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THE ROLE OF GOGAT CYCLE IN NUTRITIONAL INSECT-MICROBE SYMBIOSIS

By

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A capstone project submitted for
Graduation with University Honors

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APPROVED

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Abstract

Organisms have evolved diverse mechanisms to eliminate waste by-products through excretion. These mechanisms vary due to the organism's living conditions, and are essential to avoid the buildup of toxic ammonia. Acyrthosiphon pisum, a sap-feeding insect, reduces ammonia buildup in a distinct manner. This insect excretes ammonia in its honeydew, because unlike most animals it has lost the ability to store this waste in a less toxic form, such as urea. Moreover, unlike most animals the aphid may recycle ammonia to produce non-toxic amino donors (glutamate and glutamine) using the GOGAT cycle. Here, glutamine synthetase will recycle ammonia into glutamine, which then converts glutamine into two glutamates using glutamate synthase. The GOGAT cycle is hypothesized to be very important in allowing aphids to feed on sap, a sugar rich diet that is deficient in nitrogen. The aphid also forms an obligate mutualism with the symbiont, Buchnera, that produces essential amino acids for the aphid. It is hypothesized that the GOGAT cycle can respond to nitrogen demand and provide Buchnera with amino donors for essential amino acid biosynthesis. However, it is not known whether glutamine synthetase responds to nitrogen demand. To test this, aphids were reared on a control and lower EAA diet. Results suggest that glutamine synthetase was up-regulated in response to a lower concentration of essential amino acids in their diets.

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Table of Contents

Abstract……

Introduction……..3

Materials and Methods…….6

Results……....11

Discussion……..13

Future Directions……...14

Introduction

Insects are the most species rich group of animals on the planet and occupy many diverse niches (Foottit and Adler 2006). One such niche involves the feeding on a nutrient poor phloem sap diet of plants. Phloem sap is high in carbohydrates, however it is very low in nitrogen (Sandström and Petterson 1994). In fact phloem sap is too low in essential amino acid (EAA) concentrations to meet the nutritional demands of many sap-feeding insects (Hansen and Moran 2014). These sap-feeding insects survive on this nitrogen limited diet because it forms a mutualistic, symbiotic relationship with an obligate symbiont that provisions EAA to the insect host. A model system that is used to study these metabolic interactions is the pea aphid (*Acyrthosiphon pisum*) and its bacterial endosymbiont *Buchnera aphidicola* (hereafter referred to as *Buchnera*) (Hansen and Moran 2011). The pea aphid forms a symbiotic relationship with *Buchnera*, in which *Buchnera* encodes genes to synthesize EAA for its aphid host. In return, the pea aphid maternally transmits *Buchnera* to the next generation and provides its symbiont with required nitrogen and carbon precursors inside of specialized aphid cells called bacteriocytes (Douglas 1998).

There are many different metabolic pathways that are shared between the pea aphid and *Buchnera* and these pathways play an important role in providing resources to allow growth and reproduction for both players in the symbiotic relationship. The genomes of *Buchnera* and the pea aphid complement each other where *Buchnera* is missing genes in some of its EAA pathways and the aphid highly expresses homologs of these missing genes in bacteriocytes for the production of EAAs (Hansen and Moran 2011). Moreover, *Buchnera* is missing all of its non-essential amino acids and requires these free amino acids from its aphid's host (Shigenobu et

al. 2000). Based on previous work it has been shown that aphids display longer developmental times, use nonessential amino acids at a slower rate, and weigh much less when aphids were given antibiotics to eliminate *Buchnera* compared to control aphids that possess *Buchnera* (Prosser and Douglas 1992). Collectively, these studies highlight the importance of *Buchnera* in the biosynthesis of EAA for the pea aphid host through the process of nitrogen upgrading in which *Buchnera* will metabolize nonessential amino acids from the host and/or its diet into essential amino acids (Prosser and Douglas 1992).

One important metabolic cycle in the aphid-*Buchnera* symbiosis that may be very important in recycling waste ammonia into amino donors for *Buchnera* provisioned EAAs is the GOGAT cycle (Hansen and Moran 2011). When looking at the composition of pea aphid excreta (i.e. honeydew), it can be noted that there is no uric acid and instead ammonia is the main form of nitrogenous waste (Sasaki et al. 1990). Ammonia is known to be toxic to cells, especially in higher concentrations, so the pea aphid must encode a pathway that can reduce the concentration of ammonia to prevent the toxic build-up of ammonia in cells (Martinelle and Häggström 1993). To overcome this problem, a few insect species such as *A. aegypti* have the GOGAT cycle to help assimilate the nitrogen overflow from their diet (Scaraffia et al. 2005). It is hypothesized that the GOGAT cycle in aphid bacteriocytes recycles waste ammonia into a useable nitrogen source for the aphid, where glutamine synthetase (hereafter referred as GS) converts ammonia into glutamine and glutamate synthase converts glutamine into two glutamates (Hansen and Moran 2011). The production of glutamate is important since it serves as an amino donor for *Buchnera* to make essential amino acids (Sasaki and Ishikawa 1994).

While it is known that the GOGAT cycle can recycle ammonia within the pea aphid (Hansen and Moran 2011), it is not well known whether the enzymes in the GOGAT cycle can

respond to differences in nitrogen demand especially within aphids. To determine if GS, E.C. 6.3.1.2, responses to changes in nutrition, aphids will be fed on an amino acid artificial diet that is low in EAA compared to a control diet that has sufficient concentrations of EAA. Survival rates will be recorded for phenotypic analysis. In addition, RT-qPCR will be conducted to see if there are any differences in gene expression of the glutamine synthetase between diet treatments to ultimately determine if the GOGAT cycle can respond to changes in nutritional demands.

Material and Methods:

Aphid and plant rearing

Before artificial diet trials, three clonal sub-lines of *Acyrthosiphon pisum* (lines 1, 2, and 3) were maintained on two-week-old *Vicia faba* in an incubation chamber at 20°C with a 16:8 hr L:D photoperiod. *Vicia faba* was grown in an environmentally controlled chamber at approximately 40°C with a 16:8 hr L:D photoperiod. To stage nymphs of the same starting developmental age, reproductive adult aphids were placed on *V. faba* and were allowed to lay nymphs for 24 hours and then the adults were removed.

EAA	Original (mM)	50% EAA Diet (mM)	Nonessential Amino Acids	Original (Mg)	50% EAA Diet (mM)
Arginine	12.5	6.25	**Alanine**	5	7.5
Histidine	7.5	3.75	**Asparagine**	12.5	18.75
Isoleucine	7.5	3.75	**Aspartate**	12.5	18.75
Leucine	7.5	3.75	**Cystein Hydrate**	2.5	3.75
Lysine HCL	7.5	3.75	**Cystine**	0.2	0.0
Methionine	2.5	1.25	**Butyric Acid**	2	3
Phenylalnine	2.5	1.25	**Glutamate**	7.5	11.25
Threonine	7.5	3.75	**Glutamine**	15	22.5
Tryptophan	2.5	1.25	**Glycine**	1	1.5
Valine	7.5	3.75	**Proline**	5	7.5
Total EAA (mM)	65	32.5	**Serine**	5	7.5
			Tyrosine	0.5	0.75
			Total NEAA (mM)	68.7	103.5

Table 1: Amino acid concentrations for control and lower EAA diet. The control amino acid concentrations were derived from a previous study. 50% EAA maintained the same overall amino acid concentration by doubling the NEAA concentration. NEAA= nonessential amino acids

Artificial diet treatments

Similar to Pers and Hansen (2019) nymphs were reared on the plant until the second instar and were then transferred to artificial diet media which will help increase the survival rate of developing nymphs. Two artificial diet treatments were conducted, the control and the 50% EAA diet (Table 1). The control artificial diet media was made with the protocol described in Meyer (2004) which emulates similar concentrations of sucrose, amino acids, salts, and trace metals that are found in phloem sap of *V. faba*. In the lower EAA diet treatment, the same protocol was followed except the essential amino acid concentration was reduced by 50% and the nonessential amino acid concentration was increased to ensure that the total concentration of nitrogen was the same between both treatments. A zero-concentration essential amino acid diet was not used because previous studies have revealed that genetic polymorphisms occur in certain pea aphid biotypes that require them to have specific essential amino acid(s) to develop, even with their symbiont *Buchnera* (Srivasta et al. 1984, Wilkinson and Douglas 2002).

For artificial diet trials, a set amount of aphid nymphs per sub-line per diet treatment were reared until the 4th instar on an artificial diet, with verification of life stage with siphunculi length and the number of molts as in Pers and Hansen (2019). Bacteriocytes were then dissected from nymphs as in Hansen and Moran (2011), and were subsequently stored in RNAprotect (Qiagen, Hilden, Germany) at -80°C until RNA extractions were conducted.

Following Pers and Hansen (2019), a feeding chamber was constructed by placing two 24 well (Corning, Corning, NY, USA) plates together with parafilm separating the two (Figure 1). The top plate consisted of the artificial diet, with 400 microliters of artificial diet placed into

each well along with 1 microliter of green food coloring to verify if the aphids were feeding on both treatments. These feeding chambers were placed in a 20°C incubator with a 16:8 hr L:D photoperiod for up to 10 days to ensure that they developed into the fourth instar developmental stage. The artificial diet was replaced every 2 to 3 days and a new plate was put in every 4 to 6 days to prevent molding. The bottom plate consisted of two aphids per well and this plate was replaced weekly to prevent build-up of honeydew and mold.



Figure 1: Aphid Feeding Chamber. The aphids will reside on the bottom plate and can feed on the diet through the parafilm layer.

Phenotype and fitness measurements

To compare the survival rates between sublines and diet conditions, a Kaplan Meier survivorship curve was constructed (Kaplan and Meier 1958). The curves were compared using a

chi square test to see if there were any significant differences in survival rates between pea aphids on the lower EAA diet compared to the control diet.

RNA extraction and quantitative real-time (q-RT PCR)

To ensure there was enough RNA, bacteriocytes from 3 individual aphids were pooled together for 1 sample. These bacteriocytes were selectively obtained through the dissection of the aphid body which allowed us to isolate the bacteriocytes. There were 3 biological replicates, subline 1, 2 and 3, but data for subline 3 was not collected due to a high mortality rate for subline 3 during trials. A total of 9 samples were obtained for subline 2 with 4 samples from the lower EAA diet and 5 samples from the control diet. Subline 1 originally had 4 samples, but 1 sample did not have enough mRNA. Therefore, the lower EAA diet had 2 samples, and the control diet had 1 sample. Aphid bacteriocyte total RNA was extracted per sample using Quick-DNA/RNA Microprep Plus Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. Total RNA was then converted into cDNA using iScript Reverse Transcription Supermix for q-RT PCR (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Similar to Thairu et al. 2017, to conduct q-RT PCR, iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used on individual aphid cDNA samples on a Bio-Rad CFX96 Real-Time PCR Detection System. Gene expression values were calculated using the delta delta ct method (Livak and Schmittgen, 2001) for relative quantification and normalized to the aphid housekeeping gene EF-1a (Dunbar et al., 2007). Primers were designed for the target gene of interest in this study, GS 6.3.1.2. The primers sequences are:

F: 5'- AAACCCGAAGAACTCCCTGT -3'

R: 5'- CCTGAACGGGTCGTTAAAAA -3'

Gene expression differences of GS between diet treatments were tested as in Thairu et al. 2017 using an unpaired, one-tailed Welch's t-test. In addition, to determine the relative fold change from the q-RT PCR data compared to a housekeeping gene, EF-1a, a delta delta ct value was calculated.

Results:

Effects of Survivorship from diet:

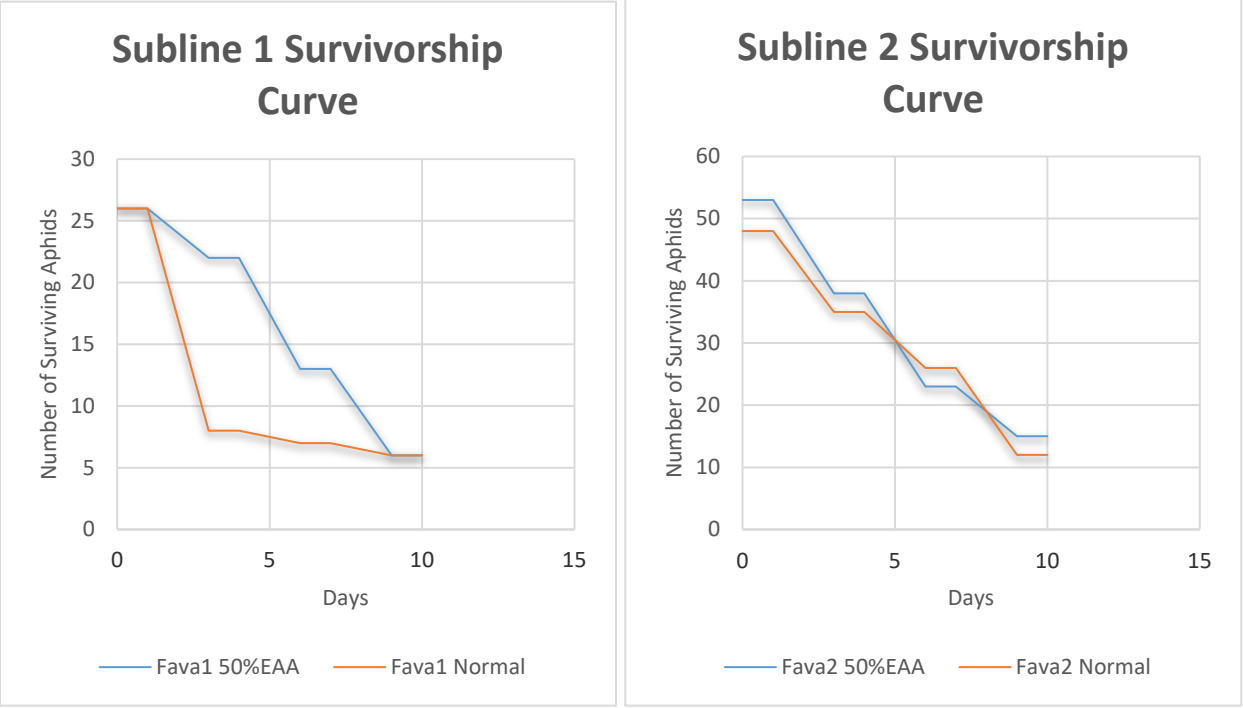


Figure 2: Kaplan Meier survivorship curve. Each line represents the number of surviving aphids at the given day. The blue line represents the 50% EAA diet group and the orange line represents the control diet group. ***(A):*** *Subline 1 data.* ***(B):*** *Subline 2 data*

To observe if there was any significant difference in survivorship caused by the differences in diet between the two groups, a Kaplan Meier survivorship curve was performed. We found that there was no significant difference in survival rates (Chi-square: χ: .4816, df: 1, p: .8266) indicating that there is no difference in survival rates between pea aphids living on the lower essential amino acid diet compared to the control diet (Figure 2).

Subline:	1	2
ΔΔct value	2.549	1.709

Table 2: ΔΔct value table. These values from sublines 1 and 2 represent the fold change difference between the expression of GS in the 50% diet compared to the control diet.

Effects of genotype expression from diet:

To investigate whether lowering the concentration of EAA had an effect on the expression levels of the enzyme GS, a RT-qPCR test was conducted. The results were normalized to an aphid housekeeping gene, eF-1a and compared to each other using a delta delta ct value (Livak and Schmittgen 2001). We observed that when aphids fed on the lower EAA diet compared to the control diet subline 1 and 2 resulted in at least a 1 fold increase in mRNA expression of GS (Table 2).

Discussion:

In this study, the GOGAT cycle, specifically GS, was investigated to determine whether or not it can respond transcriptionally in response to a lower EAA diet compared to a control diet that is higher in EAA concentrations. We observed that both sublines 1 and 2 up-regulated glutamine synthetase when developing on a low EAA diet compared to the control diet, which suggests that the GOGAT cycle is upregulated in response to a lower concentration of EAA's in its diet.

In this study aphid survival on both diets for all sublines was much lower than expected. For example, here the rate of survival was much lower compared to other studies that utilized artificial diet, which observed that at least 50% of aphid individuals survived in different nutritional treatments (Pers and Hansen, 2019; Prosser and Douglas 1991). This can be due to stress induced maternal effects because in this study aphid colonies were not controlled for density on its host plant for multiple generations before trials were conducted (Pers and Hansen, 2019). Subline 1 survival curves displayed an unexpected result where aphids feeding on the lower EAA treatment displayed higher survival than the control diet treatment. However, the sample size of Subline 1 was too low for statistical comparisons. For Subline 2, which had a larger sample size displayed survival curves that were not significantly different from one another suggesting that *Buchnera* can compensate for feeding on a diet that is lower in EAA resulting in the aphid host to have similar survival rates between both diet treatments. Furthermore, glutamine synthetase is upregulated in the lower EAA diet treatment which suggests further that the aphid is also compensating for feeding on a diet that is lower in EAA by recycling waste nitrogen into a usable form. By having higher expression of glutamine synthetase, the aphid can upgrade waste ammonia into usable amino donors such as glutamate and glutamine which are used by *Buchnera* to synthesize EAA. However, further trials need to

be conducted with more biological replicates with higher survival rates to further support this hypothesis.

Overall, the preliminary data presented here suggests that glutamine synthetase can respond to changes in EAA in its diet. This is important to understand since it can help further comprehend how aphids can respond to nutritional stress such as having lower EAA concentrations in its diet. However, due to small sample size and unexpected high mortality of aphid during trials, further studies will need to be conducted to verify that the results were not due to a bias formed by an extremely small data set.

Future Directions

The objective of this study was to find whether glutamine synthetase can respond to changes in nutrition by using a lower essential amino acid diet to test our hypothesis. While glutamine synthetase was seen to be up-regulated in both sublines, there was a few problems. One was the sample size. Subline 1 only had a total one 3 samples which makes it very likely that the data is skewed due to bias. To solve this in future studies, there needs to be a testable sample size that is greater than 10 samples per diet treatment. Additionally, there were other parameters that we were not able to measure due to the unfortunate situation. This included a test for viability. We planned to test for viability through measuring the weight of the aphids at the beginning and end of the diet treatment. By comparing the weights between the different diet groups, we can see whether the diet had an effect on its viability and would have given us useful data.

In the future, there are more ways to confirm that glutamine synthetase has a role in the aphid's response to changes in nutrition. One way is to add radioactive carbons to glutamine. This way, we can see where it goes after the aphid metabolizes it and it can help determine if most of glutamine actually does go to EAA production. Another method would be to test another variable such as a high EAA diet. With more EAA present in its diet, would the aphid need to translate normal levels of glutamine synthetase or would the levels of glutamine synthetase be down-regulated?

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