP gained by honey-fed contestants.

Chapter 5: The assessment and basis of host quality - life history, behavioural and metabolomic assays using bethylid wasps.

5.1 Abstract

Bethylids are parasitoid wasps that require hosts for reproductive success. Variations in the quality of hosts of a given species are known to affect both offspring development and the behaviour of adult parasitoids in contests for resources. However no prior investigation has examined the effects of different host species on parasitoid wasp contest behaviour, or on their metabolomic state. This chapter assesses the performance of the bethylid wasps *Goniozus legneri* and *Goniozus nephantidis* when presented with four species of lepidopteran hosts: *Corcyra cephalonica*, *Ephestia kuehniella*, *Ephestia cautella* and *Plodia interpunctella*. It also assesses whether the underlying metabolomic state of a host restricts parasitoid host range. Whilst *G. nephantidis* attacked and parasitized all four species, it rarely oviposited on species other than *C. cephalonica*. In the few cases where *G. nephantidis* did oviposit on any of the other host species, only one individual raised on *P. interpunctella* survived pupation. By comparison *G. legneri* was consistently capable of developing on all four species, whilst *G. legneri* performed best when parasitizing *Ephestia kuehniella* and *C. cephalonica* and worst when parasitizing *Plodia interpunctella*, though no individual species was advantageous for all stages of development. The effects of differing host species on parasitoid aggression between adult female *G. legneri* were also assessed. Aggression exhibited within owner-owner contests did not significantly differ across host species, and did not reflect the quality of the host as assessed by brood development. Finally both the metabolome of each host species and that of adult *G. legneri* which had developed from each host species were assessed using a combined NMR and LC-MS approach. Elevated levels of currently unidentified metabolites were observed in hosts other than *C. cephalonica*. *G. legneri* reared on *Ephestia kuehniella* exhibited elevated levels of energy rich glycerolipids, whilst wasps reared on *Plodia interpunctella* exhibited the lowest levels.

5.2 Introduction

Hymenopteran parasitoids rely on a single host organism in order to obtain resources throughout immature development (Godfray 1994). The quality of resource provided by this host can depend on a number of factors, such as its developmental instar (Kidd and Jervis 1991; Harvey et al. 1994; Vet et al. 1994; Otto and Mackauer, 1998; Karsai et al. 2006; Thiel and Hoffmeister 2009; Fand et al. 2010), size (Schmidt 1991; Hardy et al. 1992; Harvey et al. 1994; Otto and Mackauer 1998; Zaviezo and Mills 2000; Cleary and van Ginkel 2004; Ueno 2004), age at onset of parasitism (Ode and Strand 1995; Hofstetter and Raffa 1998; King 1998; Sousa and Spence 2001; Wang and Liu 2002; Ueno 2004; He and Wang 2006), disease state (Hochberg 1990), genetic composition (Kraaijeveld and Godfray 2009; Henry et al. 2010) and species (Wright and Kerr 1988; Rivers and Denlinger 1995; Pérez-Lachaud and Hardy 2001; Cleary and van Ginkel 2004; Häckermann et al. 2007; Thiel and Hoffmeister 2009). These factors may also interact e.g. viral infection may limit the quantity of diet a host is capable of acquiring.

There is a wide body of literature showing how aspects of host quality affect a range of evolutionary fitness-correlated components of parasitoid wasp life-history and behavioural decisions (Charnov and Skinner 1984; Sequeira and Mackauer 1992; Hardy et al. 1992; Peterson and Hardy 1996; Heimpel et al. 1996; Zaviezo and Mills 2000; West et al. 2001; Bezemer and Mills 2003; Häckermann et al. 2007; Ode and Hardy 2008). For example, host species has been observed to affect host acceptance (Rivers and Denlinger 1995; Pérez-Lachaud and Hardy 2001), oviposition decisions (Rivers and Denlinger 1995; Harvey 2000; Pérez-Lachaud and Hardy 2001; Cleary and van Ginkel 2004), sex ratio (Rivers and Denlinger 1995; Harvey 2000; Cleary and van Ginkel 2004), development time (Rivers and Denlinger 1995; Harvey 2000; Pérez-Lachaud and Hardy 2001), developmental morality (Pérez-Lachaud and Hardy 2001; Cleary and van Ginkel 2004; Häckermann et al. 2007) and adult offspring fitness (Wright and Kerr 1988; Rivers and Denlinger 1995; Harvey 2000; Pérez-Lachaud and Hardy 2001; Häckermann et al. 2007).

Host acceptance has previously been assessed in terms of adult parasitoid preference, with particular focus on the role of volatile host cues (Turlings et

al. 1993; Ngi-Song and Overholt 1997; Vinson 1998; Stireman 2003; Morgan and Hare 1998; Williams et al. 2008). Variations in immunological defence have also figured prominently in investigations of host preference concerning koinobiont parasitoids (Vinson and Iwantsch 1980; Götz 1985; McAllister et al. 1990; Kraaijeveld and Godfray 1997; Strand and Pech 1995). Similarly, differing weight ranges between host species have been linked to brood production (Pérez-Lachaud and Hardy 2001), though a larger host does not necessarily result in a greater brood size or bigger offspring (Sequeria and Mackauer 1992; Häckermann et al. 2007). The host comparison study conducted by Sequeria and Mackauer (1992) indicated that the quality of pea-aphid hosts within different instars was not completely dependent on host biomass, but rather on the kind and amount of nutrition available at a given time. This was supported by the findings of Häckermann et al. (2007) that similar clutch sizes are laid by the gregarious ectoparasitoid *Hyssopus pallidus* on *Cydia molesta* and *Cydia pomonella*, despite *C. pomonella* having a four times greater weight range than *C. molesta*. Furthermore, individual wasps that developed on *C. molesta* were bigger than those from broods that developed on *C. pomonella*. These studies demonstrate that the quality of nutrients available to developing parasitoids varies between host species. It can also be concluded that female parasitoids are capable detecting the quality of nutrition available from a particular host and of adjusting clutch size accordingly.

Female bethylid wasps engage in agonistic contests to acquire or defend hosts. The value that a parasitoid assigns to a host, known as the resource value (RV), can affect contest outcome (Hardy and Briffa 2013). Ownership of a host or prior contest experience significantly increases the chance of contest victory (Goubault et al. 2006; Bentley et al. 2009). Parasitoid wasps will also fight more aggressively to obtain larger hosts (Humphries et al. 2006; Stockermans and Hardy 2013); older wasps may also assign a higher RV than younger wasps (Humphries et al. 2006). Despite this extensive series of studies, the effects of variations in RV due to differing host species on contest behaviour remain unexplored. As it has been previously demonstrated that adult parasitoids in non-bethylid taxa are capable of detecting the quality of resource available from a specific host species (Sequeria and Mackauer 1992; Häckermann et al. 2007), it is possible that individuals may compete more aggressively for host species with a higher perceived RV.

In parallel, only a few studies have evaluated the biochemical basis of host nutritional quality. Visser et al. (2013) used GC-MS to evaluate correlations in fatty acid profile between the gall wasp *Diplolepis rosae* and its parasitoids/hyperparasitoids, concluding that fatty acid concentrations are remarkably similar between *D. rosae* and most of its parasitoids. Li et al. (2015) utilised a global metabolomic method to assess the effects of host diapause and diet on the metabolomic profile of the parasitoid *Nasonia vitripennis*, concluding that diapause resulted in an elevation of glycolysis derivatives in adults, whilst supplementation of the host's diet prior to parasitism with proline resulted in higher adult levels of essential amino acids and lower levels of metabolites associated with energy utilisation. A prior study by Khidr et al. (2012) used an NMR approach to detect changes in the post-paralysis quality of *Corcyra cephalonica*, a host of the bethylid wasp *Goniozus nephantidis*. Energy rich haemolymph sugars were found to decline in abundance over time, whilst waste products such as citrate and ethanol accumulated in older hosts. Adult female *G. nephantidis* defending these older hosts also competed less successfully than those defending younger hosts.

These previous studies show that the species a host belongs to directly influences its quality (Sequeria and Mackauer 1992; Pérez-Lachaud and Hardy 2001; Häckermann et al. 2007) and that parasitoids are capable of recognising the quality of resource available from individual host species. Furthermore variations in a host's biochemical state can affect the metabolomic profile of a developing parasitoid (Visser et al. 2013; Li et al. 2015). However, no previous study has examined how the biochemical profile of a given host species affects the fitness of a developing parasitoid (Snart et al. 2015). Similarly, despite parasitoids being capable recognising differences in quality between host species, no prior study has examined the effects of host species on parasitoid contest behaviour. Here the bethylid wasps *Goniozus legneri* and *Goniozus nephantidis* were reared on four lepidopteran host species, *Corcyra cephalonica*, *Ephestia kuehniella*, *Ephestia cautella* and *Plodia interpunctella*, to assay wasp reproductive decisions and brood development. Informal observations by Khidr et al. (2012) have indicated that *G. nephantidis* performs poorly when attempting to rear broods on host species other than *C. cephalonica*, by comparison *G. legneri* has been confirmed to be capable of brood rearing across the whole brood range (Khidr et al. 2012a). In addition, the effects of host species on

parasitoid contest behaviour were assessed by comparing the aggression levels of *G. legneri* when defending different host species (Lizé et al. 2012; Stockermans and Hardy 2013). This comprised of owner-owner contests in which contestants defended hosts from the same species. Aggression levels within contests were then compared between species. Owner-owner aggression contests were employed as a further behavioural assay of host quality. The metabolomes of adult female *G. legneri* reared on each species and those of individual hosts were also evaluated by a combined NMR and LC-MS approach.

5.2.1 *G. legneri* biology and host range

Goniozus legneri Gordh (Hymenoptera: Bethylinidae) is a gregarious ectoparasitoid wasp of significant ecological importance for the study of life history decisions and contest behaviour (Gordh et al. 1983; Butler and Schmidt 1985; Hardy et al. 1998, 2000; Goubault et al. 2006, 2007; Goubault and Hardy 2007; Bentley et al. 2009; Lizé et al. 2012; Stockermans and Hardy 2013; Hardy and Briffa 2013). Whilst the 'natural' host of *G. legneri* is not currently known, it is capable of parasitizing a range of hosts including *Corcyra cephalonica* Stainton, *Pectinophora gossypiella* Saunders, *Amyelois transitella* Walker, *Ectomyelois ceratoniae* Zeller, *Ephestia kuehniella* Zeller, *Ephestia cautella* Walker and *Plodia interpunctella* Hübner (Gordh and Móczár 1990; Khidr et al. 2013a). Informal laboratory observations have also found that *G. legneri* is capable of parasitizing the greater wax moth *Galleria mellonella* Linnaeus. *G. legneri* typically paralyzes hosts by injecting them with venom, and then lays a brood of 1 - 20 eggs onto the host surface over the next 24 hours (Hardy et al. 1998). Females remain in close proximity to their host throughout initial brood development and will aggressively defend hosts from any intruding females (Petersen and Hardy 1996; Humphries et al. 2006).

5.2.2 *G. nephantidis* biology and host range

Goniozus nephantidis (Muesebeck) is a gregarious larval ectoparasitoid for which many aspects of life history and behaviour have been assessed (Hardy et al. 1992; Cook 1993; Hardy and Cook 1995; Humphries et al. 2006; Goubault et al. 2007; 2007a; Khidr et al. 2012a). *G. nephantidis* is naturally associated with the coconut pest *Opisina arenosella* Walker (Venkatesan et

al. 2007), but has also been confirmed to parasitize the greater wax moth *Galleria mellonella* Linnaeus (Mohan and Shameer 2003) and the rice moth *Corcyra cephalonica* Stainton (Cook 1993; Venkatesan et al. 2007). *G. nephantidis* paralyzes any encountered host larvae but injecting venom from its ovipositor, before laying a brood of 3 - 18 eggs (Hardy et al. 1992; Petersen and Hardy 1996) on the surface of the host over the next 1 - 3 days. Eggs hatch with approximately 24 hours and developing larvae feed on the host through punctures in its integument. As with *G. legneri*, *G. nephantidis* aggressively guards its brood against intruder females who would otherwise utilize the unguarded host (Petersen and Hardy 1996).

An informal pilot study conducted prior to this investigation found that *G. nephantidis* is incapable of reliably parasitizing *E. kuehniella*, *E. cautella* or *P. interpunctella*, whilst *G. legneri* is capable of reliably parasitizing all three. However the exact physiological mechanism behind the inability of *G. nephantidis* to parasitize these species remains unknown. Through the use of a metabolomic approach it may be possible to detect differences between these species that account for this inability. As such this investigation aims to detect any elevations in detrimental compounds or any lack of essential compounds in these hosts. As both *G. legneri* and *G. nephantidis* are capable of consistent brood rearing on *C. cephalonica* this host is employed a control for metabolomic host studies (Cook 1993; Khidr et al. 2013a).

5.2.3 Aims and objectives

This chapter aims to explore whether host species influences the oviposition behaviour, brood production and contest behaviour of *G. legneri* and *G. nephantidis*. This chapter further aims to explore potential differences between the metabolomes of specific host species, and any links between the host metabolome and the metabolome of *G. legneri* reared on each host species. Specific objectives required to fulfil these aims are:

- To conduct a brood production study comparing the performance of *G. legneri* and *G. nephantidis* across four lepidopteran host species: *C. cephalonica*, *E. kuehniella*, *E. cautella* and *P. interpunctella*.
- To stage owner-owner aggression studies between *G. legneri* defending each of the four host species.

- To perform combined NMR and LC-MS analysis of the metabolomes of each host species and the metabolomes of *G. legneri* reared on each host species.

5.3 Materials and methods

5.3.1 Parasitoid origin and cultures

Four lepidopteran host species, *Corcyra cephalonica*, *Ephestia kuehniella*, *Ephestia cautella* and *Plodia interpunctella* were reared in kilner jars. All four hosts were cultured using the same artificial diet reported by Lizé et al. (2012), consisting of wheat bran, corn meal, glycerol, honey and yeast. All culturing and experimental work took place at 27 °C with a constant humidity of 60 - 70 % maintained by a water bath. A light-dark cycle of 16:8 was employed throughout culturing. The bethylid parasitoid *Goniozus legneri* Gordh (USA strain; Lizé et al. 2012) was utilised for all experiments. *Goniozus nephantidis* (Muesebeck) was utilised during initial host acceptance experiments. Both wasp species have been reared in a laboratory setting for a minimum of 9 years and are normally maintained on *Corcyra cephalonica*. All wasps selected were 3 - 5 days post emergence, a common age for female brood dispersion (Hardy et al. 1999). No female had prior experience of host ownership or contest interactions.

5.3.2 Part 1: Adult parasitoid host acceptance and offspring utilisation

In order to assess the ability of both *G. legneri* and *G. nephantidis* to accept and successfully produce broods across the selected host range, both species were presented with each of the four lepidopteran host species. Individual wasps were isolated in glass vials and allowed to parasitize one of the four lepidopteran hosts. All hosts were of a similar weight (20 ± 1 mg). Parasitoid host-pairings were assessed for the following criteria: paralysis, oviposition and the mortality of any developing broods.

5.3.3 Part 2: Effects of host species on *G. legneri* development

The results of the acceptance study found that only *G. legneri* was capable of consistently producing live offspring on all four host species. As a result development assays were limited to this species only. In order to assess the effects of host species on *G. legneri* offspring production, individual adult females were isolated in glass vials and allowed to parasitize larvae of one of the four host species. A range of overlapping larval weights were utilised (*C. cephalonica*: 7.39 - 57.70 mg; *E. kuehniella*: 7.09 - 31.69 mg; *E. cautella*: 7.64 - 23.93 mg; *P. interpunctella*: 8.71 - 29.36 mg). As *C.*

cephalonica has a greater range of larval weights than the other 3 host species, a second weight range was used in development assays for this species (7.39 - 25.15 mg) that overlapped more with the other host weight ranges. Each caterpillar was weighed to an accuracy of 0.01 mg prior to exposure.

Wasp-host pairings were observed throughout brood development for the following criteria: paralysis, oviposition, clutch size, developmental mortality, and sex ratio of emerging adults. Mothers were removed upon death (usually around offspring pupation) to prevent confusion with the developing brood. A total of 40 - 50 replicates were carried out for each host treatment.

5.3.4 Part 3: The effects of host species on parasitoid contest behaviour

Aggression contests using hosts from the same species were performed to assess the value placed on a given host species by adult female *G. legneri*. Contests were performed between *G. legneri* each possessing a host of the same species. Initial wasp-host exposure was conducted in the same manner as part 1, wasp-host pairings were maintained for 4 hours before being transferred to the contest arena (see Fig. 2.1). Contests were performed using established experimental methods (Lizé et al., 2012, Stockermans and Hardy, 2013). Wasps originating from separate broods were paired by weight (to an accuracy of 0.01 mg) to prevent any possible asymmetries. Hosts were paired to within 0.75 mg of each other, with a weight range across contests of 14.50 - 20.20 mg. Interactions between the wasps were recorded for 30 min using a digital camera and analysed using Windows Media Player. The proportion of wasp interactions that were aggressive were recorded, specific interactions were classified as follows: non-aggressive, chases, bites, stings, fights and returns to the contest arena.

5.3.5 Part 4: Metabolomic analysis of larval hosts and adult parasitoids

5.3.5.1 Host extraction protocol

Twenty individual larvae from each host species weighing 20 ± 1 mg were taken from cultures and isolated in 1 mL eppendorf tubes for 24 hours to facilitate voiding of the gastrointestinal tract. Hosts were then washed in HPLC-grade water to remove any residual culture medium or faeces prior to being snap frozen in liquid nitrogen. Frozen hosts were maintained at -80 °C for approximately two weeks prior to solvent extraction.

A modified solvent extraction protocol with a two-step solvent addition process, as employed by Wu et al. (2008), was utilised for host metabolite extraction. The higher biomass of host larvae did not require the fixed ratio approach previous adopted by this thesis, as a result a more established ratio based approach was employed. All samples were randomised during sample extraction and again prior to sample analysis. Hosts were homogenised in 4 μ L/mg of methanol and 2 μ L/mg of water using a fast prep 24 lysing matrix Z (MP-BIO®). The homogenate was transferred to a sterile 2ml borosilicate glass vial using a Pasteur pipette. The remaining matrix was washed with 4 μ L/mg of methanol and 2 μ L/mg of water, then vortexed for 30 seconds. The remaining homogenate was then transferred to the original glass vial using the original Pasteur pipette. 4 μ L/mg of methanol and 8 μ L/mg of chloroform were added for a final solvent ratio of 3/2/1 (methanol/chloroform/water). Samples were vortexed for 30 seconds then left on ice for 10 min. After this samples were centrifuged for 10 minutes at 10,000 g and left at room temperature for 5 min to facilitate phase separation.

The generated phases were separated using a Pasteur pipette, and transferred into 2 mL glass vials before storage at -80 °C. All solvents used were of a high analytical grade (LC-MS grade CHROMASOLV Sigma-Aldrich) and were kept ice cold during sample extraction. Samples were randomised before metabolite extraction and again prior to metabolomic analysis. Samples were reconstituted prior to NMR or LC-MS analysis using the protocol outlined in Chapter 3 with an added dilution step (1:10 sample-isopropanol blank) to prevent excessive peak tailing associated with column

silica saturation. Pooled quality control samples for LC-MS analysis were generated consisting of 10 μ L from each sample.

5.3.5.2 Parasitoid extraction protocol

Individual adult female *G. legneri* were allowed to parasitize hosts from each of the four host species. The resultant offspring were isolated and snap frozen in liquid nitrogen, and then stored at -80 °C prior to solvent extraction. Individual wasps were weighed to an accuracy of 0.01 mg and had a weight range of (*C. cephalonica*: 0.94 - 1.53 mg; *E. kuehniella*: 0.93 - 1.44 mg; *E. cautella*: 0.93 - 1.28 mg; *P. interpunctella*: 0.46 - 0.9 mg). A total of 20 replicates were performed for wasps that developed on *C. cephalonica*, *E. kuehniella*, *E. cautella*. Only a small number of *P. interpunctella* were available due to a short term culturing issue, as a result only 15 replicates could be performed simultaneously with the other host species.

This chapter employed the modified Bligh and Dyer (1959) biphasic lipid extraction protocol outlined in Chapter 3. All solvents were kept ice cold during extraction and were of LC-MS optima grade (CHROMASOLV Sigma-Aldrich). All samples were randomised during sample extraction and again prior to sample analysis. Generated solvent phases were separated after extraction and transferred to either a sterile and solvent resistant 1.5 mL Eppendorf ®Biopur® tube (polar/methanol phase) or a 2 mL borosilicate glass vial (non-polar/chloroform phase). Both phases were stored at -80 °C prior to analysis. Prior to sample analysis polar and non-polar phases were dried and reconstituted according to the methodology outlined in Chapter 3. Pooled quality control samples were generated consisting of 10 μ L from each sample.

5.3.5.3 NMR spectroscopy

NMR spectra were generated from individual polar samples using a Bruker Advance 800 MHz III spectrometer equipped with a 5 mm QCI Cryoprobe. 1D spectra were collected with a width of 13 ppm and signal averaged over 512 transients. A noesy 1D pre-saturation experiment was utilised to achieve water suppression. Pre-saturation frequency and power were determined prior to experimental analysis using representative samples generated under

the same solvent extraction protocol. Samples were locked using the internal D₂O signal and referenced using the internal DSS reference. Total recycling delay was 4.7 s. An exponential window function was applied to give a line broadening of 0.3 Hz prior to zero filling and Fourier transformation. In addition 2D HSQC spectra were generated using the experimental parameters outlined in wasp HSQC experiments detailed in Chapter 2 in order to aid identification of polar host metabolites and validate previous identifications reported by Khidr et al. 2012.

5.3.5.4 LC-MS analysis

The lipidomic Accela LC-Exactive MS setup outlined in Chapter 3 was replicated for both host and parasitoid non-polar extract analysis. The generated pooled sample was repeatedly injected into the LC prior to analysis to equilibrate the column, followed by intermittent injection through analysis to ascertain column stability. The MS parameters in Chapter 3 were replicated for this analysis (Ion range: 100 - 1500 m/z, ESI voltage: 3500, capillary temperature: 350°C, scan rate: 250ms). Ion identity was validated through the use of tandem mass spectrometry (MS/MS). Parasitoid extracts were pooled prior to analysis to improve metabolite yield. LC-MS/MS analysis was performed using an Accela LC coupled with a Velos Pro Dual-Pressure linear ion trap mass spectrometer as outlined in Chapter 2. Sample preparation, injection protocol, mobile phase and column conditions were replicated from initial LC-MS analysis. An ion range of 100 - 1500 m/z was monitored (ESI voltage: 3000, capillary temperature: 275 °C, scan rate: 50 ms), a collision energy of 40 V was applied during MS₂ fragmentation.

5.3.5.5 LC-MS stability validation

To validate LC-MS stability, the relative standard deviations of the peak areas and retention times of a selection of key ions outlined in Chapter 2 were assessed within the pooled QC samples of both host and parasitoid analyses. An acceptability threshold of <30 % for a minimum of 70 % of key ions was considered to establish LC-MS stability. Central clustering of QC samples within generated PCA scores plots was further required to establish LC-MS stability. As LC-MS analysis of parasitoids reared from different host species was performed alongside the wasp aging data used to validate this

LC-MS approach as presented in Chapter 3, QC key ion peak area and retention time RSD's are identical to those previously outlined in Chapter 3.

5.3.6 Data analysis

Developmental and behavioural data from parts 1, 2 and 3 were analysed with generalized linear models using the statistical package Genstat (Version 15, VSN International, Hemel Hempstead, UK). For part 1, data from both species of *Goniozus* wasp was analysed by logistic analyses of co-variance (ANCOVA) to determine whether host species and parasitoid type affected the probability of paralysis, oviposition and developmental mortality of the brood. Log-linear ANCOVAs were applied to determine whether host and parasitoid species affected clutch size. A standard ANCOVA was used to determine the effects of host and parasitoid species on adult parasitoid weight.

For part 2, data from *G. legneri* was analysed by a log-linear ANCOVA to assess the effect of host species, host weight and their interaction on clutch size. Logistic ANOVA was utilised to determine the effect of host species and host weight on the probability of host paralysis, oviposition, adult parasitoid sex ratio and developmental mortality. The possibility that the available resource per egg (host weight divided by clutch size) affects developmental mortality was also assessed. Standard ANCOVA was used to determine whether the host species, host weight and their interaction affected offspring dry weight, along with whether resource per egg, host species and their interaction affected offspring dry weight.

For part 3, a logistic ANOVA was employed to assess whether the species of host defended affects the proportion of interactions within a contest that were aggressive, following Lizé et al. (2012) and Stockermans and Hardy (2013). A multivariate analysis of variance (MANOVA) was used to assess whether the host species defended affects the overall profile of aggressive and non-aggressive interactions. As multivariate analyses assess the combined effects of profile components, individual logistic ANOVA's were performed to assess the proportion of each type of interaction. Due to multiple comparisons, the threshold for significance was adjusted using the Bonferroni correction to control type I error (Quinn & Keough, 2002).

For part 4 all NMR spectra and LC-MS chromatograms were corrected, aligned and binned (NMR) or framed (LC-MS) using the protocol outlined in Chapter 2. Generated NMR and LC-MS bin/frame tables were exported to Microsoft Excel for baseline correction and sample labelling. Frames/bins were normalised to total ion count (LC-MS) or parasitoid dry weight (NMR) respectively prior to multivariate analysis to minimise sample concentration variations. Normalised tables were individually imported into the Simca 13.0 (Umetrics) package. Sample classification was visualised using principle components analysis (PCA) and separation between classes was established using orthogonal partial least squares-discriminant analysis (OPLS-DA). Samples were mean-centred and scaled prior to analysis as outlined in Chapter 2. Potential biomarker ions were selected through a combination of weighted comparisons between experimental classes and through comparisons between PCA loading and scores plots. Selected ions were validated by one-way logistic ANOVA with Bonferroni correction to account for multiple hypothesis testing (Quinn and Keogh 2002). ANOVA's were performed using Genstat. A p-value of <math><0.05</math> was utilised when establishing significant separation between treatments. Significant parasitoid and host LC-MS ions were assigned putative identities through spectral comparisons with the Lipidmaps, Metlin and Human Metabolome databases (HMDB) and validated through comparisons of LC-MS/MS fragmentation data with known standards, as outlined in Chapter 2. Parasitoid NMR bins were identified through comparisons with 2D NMR data generated in Chapter 2. Host NMR bins were identified through comparisons between generated HSQC spectra and 2D spectral standards from the Biological Magnetic Resonance Data Bank (BMRB).

5.4 Results

5.4.1 Part 1: Adult parasitoid host acceptance and offspring utilisation

There were significant differences in clutch size ($F_{1,38} = 97.11$, $P < 0.01$, Figure 5.1a) and the mean dry weight of adult female offspring ($F_{1,38} = 17.15$, $P < 0.01$, Figure 5.1b) between *G. legneri* and *G. nephantidis* when parasitizing *C. cephalonica*. On hosts of the size presented, *G. legneri* typically produces a higher clutch size than *G. nephantidis*; however, *G. nephantidis* offspring are larger. No significant differences in the likelihood of paralysis ($G_{1,38} = 1.41$, $P = 0.235$) or developmental mortality ($F_{1,38} = 1.46$, $P = 0.243$) were found. The effects of host weight were also evaluated and found to have no significant influence on clutch size ($F_{1,38} = 0.01$, $P = 0.942$), female offspring dry weight ($F_{1,38} = 0.01$, $P = 0.972$) or mortality ($F_{1,38} = 0.20$, $P = 0.656$), likely as host weight variation was minimised experimentally.

The probability of oviposition was affected by host ($G_{3,152} = 11.58$, $P < 0.01$) and parasitoid species ($G_{1,152} = 86.87$, $P < 0.01$), though no significant interaction term was detected ($G_{3,152} = 1.09$, $P = 0.353$). Clutch size was significantly affected by host species ($G_{3,152} = 7.62$, $P < 0.01$), parasitoid species ($G_{1,152} = 346.52$, $P < 0.01$) and their interaction ($G_{3,152} = 38.29$, $P < 0.01$). Brood mortality was not significantly affected by either the host species ($G_{3,152} = 1.34$, $P = 0.267$) or the parasitoid species ($G_{1,152} = 2.94$, $P = 0.09$), however a significant interaction between the two was detected ($G_{3,152} = 9.27$, $P < 0.01$). Neither the species of host presented ($G_{3,152} = 0.94$, $P = 0.420$), the parasitoid species ($G_{1,152} = 1.09$, $P = 0.297$), nor their interaction ($G_{3,152} = 0.58$, $P = 0.628$) had a significant effect on host paralysis.

No significant differences in the probability of paralysis across all four species of host were detected between *G. legneri* and *G. nephantidis* ($G_{1,152} = 1.09$, $P = 0.297$) (Table 5.1). However, the probability of *G. nephantidis* ovipositing on hosts other than *C. cephalonica* was significantly lower than that of *G. legneri* ($G_{1,152} = 86.87$, $P < 0.01$) (Table 5.1). In total only eight

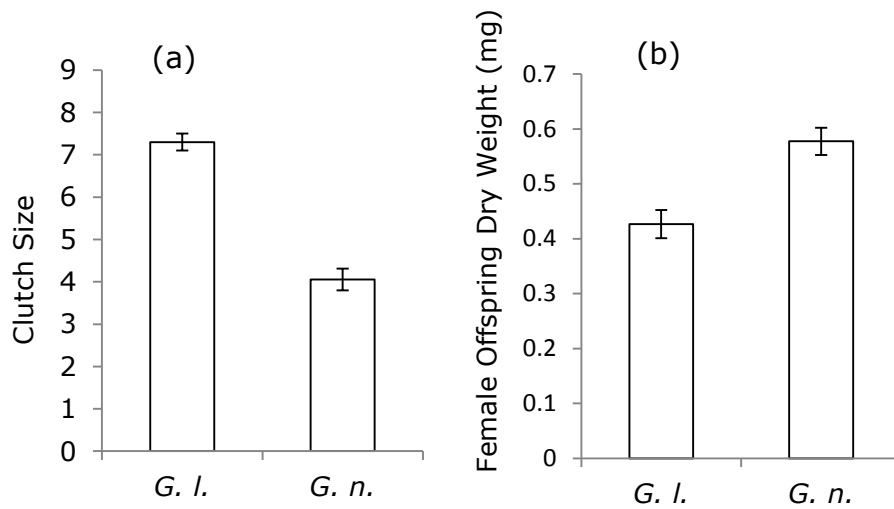


Figure 5.1. Comparisons of *G. legneri* and *G. nephantidis* (a) clutch size and (b) offspring mean dry weight when parasitizing standard sized *C. cephalonica*. *G. l.* = *Goniozus legneri*, *G. n.* = *Goniozus nephantidis*. Error bars display standard error of the mean (SEM).

broods were laid on these alternative hosts, two on *E. cautella* and three on both *E. kuehniella* and *P. interpunctella* egg. Only one brood consisting of a single egg laid on *P. interpunctella* produced a viable adult female. *G. legneri* successfully reared >80 % of hosts across all 4 host species; *G. nephantidis* was only capable of consistent brood rearing when presented with *C. cephalonica*.

5.4.2 Part 2: Effects of host species on *G. legneri* development

Host species did not significantly affect the probability of paralysis ($G_{3,170} = 1.79$, $P = 0.147$) or brood sex ratio ($G_{3,146} = 2.00$, $P = 0.112$). The probability of *G. legneri* oviposition was significantly affected by the species of host presented ($G_{3,170} = 3.41$, $P < 0.05$, $r^2 = 0.106$). Clutch size was significantly affected by the host species presented respectively. With the exception of one brood consisting of six individuals laid on *P. interpunctella*, these broods consisted of only one individual ($F_{3,142} = 20.31$, $P < 0.001$), host weight ($F_{1,142} = 51.22$, $P < 0.001$) and their interaction ($F_{3,142} = 7.43$, $P < 0.001$, $r^2 = 0.081$) (Figure 5.2a). Developmental mortality was not affected by host species

Table 5.1. Performance comparisons of two species of *Goniozus* wasps reared on four lepidoteran host species.

Parasitoid	Host	Paralysis (%)	Oviposition (%)	Pupation (%)	Live Offspring (%)
<i>G. legneri</i>	<i>C. cephalonica</i>	100	100	100	100
	<i>E. kuhniella</i>	95	85	85	85
	<i>E. cautella</i>	100	90	90	90
	<i>P. interpunctella</i>	100	100	100	100
<i>G. nephantidis</i>	<i>C. cephalonica</i>	95	95	95	95
	<i>E. kuhniella</i>	95	15	0	0
	<i>E. cautella</i>	90	10	5	0
	<i>P. interpunctella</i>	100	15	5	5

($F_{3,153} = 1.27$, $P = 0.288$), host weight ($F_{1,153} = 0.80$, $P = 0.372$), or resource per egg ($F_{1,153} = 0.72$, $P = 0.399$). The mean dry weight of adult female offspring was significantly affected by host species presented ($F_{3,142} = 3.66$, $P < 0.05$), host weight ($F_{1,142} = 24.28$, $P < 0.001$, $r^2 = 0.158$) and their interaction ($F_{3,142} = 8.25$, $P < 0.001$, $r^2 = 0.122$) (Figure 5.2b). The interaction between host species and resource available per egg significantly affected adult female dry weight ($F_{3,142} = 5.05$, $P < 0.01$). Host species ($F_{3,145} = 1.50$, $P = 0.218$) and host weight ($F_{1,145} = 1.93$, $P = 0.167$) did not significantly affect male offspring dry weight, though resource available per egg did ($F_{1,148} = 14.90$, $P < 0.001$). Male offspring dry weight decreased as the amount of per capita resources declined. Host weight did significantly affect brood sex ratio ($G_{1,148} = 6.39$, $P < 0.05$) but not the probability of paralysis ($G_{3,169} = 0.59$, $P = 0.442$) or oviposition ($G_{3,169} = 0.11$, $P = 0.735$).

When data from the restricted (and overlapping weight range) of 5 - 30mg for *C. cephalonica* was used, the probability of paralysis was not affected by either host species ($F_{3,152} = 1.66$, $P = 0.174$) or host weight ($F_{1,152} = 1.01$, $P = 0.316$). Host species ($F_{3,149} = 6.69$, $P < 0.01$), host weight ($F_{1,149} = 36.20$, $P < 0.01$) and their interaction ($F_{3,149} = 4.30$, $P < 0.01$) had significant effects on the probability of *G. legneri* ovipositing. Clutch size was also significantly affected by host species ($F_{3,111} = 9.36$, $P < 0.01$), weight ($F_{1,111} = 72.11$, $P < 0.01$) and their interaction ($F_{3,111} = 3.63$, $P < 0.05$, $r^2 = 0.051$) (Figure 5.3a). Brood mortality was significantly affected by host species

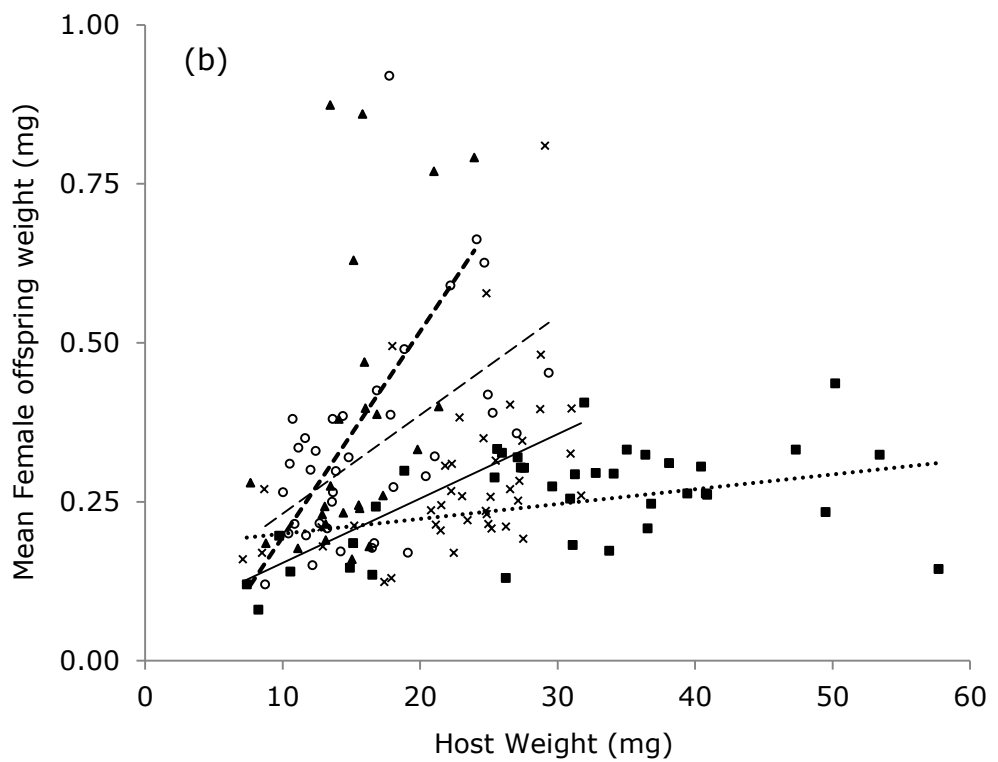
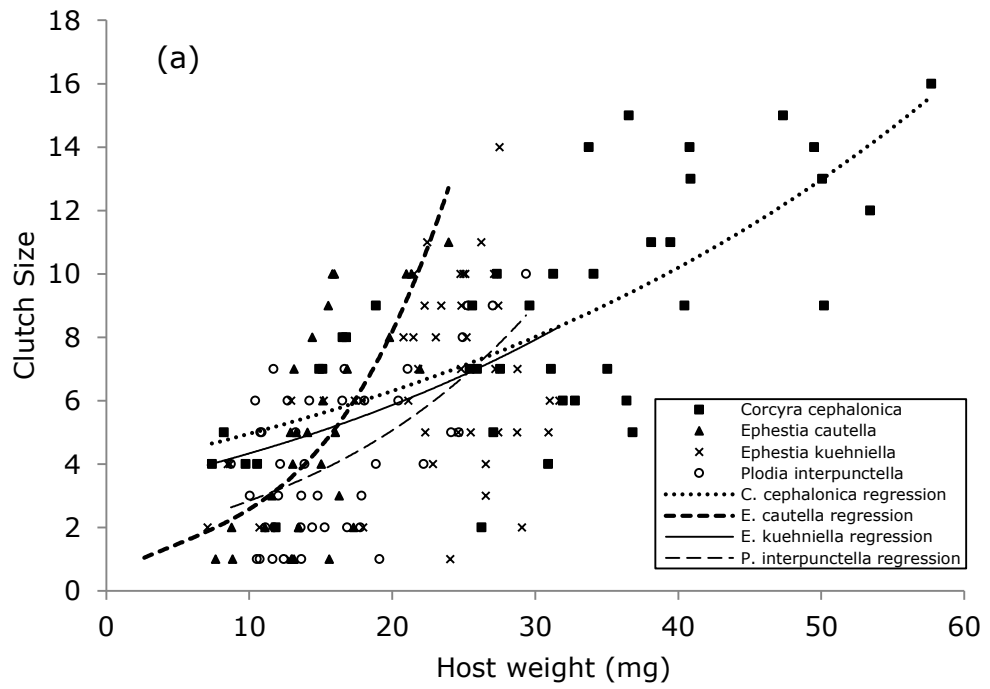


Figure 5.2. *G. legneri* reproduction in response to host weight and host species (a) *G. legneri* clutch size (b) *G. legneri* female offspring.

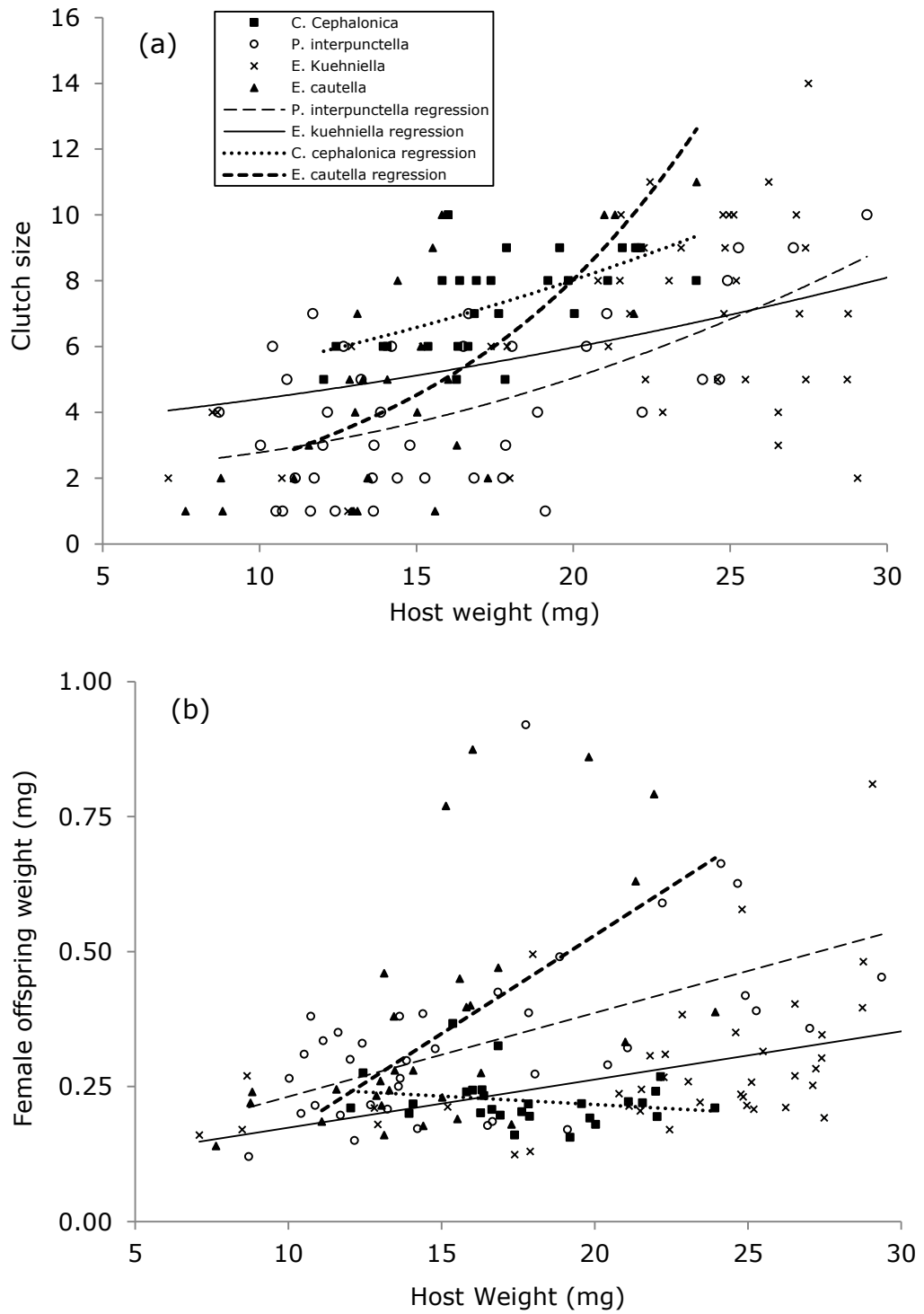


Figure 5.3. *G. legneri* reproduction in response to host weight and host species. Weight is restricted to a range of 5 - 30 mg (a) *G. legneri* clutch size (b) *G. legneri* female offspring.

Table 5.2. Average values for *G. legneri* oviposition probability, clutch size and female offspring weight. Shading indicates ranking.

Rank:	1	2	3	4
	<i>C. cephalonica</i>	<i>E. cautella</i>	<i>E. kuehniella</i>	<i>P. interpunctella</i>
Probability of paralysis	1	1	1	1
Probability of oviposition	1	0.85	1	0.87
Clutch size	8.02	8.036	6.44	4.83
Av. no. of offspring	6.9	7.6	5.93	4
Av. female offspring weight (mg)	0.22	0.53	0.25	0.42

($F_{3,111} = 11.07$, $P < 0.001$) host weight ($F_{1,111} = 9.29$, $P < 0.01$) and the available resource per egg ($F_{3,111} = 10.13$, $P < 0.01$). Adult female dry weight was significantly affected by host species ($F_{3,109} = 13.27$, $P < 0.001$), host weight ($F_{1,109} = 5.73$, $P < 0.05$) and their interaction ($F_{3,109} = 3.70$, $P < 0.05$) (Figure 5.3b). Host species ($F_{3,109} = 4.32$, $P < 0.01$) and resource per egg ($F_{1,109} = 4.89$, $P < 0.05$) significantly affected female dry weight, without significant interaction ($F_{3,109} = 0.79$, $P = 0.503$). Host species also significantly affected the available resource per egg ($F_{3,111} = 5.79$, $P < 0.01$). Adult male dry weight was not significantly affected by host species ($F_{3,111} = 0.20$, $P = 0.893$), host weight ($F_{1,111} = 0.01$, $P = 0.905$) or their interaction ($F_{3,111} = 0.36$, $P = 0.779$). Resource per egg significantly affected male offspring dry weight ($F_{1,111} = 12.54$, $P < 0.001$). Brood sex ratio was significantly affected by host weight ($F_{1,111} = 9.06$, $P < 0.01$) but not host species ($F_{3,111} = 0.80$, $P = 0.498$). The proportion of offspring that were male declined as host size increased.

Table 5.2 provides summaries of species ranks for individual aspects of parasitoid development. *G. legneri* development data included in this table used the restricted weight range previously detailed. The values for clutch size, and female offspring weight are based on a host weight of 20 mg. For each aspect of development a ranking of 1 indicates a high quality species, and a ranking of 4 indicates a low quality species. *Goniozus legneri* allocates the largest clutch sizes to *C. cephalonica*. The largest female offspring develop from *E. cautella*. *Goniozus legneri* laid the smallest clutches on *P. interpunctella*; broods developed on *P. interpunctella* also had the lowest number of surviving broods.

Table 5.3. Variation in aggression contest behaviour profile according to host species.

Type of Behaviour	Host species				ANOVAs	
	<i>Corcyra cephalonica</i>	<i>Plodia interpunctella</i>	<i>Ephestia cautella</i>	<i>Ephestia kuehniella</i>	$F_{(3,79)}$	P
Biting	0.56±0.08	0.52±0.11	0.55±0.11	0.55±0.11	0.540	0.656
Chasing	0.40±0.06	0.43±0.09	0.27±0.09	0.59±0.09	3.913	0.012
Fighting	0.26±0.05	0.26±0.07	0.22±0.08	0.30±0.08	0.354	0.787
Stinging	0.21±0.06	0.21±0.08	0.26±0.08	0.26±0.08	0.233	0.873
Non-aggressive	0.52±0.07	0.43±0.10	0.42±0.09	0.44±0.09	1.452	0.234

Relative contest behaviour count data of adult female *Goniozus legneri* (USA strain) owners of different host species. MANOVA: Wilks' Lambda = 0.7590, $F_{(15,199)} = 1.39$, $P=0.152$. Because 5 ANOVA tests were conducted the significance criterion were adjusted in respect to the Bonferroni procedure ($0.05/5=0.01$). Behavioural data underwent Log_{10} transformation prior to analysis.

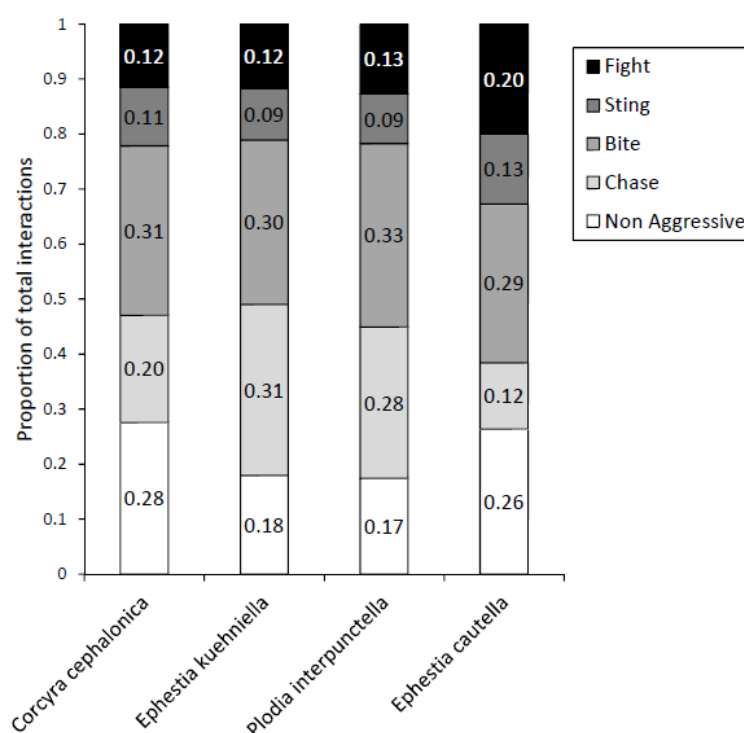


Figure 5.4. Profile of behaviour interactions during *G. legneri* aggression contests. The numbers contained within data bars represent the proportion of this behaviour out of the total behavioural interactions of a particular species.

5.4.3 Part 3: The effects of host species on *G. legneri* contest behaviour

No significant difference in the proportion of total aggressive behaviour was exhibited by *G. legneri* competing for different host species ($F_{3,79} = 0.41$, $P = 0.742$). The behavioural profile of *G. legneri* is similar when defending each of the four host species (Figure 5.4). With the exception of chasing behaviour, the number of individual behaviours did not differ across the four host species; biting ($F_{3,79} = 0.71$, $P = 0.55$), stinging ($F_{3,79} = 2.36$, $P = 0.08$), fighting ($F_{3,79} = 0.45$, $P = 0.72$), non-aggressive ($F_{3,79} = 0.23$, $P = 0.87$) and chasing ($F_{3,79} = 4.98$, $P = 0.003$). Overall no significant differences in the number of interactions were found between species (Table 5.3). There were no significant differences in the behavioural profile of contests for the four host species (Table 5.3). *G. legneri* does not appear to adjust contest behaviour according to the species of host it is competing for.

5.4.4 Part 4: Metabolomic analysis of larval and parasitoid tissue extracts

5.4.4.1 Host NMR spectral identification

Proton NMR spectroscopy of individual and pooled polar host extracts was capable of generating highly complex, high resolution spectra. In addition high resolution, readily assignable 2D HSQC spectra were generated. Comparisons of generated 1D and 2D host spectra with a combination of spectral database standards and the identifications generated by Khidr et al. (2012) resulted in the identification of a total of 27 unique metabolites (Table 5.4). These included a number of assorted sugars, amino acids and organic acids. With the exception of ethanol, citrate, 3- β -hydroxybutyrate and α -ketoglutarate these results largely verify the spectral identifications of *C. cephalonica* performed by Khidr et al. (2012).

Table 5.4. ^1H and ^{13}C chemical shift assignments of *C. cephalonica* NMR spectra.

Metabolite	^1H Chemical Shift (Multiplicity*)	^{13}C Chemical Shift
Alanine	1.47 (d), 3.77 (q)	18.84, 53.24
Arginine	1.68 (m), 1.90 (m), 3.23 (t), 3.76 (t)	26.61, 30.32, 43.19, 57.03
ATP	4.22 (m), 4.29 (m), 4.39 (m), 4.63 (m), 6.13 (d), 8.25 (s), 8.52 (s)	67.95, 73.03, 76.88, 86.68, 89.36, 142.57, 155.34,
Formate	8.46 (s)	173.88
Fumerate	6.50 (s)	138.04
Glutamate	2.04 (m) 2.12 (m), 2.34 (m), 3.75 (d)	29.68, 29.68, 36.25, 57.32
Glutamine	2.12 (m), 2.44 (m), 3.76 (t)	28.95, 33.45, 56.92
Glycerol	3.54 (m), 3.64 (m), 3.77 (tt)	65.24, 74.78
Glycine	3.54 (s)	44.14
Glycine-Betaine	3.26 (s), 3.89 (s)	56.10, 68.93
Histidine	3.12 (dd), 3.22 (dd), 3.97 (dd), 7.05 (d), 7.77 (d)	30.83, 57.53, 119.48, 138.97
Isoleucine	0.93 (t), 0.99 (d), 1.26 (m), 1.46 (m), 1.97 (m), 3.66 (d)	13.8, 17.42, 27.24, 38.63, 62.2
Lactate	1.32 (d), 4.10 (q)	22.79, 71.2
Leucine	0.96 (t), 1.71 (m), 3.72 (m)	23.6, 24.76, 26.64, 42.55, 56.17
Lysine	1.72 (m), 1.89 (m), 3.02 (t), 3.75 (t)	29.13, 32.59, 41.75, 57.17
Methanol	3.34 (s)	51.61
Proline	2.00 (m), 2.06 (m), 2.34 (m), 3.32 (dt), 3.41 (dt), 4.12 (dd)	26.51, 31.69, 31.72, 48.8, 48.8, 63.95
Sarcosine	2.72 (s), 3.60 (s)	35.36, 53.55
Succinate	2.39 (s)	36.95
Taurine	3.24 (t), 3.41 (t)	38.27, 50.11
Threonine	1.32 (d), 3.57 (d), 4.24 (m)	22.20, 63.16, 68.61
Trehalose	3.44 (t), 3.64 (dd), 3.75 (m), 3.82 (m), 3.84 (m), 3.85 (m), 5.18 (d)	63.26, 63.33, 72.42, 73.78, 74.91, 75.22, 95.96
Tryptophan	3.30 (dd), 3.47 (dd), 4.05 (dd), 7.20 (m), 7.27 (m), 7.31 (s), 7.52 (d), 7.73 (d)	29.15, 58.2, 114.62, 112.2, 121.03, 125.10, 127.8
Tyrosine	3.06 (dd), 3.19 (dd), 3.92 (dd), 6.87 (d), 7.19 (d)	38.28, 38.29, 58.94, 118.50, 133.48
Valine	0.98 (d), 1.03 (d), 2.34 (m), 3.59 (d)	19.35, 20.68, 31.69, 63.08
α -Glucose	3.39 (m), 3.52 (dd), 3.70 (t), 3.82 (ddd), 3.83 (m), 5.22 (d)	72.39, 74.13, 74.19, 75.47, 63.38, 94.8
β -Glucose	3.23 (dd), 3.39 (m), 3.46 (m), 3.75 (m), 3.88 (dd), 4.64 (d)	63.29, 76.84, 72.37, 78.59, 63.38, 98.61

*Letters m, t, d, s and q denote peak multiplicity, where m = multiplet, t = triplet, d = doublet, s = singlet and q = quartet

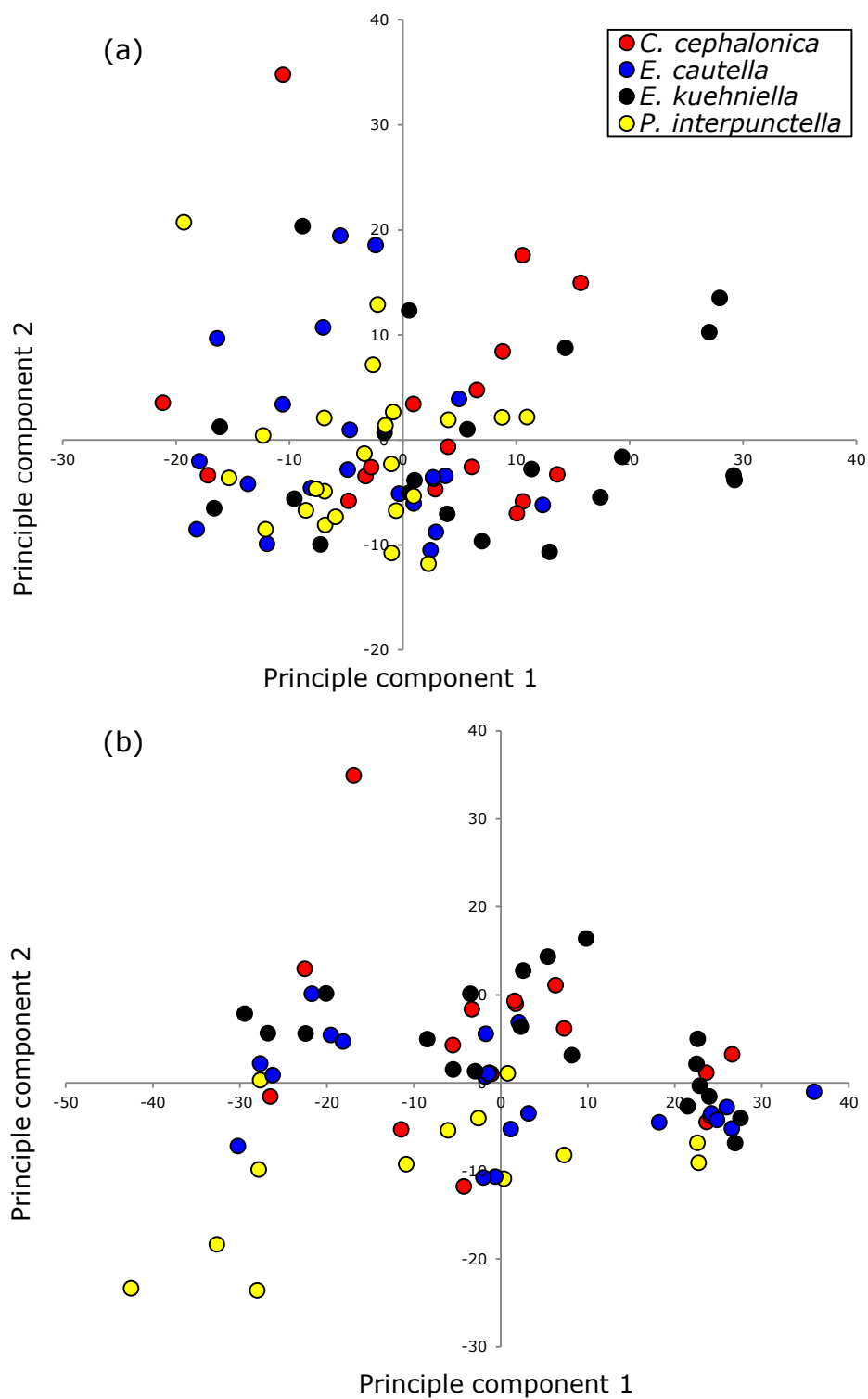


Figure 5.5. Principle components analysis of *G. legneri* and host NMR spectra (a) PCA of host polar extract NMR (PC1 = 27.2 %, PC2 = 16.7 %, PC3 = 9.2 %, $R^2X = 0.836$) (b) PCA of *G. legneri* polar extract NMR (PC1 = 44.5 %, PC2 = 9.8 %, PC3 = 8.4 %, $R^2X = 0.747$)

5.4.4.2 NMR spectroscopy

PCA comparisons of binned NMR spectra for both larval (Figure 5.6a) and *G. legneri* (Figure 5.5b) polar extracts found no clear separation between different parasitoid developmental backgrounds or different species of larvae. Partial separation between *C. cephalonica* and *P. interpunctella* was observed in parasitoid polar extracts (Figure 5.5a). OPLS-DA comparisons between these treatments indicated acceptable goodness of fit ($R^2X = 0.885$), however OPLS-DA comparisons indicated low predictive ability ($Q^2 = 0.457$). The predictive component of the generated model also demonstrated a low R^2X (0.0575). Evaluation of these model diagnostics indicated that no significant separation was present between polar NMR spectra generated from *C. cephalonica* and *P. interpunctella*.

5.4.4.3 Host species LC-MS

PCA comparisons of framed LC-MS data indicated clear separation between lipid extracts generated from each larval host species across the first three principle components (Figure 5.6a). Whilst the greatest separation existed between *C. cephalonica* and the other three host species, all four species clustered separately. Separation between experimental groups is observed across the first eleven principle components in total. Significant separation between host species was established by OPLS-DA (Figure 5.6b). Model prediction with 80% of the dataset randomly excluded generated an average correct prediction rate of 100 % for all treatment comparisons except for *E. kuehniella* vs *E. cautella* (99 %). All Fishers probabilities were below 0.01 after Bonferroni correction. Cross validation (CV) ANOVA provided a p-value of <0.00000001 for the generated OPLS-DA models. Ions heavily weighted specific for host treatments were validated by logistic ANOVA (d.f. 3, 75, $P < 0.05$). A total of 145 ions significantly separated between the four host species. These ions included multiple sodium and acetic acid adducts per unique lipid, and formed a number of sodium based clusters containing multiple metabolites. Of these, a total of 19 unique lipid identities were validated by LC-MS/MS; 15 consisted of phospholipids. These phospholipids consisted of 4 glycerophosphocholines, 7 glycerophosphoethanolamines and 4 lysophospholipids. The majority of phosphoethanolamines were elevated in *C. cephalonica* (5) though two were elevated in *E. cautella* and *P. interpunctella* respectively. Remaining

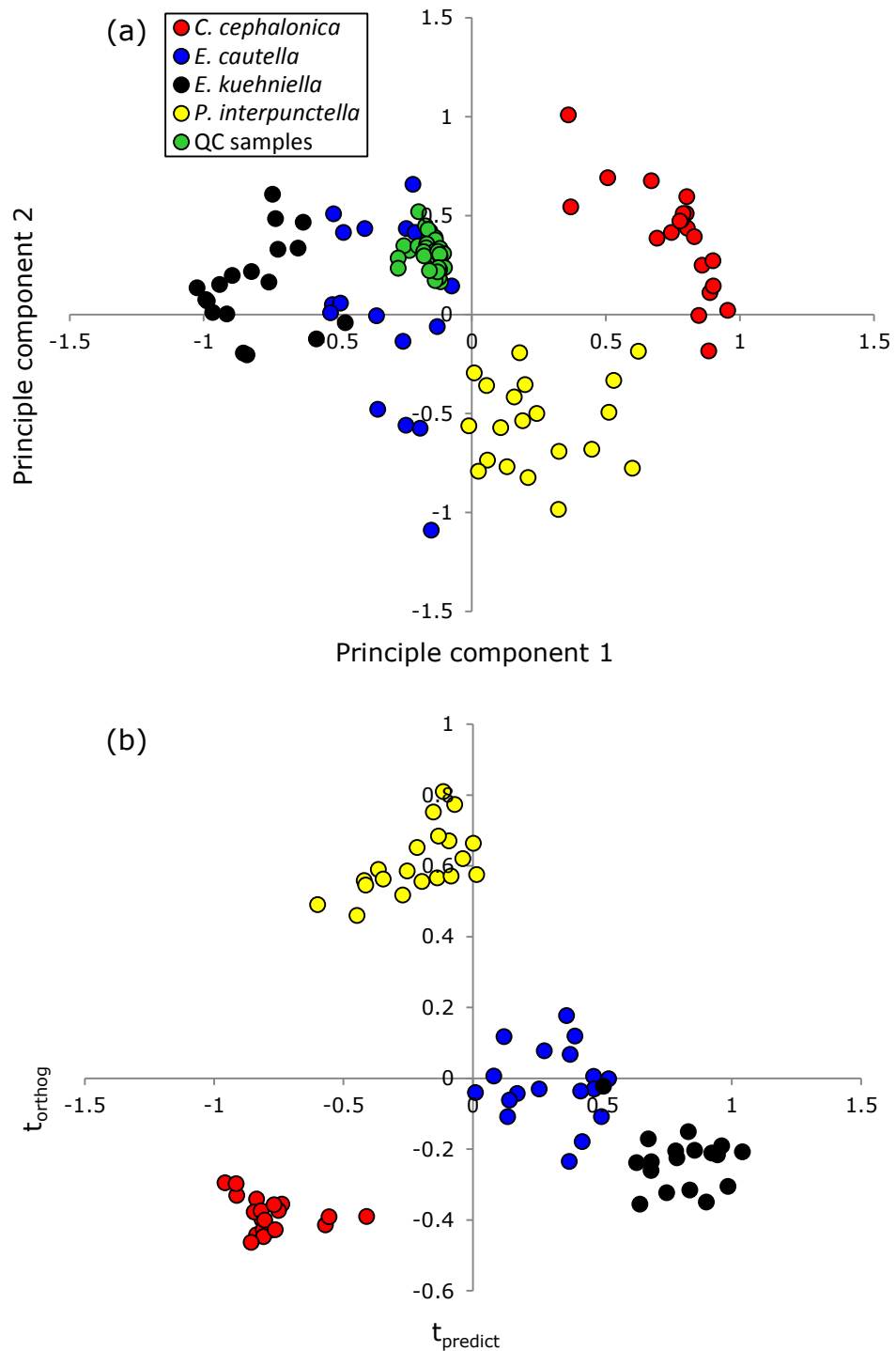


Figure 5.6. Principle components analysis of host LC-MS data (a) PCA of LC-MS data of four different host species (PC1 = 24.3 %, PC2 = 14.2 %, PC3 = 7.8 %, $R^2X = 0.784$) (b) OPLS-DA of the LC-MS data of four different host species ($R^2X = 0.555$, $Q^2 = 0.893$).

lipids consisted of four fatty acyls, palmitoleic, linolenic, linoleic and oleic acid. Fatty acyl and lysophospholipid levels were highest in *E. kuehniella* and, with the exception of oleic acid, lowest in *P. interpunctella*. Separation between *C. cephalonica* and other hosts exhibited in PCA and OPLS-DA plots largely occurred due the presence of 5 ions that occurred in the alternative hosts but were either present in low concentrations or completely absent from *C. cephalonica* lipid extracts (Figure 5.7c). Two major lipid categories were identified as potential candidates for these ions, bile acids and omega fatty acids. However, fragmentation data generated from bile acid and omega fatty acid standards did not match the fragmentation data of these thus unidentified ions. Separation between the remaining three hosts occurred due to varying levels of these unidentified compounds. Elevated metabolite levels for each host species are displayed in Figure 5.7. Bin identities, chemical shift assignments, chemical formulae, *F* ratio, *p*-value and fold changes are outlined in Table 5.5.

QC samples for host species LC-MS data clustered centrally between treatments and remained stable throughout analysis (Figure 5.6a). A total of 83.87% of peak area and retention time RSDs were within the acceptability threshold for establishing analytical stability. The average peak RSD was 26.06 % with a range of 9.24 - 65.94 %. The average retention time RSD was 2.58 % with a range of 0.46 - 21.24 %. A total of 100 % of retention time RSDs were within the acceptability threshold.

5.4.4.4 Parasitoid LC-MS

Analysis of the generated parasitoid LC-MS PCA model indicated separation between a number of wasp host backgrounds (Figure 5.8a). The greatest separation existed between wasps reared on *C. cephalonica* or *E. kuehniella* and those on *P. interpunctella* (Figure 5.8a). OPLS-DA modelling across all four species indicated separation between wasps reared on *C. cephalonica*/*E. kuehniella* and those reared on *P. interpunctella* (Figure 5.8b). Partial separation was also apparent between wasps reared on *C. cephalonica* and those reared on *E. kuehniella*. *E. cautella* exhibited loose intermediate clustering between *C. cephalonica* and *P. interpunctella*. Predictive modelling between *C. cephalonica*, *E. kuehniella* and *P. interpunctella* gave average correct prediction rates of 85 % (*C. cephalonica* vs *E. kuehniella*), 89.71 % (*C. cephalonica* vs *P. interpunctella*), 88.57 % (*E. cautella* vs

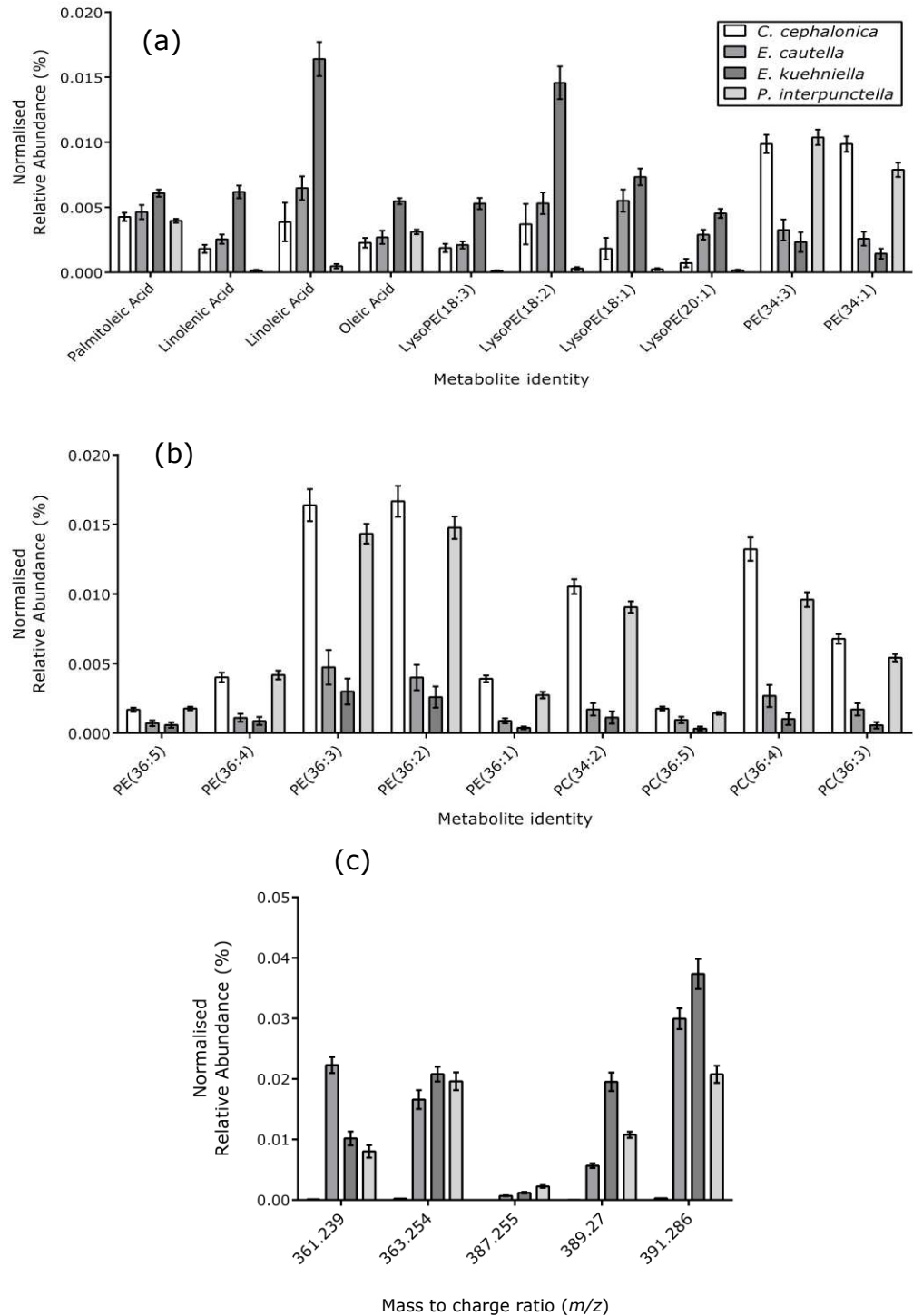


Figure 5.7. Significantly differing lipids between host species (a) fatty acids and phospholipids (b) phospholipids (c) Unidentified host lipids significantly correlated with PCA separations between different host species. These compounds were highly abundant in *E. kuehniella*, *E. cautella* and *P. interpunctella* extracts but either present at trace amounts or completely absent from *C. cephalonica* extracts. PE = phosphoethanolamine, PC = phosphocholine.

Table 5.5. Summary of non-polar biomarkers with tentative identities that significantly differ between host species extracts.
PE = phosphoethanolamine, PC = phosphocholine.

<i>M/Z</i>	<i>Average RT (min)</i>	<i>Adduct</i>	<i>ESI Phase</i>	<i>Common Name</i>	<i>Formula</i>	<i>d.f.</i>	<i>F ratio</i>	<i>P value (B)</i>	<i>Mass error (ppm)</i>
253.217	2.14	M-H	Negative	Palmitoleic acid	C ₁₆ H ₃₀ O ₂	3, 75	71.86	<0.01	0.304
277.218	1.41	M-H	Negative	Linolenic Acid	C ₁₈ H ₃₀ O ₂	3, 75	53.37	<0.01	0.696
279.233	1.70	M-H	Negative	Linoleic Acid	C ₁₈ H ₃₄ O ₂	3, 75	37.21	<0.01	0.046
281.249	2.82	M-H	Negative	Oleic Acid	C ₁₈ H ₃₄ O ₂	3, 75	15.85	<0.01	0.396
474.263	1.44	M-H	Positive	LysoPE(18:3/0:0)	C ₂₃ H ₄₂ NO ₇ P	3, 75	46.32	<0.01	1.980
476.279	1.74	M-H	Negative	LysoPE(18:2/0:0)	C ₂₃ H ₄₄ NO ₇ P	3, 75	29.92	<0.01	1.263
478.294	2.13	M-H	Negative	LysoPE(18:1/0:0)	C ₂₁ H ₄₆ NO ₇ P	3, 75	21.94	<0.01	0.087
506.325	2.05	M-H	Negative	LysoPE(20:1/0:0)	C ₂₅ H ₅₀ NO ₇ P	3, 75	41.67	<0.01	0.213
712.493	4.46	M-H	Negative	PE(34:3)	C ₃₉ H ₇₂ NO ₈ P	3, 75	32.82	<0.01	0.721
716.525	5.07	M-H	Negative	PE(34:1)	C ₃₉ H ₇₆ NO ₈ P	3, 75	57.39	<0.01	1.421
736.493	4.18	M-H	Negative	PE(36:5)	C ₄₁ H ₇₂ NO ₈ P	3, 75	12.69	<0.01	0.721
738.508	4.69	M-H	Negative	PE(36:4)	C ₄₁ H ₇₄ NO ₈ P	3, 75	31.73	<0.01	0.071
740.525	4.68	M-H	Negative	PE(36:3)	C ₄₁ H ₇₆ NO ₈ P	3, 75	40.36	<0.05	1.421
742.54	5.01	M-H	Negative	PE(36:2)	C ₄₁ H ₇₈ NO ₇ P	3, 75	59.95	<0.01	0.771
744.553	4.88	M-H	Negative	PE(36:1)	C ₄₁ H ₈₀ NO ₈ P	3, 75	65.57	<0.01	1.879
816.576	5.13	M+Hac-H	Negative	PC(34:2)	C ₄₂ H ₈₀ NO ₈ P	3, 75	105.07	<0.01	0.060
838.561	4.24	M+Hac-H	Negative	PC(36:5)	C ₄₄ H ₇₈ NO ₈ P	3, 75	13.80	<0.01	0.644
840.576	4.46	M+Hac-H	Negative	PC(36:4)	C ₄₄ H ₈₀ NO ₈ P	3, 75	69.62	<0.01	0.060
842.592	5.13	M+Hac-H	Negative	PC(36:3)	C ₄₄ H ₈₂ NO ₈ P	3, 75	77.19	<0.01	0.344

P. interpunctella) and 83.5 % (*E. cautella* vs *P. interpunctella*). Fisher's exact probabilities for all models were <0.01 after Bonferroni correction. Validation of the generated models by CV ANOVA resulted in an average p-value of 3.63×10^{-16} . Comparisons of *C. cephalonica* with *E. cautella* indicated a poor average correct prediction rate of 70% (Fishers probability = 0.224). As a result it was not possible to establish separation between *C. cephalonica* and *E. cautella*.

Validation of treatment associated ions by logistic ANOVA (d.f. 3, 68, $P < 0.05$) resulted in a total of 48 significant ions. Through a combination of database comparisons and LC-MS/MS fragmentation, it was possible to validate the identities of ions. Due to the presence of multiple adducts this corresponded to a total of 27 unique lipids. These consisted of 13 phospholipids, 13 glycerolipids and the fatty acyl, oleic acid. Phospholipids included 2 glycerophosphocholines, 8 glycerophosphoethanolamines and 3 lysophospholipids. Glycerolipids comprised of 5 diacylglycerides and 8 triacylglycerides. With the exception of the single fatty acyl, no fold changes of these lipid classes were completely correlated with one particular host species background. However, the majority of glycerolipids are present in their highest concentrations in wasps reared on *E. kuehniella* (7), compared to only 1 in those reared on *C. cephalonica* (Figure 5.9). The separation observed in PCA between *E. kuehniella* reared wasps and other host species backgrounds is accounted for by these elevated glycerolipid levels. *E. kuehniella* reared wasps also exhibited higher levels of oleic acid compared to other treatments. *P. interpunctella* reared wasps displayed the lowest concentrations of a majority of glycerolipids. Whilst *C. cephalonica* and *E. cautella* exhibit variable levels of glycerolipids, the majority of glycerolipids for these treatments were intermediate between *E. kuehniella* and *P. interpunctella*. Amongst phospholipids, 7 were elevated in wasps reared on *E. cautella*, compared 1 in those reared on *E. kuehniella*. 2 remaining phospholipids were highest in but did not separate between wasps reared on *E. kuehniella* and *E. cautella*. Separation amongst lysophospholipids was not correlated with host species background; 2 were elevated in *E. kuehniella* reared wasps and 1 was elevated in *P. interpunctella* and *E. cautella* reared wasps. Significantly elevated metabolite levels for wasps reared on each of the host species are displayed in Figure 5.9. Chemical shift assignments,

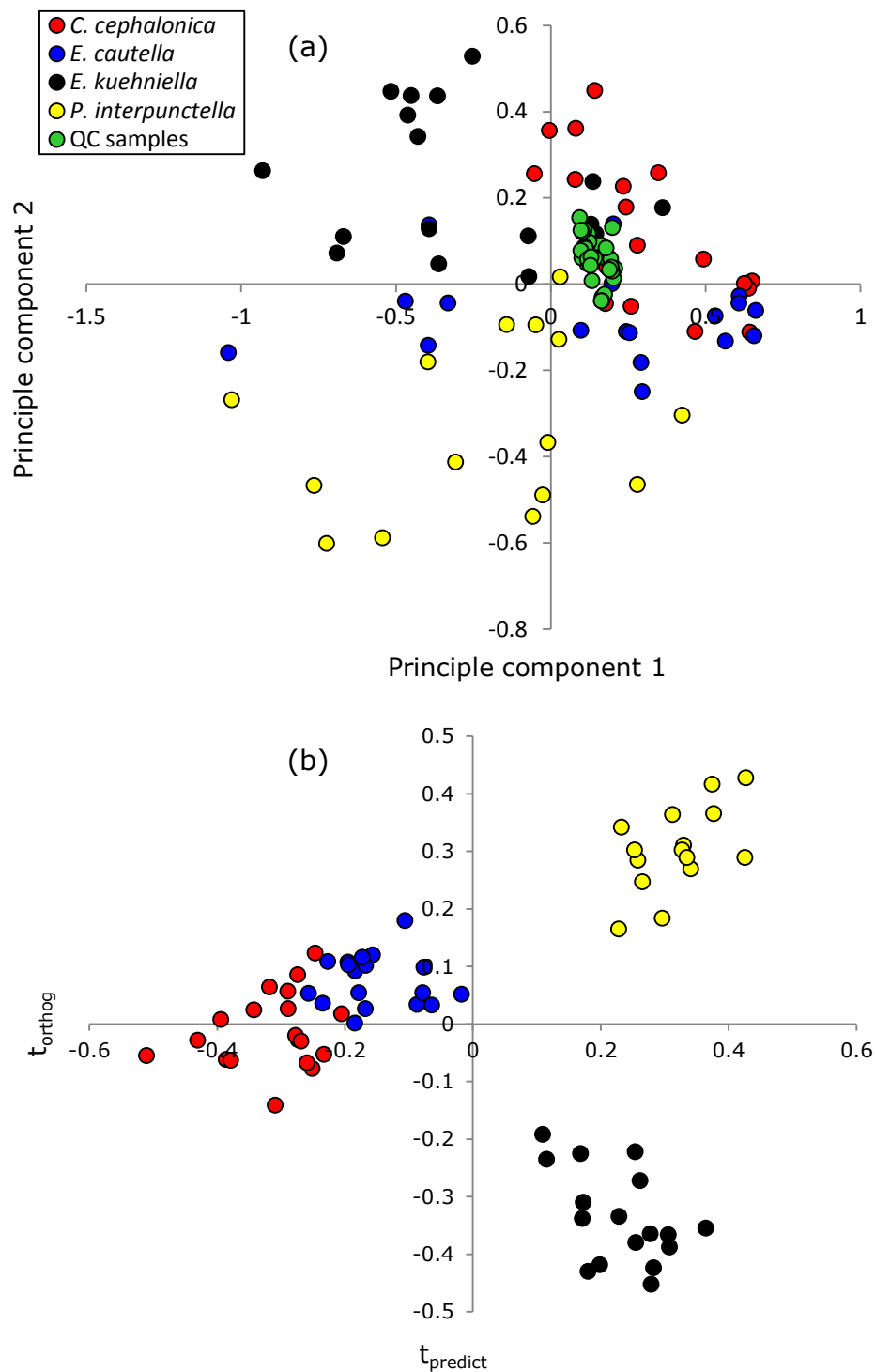


Figure 5.8. Principle components analysis of parasitoid LC-MS data (a) PCA of parasitoid lipid LC-MS data (PC1 = 33.7 %, PC2 = 11.8 %, PC3 = 10.8 %, $R^2X = 0.914$) (b) OPLS-DA of parasitoid lipid LC-MS data ($R^2X = 0.793$, $Q^2 = 0.866$).

chemical formulae, F ratio, p -value and fold changes are outlined in Table 5.6.

QC samples for LC-MS data generated from wasps reared on different host species clustered centrally between treatments and remained stable over time (Figure 5.9a). A total of 88.24% of peak area and retention time RSDs were within the acceptability threshold for establishing analytical stability. The average peak RSD was 14.05 % with a range of 2.62 - 51.39 %. The average retention time RSD was 0.9 % with a range of 0.14 - 4.3 %. A total of 100 % of retention time RSDs were within the acceptability threshold.

With the exception of oleic acid, which was elevated in both *E. kuehniella* and wasps reared on *E. kuehniella*, little correlation was observed between differences in the host metabolome and differences in that of wasps reared across the host range. Amongst hosts the highest levels of phospholipids were detected in *C. cephalonica*, whilst the highest levels of phospholipids amongst wasps were observed in those reared amongst *E. caudata*. No separation in glycerolipids levels was detected between host species, whilst glycerolipids were clearly separated in abundance between wasps reared on each host species. However the findings of the development trials did correlate with differences in the parasitoid metabolome; with wasps reared from hosts considered to be optimal for *G. legneri* development (*E. kuehniella* and *C. cephalonica*) displayed higher levels of storage glycerolipids than those considered to be poor (*P. interpunctella*). Higher levels of these glycerolipids could conceivably enhance wasp fitness by enhancing wasp lifespan as demonstrated in Chapter 4.

Table 5.6. Summary of non-polar biomarkers with tentative identities that significantly differ between wasps reared on different host species. DG = diacylglyceride, PE = phosphoethanolamine, PC = phosphocholine.

M/Z	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Mass error (ppm)
281.249	2.82	M-H	Negative	Oleic Acid	C ₁₈ H ₃₄ O ₂	3, 69	15.16	<0.01	0.396
450.263	1.62	M-H	Negative	LysoPE(16:1/0:0)	C ₂₁ H ₄₂ NO ₇ P	3, 69	18.28	<0.01	0.387
474.263	1.44	M-H	Negative	LysoPE(18:3/0:0)	C ₂₃ H ₄₂ NO ₇ P	3, 69	16.77	<0.01	0.387
476.279	1.74	M-H	Negative	LysoPE(18:2(9Z,12Z)/0:0)	C ₂₃ H ₄₄ NO ₇ P	3, 69	15.35	<0.01	0.737
517.424	5.68	M-H ₂ O-H	Negative	DG(30:2)	C ₃₃ H ₆₀ O ₅	3, 69	14.35	<0.01	1.685
559.472	6.26	M-H ₂ O-H	Negative	DG(33:2)	C ₃₆ H ₆₆ O ₅	3, 69	10.82	<0.01	0.635
585.49	5.60	M-H ₂ O-H	Negative	DG(35:3)	C ₃₈ H ₆₈ O ₅	3, 69	15.83	<0.01	1.715
638.571	5.48	M+NH ₄ , M+Na	Positive	DG(36:2)	C ₃₉ H ₇₂ O ₅	3, 69	28.64	<0.01	0.798
712.493	4.46	M-H	Negative	PE(34:3)	C ₃₉ H ₇₂ NO ₈ P	3, 69	10.91	<0.01	0.721
716.525	5.07	M-H, M+H	Negative	PE(34:1)	C ₃₉ H ₇₆ NO ₈ P	3, 69	9.04	<0.05	1.421
724.53	4.99	M-H	Negative	PE(36:3)	C ₄₁ H ₇₆ NO ₈ P	3, 69	17.32	<0.01	1.336
734.477	3.90	M-H	Negative	PE(36:6)	C ₄₁ H ₇₀ NO ₈ P	3, 69	40.73	<0.01	0.371
736.493	4.13	M-H	Negative	PE(36:5)	C ₄₁ H ₇₂ NO ₈ P	3, 69	27.43	<0.01	0.721
740.521	4.32	M+H	Positive	DG(36:5)	C ₄₁ H ₇₄ NO ₈ P	3, 69	9.24	<0.05	0.921
744.552	5.13	M+H, M+Hac-H	Positive	PE(36:4)	C ₄₁ H ₇₄ NO ₈ P	3, 69	10.21	<0.05	1.740
810.564	5.42	M+Hac-H	Negative	PE(38:4)	C ₄₃ H ₇₈ NO ₇ P	3, 69	13.14	<0.01	1.441
820.512	4.38	M+Hac-H	Negative	PE(38:7)	C ₄₃ H ₇₂ NO ₈ P	3, 69	10.14	<0.05	1.406
836.547	3.86	M+Hac-H	Negative	PC(36:6)	C ₄₄ H ₇₆ NO ₈ P	3, 69	37.73	<0.01	2.292
838.561	4.09	M+Hac-H	Negative	PC(36:5)	C ₄₄ H ₇₈ NO ₈ P	3, 69	21.12	<0.01	0.644

Table 5.6. *Cont.* Summary of non-polar biomarkers with tentative identities that significantly differ between wasps reared on different host species. TG = triacylglyceride.

<i>M/Z</i>	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Mass error (ppm)
868.738	7.22	M+NH ₄	Positive	TG(52:6)	C ₅₅ H ₉₄ O ₆	3, 69	14.5	<0.01	0.864
870.753	7.90	M+NH ₄	Positive	TG(52:5)	C ₅₅ H ₉₆ O ₆	3, 69	10.81	<0.01	1.514
872.769	8.74	M+NH ₄	Positive	TG(52:4)	C ₅₅ H ₉₈ O ₆	3, 69	11.24	<0.01	1.164
874.784	5.29	M+NH ₄	Positive	TG(52:3)	C ₅₅ H ₁₀₀ O ₆	3, 69	10.15	<0.05	1.814
894.754	7.14	M+NH ₄	Positive	TG(54:7)	C ₅₇ H ₉₆ O ₆	3, 69	58.62	<0.01	0.514
896.768	7.82	M+NH ₄ , M+K	Positive	TG(54:6)	C ₅₇ H ₉₈ O ₆	3, 69	35.05	<0.01	2.164
898.784	8.63	M+NH ₄ , M+NA, M+K	Positive	TG(54:5)	C ₅₇ H ₁₀₀ O ₆	3, 69	37.89	<0.01	1.814
901.725	7.78	M+NH ₄	Positive	TG(54:4)	C ₅₇ H ₉₈ O ₆	3, 69	34.9	<0.01	0.559

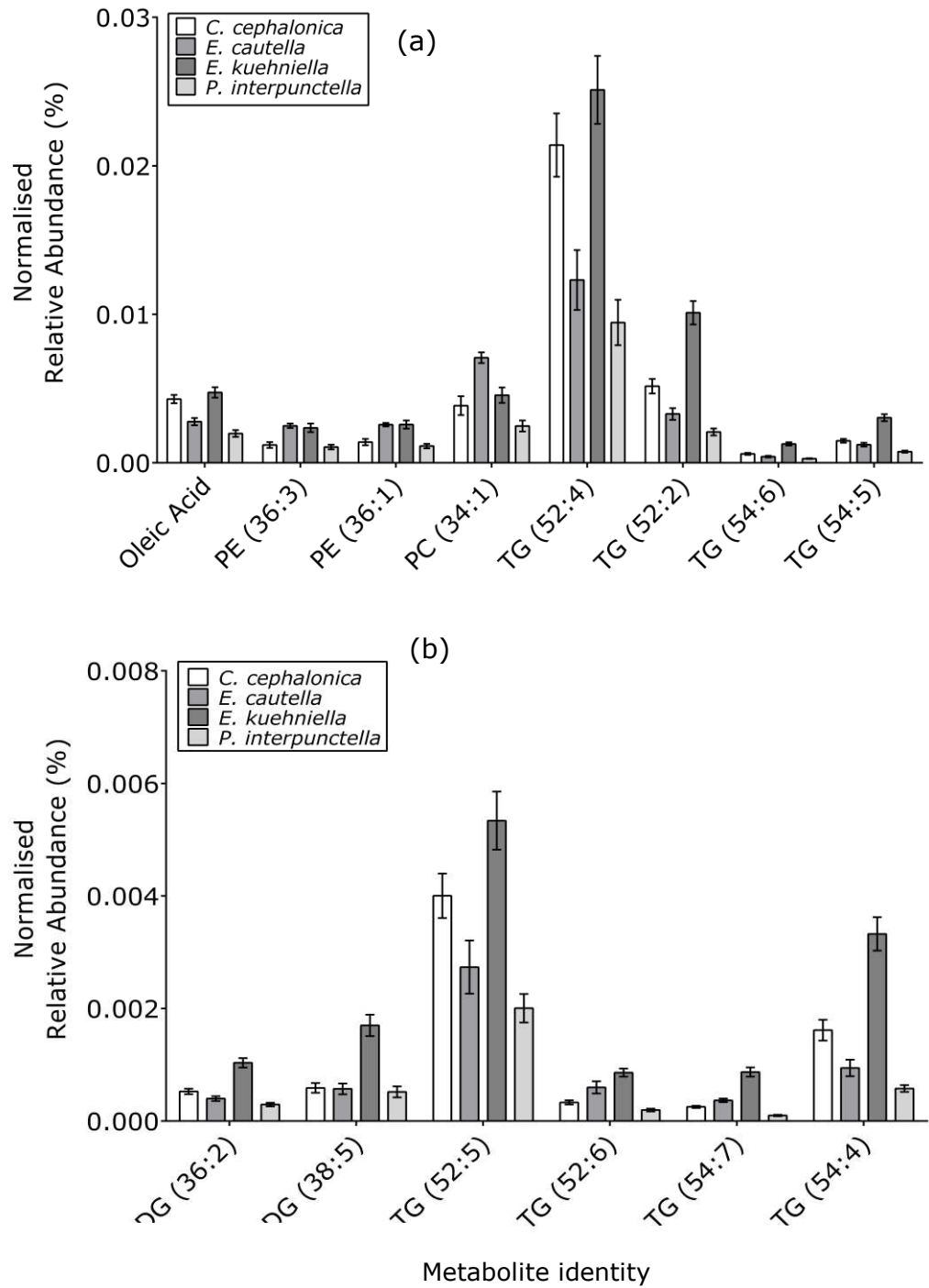


Figure 5.9. Biomarker lipids between parasitoids reared across four lepidopteran hosts (a) phospholipids and triacylglycerides (b) diacylglycerides and triacylglycerides. PE = phosphoethanolamine, PC = phosphocholine, DG = diacylglyceride, TG = triacylglyceride.

5.5 Discussion

High rates of host paralysis were exhibited by *G. legneri* and *G. nephantidis*. Oviposition behaviour indicates that *E. kuehniella* and *C. cephalonica* were accepted at the highest rate (100 % and >90 % respectively), suggesting that they are valued more highly than *E. cautella* and *P. interpunctella*. *G. nephantidis* exhibited similar brood rearing success to *G. legneri* when parasitizing *C. cephalonica*, but failed to consistently rear broods on any alternative hosts. Comparisons of performance between *G. legneri* and *G. nephantidis* when parasitizing *C. cephalonica* indicate that *G. legneri* appears to maximise total brood size throughout development at this fixed host weight, *G. nephantidis* instead selects for adult offspring fitness.

No significant differences in initial host acceptance were found between *G. legneri* and *G. nephantidis*. This demonstrates that *G. nephantidis* is at least initially capable of recognising the alternative hosts as a potential resource, though further cues required for the onset of oviposition behaviour appear to be diminished or absent. Bethyloid parasitoids have been observed to engage in host feeding prior to oviposition behaviour (Pérez-Lachaud and Hardy, 2001), as a result they may be capable of detecting hemolymph biomarkers associated with poor host quality. However, differences in metabolites crucial for parasitoid development between host species were not observed in either NMR or LC-MS analysis. The lack of separation between complex hemolymph sugars and energy rich storage lipids indicates that lack of oviposition on some host species may be due to more subtle metabolite differences.

A number of currently unidentified compounds were highly elevated in the alternative host species and were largely absent in *C. cephalonica*, it is possible that these compounds may affect *G. nephantidis* oviposition. Alternatively differences in the cuticular hydrocarbon profile between species may result in a lack of recognition by *G. nephantidis* prior to the oviposition stage. A study by Khidr et al. (2013a) has indicated that adult *G. legneri* reared on each of the four species display quantitative differences in cuticular hydrocarbon profile; however the hydrocarbon profiles of the host species themselves has not been compared. Finally, the incompatibility may be protein-mediated or due to varying immune responses between host

species, such as encapsulation (Nappi et al. 1971, Kraaijeveld and Godfray 2006), something that this study has not examined. However an immune response is unlikely due to the ectoparasitoid feeding style of *Goniozus* wasps, along with the compatibility of *G. legneri* for all host species.

One *G. nephantidis* brood reared on *P. interpunctella* generated a single live adult parasitoid, in 7 other cases wasps also laid eggs on each alternative host that failed to develop. All but one clutch initially consisted of a single egg, significantly lower than the clutch size of 3 - 18 eggs typically produced by *G. nephantidis* (Dharmaraju and Pradhan 1977; Hardy et al. 1992; Petersen and Hardy 1996). Whilst this constitutes too few cases to assess developmental performance of *G. nephantidis* when oviposition does occur, it could be possible to further assess performance by artificially generating a series of size adjusted broods. This could be achieved by transferring eggs immediately after oviposition on *C. cephalonica* to pre-paralysed alternative hosts (Hardy et al. 1992). Similarly, if the unknown compounds detected in *E. kuehniella*, *E. cautella* and *P. interpunctella* were to be identified, the role of these compounds in host acceptance could be assessed through a series of developmental trials. By injecting these compounds into pre-paralysed *C. cephalonica*, then exposing them to *G. nephantidis*, their role in host acceptance could be directly assessed. Similar injections of D₂O were utilised by Goubalt et al. (2008) to tag volatile chemicals released by *G. nephantidis* during contests. However further attempts at the identification of these unknown compounds would require the use of further spectroscopic techniques, such as 2D NMR or GC-MS.

G. legneri laid its largest clutch sizes on *C. cephalonica* and its lowest on *P. interpunctella*. *C. cephalonica* clutch sizes remain the highest across host species when both its full weight range and the overlapping weight range with other species is considered. This differentiation in clutch sizes indicates that *G. legneri* is capable of assessing host quality in terms of both size and species. Adult females with the greatest dry weight were produced on *E. cautella* and *P. interpunctella*, with the lowest produced on *C. cephalonica*. The lower clutch sizes allocated to *E. cautella* and *P. interpunctella* by *G. legneri* results in a higher amount of available resource per egg than for clutches laid on *C. cephalonica*. Resource per egg was found to significantly affect wasp dry weight, thus the increased resource per egg available from

smaller clutches laid on *E. cautella* and *P. interpunctella* likely accounts for their greater offspring dry weight. Offspring emerging from *E. cautella* were significantly larger than those emerging from *E. kuehniella*, despite similar clutches being laid on both hosts, possibly indicating that *E. cautella* is a higher quality host for *G. legneri*. Findings that host species, weight, and their interaction influence adult female offspring are consistent with those of Karsai et al. (2006). Male *Goniozus legneri* dry weight was unaffected by host species, likely due to their smaller size and lower resource requirements.

Lipid analysis of *G. legneri* reared on each of the four hosts indicated that a majority of glycerolipids are elevated in wasps reared on *E. kuehniella*, whilst elevated levels of phospholipids did not correlate with one particular host species. *G. legneri* reared on *P. interpunctella* generally displayed the lowest levels of these lipids; this is a likely explanation for why *P. interpunctella* is the least suitable host for *G. legneri* in developmental trials. *E. kuehniella* also displayed the highest levels of essential fatty acyls, whilst *P. interpunctella* displayed the lowest levels, further supporting their selection as the best and worst hosts respectively. *E. kuehniella* also exhibited the highest levels lysophospholipids, a class of phospholipids with roles in extracellular signalling and cell proliferation (zu Heringdorf and Jakobs 2006). The adult fitness of *G. legneri*, in terms of longevity, fecundity and ability to compete for hosts, was highest when reared on *E. kuehniella*, due to its larger size and lipid stores (Hardy et al. 1992; Petersen and Hardy 1996). As with most hymenopteran parasitoids (Casas et al., 2005; Jervis et al., 2008; Visser et al., 2010), *G. legneri* is incapable of *de novo* lipogenesis, adult wasps are limited to lipids gathered from the host during development (Chapter 3 and 4). Wasps emerging with a higher lipid load may possess an advantage over others, due to their greater contest performance and increased lipid reserves for egg maturation. Two glycerolipids were also elevated in *C. cephalonica*, indicating that different developmental backgrounds may also result in variations in glycerolipid profile, along with overall quantity. Unfortunately however, it is not possible to directly draw correlations between changes in the host metabolome and changes in the wasp metabolome. This is due to the lack of treatment specific correlation between significantly elevated phospholipids and specific

host species, and the currently unidentified status of a number of compounds associated with hosts other than *C. cephalonica*.

Though *G. legneri* emerging from either *E. kuehniella* or *C. cephalonica* seem to have greater adult fitness than those emerging from *P. interpunctella*, it should be noted that the host size range used for wasp metabolomic analysis was not restricted to the overlapping weight range used when assessing *G. legneri* performance. As a result elevations in glycerolipids may be due to the higher weight ranges of *C. cephalonica* and *E. kuehniella* compared to *P. interpunctella*. To assess the possibility of host weight acting as a possible confounding variable, metabolomic analysis of wasps reared on hosts limited to this range (5 - 30 mg) could be performed.

Host species did not affect the level of aggression displayed by *G. legneri* within owner-owner contests. This study focused on the aggression levels of wasps defending different host species instead of contest winner-loser outcome as it was anticipated that aggression behaviour would reflect the contestant's perceptions of host quality (Stockermans and Hardy 2013). However, as none of the species are a natural host for *G. legneri* in the field, it may be too great an assumption that they can make adaptive decisions on this scale, though their clutch size behaviour does seem to be influenced by the same considerations. Another possibility is that the host exposure period limited the amount of assessment that wasps were capable of prior to contests. A previous study by Stockermans and Hardy (2013) staged contests after 24 hours of parasitoid-host exposure; however a pilot for this current study found that oviposition routinely occurred on a majority of trial hosts in this time period. As a result the time period was reduced to five hours. An intermediate time period between these two before contest trials may have allowed for more accurate resource assessment by contestants. If wasps are incapable of such adaptive decisions, contest performance of wasps developed on differing host species could be assessed through a standard intruder-intruder winner-loser contest setup as applied in Chapter 4 of this thesis. By assessing the outcome of contests between wasps reared on *E. kuehniella* and wasps reared on *P. interpunctella*, the fitness, specifically contest ability, of wasps reared on higher quality and lower quality hosts could be assessed. As it is possible that a wasp may display higher aggression when attempting to acquire a host species matching that

on which it developed, a third host species, such as *E. cautella* or *C. cephalonica*, should be used within these contests.

5.6 Conclusions

Host species affects the oviposition behaviour and brood development of *Goniozus legneri* and *G. nephantidis*. *G. legneri* performed best when parasitizing *E. cautella* and *C. cephalonica*, and worst when parasitizing *P. interpunctella*. *G. nephantidis* exhibited similar performance to *G. legneri* when parasitizing *C. cephalonica* but was incapable of consistently parasitizing hosts other than *C. cephalonica*. These differences in host suitability may be due to differing metabolomic profiles between host species; however the key metabolites responsible for these differences remain unidentified. Adult female *G. legneri* that had developed on *E. kuehniella* and *C. cephalonica* exhibited the highest levels of high-energy storage lipids; those reared on *P. interpunctella* exhibited the lowest levels. Despite these differences in host suitability, host species did not affect the level of aggression exhibited by *Goniozus legneri* in owner-owner contests. Whilst the biochemical mechanisms underlying host suitability remain unknown, host species clearly affects the oviposition behaviour of adult *Goniozus* wasps, along with the fitness and metabolome of emerging parasitoid offspring.

Chapter 6: Host age legacy does not influence parasitoid contest performance

6.1 Abstract

The quality of a host has a significant effect on the developmental success and subsequent fitness of adult *Goniozus* wasps. Adult *Goniozus* wasps also display less aggression when attempting to acquire older hosts, along with allocating lower clutch sizes when ovipositing. The quality of a host has been demonstrated to decline post-paralysis, biochemicals associated with energy production gradually decline over time, whilst metabolic waste products accrue in host tissues. This chapter demonstrates that whilst host aging does affect the metabolomic state of parasitoid offspring, it does not significantly affect the outcome of owner-owner contests between *Goniozus legneri* reared on hosts of differing post-paralysis age. This may be attributed to the relatively young age of wasp contestants (3 - 5 days post emergence); these wasps may not yet be experiencing nutritional limiting factors associated with old age. Adult wasps emerging from older hosts exhibited decreased levels of energy rich metabolites such as diacylglycerides and triacylglycerides, these lower levels may become a significant limiting factor later in wasp lifespan. Further investigations could potentially explore contest behaviour in older host-aged wasps, along with longevity and fecundity.

6.2 Introduction

A number of previous studies have assessed how the quality of nutrition available in early life affects life-history traits and decisions in adulthood (Zwaan et al. 1991; Lindström 1999; Kaspi et al. 2002; Gorman and Nager 2004; Boggs and Freeman 2005; Royle et al. 2005; Blanckenhorn 2006; Naguib et al. 2006; Barrett et al. 2009; Hopwood et al. 2014; O'Hagan et al. 2015). Immature hymenopteran parasitoids are limited to the lipid resources obtained during larval development. As larval development is limited to a single host (Godfray 1994), the quantity and quality of resource available from a given host is of particular importance when considering a parasitoid's evolutionary fitness. In particular, host quality has been demonstrated to affect a wide range of fitness-correlated parasitoid behaviours, such as host acceptance (Rivers and Denlinger 1995; Pérez-Lachaud and Hardy, 2001), sex ratio (Rivers and Denlinger 1995; Harvey 2000; Cleary and van Ginkel 2004), brood guarding decisions (Batchelor et al. 2005; Humphries et al. 2006; Goubault and Hardy 2007), developmental mortality (Pérez-Lachaud and Hardy 2001; Cleary and van Ginkel, 2004; Häckermann et al. 2007), adult offspring fitness (Wright and Kerr 1988; Rivers and Denlinger 1995; Harvey 2000; Pérez-Lachaud and Hardy 2001; Häckermann et al. 2007) and longevity/fecundity of parasitoid offspring (Wright and Kerr 1988; Hardy et al. 1992; Otto and Mackauer 1998; Ueno 2004; Karsai et al. 2006; Lopez et al. 2009; Aruna and Manjunath 2010).

Furthermore, host quality is reliant on a range of inter-connected facets, including host size (Schmidt 1991; Hardy et al. 1992; Harvey et al. 1994; Otto and Mackauer 1998; Zaviezo and Mills 2000; Cleary and van Ginkel 2004; Ueno 2004), developmental instar (Kidd and Jervis 1991; Harvey et al. 1994; Vet et al. 1994; Otto and Mackauer 1998; Karsai et al. 2006; Thiel and Hoffmeister 2009; Fand et al. 2010), host species (Wright and Kerr 1988; Rivers and Denlinger 1995; Pérez-Lachaud and Hardy 2001; Cleary and van Ginkel 2004; Häckermann et al. 2007; Thiel and Hoffmeister 2009), disease state (Hochberg 1991), diapause status (Rivers and Denlinger 1993; 1994) and age at the onset of parasitism (Ode and Strand 1995; Hofstetter and Raffa 1998; King 1998; Sousa and Spence 2001; Wang and Liu 2002; Ueno 2004; He and Wang 2006). In addition, factors extrinsic to the host, such as diet (Harvey et al. 1995; Vinson et al. 2001; Ode 2006), previous parasitism (van Alphen and Visser 1990; Kraaijeveld 1999; Goubault et al.

2007; Moretti and Calvitti 2008), or endosymbiosis (Whitfield 1990; Carton and Nappi 1997) can also effect host quality.

Both adult wasp physiology and the quality of a given host have been established as significant determining factors in *Goniozus* wasp contest outcome (Batchelor et al. 2005; Humphries et al. 2006; Goubault et al. 2006, 2007, 2008; Goubault and Hardy 2007; Lizé et al. 2012; Stockermans and Hardy 2013; Hardy and Briffa 2013). Contest outcome is determined by two major factors, resource value (RV) and resource holding potential (RHP). RV related aspects of wasp contest performance include the age of a host; older wasps display a higher level of aggression within contests as a given host is more valuable to them than younger wasps (Stockermans and Hardy 2013). Similarly larger hosts represent a higher value resource than smaller hosts, and contestants display higher levels of aggression to both acquire and defend (Stockermans and Hardy 2013) larger hosts. Owners of a given host also demonstrate a higher probability of victory than intruders, presumably due to possessing greater information regarding the host's value (Goubault et al. 2007). Alternatively the value of a given resource may differ due to physiological status of the contestants e.g. if one competitor has eggs matured (Stokkebo and Hardy 2000). A host may also represent a greater resource investment for the owner, especially if a wasp has already oviposited (Goubault et al. 2007; Bentley et al. 2009). Other fitness related factors within wasp contests are directly related to the RHP of a wasp; larger individuals possess an advantage over smaller individuals (Petersen and Hardy 1996).

A prior study (Khidr et al. 2012) examined the effect of host post-paralysis aging on *Goniozus* wasp life history decisions. Wasp oviposition decisions were affected by the post-paralysis age of *Corcyra cephalonica* hosts, with lower clutch sizes being allocated to older hosts of equivalent size. Mortality was higher in broods developing on older hosts, and surviving adults were significantly smaller than those emerging from younger hosts. Khidr et al. (2012) also demonstrated that initial ownership of a younger host by a wasp in owner-owner contests improved its probability of contest victory. Khidr et al. (2012) utilised a novel metabolomic approach to assess the biochemistry associated with particular host ages. NMR analysis of polar host compounds indicated that levels of metabolites associated with energy production, such

as glucose and ATP, decreased with age post-paralysis. A number of waste products, such as citrate and ethanol, also increased with host age.

However, few prior studies have examined the effects of varying developmental nutrition on subsequent aggression and contest performance in adults. Royle et al. (2005) showed that the quality of nutrition available to adolescent male green swordtails (*Xiphophorus hellerii*) affected dominance status in later life. Post-eclosion nutritional variation similarly affected contest outcome in the burying beetle *Nicrophorus vespilloides*, pre-eclosion nutrition influenced body size but not contest success (Hopwood et al. 2014). This shows that the quality of developmental/juvenile nutrition received can significantly affect life history decisions; however its precise effect on an organism's RHP and RV is still unknown.

This study further explores the effects of post-paralysis rearing on offspring fitness in *Goniozus* wasps. The ectoparasitoid wasp *Goniozus legneri* was reared on a range of post-paralysis aged *Corcyra cephalonica*. Owner-owner contests involving adult parasitoids from these broods were used as a bioassay to assess the effects of differing larval nutrition on contest outcome. The biochemistry of both hosts and parasitoids was simultaneously assessed using a combined NMR and LC-MS metabolomic approach.

6.2.1 Aims and objectives

This chapter aims to explore whether developing on a hosts of variable post-paralysis age influences the contest behaviour of adult female *G. legneri*. Specific objectives required to fulfil these aims are:

- To stage owner-owner contests between adult female *G. legneri* reared on *C. cephalonica* that had undergone post-paralysis aging for 1 day and 14 days respectively.
- To perform combined NMR and LC-MS analysis of the metabolomes of 1 day, 7 days and 14 days post-paralysis aged *C. cephalonica*.
- To perform combined NMR and LC-MS analysis of the metabolomes of adult female *G. legneri* reared on 1 day, 7 days and 14 days post-paralysis aged *C. cephalonica*.

6.3 Materials and methods

6.3.1 Parasitoid origin and cultures

All parasitoid culturing and contest experiments were performed at 27 °C; with 60 - 70 % relative humidity maintained by water bath within the climate room. A 16:8 hour light:dark cycle was employed throughout culturing. Established laboratory cultures of the bethylid wasp *Goniozus legneri* Gordh (USA strain; Lizé et al. 2012) were used for all experiments. These parasitoids have been laboratory reared for >9 years and are maintained on *Corcyra cephalonica* larvae. *C. cephalonica* was cultured in kilner jars using the artificial diet reported by Lizé et al. (2012), which consisted of corn meal, wheat bran, honey, glycerol and yeast. Adult wasps were selected at 3 - 5 days post eclosion, an established age for adult female dispersal from natal broods (Hardy et al. 1999). No female had undergone prior experimental treatment or experienced hosts as an adult.

6.3.2 Part 1: The influence of nutritional legacy on *G. legneri* contest outcome

To assess the legacy of host age on parasitoid contest outcome, individual *C. cephalonica* of known weight were individually exposed to an adult female *G. legneri* in a stoppered glass vial. The wasp was discarded after initial paralysis was confirmed and before oviposition. Paralysed hosts remained in the same tube for 14 days before use in experiments; these are referred to as 'old' hosts. After 13 days a separate group of hosts were exposed individually to adult female *G. legneri*, the wasp was discarded after paralysis was confirmed. These hosts remained in their tubes for another 24 hours and are referred to as 'young' hosts. At the end of the isolation period a new set of adult female *G. legneri* were individually placed in the same tubes as the surviving hosts and allowed to engage in brood rearing behaviour. Any dead hosts were removed from experimental analysis prior to wasp exposure. This experimental design is summarised in Figure 6.1.

Emerging adult female offspring were utilised in owner-owner contests to assess the effects of developmental background on contest performance.

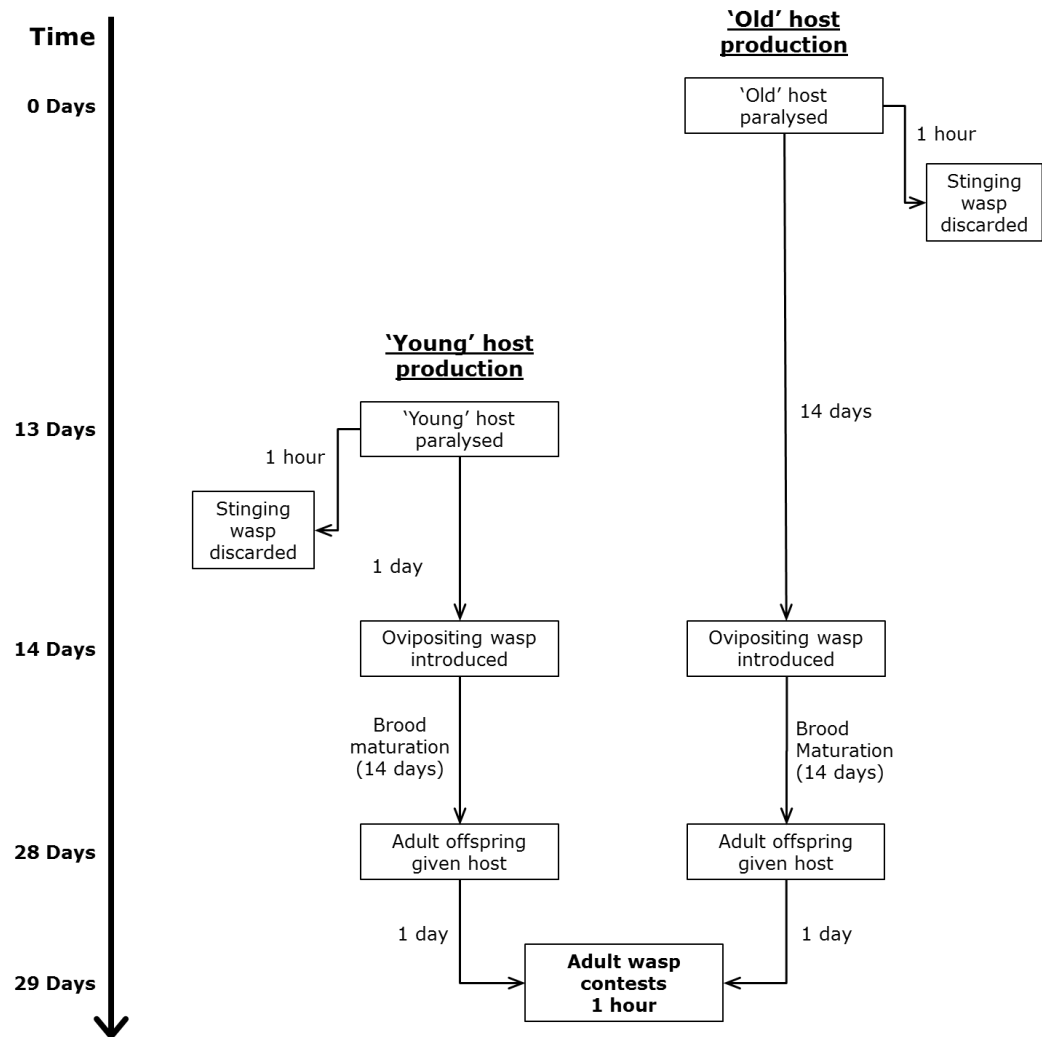


Figure 6.1. Schematic representation of the host-aging protocol prior to owner-owner contests.

Wasps were paired within contests by weight (to an accuracy of 0.01 mg) to minimise asymmetries. Hosts owned by adult offspring were paired to within 1 mg of each other, with a total weight range across all contests of 22.08 - 72.51 mg. Individual wasps were presented with *C. cephalonica* in a sealed glass vial 24 hours prior to contests which they were allowed to paralyse. Any wasps that oviposited throughout this period were removed. No wasp had prior experience of contests.

Owner-owner contests were performed using established experimental methods (Petersen and Hardy 1996; Goubault et al. 2007). Wasps were distinguished by applying a dot of red or yellow acrylic paint to the dorsal

surface of the thorax; colour coding was randomised between host age treatments to prevent visual bias. Wasp-host pairs were placed in the central chamber of a 3 chamber contest blocks, as developed by Petersen and Hardy (1996), and separated from each other by removable barriers. After a 30 minute acclimatisation period, the barriers were removed. Interactions between the wasps were recorded for 60 minutes using a digital video camera. Filmed contests were viewed using Windows Media Player to assess contest occurrence and outcome. A win or loss outcome was recorded after one wasp retreated from the central contest chamber in response to an obvious display of antagonistic behaviour. In instances where more than one interaction occurred, the wasp that had retreated from the central chamber at the end of the contest period was considered the loser (Petersen and Hardy 1996). A total of 82 contests were performed.

6.3.3 Part 2: Metabolomic analysis of larval and parasitoid tissue extracts

To assess the effects of post-paralysis aging on the host metabolome, the initial contest experimental setup summarised in Figure 6.1 was replicated with the addition of a 7 day paralysis treatment. These host treatments were referred to as 'middle-aged' hosts within the context of this study. Adult wasps were utilised to paralyse 7 day hosts under the same methodology employed for 1 day and 14 day hosts.

At the 14th day post paralysis 1 day, 7 days and 14 days old hosts were washed in HPLC-grade water to remove excess culture media and faecal matter. Hosts were weighed to an accuracy of 0.01mg and then separated into two groups. The first group were immediately snap-frozen in liquid nitrogen to halt metabolic activity. The second group were individually exposed to female *G. legneri* which were allowed to engage in brood rearing behaviour. Parasitoid offspring between 3 - 5 days old were separated and weighed to an accuracy of 0.01 mg before snap-freezing in liquid nitrogen. Host weights ranged between 31.11 and 58.82 mg. Parasitoid weights ranged between 0.62 to 1.51 mg. A total of 20 replicates were performed for each larval and parasitoid treatment. Frozen hosts and parasitoids

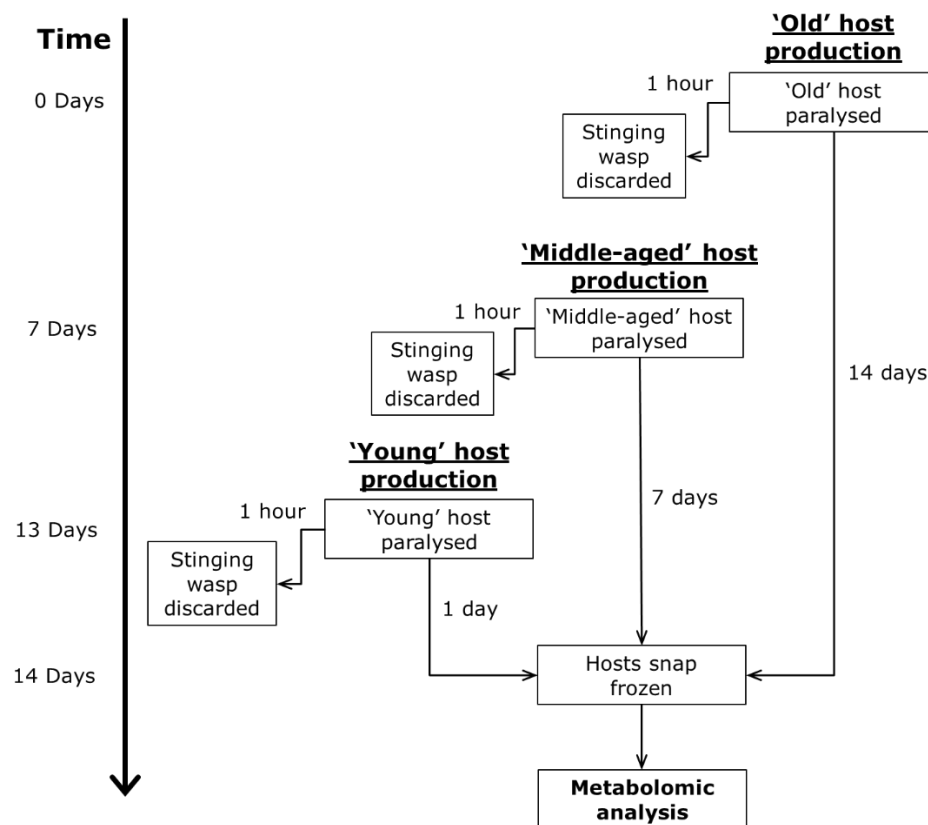


Figure 6.2. A schematic diagram outlining host-aging treatments prior to liquid nitrogen snap freezing and metabolomic analysis.

were kept at -80°C prior to solvent extraction. The host treatment setup is summarised in Figure 6.2, the parasitoid treatment setup is summarised in Figure 6.3. Tissue samples were randomised prior to metabolite extraction. All solvents used during extractions were of a high analytical grade (LC-MS grade CHROMASOLV Sigma-Aldrich) and were kept on ice throughout extraction.

6.3.3.1 Host extraction protocol

A two-step solvent addition extraction protocol, modified from Wu et al. (2008), was used for metabolite extraction. Hosts were homogenised in ice cold methanol ($4\ \mu\text{L}/\text{mg}$) and water ($2\ \mu\text{L}/\text{mg}$) using a fast prep 24 lysing matrix Z (MP-BIO®). Approximately 80% of silica spheres were removed prior to homogenisation to aid in sample reclamation. Homogenate was transferred via Pasteur pipette to a sterilised 2 mL borosilicate glass vial. The lysing matrix was then washed with $4\ \mu\text{L}/\text{mg}$ methanol and $2\ \mu\text{L}/\text{mg}$ of

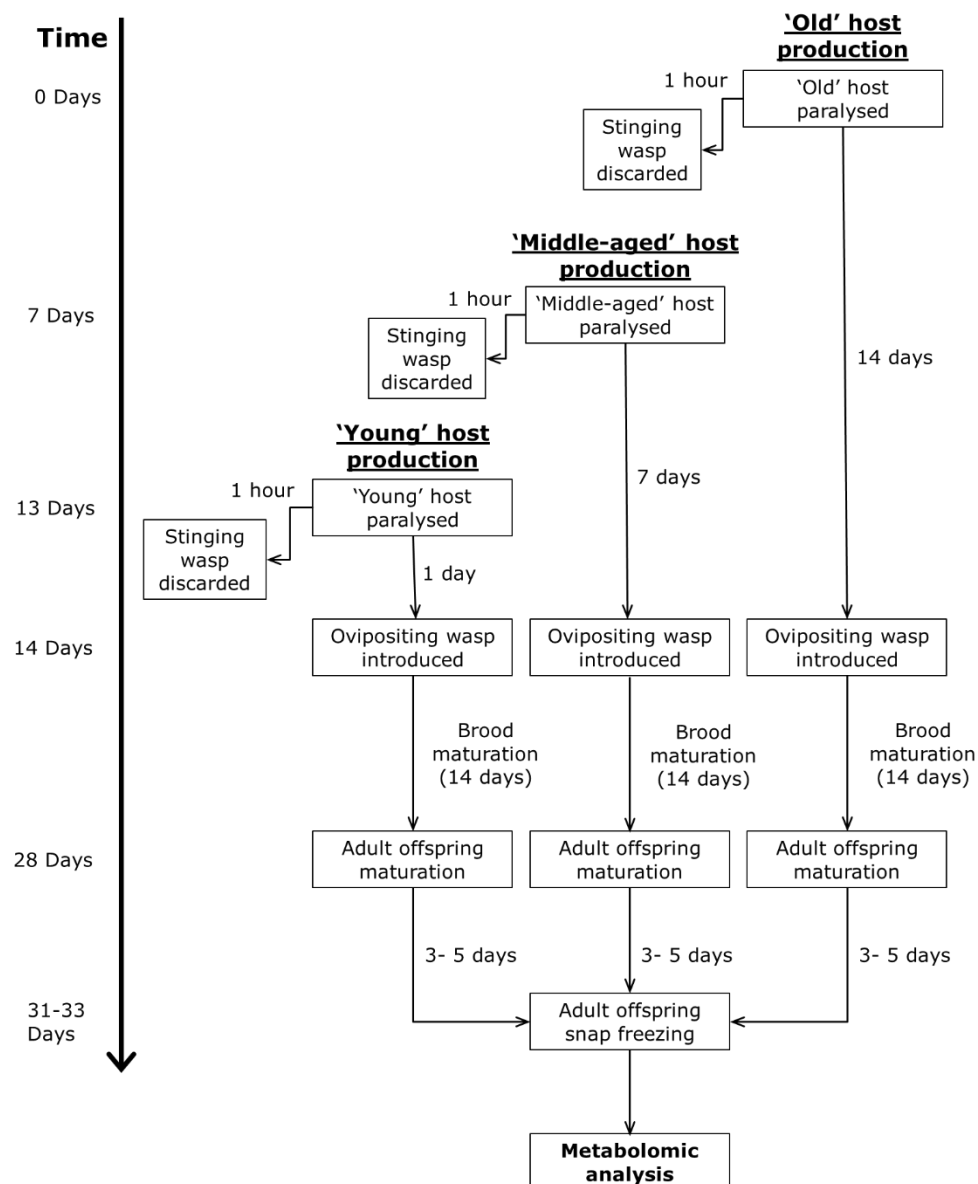


Figure 6.3. A schematic diagram outlining host-aging protocol prior to offspring parasitoid liquid nitrogen snap freezing and metabolomic analysis.

water then vortexed for 30 seconds. This extra homogenate was then transferred via Pasteur pipette to the original glass vial. A remaining 4 $\mu\text{L}/\text{mg}$ of methanol was added to homogenate along with 8 $\mu\text{L}/\text{mg}$ of chloroform. This resulted in a final solvent ratio of 3:2:1 of methanol/chloroform/water. Samples were vortexed for 30 s then maintained on ice for ten minutes. Samples were centrifuged for 10 min at 13,000 G to remove debris and set at room temperature for ten minutes. Phases were then separated using a Pasteur pipette and transferred to either a 1.5 mL solvent resistant Eppendorf Biopur® tube (polar/methanol phase) or a 2 mL glass vial (non-polar/chloroform phase). Polar and non-polar phases were randomised and

stored at -80 °C until metabolomic analysis. Samples were reconstituted prior to NMR or LC-MS analysis using the protocol outlined in Chapter 3 with an added dilution step (1:10 sample-isopropanol blank) to prevent excessive peak tailing associated with column silica saturation.

6.3.3.2 Parasitoid extraction protocol

Parasitoids were extracted using the modified Bligh and Dyer (1959) biphasic extraction protocol, as outlined in Chapter 3. Generated solvent phases were separated after extraction and transferred to either a sterile 1.5 mL solvent resistant Eppendorf Biopur® tube (polar/methanol phase) or a 2 mL borosilicate glass vial (non-polar/chloroform phase). Phases were maintained at -80 °C until analysis. All samples were randomised before extraction. Phases were dried and reconstituted as outlined under the methodology given in Chapter 3 before sample analysis. A pooled quality control sample was also generated that consisted of 10 µL from each sample.

6.3.3.3 NMR Spectroscopy

1D NMR spectra were generated from individual polar larval and parasitoid samples using a Bruker Advance 800 MHz III spectrometer equipped with a 5 mm QCI cryoprobe. A spectral width of 13 ppm was employed during spectral acquisition; spectral signal was averaged over 512 transients. A NOESY pre-saturation experiment was applied to suppress any water resonances. Representative samples generated under the same extraction protocol were used to determine pre-saturation frequency and power prior to experimental analysis. Samples were locked using the internal D₂O signal and the internal DSS signal was referenced to 0.0 ppm. Total recycling delay was 4.7 s. An exponential window function was applied to give line broadening of 0.3 Hz prior to zero filling and Fourier transformation.

6.3.3.4 LC-MS Analysis

The modified lipidomic Accela LC-Exactive MS setup outlined in Chapter 2 and 3 was replicated for analysis of both larval and wasp lipid extracts (Ion range: 100 - 1500m/z, ESI voltage: 3500, capillary temperature: 350 °C, scan rate: 250 ms). The pooled QC sample was injected repeatedly into the LC before experimental analysis to pre-equilibrate the column; this sample

was intermittently injected throughout the analytical timeframe to maintain and track column stability. A set of model samples generated under the same extraction protocol were injected in triplicate prior to analysis of experimental samples to establish technical stability between sample replicates.

6.3.3.5 LC-MS stability validation

To validate the stability of the LC-MS approach, the relative standard deviations of the peak areas and retention times of the key ions outlined in Chapter 2 were assessed within the pooled QC samples of both aged host and wasp samples. An RSD acceptability threshold of <30 % for a minimum of 70 % of key ions was considered acceptable to establish LC-MS stability. QC samples were further required to cluster centrally within the generated PCA plots to confirm LC-MS stability.

6.3.3.6 LC-MS/MS analysis

The identity of ions was validated through fragmentation using tandem mass spectrometry (MS/MS). Remaining parasitoid and larval extracts were pooled prior to LC-MS/MS analysis to improve yield. The analytical and instrumental approach outlined in Chapter 3 was replicated for fragmentation. With the exception of sample pooling, injection protocol, mobile phase gradient program and column conditions were all replicated from initial LC-MS analysis. An ion range of m/z 100 - 1500 was monitored (ESI voltage: 3000, capillary temperature: 275 °C, scan rate: 50 ms) and collision energies of 10, 25 and 40 V were employed during fragmentation.

6.3.4 Data analysis

Contest data from Part 1 of this chapter was analysed with generalized linear models using the statistical package Genstat (Version 15, VSN International, Hemel Hempstead, UK). Logistic analysis of variance (ANOVA) was employed to determine whether host age treatment affected the probability of a wasp winning. The response variable was defined as 1 = red wasp won, 0 = red wasp lost. Differences in wasp and host weights between participants were fitted as explanatory variables alongside host age treatment (Hardy and Field 1998). A fixed dispersion parameter of 1 was applied throughout

analysis (Crawley 1993). Binomial tests were carried out alongside the main data analysis using colour as a variable to check that it did not influence contest results.

For part 2 all NMR spectra and LC-MS chromatograms were corrected, aligned and binned/framed according to the protocol detailed in Chapter 2. Frames/bins were normalised to either total bin intensity (NMR) or total chromatogram ion count (LC-MS) before multivariate data analysis using Microsoft excel. Normalised tables were imported in Simca 13.0 (Umetrics) package where sample classification was visualised by principle components analysis (PCA). Mean-centring and scaling was applied to individual samples as outlined in Chapter 2. Separation between classes was established by orthogonal partial least squares-discriminant analysis (OPLS-DA).

Ions were selected by weighted comparisons between classes and through analysis of underlying PCA scores plots. One-way binomial ANOVAs were applied using Genstat v.15 (VSN International) to validate potential biomarker ions. Metabolite intensity/area was employed as numerator and total spectral intensity/chromatogram area was employed as denominator. Generated p-values were adjusted using the Bonferroni correction to account for multiple comparisons (Quinn and Keogh 2002). An adjusted p-value threshold of 0.05 was used to confirm differentiation. NMR and LC-MS metabolites were assigned initial identities through spectral database comparisons and validated by comparisons with 2D NMR identifications from Chapter 2 and LC-MS/MS fragmentation data.

6.4 Results

6.4.1 Part 1: The influence of nutritional legacy on *G. legneri* contest outcome

Out of a total of 82 contests, 58 resulted in a clear contest outcome. Host legacy had no significant effect on the probability of winning a contest ($G_{1,40} = 0.08$, $P = 0.771$) (Figure 6.4). Wasp weight difference ($G_{1,40}=1.49$, $P=0.222$), host difference ($G_{1,40} = 0.02$, $P = 0.893$) and their interaction term ($G_{1,40} = 0.3$, $P = 0.581$) had no significant effect on the probability of red wasp contest success. The interactions between red wasp legacy and wasp weight difference ($G_{1,40} = 1.56$, $P = 0.212$) and between red wasp legacy and host weight difference ($G_{1,40} = 0.26$, $P = 0.613$) had no significant effect on contest outcome. The interaction term between red wasp legacy, wasp weight difference and host weight difference had no significant effect on red wasp contest success.

6.4.2 Part 2: Metabolomic analysis of larval and parasitoid tissue extracts

6.4.2.2 Host aging NMR spectroscopy

PCA comparisons of binned NMR data for host polar extracts (Figure 6.5a) found clear separation between young and old hosts when considered across the first three principle components. Middle-aged hosts clustered between young and old host spectra and partially separated from both. Separation between experimental treatments was observed across the first nine principle components. OPLS-DA analysis established separation between young and old host treatments (Figure 6.5b). Cross validation-ANOVA of the generated model resulted in a p-value of <0.001 after Bonferroni correction. Further validation in the form of prediction modelling as outlined in Chapter 3 resulted in an average correct prediction rate of 91.76 % (Fisher's exact probability <0.0001). OPLS-DA models comparing young and middle-aged hosts ($R^2X = 0.159$, $Q^2 = 0.9$) and middle-aged and old hosts ($R^2X = 0.218$, $Q^2 = 0.813$) indicated poor goodness of fitness. As a result no separation was confirmed between these treatments.

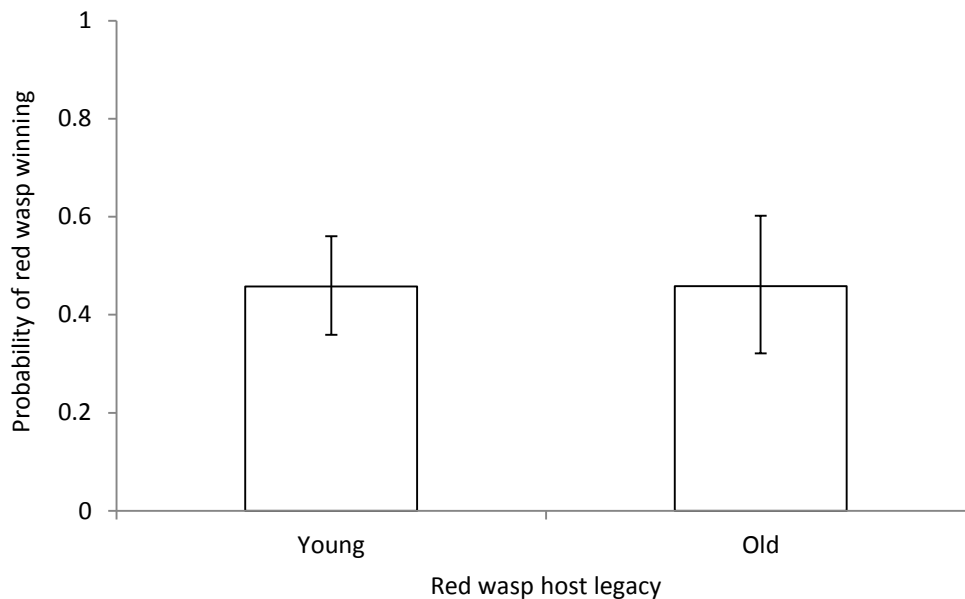


Figure 6.4. The effect of host legacy on contest outcome in owner-owner contests. Error bars represent standard error of the means (SEM). Treatment means and S.E.s were back transformed from logit-scale estimates.

Logistic ANOVA validation of treatment weighted bins resulted in a total of 91 bins that significantly differed between young and old treatments (d.f. 2, 54, $P < 0.05$). Of these, 57 were found to be either baseline fluctuations or associated with unknown or lipid spectral peaks. Of the remaining 34 bins, 28 were correlated with young hosts and 6 were correlated with old hosts. Spectral assignments of these bins indicated that a number of common haemolymph sugars were elevated in young hosts. Several amino acids were elevated in young hosts, along with the dicarboxylic acid succinate. However, histidine, lysine and sarcosine are elevated in old hosts. Metabolite levels in middle-aged host treatments generally displayed intermediate levels between young and old treatments. Significantly elevated polar metabolite levels for young, middle-aged and old hosts are displayed in Figure 6.6. Bin identities, chemical shift assignments, chemical formulae, F ratio, p -value and fold changes are outlined in Table 6.1.

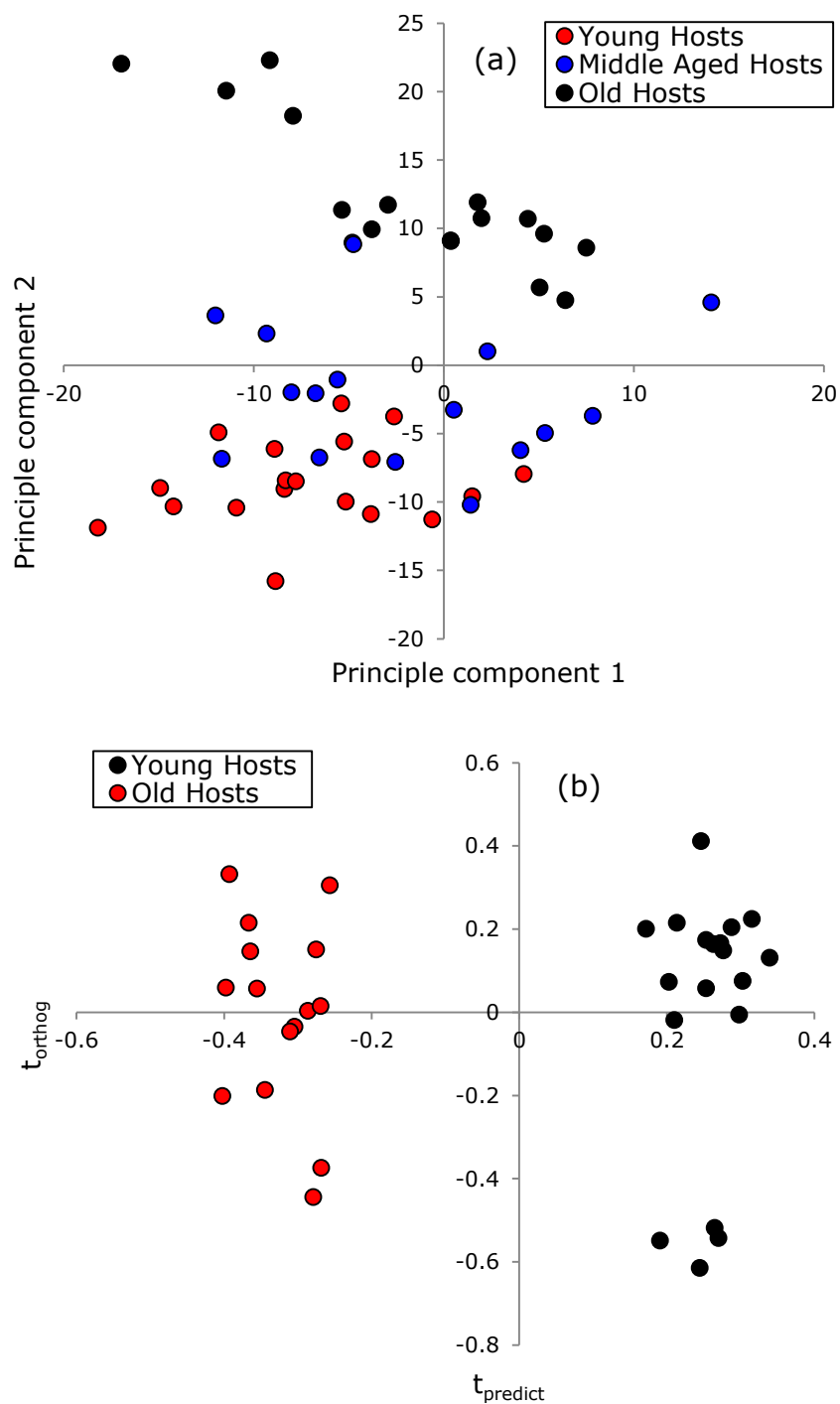


Figure 6.5. Principle components analysis of parasitoid LC-MS data (a) PCA of aged hosts NMR data (PC1 = 12.5%, PC2 = 10.1%, PC3 = 10.1 %, $R^2X = 0.621$) (b) OPLS-DA of parasitoid lipid LC-MS data ($R^2X = 0.67$, $Q^2 = 0.85$).

Table 6.1. Summary of polar biomarkers with tentative identities that significantly differ between young and old host extracts.

Common Name	Chemical shift (ppm)	d.f.	Formula	F ratio	P-value (B)	Fold change ¹
Glutamine	2.12 (m), 2.44 (m), 3.76 (t)	2, 54	C ₅ H ₉ NO ₄	24.92	<0.01	-3.48
Glycerol	3.55 (m), 3.64 (m), 3.77 (tt)	2, 54	C ₃ H ₈ O ₃	24.31	<0.01	-1.56
Histidine	3.12 (dd), 3.22 (dd), 3.97 (dd), 7.05 (d), 7.77 (d)	2, 54	C ₆ H ₉ N ₃ O ₂	30.19	<0.01	2.71
Leucine	0.96 (t), 1.71 (m), 3.72 (m)	2, 54	C ₆ H ₁₃ NO ₂	18.82	<0.01	-2.05
Lysine	1.72 (m), 1.89 (m), 3.02 (t), 3.75 (t)	2, 54	C ₆ H ₁₄ N ₂ O ₂	13.77	<0.05	1.79
Sarcosine	2.72 (s), 3.60 (s)	2, 54	C ₃ H ₇ NO ₂	13.19	<0.05	3.08
β-Glucose	3.24 (dd), 3.4 (m), 3.47 (m), 3.74 (m), 3.88 (dd), 4.64 (d)	2, 54	C ₆ H ₁₂ O ₆	29.37	<0.01	-1.77
Threonine	1.32 (d), 3.57 (d), 4.24 (m)	2, 54	C ₄ H ₉ NO ₃	38.39	<0.01	-1.86
Trehalose	3.44 (t), 3.64 (dd), 3.75 (m), 3.82 (m), 3.84 (m), 3.86 (m), 5.19 (d)	2, 54	C ₁₂ H ₂₂ O ₁₁	35.70	<0.01	-1.93
Tryptophan	3.30 (dd), 3.47 (dd), 4.05 (dd), 7.20 (m), 7.27 (m), 7.31 (s), 7.52 (d), 7.73 (d)	2, 54	C ₁₁ H ₁₂ N ₂ O ₂	36.95	<0.01	-2.34
Tyrosine	3.04 (dd), 3.18 (dd), 3.92 (dd), 6.89 (d), 7.19 (d)	2, 54	C ₉ H ₁₁ NO ₃	25.29	<0.01	-1.86
α-Glucose	3.45 (t), 3.52 (dd), 3.70 (t), 3.76 (m), 3.82 (ddd), 3.83 (m), 5.22 (d)	2, 54	C ₆ H ₁₂ O ₆	36.85	<0.01	-2.13

¹ Negative fold changes indicate metabolites that declined in abundance between 0 day old and 14 day old hosts, positive fold changes indicate metabolites that increased in abundance between 0 day old and 14 day old hosts.

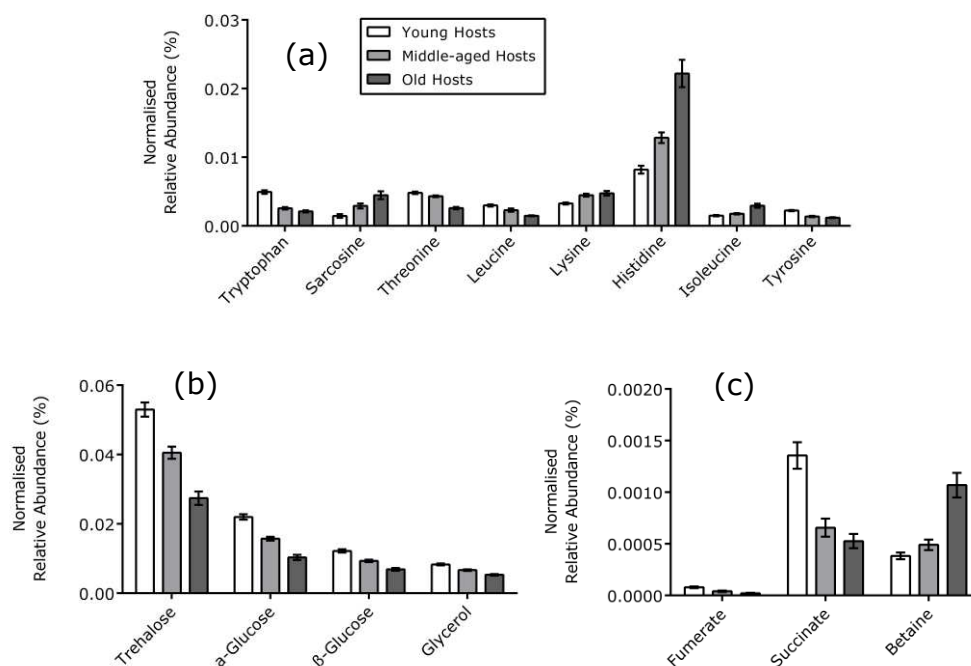


Figure 6.6. Polar metabolite abundances across young, middle-aged and old hosts (a) amino acids (b) sugars (c) organic acids and betaine.

6.4.2.3 Parasitoid NMR spectroscopy

PCA comparisons of binned parasitoid NMR spectra found no clear separation between parasitoid developmental backgrounds (Figure 6.7). However, inspection of spectra generated from internal control samples indicated that a high level of NMR tube contamination was present (Figure 6.8). NMR signals generated from this contamination broadly overlap, and in some cases, overshadow those generated in experimental samples. As a result any possible PCA differentiation between experimental treatments appears to be masked by variations in background contamination. Comparisons of control spectra with spectra generated from the previous investigation that utilised the same NMR tubes found a wide degree of spectral overlap with host polar samples generated during the host species experiments outlined in Chapter 5.

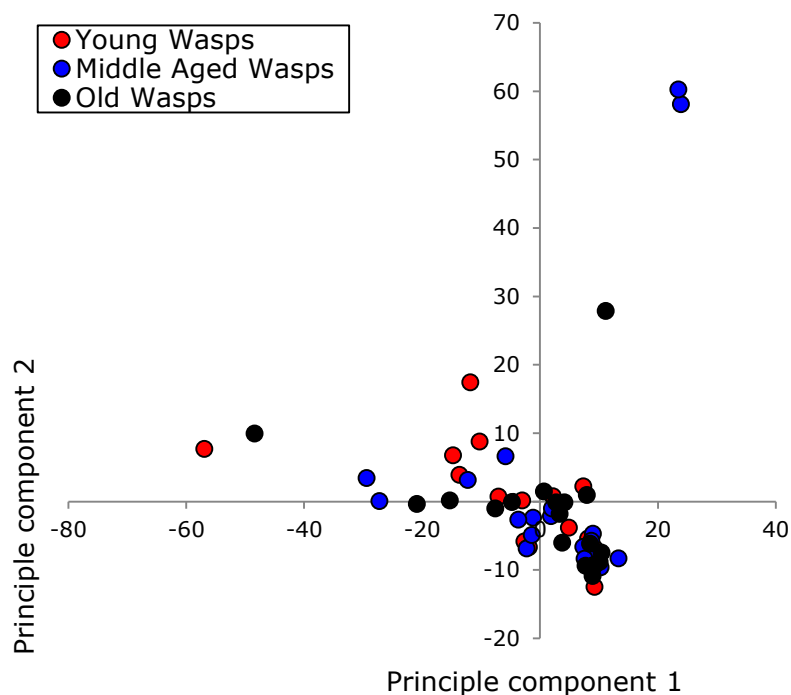


Figure 6.7. PCA of aged wasp NMR data (PC1 = 22.6%, PC2 = 18.8%, $R^2X = 0.707$).

6.4.2.4 Host aging LC-MS

Evaluation of PCA models generated from host aging LC-MS data detected poor separation between individual treatment classes across the first three principle components when two components are visualised simultaneously. However, when all three components are visualised together (Figure 6.9a), separation becomes apparent between young and old host lipid extracts. Middle-aged lipid extracts clustered between the two but do not indicate clear separation from either. OPLS-DA comparisons of young host lipid extracts and old host lipid extracts indicated good separation (Figure 6.9b). Validation of the generated model by CV-ANOVA resulted in a p -value <0.001 after bonferroni correction. Further predictive validation of the model with 80% of the dataset randomly excluded resulted in average correct prediction rate of 87.57% (Fisher's exact probability = 0.0000068).

LC-MS ions correlated with individual host treatments were statistically validated through logistic ANOVA (d.f. 2, 54, $P < 0.05$). Significant separation was detected for 18 ions between young and old aged hosts. Of these a total

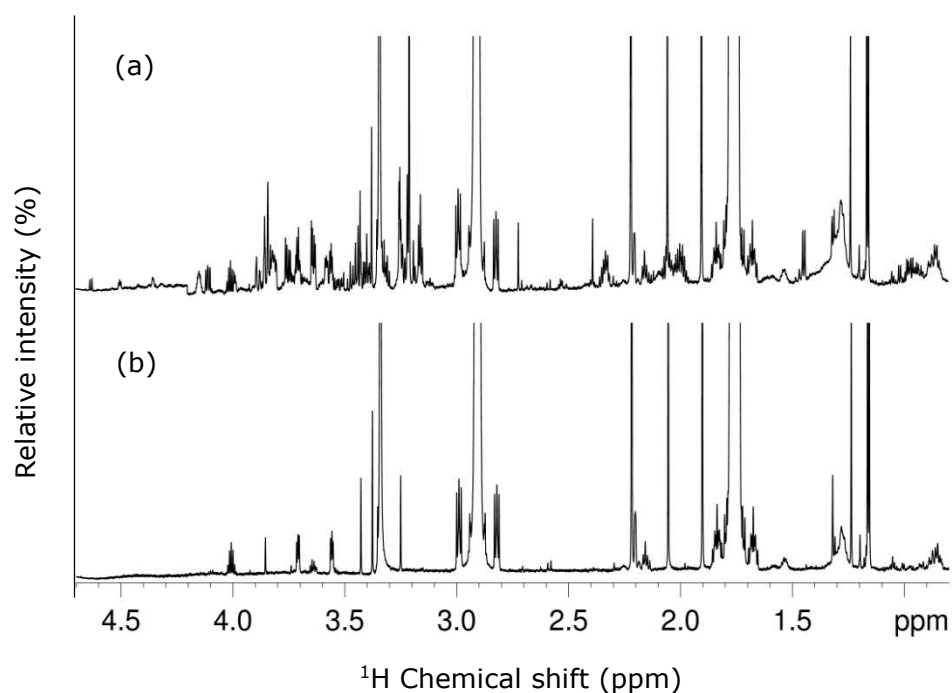


Figure 6.8. Comparison of wasp aging 1D NMR spectra with control spectra (a) Representative wasp spectra from young host reared wasps (b) Blank D_2O spectra generated alongside experimental samples. The high degree of overlap in metabolite resonances observed between wasp and control spectra indicates a high level of contamination within NMR tubes which may be obscuring differentiation between experimental classes.

of 13 were identified and successfully validated by spectral database comparisons and LC-MS/MS. Due to multiple sodium and ammonium adducts this number was further reduced to 9 unique lipids. These lipids comprised of 5 glycerophosphocholines and 4 triacylglycerides (Figure 6.10). Of these lipids, 4 glycerophosphocholines and all 4 triacylglycerides are elevated in young hosts, with a single glycerophosphocholine elevated in old hosts. Individual mass to charge ratios, identities, elution times, adducts, F values and fold changes of significant metabolites are outlined in Table 6.2.

QC samples for both host aging samples clustered centrally between treatment groups and exhibited very little drift over time. A total 93.39 % of ions displayed peak area RSDs within the acceptability threshold for analytical stability. QC ions displayed an average peak area RSD of 19.67

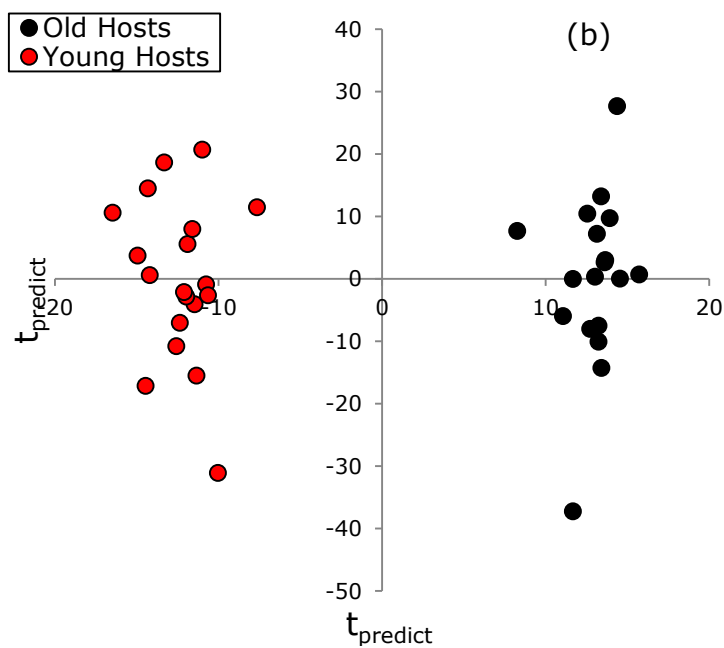
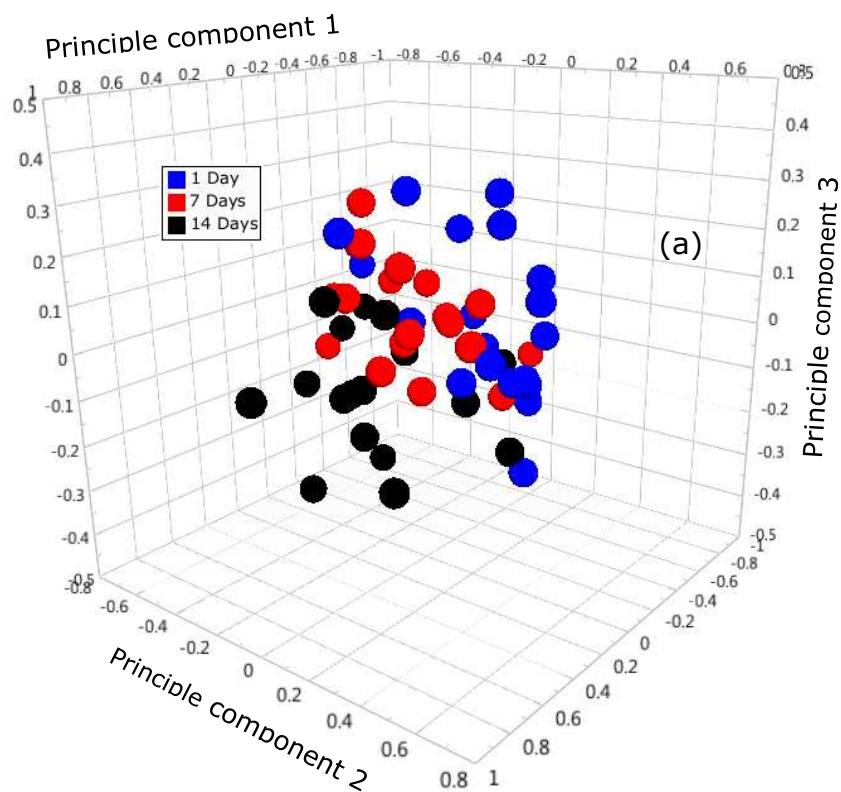


Figure 6.9. Principle components analysis of aged host LC-MS data (a) PCA of host lipid LC-MS data (PC1 = 29.1%, PC2 = 23.9, PC3 = 7.71%, $R^2X = 0.843$) (b) OPLS-DA of host lipid LC-MS data ($R^2X = 0.555$, $Q^2 = 0.893$).

Table 6.2. Summary of polar biomarkers with tentative identities that significantly differ between young and old host extracts. PE = phosphoethanolamine, PC = phosphocholine, PG = phosphoglycerol, DG = diacylglyceride, TG = triacylglyceride.

M/Z	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Fold change¹	Mass error (ppm)
718.537	4.85	M+H	Positive	PE(34:1)	C ₃₉ H ₇₆ NO ₈ P	2, 53	15.49	<0.01	-1.85	1.131
742.538	4.49	M+H	Positive	PE(36:3)	C ₄₁ H ₇₆ NO ₈ P	2, 53	13.24	<0.05	-2.18	0.131
758.569	5.38	M+H	Positive	PC(34:2)	C ₄₂ H ₈₀ NO ₈ P	2, 53	28.06	<0.01	-2.66	0.431
784.585	5.24	M+H	Positive	PC(36:3)	C ₄₄ H ₈₂ NO ₈ P	2, 53	26.42	<0.01	-2.31	0.810
818.592	6.29	M+NH ₄	Positive	PG(38:3)	C ₄₄ H ₈₁ O ₁₀ P	2, 53	14.34	<0.05	-1.56	1.442
822.754	8.82	M+NH ₄ , M+Na	Positive	TG(48:1)	C ₅₁ H ₉₆ O ₆	2, 53	22.02	<0.01	-1.63	0.514
834.754	8.29	M+NH ₄ , M+Na	Positive	TG(49:2)	C ₅₂ H ₉₆ O ₆	2, 53	35.5	<0.01	-2.66	0.514
848.77	8.79	M+NH ₄ , M+Na	Positive	TG(50:2)	C ₅₃ H ₉₈ O ₆	2, 53	25.38	<0.01	-1.51	0.164
860.771	8.20	M+NH ₄ , M+Na	Positive	TG(51:3)	C ₅₄ H ₉₈ O ₆	2, 53	18.69	<0.01	-2.00	0.836

¹ Negative fold changes indicate metabolites that were more abundant in young hosts; positive fold changes indicate metabolites that were more abundant in old hosts

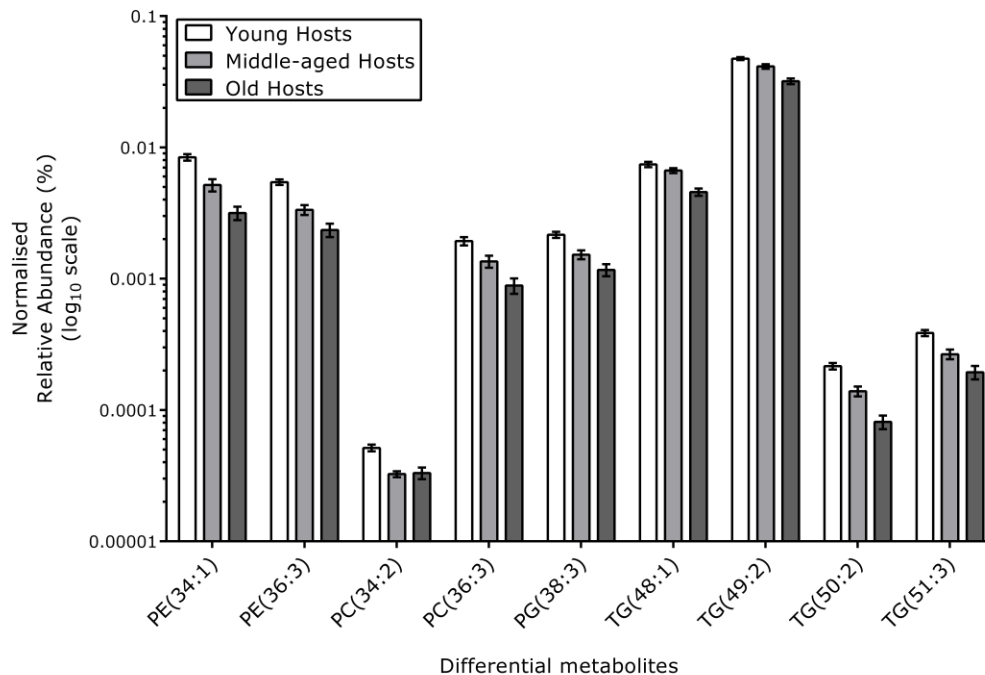


Figure 6.10. Lipid abundances across young, middle-aged and old host extracts. Metabolites are presented on a log scale to compensation for variations in scale. PC = phosphocholine, TG = triacylglyceride.

% with a range of 5.43 – 52.99 % and an average retention time RSD of 0.78 % with a range of 0.17 – 3.68 %. Assessment of retention time RSDs within QC samples found that 100 % of ions displayed RSDs within the acceptability threshold for experimental stability.

6.4.2.5 Parasitoid LC-MS

PCA visualisation of framed LC-MS data from wasp lipid extracts indicated partial separation between wasps reared on young hosts and wasps reared on old hosts (Figure 6.11a). However, clustering remained weak, with a large amount of variation being displayed within experimental classes across the first principle component. Lipid extracts from wasps reared on middle-aged hosts clustered between the two groups but also presented weak clustering. Validation of separation between young and old wasp extractions generated an OPLS-DA model with an R^2X of 0.784 and a Q^2 of 0.575 (Figure 6.11b). Cross validation of the OPLS-DA model by CV-ANOVA ($P < 0.01$ with Bonferroni correction) and predictive modelling (average correct prediction

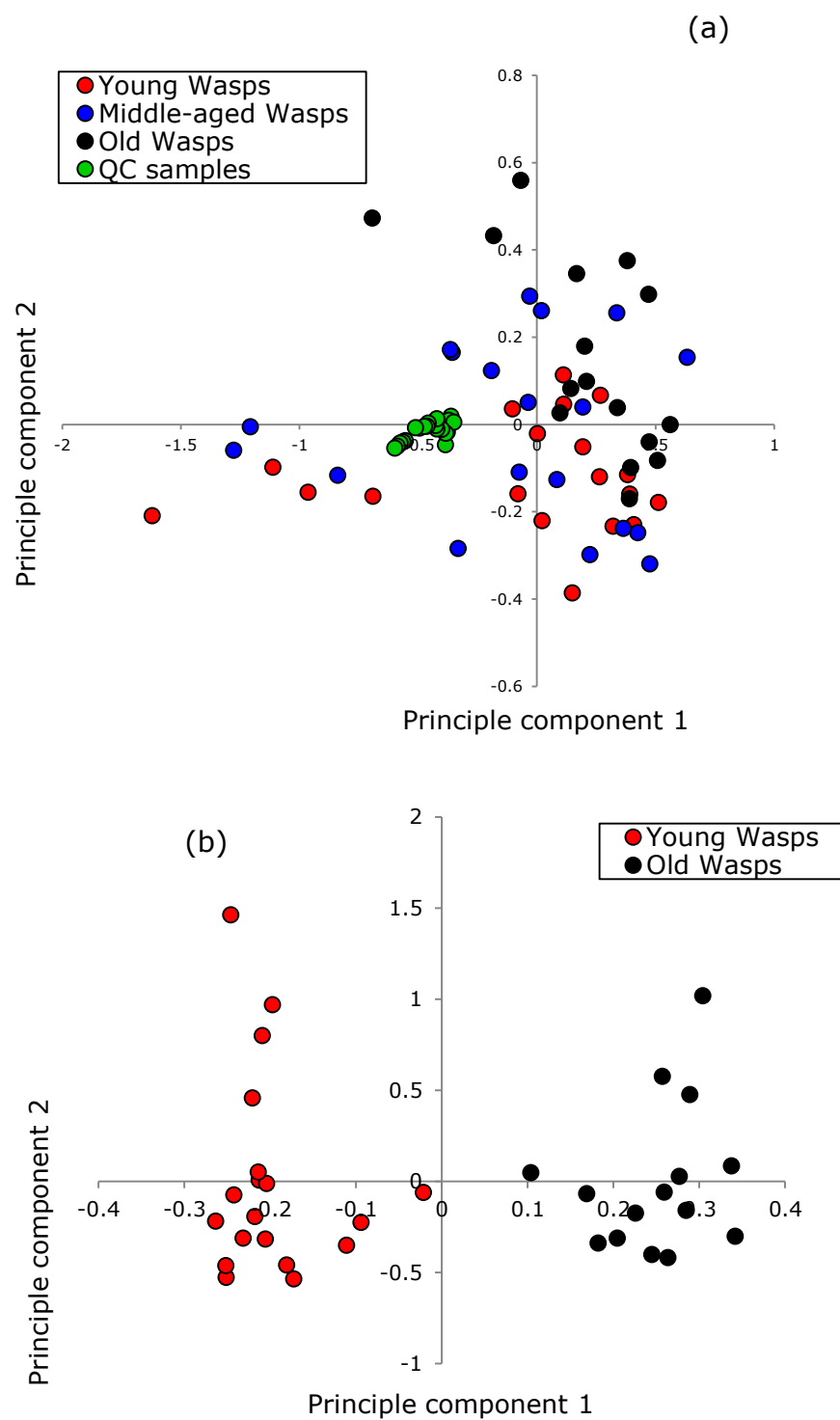


Figure 6.11. Principle components analysis of aged wasp LC-MS data (a) PCA of host lipid LC-MS data (PC1 = 60.4%, PC2 = 10.7%, PC3 = 8.71%, $R^2X = 0.915$) (b) OPLS-DA comparison of young and old aged wasps ($R^2X = 0.873$, $Q^2 = 0.719$).

Table 6.3. Summary of non- polar biomarkers with tentative identities that significantly differ between wasps reared from young hosts and wasps reared from old host extracts. DG = diacylglyceride, TG = triacylglyceride.

<i>M/Z</i>	Average RT (min)	Adduct	ESI Phase	Common Name	Formula	d.f.	F ratio	P value (B)	Fold change ¹	Mass error (ppm)
610.542	5.01	M+NH ₄ , M+Na	Positive	DG(34:2)	C ₃₇ H ₆₈ O ₅	2, 49	24.77	<0.01	-3.64	1.502
612.557	5.41	M+NH ₄	Positive	DG(34:1)	C ₃₇ H ₇₀ O ₅	2, 49	22.49	<0.01	-3.13	0.852
617.513	4.99	M+Na	Positive	DG(34:0)	C ₃₇ H ₇₂ O ₅	2, 49	22.33	<0.01	-3.35	0.951
641.513	5.05	M+Na	Positive	DG(36:3)	C ₃₉ H ₇₀ O ₅	2, 49	14.13	<0.05	-2.43	0.951
643.528	5.41	M+Na	Positive	DG(36:2)	C ₃₉ H ₇₂ O ₅	2, 49	14.96	<0.01	-2.52	0.807
654.568	5.63	M+NH ₄	Positive	DG(37:1)	C ₄₀ H ₇₆ O ₅	2, 49	13	<0.05	-1.16	3.460
792.708	7.63	M+NH ₄	Positive	TG(46:2)	C ₄₉ H ₉₀ O ₆	2, 49	15.8	<0.01	-1.67	0.436
794.724	8.44	M+NH ₄ , M+Na	Positive	TG(46:1)	C ₄₉ H ₉₂ O ₆	2, 49	23.6	<0.01	-1.50	0.786
799.68	8.44	M+H	Positive	TG(48:4)	C ₅₂ H ₉₂ O ₆	2, 49	20.54	<0.01	-1.75	1.017
818.724	7.61	M+NH ₄ , M+Na	Positive	TG(48:3)	C ₅₁ H ₉₂ O ₆	2, 49	12.7	<0.05	-1.70	0.786
820.74	8.51	M+NH ₄	Positive	TG(48:2)	C ₅₁ H ₉₄ O ₆	2, 49	17	<0.01	-1.54	1.136
844.741	7.68	M+NH ₄	Positive	TG(50:4)	C ₅₃ H ₉₆ O ₅	2, 49	12.59	<0.05	-1.75	2.136
846.756	8.44	M+NH ₄ , M+Na	Positive	TG(50:3)	C ₅₄ H ₉₈ O ₆	2, 49	17.48	<0.01	-1.58	1.486
865.727	8.95	M+H	Positive	TG(52:6)	C ₅₅ H ₉₄ O ₆	2, 49	15.56	<0.01	-1.61	0.967
872.771	8.47	M+NH ₄	Positive	TG(52:4)	C ₅₅ H ₉₈ O ₆	2, 49	12.58	<0.05	-1.56	0.836
887.781	8.51	M+K	Positive	TG(50:0)	C ₅₃ H ₁₀₂ O ₆	2, 49	14.07	<0.05	-3.65	0.061

¹ Negative fold changes indicate metabolites that were more abundant in wasps reared on young hosts; positive fold changes indicate metabolites that were more abundant in wasps reared on old hosts

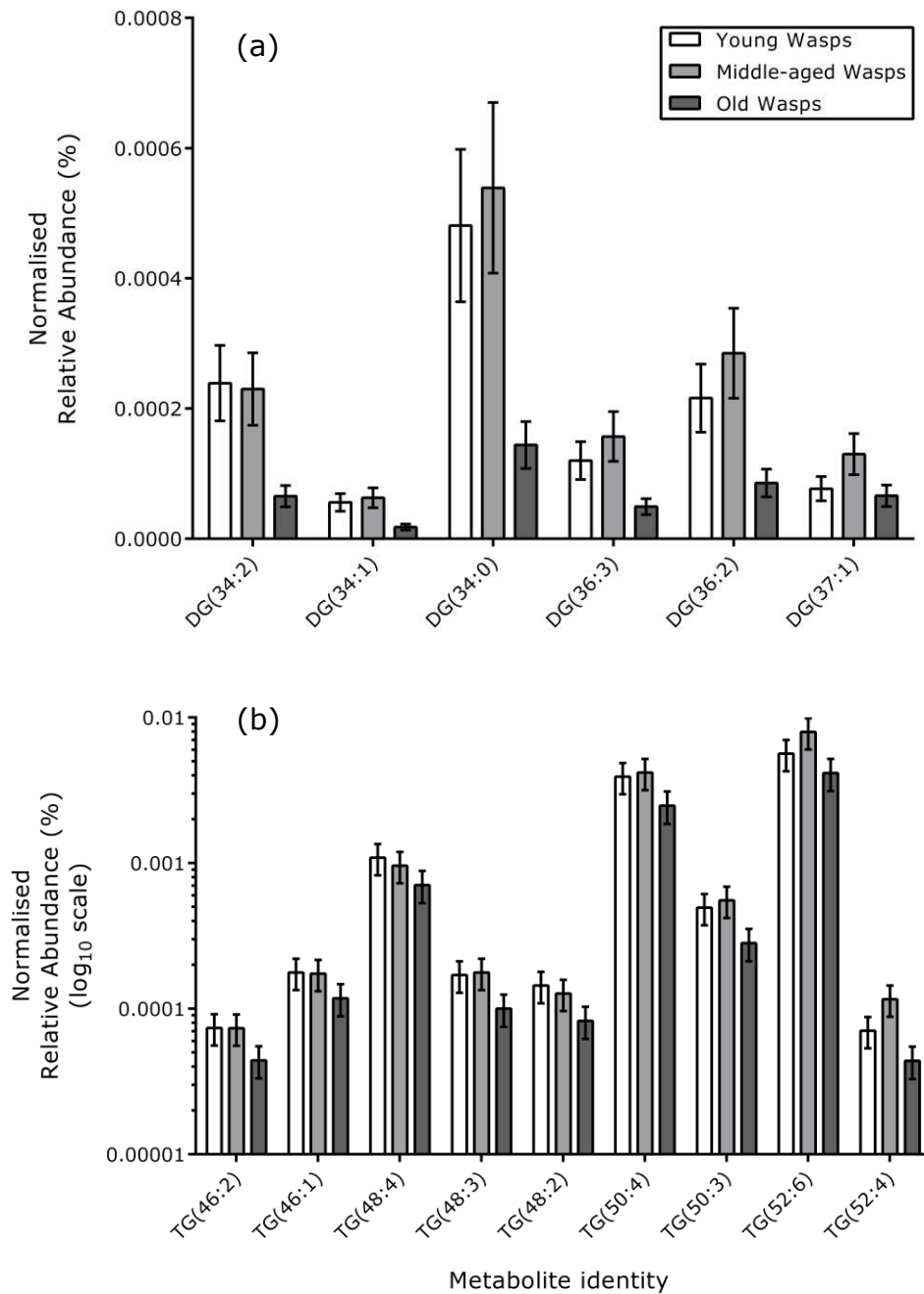


Figure 6.12. Lipid abundances for young, middle-aged and old host reared wasps (a) Diacylglycerides (b) Triacylglycerides. Note that triacylglycerides are presented on a log scale. DG = diacylglycerides, TG = triacylglycerides.

rate = 86.4%, Fisher's exact probability <0.00001) indicated good discrimination between treatment groups within the model. Validation of ions heavily correlated with young and old treatments by logistic ANOVA (d.f. 2, 49, $P < 0.05$) resulted in a total of 29 ions. The identities of 20 of these were confirmed through mass spectral database comparisons and

examination of LC-MS/MS fragmentation patterns. Due to the presence of multiple ammonium and sodium adducts this total was further reduced to 16 compounds. All 16 of these compounds were glycerolipids, including a total of 6 diacylglycerides and 10 triacylglycerides. A majority of these compounds displayed elevation in young wasp treatments compared to old wasp treatments (Figure 6.12). With the exception of a single diacylglyceride and a single triacylglyceride, middle-aged wasp treatments did not clearly separate from young wasp treatments. Ion identities, mass to charge ratios, retention times, chemical formulae, F ratios, p-value and fold changes are outlined in Table 6.3.

QC samples for wasp LC-MS samples clustered relatively centrally between treatment groups and exhibited low drift over time (Figure 6.10). A total 93.39 % of ions displayed peak area RSDs within the acceptability threshold for analytical stability. QC ions displayed an average peak area RSD of 7.27 % with a range of 1.47 – 47.27 % and an average retention time RSD of 1.21 % with a range of 0.81 – 17.51 %. Assessment of retention time RSDs within QC samples found that 100 % of ions displayed RSDs within the acceptability threshold for experimental stability.

6.5 Discussion

The results of this study suggest that the quality of a host that a parasitoid has developed on has no effect on its subsequent contest performance. Nonetheless differences in the levels of a number of glycerolipids, including diacylglycerides and triacylglycerides, were detected in LC-MS analysis between young and old host reared wasp treatments. Triacylglycerides constitute the main form of lipid storage in the insect fat body, whereas diacylglycerides are the immediate breakdown products of triglycerides and are considered key indicators of insect fat body metabolism (Arrese and Soulages 2010). From these results it can be concluded that whilst being reared on a lower quality host does result in lower nutritional reserves in adult *G. legneri*, this does not translate into an effect on contest performance. Wasps reared on middle-aged hosts exhibited little differentiation in lipid profile from wasps reared on young hosts; this combined with host LC-MS data may indicate that any decline in host quality occurs after 7 days. Alternatively wasps reared on middle-aged hosts may be able to somehow compensate for reductions in host quality.

Whilst NMR analysis of polar metabolites in wasps reared on different host treatments was rendered inconclusive by contamination, the results of NMR analysis of polar metabolites in aged hosts also found a deterioration of compounds associated with energy production. This partially confirms findings by Khidr et al. (2012) that compounds associated with energy production = decreased with time post-paralysis. Separation between LC-MS chromatograms of aged hosts treatments was observed, this was attributable to higher levels of phospholipids and a small number of triacylglycerides. However, little correlation was observed between altered lipid levels identified in aged host treatments and altered lipid levels identified in aged wasp treatments.

There are a number of potential explanations for this contrast in results between contest outcome and metabolomic analysis. Firstly, performing experiments with wasps aged 3 - 5 days may have masked nutritional legacy effects that would have been apparent had contests between older wasps been examined. Analysis of the *G. legneri* lipidome in Chapter 3 established that poor separation was apparent between newly emerged wasps and 3 day

old wasps. Results from Chapter 4 similarly indicated that no clear separation was apparent between wasps fed with honey for 3 days and those starved for 3 days. Whilst greater separation is present between the lipidomes of 3 day old young and old wasps utilised in animal contests, this separation is far less apparent than those between 0 day old and 7 day old wasps, as established in Chapter 3. This suggests the possibility that the age range utilised in this contest methodology was too short for any differences in lipid composition to become limiting factors within agonistic contests. Similar to the contest setup utilised in Chapter 5, it may be appropriate to repeat this study using 7 day old wasps in owner-owner contests. This time point is close to the maximum lifespan of *G. legneri* under these laboratory culturing conditions; by this point the lower nutritional reserves of wasps from older hosts may become a significant factor in wasp contest outcome.

Secondly, relatedness between individuals may be a compounding factor within owner-owner contests. Whilst wasps utilised within contest pairings came from different broods, the previous generation of wasps utilised to generate wasp broods from different host ages were not separated based on brood origin. It is possible as a result, that wasps within contest pairs were cousins. Lizé et al. (2012) should that close relatedness between females in owner-intruder contests results in slightly decreased aggression. Whilst these results have not been confirmed to apply within owner-owner contests, it is possible that more closely related competitors may have exhibited lower aggressiveness than expected. As a result it may be preferable to control for relatedness in any repeat of this contest setup. However this may be difficult from a logistic standpoint within such a complicated experimental setup.

Thirdly, a number of asymmetries between contestants, including host ownership, age and differences in host and wasp weight, were minimised within contests. As these factors had no significant influence on contest outcome, any advantages contestants may possess would have to be due to higher RHP. Previous results from Khidr et al. (2012) would suggest that contestants emerging from older hosts would have developed with a lower availability to energy rich metabolites. As broods developed from older hosts are characterised by smaller emerging adults and a higher mortality it is reasonable to suggest that these wasps would possess a lower RHP due to

lower fitness. However, if the results of this study are examined from the perspective of RV, it could be expected that nutritionally disadvantaged wasps (i.e. those emerging from older hosts) may assign a higher RV to a nutritionally superior host than their initial host. By comparison wasps reared in a high quality host may not assign a higher RV to a host with similar nutritional quality.

It is also possible that these combined factors, of a wasp reared on a lower quality host possessing a lower RHP, but assigning a high RV, may effectively cancel out any differences in contest performance. This result may change if a later time point was employed during contests, as a prior study by Humphries et al. (2006) indicated that older wasps are more likely to win contests than are younger wasps (i.e. discrepancy in RV overcoming discrepancy in RHP). As a result the higher RV assigned by wasps with a poor nutritional background may play a role in contest resolution. As nutritional background did not significantly affect the outcome of owner-owner contests, it is also possible that neither RHP nor RV of individual wasps differs despite their different developmental backgrounds. This may suggest that developing broods are able to compensate somehow for the lower quality of larval nutrition, and that any potential effects on contest behaviour related to host aging are limited to the resulting size of the contestants.

A large weight range for post-paralysis aged hosts was employed during metabolomic analysis (29.44 - 58.82mg). As the full polar extract is utilised in NMR spectroscopy, it is possible to correct for large variations in sample weight. However, our LC-MS approach only utilised a small and diluted proportion of the final extract (10 μ L out of approximately 200 μ L) in order to prevent saturation of the column during experimental analysis. Whilst our approach did correct internally for concentration variations between samples by normalising metabolite areas to the total sample area, the presence of real biological variations associated with larger host size remains a possibility. As host weight has been utilised as an indirect measure of larval instar in this investigation, it is possible that heavier individuals were of a higher larval instar than lighter individuals. As a result the presence of metabolomic variations in the generated data set due to differences in larval instar cannot be eliminated. The lack of correlation between altered metabolite levels in aged hosts and those in aged wasps could be due to

changes host metabolite concentrations being associated with fluctuations in larval instar, rather than host aging. If this experimental approach were used in the future it may be advisable to remove asymmetries in weight between individual hosts prior to metabolomic analysis. As host weight loss post-paralysis is variable (Khidr et al. 2012), it may be more appropriate to normalise host weight prior to paralysis across all three treatments, as normalising weights after aging may require the use of individuals from different larval instars.

This study did not detect an accumulation of waste products such as ethanol and citrate in paralysed hosts over time as reported by Khidr et al. (2012). However the volatility of ethanol reduces the possibility of detecting the compound with this experimental setup. Bins associated with these compounds did indicate statistically significant separation, but 2D NMR identifications did not feature spectral resonances associated with either ethanol or citrate. It is possible that this original identification was mistaken, though a more likely possibility is that these compounds were detected in either the faecal matter or in the culturing medium of the aged hosts analysed by Khidr et al. (2012). This study utilised a cleaning phase in which great care was taken to remove any contaminants that may have been present on the outer cuticle of the host. No such step was reported by Khidr et al. (2012), as a result the possibility of contamination remains open. However, 2D NMR spectra were only generated from 1 day paralysed hosts due to equipment time constraints; it is possible that spectral peak levels for these compounds were too low to be detected. However a number of other unidentified spectral compounds within the same spectral region are present within 2D spectra, and the same spectral peak observed in young host NMR spectra is observable in 1D spectra generated from 2D samples.

Khidr et al. (2012) have previously examined the effects of varying quality of larval nutrition on such factors as brood mortality and surviving adult size. However, the reproductive potential (i.e. fecundity) of *Goniozus* wasps subject to varying levels of larval nutrition remains unexplored. A number of prior studies have focused on the brood rearing capacity of animals that have been subjected to varying levels of developmental nutrition (Zwaan et al. 1991; Kaspi et al. 2002; Gorman and Nager 2004; Boggs and Freeman 2005; Blanckenhorn 2006; Naguib et al. 2006; Barrett et al. 2009;

Bauerfeind et al. 2009; Colasurdo et al. 2009; Kolss et al. 2009; Zajitschek et al. 2009; Dmitriew and Rowe 2011; Hopwood et al. 2014; May et al. 2015; O'Hagan et al. 2015). Kaspi et al. (2002) demonstrated that Mediterranean fruit flies (*Ceratitis capitata*) provided with a high sugar-high protein diet developed faster, had higher nutritional reserves and had a higher fecundity than flies reared on a protein deficient diet. Boggs and Freeman (2005), Barrett et al. (2009) and Dmitriew and Rowe (2011) confirmed similar effects in the butterfly *Speyeria mormonia*, the cockroach *Nauphoeta cinerea* and the ladybird beetle (*Harmonia axyridis*).

Conversely, a number of studies have observed that offspring fecundity and lifespan may be more greatly influenced by the adult reproductive environment than by initial larval nutrition. May et al. (2015) observed that in certain adult reproductive environments, adult *Drosophila* raised on a lower quality larval diet exhibited heightened reproductive performance at certain ages. This study also demonstrated increased longevity in virgin adults reared on lower quality diets. Following from these studies, a future investigation to examine the effects of host-aging on the fecundity and lifespan of *Goniozus* wasps may be appropriate.

6.6 Conclusions

The quality of a host upon which a *G. legneri* previously developed did not affect the outcome of owner-owner contests. However, wasps reared on hosts that had been paralysed for fewer days before development commenced exhibited higher levels of large storage lipids. Hemolymph sugars associated with energy metabolism were confirmed to deteriorate in hosts post-paralysis. LC-MS analysis of lipid extracts did detect separation between aged hosts; however altered levels of metabolites associated with this separation had little correlation with metabolites found in wasp lipid extractions. These results suggest that a female's biochemical state has no effect on contest ability (RHP), although it is conceivable that a reduction in RHP is compensated by an increased evaluation of the quality of the contested host (RV) by comparison to the wasp's larval environment. It is suggested that repeating the contest experiments with wasps that are older may reveal effects of nutrition that have not yet become apparent when contests occur between females aged just 3 - 5 days since eclosion as adults.

Part 3: Behavioural and biochemical mimicry by a hyperparasitoid wasp

Chapter 7: Multi-trait mimicry of ants by a parasitoid wasp

7.1 Abstract

Many animals avoid attack from predators through toxicity or the emission of repellent chemicals. Defensive mimicry has evolved in many species to deceive shared predators, for instance through colouration and other morphological adaptations, but mimicry hardly ever seems to involve multi-trait similarities. This chapter reports on a wingless parasitoid wasp that exhibits a full spectrum of traits mimicking ants and affording protection against ground-dwelling predators (wolf spiders). In body size, morphology and movement *Gelis agilis* (Ichneumonidae) is highly similar to the black garden ant (*Lasius niger*) that shares the same habitat. When threatened, *G. agilis* also emits a volatile chemical that is similar to an ant-produced chemical that repels spiders. In bioassays with *L. niger*, *G. agilis*, *G. areator*, *Cotesia glomerata* and *Drosophila melanogaster*, ants and *G. agilis* were virtually immune to spider attack, in contrast the other species were not. Volatile characterisation with gas chromatography-mass spectrometry identified *G. agilis* emissions as 6-methyl-5-hepten-2-one, a known insect defence semiochemical that acts as an alarm pheromone in ants. It is arguable that multi-trait mimicry, as observed in *G. agilis*, might be much more common among animals than currently realized.

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7.2 Introduction

Mimicry is widespread amongst plants and animals and involves the resemblance of one species to another, at least to the benefit of the mimic (Müller 1897; Howarth et al. 2004; Bates 1862; Huang et al. 2010). For instance, the Malaysian orchid mantis *Hymenopus coronatus* visually mimics flowers such that it attracts more pollinator prey than the flowers it resembles (O'Hanlon et al. 2014). To avoid attack from shared predators, prey species may also mimic morphological features of a co-existing unpalatable or toxic model species (Bates 1862), as has long been observed and studied in hoverflies, butterflies and snakes (Howarth et al. 2004; Clarke and Sheppard 1960; Kunte et al. 2014; Greene and McDiarmid 1981; Kikuchi et al. 2014). In hoverflies, which visually resemble stinging bees or wasps, colour mimicry can coincide with behavioural mimicry, such as mock stinging, wing wagging and leg waving (Penney et al. 2014; Johnstone 2002) or activity patterns (Howarth et al. 2004). Predators and prey can also be deceived via chemical mimicry: some spiders attract their lepidopteran prey through the emission of moth sex pheromones (Eberhard 1977) but chemical mimicry appears to be less frequently adopted as an anti-predator strategy (Rettenmeyer 1970; Dettner and Liepert 1994; Lorenzi et al. 1996).

Whilst behavioural mimicry often coincides with morphological similarity, mimicry only rarely seems to require convergence of a greater number of traits (Bates 1862; Pasteur 1982). The secondary hyperparasitoid *Gelis agilis* could represent an exception, as it shows several distinct similarities to sympatric ant species. *Gelis agilis* is a small (3-5 mm long) wingless, asexually reproducing parasitoid wasp that attacks several host species, including the pupae of other parasitoids (Lei and Handski 1997; Harvey 2008). Adult *G. agilis* first paralyze the host with venom and then oviposit a single egg onto the exterior of the host's body (Greene and McDiarmid, 1981). After hatching, the *G. agilis* larva feeds on the moribund host, eventually consuming all of it prior to pupation. *Gelis agilis* is extremely abundant in grassy habitats across much of Eurasia (Harvey 2008) and co-occurs and shares predators with several ant species. Its potential predators include wolf spiders, which are visually foraging diurnal hunters that attack a wide range of prey types on the ground (Nentwig and Wissel 1986; Oelbermann and Scheu 2002). In morphology (body size, colour) and

A**B**

Figure 7.1. Morphological comparisons of *Gelis agilis* Fabricius (a) (Hymenoptera: Ichneumonidae) and *Lasius niger* Linnaeus (b) (Hymenoptera: Formicidae). Photographs by Alex Kraus (a) and Rui Andrade (b).

behaviour, *G. agilis* (Figure 7.1a) closely resembles several species of ants that occur in the same habitat, including the common black garden ant, *Lasius niger* (Figure 7.1b). Moreover, when alarmed the parasitoid releases a pungent and distinctive odour. This chapter tests the hypotheses that ant-mimicry by *G. agilis* acts as a defensive strategy to reduce attack by wolf spiders and that chemical mimicry is facilitated by the emission of an ant-like alarm pheromone (Hübner and Dettner 2000).

7.2.1 Aims and Objectives

This chapter aims to explore the mimicry of *Lasius niger* by the hyperparasitoid *Gelis agilis*, and any resulting protection from predation. Specific objectives required to fulfil these aims are:

- To conduct a predation study comparing the predation rate of *G. agilis* and *L. niger* with a number of insect controls in both choice and non-choice bioassays.
- To conduct a combined APCI-MS and GC-MS study to identify any chemical emissions of *G. agilis* when stressed.

7.3 Materials and methods

7.3.1 Insect culturing

All insects were reared at 23 °C and a 16:8 h light:dark photoperiod. *Gelis agilis* and *G. areator* (both Hymenoptera: Ichneumonidae) were reared on cocoons of *C. glomerata* (Hymenoptera: Braconidae) that were reared on caterpillars of the cabbage butterfly, *Pieris brassicae* (Lepidoptera: Pieridae), on cabbage plants at the Netherlands Institute of Ecology. Adult *C. glomerata* wasps were maintained in groups of ~200 wasps in rearing cages. Cabbage leaves infested with L1 caterpillars of *P. brassicae* were presented to wasps in the rearing cages for parasitism. Parasitized caterpillars were reared in cages with 3 - 4 cabbage plants. *D. melanogaster* (Diptera: Drosophilidae) flies were reared on a baker's yeast suspension. A laboratory colony of *L. niger* (Hymenoptera: Formicidae) was established from single queens and several workers supplied by Antstore, Berlin, Germany. These colonies were supplemented by cocoons of workers collected in the field from wild colonies. Newly emerged workers are immediately 'conditioned' to ant pheromones of the host colony, in order to recognize members belonging to the same colony (Allan et al., 1996).

7.3.2 Predation bioassays

Choice and non-choice bioassays were conducted in closed Petri dishes (Petri dishes Ø 12 cm). For bioassays, wolf spiders were kept in individual Petri dishes containing water absorbed into cotton wool but were starved for 2 - 3 days after collection from the field. In the choice experiments, 2 species-combinations of insects were introduced in pairs into single Petri dishes with an individual spider. For non-choice bioassays, 3 individuals of a single species were introduced into Petri dishes with an individual spider. The dishes were left for approximately 24 h and then checked for evidence of predation. Predation was recorded only as insects that had been visibly killed and eaten, where only cuticular rudiments remained. Some insects died but were not visibly attacked by the spiders; this included very few ants and *G. agilis* wasps. These insects were excluded from the analyses, as death may have been due to natural mortality. Spiders only eat freshly killed prey and thus only those insects that were clearly attacked by the spiders were included in the analyses. Very few insects were found dead in arenas after 24 h that died naturally; no *G. agilis* and *L. niger* and only a few *C. glomerata*.

7.3.3 Statistical analyses

The number of prey of each species consumed in the no-choice experiment was compared using a Kruskal-Wallis test based on ranked data. To compare individual prey species, the ranked data were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey HSD post-hoc test.

The number of prey consumed of each of the two species in the choice experiments was analyzed using a Monte Carlo permutation test with 199 permutations. Each test randomly allocated the consumed prey in each of the replicates ($n = 61 - 121$) to one of the two species. The consumption of each of the two species in the experiment (realized) was then compared to the consumption calculated in the random permutations and a P-value was determined based on the number of times that the difference in consumption between the two species in the random permutations was more distinct than that the realized difference.

7.3.4 Chemical analyses

Chemical analysis took place at the University of Nottingham, School of Biosciences. For initial analysis, volatile chemical releases of *G. agilis* were monitored in real-time using an APCI-MS (Goubault et al. 2006). Five adults were placed individually in 20 mL glass scintillation vials and deliberately agitated for 1 minute with a paintbrush whilst positioned adjacent to the APCI-MS sampling point (Goubault et al. 2006). Agitation comprised of deliberately restricting the movement of individual wasps by pressing them against the edge of the vial. The APCI-MS sampling point draws a continuous stream of air, set up at 25 mL min^{-1} , into a heated transfer line ($160 \text{ }^\circ\text{C}$) through a deactivated silica tube ($1 \text{ m} \times 0.53 \text{ mm ID}$) before entering the APCI source. Volatiles then entered the source and were ionized by a positive ion corona discharge (4 kV), which typically forms the adduct ion $M+H^+$. Spectra were recorded using a Platform II mass spectrometer (Waters, Manchester, UK) across a mass range of 25-250 Da, with the cone voltage set to 18 V. Two major ions with the m/z of 108 and 127 were observed, consistent with the fragmentation pattern of $M = 126$ (127).

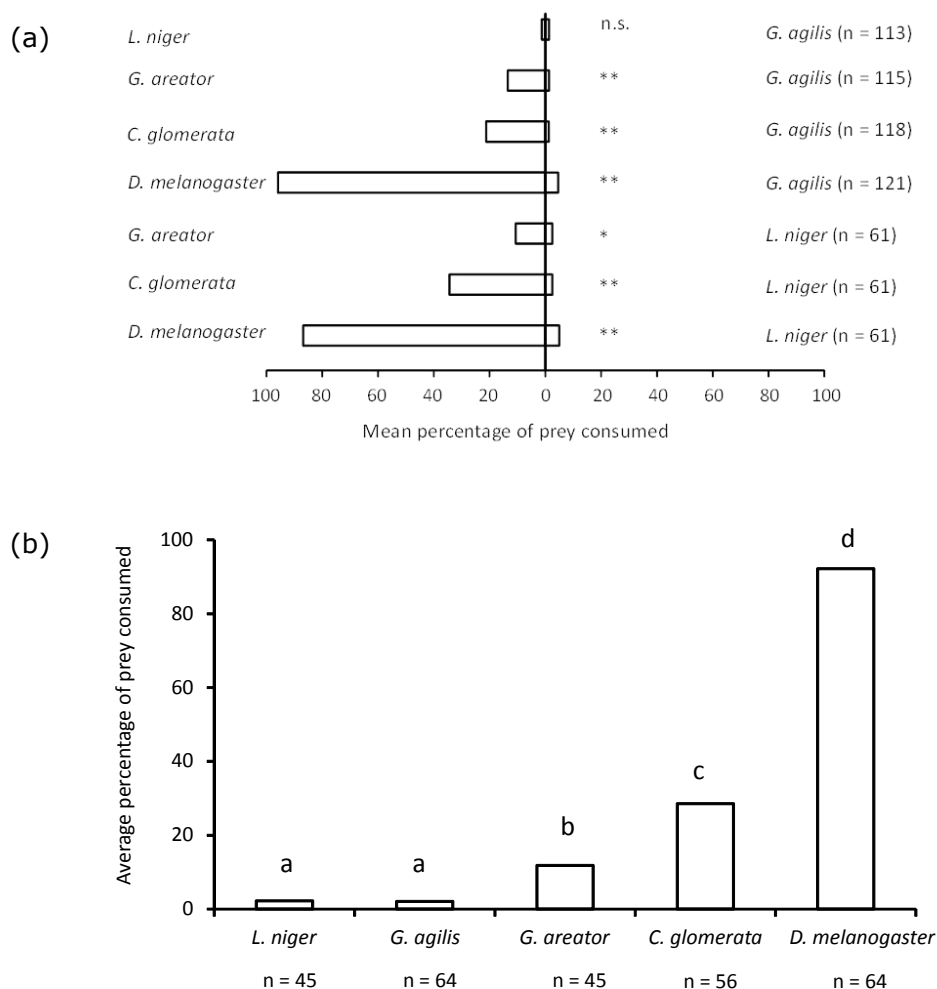


Figure 7.2. (a) Mean percentage of prey consumed by wolf spiders in paired choice tests. In A * = $P < 0.05$; ** = $P < 0.01$; in B bars with different letters are significantly different ($P < 0.05$) (b) Mean percentage of prey consumed by wolf spiders in non-choice tests.

In order to confirm the identity of the chemical released, individual *G. agilis* were placed in a 20 mL flask and deliberately agitated for 1 minute under the same protocol as the APCI analysis. Flasks were then sealed with a PTFE lined septum. Volatile compounds were transferred for GC-MS analysis using a SPME fibre (50/30 mm, assembly Divinylbenzene/Carboxen/Polydimethylsiloxane; Supelco, Bellefonte, USA), which was exposed in the flask headspace for 0.2 min at 22 °C. Desorption of volatile compounds attached to the fibre occurred in the injector at 250 °C for 2 min. Volatile compounds were transferred to the column (30 m x 0.25 mm ID, BP-5, 1.0 mM film thickness; SGE, Milton Keynes, UK) and the gas chromatogram temperature programme initialised. The GC (Trace GC

1300, Thermo, Austin, USA) temperature programme held at a temperature of 40 °C for 1 min before increasing at a rate of 8 °C min⁻¹ to 200 °C. Mass spectra were recorded using an ISQ mass spectrometer (Thermo) at 2 scans s⁻¹ from between 20 - 200 *m/z*. A prepared 6-methyl-5-hepten-2-one standard (Sigma Aldrich) was injected alongside analysis to confirm elution time and fragmentation pattern.

7.4 Results:

7.4.1 Predation bioassays

When given no choice, spiders killed and consumed virtually all *D. melanogaster*, which, in spite of possessing wings, were highly susceptible to attack. *Cotesia glomerata* and *G. areator* suffered significantly higher predation than *G. agilis* and *L. niger* ($\chi^2 = 191.7$, d.f. = 4, $P < 0.0001$; Figure 7.2). Similar patterns were obtained in choice experiments (Figure 7.2). Moreover, virtually no insects died from natural causes; they were either alive at the end of the observation period or consumed by the spiders.

7.4.2 Chemical analyses

Gelis agilis also emitted a single volatile compound when manually agitated. This volatile emission was not detected in the atmosphere of the experimental environment prior to or after agitation. Analysis of the volatile by atmospheric-pressure chemical ionization-mass spectrometry (APCI-MS) generated spectra displaying two major peaks with the molecular weights of 127 and 109 (Figure 7.3). Spectral comparisons with published literature and spectral databases of known compounds (Massbank, Massbank Project) indicated a consistency with 6-methyl-5-hepten-2-one, an unsaturated terpenoid (Tengö et al. 1982), also known as sulcatone. Fragmentation data of this emission generated by gas chromatography-mass spectrometry (GC-MS) exhibited a high degree of similarity with the spectral fragmentation and retention time of the prepared standard (Figure 7.4 and 7.5).

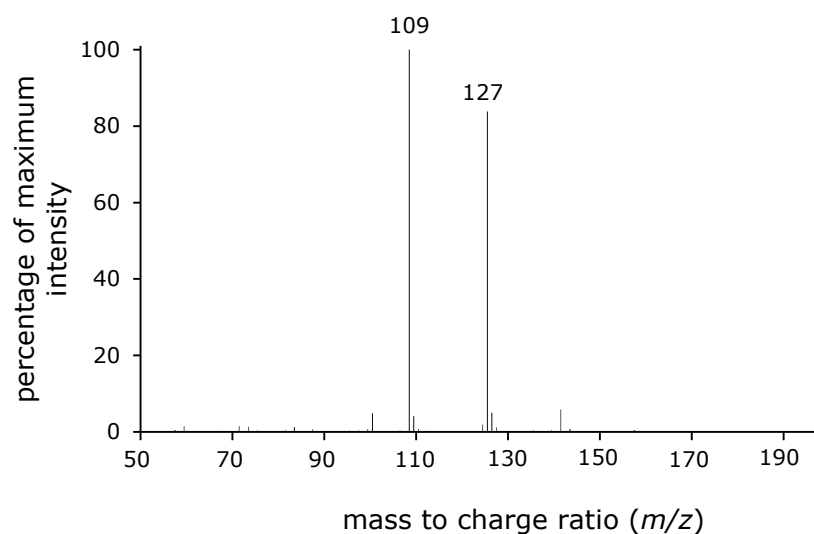


Figure 7.3. Representative APCI-MS spectrum of *G. agilis* volatile emissions. This soft ionization technique resulted in a fragmentation pattern consistent with that of 6-methyl-5-hepten-2-one (Tengö et al. 1982).

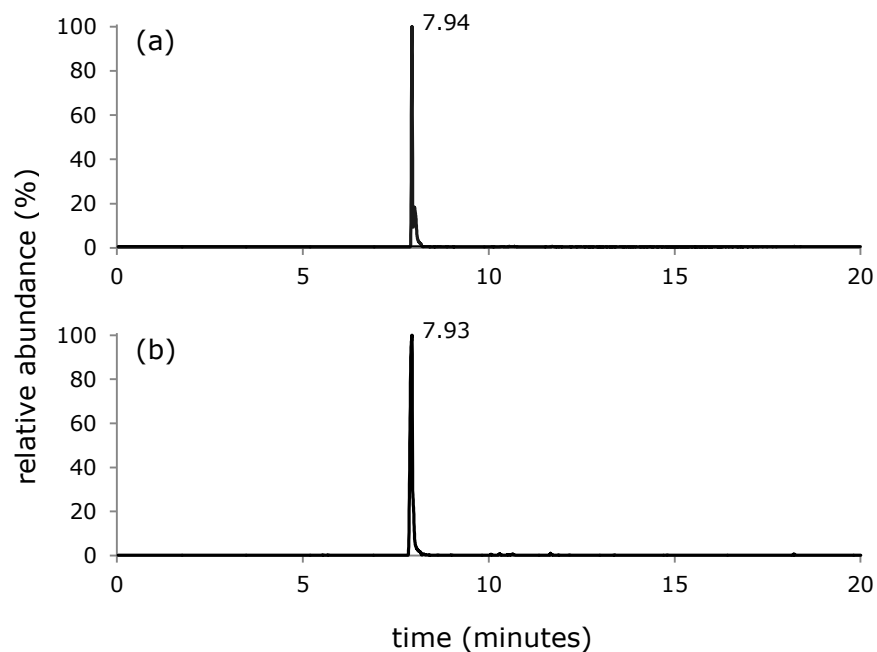


Figure 7.4. GC-MS chromatogram of *G. agilis* volatile emissions (a) GC-MS spectrum of the main peak observed (7.94 min) during *G. agilis* agitation (b) GC-MS spectrum of main peak observed (7.93 min) during analysis of a prepared 6-methyl-5-hepten-2-one standard. The two spectra display a high level of consistency in both fragmentation and retention time.

7.5 Discussion

Ants have long been known to be important drivers of selection for defensive traits in other organisms (Hölldobler 1990). Ant species are often extremely abundant and form large colonies that occur in close proximity to one another. Many species of predators co-occur with ants; cursorial spiders are among the most abundant (Samu et al. 2003). The parasitoid *Gelis agilis* shows remarkable morphological and behavioural similarity to sympatric ant species that share the same predators. When crushed, *G. agilis* and another wingless congener, *G. acarorum*, produce pungent odours that are easily detectable by human nasal olfaction. These odours are not perceived by human olfaction in other related winged hyperparasitoids that attack the same host, including *Gelis areator*, *Lysibia nana* (Hym: Ichneumonidae) and *Acrolyta nens* (Hym: Ichneumonidae) (Pers. obs. M.M, B.V. and J.A.H.).

In choice tests, *G. agilis* and *L. niger* suffered little from predator attack, indicating that *G. agilis* is an effective mimic of *L. niger*. Comparisons of attack rates on the non-pungent *G. areator* (which is similar in appearance to *G. agilis*), *C. glomerata* and the more distantly related *D. melanogaster* further revealed that morphological and behavioural mimicry by *G. areator* also may substantially reduce spider attack. Spider repellence is, however, only as effective as that of *L. niger* when behavioural, morphological and chemical mimicry are all employed, as is the case for *G. agilis*. Chemical mimicry has been observed in other parasitoid species, for instance those that attack aphids being tended by ants. *Lysiphlebus cardui* mimics the cuticular hydrocarbon profile of its ant-attended host, thereby avoiding ant aggression (Liepert and Dettner 1993; 1996). Similar to *G. agilis*, *Alloxysta brevis*, a hyperparasitoid of aphid parasitoids, releases several compounds, including sulcatone, from its mandibular glands (Völkl et al. 1994). These releases inhibit aggressive behaviour when the parasitoid is attacked by *L. niger* and repel attacks in subsequent encounters (Völkl et al. 1994). The compounds produced by *A. brevis* also confer protection against some spider species (Hübner and Dettner 2000). Hence these mandibular gland releases protect against aphid-attending ants and shared predators. Sulcatone is thus exploited by different, distantly related parasitoid species to avoid aggression from predatory spiders. What is important to stress is that chemical mimicry of ants requires an intimate evolutionary history with

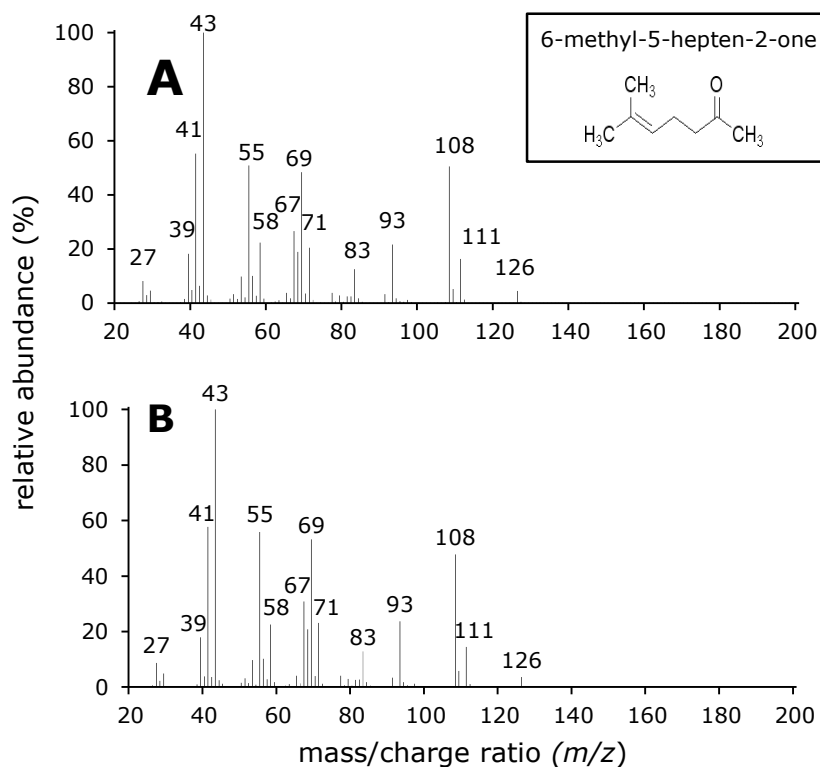


Figure 7.5. GC-MS confirmation of *G. agilis* volatile emissions (a) GC-MS chromatogram of the main peak observed during *G. agilis* agitation (b) GC-MS chromatogram of main peak observed during analysis of a prepared 6-methyl-5-hepten-2-one standard.

them. In wingless gelines, as well as in aphid primary parasitoids and hyperparasitoids, the importance of ants as selective agents is clear.

Mimicry is often imperfect, where mimics only superficially resemble their model (Sherratt 2002). Such limited resemblance may evolve, for instance, when selection on high similarity between mimic and model is reduced or relaxed, as in small hoverflies that are less favourable as prey items (Penney et al., 2012). Whilst imperfect mimicry seems to suffice in some cases, similarity in only a single trait may confer little advantage, particularly when resemblance between mimic and model is limited. These results demonstrate that the morphological and behavioural ant-mimic *G. areator* suffers less from spider attacks compared to the non-mimetic species but is still attacked at a considerably higher rate than *G. agilis* which is a morphological,

behavioural and chemical mimic (hence, the use of the term 'full spectrum mimicry'). Mimicry in morphology often coincides with similarity in behavioural traits (Howarth et al. 2004; Greene and McDiarmid 1981), but mimicry in more than two traits is rare. Studies of mimicry have focused on characters that are easily observable to the human eye; however mimicry may involve more subtle morphological, behavioural, olfactory and acoustic signals. Another case of multi-trait mimicry was recently found in a viperine snake (*Natrix maura*) that mimics the venomous asp viper (*Vipera aspis*) in terms of body size, shape, colouration, patterning and acoustic emissions (Aubret and Mangin 2014). It may be expected that the phenomenon of multi-trait mimicry, as observed in *G. agilis* and *Natrix maura*, might be much more common among animals than has been thus far realized.

7.5 Conclusions

G. agilis possesses a higher survival rate of wolf spider predation than the related hyperparasitoid *G. areator*, along with the braconid wasp *C. glomerata* and a non-hymenopteran control *D. melanogaster*. *G. agilis* survival rate indicated no statistical separation from that of *L. niger*, this similarity appears to be based on behavioural, morphological and chemical mimicry by *G. agilis* of *L. niger*. These results identified the volatile emission of 6-methyl-hepten-2-one as an essential component of spider predation survival, as demonstrated by the lower survival rate of the non-volatile emitting behavioural and morphological mimic *G. areator*. As previous studies have focused on aspects of mimicry visible to the human eye, it is possible that subtle chemical mimicry may be more common than previously realised.

Chapter 8: General discussion and conclusions

8.1 Summary of results

This project aimed to develop and validate a combined NMR and LC-MS approach capable of generating rigorous metabolomic data concerning the effects of aging, diet and host species on parasitoid (specifically *Goniozus legneri* and *G. nephantidis*) life history and further to relate changes in the metabolome to contest performance. This project also aimed to explore the role of volatile chemical emissions in parasitoid (specifically *Gelis agilis*) mimicry and predator avoidance strategies. A number of investigations were carried out to achieve these aims, the results of which are summarised as follows:

- A combined NMR and LC-MS metabolomic approach capable of detecting metabolomic changes in low biomass insect samples was rigorously optimised and validated (Chapters 2 and 3). Validation of this approach successfully detected a gradual decline in the haemolymph and lipid energy stores of adult female parasitoids.
- The availability of a carbohydrate rich honey diet significantly increases the lifespan and lipid reserves of adult female parasitoids (Chapter 4).
- Neither dietary status nor host age post-paralysis appears to affect parasitoid contest performance. It is possible that this lack of effect is due to an enhancement of RHP (from feeding on honey or being reared on young hosts) being equalised by a higher perceived value of a contested host (RV) (due to starvation or to being reared on an old host) (Chapters 4 and 6).
- Whilst *G. legneri* is capable of reliably parasitizing a range of lepidopteran hosts, *G. nephantidis* was only capable of parasitizing *Corcyra cephalonica*. *G. legneri* performed best when parasitizing *Ephestia kuehniella* or *C. cephalonica* and worst when parasitizing *Plodia interpunctella* (Chapter 5).

- LC-MS analysis of adult *G. legneri* reared across the same host range indicated that *E. kuehniella* exhibited the highest levels of energy rich glycerolipids, whilst wasps reared on *P. interpunctella* exhibited the lowest levels (Chapter 5).
- LC-MS analysis of the four host species studied found elevated levels of a number of currently unidentified metabolites in hosts other than *C. cephalonica*. Comparisons with mass spectral databases identified both bile acids and omega fatty acids as potential candidates, however comparisons with lipid standards ruled out these possibilities (Chapter 5).
- NMR analysis of 0 day, 7 day and 14 day old paralysed *C. cephalonica* confirmed previous findings (Khidr et al. 2012) that a number of haemolymph sugars decline with age. However, the previously reported increase in metabolic waste products such as citrate and ethanol was not observed.
- The hyperparasitoid ant mimic *Gelis agilis* possesses a similar survival rate to that of the common garden ant *Lasius niger*. *G. agilis*' emission of 6-methyl-5-hepten-2-one was found to be an essential component to this enhanced survival rate, as demonstrated by the lower survival rate of the non-emitting ant mimic *G. areator*.

As each empirical Chapter of this thesis includes a specific discussion, this Chapter aims to review and integrate these results in the context of the larger body of work. This Chapter considers whether the aims outlined in Chapter 1 have been achieved, discusses possible applications of this metabolomic approach within insect biology, and suggests future research avenues where appropriate. This Chapter also highlights any technical experimental issues encountered by this project, and suggests potential improvements to this work.

8.2 The applicability of a combined LC-MS and NMR low biomass insect approach

The major developmental aim of this project was the generation of a workflow capable of producing complex metabolomic datasets from extremely low biomass insect samples. Existing insect metabolomic studies have been readily capable of quantifying changes within single low biomass organisms using both LC-MS and GC-MS (Snart et al. 2015). However, no known study has previously been capable of profiling the full range of polar compounds in low biomass samples without utilising a degree of sample pooling. The combined NMR/LC-MS approach optimised by this approach was successfully capable of simultaneously generating complementary polar and non-polar metabolomic datasets from single adult female *G. legneri*. This was achieved without extensive modification of existing solvent extraction approaches (Folch et al. 1957; Bligh and Dyer 1959; Hara and Radin 1978; Wu et al. 2008). The optimised protocol was also notable for its rigorous approach in validating the stability and precision of both NMR and LC-MS analysis. Prior insect studies have suffered from a lack of consistency in reporting standards, particularly regarding validation measures (Snart et al. 2015). Through the use of quality control samples and technical replicates, this method was verified to meet current acceptability standards for both replicability and experimental stability (FDA USA; Dunn et al. 2011).

The focal organism for this project, *G. legneri*, was selected due to its previous use in the study of such varied areas as developmental mortality, sex ratio decisions, clutch size decisions and dyadic contests (Hardy et al. 2000, Bentley et al. 2009, Lizé et al. 2012, Stockermans and Hardy 2013, Khidr et al. 2013a; 2013b; 2014). The experimental approach outlined in Chapter 3 required specific considerations for this organism, in particular the presence of a thicker outer cuticle than the major insect model organism *Drosophila melanogaster*. Informal comparisons of these organisms when considering homogenisation conditions confirmed that *G. legneri* required a significantly longer homogenisation programme to achieve comparable sample breakdown to that of *D. melanogaster*. However, a maximum homogenisation time of 3 x 30 seconds produced homogenate acceptable for further analysis. Whilst this approach was specifically tailored for a disruption resistant sample, this does not limit its applicability for other insect or non-

insect samples of comparable size, as demonstrated by the lack of significant alteration required for analysis of caterpillar samples in Chapter 5 and 6. This approach may also be applicable to tougher mammalian samples, such as connective tissues (e.g. tendons and ligaments); though a degree of further optimisation of homogenisation procedures may be required.

The discussion of Chapter 3 considered the possibility of generating biomarkers associated with aging in low biomass insects, with particular focus on its applicability for malarial studies in mosquitoes. However this study purely focused on starved individuals, without taking into account potential changes in the metabolome associated with dietary intake. The findings of the dietary study reported in Chapter 4 indicate that variation in major polar biomarkers associated with parasitoid aging, such as glucose, trehalose and glycerol, is readily obscured in the presence of a honey fed diet. Furthermore it was not possible to distinguish between NMR resonances associated with undigested diet present in the digestive tract of *G. legneri* from those present in the insect haemolymph. These results further confirm the potential for confounding effects of an organism's diet in metabolomic studies (Wishart 2008). As a result any attempt to apply a similar metabolomic approach to analyse low biomass insects would be advised to also analyse any dietary components in order to detect potential spectral overlap.

8.3 The influence of the host species on the fitness of *Goniozus wasps*

Parasitoid wasps are expected to display selective preference for higher quality hosts, whether this quality be a result of size, larval instar, disease state or post-paralysis aging (Goubault et al. 2006; 2007; 2008, Goubault and Hardy 2007, Lizé et al. 2012, Stockermans and Hardy 2013, Hardy and Briffa 2013). However, only one known study has attempted to correlate parasitoid behaviour with measurable changes in host biochemistry (Khidr et al. 2012). Following the model of this study, this project found that the biochemistry of a host is significantly affected by its species, and that this in turn affects the life history decisions of ovipositing parasitoid.

Informal laboratory observations had previously noted that both *G. legneri* and *G. nephantidis* are readily capable of rearing broods through to adulthood on the Rice moth *Corcyra cephalonica*, but that only *G. legneri* was capable of consistently producing adult offspring on the Indian mealmoth *Plodia interpunctella*. Formal comparisons of the performance of *G. legneri* and *G. nephantidis* across a range of lepidopteran host species indicated that *G. legneri* is capable of parasitizing the all 4 host species, whilst *G. nephantidis* was incapable of consistently rearing live broods on any of these species other than *C. cephalonica*. Performance of *G. legneri* varied across all four species, as did the metabolome of offspring emerging from each host species. Wasps emerging from *P. interpunctella* exhibited significantly lower dry weight and lower levels of storage lipids than wasps emerging from other host species, possibly (given the generally observed positive correlation between size and fitness in parasitoids) having lower fitness as a result. As a result it could be concluded that *P. interpunctella* represents a lower quality host to *G. legneri*. However this difference was not reflected by the contest behaviour of *G. legneri* when defending each of the four species.

The lack of differences in aggression between species could be due to a number of factors. The acclimatisation period, i.e. the time period in which wasps were allowed to establish ownership of a host, within these contests was lower than that of previous staged contests using *G. legneri* (Lizé et al. 2012; Stockermans and Hardy 2013), in order to minimise the probability of contestants ovipositing prior to contest onset. As a result host owners may not have had enough time to adequately assess the quality of the host. It is possible that a repetition of these staged contests using a longer acclimatisation period may result in a more accurate assessment of the host quality by the wasp, and as a result, differences in aggression profile between wasps defending specific host species may become apparent.

Whilst host species may not affect the aggression profile of adult *G. legneri* defending hosts, the developmental and metabolomic studies suggest that wasps reared on *P. interpunctella* exhibit reduced fitness compared with wasps reared on *Ephestia kuehniella* or *C. cephalonica*. It is possible that this reduced fitness translates to reduced contest performance in *P.*

interpunctella reared wasps. *G. legneri* reared on *P. interpunctella* exhibited reduced levels of diacylglycerides compared to those reared on *Ephestia kuehniella* or *C. cephalonica*, the major form of fat mobilisation by insects (Arrese and Soulages 2010), along with reduced triacylglyceride levels. As such, further research could stage contests between wasps reared on *P. interpunctella* and wasps reared on *Ephestia kuehniella* or *C. cephalonica*. It is possible that a wasp may display higher aggression when provided with a host matching its own developmental background; as a result a separate host species from those used to rear contestants should be used within contests. It would be expected that wasps reared on *Ephestia kuehniella* or *C. cephalonica* would exhibit higher contest success than those reared on *P. interpunctella*, due to their lower energy reserves resulting in a lower RHP.

A major aim of this project was to determine if specific biomarkers associated with individual host species account for differences between the performances of *Goniozus* species. LC-MS analysis across all four lepidopteran species did detect a number of compounds that were highly elevated in the three species that *G. nephantidis* was incapable of developing on but that were only present in trace amounts, or were completely absent, in *C. cephalonica*. The identity of these compounds however remains unknown, despite the generation of structural information using LC-MS/MS. Stand compounds from two major lipid categories, bile acids and omega 6 fatty acids, were directly compared with these unknown compounds but did not generate a match for either retention time (bile acids, omega 6 fatty acids) or mass-to-charge ratio (omega 6 fatty acids). Due to the lack of any obvious alternative candidates it is not currently possible to achieve this project aim. Though time constraints prevented further identification work from being conducted, it may be possible to generate further structural information about these compounds using a combination of liquid chromatography and NMR. As these unidentified compounds have very robust retention times it may be possible to separate these compounds using LC before analysis by 1D and 2D NMR. However a level of optimisation would be required for this approach to be viable, particularly to concentrate the generated compound phases after LC dilution. A level of column contamination may also have to be accounted for. If the identity of these compounds was determined by such analysis, and if these compounds were found to be readily available or if they could be synthesised, a further

behavioural study could be conducted to determine their potential role in host acceptance. Different combinations of these compounds could be selectively injected into post-paralysed *C. cephalonica* at varying concentrations to examine whether it is possible to 'trick' *G. nephantidis* into rejecting a normally acceptable host or, if eggs were laid onto manipulated hosts, whether these compounds preclude the development of offspring to maturity.

8.4 The effects of adult wasp diet on the lifespan and contest behaviour of *G. legneri*

It is now well established that adult parasitoids are capable of utilising local nutritional sources to enhance energy stores accumulated in larval development (Jervis et al. 1983; Eijs et al. 1998; Giron et al. 2002; Giron and Casas 2003; Visser and Ellers 2008). A range of prior studies have further confirmed that the availability of a carbohydrate or lipid rich diet can enhance the lifespan and fecundity of parasitoids that lack *de novo* lipogenesis, however this does not translate to new lipid stores (Heimpel et al. 1997; Pexon and Mayhew 2002; Lee et al. 2004; Winkler et al. 2006; Wäckers et al. 2008; Gómez et al. 2012; Harvey et al. 2012). This project found that a carbohydrate diet enhances the lipid retention and lifespan of adult female *G. legneri*. It does not, however, affect contest outcome in intruder-intruder contests.

This study initially focused on the possibility of differentiation between 3 day old honey-fed, host-fed and starved wasps. This age was selected as it represented the approximate time point by which most newly emerged adult wasps disperse into their surroundings (Hardy et al. 1999, 2000). As no clear differentiation in wasp metabolomic state or contest behaviour was present between honey fed and host fed wasps at this time point, further analysis of 7 day old wasps was performed. This time point was selected as it represents the maximum lifespan (Chapter 4) of adult female *G. legneri*, as such it was hypothesised that the lack of a maintenance energy source may become a limiting factor towards the end of *G. legneri* lifespan. However, this experimental setup had the disadvantage of performing 3 day and 7 day old metabolomic analysis separately, preventing direct comparisons between

the datasets (i.e. in the same PCA plot). This study also sampled a low number of time points, as a result there is little information regarding potential fluctuations in lipid profile between the approximate time of wasp dispersal and death. As a result if this study, or another tracking metabolomic fluctuation over time, were to be reproduced it may benefit from an experimental design that unifies the metabolomic analysis of all treatments, allowing direct comparisons between individual time points. However, such an experimental design may be somewhat difficult to coordinate in *G. legneri*, due to slight variations in development time between broods (Hardy et al. 1992).

No significant differences in contest performance were observed between honey-fed and starved *G. legneri*. This thesis has previously discussed the possibility that this lack of significance may be due to the increased perceived RV of the host to the starved competitor equalising the enhanced RHP of honey-fed competitors. As this study only focused on contest outcome it is not possible to separate the effects of these two components. However, it may be possible to generate further information by observing specific agonistic behaviours within contests, as performed in Chapter 5 and previously utilised by Lizé et al. (2012) and Stockermans and Hardy (2013). This would involve conducting contests within experimental treatments i.e. honey-fed vs. honey-fed and starved vs. starved, then comparing specific aggressive behaviours (e.g. chasing, stinging, biting and escalated fighting) between treatment contests. It would be hypothesised that if starved *G. legneri* do perceive hosts as being of higher value than honey-fed wasps, they would exhibit higher levels of aggressive behaviours whilst attempting to acquire a host.

8.5 The metabolomic legacy of post-paralysis host aging on *G. legneri* offspring

Khidr et al. (2012) previously established that wasps defending younger post-paralysed hosts have a higher probability of contest success than those defending older post-paralysed hosts. This study also established that hosts decrease in value after paralysis, with energy rich metabolites declining over time. This project confirmed these initial findings, with results also indicating

that wasps reared on older hosts displayed lower levels of energy rich storage lipids. However, host background did not seem to affect the probability of contest success.

The NMR analysis of wasps emerging from young and old hosts contained in this study identified an unusually high degree of contamination, likely due to inadequate cleaning procedures. Due to time limits it was not possible to reproduce this study; as a result no information is available regarding polar wasp energy reserves. This thesis has previously discussed the possibility that short term energy stores, such as haemolymph sugars, may play a larger role in determining wasp RHP than long term energy stores, due to the short timescale of *Goniozus* wasp contests. As a result it is possible that a repeated NMR analysis of host aged wasps may generate enhanced information regarding their underlying energetic state.

The owner-owner contests performed as part of this study only examined contest behaviour between 3 - 5 day old wasps. However, these wasps may not be significantly limited by their reduced energy reserves at this age, with lower lipid stores only becoming a significant factor in contest resolution towards the end of the wasp lifespan. As a result significant differences in contest behaviour may be observable on 7 day old wasps reared on young and old hosts.

8.6 The prevalence of chemical mimicry within parasitoids

It has previously been supposed that defensive mimicry rarely involves multi-trait similarities (Bates 1862; Pasteur 1982). However, the study presented in this thesis presents an exception, the secondary hyperparasitoid wasp *Gelis agilis*. *G. agilis* is a known mimic of the black garden ant *Lasius niger*, and the two share a high degree of morphological similarity. Through a combination of no-choice and choice experiments *G. agilis* was demonstrated to possess comparative spider predation survival rates to that of *L. niger*. GC-MS analysis also confirmed that *G. agilis* emits a volatile ant defence semiochemical when agitated. This volatile release was found to be a significant factor in *G. agilis* survival, as demonstrated by the

higher predation rates of the morphologically similar but non-volatile emitting *Gelis areator*.

The GC-MS analysis conducted throughout this analysis was originally intended to directly compare volatile emission by *L. niger* with that of *G. agilis*. However specimens for this experiment were obtained remotely from VU University Amsterdam, due to a fault during shipping the contained *L. niger* died prior to GC-MS analysis. If this investigation were to be replicated it may be optimal to analyse any volatile emissions from *L. niger* alongside those of *G. agilis*. However it is unlikely that this would generate much extra information compared to the current study, as the emission of 6-methyl-5-hepten-2-one by ants as an alarm signal has already been confirmed by previous studies (Völkl et al. 1994; Allan et al. 1996).

A potential avenue of study is apparent from the results of this investigation. Whilst this study examined the predation rate of *G. agilis* exposed to common spiders, it did not explore spider behaviour in response to the release of 6-methyl-5-hepten-2-one. Prior studies have examined the spider predations of organisms that emit ant alarm pheromones (Allan et al. 1996; Hübner and Dettner 2000). The results of these studies indicate that response behaviour varies between species, some spiders are repelled by alarm signals (Hübner and Dettner 2000) whilst others utilise these signals to locate ant prey (Allan et al. 1996). Through the use of olfactometry it may be possible to determine the response of these, and potentially other, common predators of *G. agilis* emissions both with and without the visual cue of *G. agilis* morphology.

8.7 The association of metabolomic changes with parasitoid contest behaviour

A secondary aim of this project was to attempt to correlate parasitoid behaviour with changes in the underlying biochemical state. The preceding study that formed the impetus for this project, Khidr et al. 2012, found that correlations were present between the deterioration of host energy reserves, such as high concentration haemolymph sugars, was correlated with a decreased probability of contest success for its owner. This project made numerous findings on how diet and host background affects a parasitoid's

life history, the lack of significant effects by either of these factors on parasitoid contest behaviour resulted in no opportunities to directly link changes in the underlying metabolomic state to parasitoid contest performance. This project was able to draw correlations between the presence of a number of compounds in host species other than *C. cephalonica* and the poor brood-rearing performance of *G. nephantidis* on these species. However, further identification and validation of these compounds would be required for a definitive conclusion on their role to be made. This lack of correlation may be remedied, however, by any new information generated from the extra contest work outlined in this Chapter.

8.8 Final conclusions

The results of this thesis indicate that a combined low biomass NMR and LC-MS approach can be rigorously applied to the study of metabolomic changes in parasitoid wasps, such as *G. legneri*. This approach met current reporting and validation standards, an area in which previous ento-metabolomic studies have exhibited poor performance. Both polar and non-polar metabolite identities were readily assignable through a combination of 2D NMR and LC-MS/MS. This thesis further employed this combined metabolomic method to detect changes in the metabolome of *G. legneri* in response to such factors as aging, adult diet and nutritional background. This thesis further found that *G. legneri* is capable of parasitizing a wide range of hosts, though its oviposition behaviour significantly differed between hosts. Whilst correlations were drawn between the presence of a number of metabolites and the inability of *G. nephantidis* to reliably parasitize host species other than *C. cephalonica*, further identification and validation work would be required to definitively link these compounds to parasitoid behaviour. The non-significant effects of different adult diets and nutritional backgrounds on *G. legneri* contest behaviour prevented this project from directly linking contest performance with the underlying metabolomic state of the organism. Despite this, further contest work examining aggression and offspring performance may yet generate links between the metabolome and parasitoid contest performance.

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Appendix 1 - Entometabolomics: applications of modern analytical techniques to insect studies

MINI REVIEW

Entometabolomics: applications of modern analytical techniques to insect studies

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Abstract

Metabolomic analyses can reveal associations between an organism's metabolome and further aspects of its phenotypic state, an attractive prospect for many life-sciences researchers. The metabolomic approach has been employed in some, but not many, insect study systems, starting in 1990 with the evaluation of the metabolic effects of parasitism on moth larvae. Metabolomics has now been applied to a variety of aspects of insect biology, including behaviour, infection, temperature stress responses, CO₂ sedation, and bacteria–insect symbiosis. From a technical and reporting standpoint, these studies have adopted a range of approaches utilising established experimental methodologies. Here, we review current literature and evaluate the metabolomic approaches typically utilised by entomologists. We suggest that improvements can be made in several areas, including sampling procedures, the reduction in sampling and equipment variation, the use of sample extracts, statistical analyses, confirmation, and metabolite identification. Overall, it is clear that metabolomics can identify correlations between phenotypic states and underlying cellular metabolism that previous, more targeted, approaches are incapable of measuring. The unique combination of untargeted global analyses with high-resolution quantitative analyses results in a tool with great potential for future entomological investigations.

Introduction

The development of metabolomic methodologies is ongoing and has been applied to an expanding range of fields. Within the field of entomology, metabolomic techniques have been used to reveal biochemical information to aid in the understanding of physiology and behaviour. While there is an increasing amount of data available regarding gene regulation (Harshman & James, 1998; Immler & Bulet, 2005; Smith et al., 2008), transcriptomics (Pauchet et al., 2009; Mittapalli et al., 2010; Zhang et al., 2010) and proteomics within insect models (Stadler & Hales, 2002; Wolschin & Amdam, 2007; Cilia et al., 2011), information on the role of differential metabolic states in regulating insect behaviours and phenotypes has

remained comparatively scarce. Those investigations that have been conducted have indicated that the ‘-omics’ approach is increasingly promising for entomological applications (Lenz et al., 2001; Coquin et al., 2008; Kamleh et al., 2008; Aliferis et al., 2012).

Metabolomics is one of the newest ‘-omics’ technologies, and has rapidly expanded over the last decade, providing an integral new approach to the study of biological systems (Dettmer & Hammock, 2004; Rochfort, 2005). Although this field was first defined by Oliver et al. (1998) as ‘the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification’, some entomological investigations employing a recognisably metabolomic approach pre-date the adoption of the term (e.g., Thompson et al., 1990). The growth of metabolomics is associated with the recent incorporation of high-throughput methodologies, a coupling of classical analytical methodologies with automated processing technologies. This approach aims to generate as complete a

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metabolite profile within a given system as possible, and catalogue any metabolic fluctuations generated by a particular environmental condition or perturbation, with metabolites defined as molecules that are necessary for, or involved in, a particular metabolic process. The discipline has generated new insights into the subtle metabolic perturbations that exist within toxicology (Robertson et al., 2011), drug functionality (Kell, 2006), disease states (Schnackenberg, 2007), ageing (Schnackenberg et al., 2007), and overall cellular function (Nielsen, 2003).

Metabolomics possesses some advantages over the more established '-omics' approaches of genomics, transcriptomics, and proteomics. In particular its focus on 'downstream' cellular functions allows conclusions to be drawn regarding the functional metabolic phenotype of an organism. This form of analysis requires no prior knowledge of the genome of the organism under study, allowing useful biochemical data to be gathered even in the absence of full characterisation. Furthermore, the metabolomic approach provides a snapshot of the functional metabolic phenotype by detecting the full metabolome of a tissue or organism under a particular physiological state. Combined with the use of high-throughput analytical techniques, such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Reo, 2002; Dettmer et al., 2007), and the development of modern pattern recognition and multivariate data analysis software, metabolomics has become an effective way of summarising large changes in cell phenotype in terms of the fluctuations of a small number of metabolic pathways.

There are already some excellent reviews of current uses of metabolomic technologies, along with an extensive background to the field and its history (Nicholson & Wilson, 2003; Rochfort, 2005; Lindon & Nicholson, 2008; Heather et al., 2013), and several have focused on the use of metabolomics in particular fields of study, such as ecology (ecometabolomics) (Bundy et al., 2009; Jones et al., 2013; Lankadurai et al., 2013). In this review, we focus on metabolomics studies as they have been, and can be, applied to insect study systems. Although the number of insect studies employing a metabolomic approach has increased over the last decade, the total number of publications remains low (<50). Existing publications vary widely in their utilisation of data acquisition and analysis approaches, along with their reporting of technical parameters. Many of these parameters are required for independent assessments of the veracity of a particular study's findings; lack of their reporting can cast doubt on aspects such as insect rearing and collection, instrument stability, and data analysis. We aim to generate recommendations for improving this disparity by critically reviewing existing studies. As no prior review has attempted to critique

purely 'entometabolomic' studies we also briefly summarise some of the more novel applications of this methodology and provide a catalogue of current literature (Table 1).

The specific criteria for inclusion of an insect study into this review were based on whether an investigation was recognisably metabolomic in nature. As there is debate as to the exact definition of metabolomics (Oliver et al., 1998; Beecher, 2003; Ellis et al., 2007), we include studies according to the classification of recognised metabolomic approaches (including metabolomic fingerprinting and profiling) outlined by Goodacre et al. (2004). This review primarily focuses on the analytical techniques of NMR and MS, as these are the most commonly employed instruments within metabolomic studies. We begin by briefly outlining specific methodological aspects of how entometabolomic studies are carried out and then review the current range of studies employing this approach. As optimised protocols for ecometabolomics exist, the technical aspects of this review are limited to the discussion of sampling issues specific to entomological investigations. We conclude by critiquing the current state of the field and offering recommendations for future investigations.

Establishing a metabolomic workflow

A large number of reviews and methodological publications already exist outlining the major analytical and statistical steps involved in the establishment of an appropriate workflow for conducting a metabolomics, or indeed any '-omics', investigation (e.g., Fiehn, 2002; Broadhurst & Kell, 2006; Tiziani et al., 2011; Ibáñez et al., 2013; Nikolskiy et al., 2013). Furthermore, there are comprehensive reviews of ecometabolomics (Sardans et al., 2011; Rivas-Ubach et al., 2013), as well as methodological protocols optimised for insect tissues (Zhang et al., 2007; Kamleh et al., 2008). Due to the existence of these publications, we limit our discussion of the technical aspects of metabolomics to a brief consideration of specific sample collection problems entometabolomic studies may particularly encounter. We also supply a simplified workflow of a model entometabolomic study (Figure 1).

Sample preparation

Despite the existence of standardised sample preparation methodologies (Folch et al., 1957; Hara & Radin, 1978; Wu et al., 2008), problems may arise with specific organisms, in the case of insect studies this is often specifically related to low biomass (Lorenz et al., 2011; Marcinowska et al., 2011; Kim et al., 2013). As the majority of extraction methodologies are tailored for larger biomass samples, the volumes and ratios associated with these approaches may

Table 1 Insect metabolomics studies

Insect order	Species	Research topic	Sample type	Techniques utilised	Conclusions
Diptera	<i>Aedes aegyti</i>	Juvenile hormone regulation	Solvent extract	HPLC-FD*	Mevalonate and juvenile hormone pathways are highly dynamic and linked to reproductive physiology ¹
	<i>Belgica antarctica</i>	Temperature stress response	Solvent extract	GC-MS	Freezing and desiccation are associated with increases in metabolites associated with carbohydrate metabolism and a decrease in free amino acids ² . Shifts in metabolite pools are associated with changes in gene regulation related to dehydration ³
	<i>Chymomyza costata</i>	Cryopreservation	Solvent extract, biofluid	GC-MS, LC-MS	Survival of cryopreservation is associated with increased proline levels in larval tissues ⁴
	<i>Drosophila melanogaster</i>	Metabolomic profiling Temperature stress responses CO ₂ anaesthesia Bacterial infection Hypoxia	Solvent extract, biofluid	GC-MS, LC-MS	Cold shock disturbs short- and long-term cellular homeostasis ^{5,6,7,8} . Inbreeding, both in the absence and the presence of temperature stress, alters metabolic processes ⁹ . Lower rates of glycolysis occur in adapted flies undergoing hypoxia ^{10,11,12} . Age-related decline of hypoxia tolerance is linked to reduced recovery of mitochondrial respiration ¹³ . >230 metabolites profiled across four <i>Drosophila</i> subspecies ^{14,15} . Bowman-Birk inhibitor disrupts energy metabolism ¹⁶ . Long-term cold acclimation modifies the larval metabolome ¹² . Absolute quantification of 28 phospholipids ¹⁷ . Larvae with the y mutation have altered lysine metabolism ¹⁸ . CO ₂ exposure causes metabolic changes during short term recovery ¹⁹ . Infection by <i>Listeria monocytogenes</i> results in loss of energy store regulation ²⁰ . Developmental and adult cold acclimation strongly promoted cold tolerance and restored metabolic homeostasis ²¹
	<i>Drosophila montana</i>	Temperature stress responses	Solvent extract	GC-MS, LC-MS	Seasonal variations in thermoperiod are correlated with differential expression of myo-inositol, proline and trehalose ²²
	<i>Sarcophaga crassipalpis</i>	Temperature stress response	Solvent extract	GC-MS, 1D NMR	Rapid cold-hardening elevates glycolysis associated metabolites whilst reducing levels of aerobic metabolic intermediates ²³
Hemiptera	Aphids (multiple species)	Trehalose analysis Insect–bacterial symbiosis	Solvent extract, biofluid	1D NMR	High concentrations of trehalose are present in aphid hemolymph ²⁴ . Removal of bacterial–insect symbiosis reduced expression of dietary metabolites, including essential amino acids ²⁵

Table 1. Continued

Insect order	Species	Research topic	Sample type	Techniques utilised	Conclusions
Hymenoptera	<i>Apis mellifera</i>	<i>Nosema ceranae</i> infection Pesticide exposure	Solvent extract, biofluid	GC-MS, LC-MS	Exposure to infectious pathogens and neonicotinoid pesticides results in altered larval and adult metabolism ^{26,27}
	<i>Praon volucre</i>	Diapause induction	Solvent extract	GC-MS	Cold acclimation eliminated cryo-stress associated homeostatic perturbations ²⁸
	<i>Venturia canescens</i>	Temperature stress responses	Solvent extract	GC-MS	Increases in cold tolerance are associated with the accumulation of cryoprotective metabolites ²⁹
Lepidoptera	<i>Helicoverpa armigera</i>	Diapause induction	Solvent extract	GC-MS, MALDI-TOF	Diapause induces metabolic alterations associated with photoperiodic information and energy storage ³⁰
	<i>Manduca sexta</i>	Host parasitism	Biofluid	1D NMR	Insect parasitism enhances glucogenesis induction and halts lipogenesis ^{31,32} . Concentrations of small molecule metabolites change alongside larval development ³³
	<i>Spodoptera frugiperda</i> <i>Trichoplusia ni</i>	Metabolomic profiling	Solvent extract	LC-MS	Identification of major pathways associated with cellular protein productivity ³⁴
Orthoptera	<i>Chorthippus</i> (multiple species)	Metabolomic profiling	Solvent extract	GC-MS	Determination of water soluble and lipid components of abdominal secretions of grasshoppers ^{35,36}
	<i>Locusta migratoria</i>	Developmental phase transition	Solvent extract	1D NMR	Onset of solitary-group behavioural phase transitions are regulated by carnitine expression ³⁷
	<i>Schistocerca gregaria</i>	Social behaviour	Biofluid	1D NMR	Concentrations of trehalose and lipids were lower in the haemolymph of crowd-reared than in solitary-reared nymphs ³⁸
Phasmatodea	<i>Anisomorpha buprestoides</i>	Venom analysis	Biofluid	1D, 2D NMR	Stick insect defence secretions contain high levels of glucose, lysine, histidine, serotonin and sorbitol ³⁹
	<i>Peruphasma schultei</i>	Venom analysis	Biofluid	1D NMR	Individual insects produce different stereoisomeric mixtures ⁴⁰
Plecoptera	<i>Dinocras cephalotes</i>	Hypoxia	Solvent extract	1D NMR/DI-MS	Metabolic shifts associated with heat stress are more pronounced under hypoxia ⁴¹

*High-Performance Liquid Chromatography with Fluorescence Detection.

¹Rivera-Perez et al. (2014); ²Michaud et al. (2008); ³Teets et al. (2012); ⁴Košťál et al. (2011b); ⁵Malmendal et al. (2006); ⁶Overgaard et al. (2007); ⁷Malmendal et al. (2013); ⁸Williams et al. (2014); ⁹Pedersen et al. (2008); ¹⁰Feala et al. (2008); ¹¹Feala et al. (2009); ¹²Košťál et al. (2011a); ¹³Coquin et al. (2008); ¹⁴Kamleh et al. (2008); ¹⁵Kamleh et al. (2009); ¹⁶Li et al. (2010); ¹⁷Hammad et al. (2011); ¹⁸Bratty et al. (2012); ¹⁹Colinet & Renault (2012); ²⁰Chambers et al. (2012); ²¹Colinet et al. (2012a); ²²Vesala et al. (2012); ²³Michaud & Denlinger (2007); ²⁴Moriwaki et al. (2003); ²⁵Wang et al. (2010); ²⁶Aliferis et al. (2012); ²⁷Derecka et al. (2013); ²⁸Colinet et al. (2012b); ²⁹Foray et al. (2013); ³⁰Zhang et al. (2012); ³¹Thompson et al., (1990); ³²Thompson (2001); ³³Phalaraksh et al. (2008); ³⁴Monteiro et al. (2014); ³⁵Buszewska-Forajta et al. (2014b); ³⁶Buszewska-Forajta et al. (2014a); ³⁷Wu et al. (2012); ³⁸Lenz et al. (2001); ³⁹Zhang et al. (2007); ⁴⁰Dossey et al. (2006); ⁴¹Verberk et al. (2013).

require adaptation if adopted for low biomass investigations, such as in Wu et al. (2008). Furthermore, in order to prevent cross-contamination, it may be optimal to clean

the organism using high-purity water, or another appropriate solvent, prior to snap freezing. This is particularly important for entomological investigations, due to many

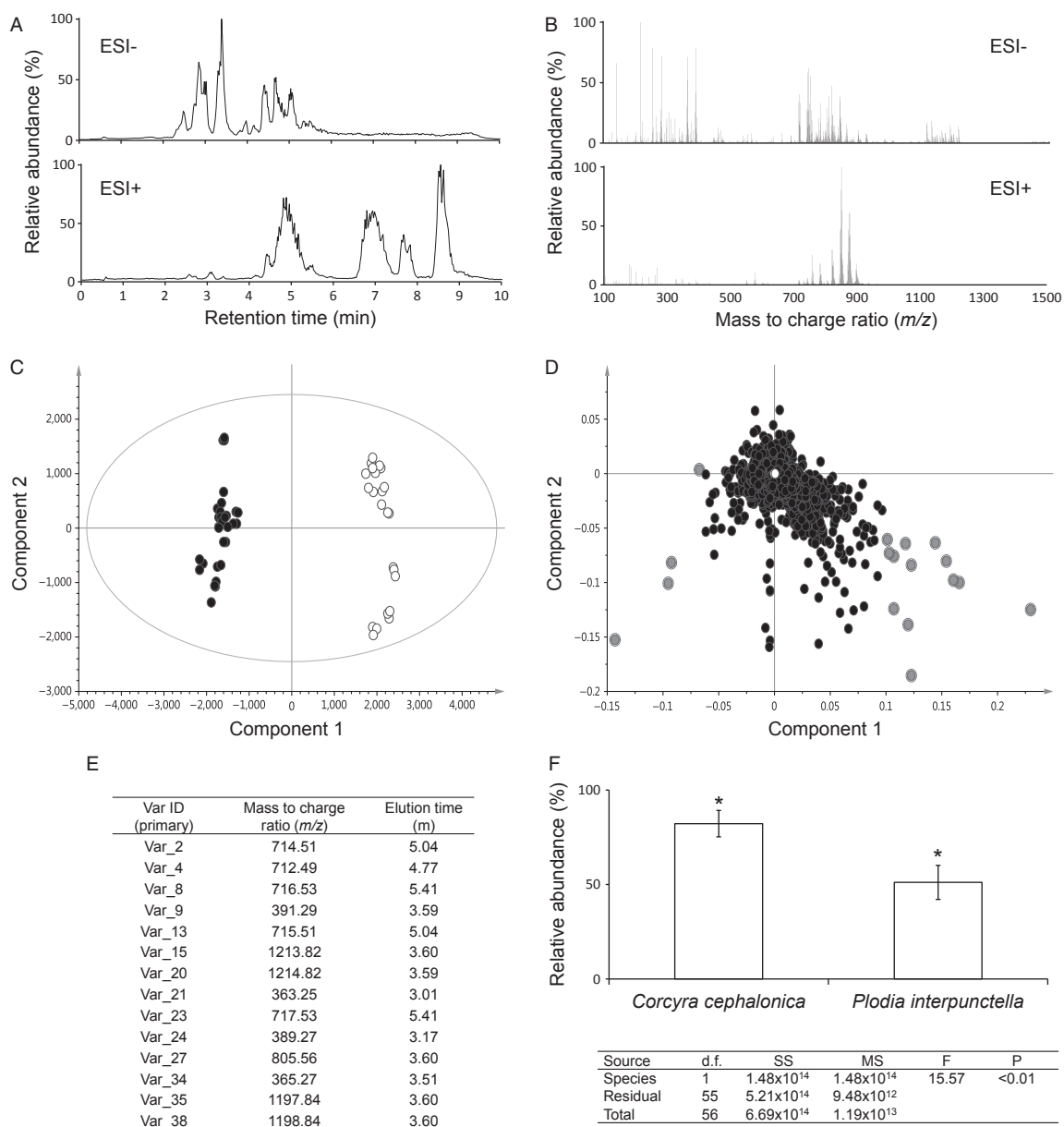


Figure 1 A sample data processing workflow. This investigation assessed differences in the larval metabolome across two pyralid moth species: rice moth (*Corcyra cephalonica* Stainton) and Indian mealmoth (*Plodia interpunctella* Hübner) (C Smart, unpubl.). Lipid extracts were generated using a modified methanol-chloroform-water extraction protocol and analysed using LC-MS (A and B). LC-MS chromatograms were aligned to a common reference sample and framed using the Thermo SIEVE (Thermo Fisher Scientific, Waltham, MA, USA) processing software. Aligned and framed data were then exported to the statistical software SIMCA 13.0.3 (Umetrics, Umeå, Sweden) and analysed using principle component analysis (PCA) (C and D). Group clustering of samples based on the two experimental groups was confirmed in the negative electrospray ionisation (ESI) mode PCA analysis (C). The two treatment groups were defined and an PLS-DA analysis was utilised to directly compare between the two groups ($R2X = 0.706$, $R2Y = 0.988$, $Q2 = 0.98$). A loadings plot was utilised to aid in identifying major differences between the two groups (D). Group-to-group comparisons were used to highlight loadings (highlighted in grey) associated with the two groups. These differential loadings were examined for their associated mass-to-charge ratios (m/z) and elution times (E). Using these values, variable ID 9 was identified as a cholesterol derivative based on consultation with online metabolite databases [LIPID MAPS and the Human Metabolome Database (HMDB)]. Further qualitative data for this metabolite were generated using the Thermo XCALIBUR software (Thermo Fisher Scientific). Mean relative abundances (± 1 SD) are shown on a bar chart (F) and ANOVA found a significant difference in metabolite level between the two groups (F).

laboratory insect populations being reared in groups where the surface of the specimen may be exposed to culture media and/or faecal matter that could affect the outcome of analysis if detected. Several common culturing components, including honey, glycerol, and ethanol, are readily detectable in metabolomic analysis, particularly in the case of ^1H NMR spectroscopy (Phalaraksh et al., 2008). Diet should also be considered, particularly as highly sensitive analytical approaches may also detect differences in gut composition. A possible approach to eliminating this issue would be to perform similar extractions and analytical profiling of the insect diet: dietary spectral data could then be compared with experimental samples, and used to rule out any observed background resonances or ions.

Current approaches to entometabolomics

Although many applications are currently only represented by relatively few studies, entometabolomics has contributed to the understanding of such topics as hypoxia (Coquin et al., 2008; Feala et al., 2008), insect–bacterial symbiosis (Wang et al., 2010), behavioural ecology (Lenz et al., 2001), parasitism (Thompson et al., 1990), development (Phalaraksh et al., 2008; Wu et al., 2012), infectious diseases (Aliferis et al., 2012), the effects of commercial pesticides (Derecka et al., 2013), and temperature-dependent stresses (Michaud & Denlinger, 2007; Michaud et al., 2008; Košťál et al., 2011a,b) (Table 1). Simultaneously, metabolomic investigations have indirectly generated information about insect life histories; particularly work focusing on plant–insect interactions (Hunt et al., 2006, 2010; Faria et al., 2007; Gattolin et al., 2008; Jansen et al., 2009; Leiss et al., 2011). The adoption and output of these approaches has steadily increased throughout the last decade, with novel applications appearing almost annually.

Many recent investigations involving insect tissues fall within the loosely defined field of ecometabolomics (Michaud et al., 2008; Sardans et al., 2011). The majority of entometabolomic studies have focused on single factor approaches, often without taking into account that numerous factors can affect the metabolome (e.g., time since the animal last fed, its health status and its age, the effects of varying the circadian rhythm). The metabolome is in fact highly dynamic, and this repeated fluctuation makes it virtually impossible to characterise every metabolite present within an individual insect (Sardans et al., 2011). Further, to obtain estimates of the natural metabolomic state, it is often desirable to perform entomological studies in the field, even though it may not be possible to regulate certain behavioural or physiological factors (e.g., diet and feeding time, photoperiod). A transition

between field conditions and final laboratory-based metabolomic analysis can also result in metabolomic perturbations. Minimising potential sources of external biological variation is critical for a metabolomic experimental design, as a result it is particularly important to consider external sources of variation that may result from such a transition (e.g., maintaining change in wild diet to laboratory-based diet, stress generated due to change in environment). Foray et al. (2013) deliberately attempted to avoid such variation by only allowing specimens to undergo short-term acclimation prior to metabolomic analysis, whilst Derecka et al. (2013) avoided any such acclimation by conducting metabolome quenching in the field. However, field quenching relies on constancy of several factors, including the availability of a quenching mechanism, sampling point consistency, and maintenance of the sample at sub-zero temperatures. In the case of laboratory studies involving laboratory cultures the existence of many established insect stocks can mitigate this, as the long-term culturing of specimens in a stable environment may largely eliminate environmental perturbations.

Metabolite profiling

The majority of MS-based insect metabolome studies have utilised the model organism *Drosophila melanogaster* Meigen (Kamleh et al., 2008, 2009; Hammad et al., 2011; Košťál et al., 2011a,b; Bratty et al., 2012; Colinet & Renault, 2012). This is not surprising, given that the combination of a large well-characterised stock of genetic mutants, genetic tractability, and a known organismal complexity make an ideal choice for generating insight into the composition and organisation of metabolic networks (Kamleh et al., 2008, 2009). The use of high-resolution analytical techniques also provides a solution to a remaining disadvantage, that of low biomass.

By combining this approach with the use of pooled samples, >200 metabolites have been identified using liquid chromatography (LC)-MS (Kamleh et al., 2008), including absolute lipid quantification (Kamleh et al., 2009) and validation (Hammad et al., 2011). These studies further indicated the practicality of LC-MS to detect differences between extremely low-biomass insect treatments, to the extent of being able to differentiate between individual *Drosophila* belonging to different subspecies or mutant types.

Many current NMR-based analyses of the insect metabolome have focused on characterising the properties of insect biofluids, with specific focus on the composition of larval and pupal haemolymph (Thompson et al., 1990; Lenz et al., 2001; Thompson, 2001; Phalaraksh et al., 2008). These studies provided expanded information regarding the composition of amino acids, organic acids,

sugars, and the role of ethanol. Perhaps the most important aspect of these early studies is the generation of an available list of common insect haemolymph metabolites (Phalaraksh et al., 2008); this is applicable for metabolite identification in both insect and crustacean investigations (Poynton et al., 2011). The list includes a large number of high-concentration molecules, the variation of which has been related to social behaviour (Wu et al., 2012) and heat stress (Michaud & Denlinger, 2007). However, the detection of alterations of metabolites present at a low concentration can be problematic, due to the over representation of many sugars within the 4–3 p.p.m. region of most NMR spectra generated from both haemolymph and full tissue extractions (Figure 2). Any attempt to assign identifications to resonances within this region would require further spectral information, such as two-dimensional (2D) NMR, an approach that has been utilised by more recent studies (Malmendal et al., 2006; Overgaard et al., 2007; Coquin et al., 2008; Hawes et al., 2008; Pedersen et al., 2008; Feala et al., 2009).

Hypoxia and anaesthesia

The use of LC-/gas chromatography (GC)-MS and NMR has generated new insights into the metabolic effects of

hypoxia in insect study systems, focusing on the regulation of glycolysis (Feala et al., 2008, 2009; Verberk et al., 2013) and fluctuations in the concentrations of free metabolites, such as proline, alanine, lactate, and acetate (Coquin et al., 2008; Košťál et al., 2011b). These studies also indicated that ageing was associated with a decline in hypoxia recovery; this recovery was linked to changes in free metabolite concentration after re-oxygenation. These investigations illustrate another benefit of high-resolution analytical techniques when experimental tissue volumes are low. The tissue of interest, the cardiac muscle, within individual flies was not present in significant quantities to be of use in biochemical investigations. This type of analysis would also face difficulty in consistently performing an appropriate dissection protocol on an insect of this size. The less direct route of metabolic modelling offered by NMR or MS allowed this limitation to be overcome.

Many entomological investigations require some form of anaesthesia to allow handling, colony maintenance, or identification (Vinuela, 1982; Nicolas & Sillans, 1989; Ashburner et al., 2005). Direct CO₂ exposure is a widespread method of anaesthesia within entomological studies, despite a number of reported side effects concerning reproductive and behavioural traits which may impact

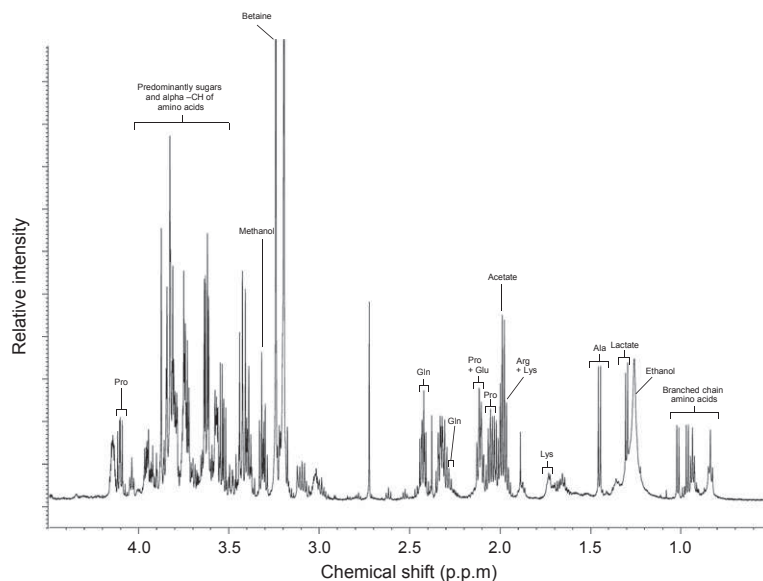


Figure 2 ¹H 600 Mz aliphatic NMR spectrum of the larvae of the rice moth, *Corcyra cephalonica*. Spectral information was generated through the use of modified Folch methanol-water-chloroform. Repeated investigations into the NMR profile of larval haemolymph have shown a remarkably conserved spectral structure (C Snart, ICW Hardy & DA Barrett, unpubl.). With the exception of the overlapping sugar-amino acid spectral region situated at 4.0–3.4 p.p.m., a high proportion of commonly observed peaks is readily assignable through simple literature comparison (Phalaraksh et al., 2008). Although exact spectral positions can vary based on the particular operating frequency of the instrument and environmental fluctuations, existing characterisations of tissue/haemolymph NMR spectra can aid in the normally extensive process of individual peak identification. Ala = alanine, Arg = arginine, Gln = glutamine, Glu = glutamic acid, Lys = lysine, Pro = proline.

physiological and metabolic traits (Nilson et al., 2006; Champion de Crespigny & Wedell, 2008). Colinet & Renault (2012) used GC-MS to investigate the metabolic effects of this exposure, both in terms of an acute exposure, and a long-term recovery, showing that CO₂ exposure resulted in acute metabolic changes that are present for 14 h. These changes were directly related to the anoxic conditions related to cardiovascular disruption. However, there was no indication of long-term alterations after a 24-h period, allowing the conclusion that CO₂ anaesthesia is an acceptable procedure when a longer recovery time is possible. With the exception of Chambers et al. (2012), the studies cited in this review either avoided the use of CO₂ anaesthesia, or accounted for this recovery period in their methodology (Overgaard et al., 2007).

Insect development and social behaviour

¹H NMR spectroscopy has been used to compare the haemolymph metabolome of nymphs of the desert locust, *Schistocerca gregaria* (Forsk.) reared under both solitary and gregarious conditions (Lenz et al., 2001). A number of metabolites varied across rearing conditions, including trehalose, lipids, acetate, and ethanol. However, later studies generated contradictory haemolymph metabolite identifications (Phalaraksh et al., 2008). A similar investigation utilised MS to examine solitary-gregarious behavioural transitions in a related locust species, *Locusta migratoria* (L.) (Wu et al., 2012). Direct comparisons of the haemolymph of solitary and gregarious phase locusts using high-performance LC-MS and GC-MS identified 319 metabolites exhibiting differential concentrations between the two phenotypes. Of these, carnitine was identified as a key differential metabolite regulating locust phase transition from solitary to gregarious, alongside its acyl derivatives. This study presents the first example of an MS approach being applied to link differences in insect behaviour with the underlying metabolomic state.

Temperature-dependant stress responses

The most common topic of insect metabolomics concerns fluctuations in the metabolome when subjects are exposed to a range of extreme temperatures (Table 1) due to both seasonal and daily cycles (Malmendal et al., 2006; Pedersen et al., 2008). Many insect species have developed biochemical, behavioural, or physiological adaptations to minimise the potential damage from these fluctuations (Michaud & Denlinger, 2007). Extensive study of the insect metabolome (particularly of *Drosophila*) under different temperature stresses has confirmed that these perturbations can have both short- and long-term effects on metabolite concentrations (Michaud et al., 2008), along with a more general effect on cellular homeostasis

(Malmendal et al., 2006). These differences in metabolite fingerprint are conserved across various temperature treatments, and indicated that the concentrations of several major metabolites were significantly altered by heat-shock, including, but not limited to, primary amino acids, ATP, acetate, and glycogen (Malmendal et al., 2006). Notably, these differences do seem to be largely conserved across a number of species (Moriwaki et al., 2003; Phalaraksh et al., 2008).

Various studies, utilising both NMR and MS, have noted a similar effect from cold-shock, with particular focus being placed on the inducement of an elevated level of glycerol. Alongside this, increases in sorbitol, proline, alanine, glutamine, pyruvate, glucose, and urea, and parallel decreases in trehalose, mannose, beta-alanine, and ornithine have been identified. Of these, the essential role of proline in surviving cold-shock has been documented using GC-MS/LC-MS in a study involving the survival of the drosophilid fly, *Chymomyza costata* (Zetterstedt), when submerged in liquid nitrogen during diapause (Košťál et al., 2011b). Similar variations were noted in regards to seasonal variation in thermoperiod (Vesala et al., 2012), whilst contrasting thermal environments during insect development indicated differentiation in the levels of glucose, fructose, alanine, and glycine, along with an accumulation of polyamines. Potential alterations in metabolites associated with energy metabolism also suggested an alteration in energy metabolism, similar to that observed after cold acclimation in *Drosophila* (Košťál et al., 2011a; Colinet et al., 2012a). This may also confirm findings by Colinet et al. (2012b), who demonstrated similar disruptions in energy metabolism under diapause in the aphid parasitoid *Praon volucre* (Haliday).

Insect-plant interactions

Some studies have indirectly used a metabolomic approach to draw conclusions about plant-insect interactions (Allwood et al., 2008; Jansen et al., 2009; Leiss et al., 2011; Misra et al., 2010). One investigation focused on the effects of herbivore by different instars of the beet armyworm, *Spodoptera exigua* Hübner. Through a combination of 1D and 2D ¹H NMR, Widarto et al. (2006) were able to differentiate significant alterations in the metabolome of *Brassica rapa* L. leaves after the initiation of feeding damage. Spectral investigation, conducted using principle component analysis (PCA), indicated an increase in the levels of glucose, ferulic acid, and gluconapin in response to feeding by second-instar *S. exigua*, compared with an increase in alanine and sinapoyl malate for fourth-instar feeding. By comparison, larvae of the moth *Plutella xylostella* L., a more specialist herbivore, elicited an increase in gluconapin, glucose, feruloyl malate, sinapoyl malate, and

threonine. This study again demonstrated some of the advantages associated with two dimensional NMR, as it was able to reduce assignment problems associated with overlapping spectral traces.

Integrated metabolomic approaches

An emerging trend within metabolomic research is to combine different high-throughput technologies to generate an integrated ‘-omics’ based approach. To date, four separate investigations have analysed insect tissues using a combination of metabolomics with either proteomics (Wang et al., 2010; Zhang et al., 2010) or transcriptomics (Teets et al., 2012; Derecka et al., 2013) and, to some extent, genomics (Derecka et al., 2013). These approaches attempted to correlate genomic/transcriptomic information with more ‘down-stream’ metabolomic or proteomic datasets. An integrated study investigated the symbiotic bacterial system present in the pea aphid, *Acyrtosiphon pisum* (Harris) (Wang et al., 2010); along with the metabolomic aspect of the investigation, the aphid proteome was also subject to analysis. Utilising dietary antibiotics to eliminate the bacterium *Buchnera aphidicola* Munson et al., metabolomic analysis indicated alterations in metabolite and protein abundance (Wang et al., 2010). These changes were dominated by decreased essential amino acid abundance and an increase in non-essential amino acids. These findings also indicated that the bacterial proteome/metabolome is more substantially affected by antibiotic treatment than by dietary manipulation. The metabolomic-proteomic approach was similarly conducted by Zhang et al. (2012) to examine the brain of larval cotton bollworm, *Helicoverpa armigera* (Hübner), concurrent with artificial induction of seasonal diapause. This integrated approach clarified the control mechanisms that underlie the pre-diapause phases, and showed that a wide range of metabolism-related proteins and metabolites differ in concentration between diapause-fated and non-diapause-fated larval brains.

A combined transcriptomic-metabolomic approach (Teets et al., 2012) was used to investigate a different aspect of extremely cold environments, where water resources may be frozen for a large portion of the year, namely that of dehydration and desiccation tolerance. Using the Antarctic midge, *Belgica antarctica* Jacobs, an insect capable of surviving the loss of over 70% of body water, Teets et al. (2012) found that changes in gene expression associated with dehydration correlated strongly with changes in the metabolite pool. This study indicated that metabolic changes induced by the processes of desiccation and dehydration were remarkably similar, with changes occurring in such metabolites as glycolytic intermediates, lactate, proline, and citrate. These findings also

indicate that metabolic responses are coordinated with changes in gene expression, a critical aspect of dehydration and desiccation responses. Transcriptomic comparisons with gene expression data derived from the arctic collembolan *Megaphorura arctica* (Tullberg), displayed little similarity in regulatory response (Teets et al., 2012), perhaps indicating that separate arthropod species have developed different compensatory mechanisms for low water availability (Teets et al., 2012).

Entometabolomics: a critique of comparative studies

The ground-breaking study by Thompson et al. (1990) on the metabolic effects of parasitism pre-dates the formalisation of metabolomics as a field and also the adoption of current technical and statistical approaches that reduce the complexity of metabolomic datasets. Our critique therefore excludes this study. We also exclude studies that, although recognisably metabolomic in their approach (Goodacre et al., 2004), are focussed on reporting the profile of insects in a single species or state. We restrict our consideration to studies that primarily investigate the underlying metabolomic change that differentiates two or more phenotypic states. There have been 37 studies that meet this criterion, 33 of which were performed since the adoption of modern (i.e., post-2006) reporting standards (Fiehn et al., 2006; Sumner et al., 2007). We suggest that improvements can be made in several areas, including sampling procedures, the countering of sampling and equipment variation, the use of sample extracts, statistical analyses, confirmation, and metabolite identification.

Sampling procedures

As previously detailed, low biomass (<1 mg) has typically required modifications to established solvent extraction methodologies. A common approach for improving yield in current entometabolomic approaches has been to pool insect tissue samples (e.g., Kamleh et al., 2008, 2009; Košťál et al., 2011a,b); pooling also appears to have been used to make individual samples more representative of a given population (e.g., a honey bee colony, *Apis mellifera* L.; Aliferis et al., 2012). Whereas pooling has largely overcome the problem of low spectral complexity, there is a trade-off with the number of replicates potentially available. Reduced replication, and hence lower statistical power during validation, increases the possibility of type II error (failing to reject an incorrect null hypothesis; Smith et al., 2011). Although low biomass may result in problems with yield, complex spectral information can be obtained from single large (>20 mg) insects (Lenz et al., 2001; Phalaraksh et al., 2008). Enhancement of the number of replicates within a treatment should typically take

precedence when spectral yield is already substantial. Of the studies we consider, 63.6% (21/33) utilised adequate replication within each treatment (>5), as defined by current reporting standards (Fiehn et al., 2006; Goodacre et al., 2007; Sumner et al., 2007). Although sample sizes do not appear to have been adversely affected by pooling in these studies, it remains important to explicitly consider its desirability prior to experimental analysis.

Tackling unwarranted variation

An organism's metabolome can be subject to fluctuations influenced by many environmental sources. Although these sources may seem minor, such as changes in diet or culture conditions, they can interfere with the composition of spectral and chromatographic data. As many insect cultures are maintained in a controlled environment, perturbations in temperature, photoperiod, and humidity from these sources are reduced. However, for a metabolomic approach to work correctly, it is also crucial to maintain uniformity across sample collection, extraction and processing conditions, along with analytical uniformity (Wishart, 2008). Sample extraction conditions in particular can have several sources of contamination and unnecessary variation. Perhaps the most vital step in initial sample collection, halting metabolomic activity, can be compromised by failing to maintain suitable experimental conditions during sample extraction and pre-processing. Failure to maintain these conditions, usually by the use of ice-cold solvents, can result in further alterations in the metabolome during extraction: this is undesirable as the metabolomic approach attempts to assess an organism's actual biochemical state. Unwanted variation can also occur if an extraction protocol requires a drying and reconstitution phase prior to analysis. For example, Li et al. (2010) utilised a rotary evaporator to dry samples at 43 °C for 3 h. Although high temperature may have affected the composition of the sample prior to analysis, it would also have advantageously decreased the time required for sample concentration. There can thus be a trade-off between processing time and sample stability, in which case it may be preferable to use less disruptive drying protocols, such as N₂ evaporation.

Imprecision of analytical equipment is another source of undesirable variation. The precision of an analytical approach is usually established through 'technical replication' which consists of separate analyses of sub-samples of each experimental replicate. The standard deviations of the measurements for key metabolites can then be assessed to evaluate the stability of the analytical methodology. Of the 33 post-reporting standards studies we are considering, only 15.2% (5/33) provided information regarding technical replication: all of these were able to adequately

summarise this information in a single sentence within their materials and methods sections. Similarly, the stability of the chosen analytical method is often established through the continuous analysis of a pooled sample during a metabolomic experiment. Sub-samples are expected to cluster centrally within any multivariate analysis and exhibit low variability throughout the analytical timeframe. Whilst this approach is commonly utilised during high-sensitivity MS, some form of quality control sample can still be utilised by NMR spectroscopy. Despite this, only one investigation (Verberk et al., 2013) explicitly stated that a pooled quality control was used. The utilisation of a randomised sampling order, in order to reduce data skew stemming from instrument drift, was similarly reported by only a single study (Foray et al., 2013). It is possible that the remaining studies did not employ these common forms of validation. A more likely scenario is, however, that this detail was unreported due to its routine nature. Nevertheless, the lack of explicit reporting of quality control methods can cast doubt on the stability of a methodology, particularly in studies with large analytical timeframes. Another stability concern, specifically related to GC-MS, is the automatisisation of derivatisation prior to analysis; a process which ensures an identical processing time for each sample. Although studies utilising GC-MS all provided information regarding derivatisation, only 18.7% (3/16) provided supporting information on this automation.

Photoperiod is one of the largest potential sources of variation in ecometabolomics (Beck, 1975; Košťál, 2006). Numerous studies have indicated that many metabolic pathways undergo photo-period dependent shifts, including the amino acid (Fernstrom et al., 1979; Gattolin et al., 2008), carbohydrate (Das et al., 1993; Seay & Thummel, 2011), lipid (Turek et al., 2005; Seay & Thummel, 2011), nucleotide (Kafka et al., 1986; Fustin et al., 2012), and even xenobiotic pathways (Claudel et al., 2007). These shifts can be correlated with the onset and cessation of major behavioural processes, perhaps most notable for entometabolomics is the control of feeding behaviour (Seay & Thummel, 2011). Despite the critical role of the circadian clock in influencing physiological and behavioural rhythms, 33% (11/33) of post-2006 studies provided no supporting information for the experimental photoperiod. Of the remaining studies, 59% (13/22) used methodology that explicitly attempted to avoid variations in sample collection time. However, this figure is influenced by a high proportion of diapause related publications (6/22) which often require photoperiod-regulated induction prior to analysis. By comparison, 50% (2/4) of pre-2006 studies provided supporting information for photoperiod and 25%

(1/4) accounted for this potential variation during sample collection.

Although there is certainly room for improvement, it must be acknowledged that limiting the effects of photoperiod may not be practical for field-based investigations that rely on very little laboratory acclimation to maintain accuracy (Foray et al., 2013). However, as previously stated, it may still be possible to harmonise sample quenching times for both individual samples and treatment groups (Derecka et al., 2013). Similarly, investigations focusing on changes in behavioural (e.g., solitary vs. gregarious) or physiological state (e.g., diapause, parasitized) rather than a specified treatment period may struggle to standardise sample collection time. A possible means of limiting variation may be to standardise time between the onset of the desired state and sample quenching.

Effective use of sample extracts

There is a notable imbalance between metabolite classes examined in the studies we consider. Most (93.9%, 31/33) investigations have focused on polar metabolites, with a subset (21.2%, 7/33) attempting to also profile the non-polar metabolites. Only one investigation (Derecka et al., 2013) focused exclusively on the non-polar or lipid fragment generated by a methanol-chloroform-water based extraction. Buszewska-Forajta et al. (2014a) primarily focused on analysing the lipid component, but these data were presented as the complement to polar data generated by Buszewska-Forajta et al. (2014b), the two publications being derived from the same study.

The general lack of analysis of lipid fragments could be a major shortfall of entometabolomic investigations. This is due to the wide range of functions performed by the insect fat body, including energy storage and regulation (Arrese & Soulages, 2010), protein and nucleic acid production (Price, 1973), amino acid and carbohydrate production (Keeley, 1985), and metamorphosis (Mirth & Riddiford, 2007). Numerous entometabolomic publications have focused on polar metabolites that are involved in these same metabolic processes (e.g., Overgaard et al., 2007; Foray et al., 2013). Whilst it is possible that analysis of the non-polar fragment did not yield differential results in these instances, lack of analysis of non-polar fragments could lead to the loss of complimentary information concerning polar metabolite concentrations. As detailed above, complimentary data on polar and non-polar metabolites can be generated by biphasic solvent extraction (Wu et al., 2008). With the recent emergence of a new sub-division of '-omics' research, known as lipidomics (Wenk, 2005), it may prove advantageous to modify current solvent extraction protocols to favour a more lipocentric approach.

Statistical analysis

As multivariate data analysis identifies potential biomarkers and underlying sources of variation, it has been widely utilised in entometabolomic studies to demonstrate appropriate separation between experimental classes. It is particularly important that any fitted statistical model is capable of describing a high degree of variation whilst possessing the capability of accurate prediction. As a result, attempts at providing a more defined set of reporting standards have emphasised the documentation of specific model parameters, particularly the 'goodness' of fit (termed R²X) and the 'goodness' of prediction (termed Q²X) (Eriksson et al., 2005). Within the studies we consider, only 11 of 33 reported an appropriate statistical model to establish separation between experimental treatments. A further four of these studies only provided rudimentary outline of the chosen statistical approach, without reporting relevant model parameters. Only 18.1% of the post-2006 publications provided supporting information for separation [i.e., R²X or Q² values for partial least squares-discriminant analysis (PLS-DA)], slightly fewer than in the pre-2006 literature (1/4, 25%).

Although the establishment of overall separation and the provision of assessment parameters are important with highly multidimensional metabolomic data, further validation is also required to establish significant differences in individual metabolites between classes. Of the previous 33 publications, 26 utilised either parametric or non-parametric statistical validation (78.8%). This is largely unchanged from pre-2006 studies (3/4, 75%). Entometabolomic studies have encountered common problems with parametric testing; perhaps the most important of these is the potential for artificial inflation of significance through multiple-hypothesis testing. Although statistical correction methods to control false discovery rates have long been available (Benjamini & Hochberg, 1995; Quinn & Keough, 2002; Verhoeven et al., 2005), they were only employed by one of the five pre-2006 publications. The percentage is higher among post-2006 studies (12/33, 36.4%). One particular recent investigation (Monteiro et al., 2014) did not apply any form of statistical testing, instead using an arbitrary cut-off to conclude whether differences were present or absent. Although it is possible that this approach could accord with results of formal hypothesis testing, this is by no means certain, especially with low numbers of replicates (Quinn & Keough, 2002).

Metabolite confirmation, identification, and function

It should also be noted that although both univariate and multivariate data analyses potentially generate lists

of many differential compounds, it is important to confirm that this differentiation is based on real peaks, rather than noise. Furthermore, it is important to recognise that it is often not only difficult to identify the full range of spectral or chromatographic peaks, but also to correlate specific changes in the global metabolome with particular physiological or behavioural states. Data from both NMR and MS contain large overlapping peaks that can mask more subtle changes that may actually govern organism condition. Identification of what is accounting for these changes can be greatly enhanced by the application of knowledge of the biochemistry of the species concerned or of insects in general. Metabolite identification should employ as targeted an approach as possible, with earlier global profiling work acting as a screening process to identify potential metabolites of interest.

The importance of accurate identification is an enduring concern in metabolomic research, resulting in entometabolomic approaches utilising confirmation methodologies even in studies prior to the 2006 adoption of reporting standards. Current reporting for metabolomic research has become highly detailed, particularly with regard to the confirmation and validation of experimental findings. As accurate identification of differential biomarkers is a vital aspect of metabolomic investigations, many studies have employed further analytical methodologies, including tandem MS-MS and 2D NMR. A number of investigations (45.5%) utilised and documented a further confirmation step, such as LC-MS-MS or 2D NMR, to identify metabolites more confidently. Including putative approaches, all but two publications (Li et al., 2010; Vesala et al., 2012) provided identification utilising comparisons with known spectral and chromatographic standards, spectral databases, or tandem MS-MS fragmentation patterns. Whilst it is possible that the remaining two studies followed a similar identification protocol, this was not reported.

One area that exhibited consistent reporting was that of metabolite function. Although a metabolomic approach is capable of generating differential information about separate organism phenotypes, the generation of robust conclusions from these data requires a thorough understanding and examination of known metabolite pathways and information. Post-2006 studies all included discussion of the potential function of differential metabolites, many correlating shifts within major pathways (e.g., glycolysis) and framing them within the context of major environmental perturbations (e.g., hypoxia) (Coquin et al., 2008). Pre-2006 studies were similarly inclusive (4/4), although somewhat brief in the case of Lenz et al. (2001).

Conclusions and recommendations

Metabolomics has been applied to a number of insect study systems in an effective manner, generating new insights into the mechanisms underlying aspects of biology including behaviour (Lenz et al., 2001), infection (Chambers et al., 2012), temperature stress responses (Malmendal et al., 2006; Li et al., 2010; Colinet et al., 2012a), CO₂ sedation (Colinet & Renault, 2012), and bacteria–insect symbiosis (Wang et al., 2010). We are sure that this list of topics will expand in the near future.

Despite this success, there are opportunities to improve standards in terms of sample preparation, analytical methodology, statistical analysis, and reporting. The success or failure of a metabolomic investigation can depend on the rigour of the planning and developmental process preceding the investigation. To develop and employ an appropriate metabolomic workflow for an entomological study system, we recommend that future entometabolomic investigations follow points 1, 2, and 3 prior to experimental analysis, and points 4 and 5 before data analysis:

- 1 Plan the experiment carefully in advance with full consultation between the biological and analytical collaborators.
- 2 Minimise possible sources of environmental contamination or variation. Although the degree of elimination can be somewhat subjective depending on the goals of the investigation, this has been widely achieved through maintenance of a sterile, sub-zero °C environment throughout sample quenching, extraction, storage, and, if possible, analysis.
- 3 Validate analytical methods. In order for an analytical method to be valid, it must be possible to demonstrate stability throughout the experimental timeframe. Recommended validation approaches include technical replication, randomisation of the sample injection order, and the use of a fixed internal standard.
- 4 Validate univariate and multivariate data analysis. Multivariate cross-validation should be employed to demonstrate the closeness of fit for a discriminant analysis, whereas appropriate test statistics (and when relevant, descriptive statistics) should be provided for any univariate validation.
- 5 Provide robust supporting information for metabolomic data. Appropriate methodological and analytical metadata should be made available in order to maximise the utility of generated data to other researchers.

Overall, it is clear that the employment of a metabolomics approach can identify correlations between a phenotypic state and the underlying cellular metabolism that older, more targeted, approaches are incapable of

measuring. This unique combination of untargeted global analysis with high-resolution quantitative analysis presents an attractive tool for future entomological investigations.

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