From Host Nutrition to Symbiont Contribution and Gene Regulation: Understanding the Symbiosis of the Reed Beetles

Master Thesis

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Statement of Authorship

I hereby declare that I am the sole author of this bachelor thesis and that I have not used any sources other than those listed in the bibliography and identified as references.

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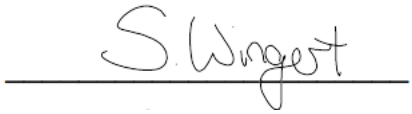

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Table of Contents

Abstract

Symbiotic relationships are contributing to insects' ability to exploit ecological niches. To understand the ecological importance of this relationship, it is crucial to understand why the relationship is kept and how it is regulated. Plant sap feeding insects are challenged with content of free essential amino acids which they cannot produce themselves. Some insects overcome this lack in amino acid through symbiotic relationships with bacteria such as reed beetles (Donaciinae). The symbionts of Donaciinae have a highly reduced genome, mainly encoding for essential amino acids biosynthesis pathways and lacking many regulatory genes. However, Donaciinae species differ in the amino acids their symbiont is encoding. Some symbionts maintain some amino acid biosynthesis pathways, while others have lost them. Here, we investigated whether a link can be made between the loss of particular biosynthesis pathways and the free amino acids in the host plants that the insect has access to. Furthermore, we wanted to understand if differences in the cocoon amino acid composition correspond to differences in the amino acid biosynthesis pathways of their respective symbionts. Additionally, we assessed the survival of symbionts in dissected symbiotic organs to afterwards investigate the regulation of amino acid biosynthesis pathways in Donaciinae symbionts by supplementing particular amino acids directly to the symbiotic organs. We show that the concentration of tryptophan in cocoons is corresponding to the presence/absence of the respective biosynthesis pathway in the symbiont's genome. Also, we show symbiont survival in symbiotic organs despite host cell death, indicating a potential for culturability of the symbiont.

1 Introduction

1.1 The Challenges of a Plant-Based Diet in Insects

Herbivorous insects face various challenges due to their specialized diet. One example are insects feeding on plant sap being challenged with a low content of essential amino acids, which the insects themselves cannot produce (Douglas, 2006). There are 10 amino acids considered as essential nutrients for insects: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Dadd, 1985). Furthermore, as tyrosine and cysteine (both not considered as essential amino acids) are synthesized from phenylalanine and methionine respectively, their requirement can also be affected by the lack of essential amino acids in nutrition (Dadd, 1985). Another example for dietary challenges can be found in leaf feeding insects. They are challenged with complex polysaccharides that require several enzymes to break down, such as Pectin (Douglas, 2009). Pectin is a particularly abundant polysaccharide in the plant cell wall that can be digested by specialized enzymes, called pectinases. In insects, these enzymes can be acquired through horizontal gene transfer from bacteria (Shelomi et al., 2016) or provided by a bacterial symbiont (Salem et al., 2017).

1.1.1 Insects Overcome Plant-Diet Challenges trough Association with Symbiotic Bacteria

To conquer the challenges of a plant-based diet, many herbivorous insects are associated with symbiotic bacteria (Douglas, 2009). Depending on the system, the insects can benefit from nutritional supplementation (Shigenobu et al., 2000; Vigneron et al., 2014) and/or enzymes to break down the plant cell wall (Reis et al., 2020). An example for herbivorous insects associated with nutritional symbionts are the (semi-) aquatic reed beetles (Choleptrea, Chrysomelidae, Donaciinae) (Stammer, 1935).

1.2 Reed Beetles (Donaciinae)

1.2.1 Life Cycle

The life cycle of reed beetles from egg to egg takes one to three years (Bienkowski, 1996; Stammer, 1935). During a period of 12-24 month, they develop five larvae stages (Bienkowski, 1996). The larvae form a cocoon in which they pupate and the imago hatches (Stammer, 1935). The development takes place during warm season.

1.2.2 Symbiosis

Reed beetles are not only challenged with one but two specific diets during their life cycle. During their larval stage they live in the soil of ponds and feed on specific host plants, presumably sucking the sap of the roots and rhizomes (Bienkowski, 1996). As adults, they live and feed on the leaves of the host plant (Bienkowski, 1996), thus facing the before mentioned challenges of digesting plant cell wall material and an amino acid poor diet. Therefore, reed beetles are associated with an obligate symbiotic Enterobacteriaceae species to conquer these two different diets (Reis et al., 2020). These bacteria provide the beetle with essential amino acids during their larval stage and pectinase during their adult stage (Reis et al., 2020).

1.2.3 Host Plant Specificity

Donaciinae is a subfamily of reed beetles that shows a pronounced specificity in the choice of host plants they are feeding on (Kölsch & Pedersen, 2008; Stammer, 1935). Prior research has shown that certain symbionts associated to Donaciinae have undergone the loss of specific amino acid biosynthesis pathways, such as the tryptophan and methionine pathways, while symbionts of other Donaciinae species have maintained these pathways (Figure 1.1) (Reis et al., 2020). The question arises whether a link can be made between the loss of these particular biosynthesis pathways and the free amino acids in the host plants that the insect has access to. Here, we investigate the composition of free amino acids in different host plants.

1.2.4 Bacterial Symbionts Contribute to Cocoon Formation in Reed Beetles

Previous research on Donaciinae has shown that beetles whose symbionts are being removed (called aposymbiotic), fail to form their cocoon (Kleinschmidt & Kölsch, 2011), implying that symbionts are involved in cocoon formation. It is suspected that cocoons are proteinaceous (Scherf, 1969), suggesting that the amino acids that are being synthesized by the symbionts are necessary for the cocoon formation. As mentioned above, some of the symbionts of different Donaciinae species have maintained some amino acid biosynthesis pathways, while others have lost them (Reis et al., 2020). For instance, the symbiont of *Macroplea mutica* is encoding for the tryptophan biosynthesis pathway while the symbiont of *Donacia marginata* has lost it (Figure 1.1). To investigate whether the symbiont is contributing to the cocoon formation, we compare the bound amino acid composition in cocoons between the species *D. marginata* and *M. mutica*.

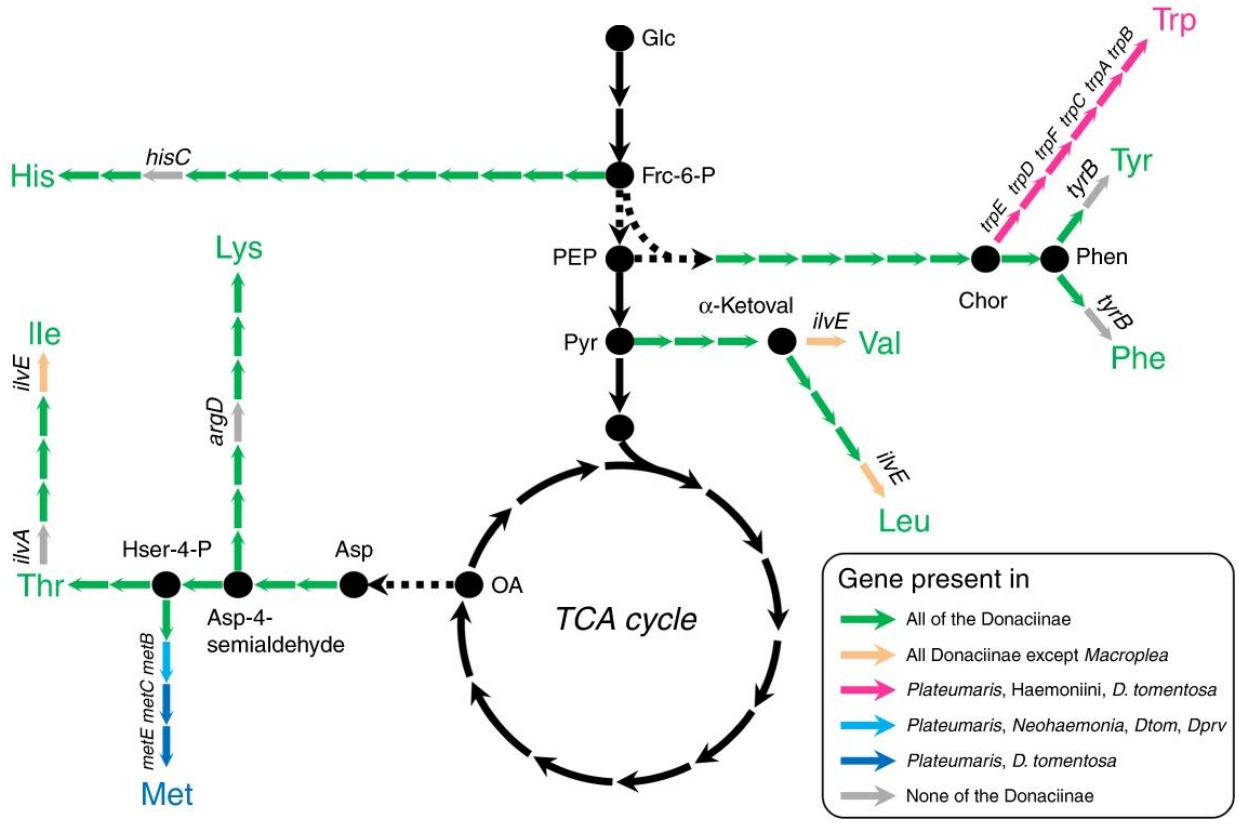

Figure 1.1: Amino Acid Biosynthesis Pathways showing Genes Encoded by the Symbionts of Donaciinae (Reis et al. 2020). *D. tomentosa*,Dtom = *Donacia tomentosa*; Dprv = *Donacia provostii*.

1.3 Genetics of Bacterial Symbionts

Genomes of bacterial obligate symbionts are on average small and show a bias towards A-T bases due to their stable intracellular, isolated environment (Anbutsu et al., 2017; Engl et al., 2018; McCutcheon & Moran, 2011; Reis et al., 2020; Vigneron et al., 2014) causing a genetic bottleneck and no possibility for horizontal gene transfer with other bacteria (Andersson & Kurland, 1998; Masson & Lemaitre, 2020; Wernegreen, 2015). Genes not beneficial for the symbiosis are lost through pseudogenization or deletions (Moran et al., 2008; Moran & Mira, 2001). The highly reduced genome of Donaciinae mainly encodes for essential amino acid biosynthesis pathways while lacking essential genes, such as regulatory genes (Reis et al., 2020).

1.3.1 Gene Regulation in Bacterial Symbionts

Beyond the qualitative presences/absence of benefits provided by the symbionts, we are interested in understanding how these benefits are regulated. In free living bacteria amino acid synthesis is tightly regulated by transcription rates and enzyme activity, primarily controlled by feedback repression and inhibition triggered by the end product (Umbarger, 1978; Wendisch, 2007). In *Buchnera*, a bacterial symbiont of aphids, genomic data show a lack in regulatory mechanisms for genes involved in amino acid synthesis. The absence of transcriptional regulators, including attenuation systems, indicates that essential amino acid production by *Buchnera* is unlikely to be governed by product-based regulation (Douglas, 2003; Shigenobu et al., 2000; Tamas et al., 2002). Research on *Buchnera aphidicola* inspected the gene expression of amino acid biosynthesis pathway genes in aphids feeding on host plants supplemented with different amino acids. They show that *Buchnera* is able to regulate the gene expression of amino acid biosynthesis pathway genes, when they encode the transcriptional regulator (Moran et al., 2005).

1.3.2 Gene Regulation in Reed Beetles

In the symbionts of reed beetles, out of roughly 450 genes only 4 are predicted to be transcription factors. Unpublished transcriptomic studies on *M. mutica* and D. thalassina symbionts have found differentially expressed genes of amino acid biosynthesis pathways in the larval stage compared to the adult stage (Carvalho, A., Wingert, S., Kirsch R., Reichelt, M., Kaltenpoth, M. [in prep.]). Specifically, symbionts of larvae show an upregulation of some amino acid biosynthesis pathways compared to symbionts of adults of the same species.

In the study mentioned above, the following genes exhibited differential gene expression in *M. mutica*: carA – which plays a pivotal role in the arginine biosynthesis pathway. Together with carB, it forms the carbamoyl phosphate synthase enzyme complex (Crabeel et al., 1980).

Genes showing differential expression in both *M. mutica* and *D. thalassina* include: aroF – encoding for chorismate synthase, this gene is a crucial component of the aromatic amino acid biosynthesis pathway (Garner & Herrmann, 1985). hisG – which encodes for ATP phosphoribosyltransferase and is involved in the histidine biosynthesis pathway (Salgado et al., 2013).

It is known that the histidine biosynthesis pathway operon hisJQMP is being regulated by the arginine repressor ArgR (Caldara et al., 2007; Chu et al., 2013; Igarashi et al., 2010), as well as the arginine biosynthesis pathway operon carAB (Caldara et al., 2006; Charlier et al., 1988, 1992). In *Escherichia coli* the hisLGDCBHAFI operon is putatively regulated by DskA-ppGpp (Lee et al., 2012). The synthesis of ppGpp by the symbionts of Donaciinae is likely but not confirmed yet. Therefore, we wondered whether the hisLGDCBHAFI operon is regulated by the arginine repressor ArgR.

1.3.3 Regulation of Amino Acid Biosynthesis Pathways in Donaciinae Symbiont

Our goal is to investigate whether we can replicate the regulation of amino acid biosynthesis pathways in Donaciinae symbionts by supplementing particular amino acids. Thus, we aim to establish a connection between the supplemented amino acid, the genes that are upregulated or downregulated as a result, and any potential involvement of putative transcription factors. Therefore, we analyse the gene expression of biosynthesis pathway genes (carA, carB, argF, hisG and aroF) and the symbiont titre in *D. marginata* symbiotic organs under an increased amino acid availability. We supplement dissected symbiotic organs with arginine and measured the gene expression of carA, carB, argF and hisG. Furthermore, we supplement L-3,4-Dihydroxyphenylalanin (DOPA) – the derivate of tyrosine – and measure the gene expression of aroF. Sustaining symbiotic bacteria alive can be challenging, even within symbiotic organs. Here, we are applying the supplementation assay method outlined in Anbutsu et al. 2017 to study the gene expression of the symbionts of *D. marginata*.

1.4 Objective of this Thesis

In this project, we zoom into the relationship between reed beetles and their symbionts, spanning from ecological aspects of the symbiosis to molecular ones. We explore connections between the amino acid content of host plants and the loss of amino acid biosynthesis capabilities in the symbiont. Furthermore, we examine how the loss of specific amino acid biosynthesis pathways in the symbiont impacts the cocoon chemistry. Additionally, our research takes a closer look at the regulatory mechanisms controlling these amino acid biosynthesis pathways within the symbiont.

By integrating information spanning from ecological to molecular facets, we aim to provide a comprehensive and precise description of these interactions. This multidimensional approach allows us to offer a more holistic view of the complex symbiotic relationship between reed beetles and their symbionts.

2 Material and Methods:

2.1 Quantification of Free Amino Acids in Host Plant Tissue

2.1.1 Collection & storage of plant samples

Root and leaves of host plant samples were collected during summer 2022 in three locations around Germany (Supplement Table 1). The samples were flash frozen in liquid nitrogen and stored at -80°C.

2.1.2 Processing Plant Samples for LC/MS

Samples were freeze dried in 50 ml falcon tubes using the freeze dryer "ALPHA 2-4 LD" (Martin Christ). Afterwards 5-7 metallic beads of two different sizes were added to the tubes and samples were grinded using the paint shaker "Skandex S-7" (Fluid Management*).* Around 5-15 mg of the dry weight was added to 1 ml 100 % Methanol and shaken for 30 minutes. 50 µl of the raw extracts were diluted with 450 µl of H_2O containing 10 µg/ml ^{13}C, ^{15}N labeled algal amino acids (Isotec, Miamisburg, USA) and 5 µg/ml of D5-tryptophan (Cambridge Isotope Laboratories, Andover, MA, USA) as internal standards for amino acid quantification.

2.1.3 LC-MS/MS

The following procedure was carried out by Michael Reichelt from the Department of Biochemistry at the Max-Planck-Institute for Chemical Ecology in Jena. The diluted extracts (see 2.1.2) were analyzed using LC-MS/MS (liquid chromatography-mass spectrometry/mass spectrometry). The analytical method was performed as described in (Crocoll et al., 2016) with following changes. The liquid chromatography system was linked to a QTRAP6500 mass spectrometer (Sciex, Darmstadt, Germany). The analysis involved the coupling of the reversed-phase liquid chromatography (LC) with electrospray ionization (ESI) in positive ionization mode to a tandem mass spectrometer operated in Multiple Reaction Monitoring (MRM) mode. The levels of individual amino acids in the sample were measured using corresponding ^{13}C and ^{15}N

labeled amino acid internal standards. However, for tryptophan, asparagine, and γ-aminobutyric acid (GABA), specific quantification methods were employed: tryptophan was measured using D5-tryptophan, asparagine was measured using ^{13}C, ^{15}N-aspartic acid with a response factor of 1.0, and GABA was measured using ^{13}C, ^{15}N-alanine with a response factor of 1.0.

2.2 Quantification of Bound Amino Acids of Cocoons

2.2.1 Collection & Storage of Cocoon Samples

Macroplea mutica cocoons were collected in Orth on the island Fehmarn (Germany) around august to September 2017 by Gregor Kölsch. *Donacia marginata* cocoons were collected in Horbach (Germany) in July 2021. Cocoons were stored at -20°C.

2.2.2 Acidic Hydrolysis of Bound Amino Acids from Cocoons

To analyze the bound amino acids of the cocoons, they needed to be hydrolyzed to remove the bounds and to be able to quantify the amino acid concentrations. To remove the free amino acids from the cocoons, samples underwent several washing steps with 80% methanol. First, cocoon samples were weighed and put into 4 ml vials. Around 1 ml of 80 % Methanol was added and the samples were incubated for 1 hour on a shaker. After removing the methanol, a new wash was performed, and fresh methanol was added. The samples were incubated for 3 days before repeating the initial methanol washing and then left to dry at room temperature under the fume hood.

Next, reaction tubes were filled with the washed cocoon samples and placed into Minivert Valve vials. In the Minivert Valve vials 500 µl of the reaction volume was pipetted as follows: 187.5 µL of HPLC-grade water, 62.5 µL of saturated Phenol solution, and 250 µL of 12M HCl. The vials were evacuated and refilled with argon three times to remove residual oxygen.

The samples were hydrolyzed for 24 hours at 110 °C. After letting it cool down, 100 µl of 80% Methanol was added to the reaction tubes, and the cocoon-methanol mix was transferred into 1.4 ml vials. Samples were diluted 100x with isotope labeled algal amino acid mixture ^{13}C, ^{15}N (Sigma-Aldrich) as internal standard, having 1ng/mL of total amino acid in 80% Methanol.

2.2.3 UHPLC-MS/MS

The following procedure was carried out by Rayko Halitschke from the mass spectrometry facility of the Max-Planck-Institute for Chemical Ecology in Jena. The diluted cocoon samples were analyzed using UHPLC-MS/MS (ultra-high-performance liquid chromatography-mass spectrometry/mass spectrometry). The analytical method was performed as described in (Schäfer et al., 2016). The separation was performed on a UHPLC ultimate 300 RS (Thermofisher Scientific) and the analysis was performed on an Elite EvoQ Triple quad-MS equipped with a HESI (heated electrospray ionization) ion source (Bruker).

2.3 Amino Acid Supplementation Assay

2.3.1 Insect Rearing, Collection and Dissection

Insect rearing was carried out in an enclosure susceptible to outside temperature and daylight cycle, using greenhouse reared *Sparganium erectum* plants. During the summer months of June to September 2022, approximately 200 adult insects were placed on the *S. erectum* plants and allowed to lay eggs.

The larvae were collected by displacing plants and inspecting soil and roots. They were placed back on *S. erectum* to be reared in water and stored in an incubator with a day/night cycle of 8 hours of light per day with a day temperature of 12°C , night temperature of 8°C at 60% humidity.

The four symbiotic organs were dissected out from larvae in a sterile phosphate-buffered saline (PBS) solution on a wax plate. This dissection was performed under a Leica M80 microscope.

2.3.2 Experimental Setup

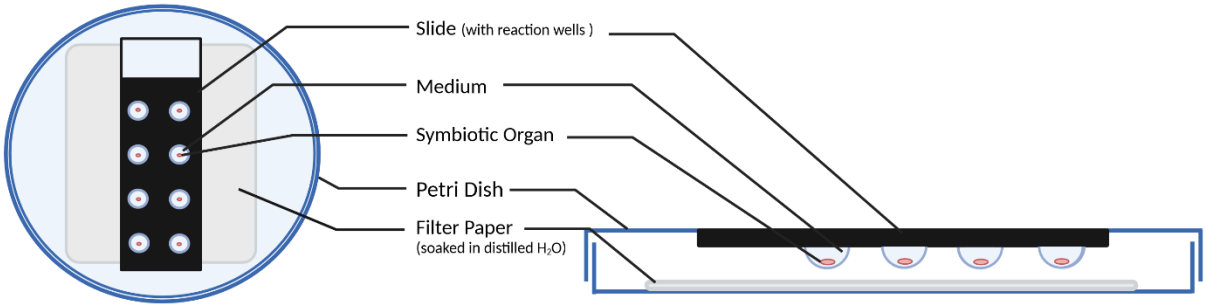

Figure 2.3.2.1: Experimental Setup for Supplementation Assay. A microscope slide with reaction wells was glued to the top of the petri dish, 15µl of medium droplets with symbiotic organs were placed into the reaction wells. Filter paper soaked with distilled water was placed on the bottom of the petri dish. The graphic was created with biorender.com.

The experimental setup including the medium solution was designed after Anbutsu et al. 2017. Four sets of conditions were prepared containing 25mM KCL, 10mM MgCl2, 250 mM Trehalose, 35mM HEPES, and different concentrations (16.7, 1.67, 0.167, 0mM) of the two treatment groups arginine or DOPA. The medium was filter sterilized under a fume hood using a 0.2 µm filter.

For the experiment, a microscope slide with 8 reaction wells (Specialty Printed Microscope Slide, 8 wells, 6mm by epredia) slide was glued to the top of a glass petri dish. The bottom of the dish was covered with filter paper soaked in distilled water. Droplets of 15 µl medium were placed into the reaction wells of the slide and one symbiotic organ per lava was placed in each droplet.

The prepared petri dish was then placed inside a polypropylene pox for eppendorf tubes filled with distilled water. This box was further placed in an incubator at 18°C.

The setup was incubated for 4 hours and afterwards the organs were flash-frozen in liquid nitrogen afterwards and stored at -80°C.

2.3.3 Symbiotic Organ Staining and Live Microscopy

Survival of the symbiotic organs in the medium described as in section 2.3.2 with 0mM of treatment (arginine or DOPA) was tested using live/dead staining. Therefore, symbiotic organs were dissected as described in section 2.3.1. Symbiotic organs were incubated in the setup for 2 hours, 4 hours or heat killed by placing the petri dish set up into a RapidFISH Slide Hybridisation

Oven (Boekel Scientific) letting it incubate at 60°C for at least 1 hour. Symbiotic organs were afterwards placed on Superfrost microscope slides (epredia) stained with a mixture of 5 µM propidium iodide (PI) and 0.835 µM SYTO9 (Thermofisher Scientific). After 5 minutes, the staining was washed away using PBS. A cover slip was placed on the slide. To avoid flattening the symbiotic organs, 2 layers of kyrogene labtape were put on the sides of the slide where the high precision cover slip was placed on. Images were taken using Leica DMi8 microscope and the imaging software Leica LAS X software. Afterwards, the symbiotic organs were slightly flattened to observe the bacteria and images were taken.

2.3.4 DNA and RNA Extraction

RNA and DNA was extracted from individual symbiotic organs using the MasterPureTM Complete DNA and RNA Kit (Epicentre). The extraction process followed the standard protocol described as follow. Symbiotic organs were homogenized in 300µl Tissue and Cell Lysis Solution (Epicentre MTC096H) using 3 metal beads with TissueLyser LT (Qiagen) at a frequency of 50 Hz for 2 minutes. 5 µl of Proteinase K (10 mg/nl) were added to the samples, incubated at 63°C for 15 minutes and placed on ice for 5 minutes afterwards. Next, 150 µl of MPC Protein Precipitation Reagent (Epicentre MMP095H) were added, the samples were vortexed for 10 seconds and the debris was pelleted by centrifugation at 14000 rpm for 10 minutes. After discarding the pellet, 500 µl of isopropanol were added to the supernatant. The samples were inverted 40 times to ensure thorough mixing and stored at -20°C over night. The following day, the samples were centrifuged (10 minutes, 14000 rpm), and the supernatant was discarded. The pellets containing both DNA and RNA Samples were resuspended in 358 µl of nuclease free H_2O and half of the samples (=179µl) were transferred into another Eppendorf tube to split them into DNA and RNA samples. RNA samples underwent DNase Treatment with DNA free Kit – Dnase Treatment and Removal (Thermofisher Scientific) with 2U of RNase free DNase I solution and 1x DNase Buffer. The DNA and RNA samples were incubated at 37°C for 15 minutes. Subsequently, 200 µl of Tissue and Cell Lysis Solution (Epicentre MTC096H) were added to the mixture, which was vortexed for 5 seconds. Then, 200 µl of MPC Protein Precipitation Reagent (Epicentre MMP095H) were added, followed by another round of vortexing before placing the samples on ice for 5 minutes. The debris was pelleted through centrifugation at 4°C (10 minutes, 14000 rpm). The pellet was

discarded, and 500 µl of isopropanol were added to the remaining samples. The tubes were inverted 40 times to ensure thorough mixing. The purified RNA was pelleted once again by centrifugation (4°C, 10 minutes, 14000 rpm), and the supernatant was discarded. To remove any remaining impurities, the RNA samples underwent two washes with 500 µl of 70% ethanol each, followed by centrifugation (10 minutes, 14000 rpm) after each wash. Finally, the extracted RNA samples were resuspended in 20 µl nuclease free H_2O and stored at a temperature of -80°C. The concentration of RNA and DNA was determined using the Nanophotometer N60 (IMPLEN).

2.3.5 Reverse Transcription of RNA into cDNA

Verso cDNA Synthesis Kit (Thermo Scientific) was used to reverse transcribe the extracted RNA into the complementary DNA (cDNA) by using the enzyme Reverse Transcriptase. Therefore, the manufacturer protocol was followed, mixing 4 µl of 5x cDNA synthesis buffer with 2 µl of dNTP mix, 1µl of a blend of random hexamers and anchored oligo-dT in a mixing ratio of 3:1, 1 µl of Verso Enzyme Mix and 11 µl of template RNA. The reverse transcription was performed in a Mastercycler epgradient S (Eppendorf) following the cycling program in table 2.3.5.1. The concentration of the generated cDNA was measured using a Nanophotometer N60 (IMPLEN) and the samples were stored at -20°C.

Table 2.3.5.1: Cycling Programm for Reverse Transcription of Extraced RNA into Complementary DNA.

	Temperature in °C	**Time**	**Number of Cycles**
cDNA synthesis	42	30 minutes	1 cycle
Inactivation	95	2 minutes	1 cycle

2.3.6 Quantification of Gene Expression

2.3.6.1 Primer Design and Primer Test

To quantify the gene expression of the target genes, primers were designed using the Primer Blast Tool by NCBI and the genome assembly of the enterobacteriaceae symbiont of *D. marginata* isolate DmarSym chromosome (NZ_CP046184.1). The primer product size was set to 150-250 base pairs and the melting temperature was set to 50 °C to 60°C. We selected primers pairs with having a GC content around 40-60 %.

Primers (table 2.3.6.1.1) were tested by performing a Polymerase Chain Reaction (PCR) in a Mastercycler epgradient S (Eppendorf) followed by a gel electrophoresis. Therefore, the "Taq 2X Master Mix" by New England BioLabs (NEB) was used as amplification kit following the manufacturer protocol, using 1 µl of forward and 1 µl of reverse primer, 1µl of purified *D. marginata* DNA as template, 25 µl of Taq 2x Master Mix and 22µl of distilled water. The following program (table 2.3.6.1.2) was used, the lid was preheated at 105°C.

Gel electrophoresis was performed in a 1.5 % agarose gel with SYBR Safe DNA Gel Stain (Invitrogen), 6X Orange dye (Thermofisher Scientific) and GeneRuler 50 bp DNA ladder (Thermo Scientific). The Gel was running at 100 V for 45-60 minutes. Pictures of the gel were taken using GeneGenius Bio Imaging System Gel Documentation UV Transilluminator (Syngene).

Primer amplicons were purified following the standard protocol of DNA Clean & Concentrator -5 (Zymo Research). Around 100 µl of primer amplificon were used as template and mixed with 500 µl of DNA Binding buffer and transferred to a Zymo-Spin™ Column in a collection tube. The samples were centrifuged for 30 seconds at 10 000 rpm. The flow-through was discarded, followed by a two washing steps by adding 200 µl of DNA Wash Buffer to the column and centrifugation for 30 seconds at 12 000 rpm after the first wash and 14 000 rpm after the second wash. The column containing the purified DNA was transferred into a 1.5 ml microtube. The purified DNA was eluted by adding 33 µl of distilled water on the column, followed by 5 minutes of incubation at room temperature and 30 seconds of centrifugation at 12 000 rpm. Concentration of the purified DNA was measured using a Nanophotometer N60 by IMPLEN. The DNA amplicons were stored at -20°C.

Table 2.3.6.1.1: List of Primers for Quantification of Gene Expression *via* qPCR.

Primer	Sequence	Direction	Target Gene
Dmar_S_carA_q_F	GGATATAGGTCATCATGGAAGTGT	Forward	carA
Dmar_S_carA_q_R	AATAGATGCATCATGTGGAC	Reverse	carA
Dmar_S_carB_q_F	AAGACCTGATGTTATTTTGCC	Forward	carB
Dmar_S_carB_q_R	TGTGAACTACTAAACAATGAGG	Reverse	carB
Dmar_S_argF_q_F2	CCTTTAGCTTAATAGAGGCA	Forward	argF
Dmar_S_argF_q_R2	TTCTCCCATAGATACCCAAA	Reverse	argF
Dmar_S_aroF_q_F	TCATTCCTTTAATATAAACAAGGGG	Forward	aroF
Dmar_S_aroF_q_R	GCTCCAATAGCTATCCAACT	Reverse	aroF
Dmar_S_hisG_q_F	AGAAGCACATGGAATTAAGG	Forward	hisG
Dmar_S_hisG_q_R	CCTATTAATGGGAAAATAGTTGGAT	Reverse	hisG
Dmar_S_GroEL_qF2	CCAAAGCTAATGATGTAGCAGGA	Forward	groEL
Dmar_S_GroEL_qR2	AGGATTCATACCAGCAGCAAC	Reverse	groEL

Table 2.3.6.1.2: Polymerase Chain Reaction Program for Testing Efficiency of Primers.

	Temperature (°C)	Time (min:sec)
Initial denaturation	95.0	0:30
40 Cycles	95.0	0:20
	53.0	0:30
	68	1:00
Final Extension	68.0	5:00
Hold	10.0	0:00

2.3.6.2 Real Time Quantitative Polymerase Chain Reaction (qPCR)

Gene expression of symbionts from supplementation assay (section 2.3.1) was measured via Real Time Quantitative Polymerase Chain Reaction (qPCR). The expression of carA, carB, hisG and argF were measured in symbiotic organs supplemented with arginine and the expression aroF was measured in symbiotic organs supplemented with DOPA. Gene Expression of groEL was used as housekeeping gene (HKG). qPCR was performed in a CFX Connect Real Time System (Bio-Rad). Therefore, Biozyms Blue S'Green qPCR Separate ROX Mix was used following the standard protocol. 10µl of 2x qPCR S'Greens BlueMix, 0.8 µl Forward, 0.8 µl Reverse Primer (table 2.3.6.1.1) and 15.52 ng of template cDNA were mixed and filled up with distilled water to up to 20µl of reaction volume. The purified amplicons from the primer test (section 2.3.6.1) were used for the standard curve in a 10-fold dilution series of 10^{-1} ng/µl to 10^{-7} ng/µl. The cycling program is given in table2.3.6.2.1.

Table 2.3.6.2.1: Quantitative PCR Cycling Program.

	Temperature (°C)	**Time (min:sec)**
Initial Denaturation	95	3:00
40 Cycles	95	0:15
	57	0:30
Melting Analysis	95	0:10
	55	0:05
	Increase from 55°C to 95°C	in 0.5°C steps

2.4 Data Analysis and Plotting

2.4.1 Plotting

All graphics were plotted with R (version 4.3.0) in RStudio (version 2023.3.1.446). Heatmaps were made using package "pheatmap" (version 1.0.12) and scatterplots were made using package "ggplot2" (version 3.4.2).

2.4.2 Data Analysis

2.4.2.1 Cocoons

In order to statistically analyze if the concentration of each amino acid differs in the two species, Kruskal-Wallis-Tests for each amino acid were performed in RStudio (see version above) followed by Bonferroni correction (p-adjust = 0.003125).

2.4.2.2 Normalization of Gene Expression

The gene expression measured via qPCR was normalized performing the Pfaffl Method (Pfaffl, 2001) using the materials provided in https://toptipbio.com/pfaffl-method-qpcr/, consulted on the 4th of June 2023. The gene expression levels of each symbiotic organ were normalized to the control organ (with 0 mM DOPA or arginine treatment) from the same specimen. First, the slope and intercept of the standard curves for each gene were calculated using R package "PCR" (version 1.2.2). Some datapoints from standard curve were excluded (Figure *2.4.2.2.1*) as they were out of line and therefore had a notable influence on the intercept and slope of the standard curve. The primer efficiency in percent was calculated using the slope of the standard curve with: primer efficiency = $(10^{-1/Slope} -1)*100$. The primer efficiency was then converted into converted primer efficiency (E) = (Primer efficiency(%)/100) +1. Delta cycle threshold (ΔCt) for gene of interest (GOI) and housekeeping gene (HKG) was calculated with using ΔCt = Ct control – Ct treatment. The Gene expression ratio (GER) was then calculated with GER = $(E_{GOI})^{\Delta Ct\ GOI}/(E_{HKG})^{\Delta Ct\ HKG}$).

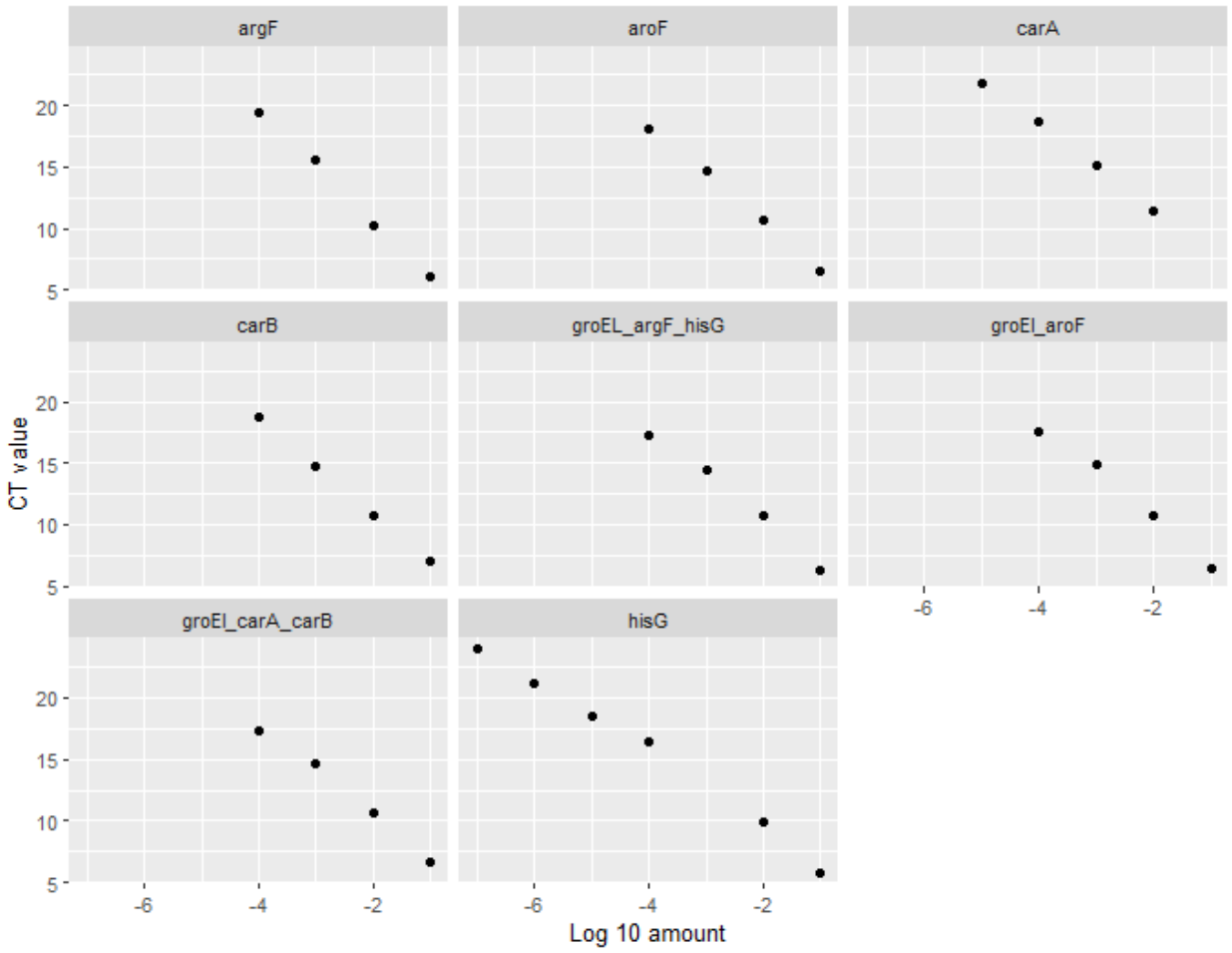

Figure 2.4.2.2.1: Calculated Standard Curves for argF, aroF, carA, carB, hisG and groEl (Housekeeping Gene). Data point missing in standard curve were excluded as they were out of line and had notable influence on intercept and slope of standard curve.

3 Results

3.1 Free Amino Acids in Roots and Leaves of Different Host Plants

All Donaciinae that have been screened for symbionts are associated with an Enterobacteriaceae symbiont (Reis et al., 2020), which is encoding for essential amino acid biosynthesis pathways. Interestingly, symbionts of some species have lost certain pathways, while others maintain them. For example, tryptophan is synthesized by the symbionts of the genus Plateumaris and the tribe Haemoniini, as well as *Donacia tomentosa* (Figure 1.1) (Reis et al., 2020). Methionine is exclusively synthesized by the symbionts of the genus Plateumaris and *Donacia tomentosa* (Figure 1.1 (Reis et al., 2020).

Donaciinae are known to be host specific in der choice of diet. We therefore wondered if there is a relation between the free amino acid concentration and the loss of certain amino acid biosynthesis pathways.

Furthermore, the different life stages of Donaciinae feed on different plant tissues with larval stage feeding on roots and rhizomes, and imago stage feeding on leaves. This raised the question whether the free amino acid concentration differed between plant tissues. Therefore, we compared the free amino acid concentrations between roots/rhizomes, and leaves of the host plants of Donaciinae. Plant samples were collected in two locations (Supplement Table 1) in Germany and free amino acids were quantified via LC/MS. The limitation of our study was the restricted sample size, which hindered our ability to employ non-parametric statistical tests.

We measured the free amino acid concentration in *Phragmites australis* and *Scirpus sylvaticus*, which are host plants of two Plateumaris species (Figure 3.1.1). Interestingly, we could not observe a great lack of tryptophan in the roots/rhizomes and leaves of these plants. Moreover, there was no obvious pattern indicating a difference in amino acids produced by the symbiont versus those not produced by the symbiont.

Additionally, we noticed that the concentration of methionine appeared to be low in both roots and leaves with no striking difference between *Phragmites australis*, *Scirpus sylvaticus* (host plants of Plateumaris who harbor symbionts encoding for methionine) and host plants of Donaciinae whose symbionts have lost methionine pathway.

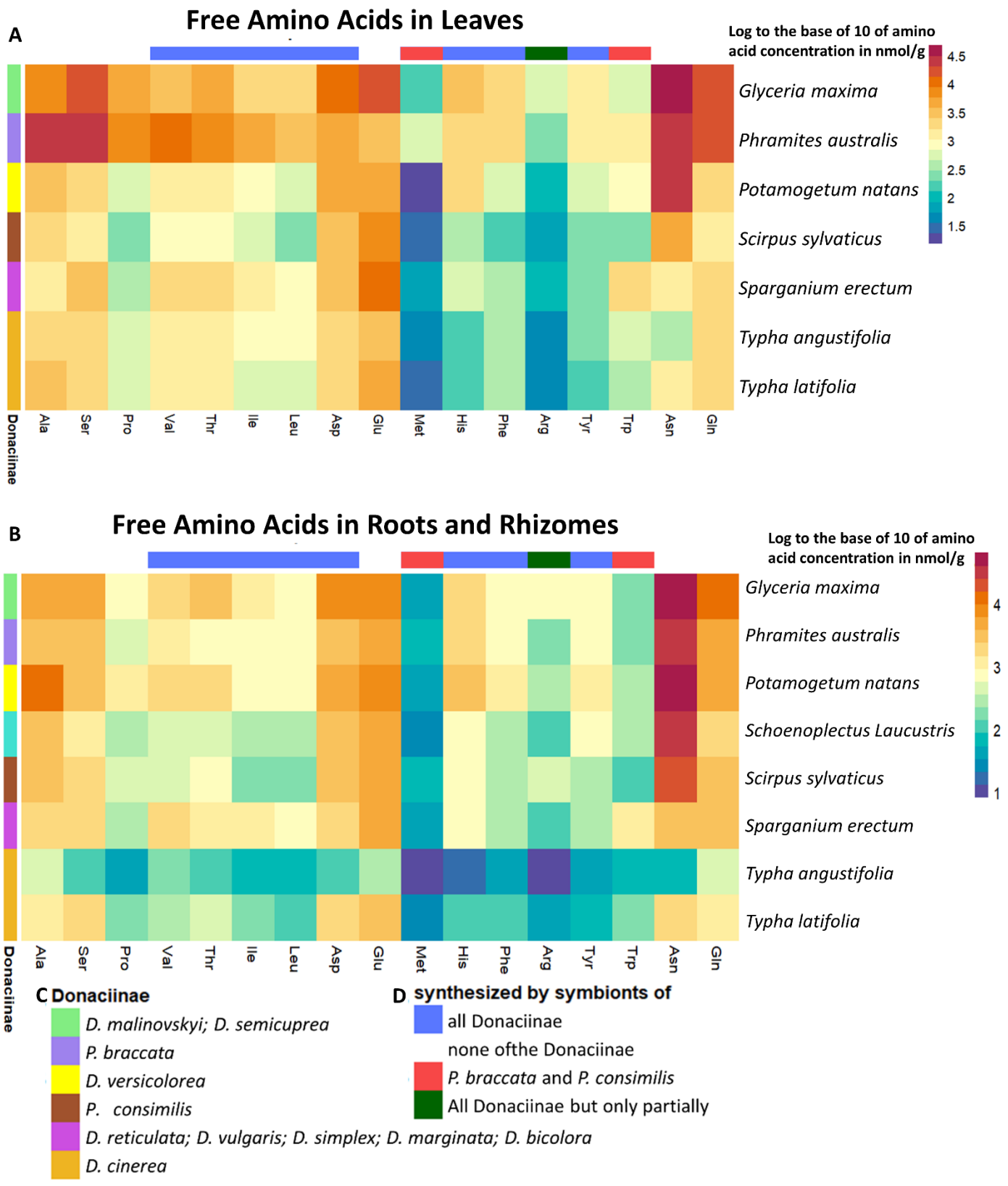

Figure 3.1.1: Free Amino Acid Concentrations in Roots and Leaves of Different Host Plants. Heatmaps show means of the free amino acid measured (log to the base of 10 amino acid concentration in nmol/g) in **A** leaves and **B** roots. Row annotations show the Donaciinae species that feed on the respective plants. Column annotation show which amino acids are being synthesized by the respective symbionts. **C** row annotation legend **D** column annotation legend.

3.2 Bound Amino Acids in Cocoons of two Donaciinae Species (*Macroplea mutica and Donacia marginata)*

Previous research conducted by Kleinschmidt and Kölsch (2011) indicated that the symbionts associated with Donaciinae play a role in the cocoon formation by contributing amino acids for the building of the cocoons.

The two Donaciinae species *Macroplea mutica* and *Donacia marginata* harbor symbionts where the former one is encoding for the tryptophan biosynthesis pathway while the latter lost it (Reis et al., 2020). Thus, we wondered whether we can observe a difference in the concentration of tryptophan between the two species.

We performed acidic hydrolysis of bound amino acids followed by LC/MS to quantify the amino acid composition. To test for differences in the amino acid concentration of each measured amino acid between the two species we performed a Kruskal-Wallis (Supplement Table 2). We observed significant differences between the bound amino acid concentration of the cocoons of the two species for arginine with a 2.5-fold (*M. mutica* mean= 160076.17 nmol/g, std = 30857.64; *D. marginata* mean = 400640.30 nmol/g, std = 28630.09) difference (Kruskal-Wallis chi-squared = 10.588, df = 1, p-value = 0.001138), for histidine with a 3.5-fold (*M. mutica* mean= 14110.67 nmol/g, std = 5790.959; *D. marginata* mean = 48877 nmol/g, std = 17915.85) difference (Kruskal-Wallis chi-squared = 9.8941, df = 1, p-value = 0.001658) and for tryptophan with a 38.9-fold (*M. mutica* mean= 770.33 nmol/g, std = 181.2817; *D. marginata* mean = 19.8 nmol/g, std= 22.39444) difference (Kruskal-Wallis chi-squared = 10.604, df = 1, p-value = 0.001129).

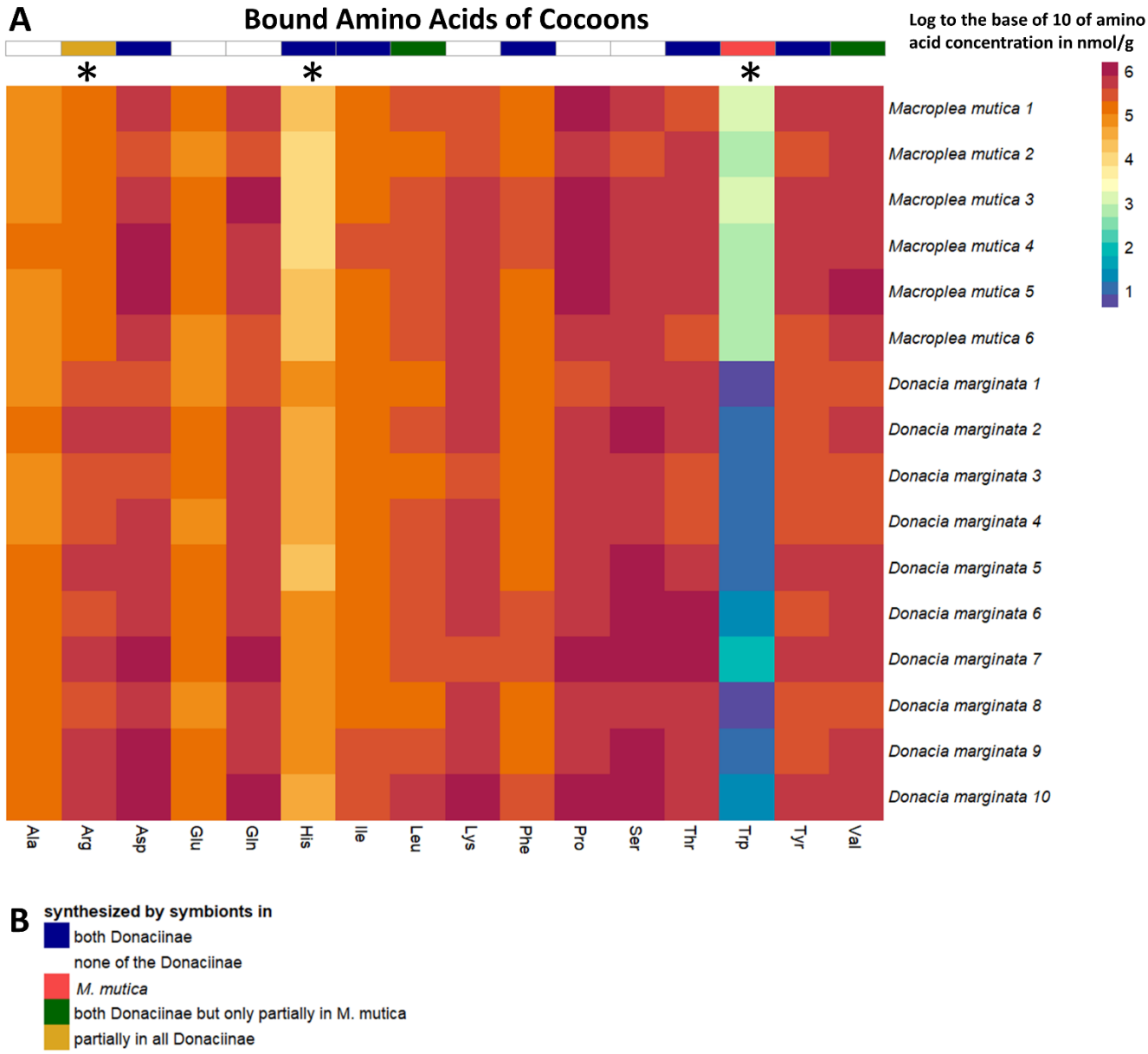

Figure 3.2.1: Bound Amino Acid Concentrations of *Macroplea mutica* and *Donacia marginata* Cocoons. Heatmaps show the bound amino acid measured (log to the base of 10 amino acid concentration in nmol/g) of *Macroplea mutica* and *Donacia marginata* cocoons. Column annotation show which amino acids are being synthesized by the respective symbionts. Significant differences between the bound amino acid concentration of the cocoons of the two species were observed for arginine (Kruskal-Wallis chi-squared = 10.588, df = 1, p-value = 0.001138), histidine (Kruskal-Wallis chi-squared = 9.8941, df = 1, p-value = 0.001658) and tryptophan (Kruskal-Wallis chi-squared = 10.604, df = 1, p-value = 0.001129) and are marked with *.

3.3 Amino Acid Supplementation Assay

3.3.1 Assessment of Survival of Symbiotic Bacteria in Symbiotic Organs

Symbionts with small genomes are often unculturable (Masson & Lemaitre, 2020) so to develop the supplementation assay, we first needed to determine how long the symbionts within the symbiotic organs can stay alive in the chemically defined medium (see section 2.3.2). For this purpose, we incubated the symbiotic organs for 2 and 4 hours at 18°C or heat killed them at 60°C. To assess the viability of the symbionts, we performed a live and dead staining with SYTO9 and PI. SYTO9 is a general nucleic acid stain that labels both live and dead cells, while PI is a nucleic acid stain that is not passing the membrane of live cells and is therefore only stains cells when they are dead and their membrane is damaged.

We imaged the symbiotic organs using microscopy at the different time points and compare them with heat-killed organs for reference. We observed host cell death while the symbionts are still being alive 4 hours post dissection (Figure 3.3.1.1). Afterwards, the organs were flattened to release the bacteria and imaged again. The images are presented in grey scale, with the merged image of PI and SYTO9 stains displayed in color. We observed that symbionts which have been mechanically released from dead host cells are still alive (Figure 3.3.1.2).

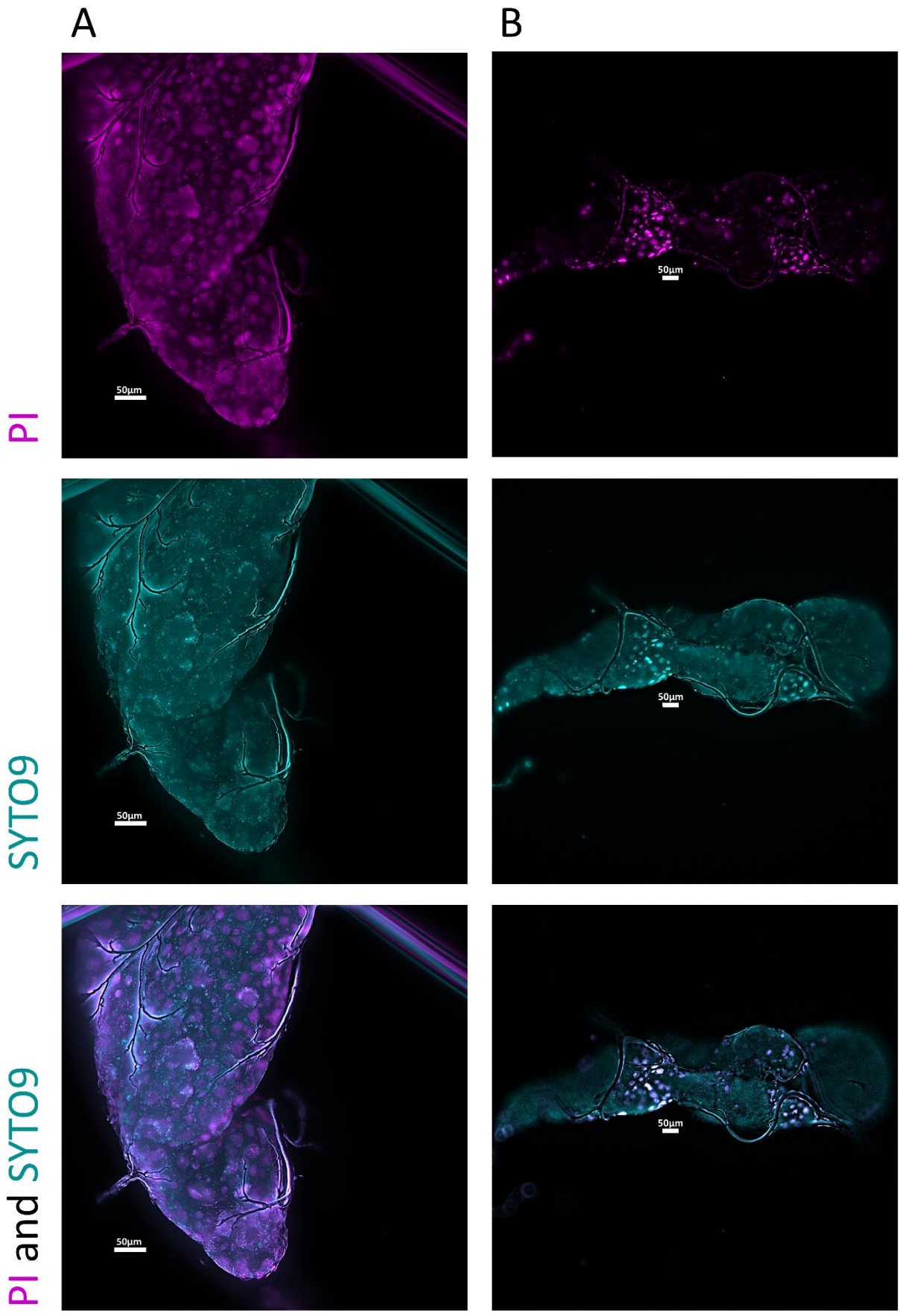

Figure 3.3.1.1: **Fluorescence Microscope Pictures of Symbiotic organs of *D. marginata*.** Pictures show symbiotic organs **A** heat killed at 60°C for 1 hour and **B** after incubation at 18°C for 4 hours. Live-dead staining with PI and SYTO9 before imaging. SYTO9 labels both live and dead cells, while PI labels only dead cells.

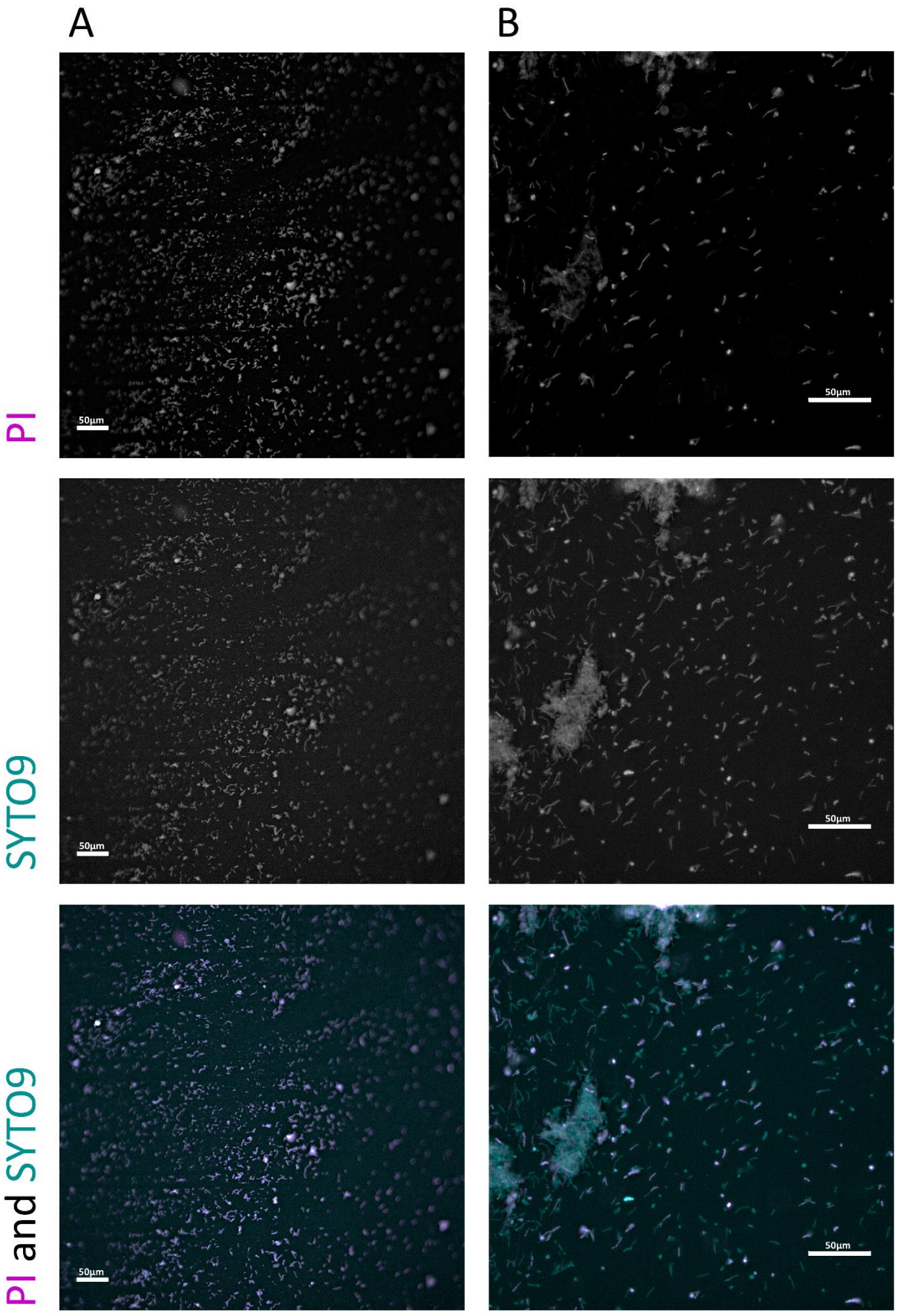

Figure 3.3.1.2: Fluorescence Microscope Pictures of Squished Symbiotic Organs of *D. marginata*. Pictures show squished symbiotic organs **A** heat killed at 60°C for 1 hour and **B** after incubation at 18°C for 4 hours. Live-dead staining with PI and SYTO9 before imaging. SYTO9 labels both live and dead cells, while PI labels only dead cells.

3.3.2 Supplementation Assay

Previous research has shown that symbionts of Donaciinae regulate the gene expression of amino acid biosynthesis pathway presumably according to the host needs in amino acids during development (Carvalho, A., Wingert, S., Kirsch R., Reichelt, M., Kaltenpoth, M. [in prep.]) To expand on this knowledge, we aimed at looking at gene expression changes when supplementing specific amino acids or derivatives.

To investigate this, we dissected symbiotic organs and directly supplemented them with different concentrations (16.7, 1.67, 0.167 mM or 0 mM as control) of either arginine or DOPA (a derivative of phenylalanine/tyrosine). As tyrosine and phenylalanine are hard to dissolve in water, we decided to supplement the derivate DOPA which can be dissolved around 3.3 g/L (Manufacturer: Sigma-Aldrich, Product Information, Product Number D 9628). For the highest concentrated treatment group, we selected a concentration of 16.7 mM, which represents the maximum solubility of DOPA in water.

In symbiotic organs supplemented with DOPA, we analyzed the gene expression of aroF (Supplement Table 3), a gene that is involved in the biosynthesis pathway of phenylalanine and tyrosine in the symbiont. We observed that the gene expression of aroF, when supplemented with 16.7 mM, 1.67 mM or 0.167 mM DOPA, appeared to be "upregulated" or close to a gene expression ratio of 1 when compared to the gene expression in symbiotic organs supplemented with 0 mM DOPA (Figure 3.3.2.1).

In symbiotic organs supplemented with arginine, we analyzed the gene expression of enzymes involved in the arginine and histidine biosynthesis pathways (carA (Supplement Table 4), carB (Supplement Table 5), hisG (Supplement Table 6), and argF (Supplement Table 7) in the symbiont (Figure 3.3.2.2).

In the symbiotic organs treated with 16.7 mM of arginine, we observed that the gene expression ratio of all four genes is similar to the gene expression ratio in symbiotic organs supplemented with 0 mM arginine (Figure 3.3.2.2). Furthermore, within the same organs, we observed that all target genes exhibited both up and down-regulation with varying intensities (Figure 3.3.2.2). It is also notable that although the symbiotic organs for each group are from the same larvae (one

organ per treatment group) they seem to have different gene expression patterns. However, as the sample size was under 5, it did not allow for any statistical test.

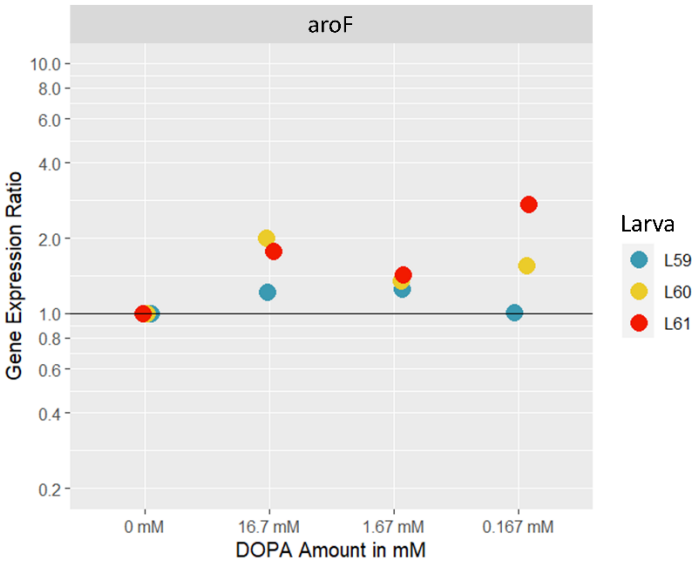

Figure 3.3.2.1: Gene Expression Ratio in DOPA Supplemented Larvae. The four symbiotic organs of *D. marginata* larvae, that are harboring the bacterial symbiont, were dissected and supplemented with 0 mM, 16.7 mM, 1.67 mM or 0.167 mM of DOPA for four hours. All four organs were distributed between the four concentrations of DOPA. Plots show the gene expression ratio of the aromatic amino acid biosynthesis gene aroF. The gene expression was normalized performing the Pfaffl Method (Pfaffl, 2001) with groEL as housekeeping gene.

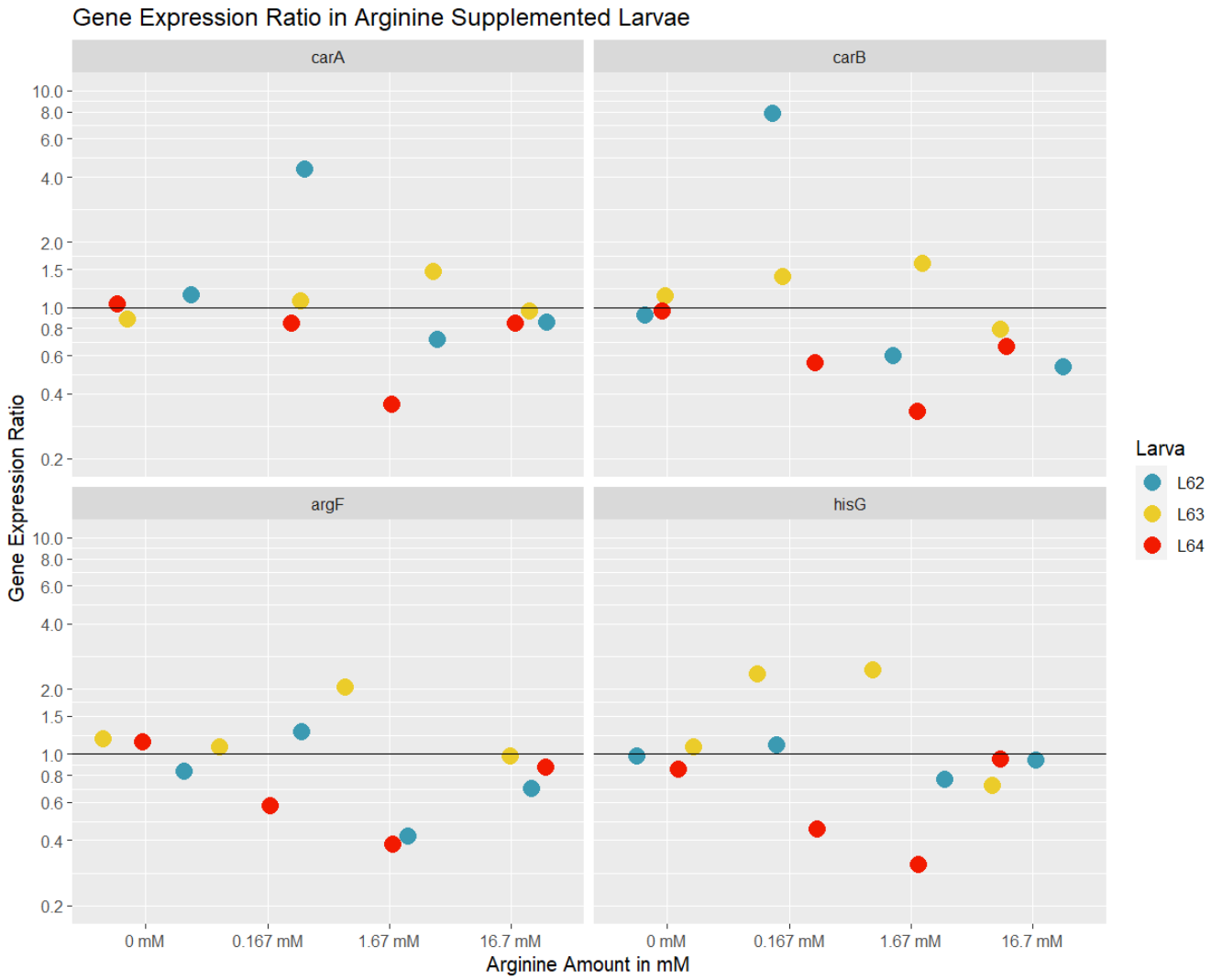

Figure 3.3.2.2: **Gene Expression Ratio in Arginine Supplemented Larvae.** The four symbiotic organs of *D. marginata* larvae, that are harboring the bacterial symbiont, were dissected and supplemented with 0 mM, 16.7 mM, 1.67 mM or 0.167 mM of arginine for four hours. All four organs were distributed between the four concentrations of arginine. Plots show the gene expression ratio of the arginine and histidine biosynthesis genes carA, carB, argF and hisG. The gene expression was normalized performing the Pfaffl Method (Pfaffl, 2001) with groEL as housekeeping gene.

4 Discussion

To understand the ecological niche of reed beetles and how its symbiotic relationship is contributing to it, it is crucial to understand why the relationship is kept and how it is regulated. In this study, we explored the complex dynamics of beetle-bacteria interactions in the reed beetles, focusing on the symbiont's role in amino acid supplementation. To achieve this, we conducted a multifaceted investigation including the analysis of free amino acid composition in various host plants, the examination of bound amino acid composition in cocoons of two Donaciinae species, and an exploration of gene expression in specific amino acid biosynthesis pathways within the bacterial symbionts, in response to different concentrations of the respective amino acids.

4.1 Apparent Low Methionine Concentration in Host Plants

Donaciinae feed on specific host plants (Kölsch & Pedersen, 2008; Stammer, 1935). Symbionts of some species have lost specific amino acid pathways throughout evolution, such as the tryptophan and methionine pathways (Reis et al., 2020). In this project we wondered if a relation between the availability of free amino acids in the host plants and the loss of these particular pathways can be observed.

A pattern in the composition of free amino acids that reflects the presence and absence of amino acid biosynthesis pathways encoded by the symbiont was not observed. Precisely, there was no evident deficiency in the concentration of tryptophan in host plants associated with beetles carrying symbionts that possess the tryptophan biosynthesis pathway, when compared to host plants of beetles whose symbionts lack this pathway. Consequently, we are not able to establish a direct link between a higher plant-based tryptophan concentration and the loss of the biosynthesis pathway in the symbionts.

Of note, it is interesting that methionine concentrations were consistently low in both root and leaf tissues across all plant specimens despite the fact that the methionine biosynthesis pathway is lost in some symbionts of Donaciinae and kept in others, e.g., in the genus Plateumaris. Further investigations are necessary to understand methionine's importance during the building of

cocoon. This would require cocoons of Plateumaris species, which however, are difficult to obtain.

Amino acid composition can vary considerably not only between plant species (Wilkinson & Douglas, 2003; Ziegler, 1975) but also between seasons (Douglas, 1993), environmental conditions (Girousse et al., 1996) and plant developmental age (Corbesier et al., 2001; Karley et al., 2002). In fact, we observed a difference in magnitude of two orders between samples from different specimen of the same species and collected tissue (Supplemental Figure 1). In *Sparganium erectum* – the host plant of several Donaciinae species – it has been demonstrated that newly established populations lean towards sexual reproduction, while established populations tend to favor vegetative reproduction (Piquot et al., 1998). Given that our plant samples were collected from the field without information about the population's establishment duration or reproductive behavior, precautions were taken to collect samples from the same species at a respectable distance from each other to avoid the collection of clones. This choice resulted in relatively small sample sizes and varying sample sizes across different plant species, which did not allow for non-parametric statistical testing nor any other statistical testing.

4.2 *D. marginata* and *M. mutica* Cocoons Differ in their Tryptophan Concentration

It has been suggested that the symbionts of Donaciinae are contributing to the formation of the presumably proteinaceous (Scherf, 1969) cocoons (Kleinschmidt & Kölsch, 2011) through the synthesis of essential amino acids. Some symbionts have maintained biosynthesis pathways while others have lost them (Reis et al., 2020), such as *M. mutica* and *D. marginata*, where the former maintains the tryptophan biosynthesis pathway while the latter lost it.

To assess the symbiont's contribution to cocoon formation, we compared the bound amino acid composition between the two species mentioned above. This allowed us to build a connection between variations in cocoon amino acid composition and the presence or absence of amino acid biosynthesis pathways in the symbiont’s genomes.

Only three of the 16 amino acids showed a statistically significant difference between the cocoons of the two species. We observed a 2.5-fold higher concentration of arginine and a 3.5-

fold higher concentration of histidine in *D. marginata*. Arginine and histidine biosynthesis pathways are present in both symbiont's genome of the two Donaciinae species. Strikingly, we observed a 38.9-fold higher concentration of tryptophan in *M. mutica* cocoons, which is only present in the symbiont's genome of *M. mutica*.

The cocoons of Donaciinae beetles possess remarkable durability due to the presence of multiple layers of a protein similar to sclerotin, providing exceptional resilience (Scherf, 1969). It is known that tyrosine and it's precursor amino acid phenylalanine are important for the cuticle melanization and sclerotization (Anbutsu et al., 2017; Arakane et al., 2016; Vigneron et al., 2014). Therefore, bacterial symbionts in various beetle species (Engl et al., 2018; Vigneron et al., 2014) are primarily encoding the shikimate pathway. This pathway plays a crucial role in supplying precursor amino acids necessary for cuticle hardening and pigmentation.

Denell et al. showed that tryptophan accumulates during development and serves as a constituent of soluble proteins or as free amino acids (Dennell, 1958). However, the mentioned study has specifically investigated the cuticles of larvae and pupae of the blue bottle fly (*Calliphora vomitoria)*, which require similar attributes such as water resistance and protective qualities. It is important to note that these cuticles differ in nature from the cocoons of reed beetles. As the exact proteins and other potential components that make up the cocoon are still unknown, further information is required to draw stronger conclusions.

M. mutica beetles are the only Donaciinae species not exclusively living in fresh water ponds but also in brackish water (Kölsch et al., 2010) thus having a higher salinity environment. Consequently, their cocoons need to be resistant to salinity. It is known that high salt concentrations in protein solutions disrupt local water structure, weaken intermolecular interactions, decrease solubility and stability, and enhance hydrophobic interactions. This ultimately leads to protein aggregation and loss of structure-function integrity (Sinha & Khare, 2014). One hypothesis for the function of the increased tryptophan concentration in the cocoons would be the adaptation to an environment with increased salt concentration. Research on proteins of other organisms dealing with high salt concentrations, namely halophilic archaea shows lower concentrations in apolar amino acids (to which tryptophan belongs) and a high

occurrence of amino acids with a low hydrophobic character (Fukuchi et al., 2003; Tadeo et al., 2009), which have polar or charged side chains, as well as a drop in lysine which is often replaced by arginine (Kastritis et al., 2007). Here, we observed a lower arginine concentration in *M. mutica* compared to *D. marginata* cocoons. However, it is important to acknowledge that cocoons are impermeable structures and a direct comparison to a living single-celled organism may not be straight forward. Thus, the ecological role of tryptophan and arginine in the cocoons of *M. mutica* requires further studies.

4.3 Amino Acid Supplementation Assay

4.3.1 Symbionts Survive in *ex*-vivo Symbiotic Organs Despite Host Cell Death

Due to the small genome of obligate symbionts (Anbutsu et al., 2017; Engl et al., 2018; McCutcheon & Moran, 2011; Reis et al., 2020; Vigneron et al., 2014) such bacteria are difficult to cultivate as they are lacking many genes essential for surviving outside the host. (Masson & Lemaitre, 2020). The smaller the genome, the more likely it cannot be cultured (Masson & Lemaitre, 2020).

However, there has been some success in culturing symbionts *in vitro* (Masson & Lemaitre, 2020; Noda et al., 2002; O'Neill et al., 1997; Welburn et al., 1987), such as *Spiroplasma poulsonii* (Masson et al., 2018) and *Sodalis glossinidius* (Hall et al., 2019). However, with a genome size of approximately 1.9 Mb (Paredes et al., 2015) and 4.3 Mb (Toh et al., 2006) respectively, they have a relatively large genome, compared to other symbionts (Anbutsu et al., 2017; Kiefer et al., 2021; Reis et al., 2020).

Currently, the technical threshold for cultivating bacteria is above a genome size of 0.5 Mb (Masson & Lemaitre, 2020). However, this threshold depends on the technology available and will probably decrease in the future. Since the genome size of *D. marginata* symbionts is roughly below 0.5 Mb, they are currently below the threshold for culturability.

In *Pachyrhynchus infernalis* the obligate symbiont *Nardonella,* despite having a genome size of 0.2 Mb was able to survive in dissected bacteriomes for a couple of hours in medium (Anbutsu

et al., 2017). We applied that same method and after 4 hours we observed that the symbionts were alive despite host cell death, even in absence of organic nitrogen source in the medium.

Many bacterial symbionts in beetles are restricted to an intracellular lifestyle, making it difficult to cultivate them outside of host cells (Masson & Lemaitre, 2020; Wernegreen, 2002). Donaciinae symbionts, although being intracellular for most of the beetle's development, are temporarily extracellular (Stammer, 1935). The symbionts are getting vertically transmitted during oviposition through a secreted coating on the laid eggs (Stammer, 1935). The eggs take 10-20 days to hatch if kept at constant 23°C (A. Carvalho, unpublished observation). The symbionts are extracellular at this stage (Stammer, 1935). The fact that the symbionts must be able to survive extracellularly for several weeks suggests that they can be potentially cultured. Nonetheless, further experiments are needed to determine whether the symbionts can be grown outside host cells.

4.3.2 Supplementation Assay

Despite only encoding for four transcription factors in *M. mutica* and *D. thalassina* (Reis et al., 2020), some amino acid biosynthesis pathway genes were upregulated in larval stage compared to adult stage (Carvalho, A., Wingert, S., Kirsch R., Reichelt, M., Kaltenpoth, M. [in prep.]). This included the genes carA only in *M. mutica* as well as aroF and hisG in both species' larvae.

In *E. coli* the histidine biosynthesis pathway operon hisLGDCBHAFI is regulated by DskA-ppGpp (Lee et al., 2012) and the operon hisJQMP by the arginine repressor ArgR (Caldara et al., 2007). Both are predicted in the symbiont as regulator. At the time of the experiment, we were unsure of the symbiont's capability to synthesize ppGpp and thus we decided to test whether ArgR could have been coopted to regulate the hisLGDCBHAFI operon as well.

A study on the obligate symbiont *Candidatus Blochmannia* from the ant tribe Camponotini has proposed that carAB, in conjunction with argI (ornithine carbamoyltransferase chain, which is annotated interchangeably with argF) could suffice for the arginine biosynthesis pathway (Williams & Wernegreen, 2010). This proposal is rooted in the understanding that the final step of this pathway, involving argininosuccinate synthase, is encoded by the host (Williams & Wernegreen, 2010). In aphids it is shown that some of the genes of amino acid biosynthesis

pathways are not encoded symbiont and are being taken over by the host (Hansen & Moran, 2011).

We aimed at investigating whether there is a difference in the gene expression of specific amino acid biosynthesis genes in the symbiont when it is supplemented with the respective amino acid or its derivative. Therefore, we supplemented symbiotic organs dissected from *D. marginata* larvae with DOPA or arginine. In symbiotic organs supplemented with arginine, we did not observe any discernible trend, whether it be an increase or decrease, in the symbiont's gene expression for either of the examined genes.

Under an increased availability of DOPA we expected a decrease in the symbiont gene expression of aroF. However, in symbiotic organs supplemented with different concentrations of DOPA we observed a trend towards an increase rather than a decrease in the gene expression although no conclusion can be made.

A previous study on the symbiotic interaction of the weevil *Sitophilus* and its symbiont shows that higher DOPA concentration accelerates the process of symbiont elimination (Vigneron et al., 2014). Research on the saw-toothed grain beetle *Oryzaephilus surinamensis* observed an increase in the symbiont titre as a response to the lack of tyrosine (the substrate for the synthesis of DOPA) (Wingert, 2020). Tyrosine is synthesized by the symbiont. Furthermore, the study showed that with DOPA being present, the symbiont titre tends to not change. Thus, it is being suggested that DOPA could be sensed by the host and could therefore be a regulatory molecule in host-symbiont regulation. A symbiont titre regulation from the host, could take place through the regulation of the symbiont's nutrition by the host. Research in weevils shows that the symbiont titre relies on the energy available through the host diet (Dell'Aglio et al., 2023). Therefore, investigations on the symbiont titres could provide more information. However, during our assessment of symbiotic bacteria survival within the symbiotic organs, we observed host cell death. Due to our inability to pinpoint the exact timing of host cell death, it remains uncertain whether a host induced change in symbiont tire could be possible within our experimental setup.

5 Conclusion and Outlook

In this project, we aimed at investigating the free amino acid composition of the host plants of Donaciinae, in order to understand why the symbionts might have lost some amino acid biosynthesis pathways throughout evolution while maintaining others. We analyzed the free amino acids in root and rhizome samples of several host plants. Interestingly, we observed a low concentration in methionine across all host species, an amino acid which is only being produced by the symbionts of some species. However, due to the small sample size, it was not possible to statistically compare the concentration of the amino acids across the host species. To gain more insight, the samples size should therefore be increased.

Furthermore, we aimed at investigating the bound amino acid composition of cocoons to observe if the composition is representing the amino acids being produced by the symbiont. We compared the composition of *D. marginata* and *M. mutica* whose symbionts differ in the production of tryptophan. Indeed, the amino acid composition differs for three amino acids (histidine, arginine and tryptophan) between the two species. The 38.9-fold higher concentration of tryptophan in *M. mutica* symbionts could reflect the biosynthesis pathway being present in the symbiont. Our results strengthen the claim that the symbiont contribute to the formation of the cocoon. While the differences in histidine and arginine are also interesting, they are both synthesized by both symbionts, so drawing any conclusions is difficult.

In order to investigate the gene expression of specific amino acid biosynthesis pathway genes under an increased amino acid availability we first verified if the symbionts survived after applying the protocol described in Anbutsu et al. 2017. As there was no previous information on how long the symbionts are able to survive in the dissected symbiotic organs, we firstly assessed the survival microscopically *via* imaging with live-dead-staining. After four hours in the medium without any organic nitrogen source, we observed host cell death while symbionts were still alive, suggesting culturability potential of this symbiont.

We supplemented dissected symbiotic organs with arginine or DOPA. The gene expression of the biosynthesis pathway genes did not show clear trends to draw definite conclusions. As already described by Stammer, we observed variability in size and shape between symbiotic organs of

the same specimen. This raised the question if this difference also reflects a different environment for the symbionts. We therefore propose to increase the sample size and distribute the symbiotic organs randomly between treatment groups.

Furthermore, we aimed at investigating the symbiont titres in supplemented organs via qPCR to assess if the titres are responding to the host need in the amino acids. This project is still ongoing. As we have only supplemented the organs for 4 hours it is questionable whether symbiont titre regulation is occurring as fast as the gene expression regulation. Different studies on pathogens have showed that DNA is still detectable in samples up to months after cell death with qPCR (Allmann et al., 1995; Josephson et al., 1993) resulting in a risk for false-positive results (Scheu et al., 1998; Wolffs et al., 2005). Within the span of a few hours differences are probably difficult to detect. A different approach could be to measure the gene expression of a gene known to be expressed on a constant level, as RNA is being degraded faster than DNA due to RNases in the environment.

In summary, we aimed at using an integrative to investigate the relationship of Donaciinae and their bacterial symbiont, spanning from ecological to molecular aspects. Understanding the ecology and life cycle of both host and symbiont can contribute for understanding both molecular (gene regulation and genomics) and physiological aspects (ex-vivo/in vitro potential of the symbiont).

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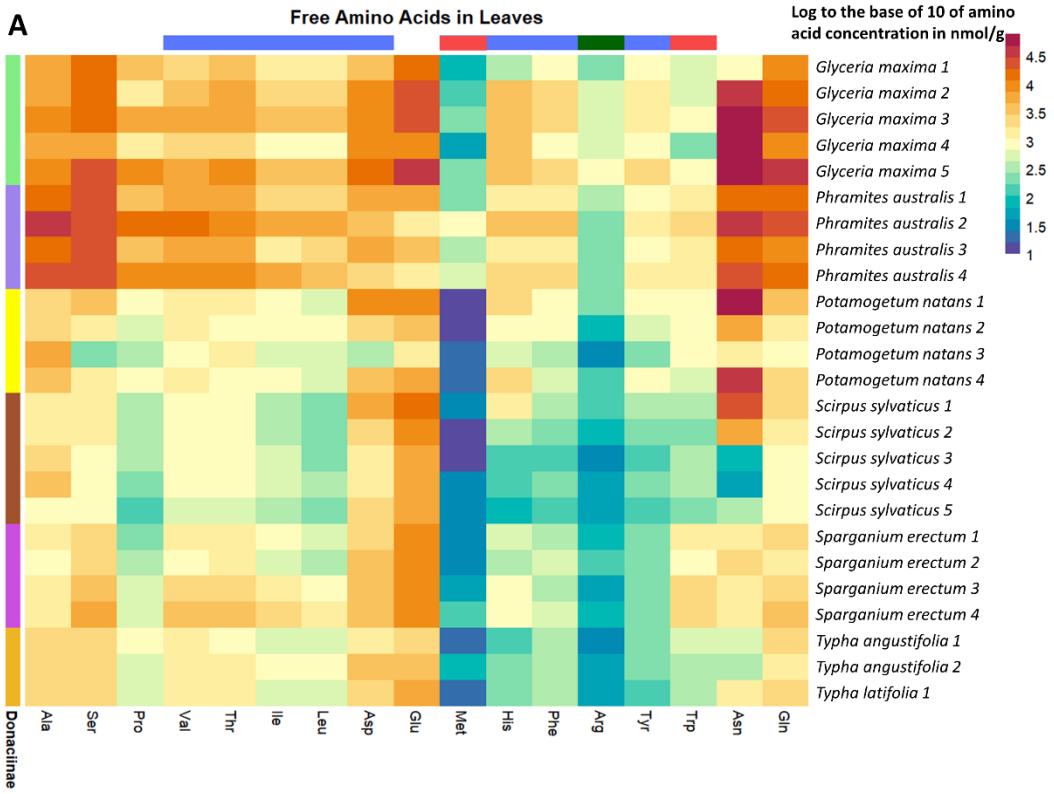
8 Supplement

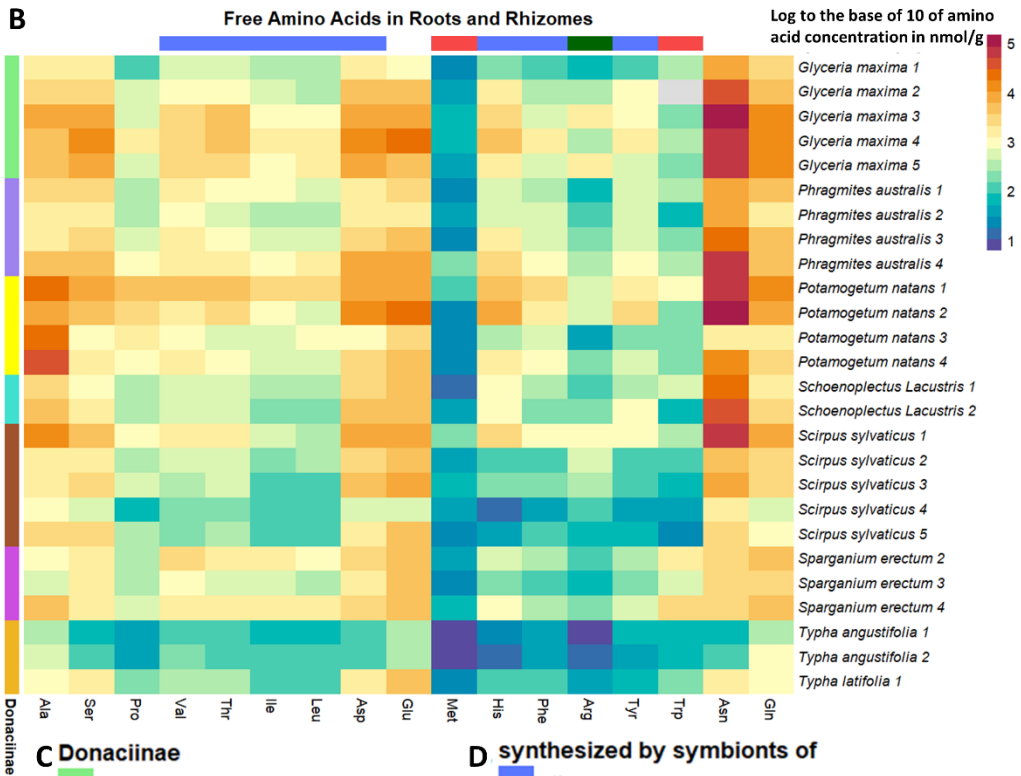
Supplement Table 1: Host Plants with Location and Time of Collection.

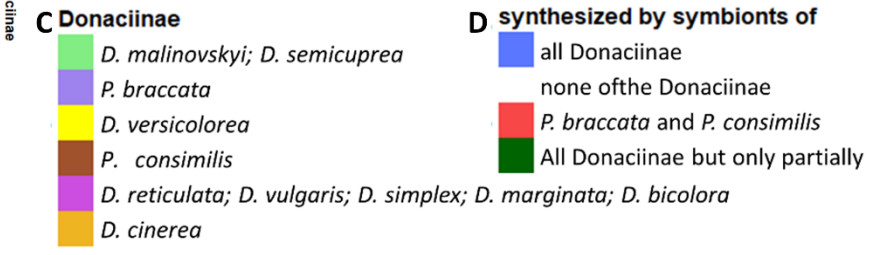
Plant Species	Collection Site	Date
Glyceria maxima	Franken, Germany	30.05.2022
Phragmites australis	Hainspitz, Germany	23.06.2022
Potamogeton natans	Truppenübungsplatz Jena Forst, Germany	11.05.2023
Schoenoplectus lacustris	Franken, Germany	30.05.2022
Scirpus sylvaticus	Franken, Germany	30.05.2022
Sparganium erectum	Franken, Germany	30.05.2022
Typha angustifolia	Franken, Germany	30.05.2022
Typha latifolia	Franken, Germany	30.05.2022

Supplement Table 2: Results of Statistical Analysis for differences in the Concentration of Bound Amino Acids in *D. marginata* and *M. mutica* Cocoons. Kruskal-Wallis-Test was performed followed by Bonferroni-correction (p-adjust = 0.003125). Significantly different results are marked with*.

Amino Acid	Chi^2	df	p-value
Ala	3.0118	1	0.08266
Arg	10.588	1	0.001138*
Asp	0.10588	1	0.7449
Glu	0.29412	1	0.5876
Gln	1.4235	1	0.2328
His	9.8941	1	0.001658*
Ile	0.75294	1	0.3855
Leu	0.29412	1	0.5876
Lys	0.011765	1	0.9136
Phe	0.57647	1	0.4477
Pro	3.8118	1	0.05089
Ser	4.2471	1	0.03932
Thr	1.4235	1	0.2328
Trp	10.604	1	0.001129*
Tyr	1.9882	1	0.1585
Val	7.3529	1	0.006695


A
Free Amino Acids in Leaves
Log to the base of 10 of amino acid concentration in nmol/g
4.5
4
3.5
3
2.5
2
1.5
1
Glyceria maxima 1
Glyceria maxima 2
Glyceria maxima 3
Glyceria maxima 4
Glyceria maxima 5
Phramites australis 1
Phramites australis 2
Phramites australis 3
Phramites australis 4
Potamogetum natans 1
Potamogetum natans 2
Potamogetum natans 3
Potamogetum natans 4
Scirpus sylvaticus 1
Scirpus sylvaticus 2
Scirpus sylvaticus 3
Scirpus sylvaticus 4
Scirpus sylvaticus 5
Sparganium erectum 1
Sparganium erectum 2
Sparganium erectum 3
Sparganium erectum 4
Typha angustifolia 1
Typha angustifolia 2
Typha latifolia 1
Donaciinae
Ala
Ser
Pro
Val
Thr
Ile
Leu
Asp
Glu
Met
His
Phe
Arg
Tyr
Trp
Asn
Gln


B
Free Amino Acids in Roots and Rhizomes
Log to the base of 10 of amino acid concentration in nmol/g
5
4
3
2
1
Glyceria maxima 1
Glyceria maxima 2
Glyceria maxima 3
Glyceria maxima 4
Glyceria maxima 5
Phragmites australis 1
Phragmites australis 2
Phragmites australis 3
Phragmites australis 4
Potamogetum natans 1
Potamogetum natans 2
Potamogetum natans 3
Potamogetum natans 4
Schoenoplectus Lacustris 1
Schoenoplectus Lacustris 2
Scirpus sylvaticus 1
Scirpus sylvaticus 2
Scirpus sylvaticus 3
Scirpus sylvaticus 4
Scirpus sylvaticus 5
Sparganium erectum 2
Sparganium erectum 3
Sparganium erectum 4
Typha angustifolia 1
Typha angustifolia 2
Typha latifolia 1
Donaciinae
Ala
Ser
Pro
Val
Thr
Ile
Leu
Asp
Glu
Met
His
Phe
Arg
Tyr
Trp
Asn
Gln


C Donaciinae
D. malinovskyi; D. semicuprea
P. braccata
D. versicolorea
P. consimilis
D. reticulata; D. vulgaris; D. simplex; D. marginata; D. bicolora
D. cinerea
D synthesized by symbionts of
all Donaciinae
none ofthe Donaciinae
P. braccata and P. consimilis
All Donaciinae but only partially

Supplementary Figure 1: Amino Acid Concentrations in Roots and Leaves of Different Host Plants. Heatmaps show the free amino acid measured (log to the base of 10 amino acid concentration in nmol/g) in **A** leaves and **B** roots. Row annotations show the Donaciinae species that feed on the respective plants. Column annotation show which amino acids are being synthesized by the respective symbionts. **C** row annotation legend **D** column annotation legend. Asparagine concentration differs by two orders of magnitude in *Potamogetum natans* root and rhizome samples as well as *Glyceria maxima* leave samples.

Supplement Table 3: AroF Gene Expression Calculation with Pfaffl Method (Pfaffl, 2001) in Symbiotic Organs Supplemented with 0 mM, 16.7 mM, 1.67 mM or 0.167 mM DOPA. Primer efficiency in percent was calculated using the slope of the standard curve with: **primer efficiency = $(10^{-1/Slope}-1)*100$** and converted into converted primer efficiency with **(E) = (Primer efficiency(%)/100) +1**. Delta cycle threshold (ΔCt) for gene of interest (GOI = argF) and housekeeping gene (HKG = groEL) was calculated with using **ΔCt = Ct control – Ct treatment**. The Gene expression ratio (GER) was then calculated with **GER = $(E_{GOI})^{\Delta Ct\ GOI}/(E_{HKG})^{\Delta Ct\ HKG}$**).

Primer efficiencies	Efficiency %	Converted efficiency E
Gene of interest (GOI) = aroF	81.88954739	1.818895474
Housekeeping gene (HKG) = groEL	84.96689963	1.849668996

Sample	GOI average Ct	ΔCt GOI	HKG average Ct	ΔCt HKG	Gene expression ratio
0 mM 59	20.04	0.00	15.47	0.00	1.00
0 mM 60	35.2	0.00	30.36	0.00	1.00
0 mM 61	22.89	0.00	17.46	0.00	1.00
16.7 mM 59	21.46	-1.42	17.18	-1.71	1.22
16.7 mM 60	34.4	0.80	30.71	-0.35	2.00
16.7 mM 61	24.28	-1.39	19.74	-2.28	1.77
1.67 mM 59	20.93	-0.89	16.7	-1.23	1.25
1.67 mM 60	33.34	1.86	29.04	1.32	1.35
1.67 mM 61	23.31	-0.42	18.45	-0.99	1.43
0.167 mM 59	21.48	-1.44	16.89	-1.42	1.01
0.167 mM 60	34.95	0.25	30.84	-0.48	1.56
0.167 mM 61	19.87	3.02	16.16	1.30	2.74

Supplement Table 4: CarA Gene Expression Calculation with Pfaffl Method (Pfaffl, 2001) in Symbiotic Organs Supplemented with 0 mM, 16.7 mM, 1.67 mM or 0.167 mM Arginine. Primer efficiency in percent was calculated using the slope of the standard curve with: **primer efficiency = $(10^{-1/Slope}-1)*100$** and converted into converted primer efficiency with **(E) = (Primer efficiency(%)/100) +1**. Delta cycle threshold (ΔCt) for gene of interest (GOI = carA) and housekeeping gene (HKG = groEL) was calculated with using **ΔCt = Ct control – Ct treatment**. The Gene expression ratio (GER) was then calculated with **GER = $(E_{GOI})^{\Delta Ct\ GOI}/(E_{HKG})^{\Delta Ct\ HKG}$**).

Primer efficiencies	Efficiency %	Converted efficiency E
Gene of interest (GOI) = CarA	92.56439157	1.925643916
Housekeeping gene (HKG) = groEL	88.80537453	1.888053745

Sample	GOI average Ct	ΔCt GOI	HKG average Ct	ΔCt HKG	Gene expression ratio
0mM 62	19.65	0.00	20.07	0.00	1.00
0 mM 63	18.2	0.00	17.1	0.00	1.00
0 mM 64	16.51	0.00	16.66	0.00	1.00
16.7 mM 62	18.29	1.36	18.52	1.55	0.91
16.7 mM 63	17.55	0.65	16.04	1.06	0.78
16.7 mM 64	16.62	-0.11	16.54	0.12	0.86
1.67 mM 62	19.05	0.60	18.89	1.18	0.70
1.67 mM 63	18.36	-0.16	17.89	-0.79	1.49
1.67 mM 64	17.71	-1.20	16.62	0.04	0.44
0.167 mM 62	20.17	-0.52	22.97	-2.90	4.49
0.167 mM 63	17.07	1.13	16.26	0.84	1.23
0.167 mM 64	16.55	-0.04	16.55	0.11	0.91

Supplement Table 5: CarB Gene Expression Calculation with Pfaffl Method (Pfaffl, 2001) in Symbiotic Organs Supplemented with 0 mM, 16.7 mM, 1.67 mM or 0.167 mM Arginine. Primer efficiency in percent was calculated using the slope of the standard curve with: **primer efficiency = $(10^{-1/Slope}-1)*100$** and converted into converted primer efficiency with **(E) = (Primer efficiency(%)/100) +1**. Delta cycle threshold (ΔCt) for gene of interest (GOI = carB) and housekeeping gene (HKG = groEL) was calculated with using **ΔCt = Ct control – Ct treatment**. The Gene expression ratio (GER) was then calculated with **GER = $(E_{GOI})^{\Delta Ct\ GOI}/(E_{HKG})^{\Delta Ct\ HKG}$**).

Primer efficiencies	Efficiency %	Converted efficiency E
Gene of interest (GOI) = carB	79.95633283	1.799563328
Housekeeping gene (HKG) = groEl	88.80537453	1.888053745

Sample	GOI average Ct	ΔCt GOI	HKG average Ct	ΔCt HKG	Gene expression ratio
0 mM 62	20.18	0.00	20.07	0.00	1.00
0 mM 63	18.88	0.00	17.1	0.00	1.00
0 mM 64	16.4	0.00	16.66	0.00	1.00
16.7 mM 62	19.22	0.96	18.52	1.55	0.66
16.7 mM 63	18.23	0.65	16.04	1.06	0.75
16.7 mM 64	17.24	-0.84	16.54	0.12	0.57
1.67 mM 62	19.98	0.20	18.89	1.18	0.53
1.67 mM 63	18.86	0.02	17.89	-0.79	1.67
1.67 mM 64	18.25	-1.85	16.62	0.04	0.33
0.167 mM 62	19.46	0.72	22.97	-2.90	9.64
0.167 mM 63	17.2	1.68	16.26	0.84	1.57
0.167 mM 64	17.2	-0.80	16.55	0.11	0.58

Supplement Table 6: HisG Gene Expression Calculation with Pfaffl Method (Pfaffl, 2001) in Symbiotic Organs Supplemented with 0 mM, 16.7 mM, 1.67 mM or 0.167 mM Arginine. Primer efficiency in percent was calculated using the slope of the standard curve with: **primer efficiency = $(10^{-1/Slope}-1)*100$** and converted into converted primer efficiency with **(E) = (Primer efficiency(%)/100) +1**. Delta cycle threshold (ΔCt) for gene of interest (GOI = hisG) and housekeeping gene (HKG = groEL) was calculated with using **ΔCt = Ct control – Ct treatment**. The Gene expression ratio (GER) was then calculated with **GER = $(E_{GOI})^{\Delta Ct\ GOI}/(E_{HKG})^{\Delta Ct\ HKG}$**.

Primer efficiencies	Efficiency %	Converted efficiency E
Gene of interest (GOI) = hisG	117.7640965	2.177640965
Housekeeping gene (HKG) = groEL	86.9558807	1.869558807

Sample	GOI average Ct	ΔCt GOI	HKG average Ct	ΔCt HKG	Gene expression ratio
0 mM 62	19.99	0.00	19.96	0.00	1.00
0 mM 63	18.63	0.00	17.06	0.00	1.00
0 mM 64	16.27	0.00	16.58	0.00	1.00
16.7 mM 62	18.79	1.20	18.35	1.61	0.93
16.7 mM 63	17.93	0.70	15.97	1.09	0.87
16.7 mM 64	16.73	-0.46	16.8	-0.22	0.80
1.67 mM 62	19.33	0.66	18.41	1.55	0.63
1.67 mM 63	18.51	0.12	18.03	-0.97	2.01
1.67 mM 64	18.14	-1.87	16.8	-0.22	0.27
0.167 mM 62	19.34	0.65	19.52	0.44	1.26
0.167 mM 63	16.99	1.64	16.04	1.02	1.89
0.167 mM 64	16.88	-0.61	16.32	0.26	0.53

Supplement Table 7: ArgF Gene Expression Calculation with Pfaffl Method (Pfaffl, 2001) in Symbiotic Organs Supplemented with 0 mM, 16.7 mM, 1.67 mM or 0.167 mM Arginine. Primer efficiency in percent was calculated using the slope of the standard curve with: **primer efficiency = $(10^{-1/Slope}-1)*100$** and converted into converted primer efficiency with **(E) = (Primer efficiency(%)/100) +1**. Delta cycle threshold (ΔCt) for gene of interest (GOI = argF) and housekeeping gene (HKG = groEL) was calculated with using **ΔCt = Ct control – Ct treatment**. The Gene expression ratio (GER) was then calculated with **GER = $(E_{GOI})^{\Delta Ct\ GOI}/(E_{HKG})^{\Delta Ct\ HKG}$**.

Primer efficiencies	Efficiency %	Converted efficiency E
Gene of interest (GOI) = argF	65.85628256	1.658562826
Housekeeping gene (HKG) = groEL	86.9558807	1.869558807

Sample	GOI average Ct	ΔCt GOI	HKG average Ct	ΔCt HKG	Gene expression ratio
0 mM 62	20.3	0.00	19.96	0.00	1.00
0 mM 63	18.97	0.00	17.06	0.00	1.00
0 mM 64	16.51	0.00	16.58	0.00	1.00
16.7 mM 62	19.09	1.21	18.35	1.61	0.67
16.7 mM 63	18.07	0.90	15.97	1.09	0.80
16.7 mM 64	17.18	-0.67	16.8	-0.22	0.82
1.67 mM 62	19.82	0.48	18.41	1.55	0.48
1.67 mM 63	18.98	-0.01	18.03	-0.97	1.83
1.67 mM 64	18.24	-1.73	16.8	-0.22	0.48
0.167 mM 62	19.7	0.60	19.52	0.44	1.03
0.167 mM 63	17.27	1.70	16.04	1.02	1.25
0.167 mM 64	17.13	-0.62	16.32	0.26	0.62