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A host-feeding wasp shares several features of nitrogen management with blood-feeding mosquitoes

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ABSTRACT

Adult feeding on hosts is common among parasitic wasps. The ingested host fluid is rich in nutrients, especially proteins. A study on *Eupelmus vuilleti* (Hymenoptera: Eupelmidae), a host-feeding parasitoid of larvae of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae), showed that the carbohydrates (maybe lipids) but not proteins, gained from host feeding accounted for the increased egg production. Thus, host protein is probably utilized for general adult metabolism, allowing conservation of carbohydrate and/or lipid resources for direct allocation to oocytes. In that case, there should be increased N excretion by female parasitoids. To test this, we studied the dynamics of excretion in *E. vuilleti* with and without host exposure. The aim of this work was threefold: (i) to identify the major N-containing compounds in adult excreta, (ii) to assess whether protein consumption during host feeding increased the amount of N excreted, and (iii), if so, to compare the increase in N excreted with the amount taken in during a single host feeding. We found that uric acid is the predominant N-containing metabolite in excreta, although small quantities of urea and traces of allantoin were also found. A calculation of the N budget showed that the extra quantity of N excreted following a host meal corresponds to the quantity ingested, confirming that host-feeding in this species offers little or no net **quantitative** benefit in N allocation to oocytes, although the allocation of specific amino acids from host feeding cannot be discounted. Interestingly, host-feeding in parasitoids appears analogous to vertebrate blood-feeding in mosquitoes, both in terms of the N-containing compounds excreted and the offset of acquired N to metabolism, rather than to oocytes. Further comparative and detailed investigations of N excretion in insects living on other N-rich fluids might establish further metabolic commonalities.

1. Introduction

Parasitoid wasps (Hymenoptera) are studied predominantly in two contexts: behavioural ecology, especially the direct links among a female's egg laying decisions, foraging behaviour and fitness (Godfray, 1994; Wajnberg et al., 2008), and their efficacy in biological control (Jervis, 2012; Heimpel and Mills, 2017). One particular area of research, covering both contexts that has received much attention is the nutritional ecology of adults, especially the respective contributions of larval (capital) and adult (income) feeding to female fitness (Rivero et al., 2001; Casas et al., 2005; Jervis et al., 2008; Strand and Casas, 2008). Many adult female parasitoid wasps wound hosts they encounter in order to feed on the resultant fluid. Such feeding can be fatal to a host, so females often oviposit and feed on different hosts, although concurrent host-feeding and oviposition on the same host is known (Jervis, 2012). The fluid ingested by host-feeding wasps is largely similar to host haemolymph, being abundant in protein and carbohydrate, with lesser amounts of lipid (Giron et al., 2002). Numerous studies have characterized the consequence of host-feeding phenomenologically; i.e., by quantifying extra eggs produced, or extra longevity, resulting from host-feeding events (e.g., Godfray, 1994; Quicke, 1997; Jervis, 2012; Fischbein et al., 2016; Heimpel and Mills, 2017). In contrast, few studies have investigated the physiology of host feeding (Rivero and Casas, 1999; Giron et al., 2002), examining what nutrients are acquired and how they influence metabolism and reproduction.

The most comprehensive understanding of the interplay between capital and income nutrient reserves and their contributions to an energy budget over the entire adult lifetime of a parasitoid wasp is that for *Eupelmus vuilleti* (Hymenoptera: Eupelmidae) a tropical solitary, host-feeding synovigenic ectoparasitoid of third- and fourth-instar larvae of

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Callosobruchus maculatus (F.) (Coleoptera: Bruchidae) (Casas et al., 2005, 2015; Richard and Casas, 2009, partially summarized in Jervis et al., 2008). Female *E. vuilleti* host feed throughout their life and use nutrients from this feeding to increase both their longevity and egg production. Somewhat surprisingly, ingested carbohydrates appear to be wholly sufficient to explain the increases in longevity and egg investment, even though substantial N is acquired in the form of proteins and amino acids (Casas et al., 2005). Since there is no indication that protein content of females increases after host feeding or that other N-containing compounds (e.g., uric acid) are retained, this suggests that the N acquired from host feeding may be metabolized for energy, with concomitant increases in excretion of N metabolites.

Most terrestrial insects studied excrete uric acid as their primary N-containing metabolite (Bursell, 1967; O'Donnell and Donini, 2017). Uricotellic excretion in insects is largely thought to be an adaptation to facilitate water conservation, since uric acid is barely soluble and its ready crystallization in the hindgut means that less water is needed for its excretion than for water-soluble products such as urea (Klowden, 2013). Moreover, the molecule is an effective vehicle for N excretion, as it contains 4 N atoms, as opposed to the two in a molecule of urea. However, while uric acid is typically the major N-containing product excreted by most terrestrial insects, it is not the only one, with many insects also excreting small amounts of allantoin, allantoic acid or urea (Bursell, 1967). Some insects even predominantly excrete allantoin and allantoic acid, both metabolites of uric acid along the urolytic pathway. The presence of small amounts of urea in excreta of terrestrial insects is interesting. In most cases, it is thought to result from the hydrolysis of arginine, but a recent study on the mosquito *Aedes aegypti* demonstrated that it was formed from uric acid through a functional amphibian-like uricolytic pathway; i.e., via allantoic acid (Scaraffia et al., 2008; Horvath et al., 2018).

To learn more about N excretion in parasitoids and its relation to host-feeding, we undertook a study on female adult *E. vuilleti*. Our aims were threefold. First, to identify the nature of the different N compounds in the excreta. Second, to assess whether protein consumption during host feeding actually increases the amount of N-excreted and thereby test the hypothesis that protein is utilized for general adult metabolism. Third, if protein is metabolized, then, to compare the increase in N excreted with the amount acquired from a single host feed.

2. Material and methods

2.1. Experimental procedures

Eupelmus vuilleti were reared on third- and fourth-instar *C. maculatus*, themselves fed on *Vigna unguiculata* (Fabaceae) seeds in a controlled environment room, with a 13:11 L:D photoperiod, a temperature regime of 33 °C (light) and 23 °C (dark), and constant 75% R.H.

Individual adult females were isolated soon after eclosion and weighed. To reduce variation in excreta amounts, only individuals with a weight between 1.8 and 2.3 mg were used in the experiment. We designed the treatments for the experiment in order to quantify over time the excreta resulting from a single complete host-feeding event, with samples containing sufficient excreta for chemical analysis (Fig. 1). For this, we started with groups of 5 females for replicates of each treatment to ensure sufficient excreta over the course of the experiment for the chemical analysis. Since we didn't want additional (to host feeding) carbohydrate for females to feed on, females were given access only to water outside of contact with hosts. This resulted in higher mortality than experienced in previous studies, such that replicates were sacrificed during the experiment to top up the number of live females (to or close to 5) in remaining replicate groups. This mixing of groups over time precluded analysis of data by mixed effect models.

In the first treatment, we starved females from the beginning of their adult life and followed the dynamics of N excretion. To collect the daily excreta, groups of five females (n = 5) were placed in 1.5 ml microcentrifuge tubes, along with a small ball of cotton wool soaked in purified water at the bottom of each tube. The top of the tube was covered with parafilm with small puncture holes to allow air circulation. Each day, females were carefully transferred to a new microcentrifuge tube, and the old tube and cotton ball, containing the excreta produced over the past 24 h, placed at −18 °C until shipped to the US for extraction and analysis (see below).

Because females need to learn how to "handle" hosts before they host feed reproducibly, we had to expose females to hosts for two days after eclosion, during which some intermittent host feeding takes place (Casas et al., 2005). To allow for any feeding during this period, we needed to use two treatments: one (HF1) in which females were only allowed access to hosts during the first two days after eclosion and the other (HF2) in which females were allowed access to hosts during the first 48 h after eclosion, then isolated from hosts for 48 h, before being allowed to feed (as an experienced feeding female) once on a host. For these treatments (both HF1 and HF2), females were allowed to experience hosts for 48 h after eclosion by placing each female in a 9 cm diameter Petri dish along with 5 cowpea seeds infested with hosts and a cotton ball with water. After 48 h, females were removed. In the HF1 treatment, groups of five females were placed in centrifuge tubes along with a saturated cotton ball and were sampled for excreta each day as for the starved treatment. In the HF2 treatment, females were left for a further 48 h (with access to water) before being allowed to feed on a host, after which they were placed in groups of five in centrifuge tubes and sampled as per the other treatments. Host feeding in this treatment was confirmed by placing the female and a host larva, inside a perforated gelatin capsule, in a Petri dish. The gelatin capsule allows observation of host feeding but precludes oviposition (Gauthier and Monge, 1999). Only females observed feeding on hosts were used in the experiment.

2.2. Excreta analyses

Prior to the experiment, we assumed uric acid to be the major N-containing metabolite in the excreta. Consequently, we designed the extraction and analytical procedures for uric acid. Although this assumption turned out to be correct in most samples (see Results), we also found an appreciable quantity of another N-containing compound, which we subsequently identified as silylated urea, based on the similarity of its mass spectrum (NIST 2014) and retention time with that of a silylated authentic sample of urea (Sigma-Aldrich, St Louis, MO). We also found trace amounts of another N-containing compound, tentatively identified as allantoin (Chen et al, 1998; based on mass spectral similarity, although no standard was available to confirm its assignment). Since we did not include an appropriate internal standard for urea prior to extraction and analysis of the samples of the urea, we used an external standard approach utilizing dilutions of authentic urea relative to the fixed amount of isotopically labeled uric acid used in the sample. However, this was carried out several months after the samples were analyzed. Due to possible drift in the calibration of the mass spectrometer over this period, we emphasize that the derived urea values should be taken as indicative rather than precise. We did not attempt to quantify the tentatively identified allantoin, as we did not have an authentic sample available and its level in samples was always either undetectable or at very low ion currents. We were unable to identify any allantoic acid in the mass chromatograms.

For the extraction and analysis of excreta, we modified an isotopic internal standard method developed for analyzing levels in human urine (Chen et al, 1998). Briefly, 75 µl of a 300 µM solution of a [1,3-$^{15}N_2$]uric acid (98% isotopic purity, Cambridge Isotope Laboratories Inc., Tewksbury, MA) in 0.25 mM KOH was added to an Eppendorf tube in which excreta had been deposited. Using clean forceps, the water-soaked cotton ball was rubbed around the inside surface of the tube and cap before the tube (and cotton ball) was vortexed for 30 s. The solution was decanted using a pipettor, squeezing as much

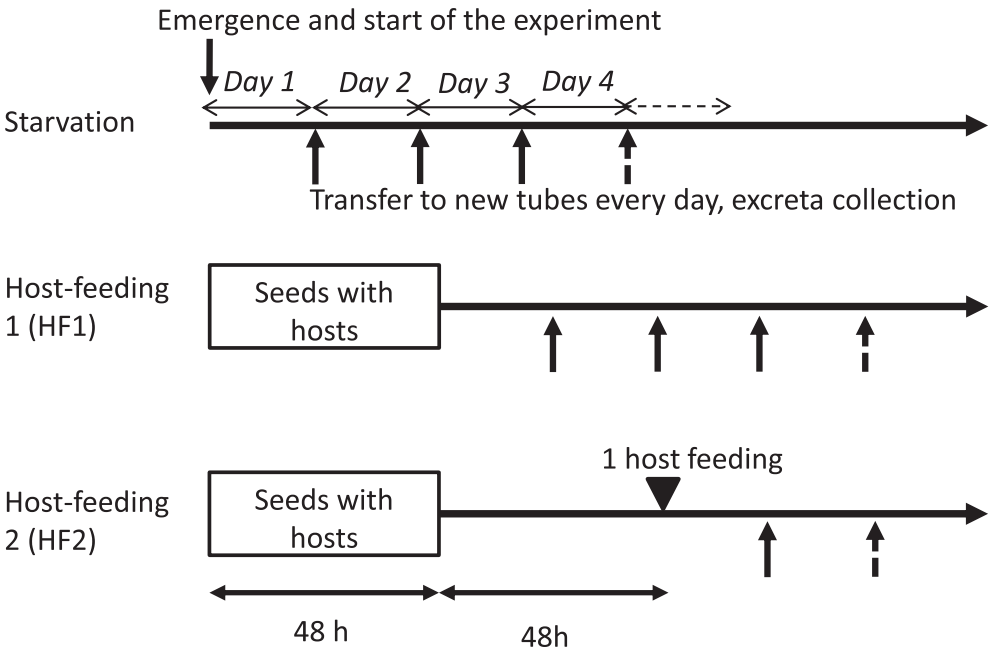

Fig. 1. Experimental set-up. In the starvation treatment, *Eupelmus vuilleti* females were deprived of all food after emergence. In HF1, females were given access to hosts for the 48 h following emergence and then deprived of food, whereas in HF2, females were given the same access to hosts for the 48 h following emergence, then deprived of food for 48 h, before being allowed to feed once on a host (at 96 h).

liquid from the cotton ball as possible and placed in a 2 ml glass autosampler vial. Then, a 75 µl aliquot of 0.25 mM KOH was added to the tube, which was vortexed, and the liquid decanted, as for the internal standard solution, into the vial. This was repeated a further time, yielding roughly 225 µl of liquid (including any remaining water for feeding on the cotton ball). In a preliminary method development, analyzing each of the three 75 ml aliquots, we found no detectable traces of urate in the third aliquot. Water was removed under vacuum in a Centrivap (Labconco, Kansas City, KS) and the residue reacted with 50 µl of a 1:1 mixture of tert-butyldimethylsilyltrifluromethanesulfonate (TBDMS; TCI America, Portland, OR): dimethyl formamide (DMF) at 120 °C for 20 min. Following reaction, excess TBDMS and DMF were evaporated under a stream of nitrogen, and the remaining TBDMS derivatives dissolved in ca. 50 µl of *n*-heptane.

The TBDMS derivatives were analyzed by coupled gas chromatography/mass spectrometry on a Hewlett-Packard 5890/5972 system, equipped with a 30 m × 0.25 mm i.d., ZB1 capillary column (Phenomenex, Torrance, CA) and a splitless injector and utilizing helium as carrier gas at a constant flow of 1.5 ml·min^{-1}. The column oven was temperature programmed at 150 °C (initial hold of 1 min) to 280° at 15 °C·min^{-1}, then to 320 °C at 20 °C·min^{-1}, and held for 15 min. The MS was operated in the electron impact ionization mode in full scan (from *m/z* 50 to 650), at 70 eV.

For quantification of urate, we extracted the characteristic *m/z* 567 (unlabeled) and 569 ($^{15}N_2$-labeled, internal standard) and calculated the number of micromoles of natural urate (i.e., from the excreta of the insects) after allowing for the contribution (using natural isotopic abundances) of the 567 (unlabeled) ion spectrum to the 569 (labeled) ion and the 2% isotopic impurity in the internal standard. The amount of uric acid is expressed as micrograms per female per day.

To roughly quantify the amounts of urea in the samples, we prepared a series of concentrations of urea in water and then added 75 µl ml of each of these to 75 µl of a 300 µM solution of a [1,3-$^{15}N_2$] uric acid to create molar ratios of urea: urate of 2:1, 1:1, 0.5:1 and 0.1:1. After TBDMS derivatization, we analyzed duplicate samples of each and extracted and integrated *m/z* 231 (for bis-TBDMS urea) and *m/z* 569 (for bis-TBDMS [1,3-$^{15}N_2$]urate). A plot of the *m/z* 231:569 ratio against the molar ratios was fairly linear ($F_{1,7} = 109.0$, $P < 0.001$, $R^2 = 0.95$) across the range, allowing us to calculate a correction factor for use in determining approximate molarities of urea in our samples.

Statistical regression analyses were performed using R version 2.13.1 freeware (R Development Team Core, 2011) with the library (lme4) on log transformed data.

3. Results

In the vast majority (> 95%) of samples, uric acid was by far the most abundant N-containing component analyzed, representing over 98% of the total mass of uric acid plus urea (allantoin was only ever detected in trace amounts in some samples and not included for quantification). However, there were a few (N = 7) samples (found in all three feeding regimens) in which the amount of urea was very high and the amount of uric acid relatively low. Because these samples had % uric acid values outside of two standard deviations from the other samples (N = 129), we calculated the % uric acid of these two groups separately. The larger group had a mean % uric acid (total uric acid plus urea) of 98.2 ± 4.6, whereas the outlying group had a percentage of only 22.2 ± 9.2.

The quantity of uric acid excreted by starved females showed a smooth exponential decline over the course of the experiment (Fig. 2a, ln(µg) per female = 1.8–0.55 days, df = 37, P < 0.0001). Females that spent the first two days with a host but did not subsequently host feed (HF1), showed a greater rate of excretion, relative to starved females, at the beginning of the experiment. The rate of excretion of uric acid then declined over time, albeit to a lesser extent than that of starving females (Fig. 2b). Females that spent the first two days with a host, were starved for 2 days and then fed on a host (HF2), showed a distinct bump in uric acid excretion rate following host feeding, after which the rate declined over the course of the experiment. A regression analysis using a linear model showed no significant interaction between time and treatment, but a significant effect of treatment (µg per female = 2.36–0.18 days for HF1 and µg per female = 3.00–0.18 days for HF2, df = 2, 55, P = 0.04), implying that a single host-feeding event results in the excretion of an extra 0.64 µg of uric acid per day.

4. Discussion

4.1. Caveat

The high variability in the dataset of females that had access to hosts contrasts with the low variability observed for the starved females. This

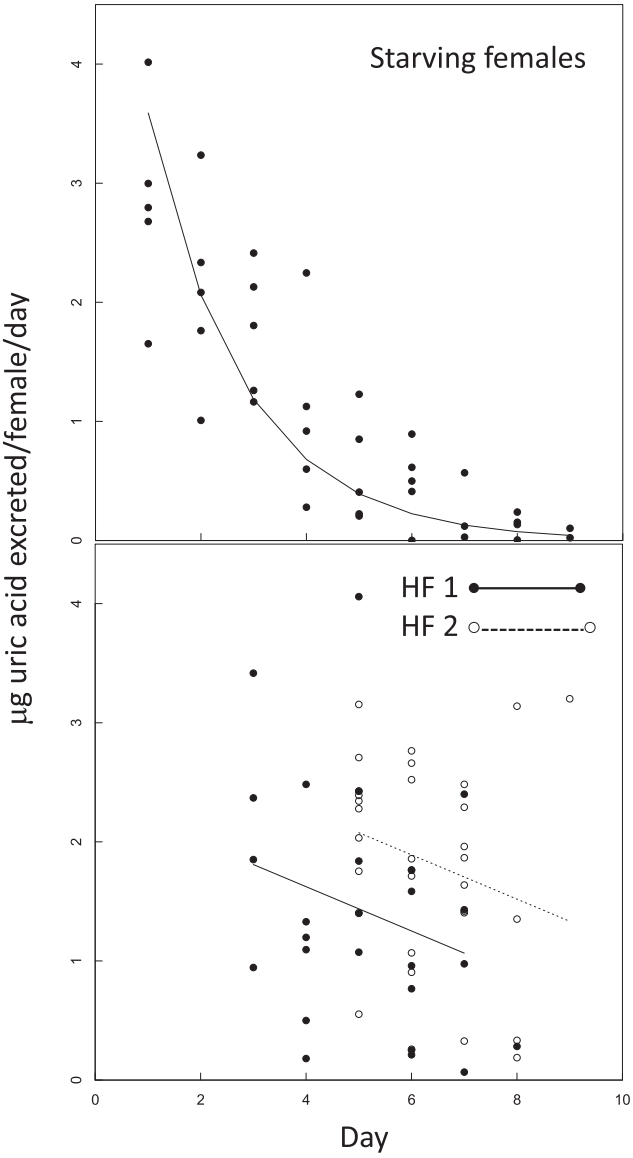

Fig. 2. Uric acid amounts over time excreted by (top) starving *Eupelmus vuilleti* females and (bottom) by females having access to hosts in the first two days after eclosion and then either starved (solid regression line, HF1) or having a single host feed on day 4 (dotted regression line, HF2).

is likely due to the large variability in the amount of fluid ingested during a given host-feeding event (Giron et al., 2004), further reinforced by the shorter time series longevity of the host-feeding treatments due to the short longevity of females.

4.2. Nature of N waste compounds and pathways for excretion

Excreta have been chemically analyzed across a wide range of insect taxa but never, according to our knowledge, for adult entomophagous parasitoids. As found for most other terrestrial insects studied, uric acid is the predominant N-containing metabolite in the excreta of *E. vuilleti*. We also found traces of allantoin and small quantities of urea, again consistent with what is known about the excreta of most terrestrial insect species studied (Bursell, 1967; Klowden, 2013; O'Donnell and Donini, 2017).

The small amount of urea in the excreta is interesting in light of the recent finding that *A. aegypti* mosquitoes produce urea via an uricoltyic pathway (i.e., they metabolize uric acid via allantoin and allantoic acid to urea), rather than through the more common hydrolysis of arginine (Scaraffia et al., 2008; Horvath et al., 2018). While we did not investigate the mechanism by which urea is produced in *E. vuilleti*, the fact that both insects share a similar N-rich food (host circulatory fluid), suggests that a similar uricolytic pathway may be present in *E. vuilleti*, especially as a few samples showed relatively high levels of urea and low amounts of uric acid. However, we caution that these samples were all from different groups and no group of females excreted high levels of urea more than once. Labeling experiments need to be carried out to show the pathway for production of urea in *E. vuilleti*.

4.3. N budget of single host-feeding events: no net gain

It is apparent from the comparison of results from HF1 and HF2 treatments that host-feeding resulted in increased N excretion by females. In fact, even the amount of uric acid excreted following the initial host experience phase, when limited feeding takes place (Casas et al., 2015), was roughly similar (Fig. 2) to that following the subsequent full host feed (i.e., in HF2). The quantification of uric acid excreted following host feeding by experienced females allows us to relate this to the known mean income of 5.7 µg protein per host-feeding event (Giron et al., 2002). Given an average of 16% N by mass in protein (Mariotti et al., 2008), this equates to an income of 0.9 µg of N. In comparison, a single host-feed event resulted in excretion of an extra 0.64 µg uric acid per day over at least 3 days (total 1.9 µg). As the N content of uric acid is 33.3% by mass, females therefore excreted 0.8 µg of N over the three days following a single host feed. That is, females subsequently excreted roughly as much N as they took in from a single host meal. There are three approximations in this rough calculation. First, we do not know the intake of free amino acids in the host fluid. While insect hemolymph contains relatively high levels of amino acids relative to vertebrate blood (Wyatt, 1961), the level is still rather low compared to protein mass. For example, in *Bombyx mori* haemolymph, amino acid mass is roughly 5% that of protein (Wyatt et al., 1956) and in *Chironomus* haemolymph ca 5–10% (Firling, 1977). Thus, its contribution to the N budget in host feeding fluid is likely to be relatively low. Second, we did not take into account the contributions of allantoin (or allantoic acid) and urea to the excreted N), both of which would add to the amount of N excreted. Finally, we used an approximate value of female longevity in each treatment, rather than integrating excretion rates over the whole lifetimes of females. A span of two days (leading to an excretion of 1.2 µg uric acid) is likely too low, while a span of four days is the maximum we observed in our study (leading to an excretion of 2.56 µg uric acid). While these approximations introduce greater uncertainty into our rough calculation, they are unlikely to change the overall conclusion that the extra amount of nitrogen excreted is similar to that ingested.

That N from host feeding is offset by increased N excretion implies that host feeding makes no net N contribution to oocyte production, although it could provide specific amino acids or affect temporal allocations, which would need to be balanced by capital N metabolism (and excretion). This complex interaction between capital reserves and income through host-feeding has been indeed observed previously using radioactive labelled amino acids (Rivero et al., 2001). Our findings appear to run counter to the general assumption that feeding on N-containing substances by adult females is driven by the need to gain extra N for allocation to oocyte production; i.e., N is limiting for oocyte production (Godfray, 1994; Quicke, 1997; Jervis, 2012; Fischbein et al., 2016). We caution that this outcome is the result of a single feeding at a specific moment; multiple host feedings and feeding at different times could result in different outcomes. Indeed, we have shown on this very species that both host-feeding dynamics and maternal investment vary over the lifetime of females (Casas et al., 2005; Muller et al., 2017). Nevertheless, our results demonstrate that in *E. vuilleti*, and possibly other host-feeding parasitoids, N from host feeding is likely mostly catabolized for energy in order to conserve carbon (probably lipids; Casas et al., 2005), for oocyte allocation early in adult life. When

summed over the entire lifetime, the N gain from proteins (and amino acids) from host-feeding leads to only a 10% increase in protein investment in oocyte production (Strand and Casas, 2008; Jervis et al., 2008). The use of proteins and amino acids from host-feeding largely for maintenance was already assumed (reviewed by Fischbein et al., 2016) and we provide here the first experimental evidence via increased excretion.

4.4. A comparative vision

Interestingly, mosquitoes again offer an comparison to our results, as a similarly low 10% of amino acids absorbed after digestion of a blood meal is actually transferred into eggs, with most catabolized for energy needs (Zhou et al., 2004; Isoe and Scaraffia, 2013; Petchampai and Scaraffia, 2016). Thus, our work identifies two physiological similarities between host-feeding parasitoids and blood-feeding mosquitoes: (1) the N metabolites excreted, and (2) the allocation of ingested N primarily to energy needs. Molecular data which would back up the results on *Eupelmus vuilleti* are unfortunately unavailable. *Nasonia vitripennis*, *N. giraulti*, and *N. longicornis* are the only parasitoids with similar life history (in particular host-feeding) for which genomes are available (Werren et al., 2010). In *N. vitripennis*, genes coding key enzymes in mosquito excretion, such as urate oxidase, an allantoinase and a probable allantoicase have been found (Werren et al., 2010). However, currently, no functional data are available and only predicted proteins can be inferred from genomic sequences. Similar conclusions can be drawn for the synthesis of a functional arginase in wasps. Further comparative and detailed investigations of N excretion in insects living on other N-rich fluids might establish further metabolic commonalities.

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