Ernst-Abbe-Hochschule Jena
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FUNCTIONAL ANALYSIS OF A PUTATIVE SUGAR TRANSPORTER (SWEET FAMILY) FROM THE POPLAR LEAF BEETLE *Chrysomela populi*

B A C H E L O R T H E S I S

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List of abbreviations

ABC	Adenosine triphosphate binding cassette
ATP	Adenosine triphosphate
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CpMRP	Chrysomela populi multidrug resistance-associated protein
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FRET	Fluorescence resonance energy transfer
fwd	Forward
GC	Gas chromatography
GFP	Green fluorescent protein
GLUT	Glucose transporter
HPLC	High pressure liquid chromatography
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
ORF	Open reading frame
PCR	Polymerase chain reaction
PCR	Significance value
qPCR	Quantitative polymerase chain reaction
rev	Reverse
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
sem	Standard error of the mean
SGLT	Sodium-glucose symporter
SLC	Solute carrier
SWEET	Sugars will eventually be exported transporter
TAE	Tris Acetate EDTA
THB	Triple helix bundle
TMH	Transmembrane helices

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1 Introduction

Carbohydrates play a key role in the flourishing and continuous development of lifeforms across the biosphere. Especially sugars are involved in essential processes like generation of energy and metabolic pathways [1)]. To maintain necessary cell functions through uptake and efflux of sugars a multitude of sugar transporters are needed. In eukaryotes, three classes of sugar transport proteins are characterized, that consist of Glucose transporters (GLUTs), sodium-glucose symporters (SGLTs) and Sugars Will Eventually be Exported Transporters (SWEETs), a recently identified class of putative low affinity sugar uniporters [1, 2)].

1.1 SWEET protein family

SWEETs are a steady object of research. Screening of *Arabidopsis thaliana* genes for the presence of previously uncharacterized polytopic membrane proteins led to the discovery of this new transporter family [2)]. Most of their function has been examined on plants, while their physiological purpose within animals is largely unknown [2-4)]. SWEET carbohydrate transport activities are associated with pollen nutrition acquirement, male fertility, nectar secretion, seed filling, pathogen susceptibility and more [2-4)]. Since then, homologues were found in several organisms, with for instance typically twenty genes corresponding to SWEET expression in plant genomes, four in fungi, seven in *Caenorhabditis elegans* and one in *Drosophila melanogaster* as well as one in humans [5, 6)]. Further bioinformatic analysis confirmed the existence of SWEET homologues within prokaryotes termed semiSWEETS. SWEETS are predicted to consist of seven transmembrane α-helices (TMHs), which include two triple helix bundles (THBs) being coupled by an inversion linker helix. SemiSWEETS, on the other hand, only contain one single THB unit [4-8)] (fig. 1).

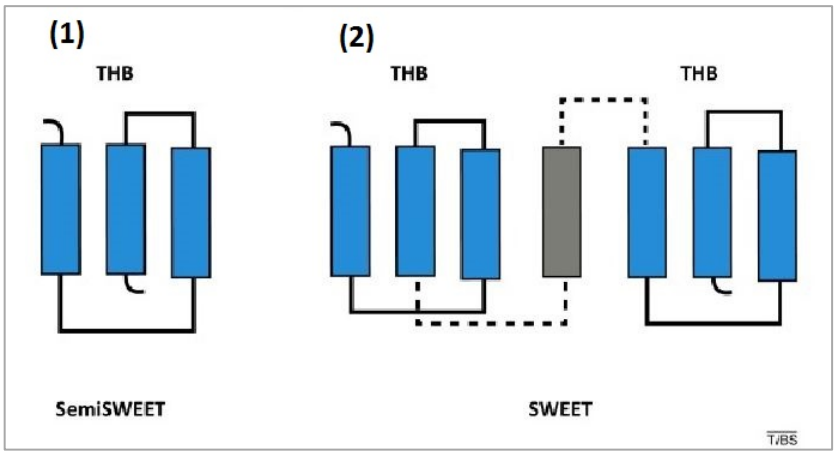

Figure 1: SWEET and semiSWEET structure. (1): triple helix bundle (THB) of semiSWEET (2): THB repeat with inversion linker helix in SWEETs [5)]

Studies based on their configuration suggest an evolutionary link between both protein families, whereby prokaryotic semiSWEETs functioned as an archetype for the development of SWEET proteins [1, 5, 6)].

Analysis of crystal structures within bacterial semiSWEETs indicated differences in their formation that correspond with open and occluded states aligned with movement observed in other sugar transporters. Further crystal structure analysis led to the conclusion that a dimer of semiSWEETs is sufficient to form a pore and therefore a SWEET monomer is thought to be adequate for transportation of substrates across membranes [1, 5, 8)]. The molecular mechanisms of sugar translocation by SWEETs are mostly unknown. Current state of research suggests mechanisms similar to that of uniporters [2, 5)]. Further functional characterization is needed to provide more information concerning their role throughout the biosphere.

1.2 *Chrysomela populi*

The leaf beetle species *Chrysomela populi* is native to forests throughout Europe and West Asia. It feeds on poplar as well as willow leaves. Like other representatives of the *Chrysomelidae* family, their occurrence is considered to have adverse effects on forestry and agriculture. Since they spend their entire life on the host plant, their presence is linked to harmful defoliation of trees [9)]. Larvae hatch twelve days after eggs have been laid and they feed for about three weeks until they get into the pupal stage, which lasts another ten days. Larvae show white coloring with black dots on their body whereas adults typically appear in bright red colors [10)]. L1, L2 and L3 serve as descriptions of larval development. Each of those stages indicates a new phase after molting [11)].

Larvae stage is followed by pupation and subsequent adult stage, where *C. populi* can mate and reproduce further (fig. 2).

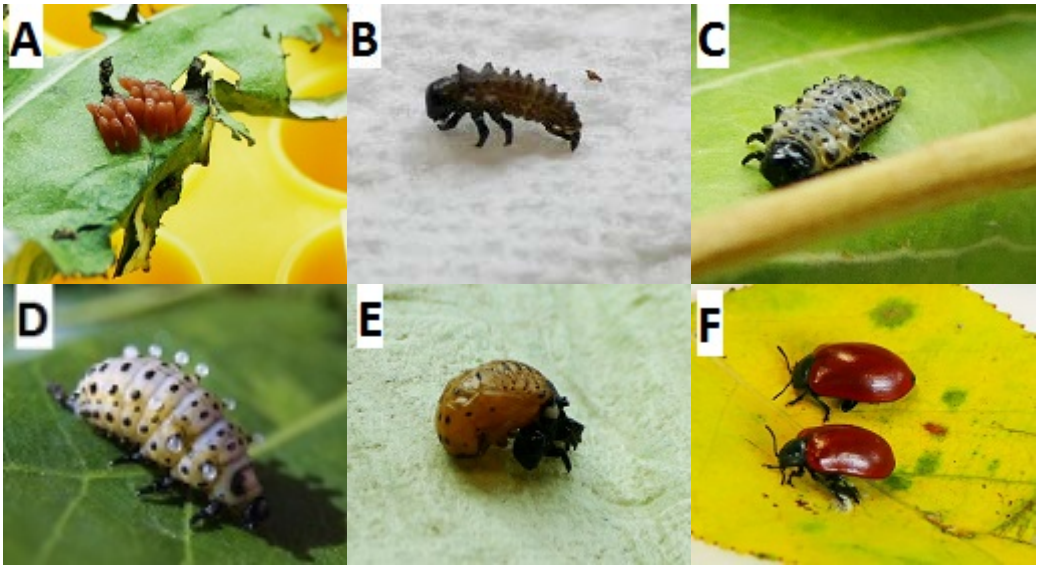

Figure 2: States of *C. populi* development. A: Eggs; B: first instar larva L1; C: second instar larva L2; D: third instar larva L3 exposing visible defensive secretion; E: Pupa; F: Adult beetle. (Source: Anja David and Anja Strauß, Max-Planck-Institute for Chemical Ecology).

During their juvenile development, *C populi* is able to excrete substances by specialized defensive glands that act as a deterrent for potential predators as well as pathogens [12, 13]. This is accomplished through evolutionary adaptation, which led to the ability of utilizing plant-derived compounds for buildup of their own chemical defense systems. Influx and efflux mechanisms ensure the translocation of glucosides through the body and into the defensive glands. There the ingested salicin is transformed into salicylaldehyde through two enzymatic steps (fig. 3). This process is referred to as sequestration, whereby exogenous chemicals are transformed into vital components of the organism [14].

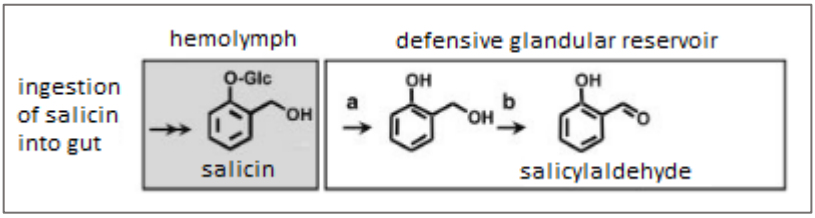

Figure 3: State of sequestered salicin throughout different tissues. a: glucosidase activity and b: oxidase activity [15].

The glucose residue of salicin is removed by the enzyme ß-glucosidase. An oxidase then finalizes the catalytic process of converting salicin into salicylaldehyde by cleavage of H_2O_2 [15].

The translocation of glucosides such as salicin through the larval body requires a network of transport proteins. Research on sequestration processes within *C. populi* indicated the presence of several transport systems that act as selective barriers along membranes [16, 17]. One such transporter, the *C. populi* multidrug resistance associated protein (*Cp*MRP), has been found to be distributed along vesicular membranes within secretory cells. There it supports the shuttling of salicin, which is imported from the hemolymph, into the defensive gland reservoir [16]. The ATP binding cassette transporter (ABC transporter) *Cp*MRP acts as a pacemaker for translocation of glucosides. *Cp*MRP possibly supports the influx of salicin into secretory cells by so far uncharacterized gradient driven transport proteins (fig. 4). Sequestration is most likely helped by an array of other still uncharacterized transporters, since the existence of an ATP dependent protein makes the additional transfer of glucose likely [18].

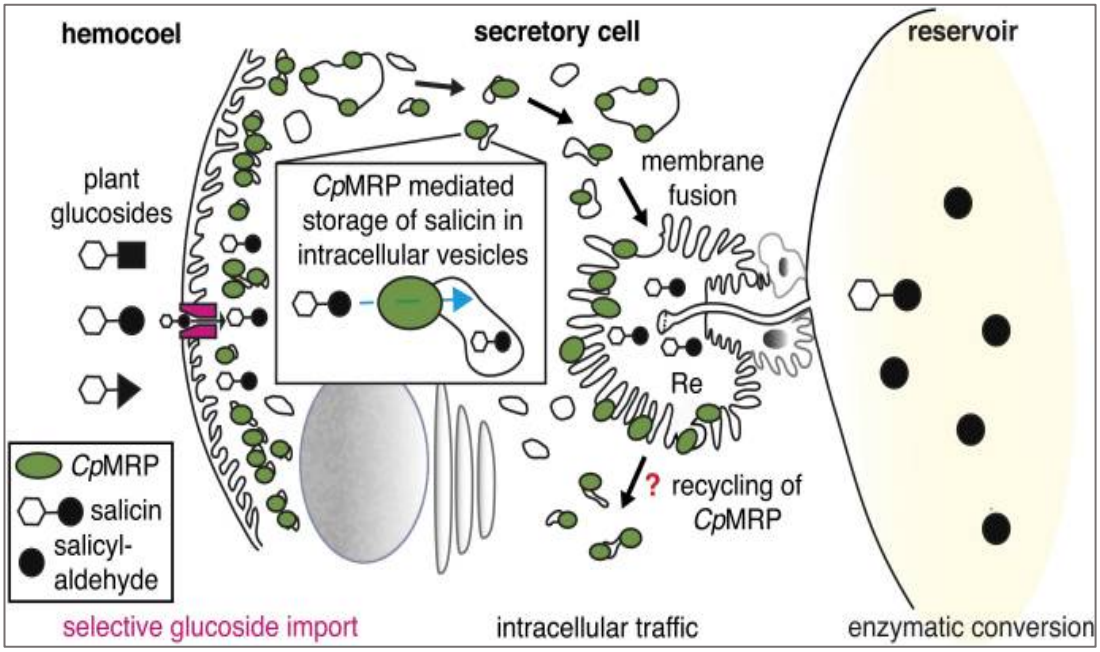

Figure 4: Model of *Cp*MRPs pacemaker function in the sequestration of salicin within the defensive glands. The ABC transporter *Cp*MRP is distributed along the membrane of vesicles within secretory cells and mediates the translocation of salicin into the defensive gland reservoir. Salicin is accumulated and stored in vesicles where it can be transported further. A putative membrane-based, gradient-driven and energy-independent transporter on the hemolymph side selectivly facilitates salicin transfer into secretory cells [16].

1.3 Potential role of SWEET proteins within *C. populi*

Transcriptomal analysis within *C. populi* helped to identify several types of potential transporters, including two sequences that correspond to the expression of SWEET proteins [19].

Previous research, as presented in the framework of a Bachelors thesis by Noreen Schuck, suggested the contribution of SWEETs to sequestration mechanisms within leaf beetles [20]. Sequestering plant derived glucosides, such as salicin, leads to the aggregation of salicylaldehyde within defensive secretion. This is done under the enzymatic cleavage of glucose within the reservoir [13, 15]. Glucose has then to be transferred against a steep gradient out of the reservoir [12]. Since SWEETs are known hexose transporters and are identified within the transcriptom of *C. populi*, it is reasonable to assume a contribution of SWEETs within the production of deterrents.

2 Aim of the thesis

Aim of this bachelor thesis is to characterize the function of a SWEET protein in *C. populi* throughout all lifecycle stages. A higher expression level within adult beetles as compared to larvae may for instance be related to a change in metabolic requirements. Expression level within different tissue samples is checked to further investigate the potential implications of SWEETs within the organism. A significantly higher rate of *sweet* expression within defensive glands would support the assumption of a role within sequestration processes. Results of expression level measurement could therefore help to shed light on a potential transporter function.
A role within the sequestration process of *C. populi* is to be checked by RNAi experiments, whereby gene expression shall be knocked down and organisms will subsequently be investigated for phenotypic changes. Those changes could potentially consist of a noticeable change in rate of growth and mortality rate, morphological changes throughout developmental stages, as well as changes within the composition of hemolymph and defensive secretion.

3 Methods

3.1 Biological Methods

3.1.1 Storage of the examined breed of *C. populi*

The leaf beetle *C. populi* originated from forests near Thrarandt and was bred in the working group in a climate chamber at a constant temperature of 20°C and 75% humidity. The beetles were kept in a ventilated box that was outlaid with pre wetted paper towels. The climate chambers were configured to illuminate the inside of the chamber during the hours of 6:00 to 22:00 at 100% light intensity. The beetles were fed a diet of poplar leaves of *Populus trichocarpa*, twice a week.

3.1.2 Preparation of the examined tissue

All examinations were performed on leaf beetle specimen throughout every stage of their life cycle. To check wether SWEET is expressed in *C. populi* in general, RNA was isolated from the whole larvae. To analyze gene expression in specific tissues, larvae were placed under a microscope in 0.9% NaCl solution. Obtained tissue consisted of Malpighian tubules, head, intestines, defensive glands and fat body tissue. The samples were then stored within a reaction tube filled with 100 µl of lysis solution and kept until further use at -20°C.

3.2 Molecular biological methods

3.2.1 RNA isolation

During the first step of RNA isolation, tissue was disrupted mechanically through the use of a pestle suitable for reaction tubes. A small amount of SiO_2 has been added to the lysis solution to facilitate tissue disruption. Previously acquired samples were either processed with the help of RNAqueous® Micro Total RNA Isolation Kit [Ambion ® Applied Biosystems ®] or RNeasy® Mini Kit [QIAGEN]. Samples were in both cases handled as described in their instructions. In case of the RNAqueous® Micro Total RNA Isolation Kit, centrifugation was always executed at 16.500 x g and elution procedure was performed in every case with an aliquot volume of 10 µl elution solution. If Qiagen RNeasy Mini Kit was used, full speed centrifugation has been performed at 15.000 x g, with every other centrifugation step performed at 8.500 x g.

3.2.2 Synthesis of complementary DNA

The synthesis of complementary deoxyribonucleic acid (cDNA) is achieved through the means of reverse transcriptase. This method offers the possibility to rewrite messenger RNA (mRNA) into DNA through the use of the enzyme SuperScript™ III Reverse Transcriptase [Invitrogen]. The process was executed in accordance with the associated First-Strand cDNA Synthesis protocol. Step five of the protocol was executed at 50°C for 60 min. The application of RNaseOUT™ was omitted. The cDNA was then stored at -20°C until it has been used as a template for further experiments.

3.2.3 Polymerase chain reaction

Finding the optimal temperature for primer annealing is one the foundations of an effective PCR. Different enzymes and buffer mixes make it necessary to run PCR programs with specified annealing step temperatures. Web based calculators were employed to establish ideal annealing temperatures suitable for specific polymerase enzymes. Tm Calculator [Thermo Fisher Scientific] for application of Phusion polymerase and BioMath calculator [Promega] for usage of GoTaq® G2 Polymerase have been utilized.

With the help of polymerase chain reaction (PCR), it is possible to amplify certain nucleotide sequences within a DNA template. This was done with the help of specific nucleotide primers and DNA polymerase enzymes. The selection consisted of two different polymerases, namely GoTaq® G2 DNA [Promega] and Phusion High-Fidelity DNA polymerase [Thermo Fisher Scientific]. Each enzyme was provided with an associated reaction buffer mix. Phusion polymerase enzyme provides a lower frequency of error and was utilized for creation of double strained RNA. To check primer functionality, GoTaq ® G2 DNA polymerase was used. Substance volumes and thermal cycler programs used are listed below. Templates consisted of cDNA, purified PCR products and plasmid cp_slc2 t-stop 1-5 [InvitrogenTM] as a control of the methodological accuracy. The PCR effectiveness can be verified by subsequent gel electrophoresis.

Table 1: Components of 20 µl PCR reaction mix for usage of GoTaq® G2 Polymerase

Substance	Volume
Nuclease free H_2O	12 µl
5x Green GoTaq® Reaction Buffer	4 µl
1:10 diluted nucleotide primermix	2 µl
Template DNA	1.5 µl
10mM dNTP mix	0.4 µl
GoTaqG2® Polymerase	0.1 µl

Table 2: Thermal cycler program for usage of GoTaq® G2 Polymerase

Step	Temperature	Time	Number of cycles
Initial denaturation	95°c	2 min	1
Denaturation	95°C	30 s	30
Annealing	58°C	45 s	
Extension	74°C	50 s	
Final extension	74°C	5 min	1

Table 3: Components of 20 µl reaction mix for usage of Phusion Polymerase

Substance	Volume
Nuclease free H_2O	11.9 µl
5x Phusion HF Buffer	4 µl
1:10 diluted nucleotide primer mix	2 µl
Template DNA	1.5 µl
10mM dNTP mix	0.4 µl
Phusion Polymerase	0.2 µl

Table 4: Thermal cycler program for usage of Phusion Polymerase

Step	Temperature	Time	Number of cycles
Initial denaturation	98°c	30 s	1
Denaturation	98°C	7 s	35
Annealing	66.4°C	10 s	
Extension	72°C	30 s	
Final extension	72°C	10 min	1

3.2.4 PCR purification

To remove remains of any previously used primers, enzymes and other possible contaminants, PCR products were treated with QIAquick PCR Purification Kit [QIAGEN]. The method was carried out by following the associated protocol. Elution was carried out by adding 30 µl elution solution to the center of the filter column membrane and a waiting period of one minute before subsequent centrifugation. This was done to ensure the maximum DNA concentration within the collected eluate. Centrifugation steps have been performed at 11.400 x g and centrifugation at maximum speed was done at 15.000 x g.

3.2.5 Real time polymerase chain reaction

Real time polymerase chain reaction, or quantitative polymerase chain reaction (qPCR), is a method that applies fluorescence measurement to quantify the amount of amplified DNA in comparison to housekeeping genes *ef1a* and *eif4a* within *C. populi*. The thermal cycler is equipped with means of detecting increasing levels of fluorescence. Fluorescence dye, which attaches to double stranded DNA, is provided within the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix [Agilent Technologies]. Depending on the utilized cDNA template it is possible to make a statement about general gene expression within the whole larval body or compare levels of gene expression within specific tissue. Real time PCR was done with at least two technical replicates to avoid contamination or errors within the composition of the mixture. A list of utilized nucleotide primers can be found under point 3.7 within the material section of this report. Gene expression in either whole larvae or specific larvae tissue was assessed through means of real time PCR. First the expression of the relevant SWEET protein was examined in whole larvae. In the next step level of expression within different tissue samples was checked. Those consisted of Malpighian tubules, head, intestines, secretory glands and fat body tissue. Primers used for this approach included pairs for the relevant SWEET protein and for housekeeping genes *ef1a* as well as *eif4a*. Housekeeping gene expression manifests itself equal throughout all cells and can therefore be used as a reference for the expression level of the relevant SWEET protein. The utilized tool for evaluation was Bio Rad CFX Manager software and Microsoft Excel. Those measurements have been done to three different biological replicates and gave a basis for further reference. The applied mathematical method for calculation was the $2^{-\Delta\Delta C}{}_T$ Method [21)].

Table 5: Components of 20 µl reaction mix with SYBR®-Green QPCR Master Mix

Substance	Volume
Nuclease free H_2O	3 µl
SYBR®-Green QPCR Master Mix	10 µl
1:10 diluted nucleotide primer mix	5 µl
Template DNA	2 µl

Table 6: Thermal cycler program used with SYBR®-Green QPCR Master Mix

Step	Temperature	Time	Number of cycles
Initial denaturation	95°c	3 min	1
Denaturation	95°C	5 s	40
Annealing	60°C	10 s	
Extension	95°C	10 s	1
Melt Curve	65°C – 95°C	5 s	1

3.2.6 Examination of primer efficiency

For nucleotide primers to be applied, it is suggested to check their efficiency by creating a standard curve through the employment of linear regression. This was executed through arranging a serial dilution starting with an aliquot of specific template DNA. This template was diluted either 1:5 or 1:2 so that a fivefold dilution series was fabricated. Depending on the quantity of nucleic acid available, at least two technical replicates have been generated. Amplification and measurement has been performed through the means of qPCR. Evaluation of the results was done with the help of CFX Manager Software [Bio Rad].

3.2.7 Synthesis of double strained RNA

The synthesis of dsRNA followed by microinjection into the organism is performed to down regulate gene expression and therefore suppress the formation of SWEET proteins. All required steps have been performed utilizing the MEGAscript® RNAi Kit [Thermo Scientific^{TM}] and its associated protocol. CDNA synthesized out of isolated RNA from *C. populi* larvae in L3 stage served as a template for standard PCR with the help of Phusion polymerase and SWEET primer pairs with attached T7 promoter sequences. As an amplification strategy, using two separate 50 µl PCR reactions with opposite T7- and gene specific primers, was used. The synthesized SWEET dsRNA had a length of around 500 base pairs (bp).

Table 7: Components of 50 µl reaction mix for usage of Phusion Polymerase with T7 primers

Substance	Volume
Nuclease free H_2O	29.75 µl
5x Phusion HF Buffer	10 µl
1:10 diluted nucleotideT7 primer	5 µl
Template DNA	3.75 µl
10mM dNTP mix	1 µl
Phusion Polymerase	0.5 µl

Table 8: Thermal cycler program for usage of Phusion Polymerase with T7 primers

Step	Temperature	Time	Number of cycles
Initial denaturation	98°c	30 s	1
Denaturation	98°C	7 s	35
Annealing	72°C	10 s	
Extension	72°C	30 s	
Final extension	72°C	10 min	1

Following the protocol, final elution was performed twice with 50 µl of 0.09% NaCl solution. The integrity of the dsRNA was checked by using gel electrophoresis and spectrometric analysis using NanoDrop^{TM} One [Thermo ScientificTM] at λ = 260 nm and λ = 280 nm.

Table 9: SWEET dsRNA results of spectrometric analysis at λ = 260 nm and λ = 280 nm

ng/µl	A260/A280	A260/A230
563.7	1.96	2.42

3.2.8 RNA interference through microinjection

RNA interference is a method that allows for research of specific protein functions in organisms by inactivating their gene expression and the subsequent study of occurring phenotypic changes. Transmission of dsRNA took place via microinjection. Two main injection lines have been established. One line consisted of L1 stage *C. populi* larvae while the other was comprised of early L3 stage larvae. Throughout every injection line, two groups have been produced. Those consisted of a GFP control group and another where SWEET gene expression was suppressed.

Table 10: Number of injected larvae

Series	# GFP(-)	# SWEET(-)
L1 series	15	15
L3 series	10	10

Injection was performed utilizing the NANOLITER2000 Injection System [World Precision Instruments]. Through thin glass capillaries 1120 ng dsRNA was injected into all examined larvae by adjusting the injection volume of the NANOLITER2000 based on the concentration of the utilized dsRNA. Point of injection was a central spot on the ventral side of the larval body.

3.3 Analytical methods

3.3.1 Gel electrophoresis

Gel electrophoresis is used to separate DNA fragments according to their size within an electric field.

Every electrophoresis analysis was done with a gel containing Tris Acetate EDTA (TAE) buffer and 1% agarose. Gels have been pre stained with Midori Green Advance fluorescence dye. 4 µl DNA sample was premixed with 1 µl 6x loading dye to ensure the visibility of DNA migration. To provide a reference concerning the size of separated nucleic acid fragments, GeneRuler 1 kb DNA Ladder [Thermo Fisher Scientific] was used. Documentation was done with the help of BioDocAnalyze [Biometra].

3.3.2 Determining concentration of nucleic acid

Concentration of either isolated RNA or purified DNA was measured by utilizing NanoDropTM One [Thermo ScientificTM] at $\lambda = 260$ nm and $\lambda = 280$ nm. This device is able to evaluate the concentration and contamination of nucleic acid by means of ultraviolet-visible spectroscopy. Cleaning of the pedestals was done with 1.5 µl of nuclease free water. A volume of 1.5 µl elution buffer has been used as blank measurement. Samples have been measured with a volume of 1.5 µl.

3.3.3 High pressure liquid chromatography followed by mass spectroscopy

High pressure liquid chromatography followed by mass spectroscopy (HPLC/MS) is a method applied for the separation and analysis of specific compounds. It has been utilized to check the composition of *C. populi* hemolymph for levels of salicin, salicortin as well as isoxazolinon 1 and 2. The samples have been weighted and dissolved in 50 µl methanol (MeOH) centrifuged and diluted with methanol in a ratio of 1:1 to be used for measurement. A volume of 1 µl 0.075 mM thiosalicin served as internal standard. Utilized device for the measurement has been the Finnigan LTQ [Thermo ScientificTM] as well as the Agilent HP1100 HPLC-system [Agilent Technologies]. Samples were measured in APCI mode with a vaporizer temperature of 450°C. Purospher® Star RP 18 endcapped (5 µm) [Merck KGaA] was the installed column. Water (solvent A) and acetonitrile (solvent B) with 0,1% formic acid have been used as solvent with a flow rate of 1 ml per minute and a maximal pressure of 350 bar.

The analytes were separated by gradient elution. Elution started with 10% solvent B and raised within 10 min to 90% solvent B. Measurement time amounted to 30 minutes.

Table 11: HPLC program for measurement of salicin, salicortin, isoxazolinon 1 / 2

Time in minutes	% of solvent B
0	10
3	10
11	90
13	10

3.3.4 Gas chromatography followed by mass spectroscopy

Gas chromatography followed by mass spectroscopy (GC/MS) is a method used for separation and detection of volatile substances. It was applied for measurement of glucose in both hemolymph as well as defensive secretion of *C. populi* larvae. The glucose was derivatized in order to make it volatile. Defensive secretion was also examined for levels of salicylaldehyde content. Utilized devices consisted of ThermoQuest Trace2000 [Thermo-Finnigan] in combination with the ThermoQuest Trace MS [Thermo-Finnigan] equipped with a ZB5 column and a 10 m Guardian End column [Phenomenex] with helium (1.5 mL/min) as carrier gas. Mass spectra were measured in electron impact (EI) mode at 70 eV. For examination of glucose levels substances had to be dried before being silylated by addition of 5 µl of pyridine and 20 µl of N-trimethylsilyl-N-methyl trifluoroacetamide (MSTFA). The substances were then mixed and put into a heat block on 70°C for 60 minutes. The samples have then been diluted 1:10 with the usage of dichloromethane (DCM) up to a total volume of 50 µl with an added volume of 2 µl sorbitol as internal standard. With a quantity of 500 µg sorbitol has been derivatized equal to the samples and was additionally dissolved in DCM so that a final concentration of 1 µg per 1 µl could be achieved. The silylated sugars were eluted under the following programmed conditions: 80°C (2 min), increase (10°C/min) to 300°C (6 min). The GC injector (split ratio 1:7), transfer line and ion source were set at 250°C, 280°C and 250°C, respectively. To research levels of salicylaldehyde in defensive secretion the substances have been mixed with 150 µl internal standard solution consisting of 50 µg/mL and dichloromethane. Dodecane served as internal standard. The following temperature program was used: 45°C (2 min), increase (10°C/min) to 200°C, increase (30°C/min) to 280°C (1 min). The GC injector (split ratio 1:10), transfer line and ion source were set at 220°C, 280°C and 250°C, respectively.

3.4 In silico methods

3.4.1 Design of primer sequences

Nucleotide primers serve as a starting point for the enzymatic replication of nucleic acid. The previously acquired sequence CL9967Contig1 has been used for primer creation. First step in primer creation was searching an open reading frame (ORF) of the desired length within the given sequence with the help of editing software Lasergene 12 [DNASTAR®]. Lasergene 12 was also used to rewrite DNA into amino acid sequences. The open reading frame was checked for its characteristics using a number of different software. Web based solutions included BlastP [NCBI] to look for similarities to other proteins, TMHMM Server v. 2.0 [DTU Bioinformatics] to determine the number of transmembrane helices and Primer 3 Plus [Bioinformatics.nl] to seek out primer sequences.

3.5 Utilized materials

The examined organism's consisted of a *C. populi* breed, that has originally been collected in forests near Tharandt.

3.5.1 Buffer and Additives

Table 12: Buffer and additives

0,1M DTT	InvitrogenTM(Darmstadt)
10mM dNTP Mix	Thermo Fisher Scientific GmbH (Bonn)
5x GoTaq® G2 Green HF Buffer	Promega (Mannheim)
5X Phusion HF Buffer	InvitrogenTM (Darmstadt)
6x DNA Loading Dye	Thermo Fisher Scientific GmbH (Bonn)
Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix	Agilent Technologies (Santa Clara, USA)
Midori Green Advance	Biozym Scientific GmbH (Hessisch Oldendorf)
Nuclease-Free Water (not DEPC-Treated)	Life Technologies Corporation (Carlsbad, USA)
Oligo (dT)12-18	InvitrogenTM (Darmstadt)
Rotiphorese® 50x TAE Puffer	Carl Roth GmbH & Co. (Karlsruhe)

3.5.2 Chemicals

Table 13: Chemicals

Dichlormethan	Carl Roth GmbH & Co. (Karlsruhe)
Ethanol	Carl Roth GmbH & Co. (Karlsruhe)
NEEO Ultra Quality Roti®garose	Carl Roth GmbH & Co. (Karlsruhe)
Silicon dioxide	Sigma-Aldrich® (Darmstadt)
β- Mercaptoethanol	Fluka Chemie AG (Buchs, Schweiz)
Methanol	Sigma-Aldrich® (Darmstadt)
MSTFA	Macherey-Nagel GmbH & Co. KG (Düren)
O-Methoxyamin-HCL	Sigma-Aldrich® (Darmstadt)
Pyridin	Sigma-Aldrich® (Darmstadt)

3.5.3 Consumables

Table 14: Consumables

1,5ml Safe-Lock Tubes	Eppendorf AG (Hamburg)
1,5ml Safe-Lock Tubes	Eppendorf AG (Hamburg)
8x0,2ml PCR optical clear flat caps	4titude® (Wotton, UK)
8x0,2ml PCR tubes	4titude® (Wotton, UK)
8xOptical Cap	Agilent Technologies (Santa Clara, USA)
8xOptical Tube	Agilent Technologies (Santa Clara, USA)
96-well Hard-Shell® PCR Plates	Bio-Rad Laboratories (München)
ClearLine® Filtered pipet tips	Biosigma (Cona, Italien)
Microseal 'B' Adhesive Seals	Bio-Rad Laboratories (München)
Pipette filter tips 1,5µl	Eppendorf AG (Hamburg)
Pipette filter tips 10µl	Eppendorf AG (Hamburg)
Pipette filter tips 100µl	Eppendorf AG (Hamburg)
Pipette filter tips 1000µl	Eppendorf AG (Hamburg)
Pipette filter tips 20µl	Eppendorf AG (Hamburg)
Pipette filter tips 200µl	Eppendorf AG (Hamburg)

3.5.4 Enzymes

Table 15: Enzymes

GoTaq®G2 DNA polymerase	Promega (Mannheim)
Phusion High Fidelity DNA-Polymerase	Thermo Fisher Scientific GmbH (Bonn)
SuperScript™ III Reverse Transkriptase	Invitrogen™ (Darmstadt)

3.5.5 Equipment

Table 16: Equipment

BioDocAnalyse	Biometra GmbH (Göttingen)
Biometra Compact M	Analytik Jena (Jena)
BP 2100 s	Sartorius AG (Göttingen)
Centrifuge 5417R	Eppendorf AG (hamburg)
Centrifuge 5424R	Eppendorf AG (hamburg)
CFX96 Touch™ Real-Time PCR Detection System	Bio-Rad Laboratories (München)
Finnigan LTQ	Thermo Fisher Scientific GmbH (Bonn)
GeneAmp® PCR System 2700	Thermo Fisher Scientific GmbH (Bonn)
HP1100 HPLC-system	Agilent Technologies (Santa Clara, USA)
Hamilton Syringe	Trajan Scientific (Victoria, Australia)
Ika® Vortex Genius 3	Sigma-Aldrich® (Darmstadt)
Mastercycler gradient	Eppendorf AG (Hamburg)
Mastercycler® pro	Eppendorf AG (Hamburg)
Micro Centrifuge	Carl Roth GmbH & Co. (Karlsruhe)
NanoDrop™ One	Thermo Fisher Scientific GmbH (Bonn)
Thermomixer comfort	Eppendorf AG (Hamburg)
ThermoQuest TRACE 2000	CE Instruments (Wigan, UK)
ThermoQuest MS	CE Instruments (Wigan, UK)

3.5.6 Kits

Table 17: Commercially available kits

MEGAscript® RNAi Kit	Invitrogen™ (Darmstadt)
QIAquick® PCR Purification KIT (50)	QIAGEN (Hilden)
RNAqueous®-Micro Total RNA Isolation Kit	Invitrogen™ (Darmstadt)
RNAqueous™-Phenol-free total RNA Isolation Kit	Invitrogen™ (Darmstadt)
RNeasy® Mini Kit (50)	QIAGEN (Hilden)

3.5.7 Oligo nucleotide sequences

Table 18: QPCR primer sequences stated from 5‘ end to 3‘ end

cp_sweet02_qpcr_fwd	TGCTTCAGCGATTCCATTCC
cp_sweet02_qpcr_rev	ACCAAGCATAGCGCAAAACC
Cp_SWEET_F666	TGGTTTTGCGCTATGCTTGG
Cp_SWEET_R741	ACCTGATGGTACCGTTGTTGG
Cp_EF1a-fwd	TCATCGGTCACGTAGATTCTGG
Cp_EF1a-rev	TTTCGATGGTACGCTTGTCG
Cp_elF4a_fwd	TTTGTAATACCCGCCGCAAG
Cp_elF4a_rev	TCCATGCATCGCAGAAACAG

Table 19: Standard PCR primer sequences stated from 5‘ end to 3‘ end

cp_sweet02_rnai_fwd	TAATACGACTCACTATAGGGCTCCT GCAACCCCATTTATTGG
cp_sweet02_rnai_rev	TAATACGACTCACTATAGGGACCAA GCATAGCGCAAAACC
cp_sweet02_genspec_fwd	CTCCTGCAACCCCATTTATTGG
cp_sweet02_genspec_rev	ACCAAGCATAGCGCAAAACC
Cp_treh_clon_f1	GAAAAAGAAGACATGAGCGACCAG
Cp_treh_clon_R+	GAACAATGCATACTCCCCAAC

Table 20: Contig sequence stated from 5‘ end to 3‘ end

CL9967Contig1
CGCATTCTAGGCGTAGATATATTCCTTGTAGATATATAAGATTTCTATGCTATGA ACTCTTCTCTTGACGGTGTATGGCCACAGAACTAAAACACTGTGAATATGTTCT GTGGCTGCTTCTGATTGGTTGTATTTCAAAATTTGAGACAAATGTAAACAAACTT CACACATTTTAAATTAATATCTCATAAATTGTTACGATATTCATAATTCATAATTC CCAATGAGACAATTTTTATAATAAATATTCTGAAATTAAAAGTAATATACCTACAT AACTCCTCAATAATTTTGTGAAATGAACTTGGGAGCTTGAGAATGGGTACTCCC GTACTGGTGCTGATTATTTAGAATTATAACTTGATGACACATGTCCTTCATTCTC AAAAGTGTTGGAAAACTTATATGAATAACACTCTGCTTCATCCAGAACCTAGTAT CATGGAAACCATATCACAAACTTTACAGCCCTATAAGAATTTAGTTGGGCAAGT TGCTAGTATTGTGACTATCTGCCAGTTTTTTTCTGGGGTCTTTGTATGTAAAGAT ATTCATGGAAAGGGAACAACAAAAGGAACTCCTGCAACCCCATTTATTGGAGGT ATTGTAATAGGGACCCTAATGCTGAAACATGGATTACTTTTAAATGATGCTGCTA TGTTGCAAGTTAACATTGCAGCCATAATTCTGAATATTATATATACAGCATTTTAT TACATATATTCTCCAGAAAAGGAGAAAGACGTTCTTAAACCTCTTGGCATCGGT GCTGCTTTGGTCGCAGTATTTTTGGGATATGCCGAATGGGAGGACCCCAATAA TTTAGAATACCGATATGGCTTGATTGTCACAATTCTGATGCTTTTATTGTTGGGA TCACCATTATTAGATGTGAAAGATATTATAGAGAAGAAAGATGCTTCAGCGATT CCATTCCCACTCACATTTATGGGTACTATTGTGACATTCTTATGGTTACTGTATG GTGTCATTTTGCTAAATGATTTCATGATTGTTCAAAATGTAATTGGTTTTGCGCT ATGCTTGGTTCAATTAGTTTTGATTTTTCTTTATCCTGGCAGACCAACAACGGTA CCATCAGGTCAGAAAACCAAAACGAAAAAGGGTGTAAAAAAAGACTAGCCTTG GAAAACTTCTTGATAATTATTGAATATTCCTAATTTTGTATTTCACATTCCAGGTC AAGATAATAATGAATGAACGAATCGTTATAAGAAAAGGATAACAGACATTACAAA TTATCGCAGAATAAACGATATTTTGTTAAATAAAAAAAA
ORF-sequence gene-specific primer qPCR primer **ORF start/stop**

3.5.8 Plasmid

Table 21: Plasmid

cp_slc2 t stop 1-5 (pCR®-Blunt II-TOPO®)	InvitrogenTM (Darmstadt)

3.5.9 Software Tools

Table 22: Software tools

BioDocAnalyse-Software	Biometra GmbH (Göttingen)
Bio-Rad CFX Manager	Bio-Rad Laboratories (München)
Lasergene 12 Core Suite	DNASTAR® (Madison, USA)
Office	Microsoft (Redmond, USA)
BLASTp	NCBI (Bethesda, USA)
TMHMM Server v. 2.0	DTU Bioinformatic (Lyngby, Dänemark)
Primer 3 Plus	Whitehead Institute for Biomedical Research (Cambridge, USA)

3.5.10 Standards

Table 23: Standards

GeneRuler 1 kb Plus DNA Ladder	Thermo Fisher Scientific GmbH (Bonn)

4 Results

At first, general expression of *sweet* within various developmental stages and also within different tissue samples was analyzed. To gain insights into the biological function of the SWEET proteins, RNAi treatment was performed on *C. populi* larvae. Morphological changes, as well as differences in the composition of hemolymph and defensive secretion were checked and compared to a control group.

4.1 Validation of *sweet* expression

Through means of standard PCR, it has been demonstrated that the SWEET protein is expressed within *C. populi*. All PCR samples have been amplified out of cDNA, synthesized from isolated *C. populi* RNA of third instar *C. populi* larvae.

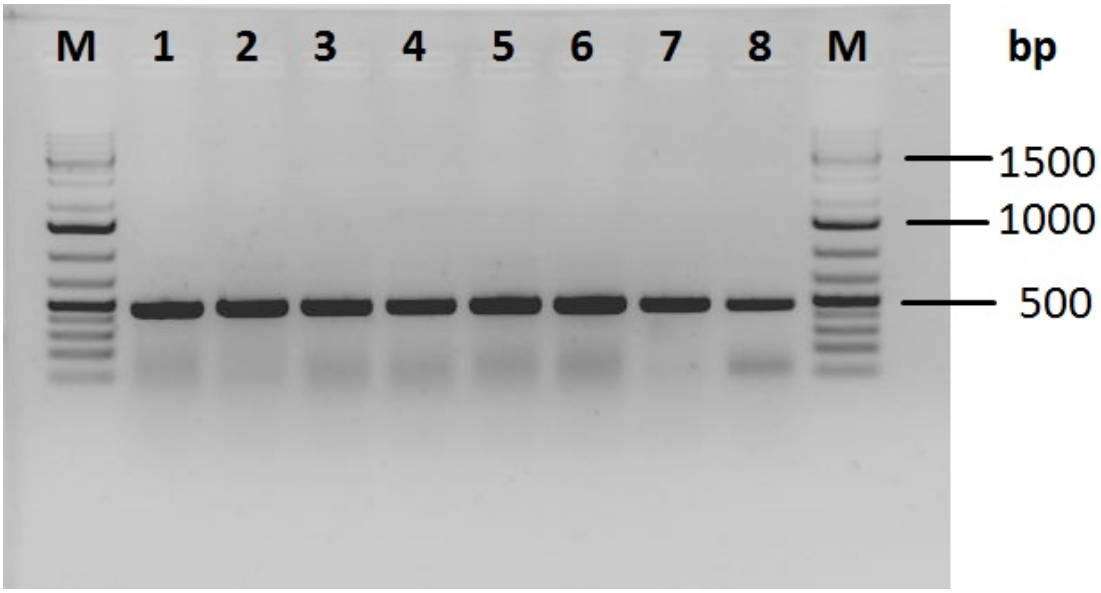

Figure 5: Agarose gel electrophoresis of amplified SWEET PCR products in *C. populi* cDNA. GeneRuler™ 1kb plus served as Marker. M: Marker; 1-8 amplified SWEET sequence from L3 stage *C. populi* larvae. Amplified sequences have a length of around 500bp.

4.2 Quantitative analysis of *sweet* expression

Quantitative PCR was utilized to measure the levels of *sweet* expression within different developmental stages of *C. populi*, as well as within different tissue samples of L3 larvae. All measurements have been carried out by using three biological replicates. Housekeeping genes *ef1a* and *eif4a* have been employed as a reference for assessing relative expression levels of *sweet* proteins.

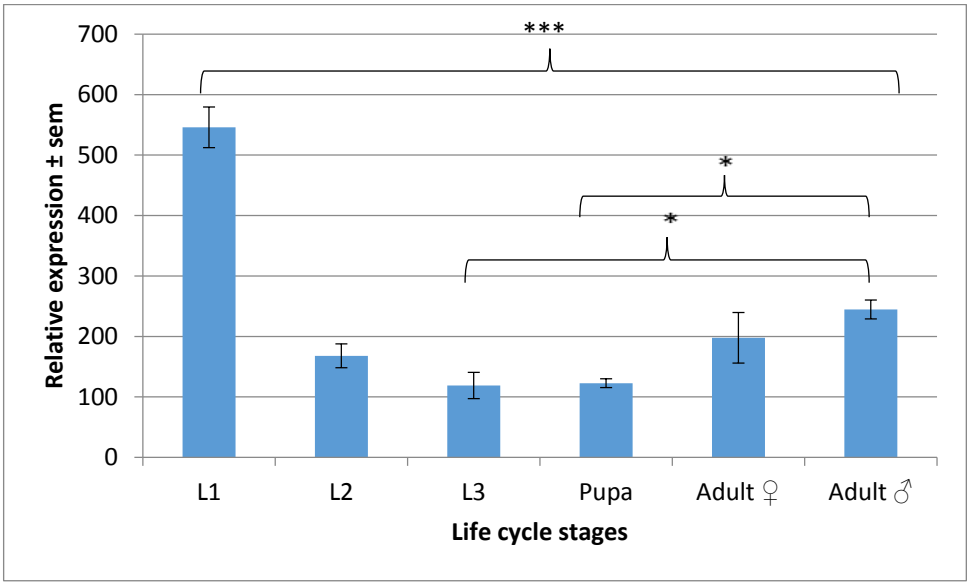

Figure 6: Relative *sweet* expression levels throughout developmental stages. Transcript levels have been quantified using housekeeping genes *ef1a* and *eif4a.* Mean expression levels of n=3 samples per life cycle stage are shown, with error bars displaying the standard error of the mean. L1-3 describes larval development stages after molting. T-test: * p<0.05; ** p<0.01; *** p<0.001.

Sweet expression could be measured throughout all developmental stages of *C. populi*. Highest expression levels have been detected in the L1 stage with a visible decline through L2 and L3 up to the pupal stage. L1 larvae show a highly significant difference ($p<0.001$) in expression levels compared to all other development stages. Within adults, expression has increased in comparison to pupal stage, with no significant difference between the sexes. Expression levels of adult males display a statistically significant difference ($p<0.05$) in comparison to pupal and L3 development stage (fig. 6).

For a better assessment of SWEET function, it is applicable to examine different tissue samples out of *C. populi* larvae. Higher expression rates in certain tissues could lead to a clearer picture concerning the role of SWEET within the organism.

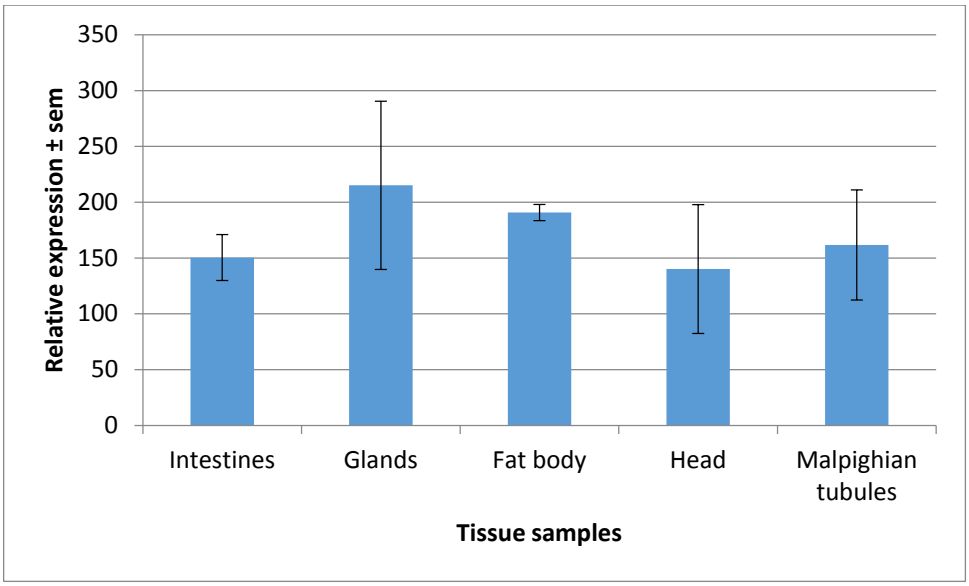

Figure 7: Relative *sweet* expression levels within tissue samples before knock down. Transcript levels have been quantified using housekeeping genes *ef1a* and *eif4a* within different tissue samples of third instar *C. populi* larvae. Mean expression levels of n=3 samples per tissue are shown, with error bars displaying the standard error of the mean. One way ANOVA: p=0.805.

Tissue samples derived from third instar *C. populi* larvae. The analyzed tissue samples consisted of intestines, defensive glands, fat body, head and Malpighian tubules. Expression of *sweet* could be measured in all examined tissue samples with no statistically significant differences in between (fig. 7).

4.3 Knock down of gene expression through RNAi

To be able to analyze potential phenotypic changes during beetle development as well as differences in composition of hemolymph and composition of defensive secretion, *Sweet* expression was suppressed by means of RNAi.

Two injection factions have been established. One included larvae injected within early L3 stage and another during L1 stage. Growth, survival rate and analysis of expression levels were examined for injected L1 larvae. As soon as they entered pupal stage, RNA isolation followed by qPCR was performed. Injected L3 larvae were constantly fed and examined for morphological differences of adult beetles. Both experimental series included larvae injected with GFP dsRNA as a control group and larvae injected with SWEET dsRNA, which should cause changes that help to discover the function of SWEET proteins. Knock down groups are referred to as GFP(-) and SWEET(-).

The SWEET(-) group displayed a drop in expression rate by a statistically significant amount ($p<0.05$) as compared to the GFP(-) group (fig. 8).

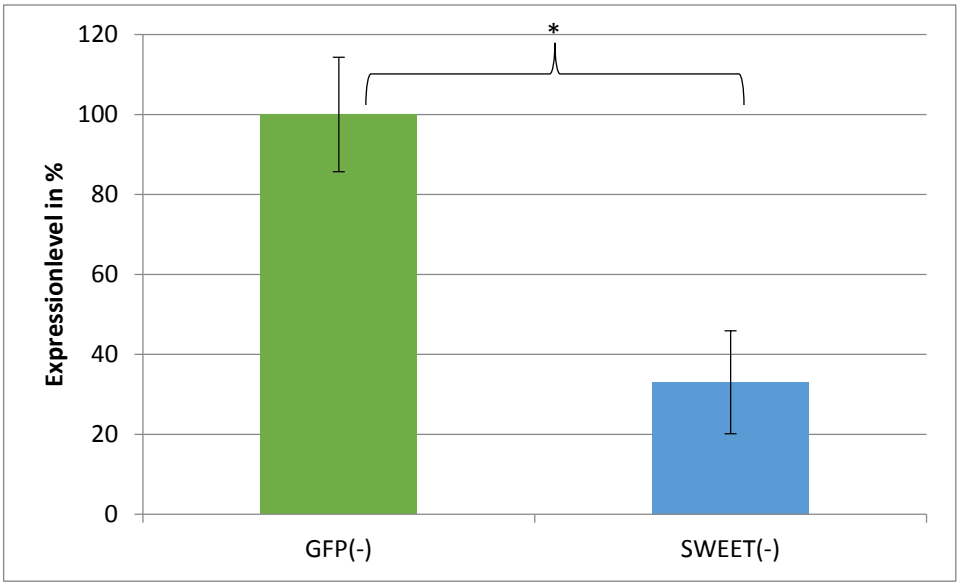

Figure 8: *Sweet* expression level in percent after knock down. Measurement was performed 12 days after injection. A drop in average expression rate of ~67% could be measured between both groups. Mean GFP(-) value is set at 100% for comparison. Expression level within SWEET(-) group amounted to an average of ~33%. Error bars display standard error of mean in percent.

Expression rate of SWEET(-) group compared to GFP(-) group decreased to ~67%. *Sweet* levels did not drop down to zero, which is why RNAi treatment is considered a down regulation of *Sweet* expression as opposed to a full stop.

4.4 Silencing effects on mortality and growth rate

Successful suppression of gene expression could hinder transport activity and make the influence of SWEET proteins measurable by comparing knock down groups to a control group. Larvae have been fed and weighed every two days after injection.

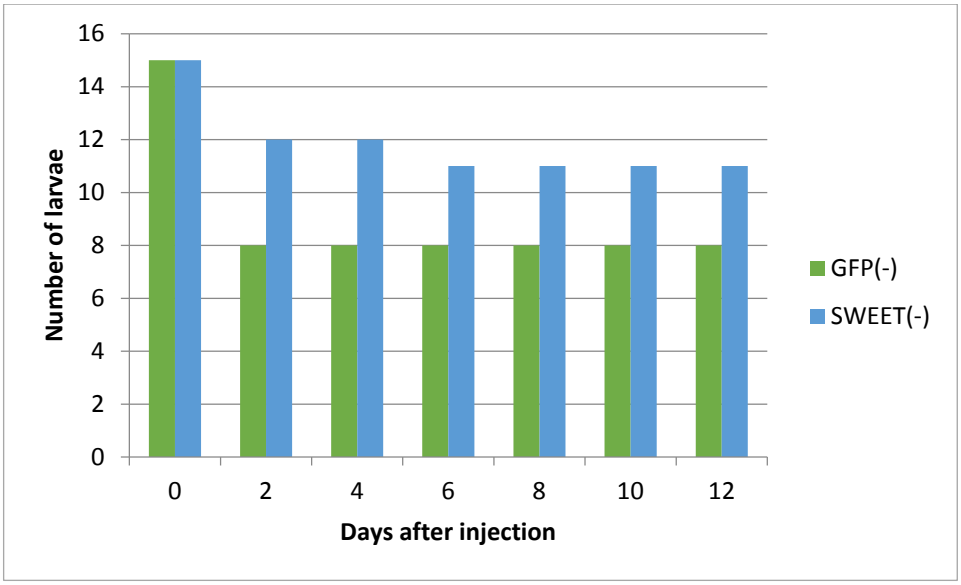

Figure 9: Number of surviving larvae. Measurement was done over the course of 12 days. Both groups consisted of 15 initially injected larvae. After 12 days, a majority of specimen went into pupation. On day 12, 11 within the SWEET(-) group and 8 within the GFP(-) group were left alive**.**

GFP(-) displayed the highest mortality rate with 7 out of 15 specimen dying, with all 7 being dead 2 days after injection. Within the SWEET(-) group, 3 died after 2 days and one more after 6 days. Afterward, no further deaths occurred (fig. 9).

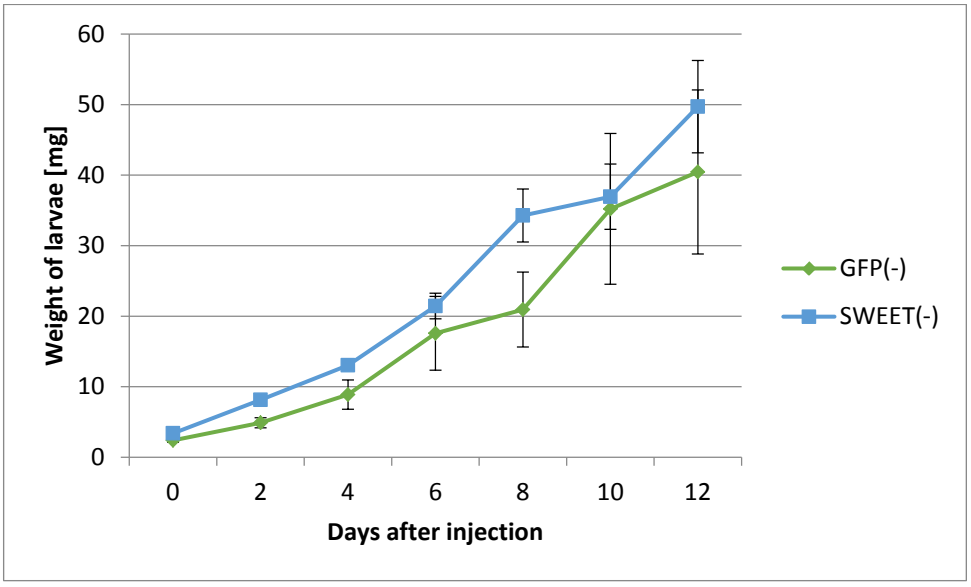

Figure 10: Body growth of larvae after dsRNA injection. Measurement was done over the course of 12 days. Weight in mg was measured until majority of specimen went into pupation**.** All surviving larvae have been fed and weighed every two days. Error bars display the standard error of the mean. No statistically significant difference in rate of growth could be measured between both groups on day 12. T-test: p=0.550.

Silencing of *sweet* had no effect on the body growth of injected larvae (fig. 10). The differences in weight on day 12 have not been statistically significant for both groups compared to each other (p>0.5).

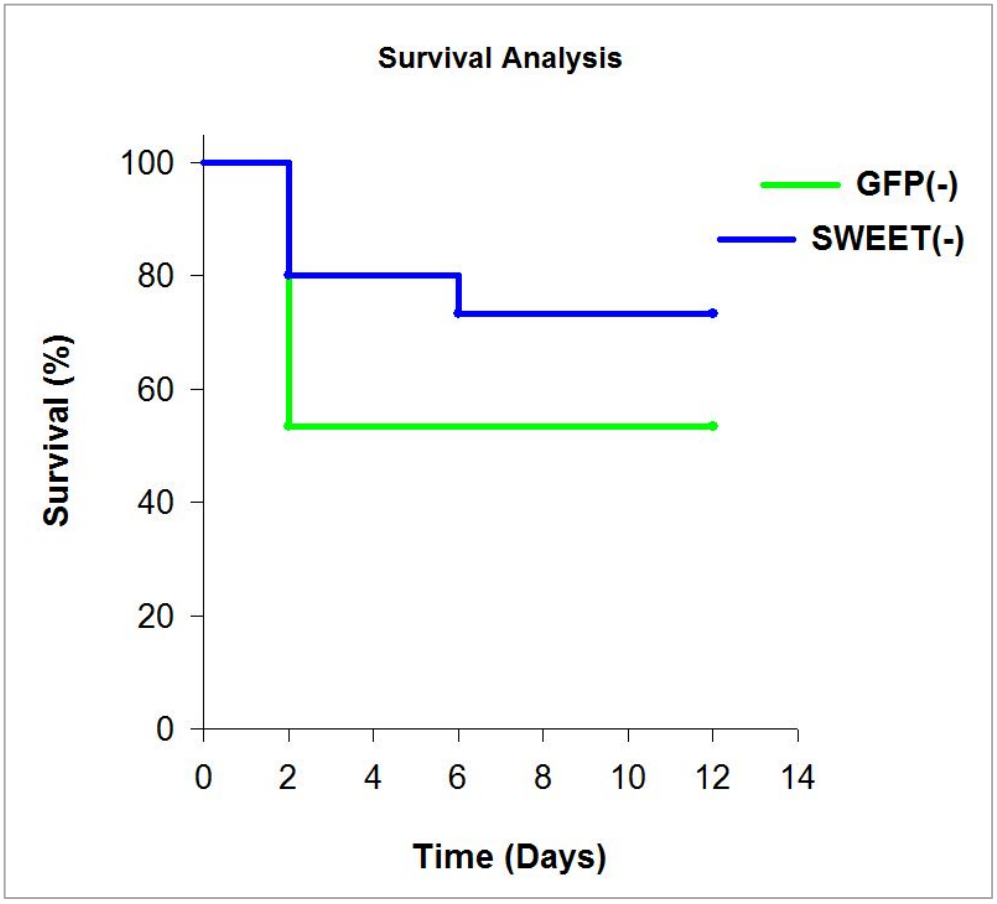

Figure 11: Kaplan-Meier curve for survival analysis. Graph displays survival rates of groups in percent plotted against days following the injection. There is no statistically significant difference between the groups (Kaplan-Meier-Survival Analysis: Log Rank: P=0.254).

Survival time analysis utilizing the Kaplan-Meier method indicated no statistically significant difference concerning mortality rates between injection groups in comparison to each other (p>0.2) (fig. 11).

Next to the L1 injection series, another injection series has been established. Injection of third instar larvae was performed equal to first instar larvae. For each group (GFP(-) and SWEET(-)), 10 individual larvae have been injected. The purpose of the L3 series, was to check their development to adult stage and to examine morphological changes due to suppression of *sweet* expression. Out of those 10, 8 survived within the L3 GFP(-) and all 10 within the L3 SWEET(-) group.

4.5 Silencing effects on hemolymph composition

To determine a potential role within the sequestration of salicin in *C. populi*, knock down of *sweet* has been performed through means of RNAi. Hemolymph serves a purpose analogous to blood in vertebrates and allows for transportation of substances through the larval body. Suppressed SWEET protein activity could lead to measurable glucose accumulation.

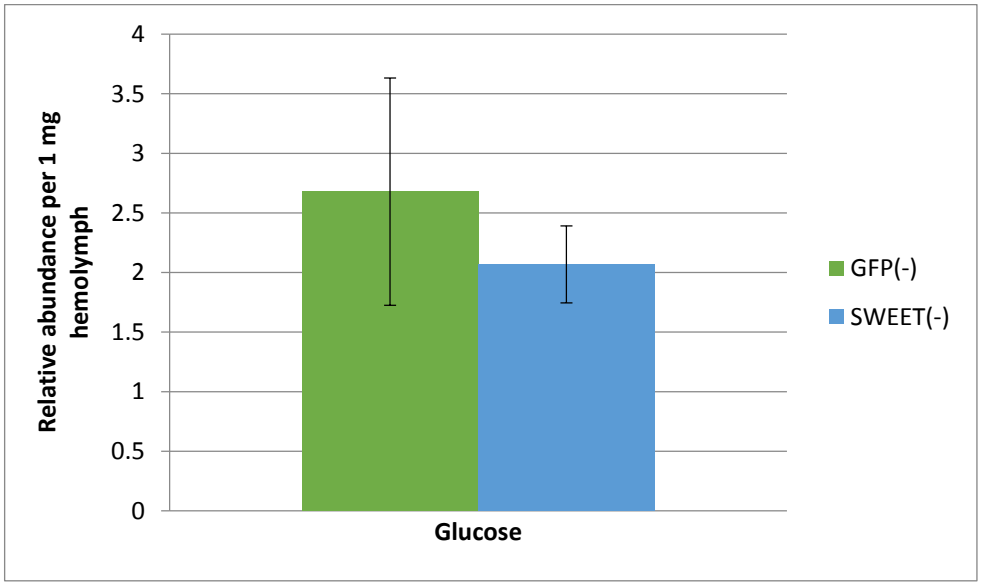

Figure 12: Relative glucose abundance in hemolymph after knock down. Measurement was performed 12 days after injection. Results of GC/MS measurement are displayed. Peak area of glucose has been divided by peak area of internal standard as well as weighed amount of hemolymph. Sorbitol served as internal standard. No statistically significant difference in glucose content between the groups could be measured (T-test: p=0.510). Groups consisted of GFP(-)(n=5) and SWEET(-)(n=9). Error bars display the standard error of the mean.

Analysis of hemolymph glucose levels was performed 12 days after injection. GC/MS measurment of SWEET(-) and GFP(-) showed no statistically significant difference between both groups (fig. 12).

To check for influence on transportation of other glucosides, levels of isoxazolinon 1 and 2, as well as levels of salicin were examined 12 days after RNAi treatment.

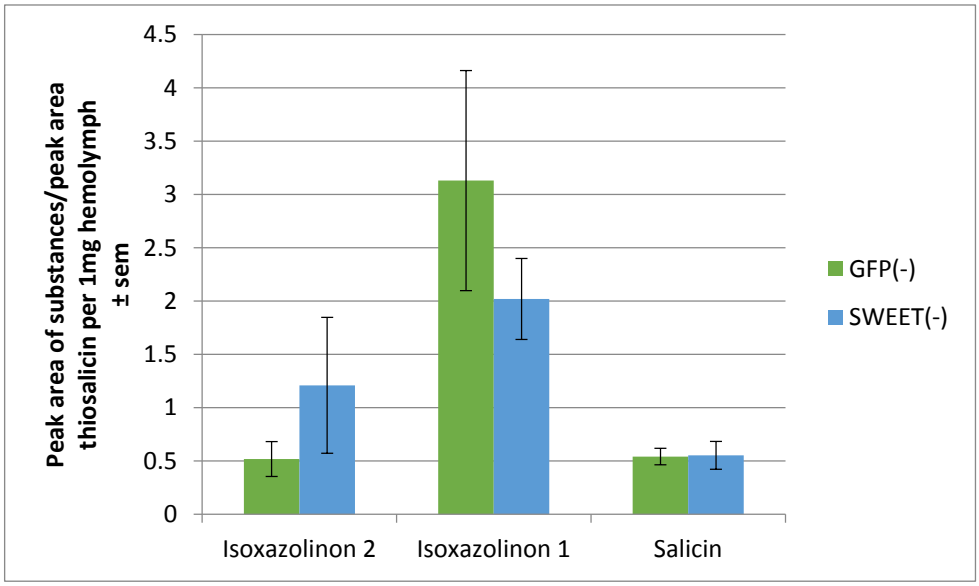

Figure 13: Detection of different sequestered glucosides within hemolymph after silencing. Measurement was performed 12 days after injection. Results of HPLC/MS measurement are displayed. Peaks of the respective substance have been divided by peak area of the internal standard as well as weighed amount of hemolymph. Thiosalicin served as internal standard. No significant difference could be measured (Mann-Whitney Rank sum test for isoxazolinon 2: p=0.594, T- test for isoxazolinon 1: p= 0.287, T- test for salicin: p=0.953). Error bars display the standard error of the mean. Groups consisted of GFP(-)(n=5) and SWEET(-)(n=9).

In general, levels of isoxazolinon 1 are higher than levels of isoxazolinon 2 and salicin. For each substanz, no statistically significant difference could be measured between groups (fig. 13). Levels of salicortin have also been measured, but could only be detected in trace amounts (not shown).

4.6 Silencing effects on defensive secretion

Successful knock down of *sweet* expression could lead to glucose accumulation within the defensive gland reservoir, where salicin is enzymatically transformed into salicylaldehyde. A change in transporter function due to RNAi treatment could therefore produce measurable differences in glucose levels within defensive secretion. Analysis is performed by GC/MS measurement 7 days after dsRNA injection. Measured samples consisted of defensive secretion that was pooled from 2 to 3 individual larvae. Only then, it could be guaranteed to have a sufficient amount of sample for analysis.

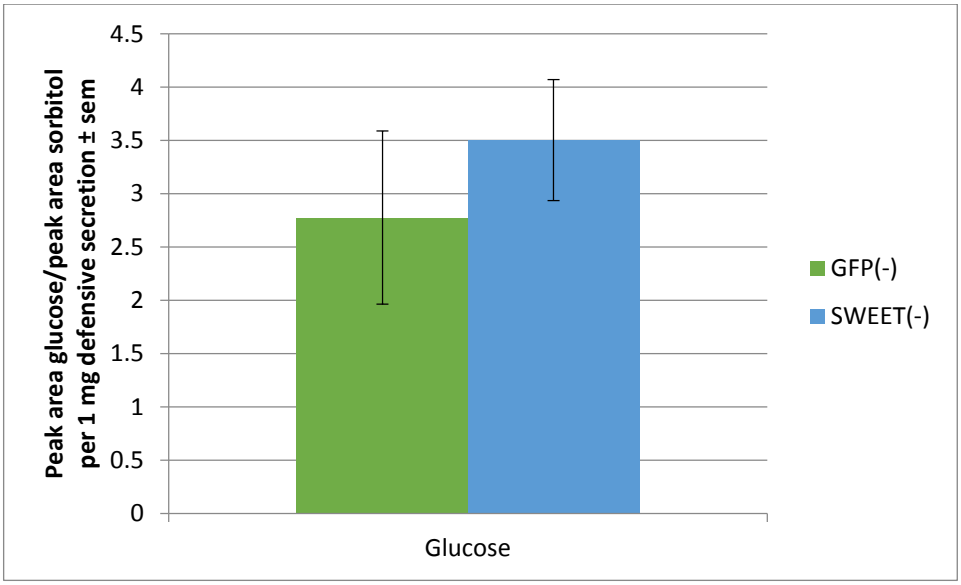

Figure 14: Relative glucose abundance within defensive secretion after knock down. Measurement was performed 7 days after injection. Results of GC/MS measurement are displayed**.** Relative differences in glucose levels between injection groups have been examined. Peak area of glucose has been divided by peak area of sorbitol as well as weighed amount of secretion. Sorbitol served as internal standard. No statistically significant difference could be measured between groups (T-test: p=0.498). Groups consisted of GFP(-)(n=6) and SWEET(-)(n=12). Error bars display the standard error of the mean. The secretion of 2 to 3 larvae has been pooled for one probe.

Both groups show roughly the same level of glucose content (fig. 14). T-test showed a p-value of 0.498 and therefore no statistically significant difference in glucose levels between both injection groups could be measured within defensive secretion.

Successful RNAi treatment could lead to the inability of transportation of glucosides into the secretory cells and therefore to the inability, to produce salicylaldehyde. This is measurable by analyzing defensive secretion.

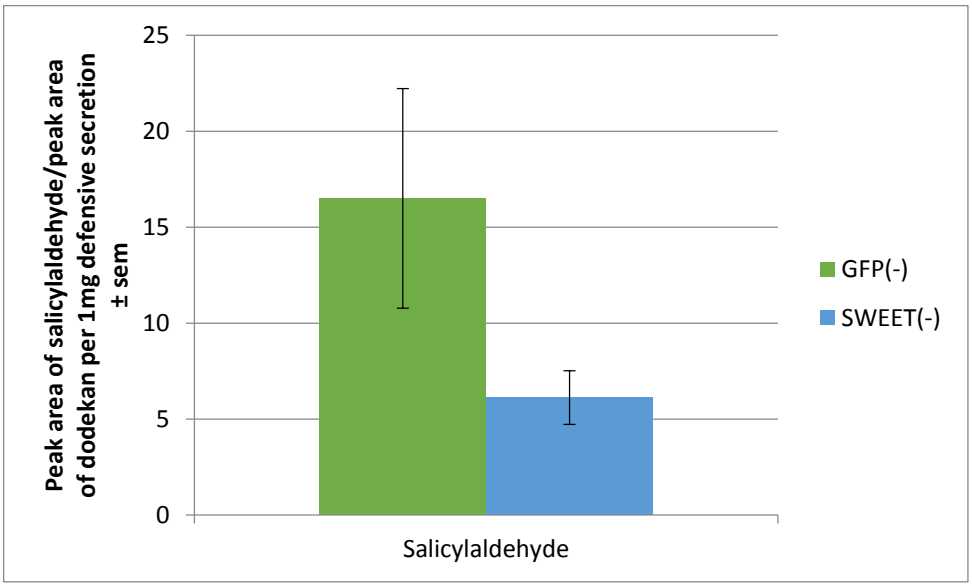

Figure 15: Relative salicylaldehyde abundance within defensive secretion after knock down. Measurement was performed 7 days after injection. Results of GC/MS measurement are displayed**.** Peak area of salicylaldehyde has been divided by peak area of internal standard and weighed amount of secretion. Dodecane served as internal standard. No statistically significant difference could be measured between groups (Mann-Whitney Rank Sum test: p=0.188). Groups consisted of GFP(-)(n=6) and SWEET(-)(n=12). Error bars display the standard error of the mean. The secretion of 2 to 3 larvae has been pooled for one probe.

Levels of salicylaldehyde in defensive secretion showed dissimilarity in averages between groups. Statistical analysis showed no significant difference in between the groups, with a resulting p-value of 0.188 (fig. 15). This is due to the high deviation in measured values.

4.7 Morphological changes as a result of RNAi treatment

Silencing effects on body growth, mortality and composition of hemolymph as well as defensive secretion have been examined on larva injected in first instar. In contrast, L3 larvae were injected to observe phenotypic changes during the adult stage.

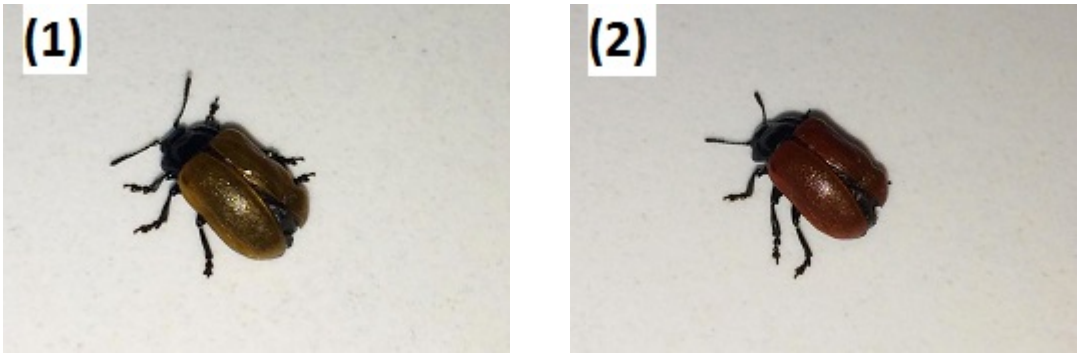

Figure 16: Change in cuticle color due to *sweet* knock down. Time between pictures amounts to 14 days. (1): light brown elytra one week after hatching; (2): typical red elytra three weeks after hatching.

Within the L3 SWEET(-) group, 9 out of 10 beetles and none in the L3 GFP(-) control group presented a change in phenotype. Within the control group, every adult beetle displayed the typical bright red elytra whereas members of the SWEET(-)group had visibly different elytra. Those beetles presented a light brown coloring. This effect wore off and after around three weeks coloring between groups was identical (fig. 16).

5 Discussion

The aim of this thesis was to examine the potential role of a SWEET protein within the leaf beetle species *C. populi*. Some members of the Chrysomelidae family, such as *C. populi*, sequester defensive compounds for example out of plant derived glucosides and utilize a network of transport proteins for that purpose [12, 13, 15]. SWEET proteins have been identified as a key player in intercellular transport of sugars in a variety of organisms [2, 13, 17]. Previous research on *C. populi* indicates a probably purpose for SWEETs within the sequestration process [12, 16, 20].

5.1 Analyzing *sweet* expression within *C. populi*

After standard PCR was used to establish general expression within *C. populi* larvae, levels of expression throughout development stages have been comparatively analyzed. This was done using qPCR with housekeeping genes *ef1a* and *eif4a* as a reference (fig. 5).

The L1 instar of *C. populi* displays the highest amount of gene expression, which can be explained by the high demand that is put on the metabolism of the larvae. For small larvae, it is necessary to start feeding and in turn accumulate fat rapidly. As *C. populi* larvae progress in growth, *sweet* expression declines until they enter

pupal stage. This finding can be explained by the steady increase of fat within the larval body and the therefore reduced need for fat accumulation. L3 and the pupal stage, show the lowest expression of *sweet*. The decline in *sweet* expression seems to correlate with the decline in the need of fat accumulation. During the pupal stage, stored fat is used as an energy source for metamorphosis. From pupal stage to adult stage, average levels of *sweet* expression increase again (fig. 6). Adult beetles are a lot more active through faster movement and flight, which puts different burdens on the metabolism. A reason for the slight increase in *sweet* expression could be the higher need for direct sugar transportation in the adult beetles.

The results of N. Schuck's bachelor thesis, whereby she demonstrated a thousand fold increase in *sweet* expression during the adult stage could not be replicated here. Different explanations can be given. Only three biological replicates have been utilized for RNA isolation with subsequent qPCR to check expression levels. This still leaves room for not excluding individual differences. Repetition of this experiment with a higher number of biological replicates could yield different results. Other than that, it could be observed that adult *C. populi* show less flight activity, the longer they stay in captivity. Through the generations, this could manifest within the genotype and lead to a decline in transport activity connected to the energy supply of flight mechanisms. This can be investigated by comparing expression levels of newly caught wild beetles with later generations of the in house breed.

Tissue samples of third instar larvae have been comparatively checked for levels of *sweet* expression. Three biological replicates have been used for each tissue sample. QPCR analysis showed a relatively similar level of occurrence throughout all examined tissue samples (fig. 7). This is in accordance with the results of N. Schucks thesis [20)]. The findings also confirm RNA sequence analysis, whereby *sweet* transcripts are present in all tissues [19)]. This leads to the assumption, that SWEET proteins fulfill a function in the entire organism and are relevant for tasks outside of sequestration processes.

5.2 Efficiency of RNAi treatment

To further look into the potential effects of SWEET proteins, gene expression has been suppressed through RNAi induction. Groups of early pupa have been checked for transcript levels after dsRNA injection.

With a p-value of 0.021, a statistically significant reduction in expression levels could be examined for SWEET(-) group in comparison to GFP(-) group, which amounts to a 67% decrease (fig. 8). This leads to the conclusion, that RNAi treatment has been successful at the mRNA level. After mRNA has noticeably been reduced, Western blot analysis could be one of the following ways to corroborate the actual decline in SWEET protein abundance [22)]. Furthermore, a ~33% SWEET activity may still be enough to perform relevant transporter functions throughout the organism. A higher concentration of dsRNA could be used to produce stronger knock down effects.

5.3 Effects of RNAi treatment

5.3.1 Body growth and survival

To measure the effects of the expression knock down on larval growth, injected beetle larvae have been weighed and fed every two days.

No statistically significant difference in weight and growth between GFP(-) group and SWEET(-) group was observed (fig. 10). A difference in average weight throughout the time of analysis between both groups is explained by the difference in average starting weight. On the day of the injection, average larval weight for GFP(-) group amounted to 2.4 mg and 3 mg for SWEET(-) group. Rate of survival was analyzed by generating a Kaplan-Meier curve. With a p-value of 0.254, this method also indicated no statistically significant difference between both groups.

This is consistent with the previous results of N. Schuck. It is likely that the deaths of individual larva occurred due to injury caused by the injection. Point of injection has been a central spot within the larval thorax. A different point of injection could lead to better results concerning their mortality rate due to lessening the amount of injury. It is also possible to compare mortality as well as growth rates between RNAi treated groups and untreated groups.

Injury could also have arisen through not enough injection practice by the performing student. Additionally, a more delicate injection capillary would possibly lessen the amount of injury done.

5.3.2 Changes in hemolymph and secretion content due to RNAi treatment

Analysis of sequestration-specific substances could lead to a clearer picture concerning the role of SWEETs within *C. populi*, since it has been demonstrated, that SWEET proteins are able to transport glucose and possibly support mediation of glucosides [1, 2, 4, 23].

Examining levels of salicin, isoxazolinon 1 and 2, as well as glucose within hemolymph, indicated no significant difference between groups (fig. 12 and fig. 13). Salicortin could only be measured in trace amounts. The lack of salicortin in hemolymph is possibly due to a lack of salicortin within leafs of *Populus trichocarpa*, which have been fed to examined *C. populi*. No significant difference in the levels of other sequestering specific substrates leads to the assumption, that SWEET proteins do not directly or indirectly influence their transportation through the organism.

The composition of defensive secretion was analyzed for levels of glucose and salicylaldehyde (fig. 14 and fig. 15). Results showed no statistically significant difference between the groups and therefore the data suggests no influence of SWEET proteins on glucose and salicylaldehyde accumulation within defensive secretion.These findings are similar to those of N. Schuck's previous work [20].

It is possible, that other proteins can take over SWEET specific tasks within the sequestration process. To confirm this, it would be necessary to check expression levels of other known transport proteins within *C. populi* after *sweet* knock down. As mentioned above, remaining levels of SWEET activity could also still be enough to perform necessary transport functions. Further knock down could therefore yield different results.

5.3.3 Morphological changes due to RNAi treatment

A second injection faction of mature larvae has been established to check for occurring phenotypic changes after metamorphosis. N. Schucks previous results could be confirmed. 9 out of 10 adult beetles within the SWEET(-) group had a change in color in comparison to the GFP(-) group. It is therefore probable that SWEET proteins have an effect on the buildup of pigmentation within the elytra.

The processes behind pigmentation of beetle exoskeletons are a continuous object of research [24, 25]. A variety of chemical pigments contribute to the coloring of insects, but their biosynthesis and genetic regulation is not well understood [24]. Research done on the ladybird beetle *Harmonia axyridis* as well as *Drosophila melanogaster* demonstrated the involvement of ABC transporters in the buildup of eye pigmentation [26, 27]. A similar mechanism of pigment transport could be responsible for the coloring of adult *C. populi* elytra. Since ABC transporters are dependent on ATP, a constant flow of glucose is necessary for energy production. This role could at least in part be fulfilled by SWEET transporters. Absorbance spectrophotometry and chromatographic measurements could be deployed, to gather further information on the pigments affected by gene knock down.

Since the brown cuticle color changed into the typical red after around three weeks, it is reasonable to assume, that RNAi effects have worn off. This could be confirmed by comparing gene expression levels of affected adults to those of a control group. Other transporters could also be able to pick up SWEET specific tasks, which can be verified by examining expression levels of other transport proteins after *sweet* suppression.

N. Schucks research also indicated a softer cuticle structure within the SWEET(-) knock down group [20]. This finding could not be replicated. Elytra within specimen of both groups displayed the same kind of rigidity after adult beetles had been fully developed. SWEET proteins could therefore have an impact on cuticle tanning, but no impact on the formation of rigid cuticle structure.

N. Schuck's results could stem from an inadequate waiting time before examination after metamorphosis, since cuticle maturation is a time- and energy consuming process [28].

6 Further perspectives

A role of SWEET proteins within the sequestration process of *C. populi* could not be validated within the framework of this thesis. Knock down of *sweet* expression has been measurable and is therefore considered successful. Analysis of both hemolymph and defensive secretion for sequestration specific substances indicated no difference in composition after gene expression has been suppressed. Analysis of growth rate as well as mortality rate also let to no conclusive evidence concerning the function of SWEET proteins.

A change in cuticle color suggests the involvement within the formation of pigmentation. Further research is suggested.

To get closer to the clarification of SWEET function within *C. populi*, different methods could be employed. Previous analysis could be redone with a higher number of replicates. An increase in examined larva could even out the error distribution and lead to a more significant results concerning the levels of salicylaldehyde within defensive secretion.

It is also possible to establish another completely untreated control group. This would be necessary especially for inquiry into growth as well as mortality rate.
Furthermore, only one SWEET related sequence has been checked. By research of both SWEET proteins, a more noticeable effect could be examined within *C. populi*. Both proteins could be interlinked with each other, since transcriptomal analysis shows the presence of the two SWEETs in all analyzed tissue samples [19)]. A knock down of one SWEET at a time, followed up by knock down of both at once, could present a more noticeable development.

Knock down should in any case be followed up by northern and western blot analysis to confirm restricted transcript and protein involvement. Transporter activity could be analyzed by utilizing known expression systems and therefore allow a more detailed examination of potential SWEET function. Fluorescence resonance energy transfer (FRET) is a method that can help to determine substrate specificity, as well as influx and efflux mechanisms of SWEET proteins.

Summary

Sugars Will Eventually be Exported Transporter (SWEET) are a family of sugar transporters that have been a relatively recent object of study. A variety of those transport proteins have been found in eukaryotes and prokaryotes, but most research activities focused on their function in plants. It was shown that SWEETs mediate the transportation of sugars in between cells. Within metazoa much of their purpose is yet to be known. Research on the leaf beetle species *Chrysomela populi* (*C. populi*) is done to provide subsequent information on the role of this protein family within animal organisms since *C. populi* utilizes plant-derived glucosides for sequestration. Previous *in silico* work showed, that two different sequences corresponding with expression of *sweets* are present within the *C. populi* transcriptome. The possible involvement of a SWEET protein within the sequestration of salicin is investigated within the framework of this thesis. At first the relevant gene expression levels within *C. populi* have been checked. It was verified through quantitative polymerase chain reaction (qPCR), that SWEET proteins are expressed throughout every development stage and also within every examined larval tissue. Silencing by RNAi suppressed gene expression of the SWEET transporter. The downregulation of *sweet* transcription was validated by use of qPCR. Following suppression of SWEET expression, the occurrence of phenotypic changes has been comparatively analyzed to a control group. No difference in composition of hemolymph and defensive secretion could be determined. Furthermore the body growth as well as mortality rate has been uninfluenced by RNAi treatment. A noticeable effect occurred in the coloring of adult beetle elytra. The SWEET knock down group displayed a light brown discoloration in contrast to the bright red cuticle of the control group. Colors of elytra adjusted between the groups with some time delay.

The expression of SWEET proteins within *C. populi* could be demonstrated within the framework of this thesis, but their specific function could not be determined clearly. The results concerning phenotypic changes indicate a role in pigmentation buildup, which has to be confirmed by further research.

Zusammenfassung

Sugars Will Eventually be Exported Transporter (SWEET) sind eine Familie von Zuckertransportern, die erst seit kurzem Gegenstand der Forschung sind. Eine Vielzahl dieser Transportproteine wurde in Eukaryoten und Prokaryoten entdeckt, wobei sich die meiste Forschungsaktivität auf ihren Zweck innerhalb von Pflanzen fokussierte. Es konnte gezeigt werden, dass SWEETs den Zuckertransport zwischen den Zellen vermitteln. In Metazoen ist noch viel ihrer Funktion unbekannt. Forschungsarbeit an der Blattkäferart *Chrysomela populi* (*C. populi*) ermöglicht es, weitere Informationen über die Rolle dieser Proteinfamilie in Tierorganismen zu gewinnen, da *C. populi* Glykoside aus ihrer Blattnahrung für den Prozess der Sequestrierung nutzt. Vorangegangene *in silico*-Arbeit hat gezeigt, dass sich zwei unterschiedliche Sequenzen im *C. populi* Transkriptom befinden, welche mit der Expression von *sweets* korrespondieren. Die mögliche Beteiligung eines SWEET Proteins in der Sequestrierung von Salicin wird im Rahmen dieser Arbeit untersucht. Zuerst wurde das Genexpressionslevel der relevanten SWEET Proteine in *C. populi* überprüft. Durch quantitative Polymerase Kettenreaktion (qPCR) konnte bestätigt werden, dass SWEET-Proteine in jedem Lebenszyklus und auch in jedem untersuchten Larvengewebe exprimiert sind. Stilllegung durch RNAi hat die Genexpression des SWEET Transporters unterdrückt. Das Herunterregulieren der *sweet* Transkription wurde durch Verwendung von qPCR validiert. Nach Unterdrückung der *sweet* Expression wurde das Auftreten von phänotypischen Veränderungen im Vergleich zu einer Kontrollgruppe analysiert. Es konnte kein Unterschied in der Zusammensetzung von Hämolymphe und Wehrsekret bestimmt werden. Des Weiteren blieb die Wachstums- wie die Sterberate durch die RNAi Behandlung unbeeinflusst. Ein deutlicher Effekt trat in der Färbung der Elytren bei adulten Käfern auf. Die SWEET *Knock down*-Gruppe zeigte eine leicht braune Verfärbung im Kontrast zu der kräftig-roten Kutikula der Kontrollgruppe. Farben der Elytren haben sich zeitverzögert zwischen den Gruppen angeglichen.

Die Expression von SWEET Proteinen in *C. populi* konnte im Rahmen dieser Arbeit nachgewiesen werden, jedoch konnte ihre spezielle Funktion nicht klar bestimmt werden. Die Ergebnisse bezüglich phänotypischer Veränderungen geben Hinweis auf eine Rolle im Pigment-Aufbau, was durch weitere Forschung bestätigt werden muss.

Bibliography

1) Chen LQ, Cheung LS, Feng L, Tanner W, Frommer WB: Transport of sugars. *Annual review of biochemistry,* **84**, 865-894 (2015).

2) Chen LQ, Hou BH, Lalonde S, Takanaga H, Hartung ML, Qu XQ, Guo WJ, Kim JG, Underwood W, Chaudhuri B, Chermak D, Antony G, White FF, Somerville SC, Mudgett MB, Frommer WB: Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature,* **468**, 527-532 (2010).

3) Bezrutczyk M, Yang J, Eom JS, Prior M, Sosso D, Hartwig T, Szurek B, Oliva R, Vera-Cruz C, White FF, Yang B, Frommer WB: Sugar flux and signaling in plant-microbe interactions. *The Plant journal : for cell and molecular biology,* **93**, 675-685 (2018).

4) Eom JS, Chen LQ, Sosso D, Julius BT, Lin IW, Qu XQ, Braun DM, Frommer WB: SWEETs, transporters for intracellular and intercellular sugar translocation. *Current opinion in plant biology,* **25**, 53-62 (2015).

5) Feng L, Frommer WB: Structure and function of SemiSWEET and SWEET sugar transporters. *Trends in biochemical sciences,* **40**, 480-486 (2015).

6) Hu YB, Sosso D, Qu XQ, Chen LQ, Ma L, Chermak D, Zhang DC, Frommer WB: Phylogenetic evidence for a fusion of archaeal and bacterial SemiSWEETs to form eukaryotic SWEETs and identification of SWEET hexose transporters in the amphibian chytrid pathogen Batrachochytrium dendrobatidis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* **30**, 3644-3654 (2016).

7) Jia B, Zhu XF, Pu ZJ, Duan YX, Hao LJ, Zhang J, Chen LQ, Jeon CO, Xuan YH: Integrative View of the Diversity and Evolution of SWEET and SemiSWEET Sugar Transporters. *Frontiers in plant science,* **8**, 2178 (2017).

8) Tao Y, Cheung LS, Li S, Eom JS, Chen LQ, Xu Y, Perry K, Frommer WB, Feng L: Structure of a eukaryotic SWEET transporter in a homotrimeric complex. *Nature,* **527**, 259-263 (2015).

9) Urban J: Occurrence, bionomics and harmfulness of Chrysomela populi L. (Coleoptera, Chrysomelidae). *JOURNAL OF FOREST SCIENCE*, 29 (2006).

10) Jiři Zahradnik lJ, Dieter Jung et al. *Käfer Mittel- und Nordwesteuropas*. Parey, Berlin (1985).

11) Ge SQ, Hua Y, Ren J, Slipinski A, Heming B, Beutel RG, Yang XK, Wipfler B: Transformation of head structures during the metamorphosis of Chrysomela populi (Coleoptera: Chrysomelidae). *Arthropod Syst Phylo,* **73**, 129-152 (2015).

12) Burse A, Frick S, Discher S, Tolzin-Banasch K, Kirsch R, Strauss A, Kunert M, Boland W: Always being well prepared for defense: the production of deterrents by juvenile Chrysomelina beetles (Chrysomelidae). *Phytochemistry,* **70**, 1899-1909 (2009).

13) Discher S, Burse A, Tolzin-Banasch K, Heinemann SH, Pasteels JM, Boland W: A versatile transport network for sequestering and excreting plant glycosides in leaf beetles provides an evolutionary flexible defense strategy. *Chembiochem : a European journal of chemical biology,* **10**, 2223-2229 (2009).

14) Duffey SS: Sequestration of Plant Natural-Products by Insects. *Annu Rev Entomol,* **25**, 447-477 (1980).

15) Kuhn J, Pettersson EM, Feld BK, Burse A, Termonia A, Pasteels JM, Boland W: Selective transport systems mediate sequestration of plant glucosides in leaf beetles: a molecular basis for adaptation and evolution. *Proceedings of the National Academy of Sciences of the United States of America,* **101**, 13808-13813 (2004).

16) Strauss AS, Peters S, Boland W, Burse A: ABC transporter functions as a pacemaker for sequestration of plant glucosides in leaf beetles. *eLife,* **2**, e01096 (2013).

17) Strauss AS, Wang D, Stock M, Gretscher RR, Groth M, Boland W, Burse A: Tissue-specific transcript profiling for ABC transporters in the sequestering larvae of the phytophagous leaf beetle Chrysomela populi. *PloS one,* **9**, e98637 (2014).

18) Stryer L. *Biochemie*. (1996).

19) Kriegbaum M. Transcriptomal analysis, regarding transmembranal sugar portars in the phytophagous beetle *Chrysomela populi*. *Fakultät für Mathematik und Informatik*. Vol. Bachelor of Science. Friedrich-Schiller-Universität, Max-Planck-Institut für chemische Ökologie, p.^pp. (2017).

20) Schuck N. Funktionelle Charakterisierung von Glukosidtransportern der "Solute carrier" - Familien 2 und 50 aus dem Pappelblattkäfer, *Chrysomela populi*. *Max-Planck-Institut für chemische Ökologie*. Vol. Bachelor of Science. Friedrich-Schiller-Universität, p.^pp. (2017).

21) Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* **25**, 402-408 (2001).

22) Mocellin S, Provenzano M: RNA interference: learning gene knock-down from cell physiology. *Journal of translational medicine,* **2**, 39 (2004).

23) Wright EM: Glucose transport families SLC5 and SLC50. *Molecular aspects of medicine,* **34**, 183-196 (2013).

24) Noh MY, Muthukrishnan S, Kramer KJ, Arakane Y: Cuticle formation and pigmentation in beetles. *Current opinion in insect science,* **17**, 1-9 (2016).

25) Bezzerides AL, McGraw KJ, Parker RS, Husseini J: Elytra color as a signal of chemical defense in the Asian ladybird beetle Harmonia axyridis. *Behav Ecol Sociobiol,* **61**, 1401-1408 (2007).

26) Dermauw W, Van Leeuwen T: The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. *Insect biochemistry and molecular biology,* **45**, 89-110 (2014).

27) Tsuji T, Gotoh H, Morita S, Hirata J, Minakuchi Y, Yaginuma T, Toyoda A, Niimi T: Molecular Characterization of Eye Pigmentation-Related ABC Transporter Genes in the Ladybird Beetle Harmonia axyridis Reveals Striking Gene Duplication of the white Gene. *Zoological science,* **35**, 260-267 (2018).

28) Arakane Y, Lomakin J, Gehrke SH, Hiromasa Y, Tomich JM, Muthukrishnan S, Beeman RW, Kramer KJ, Kanost MR: Formation of rigid, non-flight forewings (elytra) of a beetle requires two major cuticular proteins. *PLoS genetics,* **8**, e1002682 (2012).

Supplement

Relative gene expression after knock down

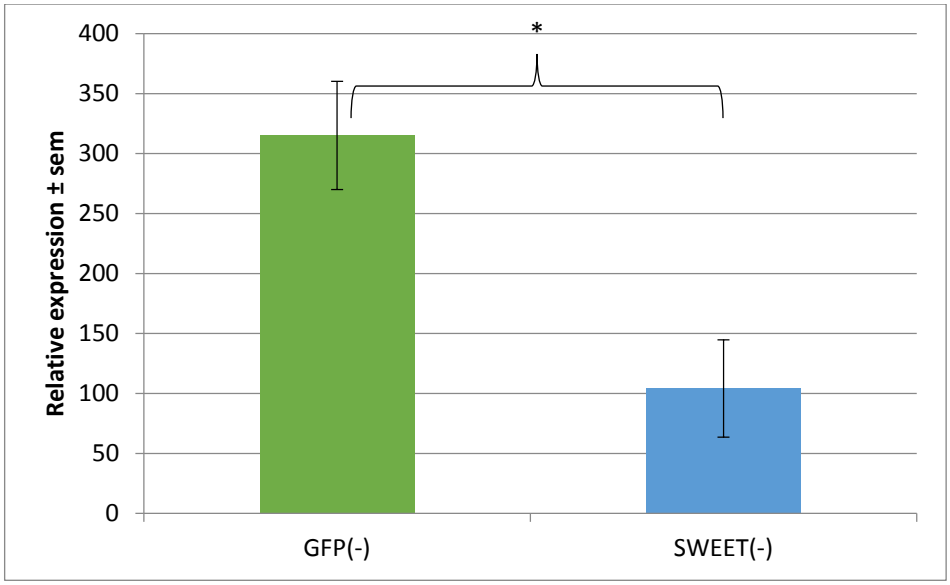

Figure 17: Relative expression of *sweet* after knock down. Measurement was performed 12 days after dsRNA injection. Transcript levels have been quantified using housekeeping genes *ef1a* and *eif4a*. Examined specimen reached the pupal stage. Error bars display standard error of mean. Level of SWEET(-) (n=5) gene expression is compared to GFP(-) (n=4) as control group. T-test: p=0.021, * p<0.05.

Adult weight

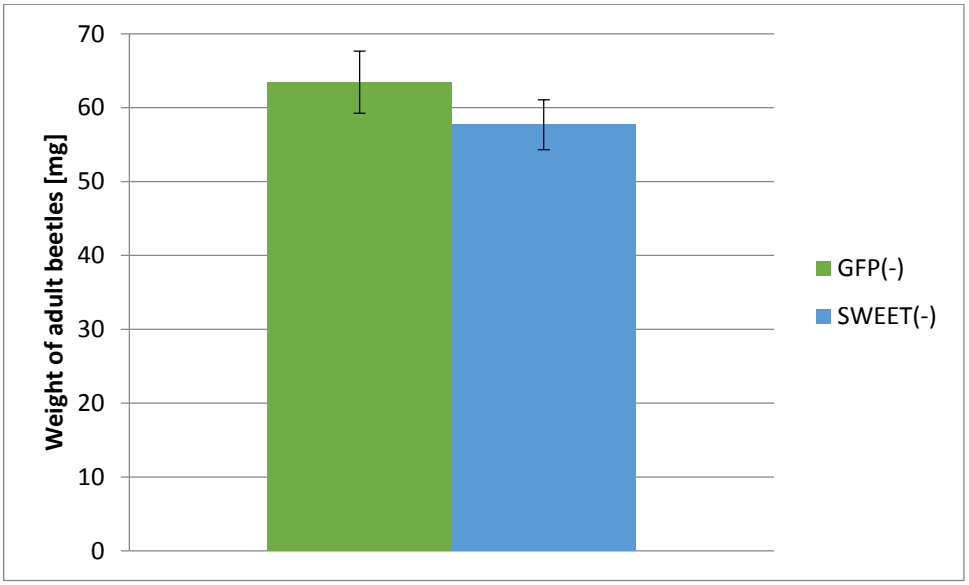

Figure 18: Weight of fully developed adult beetles after knock down. No statically significant difference is measurable between both groups. Displayed are GFP(-)(n=7) and SWEET(-)(n=9).

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Eigenständigkeitserklärung

Ich versichere, dass ich die vorliegende Arbeit selbstständig und ohne unerlaubte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Alle Stellen, die inhaltlich oder wörtlich aus Veröffentlichungen stammen, sind kenntlich gemacht. Diese Arbeit lag in der gleichen oder ähnlichen Weise noch keiner Prüfungsbehörde vor und wurde bisher noch nicht veröffentlicht.

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Jena, den _____ _____

Unterschrift

(Gerhard Albrecht)