Investigating the mechanisms of metabolic regulation in the saw-toothed grain beetle-endosymbiont interaction

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DECLARATION

I, Soumi Bhattacharyya, 18MS202, a student of the Department of Biological Sciences of the Bachelor of Science – Master of Science (BS-MS) Program of IISER Kolkata, hereby declare that this MS project entitled "Investigating the mechanisms of metabolic regulation in the saw-toothed grain beetle-endosymbiont interaction" is my own work and, to the best of my knowledge, it neither contains materials previously published or written by any other person, nor it has been submitted for any degree or diploma or any other academic award anywhere before. I have used the originality checking service to prevent inappropriate copying. I also declare that all copyrighted material incorporated into this thesis is in compliance with the Indian Copyright Act 1957 (amended in 2012) and that I have received written permission from the copyright owners for my use of their work. I hereby grant permission to IISER Kolkata to store the thesis in a database which can be accessed by anybody as permitted by the institute guidelines of IISER Kolkata.

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This is to certify that the MS project entitled "Investigating the mechanisms of metabolic regulation in the saw-toothed grain beetle-endosymbiont interaction" submitted by Ms Soumi Bhattacharyya has been carried out under my supervision. This is submitted in partial fulfilment of the requirements for the award of Bachelor of Science – Master of Science (BS-MS) degree by the Indian Institute of Science Education and Research Kolkata, and this work has not been submitted elsewhere for a degree.

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Abstract

Insects are one of the most successful organisms in this world. A wide range of insects engage in various types of interactions with other organisms that shapes their behaviour, ecology and evolution. These interactions can be mutualistic, commensalistic or even parasitic. Mutualistic or symbiotic interactions have been one of the major factors behind the successful adaptations of insects to their challenging environments and niche. Microorganisms are essential in mediating the host insect's development and growth, although our knowledge about the molecular pathways regulating the host and microbe interplay is limited. The regulatory mechanisms through which the host can actively modulate its symbiont titre are investigated in this project by studying the saw-toothed grain pest beetle, Oryzaephilus surinamensis, that cannot synthesize aromatic amino acids and needs to acquire it from its diet or other external sources. The beetle is associated with a bacterial endosymbiont, Shikimatogenerans silvanidophilus, that resides intracellularly in a specialised organ in the haemocoel of the insect body, called bacteriome This endosymbiont supplements the host with precursors of tyrosine and L-DOPA, which are important for cuticle biosynthesis and melanization in the beetles. When host beetles are exposed to glyphosate and metyrosine chemicals, that inhibit the synthesis of precursors of tyrosine and L-DOPA molecule, a change in the symbiont titer of the beetles is observed. Thus, the metabolites in the cuticle biosynthesis pathway might play an important role in signalling the host's need for tyrosine and subsequent regulation of the symbiosis. The specific molecularmechanisms governing regulation of the symbionts is poorly understood. In this project, the pathways involved in the endosymbiont regulation are investigated using RNA-seq-generated transcriptomic analysis, differential gene expression analyses and diet interference to identify differentially expressed receptors in beetles inhibited for DOPA synthesis, which might be involved in the regulation of endosymbionts. In addition to supplementing cuticle synthesis, symbionts in this beetle help the host to cope with environmental stress conditions like desiccation. When the beetles were subjected to dry environment, reduction in symbiont titers were observed indicating a potential role in host adaptation to low humidity. However, observations from this project suggest that extreme dry conditions pushes the beetle to focus more upon a quicker development and reproduction rather than improving their lifespan. This thesis provides insights into the molecular mechanisms of nutritional endosymbiont regulations in insects and the impact of humidity as a stress factor upon the phenotype of the beetles. Adiponectin receptors on the bacteriome has been found to play a critical role in the symbiont titer regulation, thus providing a direction to further investigation.

Contents

1

Introduction

There is a wide range of biodiversity on this planet, consisting of various species. Understanding the ecological significance of interspecies interactions is crucial for gaining insight into the complexity of natural ecosystems. Different types of interspecies interactions shape the dynamics of the ecosystem. Symbiotic relationships between organisms are one of the important interactions between organisms that drive the evolution of species. Symbiosis describes the extensive coexistence of different organisms. It encompasses a continuum of interactions ranging from parasitism, where one organism benefits from the other partner at its expense, to mutualism, where both partners benefit (de Bary, 1887; Frank, 1885). While symbiosis is a broadly defined concept, it is generally used to refer to mutualistic interactions (*Paracer, S., Ahmadjian, 2000*). Endosymbiosis, on the other hand, is a type of symbiosis in which one organism, often a microorganism, becomes integrated intracellularly within another organism, often within specialized host cells called "Bacteriocytes" (Buchner, 1953, Moran et al., 1998). Over time, it has been observed that microbes, in concert with environmental conditions, have directed the evolution of organisms resulting in the development of several mutualistic or symbiotic interactions between organisms. From simple microbial interactions to highly complex systems, organisms have engaged in a symbiotic relationship to survive better in their environment (Bosch et al., 2019, Daida et al., 1996). Insects being one of the majorly abundant species in the world, they co-survive with a lot of microorganisms and thus is a wonderful system to study interspecific symbiosis (Ishikawa, Book Chapter 1, 2003).

Insects exhibit a wide diversity of symbiotic relationships with various microorganisms,

such as fungi, bacteria, mites, nematodes, and others (Klepzig et al., 2009). These symbiotic associations can confer a range of benefits to both the insect host (macro-symbiont) and the micro-symbiont. These benefits may include direct or indirect nutrition, defence against plant or animal hosts, protection from natural enemies, improved development and reproduction, and communication (Klepzig et al., 2009). Additionally, micro-symbionts often benefit from transportation, protection from antagonists, and survival in extreme environments. The microsymbionts derive nutrition and a stable environment from the hosts. The outcomes of insect-symbiont interactions are often strongly influenced by other organisms and environmental factors, both existing internally and externally, of the organisms. Therefore, studying the complexities of symbiotic interactions in insects is important for comprehending various ecological aspects of nature. Endosymbiotic association with the host can lead to development of interdependence between the two organisms that can change the molecular pathways and processes over evolutionary timescale. Advantageous genetic modifications due to the ensodymbiont-host interaction can remain conserved through natural selection (Rafiqi et al., 2022)

There are various adaptations of symbiosis that have happened even in bacteria, some of which have been reported to be that of endosymbiosis. The endosymbiotic bacteria supports the host insect by supplementing the host with essential amino acids. Therefore the host insect continues to get the support of the essential nutrients even when they are living on an unbalanced diet like vertebrate blood or plant sap (Douglas 2009). The symbiotic interactions also help the insects to defend themselves against stressful environmental factors like humidity (Engl et al., 2017) and temperature (Brumin, Kontsedalov, Ghanim, 2011). Bacterial symbionts benefit from the host-derived nutrients like amino acids and sugars (Garcia et al., 2014). This mutually beneficial relationship between the insects and their microsymbionts aided the insects to survive better in diverse ecological niches. This interaction have been so important for the insects that the microorganisms have got integrated into the physiology of the host insects and the symbiotic bacteria live in the insect body in bacteriocytes.

Coleoptera is the largest and one of the most diverse order of insects (Wielkopolan et al., 2016). Coleopterans are well known for their thick and hard cuticular exoskeleton, which plays a vital role in protecting the insect against various biotic factors like predators and abiotic factors like temperature, humidity, and insecticides and thus is one of the important factors behind the evolutionary success of the beetles (Arakane 2016, Noh et al., 2016). The cuticular exoskeleton is comprised of cuticular proteins and chitins. Tyrosine amino acid is the primary precursor needed to synthesize and melanize the cuticle in insects, and tyrosine, in turn, is derived from the amino acid phenylalanine (Anbutsu et al., 2017, Vigneron et al., 2014). The cross-link between cuticular proteins and chitin, building the thick and hard cuticle that protects the insects from predators are derived by metabolizing tyrosine (Arakane 2016). Further, down the cuticle biosynthesis process, hydroxylation of tyrosine

forms 3,4-dihydroxyphenylalanine (L-DOPA), and subsequently, L-Dopamine that is further utilized in the melanization and sclerotization process (Anbutsu et al., 2017). Oryzaephilus surinamensis is the saw-toothed grain pest beetle residing in the grain storage area in high numbers as a pest worldwide, attacking cereals and grain products. These beetles, belonging to the Coleopteran order of insects, also possess a thick and hard cuticular exoskeleton. O. surinamensis have a specialized organ near the gut called a bacteriome, which houses Bacteroidetes bacterial symbionts, Shikimatogenerans silvanidophilus. This bacteria is in a symbiotic relationship with the O.surinamensis beetles and play an important role in the tyrosine supplementation to the host and defence against environmental stress factors (Engl et al., 2018).

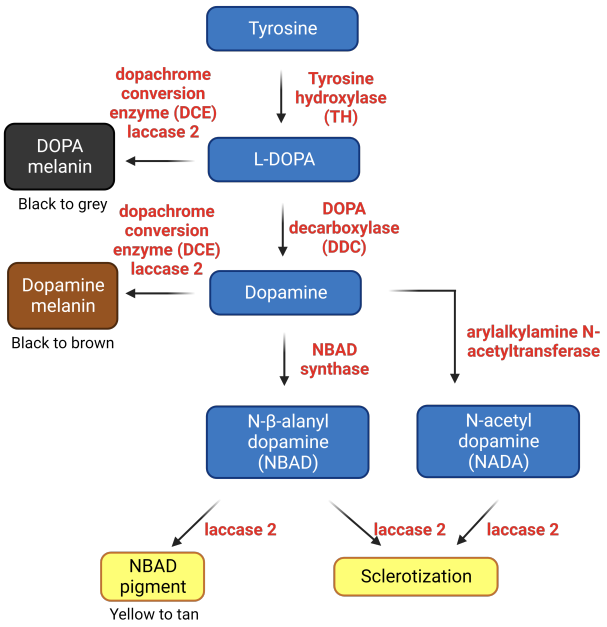

Figure 1.1: Tyrosine and L-DOPA utilization in cuticle melanization and sclerotization process. (Anbutsu et al., 2017) (Image recreated with BioRender.com)

Insects lack the ability to synthesize essential amino acids, such as tyrosine and phenylalanine, in their bodies and rely on their diet or supplementation by endosymbionts for these nutrients (Kramer Hopkins, 1987). Insufficient tyrosine and phenylalanine intake due to unbalanced diets can lead to thinner and lighter cuticles in insects, but tyrosine supplementation by endosymbionts promotes the development of thick and melanized exoskeletons, as observed in coleopteran insects like O. surinamensis (Engl et al., 2018) and cereal weevil Sitophilus (Vigneron et al., 2014), inhabiting the grain storage areas with low humidity and nutrient-deficient environments. Endosymbionts are critical in supporting the survival of beetles in their ecological niches by providing tyrosine supplementation for proper cuticle formation, which protects them from environmental stresses. The endosymbionts derive nutrition from the host and synthesize chorismate through the “Shikimate pathway” (pathway synthesizing tyrosine, tryptophan and phenylalanine), which is converted to prephenate by

the symbionts and supplied to the host. The host then synthesizes tyrosine from prephenate which is subsequently used to process with melanization and sclerotization. Insects, during their pupal stage and early adult life, develop their complete cuticle, which is the outer protective layer, while undergoing metamorphosis. During both metamorphosis and cuticle formation, insects are unable to feed and rely on stored nutrients obtained during their larval stage. The storage of tyrosine is also limited due to its toxic effects at high concentrations. Therefore the insect relies on its endosymbionts to supplement tyrosine or its precursor molecules, chorismate or prephenate, during the crucial period following emergence from pupation, when the young adult builds its thick and dark cuticle (Kiefer et al., 2021). Experimental removal of symbionts from O. surinamensis beetle (aposymbiotic beetles) resulted in less melanized and thinner cuticles, as well as increased synthesis of cuticular hydrocarbons (CHCs) in low humidity conditions and increased mortality due to desiccation stress, highlighting the crucial role of symbionts in cuticle synthesis and resistance against desiccation stress (Engl et al., 2018).

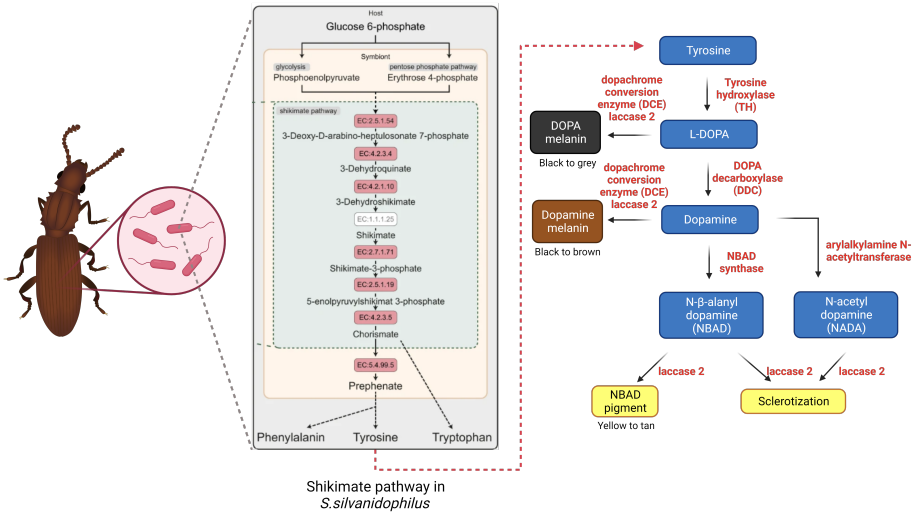

Figure 1.2: Schematic diagram of tyrosine supplementation by endosymbionts to the beetles. The endosymbionts derive nutrition from the host and through the shikimate pathway it synthesizes chorismite which is converted to prephenate by the symbiont and supplied to the host. The host then synthesizes tyrosine from prephenate and performs melanization and sclerotization. (Kiefer et al., 2021)

While endosymbionts play a crucial role in promoting the survival of insects, the maintenance of symbiont populations in bacteriocytes, or specialized organs known as bacteriomes, imposes a metabolic cost on the insects. The host has to maintain the symbionts by providing resources to them that the host could have otherwise used for its own growth and reproduction. It is reasonable to expect that such a trade-off would require an optimal balance to be maintained between the cost and the benefit. For example, In O. surina-

mensis beetles, the symbiotic females have a delayed onset of reproduction compared to aposymbiotic beetles (Engl et al., 2020). In Sitophilus, it was observed that the Sodalis pierantonius symbiont's population rises rapidly in the young adult beetles but again depletes in the later life stages of the beetles to get entirely eliminated after the completion of the cuticle synthesis (Vigneron et al., 2014). The rate of change of endosymbiont titer occurs at a faster pace in the wild weevil species S. oryzae and S. zeamais, which inhabit cereal fields, compared to the granary-associated weevil S. granarius species found in cereal storage facilities in temperate regions (Vigneron et al., 2014). These studies suggests that the endosymbiont population is tightly regulated to maintain an optimum cost-benefit balance. However, the mechanism behind such a balance is unclear. A study investigating the molecular mechanism of regulation of the symbiont titer in the weevils indicated that the L-DOPA molecule (in the cuticle biosynthesis pathway) might be the main regulatory molecule in the host-endosymbiont regulation in weevils. Supplementing the insects with L-DOPA resulted in a decrease in the symbiont population present in their bodies, and the clearance of these microorganisms occurred more quickly (Vigneron et al., 2014). However, there isn't enough evidence on the molecular level regulation of endosymbiont population in grain pest beetle, O. surinamensis yet.

To understand the regulation of symbiont titer by the host in response to its needs, the diet of the O. surinamensis beetles has been manipulated and subjected to different chemical treatments. Aromatic amino acid supplementation reduced the symbiont titer in the beetle. The availability of amino acids to the beetle dismissed their need to maintain the symbionts for aromatic amino acid supplementation and therefore resulted in the symbiont titer reduction. The host thus decreased the energy cost of symbiont maintenance (Kiefer et al., 2021).

Glyphosate is an extensively used herbicide that inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) by lowering its activity and targeting the Shikimate pathway (Sikorski et al., 1997). While being used as a herbicide, glyphosate can prove to be detrimental for insects by targeting the shikimate pathway in the bacteria in symbiotic relationships with the insect hosts (Li et al., 1987). S. silvanidophilus endosymbiont supplements the O. surinamensis beetle with tyrosine for cuticle formation through the shikimate pathway present in the bacterial symbionts. Glyphosate exposure to Oryzaephilus beetle helped to understand the direct link between the availability of tyrosine and symbiont regulation; it was also seen that impairing the tyrosine production can have a phenotypic effect on the insect as well. This treatment resulted in the beetle having a thinner and less melanized cuticle and also a reduced symbiont titer as well. It is speculated that the unavailability of tyrosine stopped the host from providing any tyrosine or nutrient to the symbionts to maintain its population in the bacteriome. Therefore, the titer reduces anyways due to a lack of proper nutrition (Kiefer et al., 2021). Metyrosine, an inhibitor of the tyrosine hydroxylase enzyme, when administered to the beetles through their diet, blocked the synthesis of

L-DOPA, resulting in an increased symbiont titer (Unpublished data).

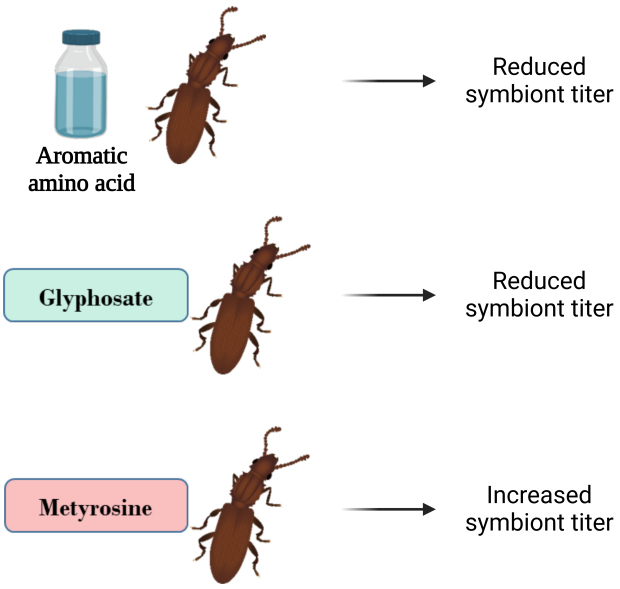

Figure 1.3: Change in symbiont titer in beetles under aromatic acid supplementation, glyphosate and metyrosine treatment. Aromatic amino acid supplementation reduces the symbiont titer in the beetle, glyphosate treatment reduces symbiont titer as well while the Metyrosine treatment increases the symbiont titer. Therefore, the symbiont titer changes when the host beetles are in different conditions. (Kiefer et al., 2021)

The possible ways to regulate the symbiont titer by the host can be -

- By controlling the production of nutrients for the endosymbiont
- By controlling the transport of nutrients from the host to the endosymbiont
- By controlling the metabolic cost to maintain symbiont population needed to be provided with nutrients

In this project, the molecular signalling pathways involved in the regulatory process have been investigated in the cuticle development stage of the O. surinamensis beetle. RNASeq data from the metyrosine-treated beetle and control treatment were analyzed to find out differentially expressed genes in the adults and the bacteriome. Further functional validation of candidate genes using drug-administered diet manipulation on beetles helped to find out receptors that might play in important role in the regulation process. The regulation of the symbiont from an ecological perspective, i.e. when the insect experiences environmental stress, has also been studied in this work. These results demonstrate a clearer idea of the molecular mechanisms of host-symbiont regulation, both in the insect developmental stage and from an ecological perspective.

2

Material and Methods

2.1 Insect rearing

Symbiotic and aposymbiotic O. surinamensis populations from Julius Kühn Institute, Berlin, were maintained for experimental use. Two hundred two-week-old beetles were collected using an aspirator and put in separate plastic boxes to maintain the populations at 28℃ temperature and 60% relative humidity. Oat flakes were used as the diet for beetles. Fluon was applied on the inner walls of the upper sides of the boxes to prevent the beetles from escaping. Symbiotic beetles needed for both pharmacological drug testing experiments and humidity experiments were taken from these main populations.

2.2 Transcriptomic Analysis - De-novo Transcriptome Assembly, Annotation, Mapping and counting reads

Samples for RNA sequencing were prepared from beetles that were fed oat flour flakes supplemented with either 10% glyphosate, 10% metyrosine or nothing as a control treatment. For each treatment, RNA extract samples were prepared from either a pool of 10 whole 5 days-old adult beetles (3 samples per treatment) or from a pool of bacteriomes dissected from 20 5 days-old adults (5 samples per treatment). The obtained RNA samples were then processed and sequenced using Illumina Next Generation Sequencing technology to perform Short read sequencing (Illumina HiSeq3000) at the Max Planck-Genome-Centre Cologne. The sequencing generated paired-end reads as output. The sequencing facility provided the

sequencing results after a quality check. The low-quality and adaptor sequence reads were removed from the RNA Sequencing output reads as described below.

From each treatment, a sequence reads library for the bacteriome and another library for the adult was prepared. The sequencing facility provided paired-end reads as RNA Sequencing outputs. Reads generated from the RNA Samples extracted from the bacteriome for metyrosine and glyphosate treatment had five replicates each, and that from the adult for each treatment had three replicates each. Some RNA samples were run in two different lanes. These RNASeq outputs were used to build a transcriptome de-novo.

2.2.1 De-novo Transcriptome Assembly

All the following steps have been done using the Galaxy online tool (https://usegalaxy.org/). For each replicate, reads were concatenated using the Concatenation tool to collate the forward and reverse reads. The quality of the concatenated files was checked using the FastQC tool. The trimmomatic tool was further used to trim the concatenated paired-end reads to maintain the good quality of the reads. Since O. surinamensis does not have a well-annotated genome, trimmed reads were further used to build de novo the transcriptome of O.surinamensis using the rnaSPAdes tool in Galaxy. An additional step of checking the quality of the transcriptome was performed using the rnaQUAST tool using Tribolium castaneum genome as reference as T. castaneum is the phylogenetically closest insect to O. surinamensis which has a well-annotated genome.

2.2.2 Transcriptome Annotation, Mapping and Counting Reads

The de novo transcriptome assembly was blasted against the Tribolium castaneum reference genome (Tcas5.2, INSDC Assembly GCA˙000002335.3 from EnsemblMetazoa). From the nucleotide BLAST output one T.cas sequence was assigned for one of our generated O. surinamensis sequence. This will help to build the reference transcriptome taking into account the reads with a high degree of homology between the two transcriptomes. Bowtie2 tool was further used to map or align each concatenated paired reads against the assembled transcriptome of O.surinamensis. The SalmonQuant tool was then used to quantify the abundance of transcript reads against the de novo transcriptome.

2.3 Differential Gene Expression Analysis

Differential gene expression analysis was performed using the DESeq2 tool in Galaxy online platform. The transcript count files were compared between (i) Bacteriome Control vs Bacteriome Metyrosine, (ii) Bacteriome Control vs Adult Control, (iii) Bacteriome Metyrosine vs Adult Metyrosine, (iv) Adult Control vs Adult Metyrosine. The DE analyses generated a list of differentially expressed genes between every two sets. The results of the

DE analyses were compared, filtered and prepared to select genes that have been highly expressed and might play a significant role in endosymbiont regulation. Nicotinic acetylcholine, Adiponectin and Kainate receptors were chosen to be targeted using agonist and antagonist drugs to be administered through the diet.

2.4 Pharmacological Drug Administration

Beetles were treated with the agonists and antagonists of Nicotinic Acetylcholine, Adiponectin and Kainate receptors. Nicotine (L-Nicotine 99+%, Thermofisher, Germany) was used as the agonist, and Bupropion (Bupropion Hydrochloride ¿98%, TCI) as the antagonist of the Nicotinic Acetylcholine receptor. For the Adiponectin receptor, only the AdipoRon (AdipoRon ¿98%, Abcam) drug, which is an agonist to the receptor, was used. Glutamate (L-Glutamic acid ¿99%, Sigma Aldrich) as the agonist and Kynurenic acid (Kynurenic acid hydrate ¿98%, TCI) as the antagonist was used to target the Kainate receptor in the beetles. The drugs were dissolved in respective solvents (Table 2.1) and water and finally mixed with the powdered form of oat flakes. The food paste was dried in the oven at 50℃. The dried food was again scraped and broken down into smaller pieces. Falcon tubes were prepared for rearing the beetles for each treatment with adequate food, and the upper sides of the falcon tubes were covered with fluon to prevent the beetles from escaping. Twenty, fourth-instar O. surinamensis larvae were transferred from the maintained beetle colonies to each falcon tube. The treatments were maintained in the climate chamber at 28℃ and 60% relative humidity. In all treatments, the larvae emerged into young adult beetles. The treatment tubes were then checked for newly emerged young adults on every alternative day, thrice a week. Adult beetles were separated into different falcon tubes with the same diet at the time they emerged and maintained for one week in the separate tubes till they were freezed at -20℃. This allowed to freeze all the beetles at the same age.

2.4.1 Cuticle Coloration Microscopy for Melanization Measurements

The frozen beetles which were under receptor-targeting drug treatments were used for measuring the melanization of the cuticle. At most, ten beetles from each treatment were used for the melanization measurement. Images of the cuticle of the beetles were recorded under the Leica microscope M165 FC fluorescent stereomicroscope. Each beetle was kept in separate Eppendorf tubes for further DNA extraction procedures.

Treatment	Drug	Solvent	Water	Oat flour
Nicotine (0.1%)	100µL	2ml Ethanol	15ml	10g
Nicotine (0.01%)	10.1µL	2ml Ethanol	15ml	10g
Ethanol (control)	–	2ml Ethanol	15ml	10g
Bupropion (0.1%)	0.01g	–	15ml	10g
Bupropion (0.01%)	0.001g	–	15ml	10g
Bupropion (control)	–	–	15ml	10g
AdipoRon (0.1%)	0.01g	2ml Ethanol	15ml	10g
AdipoRon (0.01%)	0.001g	2ml Ethanol	15ml	10g
Glutamate (0.1%)	0.01g	–	15ml	10g
Glutamate (0.01%)	0.001g	–	15ml	10g
Water (Control)	–	–	15ml	10g
Kyenurenic acid (0.1%)	0.01g	–	15ml	10g
Kyenurenic acid (0.01%)	0.001g	–	15ml	10g

Table 2.1: Composition of the drug-manipulated diet for beetles

2.4.2 Symbiont Titer Measurements

DNA Extraction

DNA was extracted from each of the beetles whose melanization was measured using the "MasterPureTM Complete DNA and RNA Kit" by Epicentre. The beetles were homogenized in 200 L of Tissue and Cell Lysis solution (epicentre MTC096H) using a nucleic acid-free sterilized plastic pestle. 100 L of TC Lysis solution was added after rinsing the pestle. 5 L of Proteinase K (10mg/nL) was added, and the samples were incubated the sample at 65°C for 15 minutes and next on ice for 5 minutes. 150 L of MPC Protein Precipitation Reagent (epicentre MMP095H / Promega a7953) was added, then vortexed the samples for 10sec and centrifuged for 10min at 14,000 rpm. The supernatant was transferred to new tubes, and the pellet was discarded. 500 L of isopropanol was added to the supernatant, and the tubes were mixed 30-40 times by inversion. The samples were stored at -20°C overnight. The DNA was pelleted down by centrifuging at 14000rpm for 10 minutes. The supernatant was discarded, and the samples were dry spun for 5 minutes at 14000 rpm to remove any traces of isopropanol. The supernatant was carefully removed and discarded. The samples were allowed to dry in the speed vac at 30°C for faster drying. The DNA was then resuspended with 50-100 L of Low TE buffer. The concentrations of all the samples were measured and stored at -20°C for further use. This DNA extraction procedure was also performed for 3 beetles collected from the main symbiotic O. surinamensis colonies from JKI that were maintained in the climate chamber to use to prepare standard dilutions later.

Amplification of 16SrRNA gene and Standard Dilution Preparation

A polymerase chain reaction (PCR) was done in TProfessional Gradient Thermocycler (Biometra) to amplify the 16SrRNA gene of the endosymbiotic bacteria S. silvanidophilus. 16SrRNA gene is present in the bacterial genome in a single copy, and therefore the copy number of the 16SrRNA gene was used to quantify the symbiont titer in the beetle. The PCR was performed using New England Biolabs MasterMix 2X with 1L sample DNA. The PCR was run for a test extracted DNA sample from the beetles to prepare the standard dilution and negative control (without any DNA and only nuclease-free water). Table 2.2 shows the protocol for preparing the PCR mix, and Table 2.3 shows the thermocycling conditions used in the PCR.

Components	***Volume (1X)***
Nuclease free water	10.5 μL
Forward primer (RRN311) (5' GGCAACTCTGAACTAGCTACGC 3')	0.5 μL
Reverse primer (RRN325) (5' GCACCCTTTAAACCCAAT 3')	0.5 μL
NEB Master mix 2X	12.5 μL
Total	24 μL

Table 2.2: Preparation of the PCR mix

STEP	**TEMPERATURE**	**TIME**
Initial Denaturation	95^oC	30 sec
30 cycles	95^oC	15 sec
	55^oC	20 sec
	68^oC	1min per kb
Final extension	68^oC	5 min
Hold	4^oC	-

Table 2.3: Thermocycling conditions

The amplified DNA samples were quantified using the Nanopore machine. The PCR amplification of the DNA was verified by running the amplified DNA samples in 1.6% agarose gel with 1µL of SYBR Green. The gel was visualized under UV Light in the gel documentation box. The amplified DNA products were then purified using the Zymogen DNA purification kit. The DNA binding buffer was added to the PCR-amplified samples in a ratio of 5:1 and vortexed briefly. The mixture was transferred to a Zymo-spin column in a collection tube and centrifuged for 30 seconds at 10,000g. 200 μL DNA wash buffer was added to the column and centrifuged at 12,000g for 30 seconds, and this step was repeated with centrifugation at 16,000g. A dry spin was given at the end for one minute at 16,000g. 14µL of DNA Elution buffer was added directly to the column matrix to elute the DNA by incubating it at room temperature for one minute. The column was transferred to a 1.5mL centrifuge tube and centrifuged for one minute at 12,000g. The DNA concentration of the purified DNA samples was quantified using a Nanopore machine. Standard dilutions for

the qPCR run were prepared from this purified DNA sample. Serial dilutions of the DNA sample were prepared till 10^9X dilution, which was used as the standard for the qPCR run to quantify the symbiont titer.

Quantification of Symbiont Titer by Quantitative PCR

Quantitative PCR (qPCR) was performed to quantify the copy number of the 16SrRNA gene in each sample of DNA extracted from each beetle. The qPCR was performed in the BioRad thermocycler using the Biozym Blue Probe qPCR Kit. A 96-well qPCR plate was used to prepare the samples for qPCR. 1µL of sample was used for amplification. The dilution series prepared as described in section 2.3.2.2 and was used to generate a standard curve for the qPCR.

Component	***1 x***
Biozym Blue Probe qPCR Kit	10 μl
Forward primer 5 µM	0.8 μl
Reverse primer 5µM	0.8 μl
Water	7.4 μl

Table 2.4: Volume measurements in qPCR

The endosymbiont concentrations measured in the qPCR run was used to determine the symbiont copy number in each insect using the following calculation -

Molecular weight of one double stranded DNA sequence is 2.02166×10^{-19} g.

$$\text{Number of 16SrRNA gene copies} = \frac{(\text{Volume of DNA extract}) \times (\text{Measured DNA concentration})}{2.02166 \times 10^{-19}}$$

2.5 Phenotypic Effects of Desiccation on Beetles

2.5.1 Insect Rearing in Low and High Humidity Conditions

One hundred O. surinamensis beetles were randomly collected from each of beetle populations from Julius Kühn Institute, maintained in 60% and 30% relative humidity (RH) conditions in small plastic boxes with oat flakes as their diet. The beetles were reared in a full factorial scheme; the parental beetles adapted to either 30% or 60% RH conditions were transferred to both 30% and 60% RH environments to oviposit and raise offsprings in the respective humidity conditions. 100 beetles from each parental population, in particular humidity conditions, were divided into two groups of 50 beetles each and one was kept at 30% RH and another in 60% RH conditions. The adult parental beetles were separated from the eggs after 3 weeks and freezed. From each treatment type, the newly emerged offspring were separated on their first day into separate boxes. The newly emerged offspring were

checked for thrice every week and isolated. One-week-old adult beetles were then frozen at -20^oC.

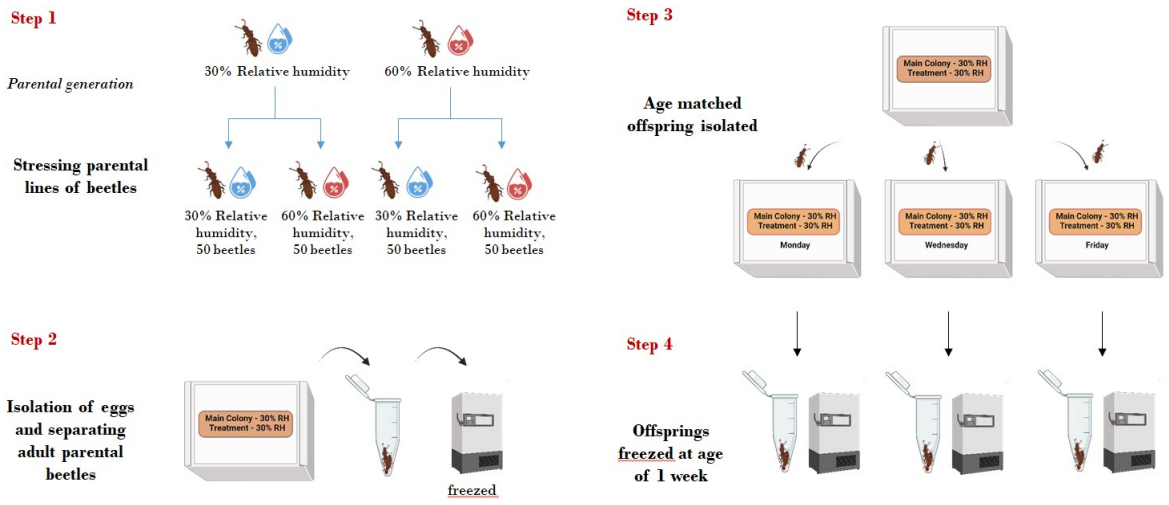

Figure 2.1: Experimental setup to study the effect of change in humidity conditions on the symbiont titer. From two colonies maintained in 30% and 60% relative humidity conditions, 100 beetles were taken from each of them. The parental lines of beetles were stressed by putting them again in low and high humidity conditions. 100 beetles from each main colony were divided into two groups of 50 beetles each and one kept under low humidity conditions and another in high humidity condition. The adult parental beetles were separated from the eggs after 3 weeks of keeping them in these humidity conditions and freezed. From each treatment type, then newly emerged offsprings are separated on their first day into separate boxes. The newly emerged offsprings were checked for thrice every week and isolated. The offsprings were then freezed at age of 1 week.

2.5.2 Cuticle Melanization Measurement

Images of frozen dead beetles were clicked as described in section 2.3.1 to measure the melanization of the cuticle of the beetles under different humidity conditions.

2.5.3 Symbiont Titer Measurement

DNA was extracted from beetle samples, and a serial dilution of 16SrRNA DNA sample was prepared as described in section 2.3.2. All the extracted DNA samples were normalized to 3ng/µL concentration. A quantitative PCR was run again to quantify the 16SrRNA DNA concentration in the DNA sample from each beetle, and the symbiont titer in each beetle was quantified using the calculation as mentioned before in section 2.3.2.3.

2.6 Statistical Analysis

2.6.1 Pharmacological Drug-administered Diet Manipulation

The symbiont titer and the cuticle melanization values of this experiment were analyzed in R using the platform Rstudio-2023.03.0-386 (http://www.R-project.org/). Violin plots were generated using the ggplot2 package (Wickham H 2016) to visualize the data. The car package (Fox J, Weisberg S 2019) and the multcomp package (Hothorn T et al., 2008) were used to perform ANOVA and the Tukey HSD analysis on the dataset based on the general linear hypothesis. The Normal distribution of the dataset was verified using the Shapiro-Wilk test, and the homogeneity of variance in the dataset using the Barlett test. Transformation of raising to fourth power was performed on the melanization data, and square-root transformation was performed on the symbiont titer values to normalize the dataset. The ANOVA test indicated the statistical significance of the difference in the symbiont titer and melanization values between the different drug treatments with the control treatment. Tukey HSD test helped to find the pair of treatments between which the difference of the values was statistically significant.

2.6.2 Phenotypic effect of ambient humidity on beetles

Rstudio-2023.03.0-386 (http://www.R-project.org/) were used to analyze the data. The ggplot2 package was used to visualize the data. To understand the impact of ambient humidity on the phenotype (symbiont titer and melanization) symbiont titer of the beetles, the data of symbiont titer and cuticle melanization (abdomen and thorax) Two-way ANOVA tests were performed on the datasets from four treatments of the experiment. Normal distribution of the datasets and homogeneity of variance between the symbiont titer of different treatments' datasets were verified to confirm that the assumption conditions of the Two-way ANOVA tests are being met. Residual vs fits plot, Shapiro Wilk test indicated that the normality of the symbiont titer dataset was violated. Therefore transformation of the symbiont titer (square-root) and melanization of the thorax (raised to the fourth power) and abdomen (squaring) data was performed to normalize it, and the homogeneity of variance and normality tests were repeated. The transformed data in the offspring generations showed an approximately fitted straight line in the residual vs fits plot. The transformation normalized the dataset, and the homogeneity of variances was maintained between the four different treatment groups. The Two-way ANOVA test performed on the transformed data of the treatments indicated the statistical significance of differences in the means of the values between the four treatments in the offspring generation and also between the offspring and the parental generation. A Tukey's HSD test was further performed to find the treatments in which the symbiont titer and the melanization were undergoing a significant change.

3

Results and Discussions

3.1 Investigation of molecular mechanisms involve in the endosymbiosis regulation in consequence of DOPA deprivation

3.1.1 Selecting candidate genes from Differential Gene Expression Analysis

The differential gene expression analysis was done to analyze the results of the metyrosine treatment against the control to find the changes in the gene expression upon inhibition of L-DOPA synthesis. 299566 transcripts were recovered from the de-novo transcriptome. In bacteriome metyrosine vs bacteriome control analysis, 1054 genes were differentially expressed out of which 150 had higher expression in bacteriome metyrosine and 954 transcripts had higher expression in the bacteriome control treatment. In adult metyrosine vs adult control analysis, 1608 genes were differentially expressed out of which 580 had higher expression in adult metyrosine and 1028 transcripts had higher expression in the adult control treatment. In bacteriome control vs adult control analysis, 65045 genes were differentially expressed out of which 29073 had higher expression in adult control and 35972 transcripts had higher expression in the bacteriome control treatment. In bacteriome metyrosine vs adult metyrosine analysis, 60910 genes were differentially expressed out of which 27734 had higher expression in adult metyrosine and 33176 transcripts had higher expression in the

bacteriome metyrosine treatment. Differentially expressed (DE) gene transcripts (p-value adjusted ¡ 0.05) were selected from the DESeq2 output files to compare the mean expression values. Highly differentially expressed genes that might have a functional significance in the beetle and the bacteriome were looked for in the list of differentially expressed genes. The impact of decrease in L-DOPA concentration was observed by comparing the bacteriome control and bacteriome metyrosine data. The idea of the specific pathway impacted by the lack of DOPA synthesis was formed from the comparison between adult beetle control and bacteriome control data. The gene enriched specifically in the adult beetle and the bacteriome were also scrutinized from the comparisons between control and the metyrosine treatment data.

Several genes were found to be significantly differentially expressed among all the treatment comparisons. For example, glutathione-S-transferase proteins, which have an important role to play in controlling oxidative stress in insects due to symbiont infection (Zug et al., 2015), were found to be expressed higher in the bacteriome of the control treatment compared to the bacteriome of the metyrosine treatment. Peptidoglycan recognition proteins (PGRPs), playing a pivotal role in the immune response in insects (Wang et al., 2018) and when symbiotic interactions improve the immune response of the host insect (Maire et al., 2019), were also found to be overexpressed in the bacteriome under metyrosine treatment. Neuropeptides like NVP-like proteins were differentially expressed as the L-DOPA was depleted in the metyrosine treatment. Trehalose transporter proteins involved in the transport of trehalose were overexpressed in the bacteriome.

Three candidate genes were chosen to target their activity and observe the respective changes in the beetles, from this list of important genes that were differentially expressed in the insect and its bacteriome. Since we focus upon identifying the signalling pathways involved in the host-symbiont regulation, selecting receptors as candidate genes was rational as receptors should be involved in sensing molecules in the cavity. Nicotinic acetylcholine receptor was the highest expressed gene in the bacteriome and the most expressed transcript in the DESeq2 results. This receptor is expressed in the bacteriome of both the control and metyrosine treatments but not in the adult insect. Specificity in the expression of this receptor led to the choice of the nicotinic acetylcholine receptor as one of the candidate genes. Adiponectin receptor was the only receptor significantly more expressed in metyrosine-treated bacteriomes than control bacteriomes and is involved in lipid regulation. To speculate whether the adiponectin receptor is involved in endosymbiont regulation through lipid metabolism, it was chosen as the second target receptor. Kainate receptors linked with glutamate signalling (Chiang et al., 2002) were also overexpressed in the bacteriome and were chosen as a candidate gene.

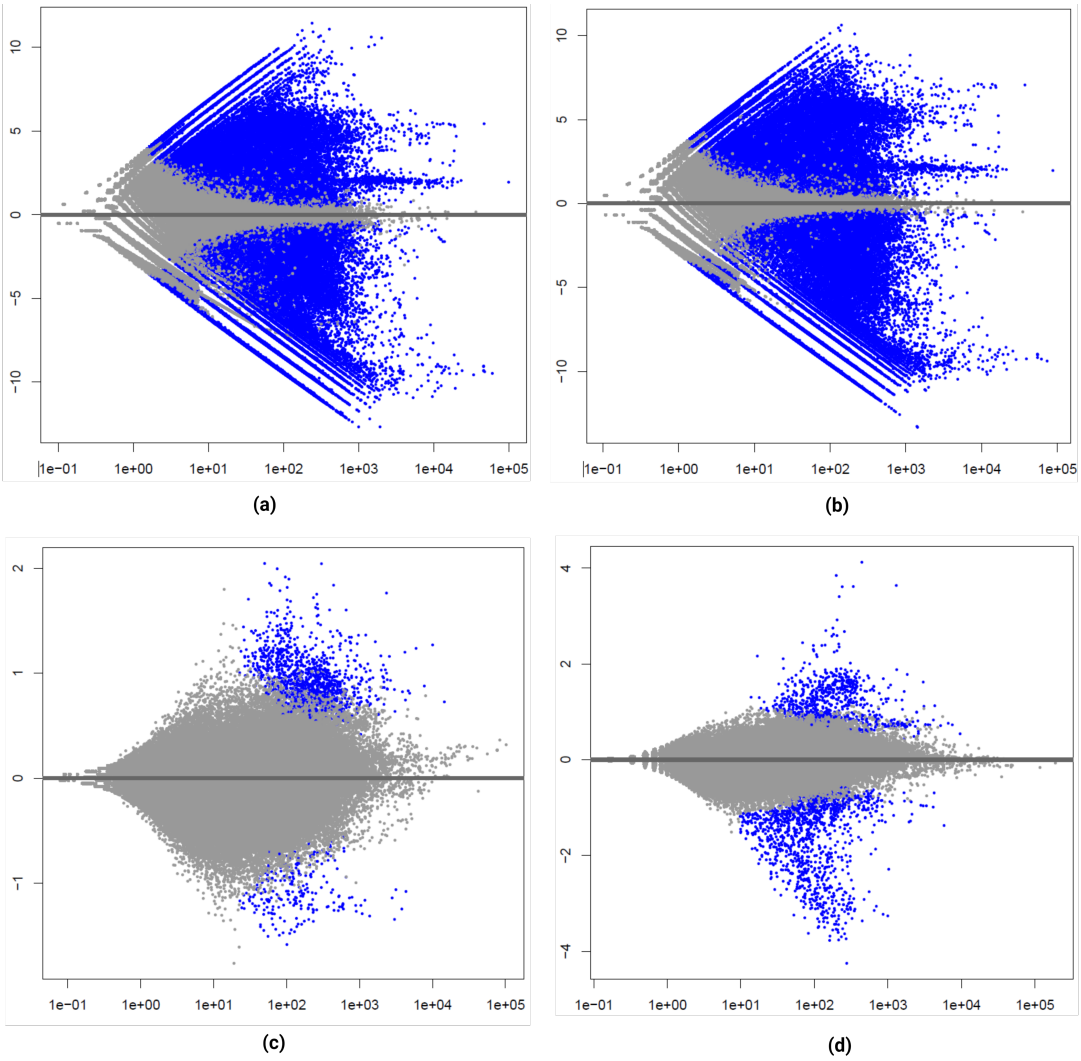

Figure 3.1: MA plots from DESeq2 analysis of log fold change for (a) Adult Metyrosine vs Bacteriome metyrosine, (b) Adult Control vs Bacteriome Control, (c) Bacteriome Control vs Bacteriome Metyrosine, (d) Adult Control vs Adult Metyrosine (blue points - over and under-expressed genes) Plots depicting the presence of differentially expressed genes between RNASeq datasets (X-axis: mean of normalized counts, Y-axis: log of fold change)

3.1.2 Impact of Triggering and Inhibiting Candidate Receptors

The adiponectin receptors were triggered by administering the AdipoRon agonist drug to the young adult beetles through their diet. Statistical analysis of the symbiont titer and cuticle melanization data obtained from the beetles under drug treatment showed that the symbiont titer increased significantly under the Adiporun treatment compared to the control. ANOVA test (p value = 7.43e-05) and Tukey's HSD test on the normalized dataset showed that AdipoRon treatments of both low and high concentration resulted in a significant increase in the symbiont titer when compared to the control (ethanol-treated) treatment. A similar and important trend in the melanization values of the abdomen and thorax of the AdipoRon treatment, although the ANOVA and Tukey HSD statistics are not significant. Observing a significant effect of triggering and inhibiting the adiponectin receptor was expected as this

receptor plays an important role in the lipid metabolism in insects (Kwak et al., 2013) and breakdown of fatty acids for assimilation (Toprak, 2020). From the DESeq2 results, the Adiponectin receptor was the only differentially expressed gene that was overexpressed in the bacteriome under metyrosine treatment. Higher expression in the bacteriome implied that the receptor would have more sensitivity to the circulating molecules and might result in a higher endosymbiont titer. The fold increase observed in the symbiont titer under the adiporon treatment is similar to the fold increase observed in the symbiont titer in the beetles under the metyrosine treatment where L-DOPA synthesis gets inhibited (Unpublished data from Aurelien Vigneron). This indicates that there might be a direct or indirect link between the adiponectin receptors and L-DOPA that holds a scope of investigation. A similar trend observed in the increase in the melanization of the cuticle in both the abdomen and thorax regions reflect that the effect of triggering the adiponectin receptor also gets translated into the cuticle melanization of the beetles. Additionally, evidence of cross-talk between the dopamine and adiponectin in humans (Borcherding et al., 2011) supports the chance of discovering a link between DOPA and adiponectin receptors. In Drosophila, dAdipoR, an adiponectin receptor homolog has been found in its insulin-producing cells (Kwak et al., 2013). Shutdown of dAdipoR resulted in an increase in the circulating levels of trehalose and glucose (Nässel et al., 2016). Trehalose is a crucial molecule that is transported as nutrients into bacteriocytes. Therefore there might be possibility of adiponectin receptor impacting the transport of trehalose into the bacteriome that can in turn affect the endosymbiont titer. Kainate receptors, being related to the glutamate signalling which is an important neurotransmission in the central nervous system in the insects, were expected to have an important effect upon triggering and inhibiting the receptor (Funada et al., 2004) (Li et al., 2016). For the Glutamate (agonist of Kainate receptor) and kynurenic acid (antagonist of Kainate receptor) treatments, the symbiont titer decreases when compared to that of the control treatment, and the melanization values follow a similar trend, but these results are again not significant. The results of the Bupropion (antagonist of Nicotine acetylcholine receptor) treatment aren't significant as well, and the Nicotine (agonist of Nicotine acetylcholine receptor) treatment has too less a sample size to conclude any result. Since nicotinic acetylcholine receptors are major neurotransmittory receptors in insects (Millar et al., 2007), tampering with the activity of these receptors was expected to have a drastic effect on the beetles. The observation of very low survival of the beetles under nicotine treatment might be probably due to the drastic effect of triggering or inhibiting the receptor, pointing at a crucial role in the homeostasis of the insect biology. This was not surprising, as these receptors are the usual target of efficient insecticides.

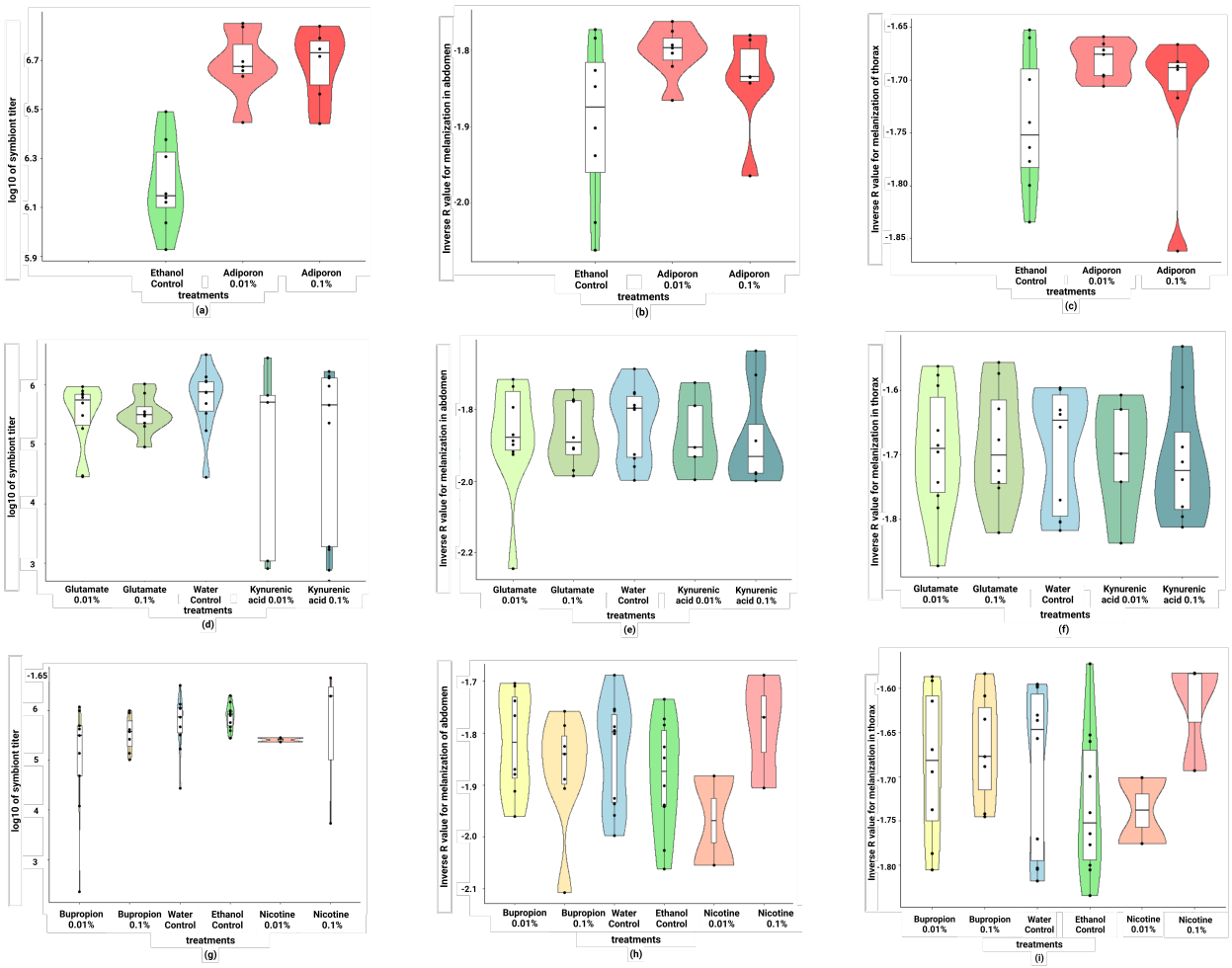

Figure 3.2: Violin plots of (a) Symbiont titer (ANOVA, p = 7.43e-05) (b) Melanization of abdomen (ANOVA, p = 0.128) (c) Melanization of thorax (ANOVA, p = 0.163) of AdipoRon treatment (d) Symbiont titer (Glutamate, ANOVA, p = 0.209; Kynurenic acid, ANOVA, p = 0.491) (e) Melanization of abdomen (Glutamate, ANOVA, p = 0.828; Kynurenic acid, ANOVA, p = 0.67) (f) Melanization of thorax (Glutamate, ANOVA, p = 0.983; Kynurenic acid, ANOVA, p = 0.944) of agonist and antagonist drug treatment targeting Kainate receptor (g) Symbiont titer (Bupropion, ANOVA, p = 0.182; Nicotine, ANOVA, p = N/A) (h) Melanization of abdomen (Bupropion, ANOVA, p = 0.596; Nicotine, ANOVA, p = N/A) (i) Melanization of thorax (Bupropion, ANOVA, p = 0.832; Nicotine, ANOVA, p = N/A) of agonist and antagonist drug treatment targeting Nicotine Acetylcholine receptor

Treatment	***Symbiont Titer (Pr)***	***Melanization in abdomen (Pr)***	***Melanization in thorax (Pr)***
AdipoRon	7.43e-05*	0.128	0.163
Glutamate	0.209	0.828	0.983
Kynurenic acid	0.491	0.67	0.944
Bupropion	0.182	0.596	0.832
Nicotine	N/A	N/A	N/A

Table 3.1: Phenotypic effect of agonist and antagonistic drugs against receptors on the beetles from results of Two-Way ANOVA analysis (Nicotine treatment produced very few beetles to perform statistics and conclude any results) (* indicates significant p value).

Treatment	*Symbiont Titer (Pr)*	*Melanization in abdomen (Pr)*	*Melanization in thorax (Pr)*
AdipoRon	7.43e-05	0.128	0.163
Glutamate	0.209	0.828	0.983
Kynurenic acid	0.491	0.67	0.944
Bupropion	0.182	0.596	0.832
Nicotine	N/A	N/A	N/A

Table 3.2: Impact of Adiporun agonistic drug targeting the adiponectin receptor on the symbiont titer and cuticle melanization of the beetles from results of the pairwise Tukey's HSD test analysis (* indicates significant p value). Results of treatment against control are presented.

3.2 Influence of Environmental Stress on Host-Symbiont Regulation

3.2.1 Influence of Humidity on Symbiont Titer in Beetles

To understand the effect of different humidity conditions on the symbiotic beetles, the symbiont titer was quantified using qPCR technique (Treatments: (a) 30% RH in parental to 30% RH in offspring generation, (b) 30% RH in parental to 60% RH in offspring generation, (c) 60% RH in parental to 30% RH in offspring generation, (d) 60% RH in parental to 60% RH in offspring generation; number of beetles per treatment (n), n = 10. It was originally hypothesized that since in a lower humidity condition, the beetles would require the protection of thick and hard cuticular exoskeleton to fight against the desiccation stress. Therefore, the beetles would invest more in maintaining a higher symbiont titer in their bacteriome to help in the cuticle formation process. But contrary to the expectation, the symbiont titer is higher in the beetles reared in 60% RH than in 30% RH conditions. When the parental generation experienced a higher humidity condition, the offspring generation has an important trend to have a higher symbiont titer, although the data is not significant. The Two-way ANOVA test performed on the square root values of the symbiont titer of the treatments indicated that the differences in the means of the symbiont titer values are statistically significantly different between the four treatments in the offspring generation and also between the offspring and the parental generation. However, there is no influence of the parental humidity conditions on the symbiont titer of the offspring generation. A Tukey's HSD test on the transformed symbiont titer values showed that the symbiont titer of the offsprings of treatment 60:60% RH is significantly higher than that of the 30:30% treatment group.

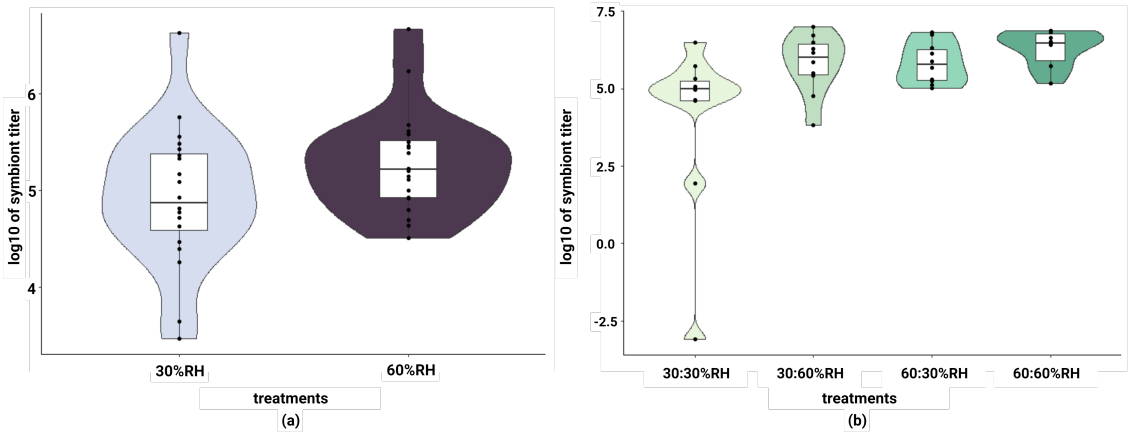

Figure 3.3: Symbiont titer variation in beetles under low and high humidity conditions (a) Symbiont titer in beetles of the parental generation reared under 30% RH and 60% RH (b) Symbiont titer in beetles of the four treatments of the offspring generation reared under 30% RH and 60% RH (in offspring generation, P = 0.00948; the difference between parental humidity conditions on the offspring generation, P = 0.03649)

3.2.2 Humidity Influence on Melanization of the Cuticle in Beetles

The cuticle development of the beetles in low and high humidity conditions was tracked by measuring the cuticle melanization of beetles of each treatment (n = 10 beetles for each treatment). The Two-way ANOVA test performed on the squared values of the melanization of the abdomen of the treatments indicated that the differences in the means of the melanization values are statistically significantly different between the four treatments in the offspring generation. Tukey's HSD test on this dataset showed that the difference in the mean values of the melanization of the abdomen was statistically significant between most treatments. The offspring generation reared in 60% RH had a more melanized cuticle than the offspring generation reared in 30% RH. This result is thus consistent with the higher symbiont titer values found in the beetles surviving at a higher humidity condition. The Two-way ANOVA test performed on the fourth power values of the melanization of the thorax of the treatments indicated that the differences in the means of the melanization values are statistically significantly different between the four treatments in the offspring generation. Tukey's HSD test on melanization of thorax data was in concert with that of the abdomen melanization. Overall, the thorax region was found to be more melanized than the abdomen in all four treatment types.

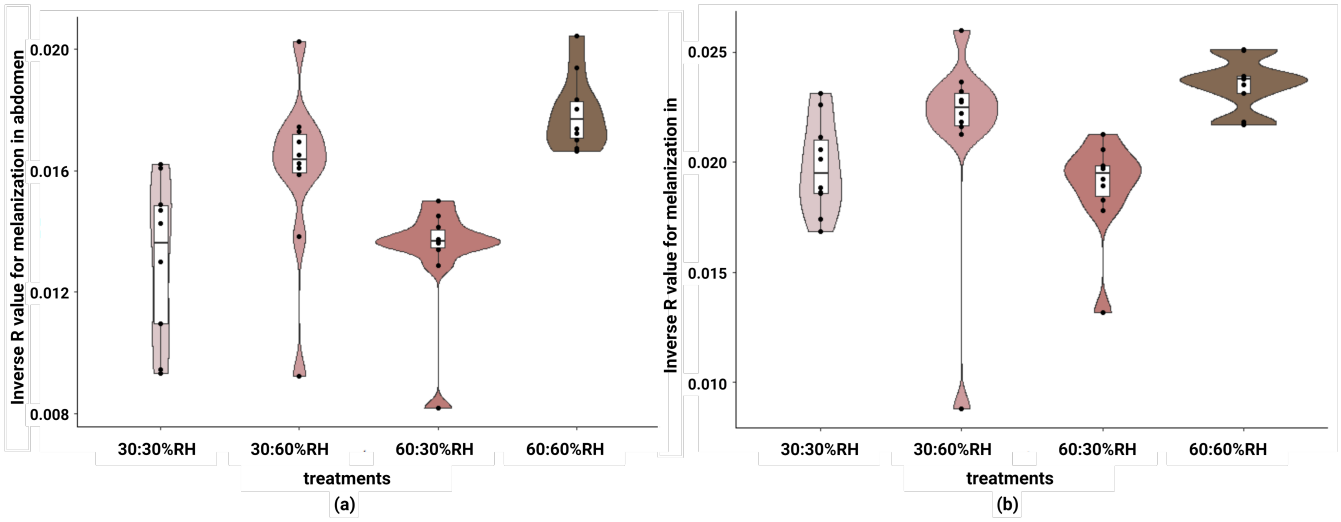

Figure 3.4: Impact on Cuticle Melanization in beetles under low and high humidity conditions (a) Melanization of the abdomen (ANOVA, P = 9.66e-07) and (b) Melanization of the thorax (ANOVA, P = 6.66e-06) of the four treatments of the offspring generation reared under 30% RH and 60% RH with a significant difference between the treatment conditions

Factors	***Symbiont Titer (P-value)***	***Melanization in abdomen (P-value)***	***Melanization in thorax (P-value)***
Influence of parental humidity	0.03649 *	0.106	0.958188
Influence of offspring humidity	0.00948 *	9.66e-07 *	0.000255 *
Interaction between parental and offspring humidity	0.76801	0.165	0.312103

Table 3.3: Impact of ambient humidity conditions on the symbiont titer and cuticle melanization of the beetles from results of Two-Way ANOVA analysis (* indicates significant p-value).

Treatment conditions	***Symbiont Titer (P-value)***	***Melanization in abdomen (P-value)***	***Melanization in thorax (P-value)***
60:30 - 30:30 %RH	0.3155135	0.9981500	0.8499684
30:60 - 30:30 %RH	0.1575169	0.0158707*	0.0740392
60:60 - 30:30 %RH	0.0070796*	0.0000305*	0.0009397*
30:60 - 60:30 %RH	0.9776880	0.0244458*	0.0105910*
60:60 - 60:30 %RH	0.3245361	0.0000515*	0.0000928*
60:60 - 30:60 %RH	0.5528908	0.1498492	0.3119512

Table 3.4: Impact of ambient humidity conditions on the symbiont titer and cuticle melanization of the beetles from results of Tukey's HSD test analysis (* indicates significant p value).

Acute desiccation stress (30% RH) on the beetles resulted in lower symbiont titer in

the beetles, quite contrary to our expectations. Although the symbionts help the beetle to survive in a desiccative environment, our experimental results suggest that the symbiotic beetles still struggle to survive under stress conditions. We observed that beetles in dry conditions had a much quicker developmental stage (approximately one week faster) than the beetles in higher humidity conditions. It is speculated that the beetles faced high stress in low humidity, and probably investing in the maintenance of symbionts was costly for the beetles. Under high stress, the beetles are investing more in completing the reproduction process instead of maintaining the symbiont population and a melanized cuticle. Therefore, we can see that the symbiont titer and, consequently, the cuticle melanization is higher in the less stressful higher humidity (60% RH) condition than the lower humidity (30% RH) environment where the beetles struggle to survive despite the presence of the symbionts.

4

Summary and Conclusion

In this project, important insights were developed into the mechanisms of metabolic regulation of the saw-toothed grain pest beetle on their endosymbionts. Amino acid precursors get supplemented to the host by endosymbionts to help in the synthesis of cuticular exoskeleton and improve the fitness of the hosts in their ecological habitat. The differentially expressed genes in the adult beetle and the bacteriome under inhibited L-DOPA synthesis, was found from the differential gene expression analysis. It helped to identify the important genes that can get affected by the L-DOPA synthesis and might have a crucial role in the host-symbiont regulatory process. The results of pharmacological drug administration tests showed that Adiponectin receptor is playing a very important role in the host-endosymbiont regulation that needs further investigation. Although we did not observe significant phenotypic changes in the cuticle of the beetle, the observation of a similar increasing trend holds the scope of repeating the experiments with a larger sample size to reach an experimental plan where significant changes could be detected. The treatments targeting the kainate and nicotinic acetylcholine receptors did not show any significant results. But observation of noticeable changes in the symbiont titer and melanization values of the cuticle gives hope that repetition of these treatments with a larger sample size might deliver significant results; thus giving a deeper insight into the signalling pathways involved in endosymbiont regulation. The transcriptome built de-novo and annotated against the T.castaneum genome helped to find out the differentially expressed genes. However, a better annotation of the transcriptome to identify more genes that might have been differentially expressed under inhibition of the L-DOPA synthesis. This will help to identify more DOPA or dopamine receptors

that might have been differentially expressed and understand their role in the regulation. To explore further about the adiponectin receptor that proved to be an important receptor in the regulation, it will be beneficial to also inhibit the receptor and see its effects on the phenotype of the beetle. RNA interference techniques can be used to knock down the adiponectin receptor as the antagonistic drug to adiponectin is not known yet. Discovering the ligand binding to the adiponectin receptor to trigger its activity will help to reveal more about the downstream signalling pathway that is delivering an effect on the symbiont titer and cuticle of the beetles. A better annotation of the transcriptome built from the transcripts from metyrosine treatment, will also help to find more information on how the adiponectin receptors are linked to the DOPA molecule in the insect. This will shed light on how the response from the adiponectin receptors get reflected upon the cuticular formation of the beetles. From the transcriptome data, we could observe a preferential expression of a gene coding for an adiponectin-like receptor in the bacteriome compared to the whole insect, pointing at the presence of these receptors in the endosymbiont-housing organ. However, the exact location of such receptor within bacteriome is still unknown. Identifying whether the receptor lies externally to the thin cellular membrane of the bacteriocytes or intracellularly associated to the vacuole surrounding the endosymbiont will help in deeper understanding of the mechanism of the underlying signaling triggered by the adiponectin receptors. Antibody-conjugation techniques such as immunohistochemistry or Western blotting could be used to pinpoint the exact location of the receptors within the bacteriome.

Although the drug administered diet manipulation resulted in changes in the symbiont titer and the cuticle of the beetles implying the regulation of the symbiont titer by the host, changes in the environmental conditions did not result in changes in the symbiont titer and the cuticle to defend the beetle against the environmental stress. The faster development but decrease in the symbiont titer of the beetles in the dry environment signifies that insects under high stress conditions focusses more upon reproduction rather than investing resources on their own better survival. An analysis of the cuticular hydrocarbon composition and cuticle thickness of the beetles under different humidity conditions will show whether the cuticle development is affected to adjust to the dessication stress.

The results from this project can be of great importance for pest management strategies in grain storage area to control the damage from the saw-toothed grain pest beetle. The regulatory pathways of the insect can be targetted to reduce the spread of the beetle. Although, association of O. surinamensis with the endosymbionts has proven to be highly beneficial by supplying the insect with amino acids precursors for cuticle biosynthesis, the symbiotic relationships are not obligatory. The presence of the symbionts is advantageous for the beetles, but the beetles can also get the essential amino acids from their diet to build a strong and thick cuticle. In terms of fitness, the symbiotic relationship provides an advantage to the insect, helping the insects to adapt to abiotic and biotic conditions in order to exploit ecological niches.

5

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6

Supplementary Material

6.1 R Script for statistical analysis of pharmacological drug administration experiment

6.1.1 Visualization Plots

```
library(ggplot2)
setwd("L:/Users/Bhattacharyya_Soumi/MS Thesis/Pharmacological test")
pharmadata <- read.csv("Pharmacological_drug_experiment_cumulated_datasheet
    ↪ .csv", sep = ",", dec=".")
str(pharmadata)
attach(pharmadata)
#box and violin plots of symbiont titer of treatments
#log of sym_titer_corr vs treatment
#customized the fill colours of the violin plot with the scale
#violin plots for symbiont titer
NicAchdata <- read.csv("Pharma exp NicACh receptor group analysis data
    ↪ sheet.csv", sep = ",", dec=".")
str(NicAchdata)
attach(NicAchdata)
p2 <- ggplot(NicAchdata, aes(x=treatment, y=log10(sym_titer_corr), fill =
    ↪ treatment))
```

```
p2 + geom_violin() + scale_fill_manual(values = c("#7CFC00", "#FEE001", "#
    ↪ FEE001", "red", "red", "red", "red"))+ geom_boxplot(width = 0.25,
    ↪ fill = "grey") + geom_point() + theme_classic()
Kainatedata <- read.csv("Pharma exp Glutamate receptor group analysis data
    ↪ sheet.csv", sep = ",", dec=".")
str(Kainatedata)
attach(Kainatedata)
p3 <- ggplot(Kainatedata, aes(x=treatment, y=log10(sym_titer_corr), fill =
    ↪ treatment))
p3 + geom_violin() + scale_fill_manual(values = c("#7CFC00", "#7CFC00", "#7
    ↪ CFC00", "lightblue", "lightblue", "red", "red", "red"))+ geom_boxplot
    ↪ (width = 0.25, fill = "grey") + geom_point() + theme_classic()
AdiRdata <- read.csv("Pharma exp Adiponectin receptor group analysis data
    ↪ sheet.csv", sep = ",", dec=".")
str(AdiRdata)
attach(AdiRdata)
p4 <- ggplot(AdiRdata, aes(x=treatment, y=log10(sym_titer_corr), fill =
    ↪ treatment))
p4 + geom_violin() + scale_fill_manual(values = c("red", "orange", "orange
    ↪ ", "red"))+ geom_boxplot(width = 0.25, fill = "grey") + geom_point()
    ↪ + theme_classic()

#violin plots of melanization of abdomen

NicAchdata <- read.csv("Pharma exp NicACh receptor group analysis data
    ↪ sheet.csv", sep = ",", dec=".")
str(NicAchdata)
attach(NicAchdata)
p2 <- ggplot(NicAchdata, aes(x=treatment, y=log10(inverse_r_abdomen), fill
    ↪ = treatment))
p2 + geom_violin() + scale_fill_manual(values = c("#7CFC00", "#FEE001", "#
    ↪ FEE001", "red", "red", "red", "red"))+ geom_boxplot(width = 0.25,
    ↪ fill = "grey") + geom_point() + theme_classic()
Kainatedata <- read.csv("Pharma exp Glutamate receptor group analysis data
    ↪ sheet.csv", sep = ",", dec=".")
str(Kainatedata)
attach(Kainatedata)
p3 <- ggplot(Kainatedata, aes(x=treatment, y=log10(inverse_r_abdomen), fill
    ↪  = treatment))
```

```
p3 + geom_violin() + scale_fill_manual(values = c("#7CFC00", "#7CFC00", "#7
    ↪ CFC00", "lightblue", "lightblue", "red", "red", "red"))+ geom_boxplot
    ↪ (width = 0.25, fill = "grey") + geom_point() + theme_classic()
AdiRdata <- read.csv("Pharma exp Adiponectin receptor group analysis data
    ↪ sheet.csv", sep = ",", dec=".")
str(AdiRdata)
attach(AdiRdata)
p4 <- ggplot(AdiRdata, aes(x=treatment, y=log10(inverse_r_abdomen), fill =
    ↪ treatment))
p4 + geom_violin() + scale_fill_manual(values = c("red", "orange", "orange
    ↪ ", "red"))+ geom_boxplot(width = 0.25, fill = "grey") + geom_point()
    ↪ + theme_classic()

#violin plots of melanization of thorax

NicAchdata <- read.csv("Pharma exp NicACh receptor group analysis data
    ↪ sheet.csv", sep = ",", dec=".")
str(NicAchdata)
attach(NicAchdata)
p2 <- ggplot(NicAchdata, aes(x=treatment, y=log10(inverse_r_thorax), fill =
    ↪  treatment))
p2 + geom_violin() + scale_fill_manual(values = c("#7CFC00", "#FEE001", "#
    ↪ FEE001", "red", "red", "red", "red"))+ geom_boxplot(width = 0.25,
    ↪ fill = "grey") + geom_point() + theme_classic()
Kainatedata <- read.csv("Pharma exp Glutamate receptor group analysis data
    ↪ sheet.csv", sep = ",", dec=".")
str(Kainatedata)
attach(Kainatedata)
p3 <- ggplot(Kainatedata, aes(x=treatment, y=log10(inverse_r_thorax), fill
    ↪ = treatment))
p3 + geom_violin() + scale_fill_manual(values = c("#7CFC00", "#7CFC00", "#7
    ↪ CFC00", "lightblue", "lightblue", "red", "red", "red"))+ geom_boxplot
    ↪ (width = 0.25, fill = "grey") + geom_point() + theme_classic()
AdiRdata <- read.csv("Pharma exp Adiponectin receptor group analysis data
    ↪ sheet.csv", sep = ",", dec=".")
str(AdiRdata)
attach(AdiRdata)
p4 <- ggplot(AdiRdata, aes(x=treatment, y=log10(inverse_r_thorax), fill =
    ↪ treatment))
```

```
p4 + geom_violin() + scale_fill_manual(values = c("red", "orange", "orange
    ↪ ", "red"))+ geom_boxplot(width = 0.25, fill = "grey") + geom_point()
    ↪ + theme_classic()
```

6.1.2 Statistical Analysis

```
    library(car)
library(multcomp)
```

6.1.3 ADIPO

```
    Adipo = read.csv("Pharma exp Adiponectin receptor group analysis data
        ↪ sheet.csv", h=T)
head(Adipo)
Adipo$treatment= as.factor(Adipo$treatment)

Adipo.symCT = Adipo[which(Adipo$treatment_num =='3'),]
Adipo.sym0.01 = Adipo[which(Adipo$treatment_num =='1'),]
Adipo.sym0.1 = Adipo[which(Adipo$treatment_num =='2'),]

##### SYM TITER ######
shapiro.test(Adipo.symCT$sym_titer_corr)
        Shapiro-Wilk normality test


data: Adipo.symCT$sym_titer_corr
W = 0.91222, p-value = 0.37

shapiro.test(Adipo.sym0.01$sym_titer_corr)
        Shapiro-Wilk normality test


data: Adipo.sym0.01$sym_titer_corr
W = 0.91595, p-value = 0.4386

shapiro.test(Adipo.sym0.1$sym_titer_corr)
        Shapiro-Wilk normality test
```

```
data: Adipo.sym0.1$sym_titer_corr
W = 0.95234, p-value = 0.7592

bartlett.test(Adipo$sym_titer_corr~Adipo$treatment)
       Bartlett test of homogeneity of variances


data: Adipo$sym_titer_corr by Adipo$treatment
Bartlett's K-squared = 3.3767, df = 2, p-value = 0.1848

aov.adipo.sym = aov(sym_titer_corr ~ treatment, data=Adipo)
summary(aov.adipo.sym)
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 5.529e+13 2.764e+13 16.88 7.43e-05 ***
Residuals 18 2.947e+13 1.637e+12
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

mcp.adipo.sym = glht(aov.adipo.sym, linfct=mcp(treatment = "Tukey"))
summary(mcp.adipo.sym)
        Simultaneous Tests for General Linear Hypotheses


Multiple Comparisons of Means: Tukey Contrasts




Fit: aov(formula = sym_titer_corr ~ treatment, data = Adipo)


Linear Hypotheses:
                        Estimate Std. Error t value Pr(>|t|)
1ADI_0.1 - 1ADI_0.01 == 0 -2310 711925 -0.003 0.999994
2NIC_CTRL - 1ADI_0.01 == 0 -3342304 662275 -5.047 0.000192 ***
2NIC_CTRL - 1ADI_0.1 == 0 -3339994 691083 -4.833 0.000370 ***
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1
(Adjusted p values reported -- single-step method)
```

```
##### THORAX ######
shapiro.test(Adipo.symCT$inverse_r_thorax^4)
> shapiro.test(Adipo.sym0.01$inverse_r_thorax^4)

       Shapiro-Wilk normality test

data: Adipo.sym0.01$inverse_r_thorax^4
W = 0.93294, p-value = 0.5762

> shapiro.test(Adipo.sym0.1$inverse_r_thorax^4)

       Shapiro-Wilk normality test

data: Adipo.sym0.1$inverse_r_thorax^4
W = 0.84202, p-value = 0.1355

> bartlett.test(Adipo$inverse_r_thorax^4~Adipo$treatment)

       Bartlett test of homogeneity of variances

data: Adipo$inverse_r_thorax^4 by Adipo$treatment
Bartlett's K-squared = 4.2042, df = 2, p-value = 0.1222

> aov.adipo.thorax = aov(inverse_r_thorax^4 ~ treatment, data=Adipo)
> qqnorm(aov.adipo.thorax$residuals)
> qqline(aov.adipo.thorax$residuals)
> summary(aov.adipo.thorax)
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 1.482e-14 7.409e-15 2.013 0.163
Residuals 18 6.626e-14 3.681e-15
20 observations deleted due to missingness
> mcp.adipo.thorax = glht(aov.adipo.thorax, linfct=mcp(treatment = "Tukey")
   ↪ )
> summary(mcp.adipo.thorax)

        Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts
```

```
Fit: aov(formula = inverse_r_thorax^4 ~ treatment, data = Adipo)

Linear Hypotheses:
                          Estimate Std. Error t value Pr(>|t|)
1ADI_0.1 - 1ADI_0.01 == 0 -3.592e-08 3.375e-08 -1.064 0.547
2NIC_CTRL - 1ADI_0.01 == 0 -6.295e-08 3.140e-08 -2.005 0.140
2NIC_CTRL - 1ADI_0.1 == 0 -2.703e-08 3.277e-08 -0.825 0.693
(Adjusted p values reported -- single-step method)

> ##### ABDOMEN ######
> shapiro.test(Adipo.symCT$inverse_r_abdomen^4)

       Shapiro-Wilk normality test

data: Adipo.symCT$inverse_r_abdomen^4
W = 0.91701, p-value = 0.406

> shapiro.test(Adipo.sym0.01$inverse_r_abdomen^4)

       Shapiro-Wilk normality test

data: Adipo.sym0.01$inverse_r_abdomen^4
W = 0.9833, p-value = 0.974

> shapiro.test(Adipo.sym0.1$inverse_r_abdomen^4)

       Shapiro-Wilk normality test

data: Adipo.sym0.1$inverse_r_abdomen^4
W = 0.91288, p-value = 0.4556

> bartlett.test(Adipo$inverse_r_abdomen^4~Adipo$treatment)

       Bartlett test of homogeneity of variances

data: Adipo$inverse_r_abdomen^4 by Adipo$treatment
Bartlett's K-squared = 1.3088, df = 2, p-value = 0.5197
```

```
> aov.adipo.abdomen = aov(inverse_r_abdomen^4 ~ treatment, data=Adipo)
> qqnorm(aov.adipo.abdomen$residuals)
> qqline(aov.adipo.abdomen$residuals)
> summary(aov.adipo.abdomen)
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 2.636e-15 1.318e-15 2.306 0.128
Residuals 18 1.029e-14 5.716e-16
20 observations deleted due to missingness
> mcp.adipo.abdomen = glht(aov.adipo.abdomen, linfct=mcp(treatment = "Tukey
   ↪ "))
> summary(mcp.adipo.abdomen)

        Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts


Fit: aov(formula = inverse_r_abdomen^4 ~ treatment, data = Adipo)

Linear Hypotheses:
                           Estimate Std. Error t value Pr(>|t|)
1ADI_0.1 - 1ADI_0.01 == 0 -1.488e-08 1.330e-08 -1.119 0.515
2NIC_CTRL - 1ADI_0.01 == 0 -2.656e-08 1.237e-08 -2.146 0.108
2NIC_CTRL - 1ADI_0.1 == 0 -1.168e-08 1.291e-08 -0.905 0.644
(Adjusted p values reported -- single-step method)
```

6.1.4 GLUTAMATE

```
   > glutamate = read.csv("Pharma exp Glutamate receptor group analysis
      ↪ data sheet.csv", h=T)
> head(glutamate)
  treatment_num treatment sym_titer_corr inverse_r_abdomen inverse_r_thorax
1 1 0GLUT_0.01 491000 0.005693691 0.01340231
2 1 0GLUT_0.01 664000 0.019247055 0.02554670
3 1 0GLUT_0.01 918000 0.013467107 0.01724614
4 1 0GLUT_0.01 776000 0.016075620 0.02178222
5 1 0GLUT_0.01 181000 0.012059962 0.01652073
6 1 0GLUT_0.01 29600 0.018374584 0.02653153
```

```
> glutamate$treatment= as.factor(glutamate$treatment)
> glu.symCT = glutamate[which(glutamate$treatment_num =='3'),]
> glu.sym0.01 = glutamate[which(glutamate$treatment_num =='1'),]
> glu.sym0.1 = glutamate[which(glutamate$treatment_num =='2'),]
> ka.sym0.01 = glutamate[which(glutamate$treatment_num =='4'),]
> ka.sym0.1 = glutamate[which(glutamate$treatment_num =='5'),]
> ######## GLU ############
> glu.glutamate = glutamate[1:28,]
> View(glu.glutamate)

> #SYM TITER#

> shapiro.test(sqrt(glu.symCT$sym_titer_corr))

        Shapiro-Wilk normality test

data: sqrt(glu.symCT$sym_titer_corr)
W = 0.96427, p-value = 0.8332

> shapiro.test(sqrt(glu.sym0.01$sym_titer_corr))

        Shapiro-Wilk normality test

data: sqrt(glu.sym0.01$sym_titer_corr)
W = 0.87966, p-value = 0.1293

> shapiro.test(sqrt(glu.sym0.1$sym_titer_corr))

        Shapiro-Wilk normality test

data: sqrt(glu.sym0.1$sym_titer_corr)
W = 0.92132, p-value = 0.4407

> bartlett.test(sqrt(glu.glutamate$sym_titer_corr)~glu.glutamate$treatment)

        Bartlett test of homogeneity of variances

data: sqrt(glu.glutamate$sym_titer_corr) by glu.glutamate$treatment
Bartlett's K-squared = 3.6626, df = 2, p-value = 0.1602
```

```
> aov.glu.sym = aov(sqrt(sym_titer_corr) ~ treatment, data=glu.glutamate)
> qqnorm(aov.glu.sym$residuals)
> qqline(aov.glu.sym$residuals)
> summary(aov.glu.sym)
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 390169 195084 1.669 0.209
Residuals 25 2921602 116864

> shapiro.test(glu.glutamate$inverse_r_thorax)

       Shapiro-Wilk normality test

data: glu.glutamate$inverse_r_thorax
W = 0.94345, p-value = 0.1353

> bartlett.test(glu.glutamate$inverse_r_thorax~glu.glutamate$treatment)

       Bartlett test of homogeneity of variances

data: glu.glutamate$inverse_r_thorax by glu.glutamate$treatment
Bartlett's K-squared = 0.030827, df = 2, p-value = 0.9847

> aov.glu.thorax = aov(inverse_r_thorax~ treatment, data=glu.glutamate)
> qqnorm(aov.glu.thorax$residuals)
> qqline(aov.glu.thorax$residuals)
> summary(aov.glu.thorax)
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 0.0000007 3.560e-07 0.018 0.983
Residuals 25 0.0005078 2.031e-05
> #ABDOMEN#
> shapiro.test(glu.symCT$inverse_r_abdomen)

       Shapiro-Wilk normality test

data: glu.symCT$inverse_r_abdomen
W = 0.9212, p-value = 0.3671

> shapiro.test(glu.sym0.01$inverse_r_abdomen)
```

```
        Shapiro-Wilk normality test

data: glu.sym0.01$inverse_r_abdomen
W = 0.90676, p-value = 0.2595

> shapiro.test(glu.sym0.1$inverse_r_abdomen)

        Shapiro-Wilk normality test

data: glu.sym0.1$inverse_r_abdomen
W = 0.89124, p-value = 0.2403

> bartlett.test(glu.glutamate$inverse_r_abdomen~glu.glutamate$treatment)

        Bartlett test of homogeneity of variances

data: glu.glutamate$inverse_r_abdomen by glu.glutamate$treatment
Bartlett's K-squared = 0.90558, df = 2, p-value = 0.6359

> aov.glu.abdomen = aov(inverse_r_abdomen ~ treatment, data=glu.glutamate)
> qqnorm(aov.glu.abdomen$residuals)
> qqline(aov.glu.abdomen$residuals)
> summary(aov.glu.abdomen)
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 0.0000050 2.495e-06 0.19 0.828
Residuals 25 0.0003288 1.315e-05
> ######### KA ##############
> ka.glutamate = glutamate[19:43,]
> View(ka.glutamate)
> #SYM TITER#
> shapiro.test(sqrt(ka.sym0.01$sym_titer_corr))

        Shapiro-Wilk normality test

data: sqrt(ka.sym0.01$sym_titer_corr)
W = 0.88977, p-value = 0.3559

> shapiro.test(sqrt(ka.sym0.1$sym_titer_corr))
```

```
        Shapiro-Wilk normality test

data: sqrt(ka.sym0.1$sym_titer_corr)
W = 0.84815, p-value = 0.05521

> bartlett.test(sqrt(ka.glutamate$sym_titer_corr)~ka.glutamate$treatment)

        Bartlett test of homogeneity of variances

data: sqrt(ka.glutamate$sym_titer_corr) by ka.glutamate$treatment
Bartlett's K-squared = 0.93699, df = 2, p-value = 0.6259

> aov.ka.sym = aov(sqrt(sym_titer_corr) ~ treatment, data=ka.glutamate)
> qqnorm(aov.ka.sym$residuals)
> qqline(aov.ka.sym$residuals)
> summary(aov.ka.sym)
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 411239 205619 0.734 0.491
Residuals 22 6162329 280106
> #THORAX#
> shapiro.test(ka.glutamate$inverse_r_thorax)

        Shapiro-Wilk normality test

data: ka.glutamate$inverse_r_thorax
W = 0.92358, p-value = 0.07951

> bartlett.test(ka.glutamate$inverse_r_thorax~ka.glutamate$treatment)

        Bartlett test of homogeneity of variances

data: ka.glutamate$inverse_r_thorax by ka.glutamate$treatment
Bartlett's K-squared = 0.18906, df = 2, p-value = 0.9098

> aov.ka.thorax = aov(inverse_r_thorax ~ treatment, data=ka.glutamate)
> qqnorm(aov.ka.thorax$residuals)
> qqline(aov.ka.thorax$residuals)
> summary(aov.ka.thorax)
```

```
          Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 2.4e-06 1.178e-06 0.057 0.944
Residuals 20 4.1e-04 2.050e-05
2 observations deleted due to missingness
> #ABDOMEN#
> shapiro.test(1/glu.symCT$inverse_r_abdomen)

       Shapiro-Wilk normality test

data: 1/glu.symCT$inverse_r_abdomen
W = 0.89379, p-value = 0.187

> shapiro.test(1/ka.sym0.01$inverse_r_abdomen)

       Shapiro-Wilk normality test

data: 1/ka.sym0.01$inverse_r_abdomen
W = 0.95939, p-value = 0.8037

> shapiro.test(1/ka.sym0.1$inverse_r_abdomen)

       Shapiro-Wilk normality test

data: 1/ka.sym0.1$inverse_r_abdomen
W = 0.83675, p-value = 0.06973

> bartlett.test(1/ka.glutamate$inverse_r_abdomen~ka.glutamate$treatment)

       Bartlett test of homogeneity of variances

data: 1/ka.glutamate$inverse_r_abdomen by ka.glutamate$treatment
Bartlett's K-squared = 0.37286, df = 2, p-value = 0.8299

> aov.glu.abdomen = aov(1/inverse_r_abdomen ~ treatment, data=ka.glutamate)
> qqnorm(aov.glu.abdomen$residuals)
> qqline(aov.glu.abdomen$residuals)
> summary(aov.glu.abdomen)
          Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 303 151.4 0.408 0.67
```

```
Residuals 20 7423 371.1
```

6.1.5 NICOTIN

```
   > nicotin = read.csv("Pharma exp NicACh receptor group analysis data
      ↪ sheet.csv", h=T)
> head(nicotin)
  treatment_num treatment sym_titer_corr inverse_r_abdomen inverse_r_thorax
1 1 2NIC_0.01 233000 0.008816399 0.01677346
2 1 2NIC_0.01 284000 0.013118367 0.01990921
3 2 2NIC_0.1 1950000 0.017023015 0.02608242
4 2 2NIC_0.1 5450 0.020533038 0.02612467
5 2 2NIC_0.1 4570000 0.012437811 0.02027164
6 3 1BUP_0.01 48700 0.017125927 0.02562000
> View(nicotin)
> nic.symCT = nicotin[which(nicotin$treatment_num ==’6’),]
> nic.sym0.01 = nicotin[which(nicotin$treatment_num ==’1’),]
> nic.sym0.1 = nicotin[which(nicotin$treatment_num ==’2’),]
> bup.symCT = nicotin[which(nicotin$treatment_num ==’5’),]
> bup.sym0.01 = nicotin[which(nicotin$treatment_num ==’3’),]
> bup.sym0.1 = nicotin[which(nicotin$treatment_num ==’4’),]
> ######## BUP ############
> bup.nicotin = nicotin[c(6:31),]
> View(bup.nicotin)
> #SYM TITER#
> shapiro.test(sqrt(bup.symCT$sym_titer_corr))

        Shapiro-Wilk normality test

data: sqrt(bup.symCT$sym_titer_corr)
W = 0.96427, p-value = 0.8332

> shapiro.test(sqrt(bup.sym0.01$sym_titer_corr))

        Shapiro-Wilk normality test

data: sqrt(bup.sym0.01$sym_titer_corr)
W = 0.95442, p-value = 0.7385
```

```
> shapiro.test(sqrt(bup.sym0.1$sym_titer_corr))

        Shapiro-Wilk normality test

data: sqrt(bup.sym0.1$sym_titer_corr)
W = 0.91643, p-value = 0.4421

> bartlett.test(sqrt(bup.nicotin$sym_titer_corr)~bup.nicotin$treatment)

        Bartlett test of homogeneity of variances

data: sqrt(bup.nicotin$sym_titer_corr) by bup.nicotin$treatment
Bartlett's K-squared = 1.7926, df = 2, p-value = 0.4081

> aov.bup.sym = aov(sym_titer_corr ~ treatment, data=bup.nicotin)
> summary(aov.bup.sym)
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 1.525e+12 7.625e+11 1.834 0.182
Residuals 23 9.563e+12 4.158e+11
> #THORAX#
> shapiro.test(bup.nicotin$inverse_r_thorax)

        Shapiro-Wilk normality test

data: bup.nicotin$inverse_r_thorax
W = 0.90268, p-value = 0.02099

> bartlett.test(bup.nicotin$inverse_r_thorax~bup.nicotin$treatment)

        Bartlett test of homogeneity of variances

data: bup.nicotin$inverse_r_thorax by bup.nicotin$treatment
Bartlett's K-squared = 0.72334, df = 2, p-value = 0.6965

> aov.bup.thorax = aov(1/sqrt(inverse_r_thorax) ~ treatment, data=bup.
   ↪ nicotin)
> qqnorm(aov.bup.thorax$residuals)
> qqline(aov.bup.thorax$residuals)
> summary(aov.bup.thorax)
```

```
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 0.175 0.0876 0.186 0.832
Residuals 22 10.374 0.4715
1 observation deleted due to missingness
> #ABDOMEN#
> shapiro.test(bup.symCT$inverse_r_abdomen)

       Shapiro-Wilk normality test

data: bup.symCT$inverse_r_abdomen
W = 0.9212, p-value = 0.3671

> shapiro.test(bup.sym0.01$inverse_r_abdomen)

       Shapiro-Wilk normality test

data: bup.sym0.01$inverse_r_abdomen
W = 0.89298, p-value = 0.2494

> shapiro.test(bup.sym0.1$inverse_r_abdomen)

       Shapiro-Wilk normality test

data: bup.sym0.1$inverse_r_abdomen
W = 0.93271, p-value = 0.5741

> bartlett.test(bup.nicotin$inverse_r_abdomen~bup.nicotin$treatment)

       Bartlett test of homogeneity of variances

data: bup.nicotin$inverse_r_abdomen by bup.nicotin$treatment
Bartlett's K-squared = 0.065865, df = 2, p-value = 0.9676

> aov.bup.abdomen = aov(inverse_r_abdomen ~ treatment, data=bup.nicotin)
> qqnorm(aov.bup.abdomen$residuals)
> qqline(aov.bup.abdomen$residuals)
> summary(aov.bup.abdomen)
           Df Sum Sq Mean Sq F value Pr(>F)
treatment 2 1.227e-05 6.136e-06 0.529 0.596
```

```
Residuals 22 2.550e-04 1.159e-05
1 observation deleted due to missingness
```

6.2 R Script for statistical analysis of ecological assay

```
    library(ggplot2) #ggplot2 will be used to build the plots
#load data
#beware of the direction of the slashbars
#calling the directory of the location of the data file
setwd("L:/Users/Bhattacharyya_Soumi/MS Thesis/Humidity experiment_Imaging
    ↪ data_Melanisation_0.Sur")

#saving the data under a name
symdata2 <- read.csv("Humidity experiment run3 cumulated data sheet.csv",
    ↪ sep = ",", dec=".")

#segregating the dataset into the offspring and parent subsets for ease
offspring2 <- subset(symdata2, treatment_num < 5)
parent2 <- subset(symdata2, treatment_num > 4)

str(symdata2) #to display the structures of the R objects in the data file
attach(symdata2) #to access the variables present in the data framework
    ↪ without calling the data frame

#box and violin plots of symbiont titer of parental populations
#log of sym_titer_corr vs treatment
p1 <- ggplot(parent2, aes(x=treatment, y=log10(sym_titer_corr), fill =
    ↪ treatment))
p1 + geom_violin() + geom_boxplot(width = 0.5, fill = "grey") + geom_point
    ↪ () + theme_classic()

#box and violin plots of symbiont titer of offspring treatments
#log of sym_titer_corr vs treatment
#customized the fill colours of the violin plot with the scale_fill_manual(
    ↪ values=c("")) command
p2 <- ggplot(offspring2, aes(x=treatment, y=log10(sym_titer_corr), fill =
    ↪ treatment))
```

```
p2 + geom_violin() + scale_fill_manual(values = c("#FA910A", "#27BBE3", "#
    ↪ FEE001", "#E68AD9"))+ geom_boxplot(width = 0.5, fill = "grey") +
    ↪ geom_point() + theme_classic()

#box and violin plots of melanization of abdomen of offspring treatments
mela <- ggplot(offspring2, aes(x=treatment, y=inverse_r_abdomen, fill =
    ↪ treatment))
mela + geom_violin() + scale_fill_manual(values = c("#FA910A", "#27BBE3",
    ↪ "#FEE001", "#E68AD9")) + geom_boxplot(width = 0.5, fill = "grey") +
    ↪ geom_point() + theme_classic()
#box and violin plots of melanization of thorax of offspring treatments
melt <- ggplot(offspring2, aes(x=treatment, y=inverse_r_thorax, fill =
    ↪ treatment))
melt + geom_violin() + scale_fill_manual(values = c("#FA910A", "#27BBE3",
    ↪ "#FEE001", "#E68AD9")) + geom_boxplot(width = 0.5, fill = "grey") +
    ↪ geom_point() + theme_classic()


#Statistical Analysis

str(symdata2)
attach(symdata2)

#Running the Anova on untransformed sym titer values to find if the
    ↪ differences in the titers are statistically significant or not
aov_sym_titer<-aov(offspring2$sym_titer_corr~offspring2$parentH*
    ↪ offspring2$offspringH)
summary(aov_sym_titer)
                                                    Df Sum Sq Mean Sq F value
                                                        ↪  Pr(>F)

offspring2$parentH 1 1.527e+13 1.527e+13 2.528 0.1206
offspring2$offspringH 1 3.317e+13 3.317e+13 5.490 0.0248*
offspring2$parentH:offspring2$offspringH 1 6.332e+10 6.332e+10 0.010 0.9190
Residuals 36 2.175e+14 6.042e+12
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1
```

```
#Checking the homogeneity of variance assumption for symbiont titer
#The residuals versus fits plot is used to check the homogeneity of
   ↪ variances. In the plot below, we cannot assume the homogeneity of
   ↪ variances as the red line is away from the mean line.
plot(aov_sym_titer,1)

#Normality plot of the residuals. In the plot below, the quantiles of the
   ↪ residuals are plotted against the quantiles of the normal
   ↪ distribution. A 45-degree reference line is also plotted.
#The normal probability plot of residuals is used to verify the assumption
   ↪ that the residuals are normally distributed.
#The normal probability plot of the residuals should approximately follow a
   ↪  straight line.



plot(aov_sym_titer,2)
#Checking the homogeneity of variance assumption for melanization of the
   ↪ abdomen
str(offspring2)

aov_melan_abdomen<-aov(offspring2$X.inverse_r_abdomen~offspring2$parentH*
   ↪ offspring2$offspringH)
summary(aov_melan_abdomen)

                                  Df Sum Sq Mean Sq F value Pr(>F)
offspring2$parentH 1 1.054e-08 1.054e-08 2.753 0.106
offspring2$offspringH 1 1.330e-07 1.330e-07 34.729 9.66e-07




offspring2$parentH: 1 7.670e-09 7.670e-09 2.004 0.165
offspring2$offspringH

Residuals 36 1.378e-07 3.830e-09
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

plot(aov_melan_abdomen,1)
plot(aov_melan_abdomen,2)
```

```
#Checking the homogeneity of variance assumption for melanization of thorax
str(offspring2)

aov_melan_thorax<-aov(offspring2$X.inverse_r_thorax~offspring2$parentH*
   ↪ offspring2$offspringH)
summary(aov_melan_thorax)

                                    Df Sum Sq Mean Sq F value Pr(>F)
offspring2$parentH 1 2.000e-17 2.000e-17 0.003 0.958188
offspring2$offspringH 1 1.349e-13 1.349e-13 16.456 0.000255 ***
offspring2$parentH:offspring2$offspringH 1 8.610e-15 8.610e-15 1.051
   ↪ 0.312103
Residuals 36 2.951e-13 8.200e-15
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

plot(aov_melan_thorax,1)
plot(aov_melan_thorax,2
#The Shapiro-Wilk Test is done to do a normality check

#Shapiro-wilk test for symbiont titer
aov_residuals <- residuals(object = aov_sym_titer)
shapiro.test(x = aov_residuals)

       Shapiro-Wilk normality test

data: aov_residuals
W = 0.8755, p-value = 0.0003989

Normality is violated

#Shapiro-wilk test for melanization of abdomen
aov_residuals <- residuals(object = aov_melan_abdomen)
shapiro.test(x = aov_residuals )

       Shapiro-Wilk normality test

data: aov_residuals
W = 0.96842, p-value = 0.3203
```

```
Normality is not violated

#Shapiro-wilk test for melanization of thorax
aov_residuals <- residuals(object = aov_melan_thorax)
shapiro.test(x = aov_residuals)

        Shapiro-Wilk normality test

data: aov_residuals
W = 0.90999, p-value = 0.003782

Normality is violated

#We, therefore, perform transformations on the data to have homogeneity in
   ↪ the variances and have normalized data and finally do ANOVA on the
   ↪ transformed data.

#For symbiont titer, the sqrt() transformation has been used. We will
   ↪ repeat the check of homogeneity of variance and normality checks for
   ↪ the sqrt() transformation of the symbiont titer.

aov_sym_titer_sqrt<-aov(offspring2$sqrt_sym_titer_corr~offspring2$parentH*
   ↪ offspring2$offspringH)
plot(aov_sym_titer_sqrt,1)
plot(aov_sym_titer_sqrt,2)

aov_residuals <- residuals(object = aov_sym_titer_sqrt)
# Run Shapiro-Wilk test
shapiro.test(x = aov_residuals )

        Shapiro-Wilk normality test

data: aov_residuals
W = 0.957, p-value = 0.1322

Normality is not violated

#levene test - to check the homogeneity of variances
```

```
#The function factor is used to encode a vector as a factor

offspring2$parentH<-as.factor(offspring2$parentH)
offspring2$offspringH<-as.factor(offspring2$offspringH)
offspring2$treatment_num<-as.factor(offspring2$treatment_num)
leveneTest(offspring2$sqrt_sym_titer_corr ~ offspring2$treatment_num)

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group 3 1.5288 0.2237
      36
P value > 0.05, This means that there is no evidence to suggest that the
   ↪ variance across groups is statistically significantly different.
   ↪ Therefore, we can assume the homogeneity of variances in the
   ↪ different treatment groups.

#ANOVA of the sqrt of sym titer data
aov_sym_titer_sqrt<-aov(offspring2$sqrt_sym_titer_corr~offspring2$parentH*
   ↪ offspring2$offspringH)
summary(aov_sym_titer_sqrt)
                                    Df Sum Sq Mean Sq F value Pr(>F)
offspring2$parentH 1 3073148 3073148 4.719 0.03649 *
offspring2$offspringH 1 4891996 4891996 7.512 0.00948 **
offspring2$parentH:offspring2$offspringH 1 57526 57526 0.088 0.76801
Residuals 36 23443210 651200
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

#TukeyHSD(aov_sym_titer)

#As the ANOVA test is significant, we can compute Tukey HSD (Tukey Honest
   ↪ Significant Differences, R function: TukeyHSD()) for performing
   ↪ multiple pairwise-comparison between the means of groups. The
   ↪ function TukeyHD() takes the fitted ANOVA as an argument.

#TukeyHSD - symtiter
TukeyHSD(aov_sym_titer_sqrt, conf.level = .95)
Tukey multiple comparisons of means
   95% family-wise confidence level
```

```
Fit: aov(formula = offspring2$sqrt_sym_titer_corr ~ offspring2$parentH *
    ↪ offspring2$offspringH)

$`offspring2$parentH`
         diff lwr upr p adj
60-30 554.3598 36.81811 1071.902 0.0364915

$`offspring2$offspringH`
        diff lwr upr p adj
60-30 699.428 181.8863 1216.97 0.009479

$`offspring2$parentH:offspring2$offspringH`
                diff lwr upr p adj
60:30-30:30 630.2055 -341.7475 1602.159 0.3155135
30:60-30:30 775.2737 -196.6793 1747.227 0.1575169
60:60-30:30 1253.7879 281.8349 2225.741 0.0070796
30:60-60:30 145.0682 -826.8848 1117.021 0.9776880
60:60-60:30 623.5824 -348.3706 1595.535 0.3245361
60:60-30:60 478.5142 -493.4389 1450.467 0.5528908









#For melanization of abdomen, the square() transformation has been used. We
    ↪  will repeat the check of homogeneity of variance and normality
    ↪ checks for the square() transformation of the melanization of abdomen
    ↪ .

str(offspring2)

aov_melan_abdomen_sq<-aov(offspring2$X.inverse_r_abdomen..2~
    ↪ offspring2$parentH*offspring2$offspringH)
plot(aov_melan_abdomen_sq,1)
plot(aov_melan_abdomen_sq,2)
```

```
#Shakiro-Wilk test
aov_residuals <- residuals(object = aov_melan_abdomen_sq)
shapiro.test(x = aov_residuals )

       Shapiro-Wilk normality test

data: aov_residuals
W = 0.96842, p-value = 0.3203

Normality is not violated

#Levene Test
leveneTest(offspring2$X.inverse_r_abdomen..2 ~ offspring2$treatment_num)

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group 3 1.5069 0.2292
      36
P value > 0.05, This means that there is no evidence to suggest that the
   ↪ variance across groups is statistically significantly different.
   ↪ Therefore, we can assume the homogeneity of variances in the
   ↪ different treatment groups.

#ANOVA of the square of melanization of abdomen data
summary(aov_melan_abdomen_sq)

                                    Df Sum Sq Mean Sq F value Pr(>F)
offspring2$parentH 1 1.054e-08 1.054e-08 2.753 0.106
offspring2$offspringH 1 1.330e-07 1.330e-07 34.729 9.66e-07 ***
offspring2$parentH:offspring2$offspringH 1 7.670e-09 7.670e-09 2.004 0.165
Residuals 36 1.378e-07 3.830e-09
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

#We can also do the transformations directly in the anova code.
aov_a2<-aov(offspring$inverse_r_abdomen^2 ~ offspring$parentH*
   ↪ offspring$offspringH)

aov_residuals <- residuals(object = aov_a2)
```

```
shapiro.test(x = aov_residuals )

        Shapiro-Wilk normality test


data: aov_residuals
W = 0.96842, p-value = 0.3203

offspring2$parentH<-as.factor(offspring2$parentH)
offspring2$offspringH<-as.factor(offspring2$offspringH)
offspring2$treatment_num<-as.factor(offspring2$treatment_num)
leveneTest(offspring2$inverse_r_abdomen^2 ~ offspring2$parentH*
   ↪ offspring2$offspringH)

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group 3 1.507 0.2292
      36

summary(aov_a2)

                                     Df Sum Sq Mean Sq F value Pr(>F)
offspring$parentH 1 1.054e-08 1.054e-08 2.753 0.106
offspring$offspringH 1 1.330e-07 1.330e-07 34.729 9.66e-07 ***
offspring$parentH:offspring$offspringH 1 7.670e-09 7.670e-09 2.004 0.165
Residuals 36 1.378e-07 3.830e-09
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1




#TukeyHSD - melanization of abdomen
TukeyHSD(aov_a2, conf.level = .95)


 Tukey multiple comparisons of means
```

```
    95% family-wise confidence level


Fit: aov(formula = offspring2$inverse_r_abdomen^2 ~ offspring2$parentH *
    ↪ offspring2$offspringH)


$`offspring2$parentH`
             diff lwr upr p adj
60-30 3.246753e-05 -7.217066e-06 7.215213e-05 0.1057511


$`offspring2$offspringH`
             diff lwr upr p adj
60-30 0.0001153129 7.562835e-05 0.0001549975 1e-06


$`offspring2$parentH:offspring2$offspringH`
                   diff lwr upr p adj
60:30-30:30 4.765597e-06 -6.976282e-05 7.929402e-05 0.9981500
30:60-30:30 8.761101e-05 1.308259e-05 1.621394e-04 0.0158707
60:60-30:30 1.477805e-04 7.325206e-05 2.223089e-04 0.0000305
30:60-60:30 8.284541e-05 8.316995e-06 1.573738e-04 0.0244458
60:60-60:30 1.430149e-04 6.848647e-05 2.175433e-04 0.0000515
60:60-30:60 6.016947e-05 -1.435895e-05 1.346979e-04 0.1498492

#For melanization of thorax, the 4th power() transformation has been used.
    ↪ We will repeat the check of homogeneity of variance and normality
    ↪ checks for the square() transformation of the melanization of thorax.

aov_melan_thorax_4th<-aov(offspring2$X.inverse_r_thorax..4~
    ↪ offspring2$parentH*offspring2$offspringH)
plot(aov_melan_thorax_4th,1)
plot(aov_melan_thorax_4th,2)
#ShapiroWilk test
aov_residuals <- residuals(object = aov_melan_thorax_4th)
shapiro.test(x = aov_residuals )

        Shapiro-Wilk normality test
```

```
data: aov_residuals
W = 0.90999, p-value = 0.003782

#levene test
leveneTest(offspring2$X.inverse_r_thorax..4 ~ offspring2$treatment_num)

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group 3 0.6752 0.5729
      36

#Anova for melanbization of thorax data
                                      Df Sum Sq Mean Sq F value Pr(>F)
offspring2$parentH 1 2.000e-17 2.000e-17 0.003 0.958188
offspring2$offspringH 1 1.349e-13 1.349e-13 16.456 0.000255 ***
offspring2$parentH:offspring2$offspringH 1 8.610e-15 8.610e-15 1.051
   ↪ 0.312103
Residuals 36 2.951e-13 8.200e-15
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

#We can also do the transformations directly in the anova code.
aov_t4<-aov(offspring$inverse_r_thorax^4 ~ offspring$parentH*
   ↪ offspring$offspringH)

aov_residuals <- residuals(object = aov_t4)
shapiro.test(x = aov_residuals )

       Shapiro-Wilk normality test


data: aov_residuals
W = 0.94422, p-value = 0.05255

offspring2$parentH<-as.factor(offspring2$parentH)
offspring2$offspringH<-as.factor(offspring2$offspringH)
offspring2$treatment_num<-as.factor(offspring2$treatment_num)
```

```
leveneTest(offspring2$inverse_r_thorax^4 ~ offspring2$parentH*
   ↪ offspring2$offspringH)

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group 3 0.8028 0.5008
      35

summary(aov_t4)

                                    Df Sum Sq Mean Sq F value Pr(>F)
offspring$parentH 1 1.510e-15 1.510e-15 0.255 0.6165
offspring$offspringH 1 1.659e-13 1.659e-13 27.986 6.66e-06 ***
offspring$parentH:offspring$offspringH 1 1.966e-14 1.966e-14 3.317 0.0771 .
Residuals 35 2.074e-13 5.930e-15
---
Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1   1
1 observation deleted due to missingness



#TukeyHSD - melanization of thorax
TukeyHSD(aov_t4, conf.level = .95)

Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = offspring2$inverse_r_thorax^4 ~ offspring2$parentH *
   ↪ offspring2$offspringH)

$`offspring2$parentH`
             diff lwr upr p adj
60-30 1.246202e-08 -3.76082e-08 6.253224e-08 0.6165326

$`offspring2$offspringH`
             diff lwr upr p adj
60-30 1.304305e-07 8.036025e-08 1.805007e-07 6.7e-06

$`offspring2$parentH:offspring2$offspringH`
                   diff lwr upr p adj
```

```
60:30-30:30 -2.783908e-08 -1.206930e-07 6.501482e-08 0.8499684
30:60-30:30 8.678499e-08 -6.068902e-09 1.796389e-07 0.0740392
60:60-30:30 1.488474e-07 5.344909e-08 2.442457e-07 0.0009397
30:60-60:30 1.146241e-07 2.177018e-08 2.074780e-07 0.0105910
60:60-60:30 1.766865e-07 8.128817e-08 2.720848e-07 0.0000928
60:60-30:60 6.206241e-08 -3.333590e-08 1.574607e-07 0.3119512

# From the ANOVA results, it can be inferred that symbiont titer and
   ↪ melanization of abdomen is influenced by both the offspring and
   ↪ parental humidity conditions. But there is no interaction between the
   ↪  offspring and parental humidity conditions.
```