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# Sustenance and Performance: Nutritional Reserves, Longevity, and Contest Outcomes of Fed and Starved Adult Parasitoid Wasps

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Dyadic contests for possession of resources occur across a wide range of animal taxa, with contest outcome often being heavily influenced by the energetic reserves of the competitors. The majority of studied parasitoid wasps lack *de novo* lipogenesis, with adult lipid reserves being limited to those acquired throughout larval development. Carbohydrate- and lipid-rich diets can increase adult parasitoid lifespan and fecundity by potentially acting as a maintenance energy store. However, the effects of such diets on fat reserve compositions and contest outcome have not been examined. This study assesses the effects of a carbohydrate rich diet (honey) on the longevity, metabolic state and contest performance of *Goniozus legneri*, a bethylid wasp. The longevity of honey-fed adults was typically more than twice that of starved wasps. Compared to similarly-aged starved wasps, honey feeding increased concentrations of common haemolymph sugars and amino acids in 3-day old and 7-day old wasps, and higher concentrations of stored lipid in 7-day old wasps. However, nutritional status did not affect the outcome of dyadic contests over host possession when both contestants were either 3 days or 7 days old. While contest outcome may be unaffected by diet, it remains possible that an enhancing effect of feeding on contest ability is matched by an effect of higher value being placed on winning by starved wasps.

Keywords: parasitoid diet, metabolomics, lipidomics, nuclear magnetic resonance, mass spectrometry, longevity, contest performance

## INTRODUCTION

Dyadic contests for possession of indivisible resources are of longstanding and ongoing interest as they occur across a wide range of animal taxa and have provided key examples for the game-theoretic approach to understanding behavioural evolution (Maynard Smith and Price, 1973; Hardy and Briffa, 2013). Two major predictors of contest outcomes are the competitive ability of individuals (resource holding potential, RHP) and the value (V) that contestants place on possession of the resource (Maynard Smith and Parker, 1976; Kokko, 2013): each of these may be affected by the nutritional status of the contestants. For example, individuals that have been well fed may be able to persist for longer during contests (RHP) due to their enhanced energy reserves (Poole, 1989; Kemp and Alcock, 2003; Martinez-Lendech et al., 2007; Briffa et al., 2013b)

while individuals with depleted reserves may compete more intensely for a contested resource, due to placing a higher value (V) on its exploitation (Riechert, 1998). Further, the availability of macronutrients (protein, fats and carbohydrates) may promote dominance-related behaviours (Davidson, 1997) while other metabolites may physiologically constrain contest behaviour. For instance, in hermit crabs and other crustaceans, metabolites related to energy reserves (glycogen and glucose) and aerobic capacity (lactic acid) as well as hormones (biogenic amines) influence contestant motivation and contest duration (Briffa and Sneddon, 2007; Briffa et al., 2013b).

In this paper we explore relationships between nutritional status and performance using the parasitoid wasp *Goniozus legneri* Gordh (Hymenoptera: Bethylidae). Female *G. legneri* parasitize host larvae that tunnel into crop tissues such as pistachio nuts, almonds and apples (Steffan et al., 2001). Attack by the wasp results in host paralysis and the female then remains in a restricted space with the host before oviposition approx. 24 h later. Additionally, *G. legneri* exhibits aggressive post-oviposition brood guarding behaviour, during which time a female may spend several days with each host both prior and post oviposition (Hardy et al., 2013). Because of the long time spent with a host, guarding females may encounter a series of conspecific intruders, with whom they compete for host possession (Bentley et al., 2009). A number of prior studies have shown that contest outcomes are affected by components of both RHP (contestant body size) and V (host size and nutritional quality, contestant age and reproductive status) (reviewed in Hardy et al., 2013). Effects of adult nutrition on *Goniozus* contest performance have, however, not been examined.

In common with other parasitoids (Ellers, 1996; Eijs et al., 1998; Casas et al., 2005; Jervis et al., 2008; Visser and Ellers, 2008), *G. legneri* adults lack *de novo* lipogenesis (Visser et al., 2010) and adults are thus limited to utilizing fat reserves accumulated throughout larval development. In many species of parasitoids, adults acquire nutrients from feeding on plant nectar, honey dew and host haemolymph (Jervis et al., 1993; Eijs et al., 1998; Giron et al., 2002; Visser and Ellers, 2008) and decline in lipid reserves can be slower when carbohydrate and lipid-rich diets are available (Ellers, 1996; Heimpel et al., 1997; Lee et al., 2004; Wäckers et al., 2006; Winkler et al., 2006; Desouhant et al., 2010; Ellers et al., 2011; Gómez et al., 2012; Harvey et al., 2012). Feeding on carbohydrates can also enhance adult parasitoid longevity (Heimpel et al., 1997; Pexton and Mayhew, 2002; Lee et al., 2004; Wäckers et al., 2006; Kapranas and Luck, 2008; Gómez et al., 2012) and preliminary laboratory observations (CJPS & AK pers. obs.) indicate that *G. legneri* fed with honey live longer than starved adults, suggesting that *G. legneri* can use dietary carbohydrates as a maintenance energy supply. Feeding on honey has the potential to counter the effect of the increased value, V, that older contestants place on host possession (Stockermans and Hardy, 2013, see also Humphries et al., 2006); if so, we might expect starved wasps to win contests against similarly aged but fed wasps. Conversely, honey-fed contestants may have enhanced contest abilities, RHP, due to higher energy reserves (Tsai et al., 2014); if so we might expect starved wasps to lose such contests.

To explore whether feeding on carbohydrates, such as honey, during the adult stage affects the subsequent energy reserves, longevity and contest performance of female *G. legneri*, we: (1) Employ a metabolomics approach (Snart et al., 2015; Kapranas et al., 2016) to assess how feeding on a carbohydrate-rich honey diet affects the concentrations of lipids, carbohydrates and other metabolites in individual parasitoids; (2) Compare the lifespans of starved wasps with those of wasps provided with carbohydrate-rich honey diet; (3) Stage contests for host possession between pairs of females, either both young or both old, in which one contestant was fed on honey and the other one was left unfed.

## MATERIALS AND METHODS

### Parasitoids and Hosts

*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. 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 contest studies (Goubault et al., 2006; Bentley et al., 2009; Stockermans and Hardy, 2013).

In Parts 1 and 3 we respectively examine the metabolomic state and contest behaviour of females aged 3 and 7 days. These ages were chosen because females typically disperse from their natal broods 3 days after eclosion from their cocoons (Hardy et al., 2000) and 7 days is the typical lifespan of unfed females in our laboratory conditions: see Part 2.

### Part 1: Effects of Feeding on Metabolomic Profiles

We employed a combined NMR and liquid chromatography–mass spectrometry (LC-MS) metabolomics approach (Kapranas et al., 2016) to assess simultaneously differences in individual lipids across multiple lipid classes and complementary information on polar metabolite concentrations, including haemolymph sugars, amino acids, and organic acids.

Adult female wasps that had emerged from their cocoons within 12 h were isolated in 0.5 ml Eppendorf vials. Half of the vials, chosen randomly, had been streaked on the inside wall with a raw honey droplet as a source of carbohydrates. Honey droplets were weighed to approximately 1 ± 0.1 mg prior to provision. The remaining vials contained no dietary provision (including water). Wasps were maintained in isolation for either 3 or 7 days after eclosion. Observations of the tube at the end of this period indicated that no wasp had successfully exhausted its honey droplet, as such honey provision was considered to have been ad infinitum. At the end of this time individual wasps were weighed to an accuracy of 0.01 mg and then snap frozen in liquid nitrogen and stored at −80°C prior to solvent extraction. There were 20 replicates (individual females) for each of the starved and honey-fed treatments. In the 7-day treatments, four starved wasps died prior to snap freezing; these replicates were excluded from further analysis.

Polar and non-polar extracts of intact, individual wasps were obtained using a modified Bligh and Dyer (1959) biphasic extraction approach (Kapranas et al., 2016). An additional reference sample was generated alongside wasp extracts by replacing the wasp with 1 mg of raw honey to aid spectral comparisons. Polar phases were transferred to a sterile 1.5 ml solvent resistant Eppendorf tube (Biopur) and non-polar phases were transferred to a sterile 2 ml borosilicate glass vial. Sample order was randomised prior to extraction and again before sample analysis. Solvent phases were dried and reconstituted for experimental analysis (Kapranas et al., 2016). 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 proton nuclear magnetic resonance ($^1$H NMR) spectroscopy, whereas reconstituted non-polar phases were analysed by LC-MS.

### NMR Spectroscopy

1D NMR spectra were generated from individual parasitoid polar phases using a Bruker Advance 800 MHz III spectrometer equipped with a 5 mm QCI cyroprobe. A spectral width of 13 ppm was utilised for spectral acquisition; the spectral signal was averaged over a total of 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 $D_2O$ signal was utilised for sample locking, with a secondary DSS signal (0.5 mM) 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. To further establish metabolite identity 2D NMR spectra were generated from a pooled extract of 20 wasps produced by the same method as previous extracts. These spectra were acquired using the method described by Kapranas et al. (2016); briefly a heteronuclear single-quantum correlation (HSQC) experiment was employed. Spectral signal was averaged over a range of 40–160 transients with between 400 and 512 points in t1 and 4,096 points in t2. Spectra covered a $^1$H width of 8484 Hz and a $^{13}$C width of 33339 Hz.

### LC-MS Analysis

Lipidomics analysis of non-polar extracts used an Accela LC coupled to a Thermo Scientific Exactive MS. An Ace Excel 2 Super C18 (2 µm particle size, 2.1 × 50 mm) column (Hichrom, UK) was maintained at 40°C throughout the analysis. The pooled quality control (QC) sample was analysed throughout the experimental timeframe to assess the quality of the analysis. LC gradient program and MS scan parameters were as described by Kapranas et al. (2016), briefly: ion range, $m/z$ 100–1,500; ESI voltage, 3,500 V; capillary temperature, 350°C; scan rate, 250 ms).

Further MS-MS analysis was undertaken to identify lipids which were found to change significantly between groups (see section on data analysis). For this purpose, extracts were pooled prior to LC-MS/MS analysis to improve sensitivity. Analysis was performed utilising an Accela LC coupled with an LTQ Velos Pro Dual-Pressure linear ion trap mass spectrometer (Thermo Fisher Scientific, USA) as described by Kapranas et al. (2016). Briefly, an ion range of $m/z$ 100–1,500 was monitored (ESI voltage, 3,000; capillary temperature, 275°C; scan rate, 50 ms) and a collision energy of 40 V was employed during fragmentation.

The quality of the lipidomics analysis was confirmed by assessing the relative standard deviations (RSD) of the peak areas and retention times of a range of key lipids in the pooled QC samples. An RSD acceptability threshold of <30% for a minimum of 70% of key ions was considered acceptable to establish LC-MS stability (see Supplementary Material).

## Part 2: Effects of Feeding on Longevity

A total of 100 newly emerged wasps were weighed to an accuracy of 0.01 mg then isolated in 0.5 ml Eppendorf vials, half of which contained a droplet of honey, as above. Eppendorf lids were pierced to provide ventilation. Wasps were then maintained in isolation until death. Each vial was inspected daily for signs of life (movement of the body in response to gentle shaking of the vial, movement of the mandibles or legs).

## Part 3: Effects of Feeding on Contest Outcomes

Newly emerged female wasps were weighed to an accuracy of 0.01 mg then isolated for either 3 or 7 days in 0.5 ml Eppendorf vials, half of which contained honey, as above. Pairs of females were chosen for staged contests on the basis of closely matching weight (the largest difference between a pair of contests 0.04 mg) and within each dyad one female had been fed and one starved. Further, females within dyads were the same age (either 3 days or 7 days old), were non-siblings that had emerged from different broods (Lizé et al., 2012) and were distinguishable by red or yellow acrylic paint dotted onto the dorsal surface of their thoraxes (Petersen and Hardy, 1996). There were 80 contests set up between pairs of females aged 3 days and 60 between pairs of 7-day old females.

Contests were staged in a plastic contest block consisting of three 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 host of known weight (range: 30.11–38.95 mg) that had been stung and paralysed by a female wasp (which was then discarded) was placed into the central chamber. Contestants were placed separately in the lateral chambers, with the barriers closed. Thus, neither contestant had encountered or taken possession of the host prior to the contest. We staged such "intruder-intruder" contest setups (following Lizé et al., 2012) rather than "owner-owner" or "owner-intruder" setups (Hardy et al., 2013) to prevent females from being able to feed on host haemolymph prior to the contest. After a 60 min acclimatisation period, the barriers were withdrawn sufficiently to connect the three chambers. Parasitoid contests were recorded from above for 60 min using a SONY HDR-CX 190 digital camcorder. A contest winner was defined as the individual

that remained within the immediate vicinity of host at the end of the observation period. Aggressive contest behaviour was additionally confirmed to have occurred by reviewing the video footage (Petersen and Hardy, 1996). For the purposes of this study, aggressive behaviour was considered to include any of the following categories: chasing, biting, stinging and fighting (mutual grappling), as outlined by Stockermans and Hardy (2013). Contest trials that did not result in aggressive behaviour were considered not to be clearly resolved.

## Data Analysis

Metabolomics data were analysed using a combination of multivariate and non-parametric data analysis as outlined in Kapranas et al. (2016). Briefly, generated NMR bins were baseline-corrected and normalised to wasp dry weight prior to multivariate analysis, whilst LC-MS data was framed using Sieve 2.0 (Thermofisher Scientific) and normalised to total chromatogram ion count. Principle components analysis (PCA) was employed to highlight any differences between dietary treatments, identified key frames/bins were evaluated by Kruskall-Wallis tests using GenStat v.15 (VSN International, Hemel Hempstead). Multiple-comparisons were accounted for by Bonferroni correction (Quinn and Keough, 2002); an adjusted *P*-value of 0.05 and an arbitrary fold change of >1.5 were used as significance between treatments. Lipid identities were assigned through comparisons with the Lipidmaps, Metlin, Lipid blast (Kind et al., 2013), and Human Metabolome databases (HMDB), along with previously generated LC-MS/MS datasets (Kapranas et al., 2016). NMR bins were identified through comparisons with 2D NMR spectra generated alongside 1D NMR spectra (Kapranas et al., 2016).

Longevity and contest outcome data were analysed using generalised linear models in GenStat. The effect of feeding on longevity was assessed using parametric cohort survival analysis (Crawley, 1993) which also evaluated age-dependent survival rates and the influence of female weight. Logistic analyses of covariance were used to explore influences on the probability of a contest being clearly resolved, fitting the age of the contestant females as a factor with two levels and the magnitude of the weight difference between contestants and the weight of the contested host as covariates. Dietary treatment was not included in this analysis as it was invariant across replicates: contests were always between one fed and one starved female. For resolved contests, influences on the probability of a given female (nominally, the red-marked individual) winning a contest were explored by fitting dietary treatment and contestant age as factors (each with two levels) and the size difference between contestants (calculated as red wasp weight minus yellow wasp weight: Goubault et al., 2006; Bentley et al., 2009; Lizé et al., 2012) as a covariate. Host weight was not included as an explanatory variable as the same host was contested by both females and neither had had the opportunity to assess its quality prior to the contest. All possible interactions between fitted main effects were included in the initial statistical models of contest outcomes. Simplification to the minimal adequate model was achieved by backward stepwise procedures (Crawley, 1993; Hardy and Field, 1998; Briffa, 2013a).

# RESULTS

## Part 1: Effects of Feeding on Metabolomic Profiles

### NMR Spectroscopy

Raw spectral comparisons of different treatment groups identified a consistent sugar signature between 4.15 and 3.2 ppm across the majority of both 3-day old and 7-day old honey-fed wasps. There was a high degree of overlap with the equivalent region of the spectra collected from raw honey samples (Figure S1); this resonance overlap is most likely due to detection of honey within the intestinal tract of honey-fed wasps. *G. legneri* were observed directly feeding on the provided honey throughout isolation, additionally females displayed extensive self-cleaning behaviour after accidental honey contact, allowing us to exclude the possibility that the observed honey resonance was a result of widespread surface contamination (CJPS & AK pers. obs.). Two 3-day old and three 7-day old honey-fed females did not display these resonances, moreover these samples clustered alongside starved wasps in PCA score plots, likely due to the lack of ingestion of honey by these individuals and thus these replicates were removed from further NMR and LC-MS data analysis.
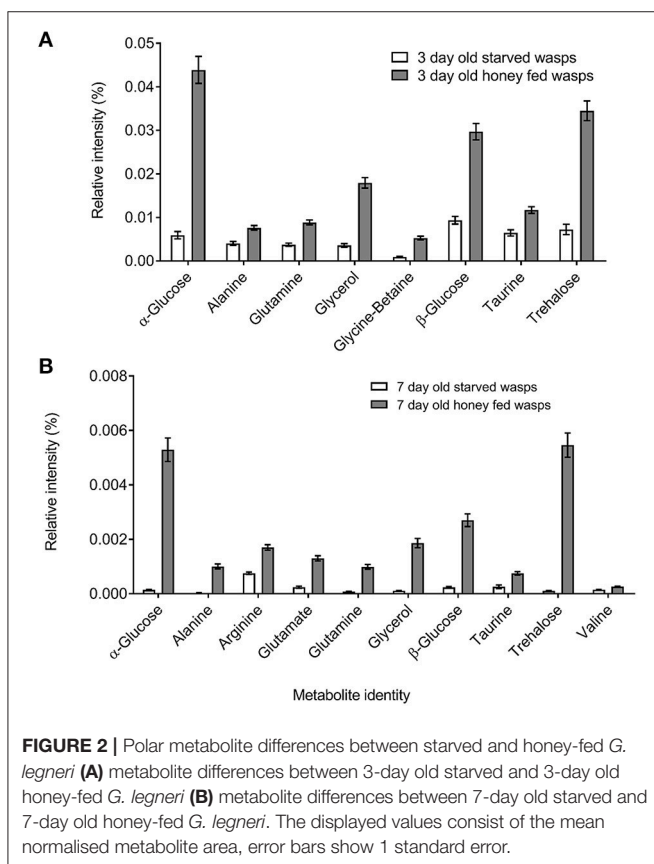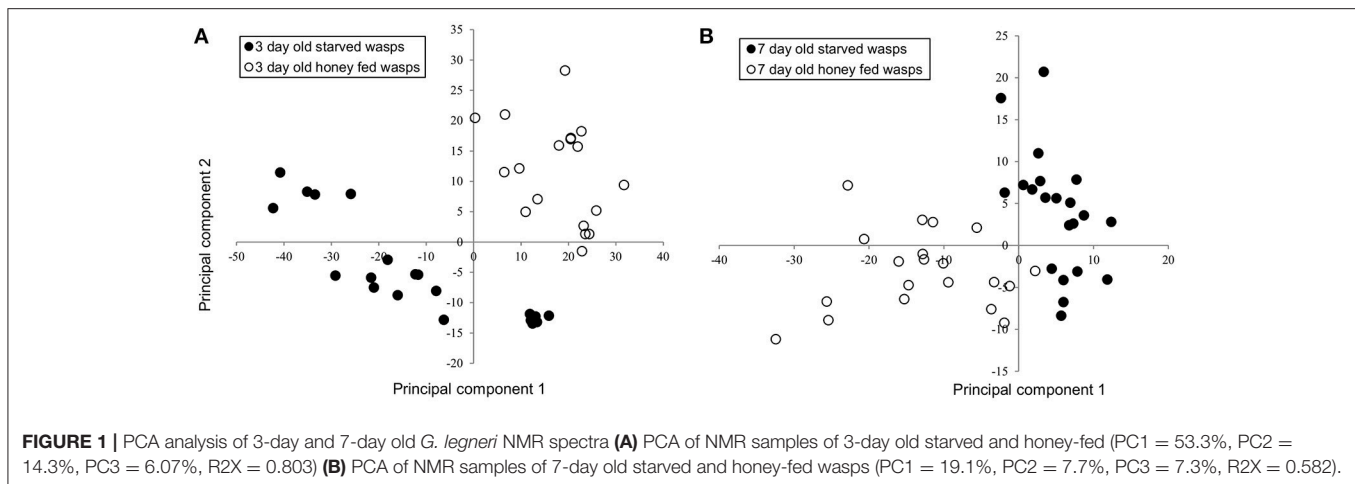
NMR PCA scores plots showed clear separation between honey-fed and starved treatments for both 3-day and 7-day old wasps (**Figure 1**). Validation of weighted scores plots for 3-day old wasps by Kruskal-Wallis tests (d.f. = 1, P < 0.05) found that 166 bins differed between treatments. Of these spectral identities were assignable to 44 bins, comprising of 15 unique metabolites associated with honey-fed wasps and 3 associated with starved wasps. Further validation of pooled bins associated with these metabolites by Kruskal-Wallis tests (d.f. = 1) found a total of 8 metabolites that were significantly elevated in honey-fed wasps (**Figure 2A**), whilst no metabolite associated with starved wasps met our criteria for significance (P < 0.05, fold change >1.5).

For 7-day old females, a total of 79 bins differed between treatments (d.f. = 1, P < 0.05). Of these spectral identities were assignable to 32 bins, comprising of 11 unique metabolites associated with honey-fed wasps. Further validation of pooled bins associated with these metabolites by Kruskal-Wallis tests (d.f. = 1) found a total of 10 metabolites that were significantly elevated in honey-fed wasps (**Figure 2B**). All bins associated with starved wasp treatments were identified as baseline fluctuations.

Bins associated with honey feeding for both 3-day and 7-day old wasps included common insect haemolymph sugars, along with amino acids that varied between 3-day and 7-day treatments. Further information regarding specific spectral identities is outlined in **Table 1**.

### LC-MS Analysis

PCA plots of LC-MS data indicated poor separation between the fed and starved treatments at 3 days (**Figure 3A**) but greater separation at 7 days (**Figure 3B**). Further validation of weighted PCA scores plots found that 63 ions differed significantly between 7-day old starved and fed wasps (Kruskal-Wallis tests, d.f. = 2,

**FIGURE 1 |** PCA analysis of 3-day and 7-day old *G. legneri* NMR spectra **(A)** PCA of NMR samples of 3-day old starved and honey-fed (PC1 = 53.3%, PC2 = 14.3%, PC3 = 6.07%, R2X = 0.803) **(B)** PCA of NMR samples of 7-day old starved and honey-fed wasps (PC1 = 19.1%, PC2 = 7.7%, PC3 = 7.3%, R2X = 0.582).



**FIGURE 2 |** 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 1 standard error.

$P < 0.05$, fold change > 1.5). Tentative identities were assigned to 40 ions through metabolite database comparisons and 37 of these were validated by LC-MS/MS; due to the presence of multiple adducts this corresponded to 24 unique lipids. Lipid identities comprised eight lysophospholipids, two diacylglycerides, one phospholipid and 13 triacylglycerides. All 24 lipids were elevated in honey-fed wasps (**Figure 4**). Specific lipid identities are detailed in **Table 2**.

## Part 2: Effects of Feeding on Longevity

Survival data were initially assessed by comparing two models of longevity: an exponential model (estimating the rate of mortality) and a Weibull model (additionally estimating variation in mortality rate over time). The Weibull model resulted in a significantly better fit ($G_1 = 58.3$, $P < 0.001$), indicating that the daily mortality rate increases with age. Adding the weights of individual wasp weights to the Weibull model showed that longevity was positively related to size ($G_1 = 4.91$, $P < 0.05$). Diet also affected longevity ($G_1 = 533.72$, $P < 0.001$): the majority of starved females died aged around seven days and none lived beyond 10 days. In contrast, honey-fed wasps lived at least 17 days and some lived beyond 30 days (**Figure 5**).

## Part 3: Effects of Feeding on Contest Outcomes

Out of the 80 contests between 3-day old wasps, 54 resulted in a clear contest outcome (a winner and a loser). For females aged 7 days, 31 of 60 contests were resolved. The probability of a contest being resolved was not significantly affected by host weight or the difference in weight between contestant wasps (**Table 3**). The age of the contestant wasps had a marginally non-significant effect on the probability of contest resolution ($G_1 = 3.56$, $P = 0.059$, %Deviance explained 1.93, **Table 3**): as probability estimates in logistic analyses are inexact (Crawley, 1993) and as over-reliance on $P$-values be generally inadvisable (Nakagawa and Cuthill, 2007), we interpret this as suggestive that contests between younger wasps are typically more likely to be resolved than those between older wasps.
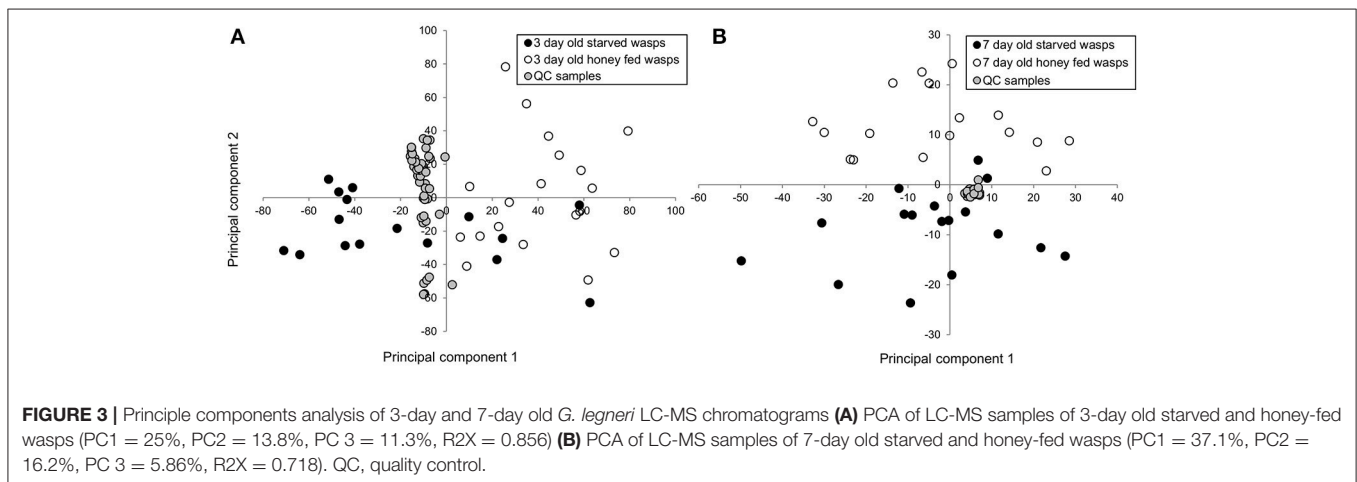
Among contests that were resolved, the probability of the focal (red marked) was winning was not significantly affected by diet, wasp age or the size difference between contestants (**Table 4**). Similar results were obtained whether analysing data from contests between 3-day old females and between females aged 7 days separately or in combination (**Table 4**). The proportion of contests won by the fed wasp was 0.42 ($\pm SE = 0.05$) overall and there were no significant effects of any of the measured variables on the probability of the contest being won by the wasp that had been fed honey.

**TABLE 1 |** Polar biomarkers.

| Common name | Formula | 1D Chemical shift (ppm) | 3 days | | | | 7 days | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | d.f. | H | P (B) | Fold change[a] | d.f. | H | P (B) | Fold change[a] |
| α-Glucose | $C_6H_{12}O_6$ | 3.45 (t), 3.52 (dd), 3.70 (t), 3.76 (m), 3.82 (ddd), 3.83 (m), 5.22 (d) | 1 | 27.69 | <0.01 | −7.42 | 1 | 23.18 | <0.01 | −37.93 |
| Alanine | $C_3H_7NO_2$ | 1.47 (d), 3.77 (q) | 1 | 16.28 | <0.01 | −1.89 | 1 | 23.18 | <0.01 | −30.94 |
| Arginine | $C_6H_{14}N_4O_2$ | 1.67 (m), 1.91 (m), 3.23 (t), 3.76 (t) | 1 | 3.18 | NS | NS | 1 | 22.82 | <0.01 | −2.26 |
| Glutamate | $C_5H_9NO_4$ | 2.04 (m) 2.12 (m), 2.34 (m), 3.75 (d) | 1 | 3.61 | NS | NS | 1 | 23.18 | <0.01 | −5.41 |
| Glutamine | $C_5H_{10}N_2O_3$ | 2.12 (m), 2.44 (m), 3.76 (t) | 1 | 22.71 | <0.01 | −2.37 | 1 | 23.18 | <0.01 | −13.00 |
| Glycerol | $C_3H_8O_3$ | 3.55 (m), 3.64 (m), 3.77 (tt) | 1 | 27.39 | <0.01 | −5.02 | 1 | 23.18 | <0.01 | −17.57 |
| Glycine-Betaine | $C_2H_5NO_2$ | 3.54 (s) | 1 | 27.69 | <0.01 | −5.67 | 1 | 6.91 | NS | NS |
| ß-Glucose | $C_6H_{12}O_6$ | 3.24 (dd), 3.4 (m), 3.47 (m), 3.74 (m), 3.88 (dd), 4.64 (d) | 1 | 27.08 | <0.01 | −3.18 | 1 | 23.18 | <0.01 | −11.53 |
| Taurine | $C_2H_7NO_3S$ | 3.23 (t), 3.39 (t) | 1 | 14.44 | <0.01 | −1.81 | 1 | 15.87 | <0.01 | −2.92 |
| Trehalose | $C_{12}H_{22}O_{11}$ | 3.44 (t), 3.64 (dd), 3.75 (m), 3.82 (m), 3.84 (m), 3.86 (m), 5.19 (d) | 1 | 26.18 | <0.01 | −4.76 | 1 | 23.18 | <0.01 | −51.82 |
| Valine | $C_5H_{11}NO_2$ | 0.99 (d), 1.04 (d), 2.34 (m), 3.6 (d) | 1 | 4.43 | NS | NS | 2 | 17.73 | <0.01 | −1.77 |

*Summary of polar biomarkers with tentative identities that significantly differ between starved wasps and honey-fed wasp extracts at 3 days old and 7 days old. NS, not significant for this treatment.*
*[a]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 3 |** Principle components analysis of 3-day and 7-day old *G. legneri* LC-MS chromatograms **(A)** PCA of LC-MS samples of 3-day old starved and honey-fed wasps (PC1 = 25%, PC2 = 13.8%, PC 3 = 11.3%, R2X = 0.856) **(B)** PCA of LC-MS samples of 7-day old starved and honey-fed wasps (PC1 = 37.1%, PC2 = 16.2%, PC 3 = 5.86%, R2X = 0.718). QC, quality control.
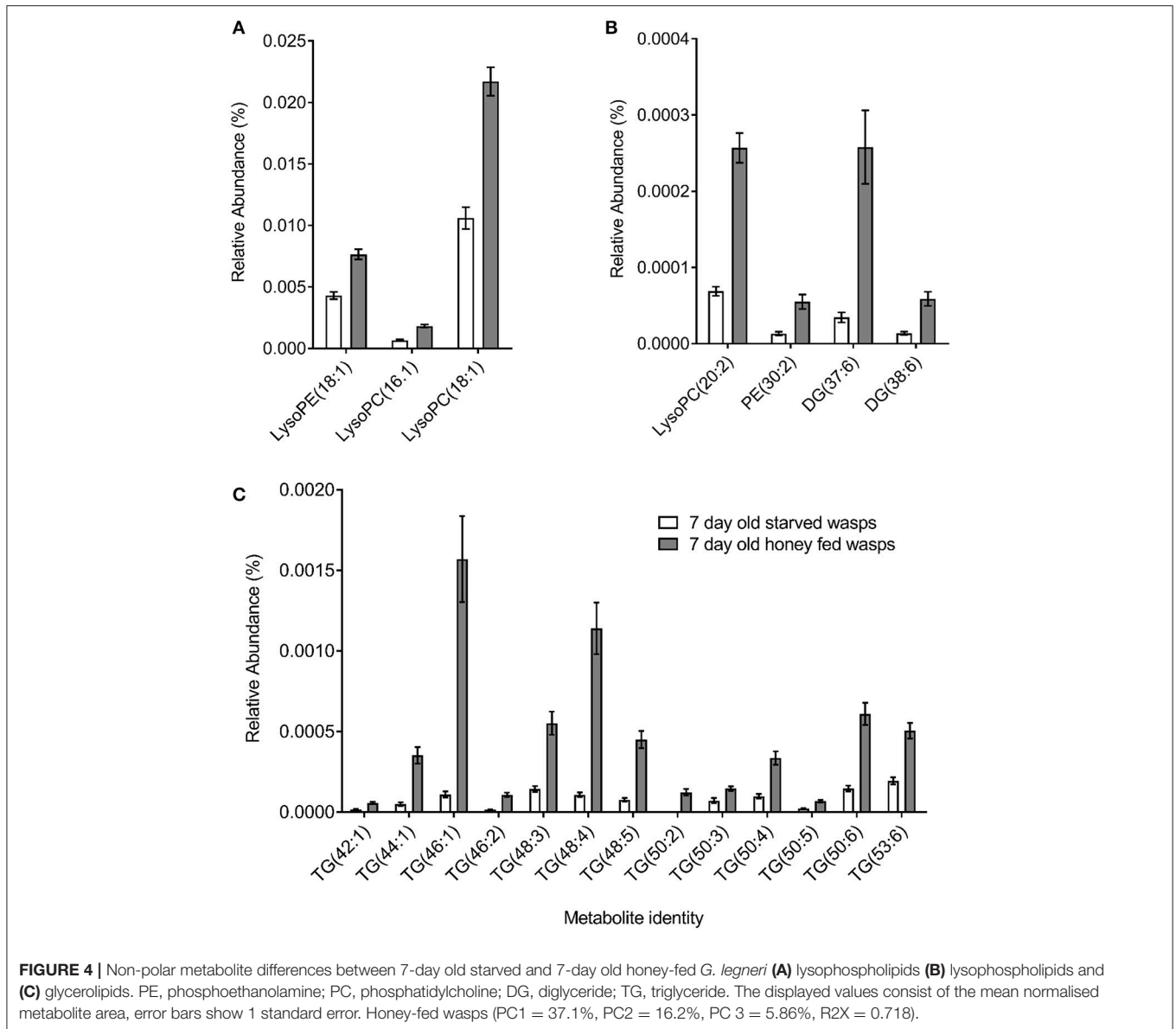
# DISCUSSION

While several previous studies have considered the metabolomic effects of dietary intake in a range of organisms (German et al., 2002, 2003; Davis and Milner, 2004; Zeisel et al., 2005; Wang et al., 2009; Ametaj et al., 2010; Primrose et al., 2011), ours is the first to consider dietary effects on the metabolome in the context of animal resource competition. Results from our dyadic contest setup indicate that dietary intake does not influence the outcome of contests between *G. legneri* females, while we also find that feeding enhances female longevity considerably and that a large number of polar and non-polar metabolites associated with energy storage and metabolism were elevated in older fed wasps. There are a number of possible implications of these findings for the

understanding of parasitoid behaviour and life-histories and we discuss these in turn along with suggestions for further investigations.

The energetic costs of animal contests have previously been examined in red deer (Clutton-Brock et al., 1979), cichlid fish (Neat et al., 1998a,b), hermit crabs (Briffa and Elwood, 2000, 2001, 2004, 2007), shore crabs (Sneddon et al., 1999), swimming crabs (Thorpe et al., 1994), and spiders (Prestwich, 1983a,b, 1988; DeCarvalho et al., 2004; Elwood and Prenter, 2013). 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). Although fed wasps contained higher levels of polar-metabolites associated with the rapid mobilization of energy (e.g., glucose, trehalose, glutamate, glycerol) than did

**FIGURE 4 |** Non-polar metabolite differences between 7-day old starved and 7-day old honey-fed *G. legneri* **(A)** lysophospholipids **(B)** lysophospholipids and **(C)** glycerolipids. PE, phosphoethanolamine; PC, phosphatidylcholine; DG, diglyceride; TG, triglyceride. The displayed values consist of the mean normalised metabolite area, error bars show 1 standard error. Honey-fed wasps (PC1 = 37.1%, PC2 = 16.2%, PC 3 = 5.86%, R2X = 0.718).

starved wasps, this does not appear to have enhanced their resource holding potentials (RHP); an affect that might be expected if contest interactions are energetically demanding. In a study on dyadic contests between male parasitoid wasps, Tasi et al. (2014) also found no effect of honey feeding on outcome when wasps were young (1 day old) but they did find that feeding by 4-day old males enhanced their contest success (although it was not possible to conclude whether this likely effect on RHP was operating via increased body mass after ingestion or improved energetic reserves). It may be that the *Goniozus* contest interactions we observed were too brief (typically <60 s of agonistic interactions, including just a few seconds of fully escalated fighting, Petersen and Hardy, 1996) for energetic constraints on RHP to become apparent. Experiments that force *G. legneri* females into more

sustained interactions, or that use bethylid species in which contests appear to be of naturally longer durations (Batchelor et al., 2005), may prove useful to explore this possibility further.
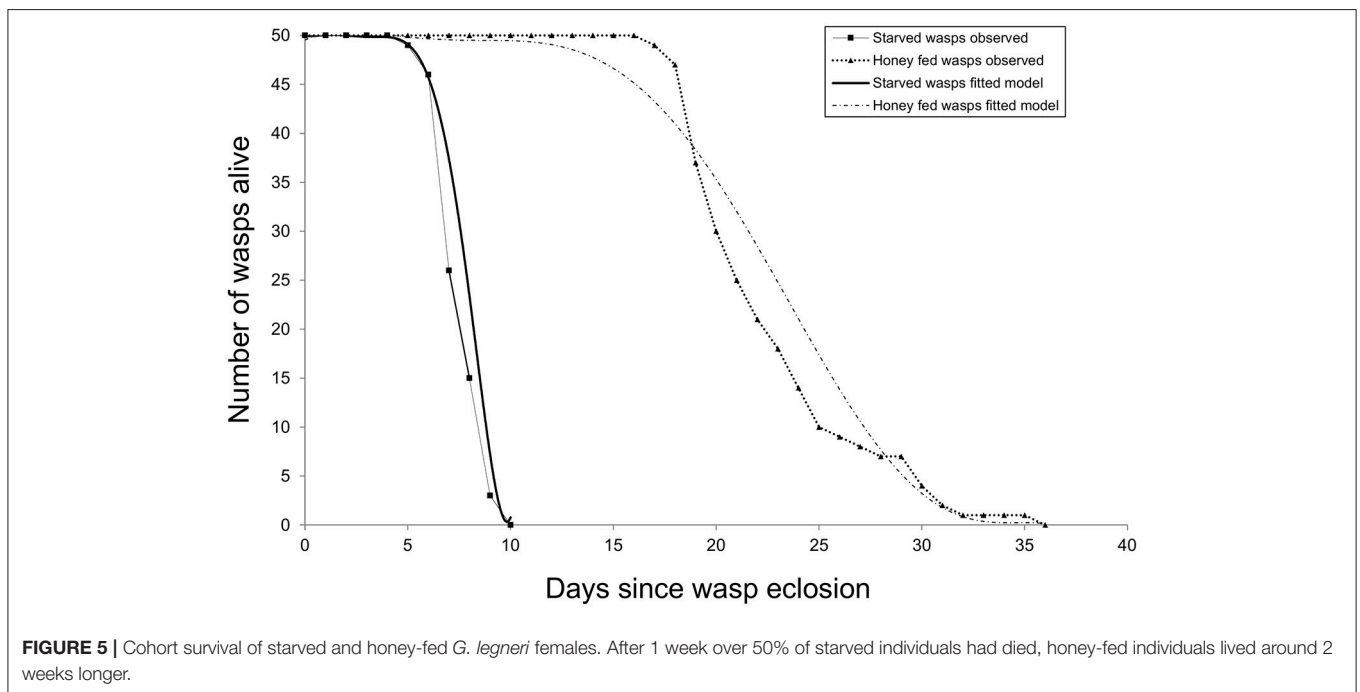
Our study also assessed the levels of non-polar metabolites, typically associated with long-term energy storage. As for the carbohydrates, the higher levels of glycerolipids (storage lipids) and diacylglycerides (the major form of fat mobilisation in insect haemolymph (Arrese and Soulages, 2010) and one of the primary energy sources for insect flight (Vogt et al., 2000; Harrison and Fewell, 2002) of 7-day old honey-fed wasps suggest that these contestants could have greater available energy and thus higher RHPs than their starved opponents. However, starved wasps could potentially place a greater value (V) on winning possession of the contested host than would honey-fed wasps,

**TABLE 2 |** Non-polar biomarkers.

| M/Z | Average RT (min) | Adduct | Common name | Formula | d.f. | H | P (B) | Fold change[a] | Mass error (ppm) |
|---|---|---|---|---|---|---|---|---|---|
| 452.277 | 1.51 | M+Na | LysoPE(0:0/16:1) | $C_{21}H_{44}NO_7P$ | 1 | 21.309 | <0.01 | −2.26 | 0.737 |
| 474.263 | 1.50 | M+H | LysoPE(18:2/0:0) | $C_{23}H_{42}NO_7P$ | 1 | 21.901 | <0.01 | −2.25 | 0.387 |
| 476.277 | 1.32 | M+H, M+NA | LysoPE(0:0/18:3) | $C_{23}H_{42}NO_7P$ | 1 | 17.393 | <0.05 | −1.85 | 1.263 |
| 480.308 | 2.01 | M+H, M+Na | LysoPE(18:1) | $C_{23}H_{46}NO_7P$ | 1 | 19.023 | <0.01 | −1.78 | 0.425 |
| 494.324 | 1.44 | M+H | LysoPC(16.1) | $C_{24}H_{48}NO_7P$ | 1 | 21.016 | <0.01 | −2.70 | 0.115 |
| 522.355 | 1.98 | M+H, M+Na | LysoPC(0:0/18:1) | $C_{26}H_{52}NO_7P$ | 1 | 21.309 | <0.01 | −2.06 | 0.416 |
| 540.305 | 1.31 | M+Na | LysoPC(0:0/18:3) | $C_{26}H_{48}NO_7P$ | 1 | 19.023 | <0.01 | −1.98 | 1.057 |
| 548.37 | 2.09 | M+H | LysoPC(20:2/0:0) | $C_{28}H_{54}NO_7P$ | 1 | 24.665 | <0.01 | −3.74 | 1.066 |
| 659.522 | 5.76 | M+CH₃OH+H | DG(37:6) | $C_{40}H_{66}O_5$ | 1 | 17.263 | <0.05 | −7.49 | 2.514 |
| 682.448 | 6.32 | M+Na | PE(30:2) | $C_{35}H_{66}NO_8P$ | 1 | 15.333 | <0.05 | −4.20 | 0.8 |
| 687.553 | 6.33 | M+CH₃OH+H | DG(38:6) | $C_{42}H_{70}O_5$ | 1 | 15.836 | <0.05 | −4.34 | 2.814 |
| 743.616 | 7.37 | M+Na | TG(42:1) | $C_{45}H_{84}O_6$ | 1 | 16.347 | <0.05 | −3.41 | 0.008 |
| 766.691 | 7.88 | M+NH₄, M+Na, M+K | TG(44:1) | $C_{47}H_{88}O_6$ | 1 | 21.309 | <0.01 | −7.26 | 0.914 |
| 769.631 | 7.21 | M+Na | TG(44:2) | $C_{47}H_{86}O_6$ | 1 | 17.019 | <0.05 | −6.06 | 0.658 |
| 792.707 | 7.87 | M+NH₄ | TG(42:2) | $C_{49}H_{90}O_6$ | 1 | 22.501 | <0.01 | −6.06 | 0.564 |
| 794.722 | 8.98 | M+NH₄, M+Na, M+K | TG(46:1) | $C_{49}H_{92}O_6$ | 1 | 20.725 | <0.01 | −14.28 | 1.214 |
| 797.663 | 7.97 | M+Na | TG(46:2) | $C_{49}H_{90}O_6$ | 1 | 20.15 | <0.01 | −5.76 | 0.041 |
| 818.723 | 7.86 | M+NH₄, M+Na | TG(48:3) | $C_{51}H_{92}O_6$ | 1 | 19.865 | <0.01 | −3.89 | 0.214 |
| 820.739 | 8.94 | M+NH₄, M+Na, M+K | TG(48:2) | $C_{51}H_{94}O_6$ | 1 | 18.746 | <0.05 | −6.56 | 0.136 |
| 844.739 | 8.19 | M+NH₄, M+Na, M+K | TG(50:4) | $C_{53}H_{94}O_6$ | 1 | 16.091 | <0.05 | −3.76 | 0.136 |
| 846.753 | 8.92 | M+NH₄, M+K | TG(50:3) | $C_{53}H_{96}O_6$ | 1 | 16.091 | <0.05 | −3.92 | 1.514 |
| 849.694 | 7.93 | M+NH₄ | TG(50:2) | $C_{53}H_{98}O_6$ | 1 | 17.928 | <0.05 | −3.47 | 0.259 |
| 863.653 | 7.27 | M+K | TG(50:5) | $C_{53}H_{92}O_6$ | 1 | 18.199 | <0.05 | −3.09 | 0.451 |
| 887.781 | 8.92 | M+K | TG(50:0) | $C_{55}H_{108}O_5$ | 1 | 17.659 | <0.05 | −2.59 | 1.835 |

*Summary of non-polar biomarkers with tentative identities that significantly differ between 7-day old honey-fed and starved wasp extracts. PE, phosphoethanolamine; PC, phosphatidylcholine; DG, diacylglyceride; TG, triacylglyceride.*
[a]*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 5 |** Cohort survival of starved and honey-fed *G. legneri* females. After 1 week over 50% of starved individuals had died, honey-fed individuals lived around 2 weeks longer.

due to their lower life-expectancy (Humphries et al., 2006; Stockermans and Hardy, 2013) and/or the opportunity to host-feed, whereas fed wasps may perceive a host as having lower value due to their more abundant reserves. Thus, it remains possible that diet influences *G. legneri* contests between starved and fed wasps, but via opposing and coincidentally equal effects.

Further investigations could assess individual contest behaviours, rather than contest outcome. Older *G. legneri* females behave more aggressively during contests (Stockermans and Hardy, 2013), due to RV increasing as females age; thus

increased aggression in contests between two starved wasps compared to two honey-fed wasps may similarly indicate that starved wasps value hosts more greatly, while higher aggression among fed wasps may indicate that diet affects RHP, and both influences may interact with female age. Preliminary observations on 3-day old female *G. legneri*, however, suggest that feeding does not affect behavioural interactions during contests (A.K. unpublished data). In other words, starved wasps could be on one hand weaker, due to lack of energetic reserves (lower RHP), but may on the other hand be bolder due to their limited future reproductive opportunities, and as a result fight more fiercely than honey fed wasps (higher RV). Similarly, Tasi et al. (2014) found no effect of honey feeding on the degree of agonism during male-male contests, despite feeding by older males affecting the outcome of contests.

While we have found no overall relationships between metabolome of wasps and contest outcomes, we have found relationships between wasp age and metabolomic state. At 3 days, the lipid profiles of honey-fed and starved wasps were similar but differed clearly among 7-day old wasps. This indicates that effects of dietary provision only become important as individuals approach the life-expectancy of unfed wasps. The greatly increased longevity of honey-fed wasps compared to starved wasps suggests that fed wasps are capable of preserving long-term lipid stores by using dietary carbohydrates as a maintenance source for energy metabolism. However, the presence of higher levels of diacylglycerides in 7-day old honey-fed females than in starved females suggests that carbohydrate-rich dietary feeding did not entirely supress lipid reserve mobilisation throughout

**TABLE 3 |** Potential influences on contest resolution.

| Source | d.f. | G | P |
|---|---|---|---|
| Wasp age | 1 | 3.56 | 0.059[a] |
| Contestant weight difference | 1 | 0.19 | 0.662 |
| Host weight | 1 | 0.00 | 0.991 |
| Wasp age × host weight | 1 | 0.17 | 0.684 |
| Wasp age × contestant weight difference | 1 | 1.32 | 0.250 |
| Contestant weight difference × host weight | 1 | 2.21 | 0.137 |
| Wasp age × contestant weight difference × Host weight | 1 | 0.00 | 0.962 |
| Residual | 130 | | |
| Total | 137 | | |

*Results are from backwards stepwise logistic ANCOVAs on the probability of a contest being clearly resolved. Note that contestant weight differences (heaviest wasp–lightest wasp) were experimentally minimised.*
[a]*Note marginal non-significance: see main text.*

**TABLE 4 |** Potential influences on outcomes of resolved contests.

| | 3 days | | | 7 days | | |
|---|---|---|---|---|---|---|
| Source | d.f. | G | P | d.f. | G | P |
| **(A) RESOLVED CONTESTS ANALYSES SEPARATELY BY CONTESTANT AGE** | | | | | | |
| Red wasp diet | 1 | 1.77 | 0.183 | 1 | 0.12 | 0.727 |
| Contestant weight difference | 1 | 0.18 | 0.670 | 1 | 0.32 | 0.569 |
| Red wasp diet × contestant weight difference | 1 | 1.21 | 0.270 | 1 | 0.04 | 0.837 |
| Residual | 50 | | | 27 | | |
| Total | 53 | | | 30 | | |
| **(B) ALL RESOLVED CONTESTS** | | | | | | |
| Source | | | | d.f. | G | P |
| Red wasp diet | | | | 1 | 1.75 | 0.186 |
| Wasp age | | | | 1 | 0.33 | 0.568 |
| Contestant weight difference | | | | 1 | 0.15 | 0.697 |
| Red wasp diet × wasp age | | | | 1 | 0.00 | 0.964 |
| Red wasp diet × contestant weight difference | | | | 1 | 1.44 | 0.229 |
| Contestant weight difference × wasp age | | | | 1 | 0.01 | 0.917 |
| Red wasp diet × contestant weight difference × wasp age | | | | 1 | 0.11 | 0.735 |
| Residual | | | | 77 | | |
| Total | | | | 84 | | |

*Results are from backwards stepwise logistic ANCOVAs on the probability of a focal (red painted) wasp winning. Note that contestant weight differences (red wasp–yellow wasp) were experimentally minimised.*

the experimental time-frame. This result suggests that lipid store deterioration is merely delayed, rather than prevented, in honey-fed wasps.

Parasitoid fecundity has not been assessed in this study, but it is likely that the use of dietary carbohydrates to maintain vital body processes would allow greater allocation of lipid reserves toward egg production and maturation, as demonstrated by a number of studies on other parasitoid species (Jervis and Kidd, 1986; Heimpel et al., 1997; Winkler et al., 2006; Kapranas and Luck, 2008; Harvey et al., 2012). Indeed, it has already been shown for the congener *Goniozus nephantidis* that, as adult wasps aged, the egg loads of honey-fed females rose higher and declined later than those of starved females (Stokkebo and Hardy, 2000). Provision of such a diet could also conceivably aid parasitoid dispersion, as two of the three main circulating metabolites used during flight (trehalose and lipids) were found to be elevated in wasps that had consumed a honey diet (Beenakkers et al., 1984; Vogt et al., 2000; Harrison and Fewell, 2002). As such it is plausible that a parasitoid with access to a carbohydrate rich diet could exploit a larger field range in search of potential host larvae, and additionally achieve greater exploitation through decreased egg resorption.

Although it has antecedents in contest studies that focussed on a small sub-set of metabolites (e.g., Briffa and Elwood, 2007) and in studies of the effect of feeding on parasitoid contest behaviour without the associated metabolic analysis (Tsai et al., 2014), ours is the first study to explicitly couple the untargeted metabolomics approach (Snart et al., 2015) with the study of dyadic animal contests (Hardy and Briffa, 2013). Despite the small size of the organisms involved, this methodology is capable of reliably producing identifiable NMR spectra associated with different diet treatments (Kapranas et al., 2016) and as such has potential for application to a wide range of studies of the behaviour and ecology of small insects and other organisms. The approach can identify experimental outliers, in this instance individuals that had not ingested the provided diet were detected by absence of the highly conserved honey signal in the NMR spectra of fed wasps. However, as the whole insect is sampled, the method cannot distinguish between resonances generated from insect haemolymph from those generated from diet within an insect's digestive tract (similarly, Tsai et al., 2014 were unable to determine whether mass gained by honey-fed wasps indicated metabolism or simply ingestion). Hence, further experimentation, such as our longevity study, is required to confirm that parasitoids are capable of utilising a particular dietary resource other than merely ingesting it.

## CONCLUSIONS

This is the first study to explicitly couple the untargeted metabolomics approach with the study of dyadic animal contests. Provision of a carbohydrate-rich diet affected the metabolomic state of *G. legneri* and enhanced its longevity but did not affect its performance in dyadic contests. While honey-fed wasps had higher levels of energy rich metabolites, these may not have been sufficient to enhance RHP substantially. Alternatively, an effective increase in RHP among fed females may have been countered by an increase in the host's perceived value, V, by starved competitors.

## ETHICS STATEMENT

The research reported here using insects is in full compliance with the principles of Replacement, Reduction and Refinement as set by ARRIVE (Animal Research: Reporting of *in vivo* Experiments) guidelines and enforced by the University of Nottingham Animal Welfare and Ethical Review Body (AWERB).

## AUTHOR CONTRIBUTIONS

CS, AK, IH, and DB: Conceived and designed the experiments; CS, AK, IH and HW: Performed the experiments; CS, AK, IH, and HW: Analysed the data; IH, DB, and HW: Provided resources; CS, AK, DB, and IH: Wrote the paper; All authors contributed to final draft approval.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00012/full#supplementary-material

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