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FELLOWSHIP FINAL REPORT

Stable isotope methods for insect physiology

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REPORT INFO

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ABSTRACT

Insects are successful largely because they are highly efficient at optimizing nutrients for reproduction. To understand this efficiency, we have used the stable isotope tracer-tracee-based technique mass isotopomer distribution analysis (MIDA) to follow metabolic allocation in insects in vivo. Based largely on application of these techniques, we had three aims during the fellowship:

a) To adapt MIDA to study allocation of carbohydrates acquired during host feeding by the parasitoid Eupelmus vuiletti to fat production.
b) To study sex pheromone storage in a moth (Bombyx mori).
c) To write a significant, high impact review on insect physiology.

We successfully adapted MIDA to study fat acquisition in E. vuiletti. Essentially, females allowed to feed on a glucose drop, turned over their hemolymph trehalose substantially (ca. 30-40%). However, very little of this acquired sugar was converted to fat. Moreover, isotopic enrichment of fat was substantially less than that of the trehalose, indicating that other (non-labeled) sources of precursor are used for this fat synthesis. This supports the finding that parasitoids can synthesize fat, but only in very small amounts in comparison to their carbohydrate acquisition. These techniques were transferred to staff and students at IRBI.

Problems with supply of insects meant that we could not fully study sex pheromone storag in B. mori. However, we were able to demonstrate that females, when synthesizing pheromone, stored most pheromone on the gland cuticular surface, rather than intracellularly, thereby facilitating emission of pheromone.

Finally, we wrote and submitted a proposal for a review on insect exocrine glands for the highly prestigious Annual Review of Entomology (2022 IF = 23.8). We were notified of the success of our proposal in December 2022. Thereafter , a considerable portion of the visit was dedicated to researching, synthesizing, and writing this review. The manuscript will be submitted in January 2024 and, hopefully, published in January 2025

1- Introduction

Insects are probably the most successful group of animals on the planet (17). One reason they are so successful is their efficiency in utilizing nutrients to optimize their reproductive output. Most studies on this haver been ecological in nature, feeding insects manipulated diets and assessing the effects on fecundity (1) (3). However, there has been surprisingly little mechanistic study on nutrient intake and its allocation to physiologies, e.g., (2). In large part this is because such studies can be complex and require specific methods to quantify nutrient allocation. Stable isotopes have been used extensively in biomedical research to study metabolic disorders (23). Their great advantage for biomedical research is that they are perfectly safe, unlike radioisotopes, and can be injected into patients and sampled (in blood) over time.

We have adapted some of these methods to use to study physiological processes in insects. In particular, we have used mass isotope distribution analysis (MIDA) [15, 16], to quantify nutrient allocation in moths to sex pheromone production (5) (7). MIDA is a combinatorial solution for calculating precursor enrichment (*PE*; proportion of a labeled monomer) in a polymeric product (15). ***One of the major aims of the fellowship was to transfer these stable isotope tracer-tracee techniques to the scientific host (JC) and cooperators, so that these techniques could be used at IRBI.***

One project in which stable isotope tracer-tracee techniques might prove useful is host-feeding in the parasitoid *Eupelmus vuiletti*. In this species, females feed on larvae of the bruchid beetle *Callosobruchus maculatus* by establishing a feeding tube that samples body fluid (primarily hemolymph) from the larva (14). The fate of sugar in the fluid is particularly intriguing, since adult parasitoids, unlike other animals have been previously thought unable to synthesize fat from carbohydrate (13; 22). A recent study on another parasitoid wasp *Leptopilina heterotoma* found that the ability to synthesize fats had not been lost completely but was turned on only when females fed on low fat hosts (19). We hypothesized that MIDA could be used to follow and quantify carbohydrate metabolism in *E. vuiletti*.

Another project we wished to use stable isotope tracer-tracee techniques at IRBI was to follow pheromone biosynthesis and storage in moths, complementing my previous work in the USA (7-12). I established that aldehyde pheromone components were stored exclusively on the cuticle of the pheromone gland cutcile, while their alcohol precursors were stored primarily in gland cells (9). In Tours, I wanted to use these technqiues to quantify where acetate ester or alcohol pheromone components were stored. We suspected that they were stored primarily in gland cells (where they are produced) and were translocated slowly to the cuticle prior to emission. Finally, while at IRBI, my host and I wished to co-write a significant review on some aspect of insect physiology. The precise topic and journal would be determined after my arrival in Tours.

In this report we address the three aims of the fellowship:

a) To adapt MIDA to study allocation of carbohydrates acquired during host feeding by the parasitoid *E. vuiletti* to fat production.

b) To study sex pheromone storage in a moth (*Bombyx mori*) (4).

c) To write a significant, high impact review on insect physiology.

2- Experimental details

Insects

A colony of E. vuilletti was available at IRBI. Adult females were harvested regularly from the colony and placed in Eppendorf centrifuge tubes for use in experiments in the same controlled environment chamber (30 oC, 12:12 L:D) as they were reared.

We had difficulty obtaining a colony of suitable moths on which to work. In the end, we settled on using silkworm moths. These were shipped to us from a commercial supplier as pupae (in cocoons) and placed in a controlled environment at 25 ºC, 14:10 (L:D). Newly emerged adults were sexed, and females placed in individual containers and used the same day. Unfortunately, the supply and quality of insects was erratic, which hindered progress on this work.

Insect treatments

We considered various approaches for introducing the stable isotope label (as U-^{13}C-glucose) into insects, including microinjection into the parasitoid or host, and pipetting into the feeding tube. Because of the small size of *E. vuilletti* and the delicate nature of the feeding tube, these were rejected as not feasible. In the end, we allowed the parasitoid to feed ad libitum on a small (5 ml) drop of 30% U-^{13}C-glucose solution at the bottom of the centrifuge tube. After the insect and tracer were placed in

the tube, the tube was sealed and left for various times (0-7 d) before insects were sampled.

For fat, an insect was homogenized with a mortar and pestle in ca 50 ml of dichloromethane/methanol (2:1) along with tripentadecanoin as internal standard. The liquid layer was decanted into a vial and the solvent evaporated by a nitrogen stream. The fats were converted to methyl esters (FAME) by base methanolysis (using methanolic KOH), which was neutralized with aqueous HCl, and extracted with 50 ml of *n*-heptane (16). For analysis of hemolymph sugars, insects were homogenized in water along with sorbitol as an interval standard. The liquid was decanted into a vial and dried in a Speedvac. Then, a mixture of toluene and acetic anhydride was added and the sugars acetylated at 100 ºC. After evaporation of surplus reactants, the acetylated sugars were extracted with dichloromethane (6).

Female *B*. *mori* were analyzed for pheromone by two methods. In the first, we immobilized a female while placing pressure on the abdomen to force out the ovipositor and evert the gland. The exposed gland was rinsed with ca 50 ml of *n*-heptane which was collected in a vial. Next, the rinsed gland was excised and placed in ca 50 ml of *n*-heptane and left to extract for ca. 60 min. This gave us two samples for each female: the rinse, which contained pheromone from the gland surface, and the extract, which gave us pheromone from gland cells. Both samples had a known amount of bombykal, as an external standard, added prior to analyses by gas chromatography/mass spectrometry (GC/MS).

GC/MS analysis

Samples were analyzed on an Agilent 7890-7000 mass spectrometer. The GC was equipped with a DB5 column (Agilent Technologies) and used helium as carrier gas,. The MS was run either in scan or selected ion modes, depending upon the analysis.

MIDA and Enrichment analyses

For fat synthesis, we analyzed methyl hexadecanoate (16:Me) , the FAME product of de novo fatty acid synthesis (21) and one of the most abundant fats in *E*. vuiletti. GC/MS is used to determine the intensities of several isotopomers of this polymer, an octomer of acetyl CoA (produced in part from the U-^{13}C-glucose). Then, tracer to tracee (TTR) ratios are calculated, allowing for the natural abundances of stable isotopes and the overlap of isotopomer spectra (23).

(i) $TTR(M+1) = (M+1/M+0)_{post} - (M+1/M+0)_{pre}$

(ii) $TTR(M+2) = (M+2/M+0)_{post} - (M+2/M+0)_{pre} – dT_1 \times TTR(M+1)$

The terms ‘pre’ and ‘post’ indicate intensities before and after label enters the system, while dT_1 accounts for spectral overlap of (M+1) and (M+2) isotopomers.

Precursor enrichment is then calculated:

(iii) $PE = 2 \times [TTR(M+2)/TTR(M+1)] \div [(n-1) + TTR(M+2)/TTR(M+1)]$

For analysis of trehalose (as trehalose octaacetate), we used *m/z* 331 and 337, representing fragments of trehalose with intact C_6 glucose rings with zero and six ^{13}Cs, respectively. We calculated the atom percent enrichment (APE) of trehalose by:

$APE = Intensity_{331}/(Intensity_{337}+1)$.

3- Results and Discussion

a) Adapt MIDA to study allocation of carbohydrates acquired during host feeding by the parasitoid E. vuiletti to fat production.

We found that females could survive ca. 5-7 days while feeding on a drop of 30% glucose (labeled or unlabeled). We set up time course experiments and sampled females for enrichment of trehalose and fat (hexadecanoate).

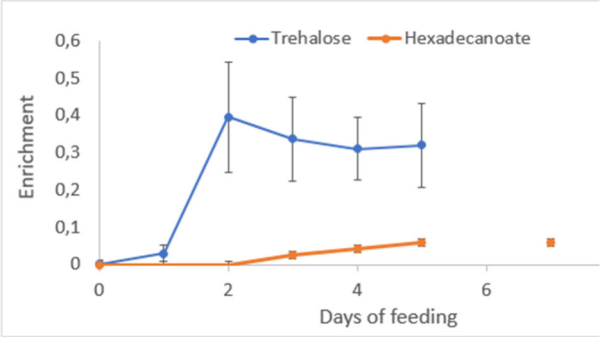

Figure 1. Enrichment of trehalose and hexadecanoate of *Eupelmus vuiletti* females after feeding on U-^{13}C-glucose for up to 7 days.

Within 2 days after feeding on U-^{13}C-glucose, a substantial proportion (ca. 30-40 %) of the hemolymph trehalose had been turned over (i.e., replaced by the labeled glucose). Thus, females appear to feed substantially on the glucose at least during the first 2 days of the experiment, but not to a significant degree after this. By contrast, newly synthesized hexadecanoate (extant hexadecanoate at the start of the experiment cannot incorporate label) reached a peak enrichment of ca. 5%, even after 7 days of potential feeding. These results tell us that very little hexadecanoate was synthesized during the experiment (only ca. 2-3% of the total hexadecanoate in the insect was synthesized by the end of the 7 d) and that this was synthesized preferentially from an acetyl CoA pool other than that of the hemolymph trehalose; if it were synthesized entirely from hemolymph trehalose, its enrichment should be ca. 30%. There are several possibilities for this (non-enriched) other pool, stored glycogen in the fat body, fatty acids or protein. Of these, we can rule out glycogen since this would pass through the hemolymph trehalose pool. Therefore, the small amount of fat made during the experiment came preferentially from either oxidized fat or protein.

These techniques and the quantitative analysis procedure were transferred to a student intern (M. Paul Bernard) and a faculty member (Dr. Vincent Foray) of IRBI. M. Bernard produced a short dissertation on their application.

b) To study sex pheromone storage in a moth (Bombyx mori)

As mentioned previously, we had trouble with insect supply. We received insects only two times and, in one of those, insects were of very poor quality as they had been reared on an artificial diet. Nevertheless, we were able to analyze females for storage of the pheromone bombykol. We found, after females emerged, that the amount of bombykol increased in the gland. Surprisingly, most (ca. 75%) of pheromone was stored on the gland cuticular surface, with the balance stored in gland cells. Since females are releasing pheromone ("calling") during this period (20), this indicates that the pheromone, after synthesis, is quickly translocated to the gland surface for emission. Our attempts to sample the emitted pheromone using a collection apparatus (18) were not successful. This work was carried out in collaboration with Dr Mourad Jaffer-Banerjee, a former PhD student of JC and currently a fellow at the Mx Planck Institute of Colloids and Interfaces in Potsdam, Germany.

c) To write a significant, high impact review on insect physiology.

During my first two months at IRBI, Prof. Casas and I discussed writing a major review on some aspect of insect physiology. Eventually we decided to write an article on exocrine glands, which generally release chemicals to the outside epithelium of an insect, although salivary glands and reproductive accessory glands also release to an epithelium inside the insect. We decided to target Annual Review of Entomology, the highest-ranking journal (IF = 23.8) in insect science. However, it is highly competitive to get published in Annual Review of Entomology, and one must first submit a proposal/outline for review by the editors. We wrote a proposal and were notified around December 2022 that it was successful. At this point, we decided to focus much of the rest of my visit on writing this chapter (due in January 2024, for publication in January 2025), especially as it was such a broad topic. We undertook a major literature survey, reading >600 published articles, and synthesized this information to develop a working model for

how insect (in theory, all, including mammalian) exocrine glands works. This was a large task, greatly helped by Prof. Casas and I being in close proximity to discuss literature and ideas in person as we developed our idea, which evolved considerably from our initial proposal. We are currently, settling on a final draft of the manuscript, which we will submit in time for the deadline.

4- Conclusion

This turned out to be a highly stimulating and productive visit to Tours. We achieved our objective to study fat synthesis and transfer appropriate chemical tracer/tracee techniques to staff and students at IRBI and partially achieved our objective of studying pheromone storage in a moth. However, the greatest achievement was the formulation and success of a proposal to write a chapter on insect exocrine glands to Annual Review of Entomology. The success of this proposal and the necessity to commit a larger amount of time to the review, diverted a considerable amount of my personal effort from the other two objectives. The writing of this review was greatly facilitated by my being in Tours, where not only could I interact regularly with Prof Casas, but also with other faculty and students at IRBI, who had knowledge on this topic.

5- Perspectives of future collaborations with the host laboratory

My collaboration with Prof. Casas has been ongoing since 2015 (last time I was on sabbatical in Tours). Although I am now back in the USA, it is continuing as we finish the writing of the Annual Review chapter, to be submitted in January 2024. I also interact informally with his current and former graduate students, providing advice on an ad hoc basis. The collaboration will be ongoing, hopefully with reciprocal visits, otherwise through regular Zoom calls. My two visits to Tours (in 2015 and 2022/230, both assisted by Le Studium, have expanded my range of skills and helped me develop a new, quantitative, perspective at looking at scientific problems.

6- Articles published in the framework of the fellowship

The Annual Review of Entomology article will be the most important article published from this visit. Its tentative working title is “How insect exocrine glands work” and, if accepted, will be published in January 2025.

7- Acknowledgements

I am indebted to the Le Studium staff and other fellows for making this such a productive, smooth, and enjoyable stay in Tours. I am also very grateful to Prof J. Casas for his great discussion and his generosity of time and resources. Finally, I would like to thank all the staff at IRBI, but especially Dr. E. Perdereau in the chemistry platform, for their help and support.

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