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Using Thermochemolysis Biomarker Techniques to Characterize White Grub Feeding Strategy

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May 8th, 2020

Introduction

The immature stages of some species of the geophagous beetles of the family Scarabaeidae (Coleoptera: Scarabaeoidea) are among the most important economic pests in the United States. The larval stage of these beetles often occupies the majority their life cycle. Their larvae are collectively called the white grubs, most of which are about 20 to 45 mm long, with white C-shaped bodies, brown heads, and three pairs of legs. They take underground roots and soil organic material as their main food resource. Such characteristics make them an important threat to the turf grass industry. Of all the white grubs, the Japanese beetle (Popillia japonica) is undoubtedly the most feared and destructive member in the United States (Timothy Gibb, 2019). This beetle, which is native to eastern Asia, was spread to the United States around the 1910s. The cost of controlling Japanese beetles alone is as high as $460 million (US) per year throughout the United States (USDA, 2015). With years of adaptation and spread, according to USDA statistics at the end of 2018, the Japanese beetle is currently distributed in 36 states across the United States and has caused more serious damage.

The interaction between white grubs and soil becomes very important. Although there are some studies that have verified the relationship between other similar species of white grub and soil physical properties (Millas and Carrillo, 2010; Li and Brune, 2005), no one has ever regarded Japanese beetle-related experiments, and there is yet no explanation for this problem from the

perspective of soil chemistry. As of now, no research has been able to accurately summarize the eating habits and nutritional composition of Japanese beetle larvae. We have proposed the following hypothesis: that white grubs have a preference when it comes to grazing grass roots. Therefore, we hope to reveal the feeding ecology of Japanese beetles through experiments.

In order to further understand the eating habits and nutritional composition of the Japanese beetle, we used a micro-forensic organic geochemical tool (tetramethyl ammonium hydroxide thermal chemical decomposition method) to examine the fecal matter of Japanese beetle larvae. Using this method, we aim to clarify the dietary components and subsequent transformation products related to the feeding behavior of these insects. At the same time, we also extracted the digestive tract residue from some southern masked chafer (Cyclocephala lurida), a native white grub species, and compared it with the Japanese beetle. We hope to start from the differences between the feeding strategies of these two different species to explain why the Japanese beetle is so good for adapting to the local environment throughout this experiment.

Method

In response to our hypothesis, we designed two experiments. First, we had designed an analysis of chemical substances in samples collected in the field for visually judging the ingredients contained in white grub food and determine

whether they have tendency to eat in certain part when they eat plant roots. Next, we conducted a feeding assay experiment to measure the changes in food composition of Japanese beetle larvae over time in an artificial environment.

Study Site: William H. Daniel Turfgrass Research and Diagnostic Center is located in 1340 Cherry Lane, West Lafayette. The area was created in 1999 and has 22 acres of land dedicated to turf research. This area not only has all our research subjects but is a monoculture area that grows only Kentucky bluegrass, so there was no chance of the grubs eating any other type of plant there. This ensures that we minimize the amount of sample interference.

Sample Collection and Analyses: For the overall preparation section, we collected large numbers of third instar Japanese beetle larvae and some southern masked chafer third instar larvae from the Daniel Center. Afterwards, we collected a large amount of Kentucky bluegrass (Poa pratensis) in the same time and same location. We tried to keep their roots intact when we pull them from the soil. After returning to the laboratory, we took out the food residues from the two species of white grubs by putting each grub into its own glass vial and allowing it to void its gut contents (frass). The frass was collected the next day from each vial, then we bottled them, and dried them in the oven under 80° C for 12 hours. Then, we put the collected Kentucky bluegrass at room temperature and dried them. After it was completely dry, we carefully separated the roots of different root part using the root-order method under the microscope. Root-order

method is a method to reveal the difference of morphology and physiology of plant roots (Ying Liu, Guoliang Wang, 2018), it lists the epiphytic roots of each level on the main root as different orders to record the root position. In this way, we collected grass roots from the first, second, and third root orders, and the parts that are lower than the third root order were merged into the third root order during the separation. This is for the convenience of the experiment, because the grass roots lower than order 3 are very difficult to distinguish by light microscope alone and hard to separate, under our current conditions, it is difficult to collect enough quantities for one test.

For the feeding assay experiment, Japanese beetle grubs that we collected from Daniel's Center were fed only soil organic matter in the lab for 2 weeks. Then they were placed in individual clean glass vials and fed Kentucky bluegrass 2nd-order roots for 96 hours. Every 12 hours, the frass they produced was collected from each vial and pooled per 12-hour period. Finally, we oven-dried them as mentioned above.

When all the samples had been dried, we ground them, because we need all samples to be granulated into small granule to meet the needs of the test. The instrument we chose to use at the beginning was a Retsch MM400 ball mill. We used 30rps to grind the samples for two minutes. But over time, we found that during the use of the instrument, a large amount of sample powder will adhere

to the container wall, which will cause sample waste. To solve this problem, we replaced the ball mill with a Spex 67/50 freezer mill during the subsequent grinding process. We set the parameters of freezer mill to a 15-minute precool phase, a two-minute 20rps grinding phase, and a one-minute recooling phase between two grinding phases. By changing the instrument, we effectively solved the problem of sample waste and got sufficient amount of sample powder.

When testing samples, we used Pyrolysis Gas Chromatography- Mass Spectrometry (Pyr-GCMS) as the main detection method. Pyr-GCMS is a method of chemical analysis in which the sample is heated to decomposition to produce smaller molecules that are separated by gas chromatography and detected using mass spectrometry (McEwen, C. N, 2006). In this set of experiments, we used Tetramethylammonium hydroxide (TMAH) as the extraction solvent. We put 0.1 mg sample weighed into a platinum pyrolysis boat. 5μL TMAH and 1μL eicosane (internal standard) added to boat and allowed to derivatize for 15 minutes. Samples were pyrolyzed at 350° C and analyzed on a Shimadzu GCMS-QP5050A. The analysis method used was a temperature ramp of 65 to 300° C at 8° C per minute, with a 10 minute hold time (the time that it sits at 300° C at the end of the run to make sure that everything is out of the column and has had a chance to reach the detector). Mass spectra were compared to NIST (National Institute of Standards and Technology) and internal lab databases for compound similarity in order to identify compounds in the sample.

Also, in order to determine the proportion of carbon and nitrogen in each sample, we ran an elemental analysis, which 3 mg sample weighed into tin capsules and analyzed on a Sercon EA-CN1 elemental analyzer coupled with a Sercon 20-22 stable isotope ratio mass spectrometer.

Result

First, based on the results of the Pyr-GCMS test of Daniel Center's sample, we obtained the ratios of various compounds in the samples collected in the field. We selected the most representative aromatic (Ar), aliphatic (Al) and sugars (S) substances as well as the carbon and nitrogen content in each group of samples as the main reference and obtained Figure 1. The aromatic and aliphatic compounds are assumed to maintain their structure when they pass through the grub gut. So, we expect them to represent the aromatic and aliphatic concentrations in whatever they were eating. The very first thing that we found is the relative proportions of aromatic and aliphatic compounds are varying across different root branch orders, and from that we believe we can conduct more in-depth analysis. From Figure 1 we can conclude that the ratios of aromatic to aliphatic compounds (Ar: Al) is decreasing from 1st, 2nd to 3rd root branch orders, and the Japanese beetle frass Ar: Al ratio is about half-way between that of 2nd and 3rd root branch orders. The southern masked chafer frass Ar: Al is also between 2nd and 3rd branch orders, however, the result showed that the ratio is closer to 2nd branch order than to 3rd. Also, we conducted the

ratio of sugars to non-sugars (S: NS) for both Japanese beetle and southern masked chafer frass, and both of these frass show a much lower S: NS ratio than all the branch orders. From that we conclude that the sugars may be a substantial nutrient source for both of the grubs.

From the results of the elemental analysis of Daniel Center's field-collected sample, we hope to reveal the source of the ingredients of two different beetle frass from a more macro-element perspective. So, we calculated the carbon to nitrogen ratios (C: N) for all the three root branch orders, soil and two different kind of beetle frass. All these data make up figure 2. From figure 2, first of all, the most intuitive result we get is C: N ratio in soil and grass roots is completely different, this value is higher for plant material, lower for soil. Then we found out that frass C: N for both JB and MC is pretty low and is somewhere between root branch orders and soil C:N ratio. So, we believe that grub frass is likely incorporating quite a bit of soil in addition to root material.

Using the data from the elemental analysis of the feeding assay (Japanese beetle grubs from TPAC fed 2nd order roots in the lab for 4 days), we have made the figure 3 also with the C: N ratio. From this figure, the first thing we can observe is the changing C: N ratio of the Japanese beetle frass over time. We think this may prove that there are significant changes in the substance content of Japanese beetle larvae at different digestion stages. In addition, if we compare

the C: N ratio of Japanese beetle white grub in Figure 2, we find that they are very different. This may also proves that larvae in the wild cannot take only grass roots as food resource, and their diet structure is likely to contain part of the soil.

Discussion

First of all, although our experimental results have not yet been able to piece together all the feeding strategies of white grubs in our region, at least we can first confirm our assumptions before the experiment, for example, white grubs have a preference for root branch orders when feeding. At the same time, we also provide evidence that white grubs may also eat the soil itself while eating plant organic matter in the soil. We believe that such findings are sufficient to prove that micro-forensic organic geochemical technology can play an excellent role in exploring the eating habits and nutritional composition of white grubs.

Although our findings so far are not comprehensive and are only at the initial stage, we believe that if we analyze the current results, we can make many constructive assumptions from them. First of all, by using different Ar: Al ratio in different root orders and different kinds of beetle frass for comparison, we can more effectively determine the feeding preference of the white grub for different root parts. If we analyze this problem from a vertical perspective, we may be able to get a new inspiration, because a larger root order means finer roots, and such roots tend to be distributed in deeper soil layers. So maybe in the future, we

can establish a more complete coordinate system, and directly determine the soil depth of larval activity according to the Ar: Al ratio in white grub frass. In addition, based on the results of the Pyr-GCMS test and elemental analysis, we obtained the overall ratio of the important element of both Japanese beetle and southern masked chafer. Based on our findings, we can say they likely share a similar feeding strategy. Despite this, we can still see that there are some subtle differences in the feeding strategies of these two species. Although we cannot explain the reasons for these differences at present, we think this will be an important research direction in the future.

Judging from the results of the feeding assay, we can clearly see that after the Japanese beetles eat, the proportion of their chemical components in the frass changes continuously with time. We think it is very likely that the Japanese beetle white grubs will undergo different stages of digestion. In the future, if there is more research from the perspective of digestion physiology to explain this phenomenon, then using Pyr-GCMS technology, we can directly determine where the white grub collected in the field is digesting food. From a longer-term perspective, this may mean that we can more accurately estimate the feeding time of Japanese beetle white grub sample. This may have important value for future research on all kind of white grub.

Of course, we need to admit that our current sample data is too narrow, which

may mean that our results lack universality and representativeness. In the future, we hope to investigate Japanese beetle white grub and southern masked chafer white grub in more regions. At the same time, we plan to investigate more types of grass and white grub to enrich our sample library. But in general, we think that micro-forensic organic geochemistry is a new perspective for studying feeding habits and nutritional composition of the white grub diet, and our results are sufficient to prove the high potential of this research method for the future.

Acknowledgements

I would particularly like to thank Professor Douglas Richmond for his guidance and encouragement throughout the process, as well as Gordon Macleod for his meticulous help throughout the experiment and the completion of this article.

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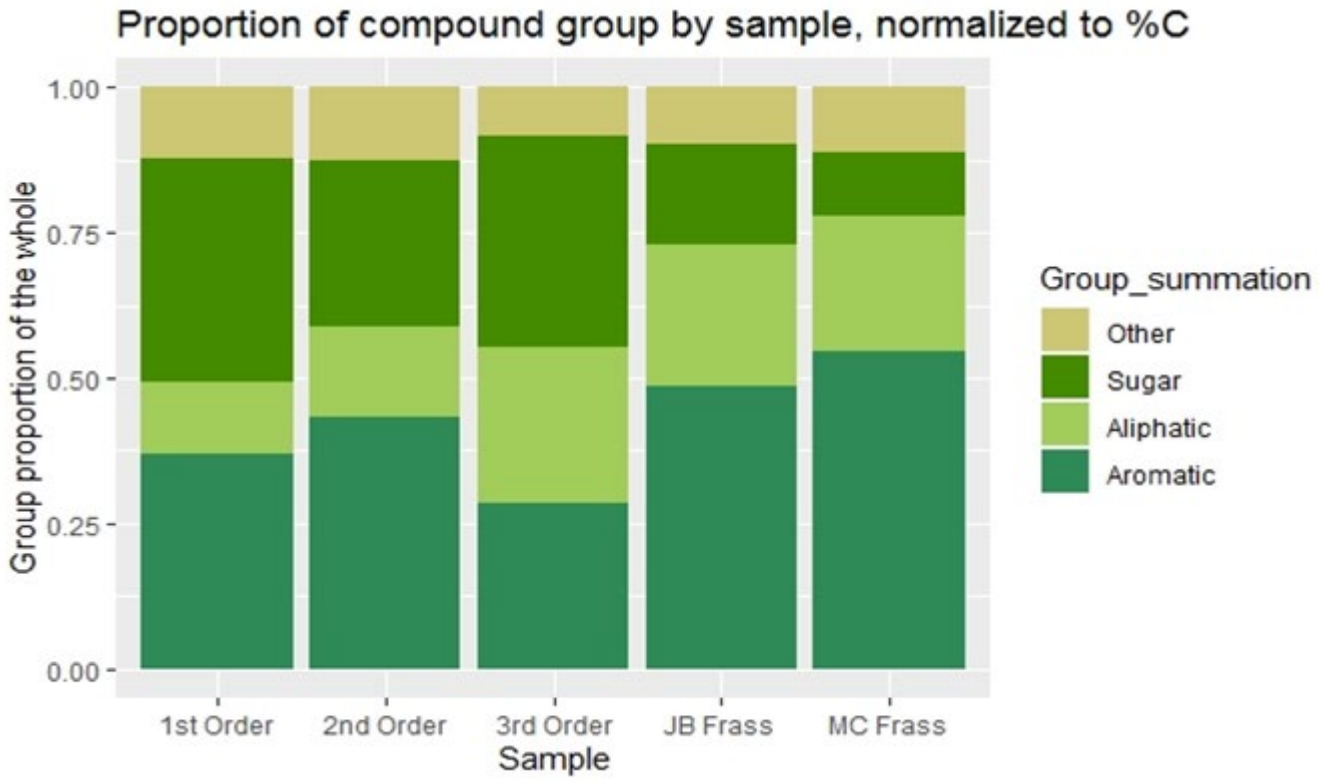
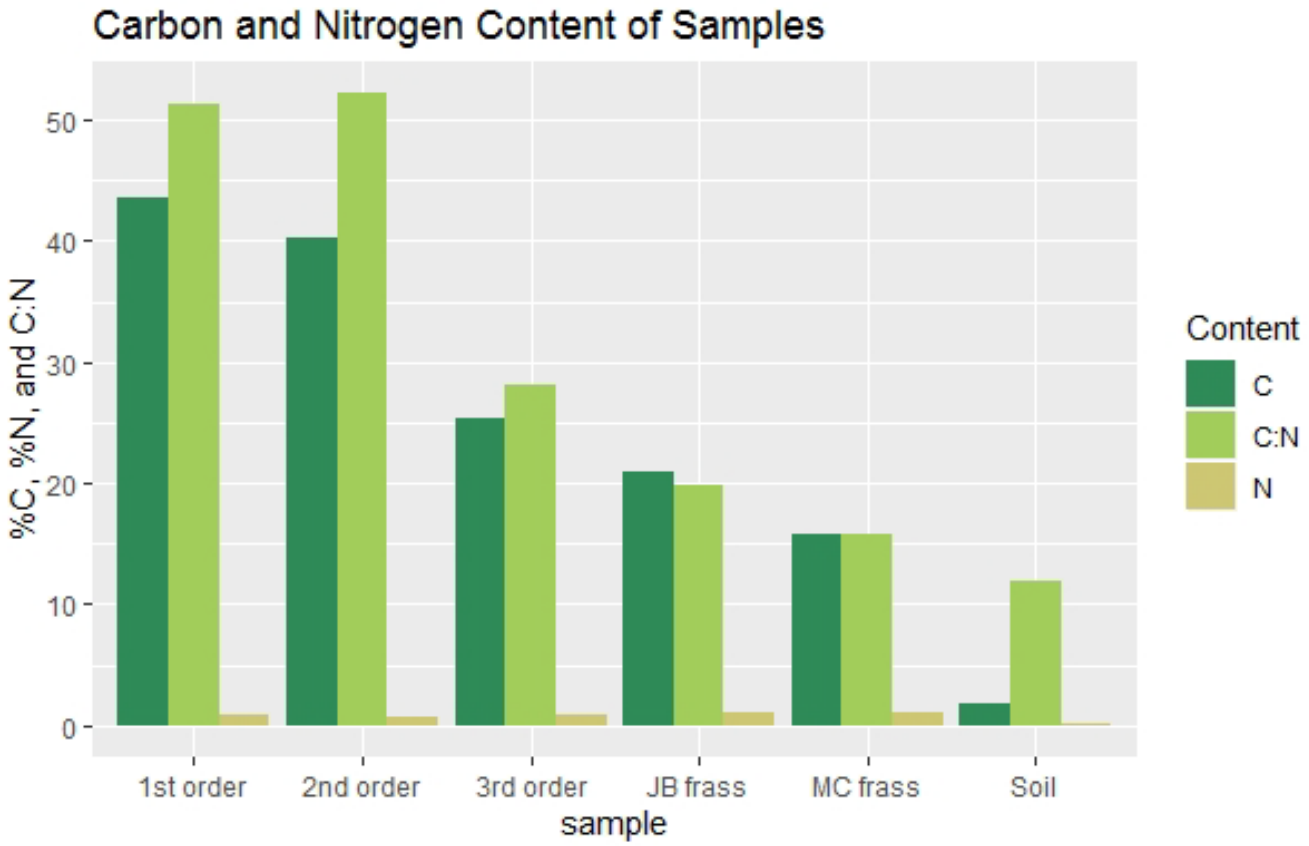
Figures

Figure 1: Proportion of aromatic and aliphatic compounds, sugars and unidentified componenets in 1st, 2nd, and 3rd order roots of Kentucky bluegrass, and frass of 3rd instar larvae of the Japanese beetle and southern masked chafer.



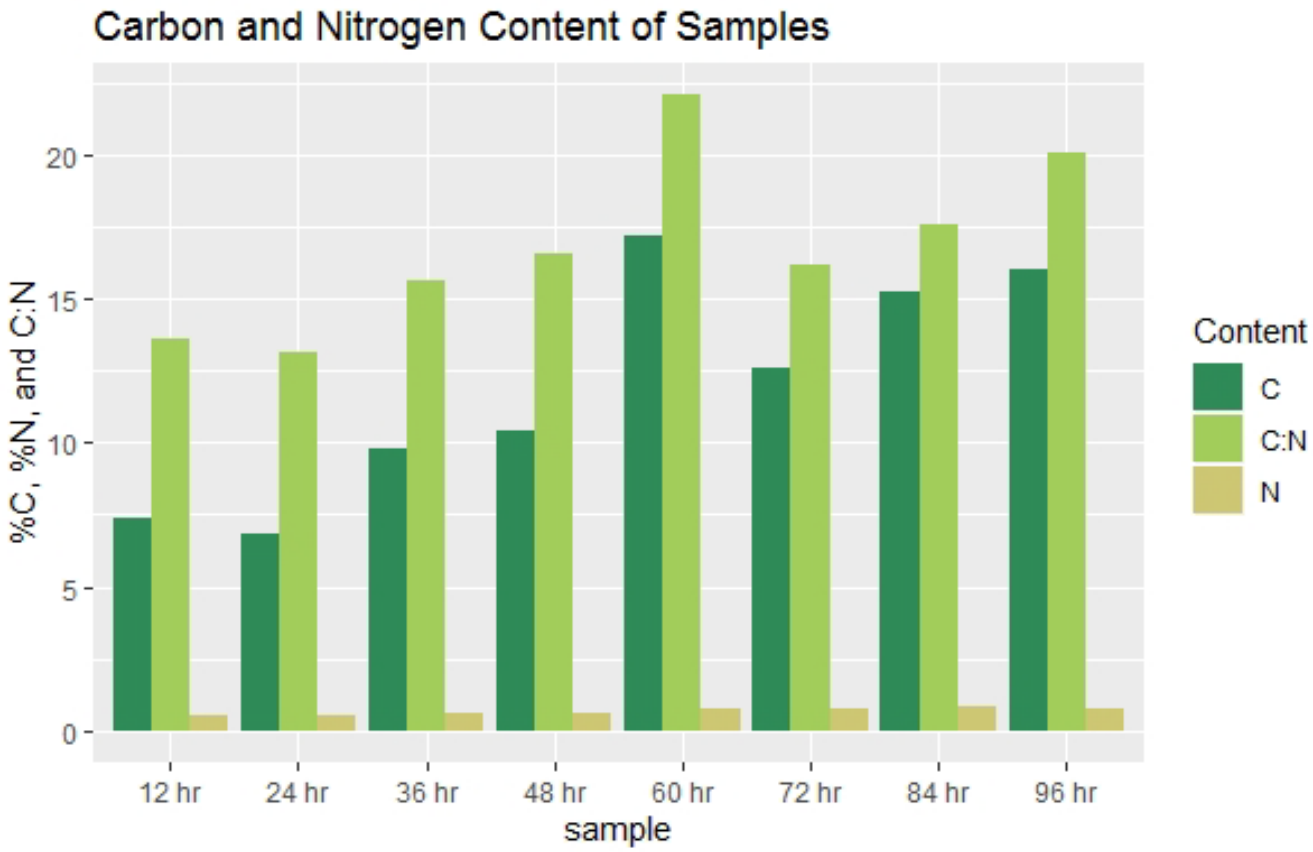
Ar:Al	3.08	2.83	1.05	1.97	2.36
S:NS	0.79	0.49	0.65	0.23	0.14

Figure 2: Proportion of carbon element, nitrogen element, and carbon: nitrogen ratio in 1st, 2nd, and 3rd order roots of Kentucky bluegrass, and frass of 3rd instar larvae of the Japanese beetle and southern masked chafer.



%C	43.59	40.18	25.45	20.94	15.74	1.85
C:N	51.28	52.18	28.15	19.91	15.81	11.91
%N	0.85	0.77	0.90	1.05	1.00	0.16

Figure 3: Proportion of carbon element, nitrogen element, and carbon: nitrogen ratio in the frass of 3rd instar larvae of the Japanese beetle of the feeding assay from every 12 hours.



%C	7.34	6.83	9.82	10.43	17.20	12.62	15.25	16.01
C:N	13.59	13.13	15.59	16.56	22.05	16.18	17.53	20.01
%N	0.54	0.52	0.63	0.63	0.78	0.78	0.87	0.80