RNA interference:

Process and Application to Pest Control

Dissertation

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Declaration

I hereby declare that the submitted doctoral thesis entitled “RNA interference: Process and Application to Pest Control” is my own work and was prepared without further sources and aids other than acknowledged, and that the submitted thesis was not part of any previous examination procedures.

For my family

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General abbreviations

α-SNAP	alpha-soluble NSF attachment protein
ACC	acetyl coenzyme A-carboxylase
act	actin
Ago2	Argonaute2
ANOVA	analysis of variance
Armet	Arginine rich, mutated in early stage of tumors
ATP	adenosintriphosphate
BiP	binding immunoglobulin protein
bp	base pair(s)
BUSCO	Benchmarking Universal Single-Copy Orthologs
cact	cactus
chc	clathrin heavy chain
CHS	chitin synthase
ChUP	Cholesterol Uptake associated
CI	confidencel interval
CRISPR	clustered regularly interspaced short palindromic repeats
Cry	crystalline protein
d	day(s)
Dcr2	Dicer2
diap1	death-associated inhibitor of apoptosis protein1
ds	double-stranded
e. g.	exempli gratia (for example)
ET50	effective time for 50% effect
etc.	et cetera
EW	emulsifier W
Fig.	Figure
FOXO	forkhead box subgroup O
GAL4-UAS	galactose-responsive transcription factor 4 - upstream activating sequence
GFP	Green Fluorescent Protein
GM	genetically modified
gw	gawky
HMGR	3-hydroxy 3-methylglutaryl coenzyme A reductase
hsc70-3	heat shock 70kDa protein cognate 3

Hsp	Heat shock protein
IAP	Inhibitor of Apoptosis
i. e.	id est (that is)
IMPI	inducible metalloproteinase inhibitor
inr-a	inverse regulator-a
IPM	integrated pest management
MID	middle
mg	milligrams
miRNA	micro RNA
mm	millimeter
mM	millimolar
MoA	mode of action
mRNA	messenger RNA
N/A	not applicable
n. d.	not determined
ns	not significant
PAZ	Piwi/Argonaute/Zwille
piRNA	piwi-interacting RNA
PIWI	C-terminal P-element Induced Wimpy testis
PP1-α	protein phosphatase 1 alpha
pros-α2	proteasome subunit alpha2
PTGS	posttranscriptional gene silencing
RT-qPCR	quantitative Real Time Polymerase Chain Reaction
RDE-4	RNAi defective-4
RdRP	RNA-dependent RNA polymerase
REase	RNAi efficiency-related nuclease
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNAse	ribonuclease
rop	ras opposite
rpn7	regulatory particle non-ATPase7
rpt3	regulatory particle triple-A ATPase3
SEM	standard error of the mean

shi	shibire
SID	systemic RNAi defective
Sil	SID-like
siRNA	small interfering RNA
snf7	sucrose non-fermenting 7
SR	Scavenger receptor
srp54k	signal recognition particle protein 54k
StauC	StaufenC
Ta	annealing temperature
UV	ultraviolet
vATPase	vacuolar H^+ ATPase
vha16	vacuolar H^+ ATPase 16 kDa subunit

Abbreviations of Species

A. aegypti	*Aedes aegypti* (Diptera: Culicidae), yellow fever mosquito
A. bipunctata	*Adalia bipunctata* (Coleoptera: Coccinellidae), two-spotted lady beetle
A. eugenii	*Anthonomus eugenii* (Coleoptera: Curculionidae), pepper weevil
A. glabripennis	*Anoplophora glabripennis* (Coleoptera: Cerambycidae), Asian long-horned beetle
A. grandis	*Anthonomus grandis* (Coleoptera: Curculionidae), cotton boll weevil
A. mellifera	*Apis mellifera* (Hymenoptera: Apidae), honey bee
A. pisum	*Acyrthosiphon pisum* (Hemiptera [Sternorrhyncha]: Aphididae), pea aphid
A. planipennis	*Agrilus planipennis* (Coleoptera: Buprestidae), emerald ash borer
A. tumida	*Aethina tumida* (Coleoptera: Nitidulidae), small hive beetle
B. aeneus	*Brassicogethes aeneus* (Coleoptera: Nitidulidae), pollen beetle
B. dorsalis	*Bactrocera dorsalis* (Diptera:Tephriditae), oriental fruit fly
Bt	*Bacillus thuringiensis* (Bacillales: Bacillaceae), (entomopathogenic bacterium)
B. terrestris	*Bombus terrestris* (Hymenoptera: Apidae), large earth bumblebee
C. capitata	*Ceratitis capitata* (Diptera: Tephritidae), medfly
C. carnea	*Chrysoperla carnea* (Neuroptera: Chrysopidae), common green lacewing
C. elegans	*Caenorhabditis elegans* (Rhabditida: Rhabditidae), (nematode)
C. maculata	*Coleomegilla maculata* (Coleoptera: Coccinellidae), pink spotted lady beetle
CPB	Colorado potato beetle (Coleoptera: Chrysomelidae)
C. populi	*Chrysomela populi* (Coleoptera: Chrysomelidae), red poplar leaf beetle

C. puncticollis	*Cylas puncticollis* (Coleoptera: Apionidae), African sweet-potato weevil
C. septempunctata	*Coccinella septempunctata* (Coleoptera: Coccinellidae), 7-spotted lady beetle
D. frontalis	*Dendroctonus frontalis* (Coleoptera: Curculionidae), southern pine beetle
D. maculatus	*Dermestes maculatus* (Coleoptera: Dermestidae), common hide beetle
D. melanogaster	*Drosophila melanogaster* (Diptera: Drosophilidae), common fruit fly
D. undecimpunctata	*Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae), southern corn rootworm
D. valens	*Dendroctonus valens* (Coleoptera: Curculionidae), red turpentine beetle
D. v. virgifera	*Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), western corn rootworm
E. heros	*Euschistus heros* (Hemiptera [Heteroptera]: Pentatomidae), Neotropical brown stink bug
E. postvittana	*Epiphyas postvittana* (Lepidoptera: Tortricidae), light brown apple moth
F. occidentalis	*Frankliniella occidentalis* (Thysanoptera: Thripidae), Western flower thrips
H. armigera	*Helicoverpa armigera* (Lepidoptera: Noctuidae), cotton bollworm
H. halys	*Halyomorpha halys* (Hemiptera [Heteroptera]: Pentatomidae), brown marmorated stink bug
H. vigintioctopunctata	*Henosepilachna vigintioctopunctata* (Coleoptera: Coccinellidae), 28-spotted lady beetle
H. virescens	*Heliothis virescens* (Lepidoptera: Noctuidae), tobacco budworm
H. zea	*Helicoverpa zea* (Lepidoptera: Noctuidae), corn earworm
L. decemlineata	*Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), Colorado potato beetle
L. dispar	*Lymantria dispar* (Lepidoptera: Erebidae), gypsy moth
L. migratoria	*Locusta migratoria* (Orthoptera: Acrididae), migratory locust
M. persicae	*Myzus persicae* (Hemiptera [Sternorrhyncha]: Aphididae), green peach aphid
M. u. undatus	*Myllocerus undecimpustulatus undatus* (Coleoptera: Curculionidae), Sri Lanka weevil
N. viridula	*Nezara viridula* (Hemiptera [Heteroptera]: Pentatomidae), southern green stink bug
O. rhinoceros	*Oryctes rhinoceros* (Coleoptera: Scarabaeidae), (coconut) rhinoceros beetle
P. cochleariae	*Phaedon cochleariae* (Coleoptera: Chrysomelidae), mustard leaf beetle
P. foveolatus	*Pediobius foveolatus* (Hymenoptera: Eulophidae) (parasitoid wasp)

P. putida	*Pseudomonas putida* (Pseudomonadales: Pseudomonadaceae), (soil and insect-gut commensal bacterium)
P. versicolora	*Plagiodera versicolora* (Coleoptera: Chrysomelidae), willow leaf beetle
P. xylostella	*Plutella xylostella* (Lepidoptera: Plutellidae), diamondback moth
S. frugiperda	*Spodoptera frugiperda* (Lepidoptera: Noctuidae), fall armyworm
S. gregaria	*Schistocerca gregaria* (Orthoptera: Acrididae), desert locust
S. littoralis	*Spodoptera littoralis* (Lepidoptera: Noctuidae), Egyptian cotton leafworm
T. castaneum	*Tribolium castaneum* (Coleoptera: Tenebrionidae), rust red flour beetle
T. evanescens	*Trichogramma evanescens* (Hymenoptera: Trichogrammatidae) (parasitoid wasp)
T. urticae	*Tetranychus urticae* (Acarida: Tetranychidae), two-spotted spider mite
V. destructor	*Varroa destructor* (Acarida: Varroidae), *Varroa* mite
WCR	Western corn rootworm (Coleoptera: Chrysomelidae)

Abstract

Innovation is one of the keys to ensure effective insect pest control in crop protection. A much discussed novelty to insect pest management is represented by RNA interference (RNAi). Employing this conserved virus defense mechanism and diverting it to turn against its own host resulting in insect death has sparked research in many insect pests with regards to susceptibility, suitable target genes and delivery strategies.

Finding new lethal genes universal to insect pest management would reduce the work invested in researching putative target genes and simplify the adjustment of dsRNA sequences to target adaptable pest species spectra. A large-scale RNAi screen in *Tribolium castaneum* identified eleven highly lethal target genes that could serve this purpose. The next step was the transfer to other pest species. To this end, the herbivorous mustard leaf beetle *Phaedon cochleariae* was selected in this study. The transcriptome of *P. cochleariae* revealed nine orthologs to the highly lethal genes described in *T. castaneum* and key players of the RNAi mechanism. Employing a similar procedure as the *T. castaneum* screen, injection of dsRNA induced high levels of mortality confirming that these target genes can be successfully used for the control of other pest species. However, injection is not a viable option for pest control. Therefore, an experimental set-up as close as possible to a spraying application while still maintaining a small-scale screening procedure was established to test the efficacy of oral RNAi. In this set-up, *P. cochleariae* could serve as a screening model to test sprayable RNAi applications. Five target genes induced high mortality rates accompanied by feeding cessation and stunted growth across three tested dsRNA concentrations demonstrating a good transfer rate of 56% of highly lethal targets. Previously proposed reference genes for *P. cochleariae* were confirmed for use in RT-qPCR across developmental stages, facilitating expression data analysis for this and future studies. Employing these reference genes, target gene knockdown was observed for most dsRNAs inducing high mortality rates upon dsRNA injection and feeding confirming the gene specificity of this pest control strategy. In summary, this study supports and validates the value of the highly lethal target genes identified in the *T. castaneum* screen for the application in pest management.

Apart from target gene efficacy, the success of RNAi as an insect pest control measure depends on a uniform RNAi response within a species. Very few studies so far questioned whether different natural populations of a species vary in their response towards dsRNA. Instead, many studies rely on a single laboratory population. This work expands the knowledge on RNAi response variability by comparing fourteen populations of the Colorado potato beetle *Leptinotarsa decemlineata* collected in nine European countries. Spray application of dsRNA targeting the *actin* gene in a diagnostic dose based on a dose-response experiment in a German inbred strain was used for comparison of all populations.

Overall, only minor variability between European populations was observed in terms of mortality, target gene conservation, growth and developmental retardation. These results denote RNAi as a suitable control measure for this species. Nevertheless, the RNAi responses were marked by significant differences in their temporal onset dependent on the population. Additionally, one Spanish population stood out by its relative tolerance to the oral dsRNA treatment. Neither observation was explained by *actin* nucleic acid sequence divergence, its knockdown or the differential expression of the target gene or molecular participants of the RNAi response between populations. Therefore, integrated pest management should take these data into consideration for the implementation of the RNAi technology as an insect control strategy. Lastly, strong correlation of gene expression was found between two RNAi core machinery genes, *dicer2* and *argonaute2a*, and the recently identified factor of the RNAi response, *staufenC*. Evidently, research on the underlying mechanism of RNAi in insects is not complete yet which provides incentive for further basic research.

1. Introduction

1.1. Insect pests in agriculture

Pre- and postharvest damage of agricultural crops by insect pests can dramatically reduce yields and result in major economic losses (Bradshaw et al., 2016; Oerke, 2006). These pest insects cause 18% of crop loss, though estimated yield losses without preventive measures are predicted to be much higher and are expected to increase due to global warming (Lehmann et al., 2020; Oerke, 2006).

Insect control in the last century heavily relied on chemical insecticide use, yet the appearance of resistance in many insect species to one or more active ingredients (Figure 1.1) have called for the development and launch of new insecticides, preferentially with novel modes of action (Borel, 2017; Casida and Bryant, 2017; Oerke, 2006; Sparks et al., 2019; Sparks and Nauen, 2015; Tabashnik and Carrière, 2017).

To date, at least 57 chemical classes of insecticides grouped in 32 known modes of action (MoA) as well as five classes of compounds with unresolved MoA were distinguished by the Insecticide Resistance Action Committee (IRAC, 2020). One alternative to synthetic insecticides was found in isolates of the insecticidal *Bacillus thuringiensis* (Bt) endotoxin (MoA class 11) which was readily adopted for pest management in sprayable and later in 1996 in transgenic trait applications (Mendelsohn et al., 2003; Sansinenea, 2012). Multiple improved and modified versions of these so-called crystalline (Cry) proteins with different target spectrums were isolated, developed and combined in mixtures or genetically modified (GM) crops to combat pests from various orders such as Lepidoptera (eg. *Helicoverpa zea*, *Spodoptera frugiperda*), Coleoptera (e. g. *Diabrotica spp*.) or Diptera (e. g. *Ceratitis capitata*) (Badran et al., 2016; de Maagd et al., 2001; Head and Greenplate, 2012; Sansinenea, 2012; Vidal-Quist et al., 2010; Ward et al., 2005; Zhong et al., 2000). Beginning in 2002, first cases of resistance were reported which by 2019 affected seven traits in GM crops (Figure 1.1) (Ali et al., 2006; Sparks et al., 2019; Tabashnik and Carrière, 2017).

New and safe pest control agents are needed due to a number of issues with conventional methods: difficulties with resistance, environmental and toxicological concerns despite spurring improved ecotoxicological profiles of new synthetic insecticides such as flupyradifurone (Nauen et al., 2015), rising caution of consumers regarding chemical residues and resulting higher (re-) registration standards for agrochemicals especially according to the hazard-based risk assessment of the European Union (Corsi and Lamberth, 2015). In recent years, the utility of RNAi (see following sections) as a new, species-specific and ecofriendly insect control measure was presented and is continually being explored (Baum et al., 2007; Borel, 2017; Liu et al., 2020; Mao et al., 2007).

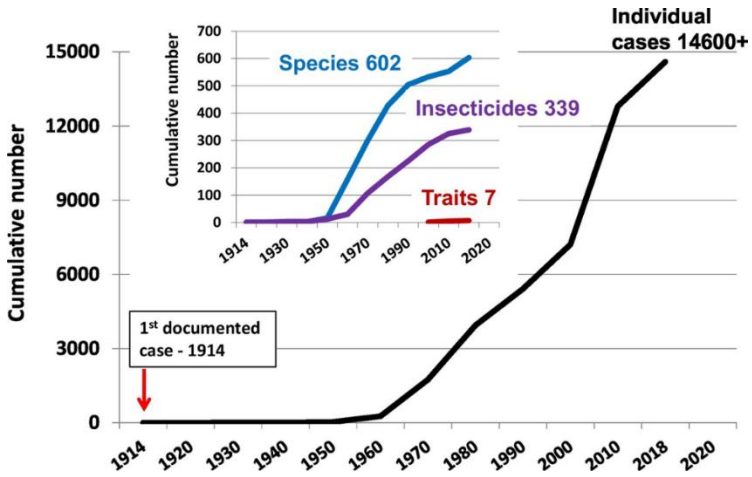

Figure 1.1: The replicated graph from Sparks et al. (2019) shows the temporal development of insecticide resistance. Total numbers of resistance reports (black), insect species with resistant populations (blue), insecticides with reduced efficacy due to resistance (purple) and insecticidal traits in GM crops (red) are shown.

1.2. RNAi in pest management

For the use of RNA interference (RNAi) in pest insect control, double-stranded RNA (dsRNA) must be made available to the insects for oral uptake. Shortly after a first study in *Epiphyas postvittana* successfully demonstrated knock-down of target genes upon dsRNA feeding in insects (Turner et al., 2006), dsRNA was shown to induce stunted growth in *Helicoverpa armigera* (Mao et al., 2007) and to trigger insect mortality in three beetle species upon oral exposure leading to a wider and more detailed investigation of RNAi as a pest insect management tool (Baum et al., 2007). This motivated researchers to test for more amenable insect species, to establish and refine delivery strategies and to find suitable target genes as outlined in the following sections.

1.2.1 dsRNA delivery methods

Multiple approaches for field application with varying advantages and disadvantages were explored. One of the easiest options is sprayable (exogenous) RNAi. Here, the dsRNA is sprayed on crops in an aqueous solution, similar to formulated synthetic insecticides, depositing the dsRNA on the leaf surface for the insects to feed on. Despite the ease of this delivery format in the field, it is more challenging to simulate field-realistic exposure scenarios under laboratory conditions where spraying is often replaced by manually spreading dsRNA solution on leaves (Gogoi et al., 2017; Xu et al., 2019b; Zhu et al., 2011), direct feeding of droplets of dsRNA solution (Rodrigues et al., 2017b; Turner et al., 2006) or diet overlay assays (Baum et al., 2007). Sprayable RNAi is mostly applicable for leaf-feeding, chewing

insects. Another application strategy is irrigation of plants with dsRNA solution. Not only would this extend the range of targeted pests to soil-borne insects, but also piercing/sucking insects feeding on shoots/leaves since roots take up dsRNA and distribute it along their vascular system (Brosnan et al., 2007; Hunter et al., 2012; H. Li et al., 2015; Voinnet et al., 1998). However, this method exposes dsRNA to the plant RNAi machinery (H. Li et al., 2015) so that dsRNA is partially processed and thus unavailable for insect dsRNA uptake. Both foliar and irrigation applications expose dsRNA to biotic and abiotic factors that lead to quick decay of dsRNA, e. g. by UV-light and soil organisms (Dubelman et al., 2014; H. Li et al., 2015; San Miguel and Scott, 2016). A transgenic crop approach protects the dsRNA from degradation in the environment coupled with long-term plant protection. It was one of the earliest demonstrated strategies for insect control via RNAi (Baum et al., 2007; Head et al., 2017; Hu et al., 2016; Hussain et al., 2019; Ibrahim et al., 2017; Z. Wang et al., 2018). Nevertheless, dsRNA is processed (and degraded) by the plant RNAi machinery if ubiquitously expressed in plant tissues. However, this can be prevented by the transgenic expression of dsRNA (for example as hairpin RNA) in chloroplasts which lack an RNAi machinery (Bally et al., 2016; Burke et al., 2019; Jin et al., 2015; Zhang et al., 2015). Plant transformation however is a time-consuming process and genetically modified crops are not generally accepted by consumers. Lastly, trunk injection of dsRNA solution is an additional treatment means suited for trees and other perennial plants (Dalakouras et al., 2018; Hunter et al., 2012).

Therefore, the decision which dsRNA delivery method is used must consider the crop, the targeted pest species and their feeding mode, its overall efficiency and the regulatory framework of the country/region where the product is supposed to be launched.

1.3. RNAi mechanism

Before commercialization of new insect control agents, knowledge of their mode of action may facilitate incorporation into integrated pest and resistance management strategies or help with registration. Here, the RNAi mechanism represents the MoA.

RNA interference (RNAi) is an ancestral immune response of eukaryotic organisms to combat viral infections, transposable elements and to regulate expression of endogenous genes (Cerutti and Casas-Mollano, 2006; Dowling et al., 2016; Shabalina and Koonin, 2008). First evidence for this pathway was discovered in petunia plants which despite the overexpression of an anthocyanin biosynthesis enzyme displayed white or spotted flowers instead of an expected intense color (Napoli et al., 1990). This and further research in plants discovered the involvement of RNA and its importance for virus resistance, the spreading of the (RNA) signal and the depletion of homologous mRNA which lead to the term “posttranscriptional gene silencing” (PTGS) (Angell and Baulcombe, 1997; Napoli et al., 1990; Palauqui

et al., 1997; Voinnet et al., 1998; Waterhouse et al., 1998). Similarly, observations in fungi also relied on unexpected discrepancies in phenotypes and first genetic basics of the phenomenon (in this field named “quelling”) were described (Cogoni et al., 1994; Cogoni and Macino, 1997). However, the breakthrough was signified by the identification of the exact nature of the causative agent as double-stranded RNA (dsRNA) in *Caenorhabditis elegans* which was immediately reinforced by similar findings in *Trypanosoma brucei* (Fire et al., 1998; Ngô et al., 1998). These discoveries enabled the molecular characterization of the RNAi mechanism, its diversification into e.g. microRNA (miRNA), small interfering RNA (siRNA) and piwi-interacting RNA (piRNA) pathways and their corresponding functions (Dowling et al., 2016; Mongelli and Saleh, 2016). Since this study focuses on the application of exogenous dsRNA to trigger the siRNA pathway in insects, only this route will be further elaborated in the following sections.

1.3.1 siRNA machinery

Two major steps define the siRNA pathway, namely the cleavage of the original long dsRNA into smaller fragments termed “dicing” and the recruitment and subsequent cleavage of RNA sequences homologous to the small dsRNA fragments called “slicing” (Hammond, 2005).

The initial step is mediated by Dicer2 (Dcr2) protein first identified in *Drosophila melanogaster*, a ribonuclease (RNAse) belonging to clade III of ribonucleases (Bernstein et al., 2001). It typically contains two helicase and two RNAse domains, a Piwi/Argonaute/Zwille (PAZ) domain (meant for the recognition of siRNAs), another dsRNA binding motif, a Dimer domain as well as either a DEAD box or a ResIII domain depending on the insect species (Bernstein et al., 2001; Davis-Vogel et al., 2018a; Tomoyasu et al., 2008). Long dsRNA is recognized by Dcr2 and subsequently (energy-dependently) cut into siRNAs of 21-23bp length with two to three 3´-nucleotide overhangs (Figure 1.2) (Elbashir et al., 2001a; Sinha et al., 2018; Zamore et al., 2000).

In order to proceed to the next step, siRNAs are recognized by an RNA-binding protein R2D2 corresponding to RDE-4 from *C. elegans* (Liu et al., 2003). R2D2 binds the 5´-phosphate of the thermodynamically more stable end of a siRNA defining its passenger strand (Tomari et al., 2004). Together with Dcr2 binding the other end of the siRNA, these two proteins determine as a heterodimer the guide strand of the siRNA for following steps (Tomari et al., 2004).

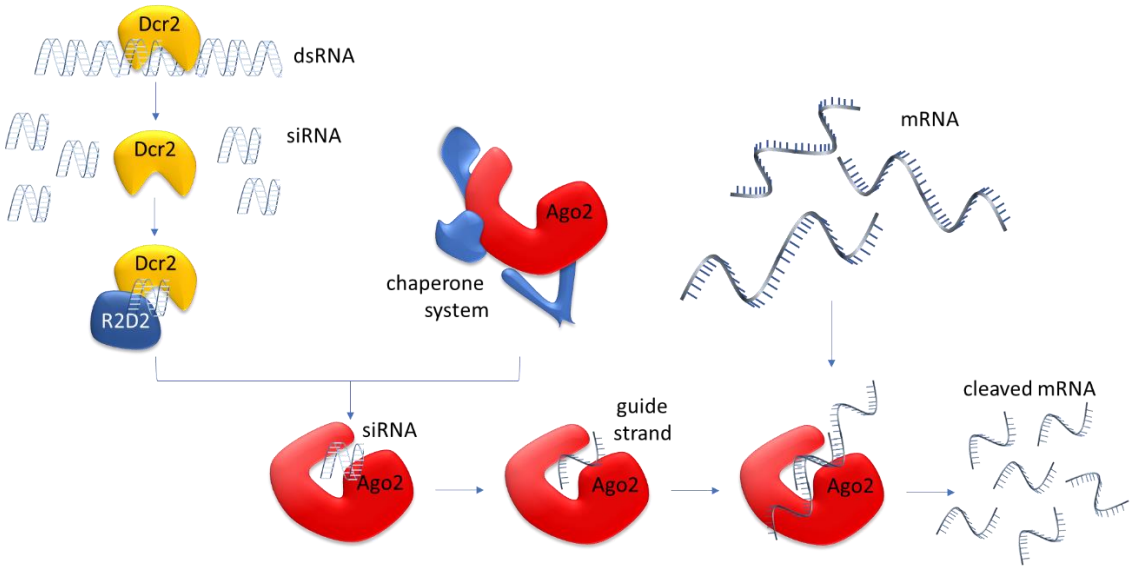

Figure 1.2: Schematic representation of the siRNA pathway with its major constituents.

The by Dcr2 and R2D2 oriented siRNA is passed on to form – together with other constituents - the RNA-induced silencing complex (RISC) with its catalytic protein component Argonaute2 (Ago2) (Hammond et al., 2001, 2000; Iwasaki et al., 2015; Rivas et al., 2005). Ago2 is held in open conformation by the Heat shock protein 70 (Hsp70) and Hsp90 systems and other chaperones (Iwasaki et al., 2010; Tsuboyama et al., 2018) to receive the 3´-end of the siRNA guide strand with its PAZ domain and the 5´-end with its middle (MID) domain (Boland et al., 2010; Cerutti et al., 2000; Ma et al., 2005; Song et al., 2003). The C-terminal P-element Induced Wimpy testis (PIWI) domain nicks the passenger strand between the 9th and 10th nucleotide from the 5´-end so that the RISC complex remains with a single stranded guide RNA after dissociation of the two halves of the passenger strand (Cerutti et al., 2000; Cox et al., 1998; Elbashir et al., 2001b; Matranga et al., 2005). In this mature RISC complex, the positively charged groove formed by the domains of Ago2 is free to fit RNA complementary to the bound guide strand (Song et al., 2004). Target messenger RNA (mRNA) cleavage is performed by the PIWI domain (Elbashir et al., 2001a; Liu et al., 2004; Rivas et al., 2005). Multiple cycles of cleavage are possible due to the release of the cut mRNA strands from the complex which is ATP-dependent in *D. melanogaster* (Haley and Zamore, 2004). Thus cut mRNA is degraded rapidly and fails to produce functional protein, leading to the gradual depletion of the targeted protein (Bolognesi et al., 2012; Cogoni et al., 1994; Fire et al., 1998; Napoli et al., 1990; Vélez et al., 2019).

The RNAi machinery in insects shows some differences compared to other organisms. One of them is the lack of a RNA-dependent RNA polymerase (RdRP) (Li et al., 2018; Tomoyasu et al., 2008) which amplifies and produces dsRNA to generate secondary siRNAs augmenting the original RNAi response

observed in plants (Dalmay et al., 2000; Mourrain et al., 2000; Vaistij et al., 2002) and *C. elegans* (Sijen et al., 2001; Smardon et al., 2000). Instead, insects only rely on the starting dsRNA material which also limits the pool of available siRNAs to the region covered by the original dsRNA and spreading to other stretches of the targeted mRNA is not observed (Li et al., 2018). Another difference between insects and other taxa was described very recently; Coleopterans possess an additional factor called StaufenC (StauC) potentially involved in the RNAi machinery (Yoon et al., 2018). Although the related Staufen protein is known for its RNA- and even dsRNA-binding capacities in *D. melanogaster* and mammals required for intracellular mRNA transport, it was not functionally linked to RNAi (St Johnston et al., 1992, 1991; Wickham et al., 1999; Yoon et al., 2018). Recently in beetles it was shown that StauC was required for Dcr2 function in dsRNA cleavage, although the protein is not fully characterized yet, demonstrating the need for ongoing fundamental research on RNAi (Yoon et al., 2018).

1.3.2 dsRNA uptake

Before execution of the RNAi mechanism, the dsRNA first must be taken up by the cell and translocated to the cytosol. Apart from viruses, herbivorous insects face natural dsRNA species from their plant diet and process it into siRNAs (Ivashuta et al., 2015). For an insect pest control approach aiming to utilize RNAi, exogenous insecticidal dsRNA is considered to be delivered into the insect by ingestion of the dsRNA together with their host plant when they begin to damage the crop. As such, the dsRNA enters the insect body via its alimentary system in which the midgut is considered to be the main route of uptake because it is not lined by cuticle (Ivashuta et al., 2015; Shukla et al., 2016). This route of uptake of external dsRNA is called environmental RNAi (Figure 1.3), i. e. the uptake of dsRNA by cells from surrounding fluids (Whangbo and Hunter, 2008). Further, distribution of the dsRNA from cells to neighboring cells and within the insect body via hemolymph characterizes systemic RNAi (Dong and Friedrich, 2005; Tassetto et al., 2017; Tomoyasu et al., 2008). The extent of dsRNA spread depends on the insect species, ranging from almost no dsRNA spread (cell-autonomous RNAi, see Figure 1.3) to even being passed on to the next generation by parental RNAi (Bucher et al., 2002; Li et al., 2018; Miller et al., 2008; Ramaseshadri et al., 2013; Tomoyasu et al., 2008; Whangbo and Hunter, 2008; Xiang et al., 2016).

Recent research has shed some light on several aspects of the molecular mechanisms of dsRNA uptake. One prerequisite that dsRNA needs to fulfill for cellular uptake relates to its length. In insects such as *Diabrotica undecimpunctata, Diabrotica virgifera virgifera* or *T. castaneum*, dsRNAs shorter than 60bp,

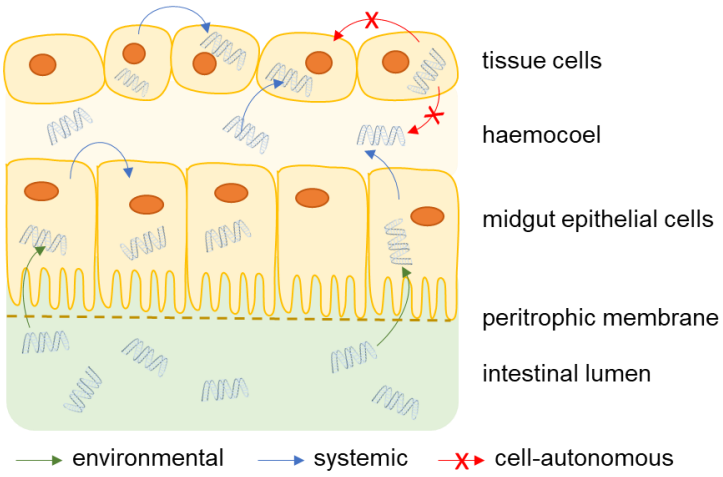

Figure 1.3: Schematic illustration of dsRNA uptake and distribution modes of dsRNA denoting environmental (green arrows), systemic (blue arrows) and cell-autonomous (red arrows) RNAi. The design is based on figures from Whangbo and Hunter (2008) and Joga et al. (2016).

including siRNAs, are not taken up while longer dsRNAs are internalized and, when covering at least 240bp of the target gene, are more efficient in target gene suppression (Bolognesi et al., 2012; Ivashuta et al., 2015; Miller et al., 2012; Wang et al., 2019).

Several pathways are discussed to be relevant during the dsRNA uptake and/or transport process. A first uptake mechanism was found in *C. elegans* where systemic RNAi defective 1 (SID1) acts as a membrane-spanning dsRNA-specific channel protein (Feinberg and Hunter, 2003; W. Li et al., 2015; Shih and Hunter, 2011; Winston et al., 2002). However, no direct SID1 ortholog was found in insects (Tomoyasu et al., 2008). Instead, identified SID1-like (Sil) proteins showed more homology to the *C. elegans* Tag-130 /ChUP1 protein which does not contribute to the RNAi response in *C. elegans* (Tomoyasu et al., 2008; N. Wynant et al., 2014). Nevertheless, Sil proteins were tested for their impact in systemic RNAi in different insect species with varying results. In *Apis mellifera*, a Sil-protein was upregulated after dsRNA exposure unlike in other studies (Aronstein et al., 2006) and in *L. decemlineata*, minor contribution to systemic RNAi were attributed to one of its Sil proteins (SilC but not SilA) (Cappelle et al., 2016; Yoon et al., 2016) while the strongest participation of Sil proteins was found in *D. v. virgifera* (Miyata et al., 2014; Pinheiro et al., 2018). In *T. castaneum*, all three identified Sil proteins were irrelevant for systemic RNAi (Tomoyasu et al., 2008) and similar results were obtained for *Schistocerca gregaria* (N. Wynant et al., 2014), *Plutella xylostella* (Wang et al., 2014) and *Locusta migratoria* (Luo et al., 2012).

As part of the mechanism for SID2 dsRNA receptor mediated cellular dsRNA entry from gut lumen, endocytosis was required in *C. elegans* (McEwan et al., 2012). First indication that endocytosis is relevant for insects was presented in a *D. melanogaster* cell line (Saleh et al., 2006; Ulvila et al., 2006). More specifically, involvement of clathrin-dependent endocytosis exemplified with Clathrin heavy chain (Chc) and vacuolar H^+ ATPase 16 kDa subunit (Vha16) was demonstrated for several insect species such as *L. decemlineata* (Cappelle et al., 2016), *Bactrocera dorsalis* (X. Li et al., 2015), *T. castaneum* (Xiao et al., 2015), *D. v. virgifera* (Pinheiro et al., 2018) and *S. gregaria* (N. Wynant et al., 2014). In these studies, the influence of clathrin-mediated endocytosis often outweighed the contribution of Sil proteins indicating the presence of other proteins helping dsRNA to cross to the cytosol. Putative dsRNA receptors were proposed to be pattern-recognition receptors (Saleh et al., 2006) previously only associated with bacterial infections in *D. melanogaster*, Scavenger receptors (SR) SR-CI and Eater (Kocks et al., 2005; Rämet et al., 2001; Ulvila et al., 2006). SRs were also found to be relevant for RNAi in *S. gregaria* and *L. decemlineata* (N. Wynant et al., 2014; Yoon et al., 2016) and were upregulated after dsRNA exposure in honey bees (Brutscher et al., 2017; Flenniken and Andino, 2013). The presence of additional dsRNA receptors should still be considered, for example the involvement of human Stabilin-1 and -2 functioning as antisense oligonucleotide receptors were not yet investigated in insects (Miller et al., 2016).

After uptake into intracellular vesicles, dsRNA is believed to follow endosomal maturation until the late endosomes or multivesicular bodies and is assumed to escape to the cytosol before fusion with lysosomes (Lee et al., 2009; Saleh et al., 2006; Shukla et al., 2016; Xiao et al., 2015; Yoon et al., 2016). In *Spodoptera frugiperda*, dsRNA was colocalized with and accumulated in early and late endosomes, indicating that dsRNA was unable to escape from the endosome (Yoon et al., 2017). In other insects, acidification of endosomes by vacuolar ATPase appears to play a crucial part in dsRNA endosome escape (Saleh et al., 2006; Yoon et al., 2016). However, the exact mechanism of how and when exactly dsRNA leaves the endosome still requires more detailed examination. Upon release, dsRNA can interact with the components of the RNAi machinery localized in GW-bodies (subcellular structures for RNA degradation) that were found to be associated with late endosomes to initiate the RNAi response (Lee et al., 2009).

1.4 Selection of suitable target genes

Following uptake of the provided dsRNA and its processing by the RNAi machinery, the effect of the RNAi response can be evaluated. Success of RNAi as an insect control measure strongly depends on the importance of the selected target gene for insect survival. Frequently, target genes were selected

based on their known essential function. Such genes represent for example *actin*, *α-tubulin* or different subunits of *V-ATPase* which were often employed to demonstrate functional RNAi in the respective insect species by the expression of a lethal phenotype (e. g. Baum et al., 2007; Castellanos et al., 2019; Mogilicherla et al., 2018; Riga et al., 2020; Upadhyay et al., 2011; Whyard et al., 2009; Yao et al., 2013; Zhu et al., 2011). In another strategy, genes known as insecticide targets such as chitin synthase (CHS), acetyl coenzyme-A carboxylase (ACC), 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMGR) or acetylcholineesterase were targeted with variable success (Galdeano et al., 2017; Kumar et al., 2009; Riga et al., 2020; Shi et al., 2016; Wang et al., 2013; Zhang et al., 2010). This type of target genes partially overlaps with the next interesting targets represented by (more or less) insect specific genes since they are meant to increase specificity and to diminish possible risks for consumers and non-target organisms. Such targets include CHS as mentioned above, but also the ecdysone receptor involved in development and molting of insects (Christiaens et al., 2014; Hussain et al., 2019; Israni and Rajam, 2017) or genes involved in juvenile hormone pathways (Van Ekert et al., 2014; Zhou et al., 2008). Selectivity can be narrowed even to certain insect groups, as shown for 3-hydroxykynurenine transaminase which is mosquito-specific (Kumar et al., 2013). However, the identification of such genes requires extensive preliminary research.

In a reverse genetics approach, lethal target genes could also be identified in so-called targeted RNAi screens. First approaches of screening tactics started with a large set of selected genes, as described by Baum et al. (2007) who screened 290 genes of essential function in *D. v. virgifera* of which 67 showed mortality or growth defects at a low dsRNA concentration of 5.2ng/cm^2. Bai et al. (2011) tested all 111 identified G-protein-coupled receptors of *T. castaneum*, albeit at relatively high concentrations, and ended up with eight putative lethal target genes. In order to expand the number of novel potential target genes suitable for pest control, an unbiased screening approach is required, preferably in a relevant species with a well assembled and annotated genome. A genome-wide RNAi library was constructed for *D. melanogaster* which enabled high-throughput cell-based screens to find new or consolidate known players in various pathways regulating for example cell proliferation, epigenetics, hypoxia or even the RNAi mechanism itself (Boutros et al., 2004; Dekanty et al., 2010; Dorner et al., 2006; Müller et al., 2005; Umer et al., 2019). Despite the advanced screening platform, *D. melanogaster* as a model for lethal target identification is only partially suited due to its insensitivity towards systemic RNAi and its taxonomic distance to the insect orders which comprise many pest species, like for example Coleoptera.

Therefore, another unbiased effort initiated in the highly RNAi-sensitive beetle *T. castaneum* was performed, where roughly 5000 genes were targeted upon injection of dsRNA into pupae and/or larvae (Schmitt-Engel et al., 2015). The obtained data were stored in a public database to open new avenues

for e. g. research of insect development (Dönitz et al., 2018, 2015; Schmitt-Engel et al., 2015). In relation to insect pest control, this screen identified eleven highly lethal target genes that were even more efficient than other established lethal targets such as *V-ATPase*, demonstrating the power of the screening approach (Ulrich et al., 2015). These novel targets were rapidly incorporated into current research and tested in a variety of insects (Castellanos et al., 2019; Dhandapani et al., 2020; Knorr et al., 2018; Kyre et al., 2019; Mogilicherla et al., 2018; Rodrigues et al., 2018; Xu et al., 2019b; Zhang et al., 2019) or used as a starting point for other lethal target genes (Bingsohn et al., 2017).

Apart from directly eliciting mortality, target genes could also be chosen for other features. For example, RNAi could be used to revert susceptibility of pest insects to insecticides or plant secondary metabolites by targeting genes of detoxification enzymes known to confer resistance (Bautista et al., 2009; Bona et al., 2016; Mao et al., 2007; Yu and Killiny, 2018). A different strategy aims to reduce fecundity or to exploit parental RNAi by inducing embryo lethal phenotypes without affecting adult survival, but targeting the next generation instead (Coelho et al., 2016; Dong et al., 2016; Fishilevich et al., 2016; Khajuria et al., 2015; Niu et al., 2017).

1.5. Population variability towards RNAi

Tests on the efficacy of suitable dsRNAs have often been conducted in laboratory strains of a species. However, if dsRNA is released as an insecticidal product under field conditions across countries, it faces the entire spectrum of natural genetic variation within the target species. Differences in the RNAi response may influence the performance of the dsRNA between populations of the same species, and if less susceptible populations already exist, this may quickly select for resistance. Still, only few studies so far have explored the possibility of inter-population variability. Populations of *T. castaneum* from China did not display significant differences in the RNAi response (H. Wang et al., 2018), while some Japanese populations of *L. migratoria* exhibited RNAi tolerance depending on the sampling location (Sugahara et al., 2017). In another study performed in two *T. castaneum* laboratory strains, despite the lack of RNAi sensibility issues, the same dsRNA targeting *α-importin* produced opposite developmental defects probably due to differences of target gene expression and sequence (Kitzmann et al., 2013). These results demonstrate that the RNAi response may vary between populations, but the extent and underlying mechanisms are still mostly elusive.

1.6. Resistance

Just like any insect control technique, RNAi also faces the threat of resistance development. The accumulation of polymorphisms within the targeted sequence was considered as a possible resistance mechanism though with moderate risk (Kunte et al., 2020). Other studies speculated that dsRNA resistance may evolve by reduced expression levels of genes of the RNAi machinery, such as *dcr2*, *ago2* or *stauC* (X. Li et al., 2015; Vélez et al., 2016b; Yoon et al., 2016, 2018), while a third option suggested alterations in the dsRNA uptake mechanism (Yoon et al., 2016). Recently, Khajuria et al. (2018) successfully generated a dsRNA resistant strain of *D. v. virgifera* whose RNAi response was not only impaired for the selective dsRNA targeting *snf7* (sucrose non-fermenting 7) but also for dsRNAs targeting other genes (Khajuria et al., 2018). The resistant beetles were shown to overcome RNAi as an insect control measure by a mechanism blocking dsRNA uptake, though the molecular details are not yet resolved (Khajuria et al., 2018).

1.7. Susceptibility of insect orders

Apart from the risk of selection for resistance, RNAi in insect pest control is limited by the variability of RNAi responses between different insect species or different orders.

Common opinion in insect RNAi research particularly considers coleopteran pest species as the most sensitive insects towards dsRNA treatment. Functional RNAi was demonstrated for a wide range of beetle species such as *T. castaneum* (e. g. Brown et al., 1999; Bucher et al., 2002; Bucher and Klingler, 2004; Schmitt-Engel et al., 2015), *D. v. virgifera* (e. g. Baum et al., 2007; Hu et al., 2016; Niu et al., 2017; Vélez et al., 2019), *D. undecimpunctata* (Baum et al., 2007; Bolognesi et al., 2012), *L. decemlineata* (Baum et al., 2007; Shi et al., 2016; Zhu et al., 2011), *Aethina tumida* (Powell et al., 2017), *Agrilus planipennis* (Rodrigues et al., 2017b, 2018), *Anoplophora glabripennis* (Rodrigues et al., 2017a), *P. cochleariae* (Bodemann et al., 2012; Frick et al., 2013; Stock et al., 2013), *Chrysomela populi* (Strauss et al., 2013), *Anthonomus grandis* (Coelho et al., 2016), *Anthonomus eugenii* (Wu et al., 2019), *Brassicogethes aeneus* (Knorr et al., 2018), *Dendroctonus frontalis* (Kyre et al., 2019), *Cylas puncticollis* (Prentice et al., 2017), *Plagiodera versicolora* (Xu et al., 2019b; Zhang et al., 2019), *Myllocerus undecimpustulatus undatus* (Pinheiro et al., 2020), *Henosepilachna vigintioctopunctata* (Chikami et al., 2019; Lü et al., 2020), *Dermestes maculatus* (Xiang et al., 2016), *Oryctes rhinoceros* (Watanabe et al., 2020), *Coccinella septempunctata* and *Adalia bipunctata* (Haller et al., 2019). Ingested dsRNA was detected in the hemolymph of *L. decemlineata* after 10h of dsRNA exposure and circulated in the hemolymph for at least three days (Shukla et al., 2016). In *D. v. virgifera* adults, target gene suppression could be observed as fast as within 10h of oral dsRNA exposure (Wu et al., 2018a). This response was

sustained for up to 20-40 days after the end of dsRNA exposure, when non-lethal target genes were applied (Wu et al., 2018a). A dsRNA response could also be achieved across developmental stages as seen in *T. castaneum* where injection into larvae could affect pupae and adult stages (Tomoyasu and Denell, 2004). Taken together, these studies supported the application of RNAi as an attractive new insect control measure, especially for coleopteran pests.

Nevertheless, even with beetles there are some obstacles. Although RNAi was demonstrated to work well in principle for several beetle species upon injection, oral RNAi in the same species sometimes failed to produce similar effects. Such cases are represented by *A. grandis*, *A. eugenii* and *C. puncticollis*. Nucleases expressed in the midgut were identified to cause rapid degradation of dsRNA essentially preventing uptake of sufficient dsRNA amounts (Garcia et al., 2017; Prentice et al., 2019, 2017; Wu et al., 2019). Interestingly, such nucleases were also found in insects where oral RNAi works well, such as *L. decemlineata* and *A. planipennis* (Singh et al., 2017). Although no information on the contribution of nucleases in *A. planipennis* is available, nuclease activity appeared to impair dsRNA efficiency in *L. decemlineata* (Spit et al., 2017). dsRNA degradation within 10 min in the midgut was observed, though the effect was more pronounced in adult beetles than in fourth instar larvae in accordance with higher nuclease expression levels in adults compared to larvae (Spit et al., 2017). This discrepancy between developmental stages may even occur in earlier larval instars since RNAi was more efficient in younger life stages, although dsRNA concentration was not adjusted to varying body size in this study (Guo et al., 2015). For *T. castaneum*, contrasting results on oral RNAi exist. While some studies show the amenability of this insect towards dsRNA feeding (Abd Halim et al., 2016; Whyard et al., 2009), other studies show the presence of nucleases and report unsuccessful feeding experiments (Miyata et al., 2014; Singh et al., 2017; Spit et al., 2017).

Therefore, if dsRNA is used as an insecticide, the applied rate should consider which species and developmental stages are targeted and adjust the rates appropriately, just as it is done for conventional insecticides.

Unlike beetles, most other insect orders that were tested for RNAi showed varying levels of sensitivity. Most prominently, Lepidoptera were often reported to be recalcitrant to RNAi or to display rather weak responses (Terenius et al., 2011). Multiple explanations for this observation were described. Firstly, the midgut of lepidopteran insects, along with several orthopteran or hymenopteran species, is alkaline which destabilizes dsRNA and thus facilitates degradation (Dow, 1992; Ortego, 2012; Wu et al., 2016). Additionally, Lepidoptera-specific RNAi efficiency-related nuclease (REase) and other dsRNAses expressed in the midgut degrade dsRNA before it can be taken up and processed by the RNAi machinery (Arimatsu et al., 2007; Furusawa et al., 1993; R. Guan et al., 2018; R.-B. Guan et al., 2018;

Liu et al., 2012). Quite often, dsRNA is eliminated in their body fluids prior to processing, be it in the midgut and hemolymph of Lepidoptera, Hemiptera and Orthoptera, or saliva of Hemiptera and in many cases nucleases are suggested the primary cause of RNAi tolerance (Allen and Walker, 2012; Christiaens et al., 2014; Garbutt et al., 2013; Ghodke et al., 2019; Luo et al., 2017; Mogilicherla et al., 2018; Shukla et al., 2016; Singh et al., 2017; Vatanparast and Kim, 2017; Niels Wynant et al., 2014). Contrarily, dsRNA was relatively stable in midgut and hemolymph of Dictyoptera, e. g. cockroaches (Garbutt et al., 2013; Wang et al., 2016). Additional to nucleases in Lepidoptera, dsRNA was shown to be trapped in endosomes of *Heliothis virescens* and *S. frugiperda* cell lines and *S. frugiperda* tissues, thus blocking further cleavage to siRNAs (Shukla et al., 2016; Yoon et al., 2017).

1.8. Insect pest species relevant to this study

1.8.1 *Tribolium castaneum*

The red flour beetle *Tribolium castaneum* belongs to the Tenebrionidae family of the order Coleoptera. It is known as a serious pest of stored products such as nuts, wheat, rice and other grains and has spread worldwide due to global trading of these goods (Crop Protection Compendium, 2019; Klingler, 2004; Sokoloff, 1977). Larvae of this species (Figure 1.4) pass through a variable number of molts ranging between six to twelve before pupation and the entire life cycle can be completed in less than two months with each female adult contributing to propagation with up to 1000 eggs in total (Institut für Schädlingskunde, 2020). As observed with other pests when frequently treated with insecticides, *T. castaneum* has evolved resistance to a multitude of active ingredients including commonly used pyrethroid insecticides and even phosphine gas (Champ and Dyte, 1977; Dyte and Blackman, 1970; Jagadeesan et al., 2012; Schlipalius et al., 2012; Zettler, 1991; Zhu et al., 2010).

Apart from its importance as a pest species, *T. castaneum* is an established model organism for the study of gene function in insects in general and beetles specifically. Fast reproduction throughout the year in large numbers paired with easy rearing conditions enable its role as a model insect (Sokoloff, 1977). Its genome is well annotated and is continuously updated (Herndon et al., 2020; Tribolium Genome Sequencing Consortium, 2008). Additionally, a wide variety of methods for the study of gene function are established, such as transposon-based genetic transformation (Berghammer et al., 1999; Lorenzen et al., 2003; Trauner et al., 2009), the GAL4-UAS system enabling transgene expression (Schinko et al., 2010), experimental gene silencing by RNA interference (Brown et al., 1999; Bucher et al., 2002) and more recently genome editing by CRISPR (clustered regularly interspaced short palindromic repeats) (Gilles et al., 2015; Rylee et al., 2018).

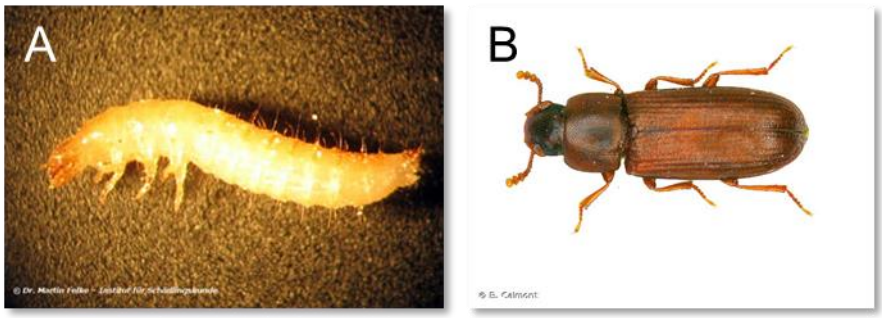

Figure 1.4: Representative pictures of larval **(A)** and adult **(B)** stages of *Tribolium castaneum*. The picture of the larva was obtained from https://schaedlingskunde.de/schaedlinge/steckbriefe/kaefer/rotbrauner-reismehlkaefer-tribolium-castaneum, for the adult from https://inpn.mnhn.fr/espece/cd_nom/244669?lg=en

1.8.2 *Phaedon cochleariae*

Phaedon cochleariae, the mustard leaf beetle, belongs to the Chrysomelidae family of Coleoptera and is spread across the Northern hemisphere (Bogdanov-Katjkov, 1923). It feeds on a multitude of Brassicaceae plant species including crops such as cabbage, mustard, watercress or turnips (Bogdanov-Katjkov, 1923). Metallic blue-green to black female beetles chew small cavities into host plant tissue to deposit typically 1-22 individual yellow eggs per day (Bogdanov-Katjkov, 1923; Hamnett, 1944). Brownish-black larvae feed on foliage and go through three larval stages ending in a prolonged prepupal stage (Figure 1.5) (Bogdanov-Katjkov, 1923; Hamnett, 1944). Eversible defensive glands on adults and the larval dorsal thorax and abdominal segments (Figure 1.5) produce deterrent compounds based on plant metabolites as a defense against natural enemies (Bodemann et al., 2012; Hamnett, 1944; Pasteels and Rowell-Rahier, 1989). Similar to *T. castaneum*, short generation time of 1-2 months (Bogdanov-Katjkov, 1923; Hamnett, 1944) and uncomplicated rearing allow the use of this species as a model organism as well, for example for insecticide discovery by agricultural companies (e. g. Andersch et al., 2001).

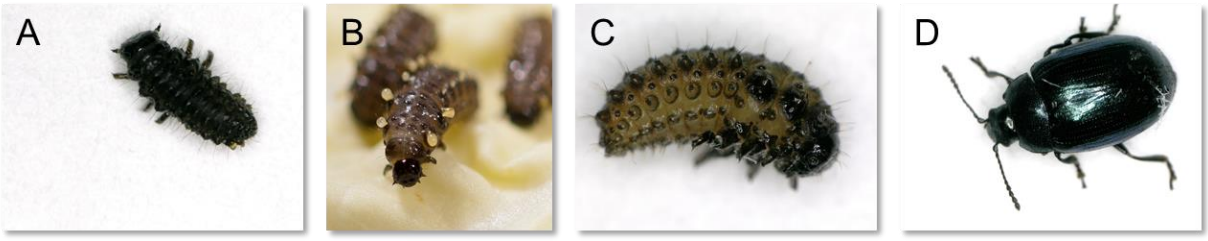

Figure 1.5: Representative pictures of third instar larvae without **(A)** and with **(B)** protruding defensive glands, prepupa **(C)** and adult **(D)** stages of *Phaedon cochleariae*. The image of larvae with visible defensive glands was obtained from https://www.mpg.de/6984000/Terpen-Stoffwechsel-Insekten

1.8.3 *Leptinotarsa decemlineata*

For more than 150 years, the Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) has been known as a notorious pest of potato and other solanaceous plants such as eggplants and tomatoes (Edgerton, 1861; Walsh, 1865; Weber, 2003). Both larval and adult stages (Figure 1.6A-C) are voracious feeders consuming 40 cm^2 in total during larval development and 10 cm^2 leaf area per day during adulthood, respectively, with accelerating rates from first to fourth instar (Ferro et al., 1985; Tamaki and Butt, 1978). Late fourth instar larvae burrow into soil for pupation and after its completion, emerging adults either migrate, start mating after which females attach eggmasses to leaves and stalks, or they enter diapause depending on environmental circumstances (Alyokhin et al., 2013).

From its origins in Mexico, it first spread to the central plains of North America probably by following the expansion of its non-crop host plants (e.g. *Solanum rostratum*) where it adapted to its new host plant potato (*Solanum tuberosum*) (Alyokhin et al., 2013; Izzo et al., 2018). Since then, its distribution expanded from Northern America over Europe and China across the Northern hemisphere (Figure 1.6D) (EPPO/OEPP, 2012; Izzo et al., 2018; Johnson, 1967; Wang et al., 2020). Its establishment as an insect pest prompted the first large-scale use of insecticides (Casagrande, 1987). Over time and across a variety of insecticides, *L. decemlineata* proved highly adaptable and developed resistance often quite quickly (Alyokhin et al., 2008; Casagrande, 1987).

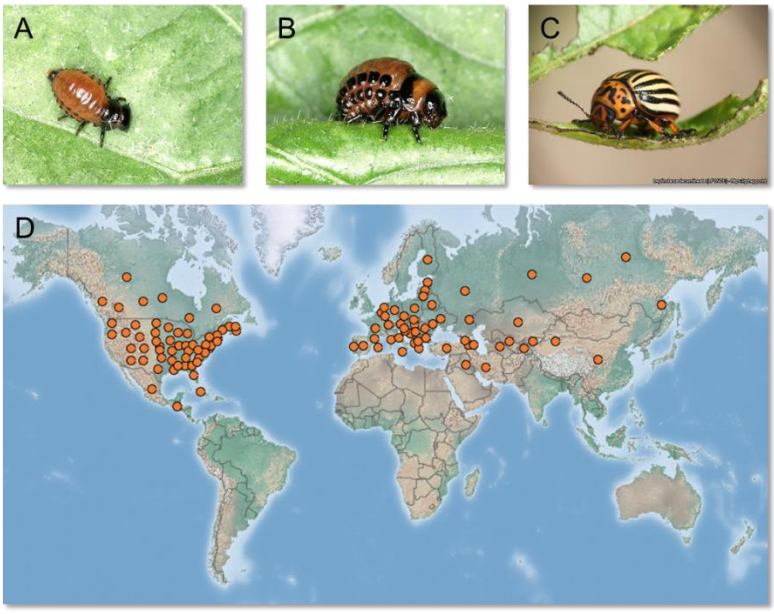

Figure 1.6: Representative pictures of young third **(A)** and fourth instar larvae **(B)** and adult **(C)** stages of *Leptinotarsa decemlineata*. The picture of the adult was obtained from https://gd.eppo.int. The lower panel **(D)** depicts a map of the worldwide distribution of CPB taken from the Invasive Species Compendium (https://www.cabi.org/isc/datasheet/30380).

1.9. Aims and scope

The main objective of the present thesis was to further explore of the feasibility of RNAi as a pest management tool for herbivorous beetle pests.

The first part explores in how far target genes detected in a large-scale screen in *T. castaneum* (Ulrich et al., 2015) could be transferred to other species and how efficient oral RNAi is. To test this, *P. cochleariae* was selected as an easily manageable, leaf-feeding pest insect and was used to test the orthologs of eleven RNAi target genes recently described from *T. castaneum*. Amenability to RNAi and the lethality upon knockdown should first be tested by injection followed by dsRNA feeding experiments. Target gene downregulation for each dsRNA target gene should be assessed as a confirmation for a specific RNAi response along with phenotypical characterizations. This work should on one hand identify the best RNAi target genes for a pest species and on the other hand provide an experimental basis for judging the transferability of RNAi target genes from one species to the other.

The second part of my thesis questions in how far natural variability in the RNAi response could possibly interfere with application in pest control. *L. decemlineata* represents both a serious pest species and is highly susceptible towards oral dsRNA exposure (Alyokhin et al., 2013; San Miguel and Scott, 2016; Zhu et al., 2011). Additionally, it was not implicated in RNAi response variability studies before. Populations of this species collected in various European countries should be fed with dsRNA targeting the conserved *actin* gene and monitored for differences in susceptibility expressed as variations in mortality or developmental retardation. Putative polymorphisms in the dsRNA target sequence as well as differences in RNAi machinery expression should be examined among different populations to develop a better understanding of variability in RNAi sensibility.

2. Manuscript 1: The mustard beetle, *Phaedon cochleariae*, as a screening model for exogenous RNAi-based pest control

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Status:

in preparation

Journal:

will be submitted to “Insect Biochemistry and Molecular Biology”

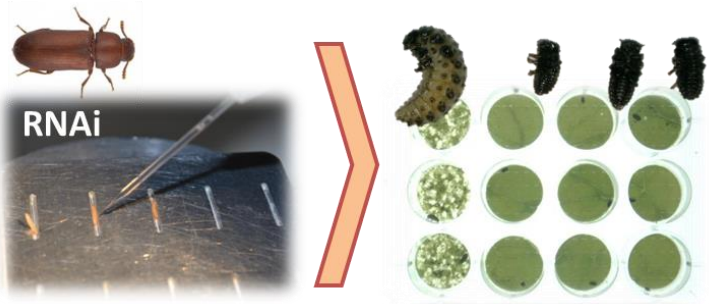
My contributions:

- Performed experiments (apart from injection procedure by Julia Ulrich and transcriptome library preparation of *P. cochleariae* by Bettina Lüke)
- Analyzed experiments (apart from transcriptome assembly and its analysis)
- Writing of the draft manuscript
- Preparation of Figures (apart from Figure 2.4 [partial contribution] and the Graphical Abstract by Ralf Nauen)

Individual author contributions: see page 42

2.1. Abstract

Research aiming for the identification of suitable target genes for RNA interference for the application in insect pest control has received much attention in recent years. In a screen in *Tribolium castaneum*, eleven novel highly lethal target genes were discovered. The implementation of a screening procedure proves difficult for many pest insects, therefore we present *Phaedon cochleariae* as a low-maintenance model beetle for sprayable RNAi. Nine homologs as well as key RNAi machinery genes were identified in the transcriptome of *P. cochleariae*. A feeding biotest was developed and demonstrated functional RNAi upon oral dsRNA application in this species. For later target knockdown analysis via RT-qPCR, three reference genes were verified across developmental stages. Upon oral dsRNA application, five of nine tested dsRNAs elicited strong target gene knock-down resulting in up to 100% mortality in all three tested dsRNA amounts of 3 µg, 1 µg and 0.3 µg per leaf disc equivalent to 95.5 g/ha, 31.8 g/ha and 9.6 g/ha, respectively. These results denote a high transfer rate of the RNAi effect of the target genes from one species to another, reinforcing *P. cochleariae* as a new model insect species for RNAi research.



Graphical abstract

Highlights

- Key RNAi pathway genes are present in the *Phaedon cochleariae* transcriptome
- Functional RNAi response in *P. cochleariae* larvae upon dsRNA feeding
- five of nine *Tribolium castaneum* lethal genes originating from a dsRNA screen were confirmed to be highly lethal in *P. cochleariae*, demonstrating a transfer rate of 56%
- three reference genes were confirmed for the use for RT-qPCR across developmental stages

Keywords

RNA interference, *Phaedon cochleariae*, lethal dsRNA, reference genes, *Tribolium castaneum,* insect control

2.2. Introduction

RNA interference (RNAi) in pest management exploits an ancestral eukaryotic immune defense system designed to combat viruses (Bronkhorst and van Rij, 2014; Wang et al., 2006). In insects, the small-interfering RNA (siRNA) pathway recognizes long double-stranded RNA (dsRNA) which is cleaved by Dicer2 (belonging to the ribonuclease III family) into small, 21-23 bp long RNA duplexes (Bernstein et al., 2001; Zamore et al., 2000). With the help of RNA-binding protein R2D2, the resulting siRNAs are oriented and loaded into a multi-protein complex called RISC (RNA induced silencing complex) (Liu et al., 2003; Tomari et al., 2004). Its major catalytic constituent Argonaute2 (Ago2) cleaves complementary sequences such as targeted messenger RNAs (Hammond et al., 2001; Song et al., 2004) which can turn this mechanism against its own host by reduction in the transcript levels of essential genes. Subsequent lethal effects due to depletion of protein by the application of long dsRNA make RNAi technology a good strategy for pest management.

Unlike in plants (Dalmay et al., 2000; Mourrain et al., 2000; Vaistij et al., 2002) and worms (Sijen et al., 2001; Smardon et al., 2000), siRNAs are not amplified with enzymes like RdRP (RNA-dependent RNA polymerase) to promote the RNAi response in insects (Li et al., 2018). Homolog proteins or other proteins taking over its role were not found in insects so that siRNAs are not propagated; instead, only the initial, exogenous dsRNA is responsible for the RNAi response (Li et al., 2018; Tomoyasu et al., 2008). The intended effect of insecticidal dsRNA is therefore directly linked to the starting amount of dsRNA the insect is exposed to. Additionally, diminished dsRNA levels after exposure to the environment (Dubelman et al., 2014; Fischer et al., 2016; San Miguel and Scott, 2016) as well as low application rates to minimize production costs would further promote the selection of highly lethal dsRNAs that are still effective at low amounts to ensure pest insect control.

With this in mind, early as well as more recent studies tended to rely on target genes with known vital functions and tested them for mortality (eg. Abd Halim et al., 2016; Baum et al., 2007; Hu et al., 2016; Kumar et al., 2013; Whyard et al., 2009). Discovery of novel, less well studied targets is impeded in this strategy. An unbiased, undirected approach would be able to uncover target genes outside the previous scope of research. In a screen initiated in the model insect *Tribolium castaneum* approximately half of the genes encoded in its genome were targeted by individual dsRNAs and the observed phenotypes, including mortality, were deposited in an online database (Dönitz et al., 2015; Schmitt-Engel et al., 2015; Ulrich et al., 2015). From all tested lethal genes from the initial screen, a subset of eleven genes proved to be lethal down to 3ng/µl upon dsRNA injection (Ulrich et al., 2015). These novel highly lethal genes were promptly adopted into research and tested in a variety of insect species, such as *Diabrotica virgifera virgifera* (Knorr et al., 2018), *Brassicogethes aeneus* (Knorr et al., 2018), *Agrilus planipennis* (Rodrigues et al., 2018), *Halyomorpha halys* (Mogilicherla et al., 2018),

Dendroctonus frontalis, *Anoplophora glabripennis* (Dhandapani et al., 2020), *Plagiodera versicolora* (Xu et al., 2019b; Zhang et al., 2019) and *Euschistus heros* (Castellanos et al., 2019). Each study reported at least one of the eleven genes as lethal confirming the proposed hypothesis of the transferability of lethal target genes from model to pest insect species.

Here, we propose a different coleopteran species, the mustard beetle *Phaedon cochleariae* as an intermediate model species to screen for lethal target genes. *P. cochleariae* is an up to 4.5 mm sized member of the Chrysomelidae family infesting a variety of plants and crops of the Brassicaceae family such as cabbage, turnip or its namesake mustard (Bogdanov-Katjkov, 1923). Unlike *T. castaneum*, *D. v. virgifera* or piercing and sucking insects, both larval and adult stages feed on foliage enabling the direct testing of sprayable RNAi without the need to resort to artificial diets. Functional RNAi by dsRNA injection in this species was recently demonstrated as a reverse genetics tool for the characterization of proteins involved in terpene and defensive compound biosynthesis as well as sugar transport (Bodemann et al., 2012; Frick et al., 2013; Stock et al., 2013). Moreover, it is easy and resource-efficient to rear in large quantities all year around unlike i.e. the large Colorado potato beetle *Leptinotarsa decemlineata* or *B. aeneus* with a single generation per year. For these reasons, it was already used for the discovery of insecticidal compounds (Andersch et al., 2001; Funke et al., 2014; Raemaekers et al., 2015).

In this study, we explore the amenability of *P. cochleariae* to oral RNAi in an experimental set-up appropriate for screening. In order to further test the hypothesis that lethal genes can be functionally transferred from a model to another species and the capacity of *P. cochleariae* to fulfill that role, we concentrated on the eleven genes proposed by Ulrich et al. (2015).

2.3. Materials and Methods

2.3.1. Insect rearing and chemicals

Phaedon cochleariae insects were maintained on kale leaves (*Brassica oleracea* subsp*. sabellica*) at 24°C and continuous light during the week and at 16°C and continuous dark over weekends. Experiments were conducted at 24°C and 12 h light/12 h dark conditions using Chinese cabbage (*Brassica oleracea* subsp. *pekinensis*). Unless otherwise specified, chemicals and reagents were acquired from Sigma (Munich, Germany). The detergent used for dsRNA spraying solutions emulsifier W (EW; CAS No. 104376-72-9) was purchased from Lanxess (Leverkusen, Germany). *Coccinella septempuntata* eggs were purchased from Katz Biotech AG and reared on *Myzus persicae* aphids until use for transcriptome generation.

2.3.2. *de novo* transcriptome assembly

40 adult *P. cochleariae* beetles of mixed gender were snap-frozen, ground in a laboratory ball mill (Retsch) and RNA was prepared with the RNeasy Plus Universal Mini Kit (QIAGEN) according to manufacturer`s instructions. RNA quality was assessed on a 2100 Bioanalyzer (Agilent) with an RNA 6000 Nano Kit (Agilent). The library preparation was carried out with a TruSeq® Stranded mRNA LT Kit (Illumina) according to manufacturer`s instructions with 1 µg of total RNA as starting material and quality was confirmed on a 2100 Bioanalyzer (Agilent) with a High Sensitivity DNA Kit (Agilent). Paired-end sequencing was performed on a NextSeq500 using a NextSeq Mid-Output kit v2 (300 cycles) (Illumina). The transcriptome was assembled with Trinity 2014-04-13p1. Key parameters of the transcriptome are listed in Table 2.S1. The same procedure applied for *Coccinella septempunctata* transcriptome assembly, except that RNA of eleven starved adult beetles of mixed gender was extracted individually and later combined into four library preparations, and the resulting sequencing data was united into one transcriptome. Key parameters can be accessed in Table 2.S2.

2.3.3. Identification of orthologous genes and RNAi machinery

The transcriptome assembly of *P. cochleariae* was translated using a TransDecoder v2.0.1 pipeline (Haas et al., 2013). First, longest open reading frames (ORFs) with minimal length of 30 amino acids were extracted using TransDecoder.LongOrfs tool using a universal genetic code. Homology of ORFs to known proteins was taken out by NCBI-BLASTP search v2.3.0+ against SWISSPROT database and PFAM domain prediction using HMMER v3.1b2 (Altschul et al., 1997; Bairoch and Apweiler, 1997; HMMER website). The most likely predicted ORFs were taken out using TransDecoder.Predict and the longest ORF for each transcript was retained. Orthologous genes to *T. castaneum* official gene set 3 (OGS3)

from genome assembly Tcas2 (REF) were predicted using OMA v2.1.1 using default parameters. Pairwise orthologous genes of 11 *T. castaneum* lethal genes found by Ulrich et al. (2015) as well as genes essential for the *T. castaneum* RNAi machinery (Knorr et al., 2018; Tomoyasu et al., 2008) were identified. ORFs were manually curated based on alignments of protein and nucleic acid sequences that were carried out in Geneious® 11 software (Biomatters) with Clustal W 2.1. Similar procedures applied to the *C. septempunctata* transcriptome excluding the search for RNAi machinery genes and manual curation of sequences.

2.3.4. dsRNA preparation

For template generation, RNA of *P. cochleariae* 2nd instar larvae was extracted using RNeasy Mini Kit (QIAGEN) according to manufacturer`s instructions including DNAse digestion. 1 µg total RNA were applied in cDNA synthesis using the SuperScript™ II Reverse Transcriptase Kit (Thermo Fisher Scientific) with oligo-dT primers (Thermo Fisher Scientific) according to manufacturer`s instructions. Gene-specific amplification from 1 µl 1:10 diluted cDNA was carried out following manufacturer`s guidelines using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) in a 50 µl reaction with a final concentration of 0.5 µM forward and reverse primers, respectively (program: 98°C 3 min, 35 x (98°C 1 s, Ta as indicated in Table 2.S3 5 s, 72°C 15s), 72°C 1min, 12°C on hold). Primers for dsRNA targeting *P. cochleariae* sequences were designed to approximately match the dsRNA position on *T. castaneum* target genes (Table 2.S3, Fig. 2.S1). Purification of the PCR product after agarose gel electrophoresis was carried out using the NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel) according to manufacturer`s instructions and 500ng PCR product were used as template for dsRNA production with the MEGAscript™ T7 Transcription Kit (Thermo Fisher Scientific). The dsRNA purification step using LiCl solution was modified by the extension of centrifugation steps to 30 min and the addition of two ethanol wash steps. Nuclease-free water was used to resuspend the dsRNA. *GFP*-based dsRNA was kindly provided by GreenLight Biosciences and purified with LiCl solution as described above. DNA and dsRNA concentrations were measured with a NanoQuant Plate™ on a Plate reader Infinite 200 PRO (Tecan Life Sciences).

2.3.5. dsRNA delivery by injection

Approximately 150 ng target dsRNA was injected into the lateral abdomen of nine ice-sedated 2nd instar larvae of *P. cochleariae* per replicate with a Microinjector FemtoJet® (Eppendorf) with pulled borosilicate glass capillaries (Hilgenberg) and repeated three times. *GFP* dsRNA served as negative control. After a short recovery time, larvae were placed into 9 cm Petri dishes containing leaves on

moistened filter paper. On day one, larvae that died of disruptive injection were replaced by spare injected larvae. For expression analysis, larvae were frozen in liquid nitrogen on the third day. Leaves were exchanged every one or two days and mortality was monitored over 10 days. On day 7, representative pictures of the observed phenotypes were taken at 20x magnification with a Keyence VHS-5000 series digital microscope (Osaka, Japan).

2.3.6. dsRNA oral application

Feeding bioassays were adopted to suit an industrial screening situation (Benting and Nauen, 2004). Application of target dsRNA diluted in 0.1% EW to leaf discs (Ø=2 cm) placed on 0.75% agar in a 12-well plate system was performed by a custom-built spraying device in rates ranging from 0.3 µg, 1 µg to 3 µg dsRNA per leaf disc. Spraying of 0.1% EW alone and 3 µg/leaf disc of ds*GFP* served as surfactant and dsRNA negative controls, respectively. Per plate, always one of the two controls as well as all three concentrations of one of the nine dsRNAs were included. Two young 2nd instar *P. cochleariae* larvae were placed in each well and allowed to feed for three days. Afterwards, one larva was frozen in liquid nitrogen for usage in expression analysis while the other larva was monitored over 10 days in total with untreated leaf discs exchanged on days 3, 5, 6 and 7. Each treatment consisted of 18 larvae; nine for the bioassay and nine for expression analysis. Time lapse videos were recorded with a purpose-built image acquisition device. Representative pictures of larvae of each treatment were taken on the seventh day using a Keyence VHS-5000 series digital microscope (Osaka, Japan) at 20x magnification. Mortality was analyzed and corrected for control mortality according to Abbott (1925) using Excel 2010 (Microsoft) and Prism 5 (GraphPad) software.

2.3.7. quantitative Real-Time PCR (RT-qPCR)

Verification of target gene knock-down was tested by expression analysis by RT-qPCR. Individual snap-frozen larvae were ground in a MM300 laboratory ball mill (Retsch) at 20-30 Hz within 2 x 20 s pulses using two 3 mm stainless steel beads. The samples were vigorously mixed with 0.5 ml TRIzol™ Reagent (Thermo Fisher Scientific) and the aqueous phase was isolated as described in the manufacturer`s manual: After 5 min incubation at RT, 100 µl chloroform were added and the tubes inverted for 15 s. Phases were allowed to separate for 3 min at RT, then by centrifugation at 12000 x g, 15 min, 4°C. RNA from the aqueous phase was purified with the Agencourt RNAdvance Tissue Kit (Beckman-Coulter) according to manufacturer`s instructions including a DNAse I digest on a CyBio® FeliX pipetting platform (Analytik Jena) and quantified with a NanoQuant Plate™ on a Plate reader Infinite 200 PRO (Tecan Life Sciences). Examination of gDNA contamination was carried out using the QIAxcel DNA

Screening Kit (QIAGEN) after a PCR on RNA (20 µl reaction with 2x JumpStart™ Taq Ready Mix (Thermo Fisher Scientific), 0.3 µM final concentration *rps6* forward and reverse primers (Table 2.S3), 1 µl template; program: 98°C 3 min, (98°C 30 s, 60°C 30 s, 72°C 30 s) x 40, 72°C 3 min).

After confirming RNA quality using a QIAxcel RNA QC Kit v2.0 (QIAGEN), 250 ng total RNA were used in iScript™ cDNA Synthesis Kit (Bio-Rad). Gene-specific primers were designed with Geneious® 10 software (Biomatters) without overlap to the respective dsRNAs (Table 2.S3) except for *Pcrpt3* where partial overlap was accepted due to very low GC-content of the gene and thus difficulties in primer design. Primer sequences for *Pcrps3*, *Pcrps6* and *PcRP-L8* were adopted from Stock et al. (2013) and Strauss et al. (2013) (Table 2.S3). Their stability as reference genes was further tested across developmental stages. For this, 10 insects per stage and replicate were pooled and cDNA synthesis was carried out for four replicates with 500 ng total RNA.

RT-qPCR was run in triplicates in 10 µl reactions with 1 µl cDNA (1:10 diluted for target knockdown validation, 1:20 diluted for reference gene stability test to achieve similar starting material in reactions) as template, gene-specific primers at a final concentration of 0.3 µM and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) according to manufacturer`s instructions in a CFX384™ cycler (Bio-Rad). Primer efficiencies were tested with five concentrations in a 1:5 serial dilution. The highest concentration was a 1:20 dilution from cDNA with 500 ng RNA starting material. Expression analysis was carried out in qBase+ 3.2 software (Biogazelle) (Hellemans et al., 2007) and reference genes were evaluated using the integrated geNorm function (Vandesompele et al., 2002). Target gene expression was normalized to the three reference genes *Pcrps3*, *Pcrps6* and *PcRP-L8* and shown relative to ds*GFP* treatments.

2.4. Results

2.4.1. Identification of orthologs in *Phaedon cochleariae*

A large-scale RNAi screen in *Tribolium castaneum* revealed new targets for insect pest management displaying high mortality upon injection despite using low concentrations of dsRNA (Ulrich et al., 2015) which were adopted for examination in *P. cochleariae*.

For identification of orthologous sequences in the screening model Coleopteran, *Phaedon cochleariae*, the transcriptome was searched for the 11 lethal sequences from *T. castaneum*. *In silico* analysis revealed one ortholog for nine sequences (Table 2.1, Figure 2.S1). Since only the best hits were considered for ortholog identification, the presence of alternative splicing variants or multiple copies can not be excluded. Only truncated transcripts of orthologs to *Tcgawky* and *Tcinr-a* were detected in the *P. cochleariae* transcriptome. However, this may be attributable to low expression or incomplete sequencing despite the BUSCO score of 82% complete BUSCOs from the endopterygota set (Table 2.S1) and lack of a reference genome for comparison.

Table 2.1: Identification of *Phaedon cochleariae* genes with indicated nucleotide and protein identity based on lethal orthologous genes recently described for *Tribolium castaneum* (Ulrich et al., 2015).

No.[1]	Gene name (*Dm*)	Symbol	*Tc* gene ID.	*Pc* ortholog gene ID.	Nucleotide identity *Tc*	Protein identity *Tc*
L10	Cactus	*cact*	TC002003	MT263930	54.6	47.2
L11	Signal recognition particle protein 54k	*srp54k*	TC002574	MT263931	79.5	94.9
L44	Ras opposite	*rop*	TC011120	MT263932	73.3	88.3
L47	*alpha* soluble NSF attachment protein	*α-SNAP*	TC013571	MT263933	76.7	90.4
L50	Shibire	*shi*	TC011058	MT263934	75.4	92.7
L55	Protein phosphatase 1 *alpha* at 96a	*PP-α*	TC015321	MT263935	79.9	99.1
L67	Inverse regulator a	*inr-a*	TC008263	N/A		
L76	Heat shock 70-kDa protein cognate 3	*hsc70-3*	TC004425	MT263936	64.0	65.3
L80	Regulatory particle non-ATPase 7	*rpn7*	TC006375	MT263937	74.4	90.2
L82	Gawky	*gw*	TC006679	N/A		
L84	Regulatory particle triple-A ATPase 3	*rpt3*	TC007999	MT263938	76.7	96.8

[1]Gene identifier number according to Ulrich et al. (2015); Abbreviations: Dm: Drosophila melanogaster; Tc: Tribolium castaneum; Pc: Phaedon cochleariae

2.4.2. RNAi machinery presence

For dsRNA to act as a pest control agent, a functional RNAi machinery is required in the targeted organism. Recent publications showing operative RNAi in *P. cochleariae* via dsRNA injection (Bodemann et al., 2012; Frick et al., 2013; Stock et al., 2013) suggest the presence of the necessary proteins, but their sequences were not yet presented.

An *in silico* search of the transcriptome with *T. castaneum* sequences as template revealed copies of core siRNA machinery proteins such as Dicer2, R2D2 and Argonaute2 in *P. cochleariae* (Table 2.2, Figure 2.S2). One ortholog for Systemic RNA Interference Deficient (SID)-like proteins was identified as well (silC). Additionally, proteins associated with clathrin-mediated endocytic dsRNA uptake, clathrin heavy chain (CHC) and vacuolar H^+-ATPase subunit 16 (Vha16), were found. All in all, the presence of the siRNA core machinery and proteins probably involved in dsRNA uptake could be confirmed for *P. cochleariae*, but statements on copy numbers require corroboration with genomic data.

Table 2.2: Identity of selected RNAi pathway proteins of *Phaedon cochleariae* (*Pc*) compared to *Tribolium castaneum* (*Tc*) orthologs.

Gene name (*Dm*)	Symbol	*Tc* protein ID.	*Pc* protein ID	Protein identity to *Tc*
Dicer2	*Dcr2*	EEZ99277.1	MT263939	47.3
Argonaute 2a	*Ago2*	EFA11590.1	MT263940	49.6
R2D2	*R2D2*	EFA05903.1	MT263941	42.2
Sid-like C	*silC (SID)*	EFA05674.2	MT263942	61.7
Clathrin heavy chain	*CHC*	EFA04947.1	MT263943	95.6
Vacuolar H^+ ATPase 16	*Vha16*	EEZ97227.1	MT263944	89.9

Dm: Drosophila melanogaster; Tc: Tribolium castaneum; Pc: Phaedon cochleariae

2.4.3. Injection of dsRNA

Knowledge of functional RNAi upon dsRNA injection into *P. cochleariae* denoted this delivery method as a good tool to obtain a first impression on the performance of putatively lethal dsRNAs in *P. cochleariae*.

A single dose equivalent to approximately 150ng per larva was injected for each of the nine dsRNAs. ds*rp54k*, ds*rop*, ds*α-SNAP*, ds*rpn7* and ds*rpt3* achieved 100% mortality and only 7.6% of ds*hsc70-3* treated larvae survived by day 10 (Figure 2.1B-D, G-I). This was accompanied by a rapid decline in survival between days 4-7 (Figure 2.1B-D, G-I) and a reduction in larval growth (Figure 2.3A). Although the effects of ds*cactus*, ds*shibire* and ds*PP-α* started in the same time range, survival was not equally reduced; ds*cactus* treatment lead to 59.3% survival, ds*shibire* to 37% survival and ds*PP-α* dsRNA to 52% survival (Figure 2.1A, E, F). Visual inspection of larval growth showed a reduction to some extent in these three treatments compared to the ds*GFP* control, but not as severe as with for example with ds*rpn7* (Figure 2.3A). In total, six of nine dsRNAs elicited high mortality rates in *P. cochleariae* upon injection.

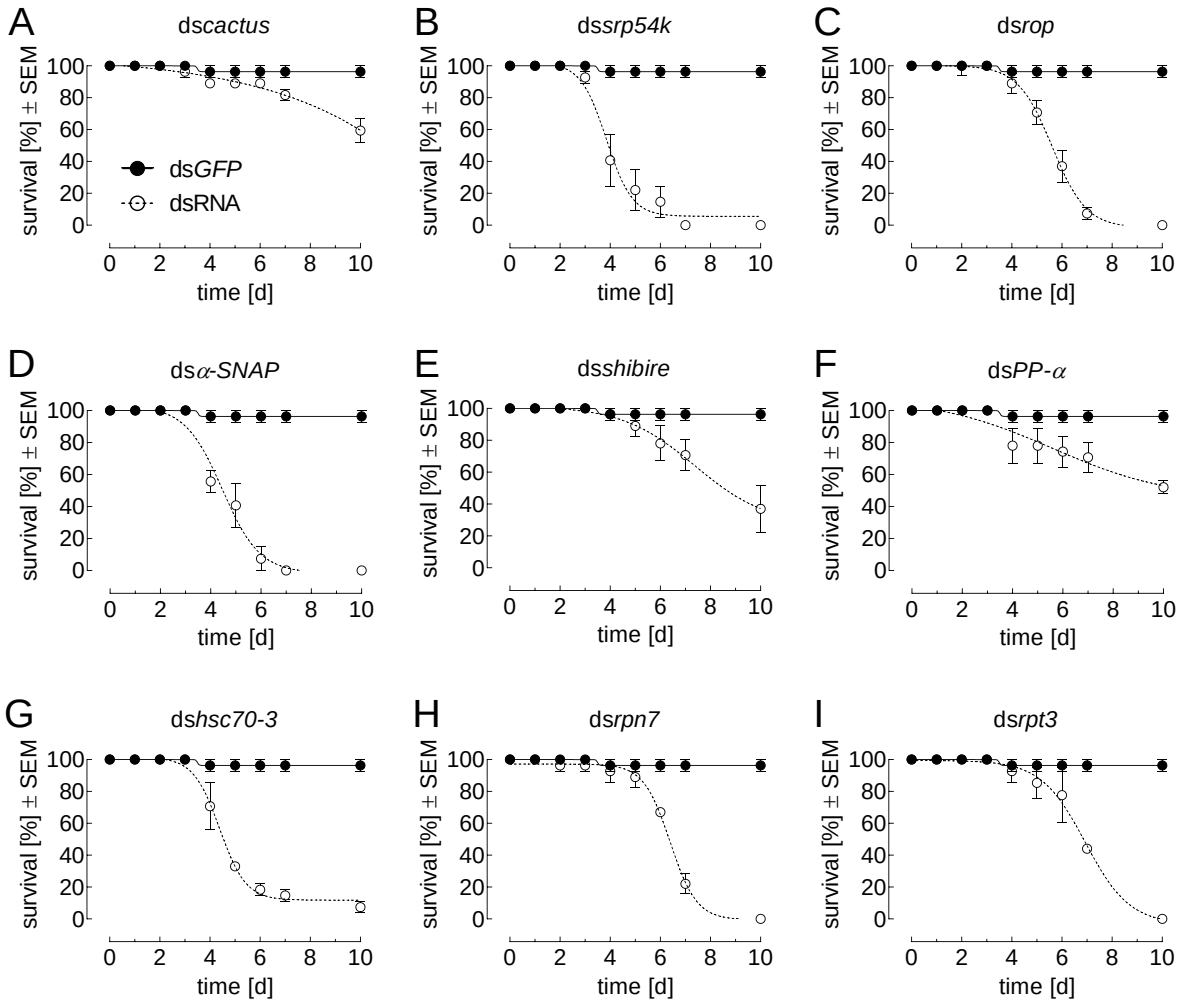


Figure 2.1: Survival [%] at different elapsed time intervals of 2nd instar *P. cochleariae* larvae after injection of dsRNA targeting *cactus* (**A**), *srp54k* (**B**), *rop* (**C**), *α-SNAP* (**D**), *shibire* (**E**), *PP-α* (**F**), *hsc70-3* (**G**), *rpn7* (**H**) and *rpt3* (**I**) at approximately 150 ng/larva. Injection of 150ng *GFP* dsRNA served as negative control. Data are mean values ± SEM (n=3).

2.4.4. Feeding bioassay

The exploration of lethal dsRNAs for industrial purposes requires the oral application of the dsRNA as well as development and integration of a suitable method into existing screening procedures. Three rates of each of the nine putatively lethal dsRNAs were tested on 2nd instar *P. cochleariae* larvae. These dsRNA amounts of 3 µg, 1 µg and 0.3 µg per leaf disc are equal to about 95.5 g/ha, 31.8 g/ha and 9.6 g/ha, respectively. The total amount of dsRNA from the lowest rate in oral application approximately matched the amount of injected dsRNA under the assumption that both larvae fed equally on the treated leaf material. Five of the nine tested dsRNAs targeting *srp54k*, *rop*, *α-SNAP*, *rpn7* and *rpt3* showed highly lethal effects in all three rates after 5 to 6 days (Figure 2.2B,C,D,H,I). Mortality

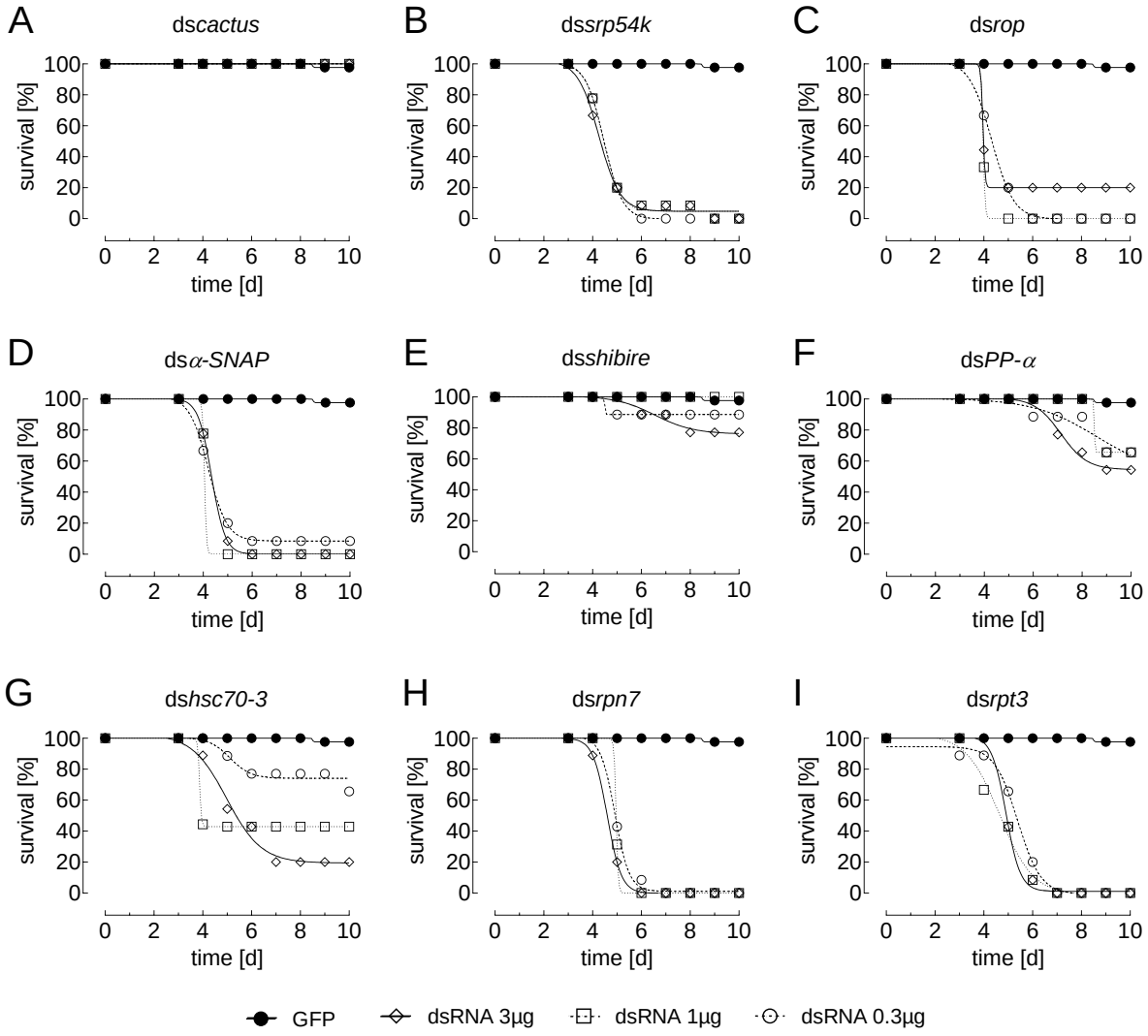


Figure 2.2: Survival [%] at different elapsed time intervals of 2nd instar *P. cochleariae* larvae after foliar application of dsRNA targeting *cactus* (**A**), *srp54k* (**B**), *rop* (**C**), *α-SNAP* (**D**), *shibire* (**E**), *PP-α* (**F**), *hsc70-3* (**G**), *rpn7* (**H**), *rpt3* (**I**) at 3 µg, 1 µg and 0.3 µg per leaf disc. *GFP* dsRNA (3 µg/leaf disc) served as negative control. Survival rates were Abbott corrected using controls feeding on leaf discs treated with emulsifier W (0.1%).

rates of 100% were recorded by days 6 or 7 in these dsRNA treatments, and latest on day 9 for 3 µg and 1 µg ds*srp54k*. Exceptions pose 3 µg ds*rop* and 0.3 µg ds*α-SNAP* in which survival remained at 20% and 8.6% until day 10, respectively. Examination of the phenotype of larvae treated with highly lethal dsRNAs revealed size reduction of the larvae compared to the negative controls (Figure 2.3B) correlating with relatively quick deaths (Figure 2.2) and preceding feeding cessation (Figures 2.4, 2.S3). For d*shsc70-3*, a dose dependent response could be observed. The highest rate of 3 µg/leaf disc achieved 80% mortality which diminished to 34.3% in the lowest rate (Figure 2.2G). This is also

reflected in the phenotype, in which lower rates approximate phenotypes of the control treatments (Figure 2.3B). For ds*shibire* and ds*PP-α*, mortality did not exceed 50% (maximum: 45.7% for 3 µg/leaf disc ds*PP-α*) and ds*cactus* had no effect on survival at all (Figure 2.2A,E,F). Similarly, no effect on the phenotype could be observed in these treatments except for ds*PP-α* in the highest rate where a growth delay could be detected (Figure 2.3B). In summary, functional RNAi upon oral dsRNA exposure was demonstrated for *P. cochleariae* with five of the six dsRNAs that showed high efficacy upon injection.

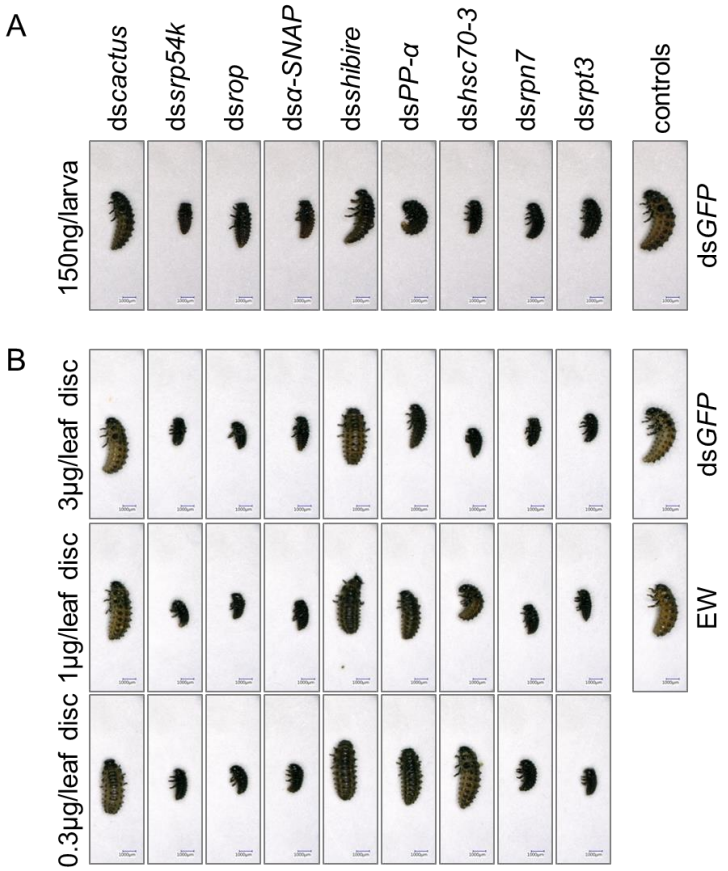


Figure 2.3: Phenotypic variation of *P. cochleariae* larvae seven days after injection of dsRNA in 2nd instar larvae at approximately 150 ng/larva (**A**) and foliar application of dsRNA at 3 µg, 1 µg, and 0.3 µg per leaf disc (**B**) targeting *cactus*, *srp54k*, *rop*, *α-SNAP*, *shibire*, *PP-α*, *hsc70-3*, *rpn7*, *rpt3* sequences. *GFP* dsRNA (150 ng/larva and 3 µg/leaf disc in injection and feeding assays, respectively) and emulsifier W (0.1%) served as negative controls. Scale bars in images represent 1mm.

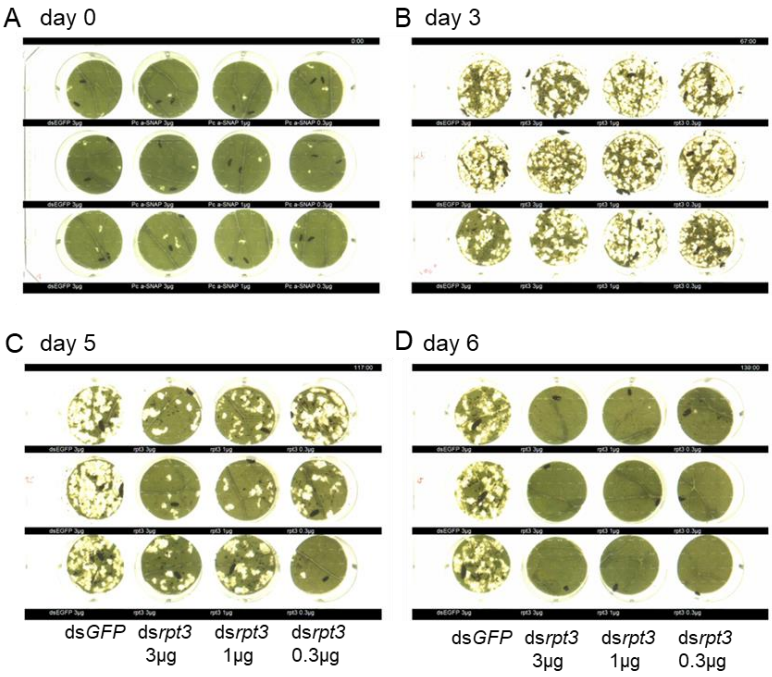
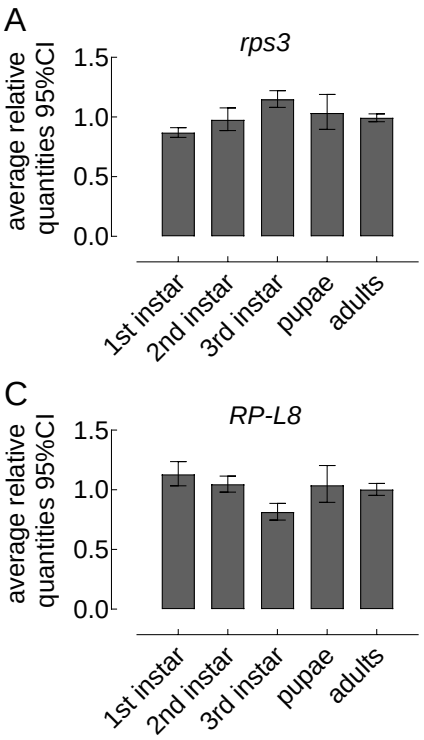


Figure 2.4: Still pictures taken from time lapse videos recorded from 2nd instar *P. cochleariae* larvae in 12-well plates feeding on leaf discs treated with ds*GFP* (3 µg) and ds*rpt3* at rates of 3 µg, 1 µg and 0.3 µg. Treated leaf discs were replaced with untreated leaf discs on days 3, 5 and 6. The full time-lapse videos can be accessed in the supplementary material. On day 3, one of the two larvae per well was removed for gene expression analysis by RT-qPCR. The pictures reflect the leaf disc condition at the start of the experiment (**A**) and before exchange of leaf discs on day 3 (**B**), day 5 (**C**) and day 6 (**D**).

2.4.5. Reference gene stability across developmental stages

rps3, *rps6* and *RP-L8* were used as reference genes for RT-qPCR in recent publications and while *rps3* and *rps6* were implicated to be stable in RNAseq analysis (Stock et al., 2013), changes of reference gene stability throughout development was not studied. Examination of the expression pattern in all three larval instars, pupa and adult stages revealed relatively stable expression across the developmental stages (Figure 2.5A-C). Analysis with geNorm to assess the group variance of these reference genes revealed M-values below the maximal recommended M-value of 0.5 (Figure 2.5D). The pairwise variation V which in this case had to be operated in minimal constellation of V2/3 obtained a value of 0.09, well below the maximal value of 0.15 (Figure 2.5D). These results confirm *rps3*, *rps6* and *RP-L8* as suitable reference genes for *P. cochleariae* and suggest that the use of two of the genes would be sufficient for RT-qPCR normalization.



D

geNorm analysis	
M-value *rps3*	0.225
M-value *rps6*	0.205
M-value *RP-L8*	0.243
pair-wise variation V	0.090

Figure 2.5: Reference gene stability across developmental stages of *P. cochleariae*. Average relative quantities are shown with 95% confidence intervals (CI) for *rps3* (**A**), *rps6* (**B**) and *RP-L8* (**C**) transcripts and calculated for different larval instars, pupae and adults (n=4). Group-wise reference gene stability was analyzed using geNorm qBase Plus (**D**) showing the M-values and pair-wise variation V. For further details refer to Material and Methods.

2.4.6. Confirmation of target gene knock-down

RNAi is based on the assumption that the targeted mRNA is downregulated. Examination of expression levels was performed by RT-qPCR. Three days after injection of seven out of nine dsRNAs, a decrease in transcript level could be observed (Figure 2.6B, D-I). *rpt3* showed the greatest reduction of 94.5%, followed by *srp54k* with 94.1%, *rpn7* with 93.9%, *α-SNAP* with 84.9%, *shibire* with 81.3%, *PP-α* with 80.5% and *hsc70-3* with 75.9%. For *cactus* and *rop* dsRNA treatments, no significant difference between control and target gene treatments could be observed (Figure 2.6A, C). Upon oral application of *cactus* dsRNA (Figure 2.7A), no significant target gene reduction was detected either whereas *rop* was suppressed by 84%, 82.9% and 73.1% in the 3 µg/leaf disc, 1 µg/leaf disc and 0.3 µg/leaf disc ds*rop* treatments, respectively (Figure 2.7C). In general, no significant difference could be found between the two negative controls ds*GFP* and emulsifier W. Significant differences among the three rates of oral dsRNA treatments were only observed with ds*PP-α* (Figure 2.7F). In the case of *shibire* dsRNA exposure, dose dependent target gene suppression could be observed with 3 µg/leaf disc accounting

for 64.4% transcript reduction, 1 µg/leaf disc for 52.4% and 0.3 µg/leaf disc for 35.6% (Figure 2.7E). Feeding of the remaining five dsRNAs resulted in a decrease in target gene expression in all three dsRNA rates compared to both control treatments (Figure 2.7B, D, G-I). The strongest target gene suppression could be observed for *rpt3* with 96.1%, 95.4% and 94.3% from highest to lowest rate, followed by *rpn7* (95.2%, 93.3%, 91.5%), *srp54k* (92.6%, 94.4%, 92.9%), *PP-α* (88.9%, 82.2%, 79.1%), *α-SNAP* (87.7%, 88.5%, 88.2%), and *hsc70-3* (74.5%, 72.3%, 75.0%). Specific target gene downregulation due to dsRNA treatment could therefore be confirmed for eight of nine genes.

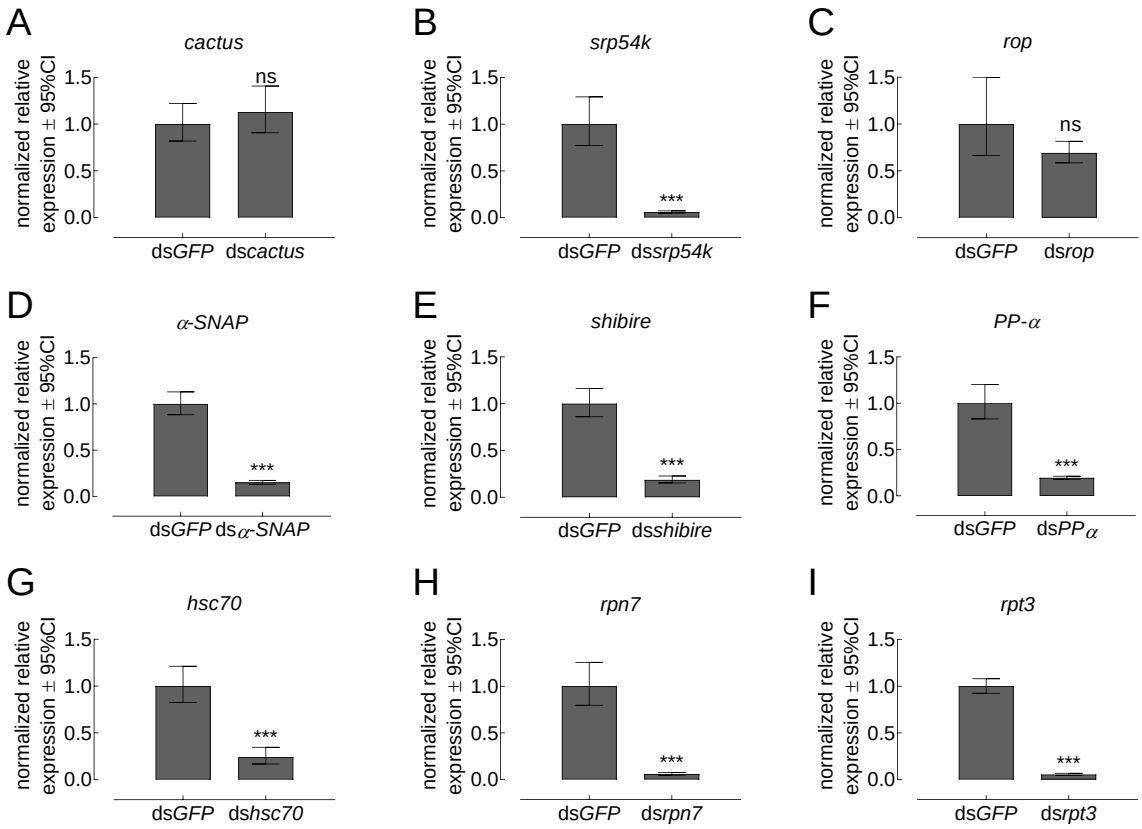


Figure 2.6: Target gene expression in *P. cochleariae* larvae three days after injection of 150ng dsRNA targeting *cactus* (**A**), *srp54k* (**B**), *rop* (**C**), *α-SNAP* (**D**), *shibire* (**E**), *PP-α* (**F**), *hsc70-3* (**G**), *rpn7* (**H**), *rpt3* (**I**). The expression level was normalized to reference genes *rps3*, *rps6* and *RP-L8* and relative to ds*GFP* treatment. Expression levels are displayed as ± 95 % confidence intervals (CI) (n=9-12). Two-tailed unpaired t-tests were carried out to test for significant differences between treatments (*** $p<0.001$, ns $p>0.05$).

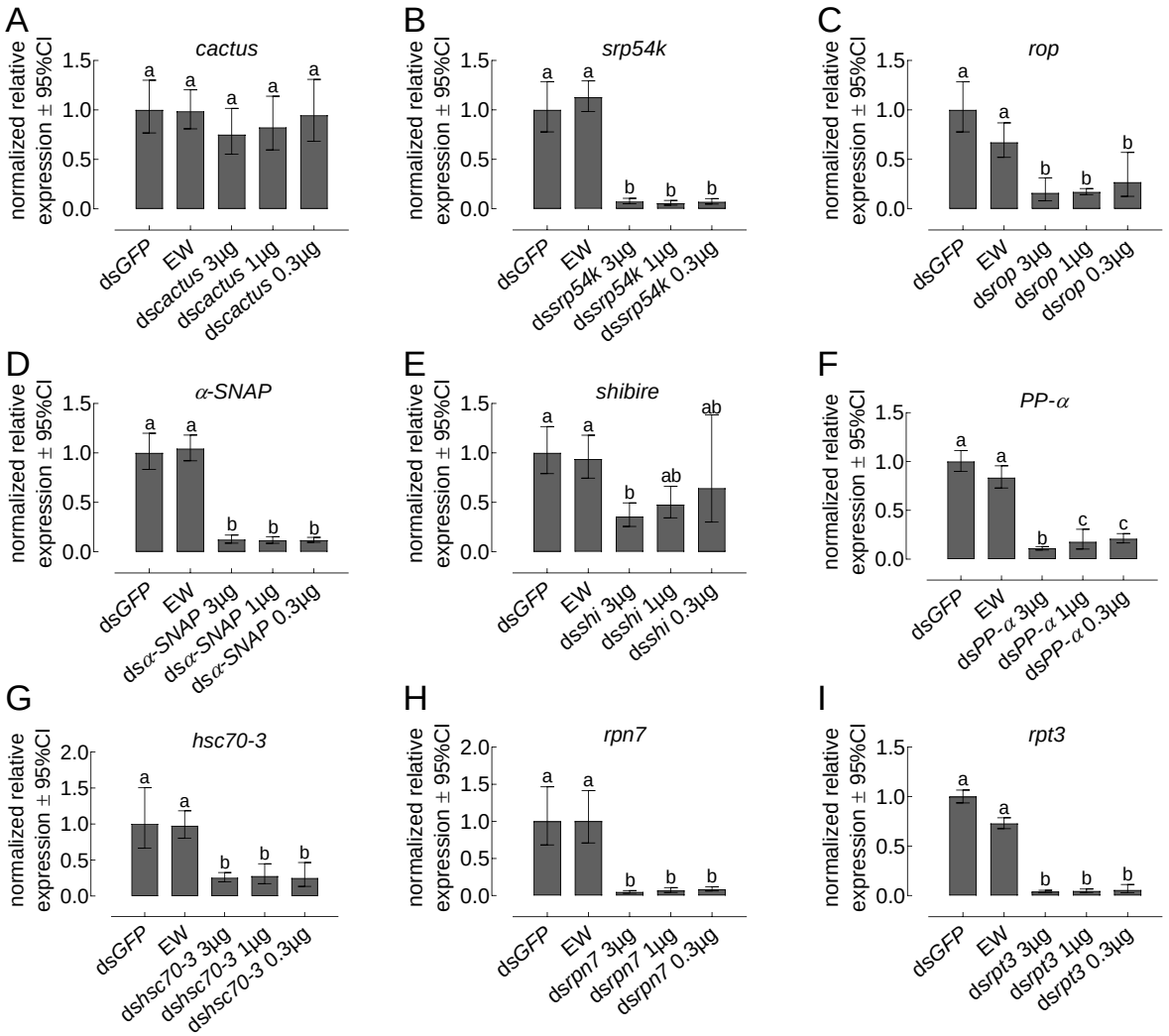


Figure 2.7: Target gene expression in *P. cochleariae* larvae three days after foliar exposure to dsRNA targeting *cactus* (**A**), *srp54k* (B), *rop* (**C**), *α-SNAP* (**D**), *shibire* (**E**), *PP-α* (**F**), *hsc70-3* (**G**), *rpn7* (**H**), *rpt3* (**I**). The expression level was normalized to *rps3*, *rps6* and *RP-L8* reference genes and relative to ds*GFP* treatment. Emulsifier W (0.1%) treatments served as non-dsRNA controls. Expression levels are shown ± confidence intervals (CI) (n=5-9). One-way ANOVA followed by Tukey post-hoc test was carried out ($p<0.05$). Different letters indicate significant differences between treatments.

2.4.7 Comparison of dsRNA sequences to beneficial insects

Apart from the efficiency of the dsRNA on the pest insect, possible consequences for non-target organisms such as beneficial insects need to be kept in mind in a pest control scenario. *In silico* analysis of target sequences offers a first hint on possible negative effects. Comparison of the nine target genes of *P. cochleariae* to honey bee *Apis mellifera* and lady beetle *Coccinella septempunctata* homologous nucleic acid sequences revealed different levels of conservation from 46.6% (*cactus*) to 76.5% (*rpt3*) in

A. mellifera and 51.8% (*cactus*) to 81.8% (*srp54k*) for *C. septempunctata* (Table 2.3, Figures 2.S4+2.S5). The identity within the dsRNA region rarely differed much from the overall identity for either species (Table 2.3). Stretches of at least 21bp identity signifying the possible generation of specific siRNAs in the beneficial species from the dsRNA were found for two of the nine genes namely *srp54k* and *rpn7* in *A. mellifera* and *shi* and *rpn7* in *C. septempunctata* (Table 2.3). However, each time a single and not multiple regions were responsible for putative siRNA generation (Figures 2.S4+2.S5). The longest consecutive dsRNA stretch was found in *C. septempunctata* for *shi* with 29bp (Table 2.3, Figure 2.S5).

Table 2.3: Nucleotide identity [%] of homologous sequences from the beneficial insects *Apis mellifera* and *Coccinella septempunctata* compared to *Phaedon cochleariae* (Pc) coding and dsRNA sequences. The number of possible 21mers in each beneficial is indicated together with the longest found stretch of overlap for each gene.

	Apis mellifera					*Coccinella septempunctata*				
	Accession no.	Overall identity to Pc [%]	identity to Pc dsRNA region [%]	longest overlap in dsRNA region [bp]	no. of independent 21mer sites	Accession no.*	Overall identity to Pc [%]	Identity to Pc dsRNA region [%]	longest overlap in dsRNA region [bp]	no. of independent 21mer sites
cactus	NM_001163712.1	46.6	47.2	8	0	c21647_g1_i1	51.8	52	13	0
srp54k	XM_624494.5	74.4	80.1	26	1	c23499_g1_i1	81.8	81.9	15	0
rop	XM_016911186.2	68.2	68.1	11	0	c25247_g1_i1	73.5	74.1	19	0
α-SNAP	XM_006566736.3	74.6	79.2	17	0	c16907_g1_i1	75.8	79.8	20	0
shibire	XM_006568411.3	67.9	73.6	20	0	c26107_g1_i2	68.7	75.9	29	1
PP-α	XM_392943.7	71.5	72.6	14	0	c21247_g1_i1	77.9	77.7	12	0
hsc70-3	NM_001160052.1	57.7	54.8	8	0	c26790_g1_i1	66.6	67.1	15	0
rpn7	XM_006568751.3	68.3	72.1	23	1	c21317_g1_i1	73.6	74.3	25	1
rpt3	XM_003251401.4	76.5	76.9	20	0	c24553_g1_i1	79.6	78.8	14	0

*transcriptome identifier. Sequences are submitted to NCBI, but official Accession numbers are not assigned yet. Pc: *Phaedon cochleariae*

2.5. Discussion

In view of development of resistances to conventional insecticides in many pest insect species, RNAi is debated as a new strategy for integrated pest management. However, the implementation of RNAi still faces several challenges in various stages along the development pipeline, one of which poses the selection of suitable target sequences. As a result of a large-scale RNAi screen in *Tribolium castaneum*, eleven new highly lethal sequences were identified (Ulrich et al., 2015) and recently tested in several insect pest species (Castellanos et al., 2019; Dhandapani et al., 2020; Knorr et al., 2018; Kyre et al., 2019; Mogilicherla et al., 2018; Rodrigues et al., 2018; Xu et al., 2019b; Zhang et al., 2019). Here, the utility of *Phaedon cochleariae* as a screening model pest insect for oral RNAi was explored using the same gene set.

The presence of the RNAi machinery or, more precisely, the siRNA pathway genes, is indispensable with regards to functional RNAi. Conserved sequences of such proteins were not reported for *P. cochleariae* prior to this study. The copy number of RNAi machinery genes however remains unclear. In *T. castaneum* (Tomoyasu et al., 2008) and other Coleopterans like *B. aeneus* (Knorr et al., 2018) and *L. decemlineata* (Schoville et al., 2018; Yoon et al., 2016), two Ago2 paralogs are reported. In contrast, only one ortholog was found in the genome of *Anoplophora glabripennis* (Rodrigues et al., 2017a) while contradictory reports exist for *D. v. virgifera* (Knorr et al., 2018; Miyata et al., 2014), demonstrating variable copy numbers between insect species. Sequencing of the genome for comparison to the transcriptome could help resolve this question for *P. cochleariae*. Molecular characterization to confirm that the identified candidates correspond functionally to the siRNA pathway genes would require a dedicated study in the future, for example in an RNAi on RNAi approach (Yoon et al., 2016).

Systemic and environmental RNAi are prerequisites for the use of dsRNA in pest management. Therefore, SID-like proteins, CHC and Vha16 were included in the *in silico* analysis for their possible involvement in dsRNA uptake (Aronstein et al., 2006; Cappelle et al., 2016; Miyata et al., 2014; Yoon et al., 2016). Although no direct orthologs for the cellular dsRNA uptake and distribution protein SID1 from *C. elegans* (W. Li et al., 2015; Winston et al., 2002) were identified in insects, several homologous proteins termed SID-like proteins were discovered. While orthologs to *Tc*silA and *Tc*silB were missing from the *P. cochleariae* transcriptome, an ortholog to *Tc*silC was identified (Tomoyasu et al., 2008). Contrasting evidence on the importance of SID-like proteins in insect RNAi and suggestion of indirect participation in dsRNA uptake due to membrane cholesterol regulation were reported (Aronstein et al., 2006; Cappelle et al., 2016; Miyata et al., 2014; Tomoyasu et al., 2008; N. Wynant et al., 2014). Involvement of clathrin-dependent endocytosis at times exceeding the importance of SID-like proteins has been demonstrated for example for *D. melanogaster* (Saleh et al., 2006; Ulvila et al., 2006)

L. decemlineata (Cappelle et al., 2016) and *D. v. virgifera* (Pinheiro et al., 2018). Orthologs for both CHC and Vha16 were found in *P. cochleariae* enabling further research of their function in RNAi.

Nine of the 11 highly lethal target genes from *T. castaneum* were identified in the *P. cochleariae* transcriptome and tested for performance upon dsRNA injection and feeding. Upon injection, six of the nine dsRNA displayed high mortalities, namely ds*srp54k*, ds*rop*, ds*α-SNAP*, ds*hsc70-3*, ds*rpn7* and ds*rpt3* (Figure 2.1B-D, G-I). Five out of these dsRNAs (missing ds*hsc70-3*) also showed high mortalities in three rates in a feeding situation as well (Figure 2.2B-D, H, I), which coincided with the specific downregulation of the target genes (Figure 2.7B-D, H, I). Therefore, specific dsRNA effects were passed on successfully from injection to feeding treatments, additionally demonstrating functional oral RNAi in *P. cochleariae*. The good transfer rate of highly lethal target genes of 67% in injection and 56% in oral dsRNA treatments indicates that the target gene set presented by Ulrich et al. (2015) is indeed a good source for dsRNA targets in insect pest management.

Higher mortality rates are typically expected upon dsRNA injection compared to oral application. By implication, dsRNA feeding represents a more stringent screening procedure. In a direct comparison between delivery strategies, the lowest oral dsRNA rate was adjusted to correspond to the injection dose in total amounts of RNA. In practice however, the fed larvae most likely received a lower dose when taking incomplete consumption of the treated leaf disc (Figures 2.4B, 2.S3), increased time for uptake of the same dsRNA amount and possible degradation of the dsRNA on the leaf disc and in the insect midgut into account. Therefore, it is not surprising to find higher mortalities upon injection, which circumvents these impediments (Figures 2.1+2.2). Still, the greatest spread in mortality rates between delivery strategies was observed in targets that were already not highly effective upon injection, such as *shibire* or *PP-α* while the highly lethal genes like *rpn7* or *rpt3* retained their efficacy. Therefore, dsRNAs showing even slight deficits in mortality rates upon injection as with *hsc70-3* are not recommended for testing in future oral dsRNA application studies in *P. cochleariae*.

Despite the possible advantage of direct dsRNA delivery that injection offers, it is not applicable for RNAi in pest management. Additionally, the dsRNA effects on survival did not profit from earlier onset compared to feeding. This suggests that the initiation of the RNAi response as well as target protein depletion are the limiting factors for RNAi speed of action in *P. cochleariae* rather than uptake from ingested material. In *L. decemlineata*, dsRNA was detected in hemolymph within 10h after oral dsRNA exposure (Shukla et al., 2016), while the induction of RNAi machinery genes like Dcr2a+b and Ago2a+b within 6h implies even faster distribution of the dsRNA within the insect body (Guo et al., 2015). The implied fast uptake of ingested dsRNA may explain the similar onset of mortality between delivery strategies.

Nonetheless, the dynamics between delivery strategy speed of action may be more obvious on transcript level since it disregards protein quantity. For example, *rop* was strongly downregulated upon oral dsRNA application but not upon injection (Figures 2.6C+2.7C). However, the knockdown of *rop* upon dsRNA feeding implies that the mortality observed in injected larvae were specific and not because of off-target effects. A similar situation was described in *Acyrthosiphon pisum*, although knockdown was observed upon dsRNA injection, but not upon feeding while aphids were affected in both treatments (Cao et al., 2018). While lack of target gene reduction in *A. pisum* was attributed to general activity of extracellular nucleases, ds*rop* itself may have a faster turnover compared to other dsRNAs. Different temporal profiles of dsRNA-mediated target gene knockdown were described in *D. v. virgifera* (Wu et al., 2018b). Together with the fact that dsRNA is not amplified in insects (Li et al., 2018), ds*rop* may be depleted more quickly while still sufficing to disturb protein homeostasis enough to cause insect death upon injection. In a feeding situation, the insect is continuously exposed to dsRNA for three days – even though dsRNA is not replenished in that time - probably keeping transcript abundance of *rop* at low levels. For more detailed temporal resolution to test this hypothesis, a second, non-overlapping dsRNA could be tested while expression levels of *rop* should be monitored in short time intervals.

Two more examples showing dynamics in the RNAi response were found. Upon feeding of ds*hsc70-3*, transcript levels were reduced by more than 70% in all three doses yet a dose-dependent response in mortality was observed (Figures 2.2G+2.7G). Contrarily, target gene expression was dose-dependently suppressed upon oral ds*shibire* exposure which was not reflected in survival rates (Figures 2.2E+2.7E). As already mentioned, dsRNAs may show differences in transient target gene downregulation and recovery time (Wu et al., 2018b). Together, these three examples demonstrate that the dynamics of environmental RNAi is not yet fully understood and requires more research if long-lasting dsRNA effects are desired for insect pest control.

In the case of ds*PP-α*, functional redundancy as well as target protein stability and half-life exceeding the duration of the dsRNA effect may be reasons for high survival rates despite strong target gene repression. Testing of this hypothesis would require the generation of specific antibodies which are not available yet.

The only dsRNA that failed to reduce target gene expression was ds*cactus* (Figures 2.6A+2.7A) which suggests that the mortality observed upon dsRNA injection may be a result of off-target effects (Figure 2.1A). Alternatively, target gene downregulation may have occurred prior to sampling, with high protein stability or functional redundancy mitigating the dsRNA effect. Splicing variants of *cactus* exist for example in *D. melanogaster* and are expressed in its larval stages (Geisler et al., 1992; Kidd,

1992). Since *cactus* dsRNA was produced on larval material implying sufficient expression of the targeted isoform, possible splicing variants of *cactus* in *P. cochleariae* should only have attenuated target gene suppression, not completely abolished it. Testing dsRNA based on a different stretch on *cactus* mRNA may to help resolve the difficulties with target gene knockdown. However, cactus was also the least conserved protein (Table 2.1) which may indicate that it is not as essential in *P. cochleariae* as in *T. castaneum* or that its function is supported by other structurally, but not necessarily sequence-related proteins. Additionally, poor performance in other insect species such as *Euschistus heros*, *Agrilus planipennis* and *Plagiodera versicolora* together with the results from *P. cochleariae* suggest that *cactus* is not a good target gene for pest control in general (Castellanos et al., 2019; Rodrigues et al., 2018; Zhang et al., 2019).

All analysis of transcript levels by RT-qPCR, as presented above, relies on stable reference genes. For *P. cochleariae*, three reference genes were used in recent publications (Stock et al., 2013; Strauss et al., 2013). Here, they were validated across developmental stages facilitating future studies in other stages of *P. cochleariae*, especially in terms of RNAi which often requires tests for specific target gene knockdown. For this study, it confirmed that the reference genes were stable between 2nd and 3rd instars which correspond to the mixed developmental stages of larvae when they were collected for transcript analysis (personal observation). Additionally, two reference genes were enough for normalization keeping RT-qPCR experiments simple. This is another point in favor of *P. cochleariae* as a model insect for oral RNAi.

For RNAi to count as an attractive insect control strategy, limited damage on crops after dsRNA treatments of paramount importance. Until RNAi-mediated mortality is induced, insect pests can potentially continue to feed on the crop which may lead to yield losses. Therefore, not only high mortality rates but also feeding cessation is an important indicator for the efficacy of individual dsRNAs. The presented experimental set-up for oral RNAi in *P. cochleariae* together with time-lapse videos allows for individual assessment of leaf consumption of larvae. Feeding on leaf material stopped three (ds*α-SNAP*) to four (ds*rpt3*) days after experiment start (Figure 2.S3) while high mortality rates were only observed two days later (Figure 2.2D,I). In the control treatment, feeding was diminished only after the sixth day (Figure 2.S3) which correlated with the onset of the naturally inactive prepupal stage (Figure 2.3B). Feeding cessation of *P. versicolora* larvae on willow leaves was observed after four days of dsRNA exposure (Zhang et al., 2019). Consumption of potato leaves was already significantly decreased within 24h exposure of *Henosepilachna vigintioctopunctata* larvae to *diap1* (death-associated inhibitor of apoptosis protein1) dsRNA (Chikami et al., 2019). Early feeding cessation of chewing insects can thus indicate good performance of the dsRNA to users and should be included in studies for effective target genes for pest management purposes.

Before effective dsRNAs are released for commercial use, their possible ecological impact needs to be assessed. Part of this risk assessment pertains the influence on beneficial insects such as honey bee (EFSA, 2013b). Functional oral RNAi in *A. mellifera* was demonstrated in 2nd instar larvae fed with dsRNA targeting *vitogellin* by a strong decrease in target gene expression (Nunes and Simões, 2009). Avoidance of 21nt matches between the ds*snf7* sequence of *D. v. virgifera* and non-target species sequences was accompanied by high survival rates in honey bee larvae and adults (Bachman et al., 2016; Tan et al., 2016). Somewhat contrarily, oral administration of dsRNA targeting *vATPaseA* based on *D. v. virgifera* and even honey bee sequence itself offering full sequence homology did not induce mortality or differences in adult eclosion and mild target gene suppression was only observed in treated adults (Vélez et al., 2016a). Due to these discrepancies, caution would call for the elimination of any 21nt matches in the dsRNA as is the case for *shi* and *rpn7* dsRNAs in *P. cochleariae* (Table 2.3) in future studies so long as the reasons for variability in RNAi responses are not fully described.

Other beneficials such as the predatory seven-spotted lady beetle *Coccinella septempunctata* are part of the same insect order as *P. cochleariae* and are as such thought to be more likely affected by insecticidal dsRNA present in the environment. A sequence comparison of the eleven highly lethal genes of *T. castaneum* to the pink spotted lady beetle *Coleomegilla maculata* revealed high sequence conservation of at least 68% on nucleotide level and several putative sites albeit with variable frequency for siRNA generation along the entire genes (Allen, 2017). Recently, neonates of *C. septempunctata* and another lady beetle species *Adalia bipunctata* proved amenable to dsRNA in a feeding approach (Haller et al., 2019). When fed *D. v. virgifera*-based ds*vATPaseA*, more strongly decreased survival in *C. septempunctata* coincided with a higher number of 21nt matches in *C. septempunctata* compared with *A. bipunctata* (Haller et al., 2019). Despite the use of high dsRNA amounts these species are unlikely encounter in the environment, this exemplifies the need to avoid too high homology of dsRNA sequences between pest and beneficial insects. In the case of *P. cochleariae*-based dsRNAs where siRNAs were possible in *C. septempunctata* in two dsRNA (Table 3), future studies could profit from either narrowing down the dsRNA sequences or shift the entire sequence so that sequence overlaps are prevented.

2.6. Conclusions

Unbiased screening is an opportunity for the identification of suitable lethal dsRNAs for pest management. While dsRNA screening by injection was easily feasible in *T. castaneum*, broadening the scope to field conditions is impracticable with this species due to its nature as a storage pest. *P. cochleariae*, which is already known as a screening model Coleopteran for sprayable insecticides in industry, was tested as an alternative. Five out of nine dsRNA targets that were found to be lethal in *T. castaneum* were confirmed to be lethal in *P. cochleariae* demonstrating a high transfer rate of lethal genes upon dsRNA feeding. Despite the sometimes – albeit expected – decrease in mortality compared to injection, the onset of the RNAi response was not delayed due to oral dsRNA delivery which speaks for *P. cochleariae* as a robust model for sprayable RNAi.

2.7. Acknowledgements

We thank the staff of Bayer AG for insect rearing, provision of plant material and the opportunity to use all required devices. Moreover, we thank GreenLight Biosciences for the provision of *GFP*-based dsRNA.

2.8. Author contributions

Experiments were conducted by SM and JU. BL performed the RNA extraction and library preparation for transcriptome sequencing for *P. cochleariae*. The transcriptomes were assembled by FM and BB. Orthologous sequences were obtained by BB, FM and SM. The publication draft was written by SM. The published version will have major contributions of RN to the draft manuscript, and will be revised by RN, BB, FM, JU, GB and SG.

2.9. Funding

The work was funded by Bayer AG.

2.10. Declaration of interest

BB, FM, BL, SG and RN are employees of Bayer AG.

2.11. Appendices

Table 2.S1: Statistics of the *de novo* Illumina RNAseq assembly of the *Phaedon cochleariae* (adults, mixed sex) transcriptome. Benchmarking Universal Single-Copy Orthologs (BUSCO) scores were assessed according to Simão et al. (2015) using the endopterygota_odb9 dataset.

stage	raw reads	assembled bases	assembled genes	assembled transcripts	BUSCOs				
					complete	complete + single-copy	complete + duplicated	fragmented	missing
adults	20,828,684	50,483,672	79,623	103,441	82%	53%	28%	10%	8%

Table 2.S2: Key parameters of the *de novo* Illumina RNAseq assembly of the *Coccinella septempunctata* (adults, mixed sex) transcriptome.

stage	Average raw reads	assembled genes	assembled transcripts	Contig N50*	Median Contig Length*	Average Contig Length*
adults	17,671,717	36,507	48,808	1785	419	913

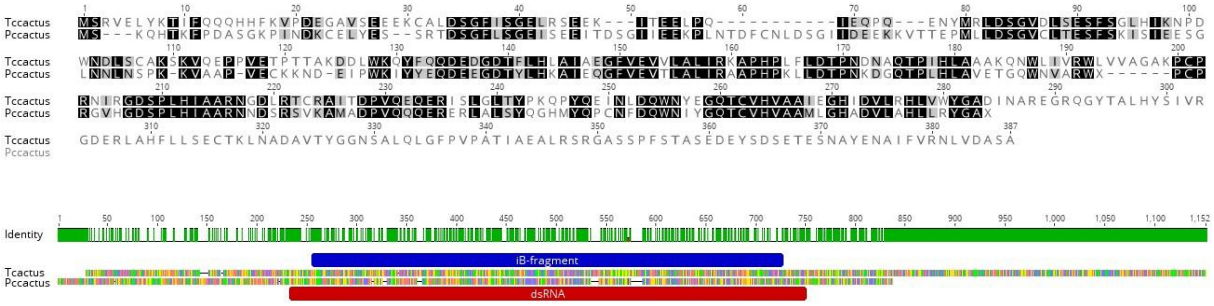
*based on longest isoform

Table 2.S3: Primer sequences for dsRNA production and RT-qPCR. Lower case letters indicate T7 promoter sequences. (n.d.= not determined)

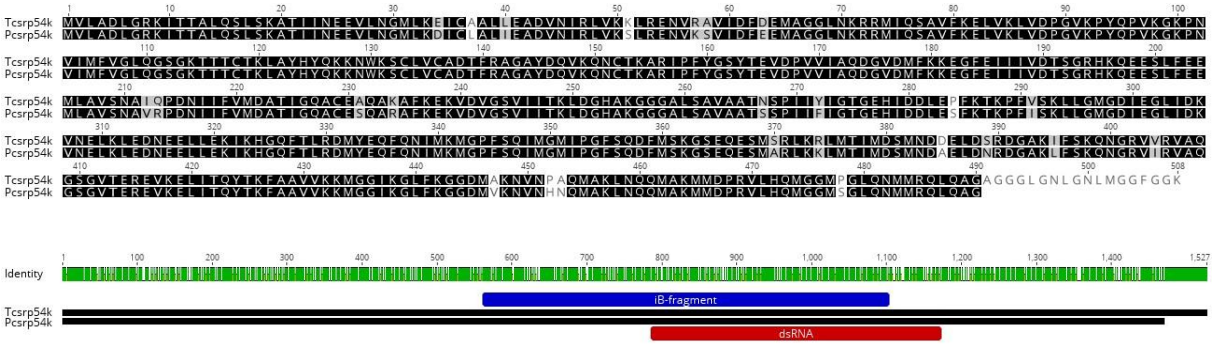
Target	dsRNA primers	Ta [°C]	dsRNA [bp]	qPCR primers	qPCR amplicon [bp]
cact	F - taatacgactcactataggGGACAGTGGCGTGTGTCTTA R - taatacgactcactataggACTGGTCAAAATTGCACGGC	65.0	517	F - TCCCTTGTGAATGCTAGTGC R - TCCTTCCTTCTGAACCCTGC	110
srp54k	F - taatacgactcactataggAGTGGCAGCAACCAGTAGTC R - taatacgactcactataggTCCCTGTTGTCCAACTCTGC	65.2	387	F - ATGGCGAAGATGATGGACCC R - ACCGAATCACTTGTTGCCGA	148
rop	F - taatacgactcactataggAACTGCGTCACCAGCACATT R - taatacgactcactataggACAATCCTCCATGACATCTTTG	61.6	599	F - TTGGTTGGAAGAGCCCAGAG R - CCACCACGAAGACGATGAGG	124
α-SNAP	F - taatacgactcactataggGACATGGGTAGGTTTACCATG R - taatacgactcactataggACATCAACACATAAGTGGCA	60.7	317	F - CCAGAGGATTCTTTGGATCG R - ACTTCCTGCTGAATCCCAAC	119
shi	F - taatacgactcactataggATCAAATGTTGTAAGAATGTGTAC R - taatacgactcactataggTACATTCCTGCCATCGGGAT	58.9	481	F - CCCAACAGGTTCAGATGGCT R - TCAATTTGGTCTGCCAGGCT	146
pp1α-96a	F - gaattgtaatacgactcactataggTTCCCCCCGGAATCCAACTA R - gaattgtaatacgactcactataggTGCAGGAACTTGCCCACCAC	65.9	470	F - TCCTTCCTAATCCGAGCCATC R - CAATTTGTCTGCTTCCGCCA	96
hsc70-3	F - taatacgactcactataggCCATCGCCTACGGCATGGAC R - taatacgactcactataggGCACCTTCGGGATACGAGTAG	64.8	498	F - CAAGCAGCAGAAGAAGCAGC R - CCTTCAGCTCGTCCTCATCC	119
rpn7	F - taatacgactcactataggGCATATTGCATGGCTGTGAG R - taatacgactcactataggACAATGCCTCCCACTCTGTC	62.6	506	F - TCTTTGGGTCACCGGCTAG R - TCCCAGTCTCCTCCCTCTTC	128
rpt3	F - taatacgactcactataggGCACATCACACTACAGCTGC R - taatacgactcactataggCAGCCTCTTGACAGATGGCA	64.4	490	F - CATGCTGGCAAAGGCTGTAG R - ACCATTCGTGGACCTTCACC	102
rps3	- -		-	F - AAGGCTGTGAAGTTGTGGTATCTGG R - ATGGCGGGTGGCAGTGTCTAC	n.d.
rps6	- -		-	F - CAAGACAGGATCAGACCTAGAC R - CACTCTCTTTCCTTTGTGGGAAC	n.d.
RP-L8	- -		-	F - CATGCCTGAAGGTACTATAGTGTG R - GCAATGACAGTGGCATAGTTACC	n.d.

Figure 2.S1: Clustal W protein (upper panel) and nucleotide (lower panel) alignments of *Tribolium castaneum* (Tc) and *Phaedon cochleariae* (Pc) orthologous sequences for cactus (**A**), srp54k (**B**), rop (**C**), α-SNAP (**D**), shibire (**E**), PP-α (**F**), hsc70-3 (**G**), rpn7 (**H**) and rpt3 (**I**). *T. castaneum* dsRNA used in the original iBeetle screen (iB-fragment, blue) and *P. cochleariae* dsRNA positions (red) are indicated in the nucleotide alignments (lower panel).

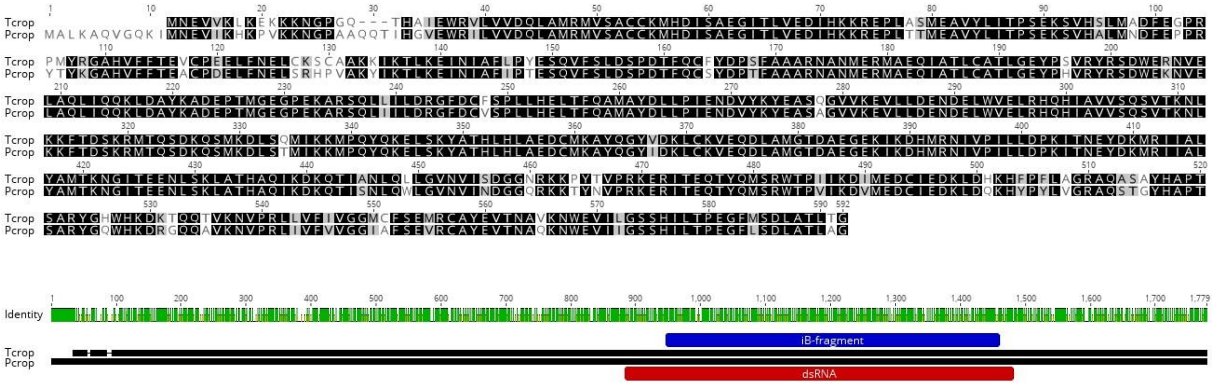
A



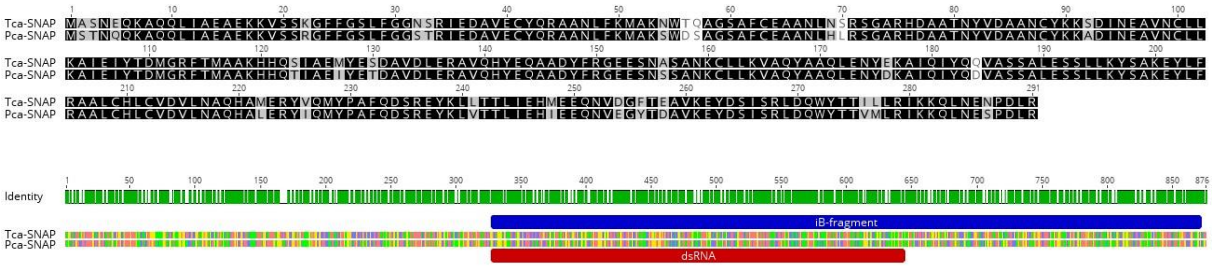
B



C



D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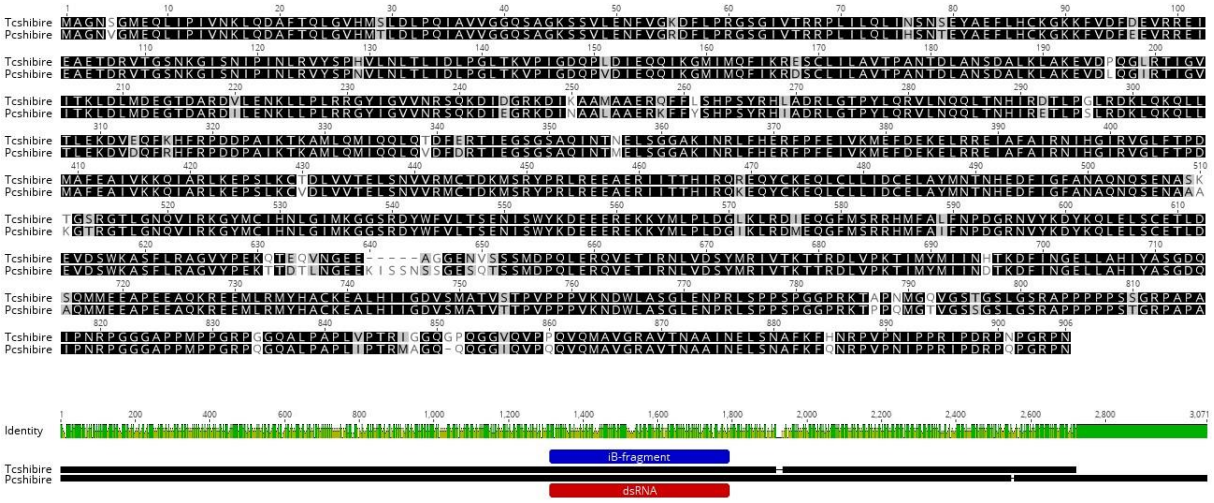
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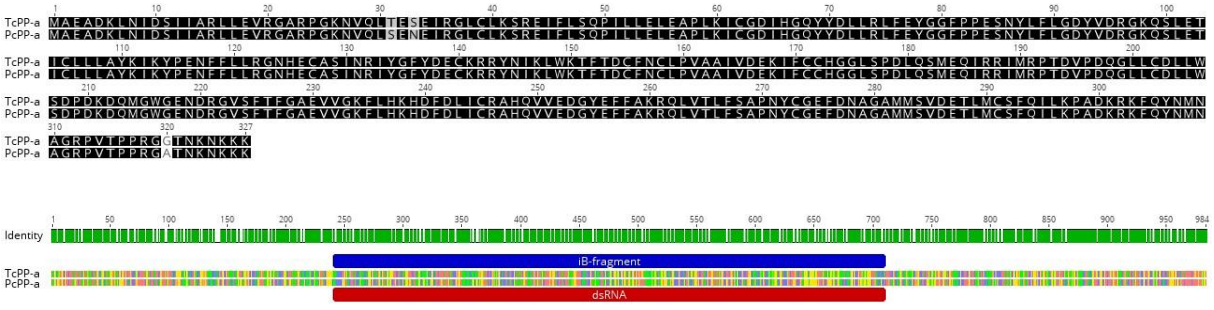
TcPP-a
PcPP-a
Identity
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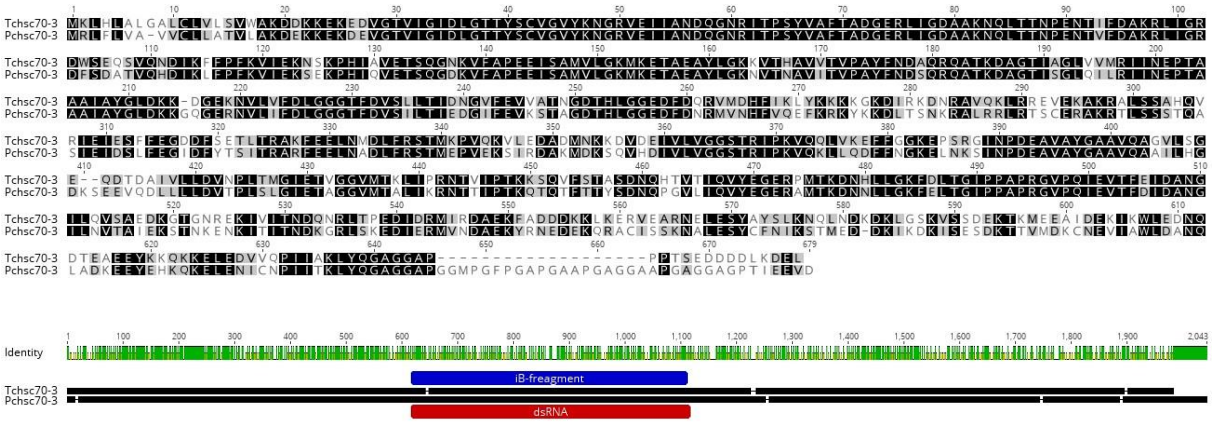
Tchsc70-3
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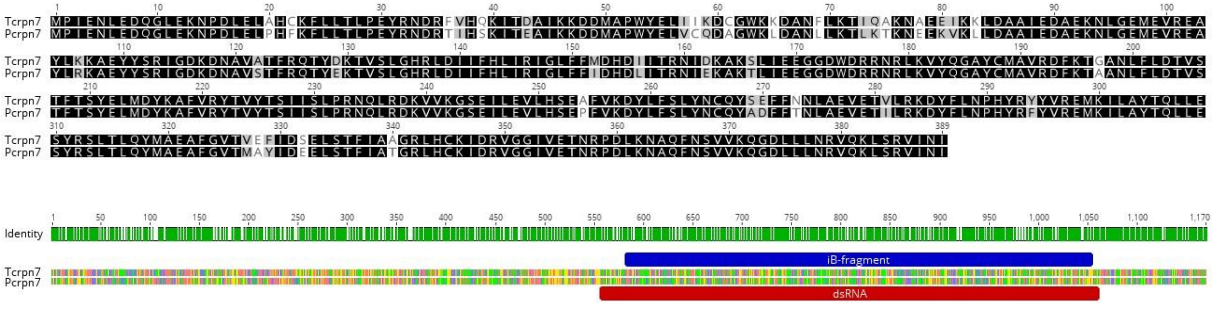
Tcrpn7
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Identity
iB-fragment
dsRNA

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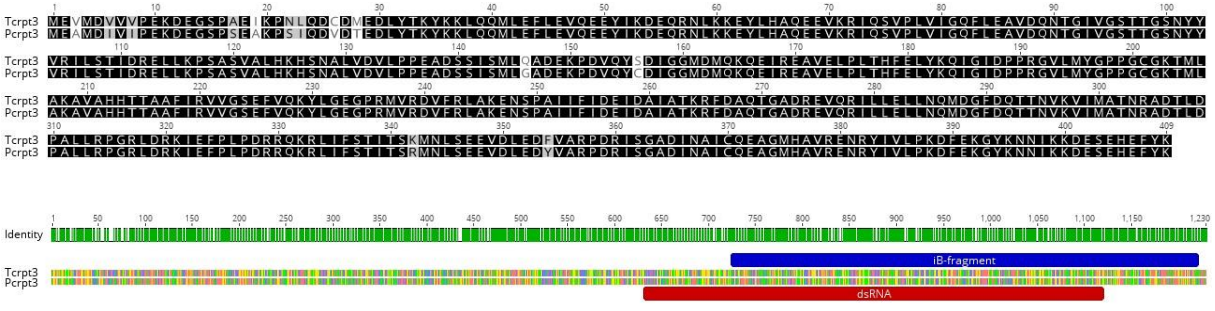
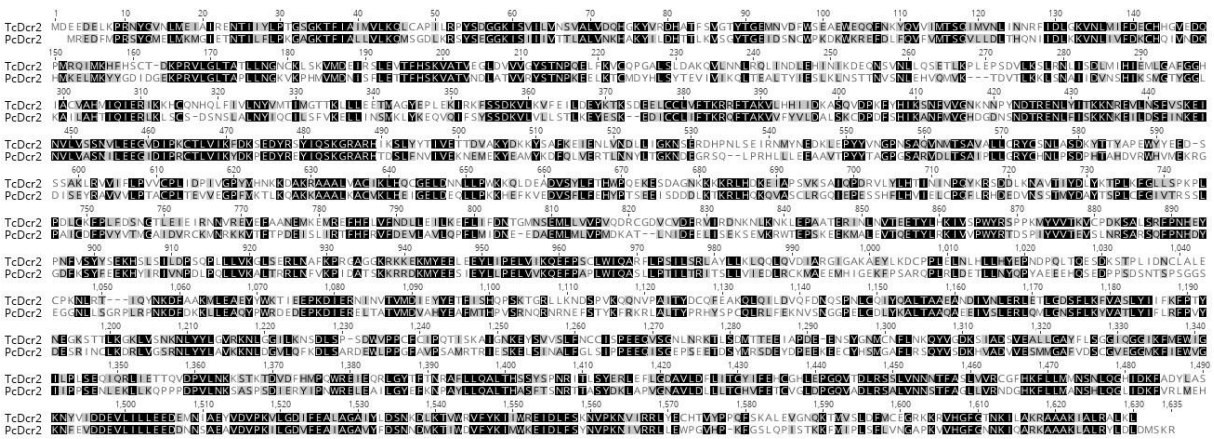

Tcrpt3
Pcrpt3
Identity
iB-fragment
dsRNA

Figure S2. Clustal W protein alignments of *Tribolium castaneum* (Tc) and orthologous *Phaedon cochleariae* (Pc) sequences for siRNA core machinery components Dicer-2 (**A**), Argonaute-2 (**B**), R2D2 (**C**), silC (**D**), CHC (**E**) and vha16 (**F**).

A



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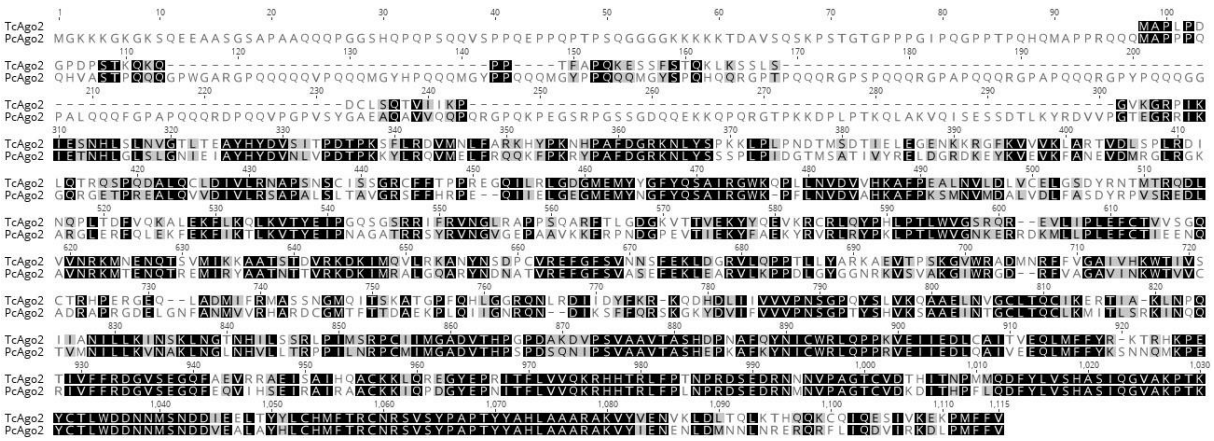

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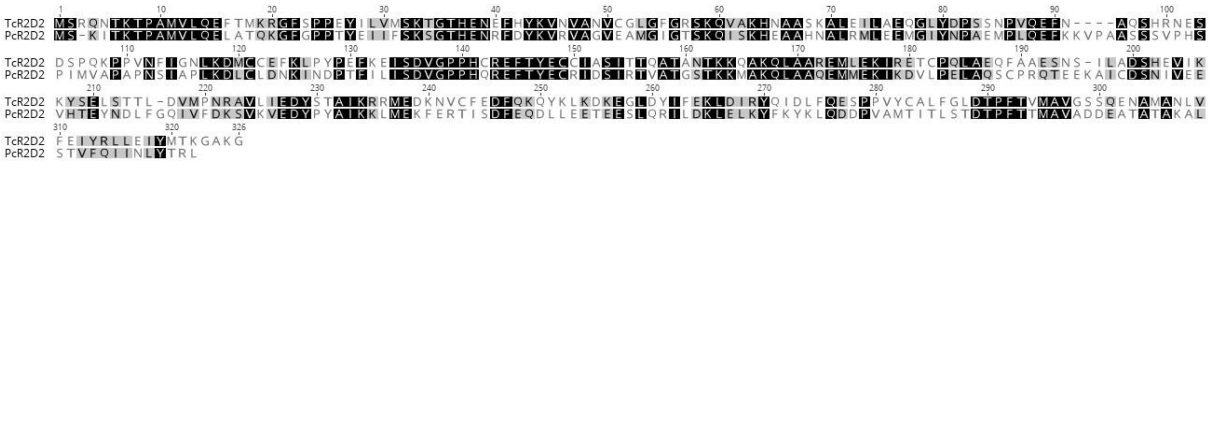

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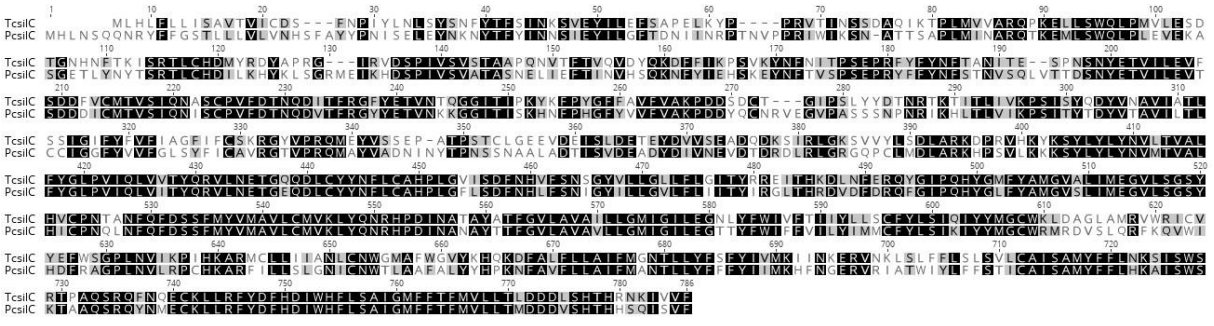

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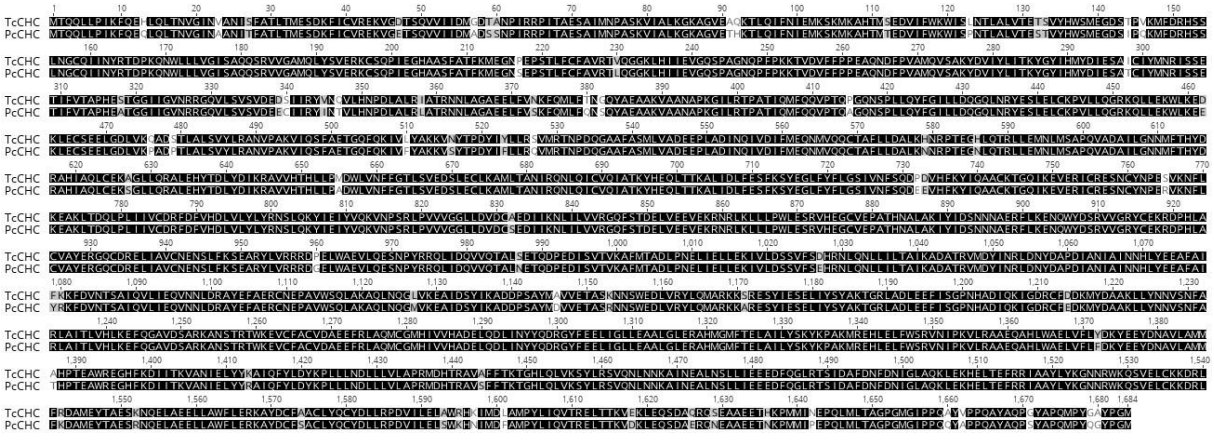


F

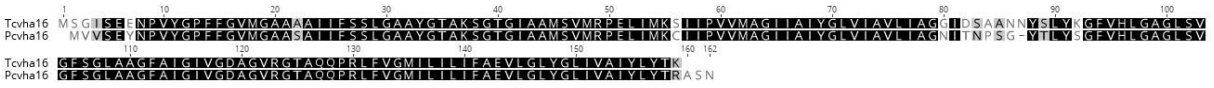
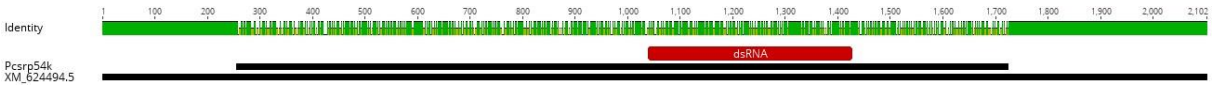


Figure 2.S3: Time lapse video recording over seven days. Second instar *P. cochleariae* larvae in 12-well plates feeding on leaf discs treated with ds*GFP* (3 µg) and ds*αSNAP*/ds*rpt3* at rates of 3 µg, 1 µg and 0.3 µg. Treated leaf discs were replaced with untreated leaf discs on days 3, 5 and 6. On day 3 one of the two larvae per well was removed for gene expression analysis by RT-qPCR. The time lapse videos need to be watched in presentation mode.

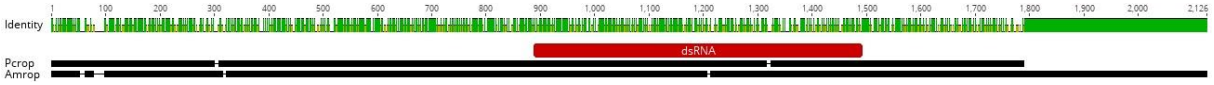
Figure 2.S4: Clustal W nucleotide sequence alignment of *Phaedon cochleariae* and *Apis mellifera* orthologs for *cactus* (**A**), *srp54k* (**B**), *rop* (**C**), *α-SNAP* (**D**), *shibire* (**E**), *PP-α* (**F**), *hsc70-3* (**G**), *rpn7* (**H**) and *rpt3* (**I**). Positions of *P. cochleariae* dsRNAs are marked in red.

A

B



C



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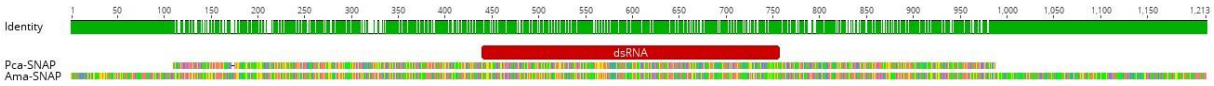

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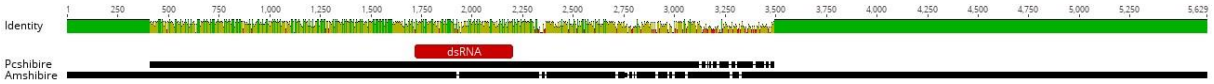

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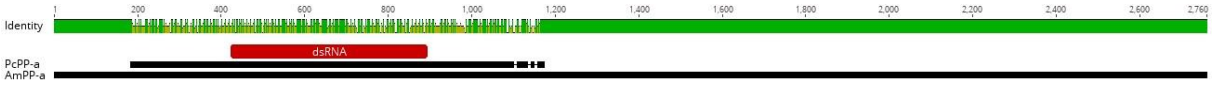

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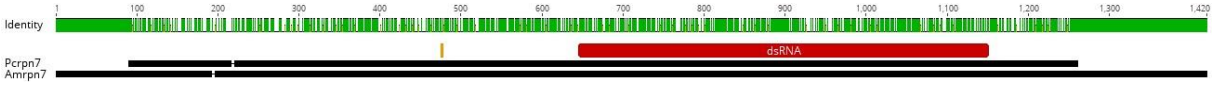

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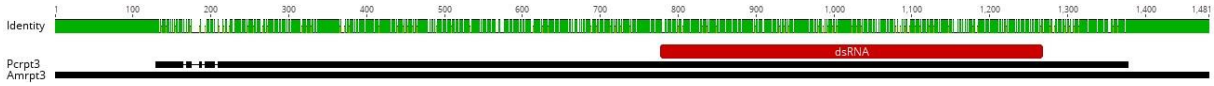
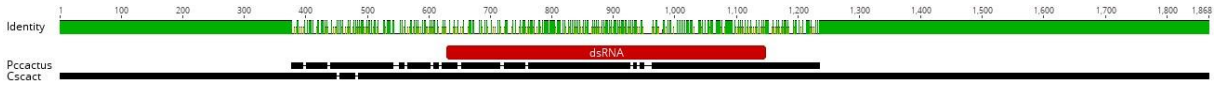
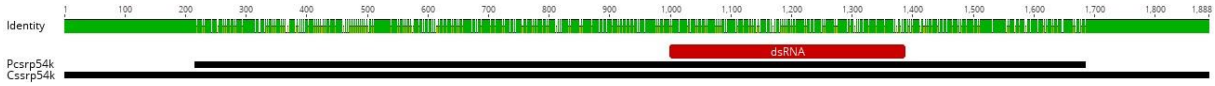


Figure 2.S5: Clustal W nucleotide sequence alignment of *Phaedon cochleariae* and *Coccinella septempunctata* orthologs for *cactus* (**A**), *srp54k* (**B**), *rop* (**C**), *α-SNAP* (**D**), *shibire* (**E**), *PP-α* (**F**), *hsc70-3* (**G**), *rpn7* (**H**) and *rpt3* (**I**). Positions of *P. cochleariae* dsRNAs are marked in red.

A



B



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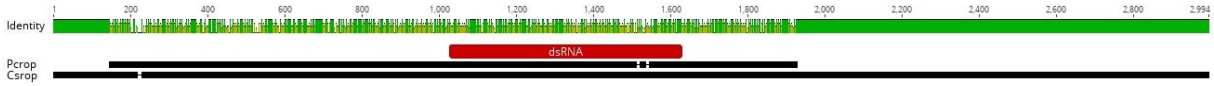

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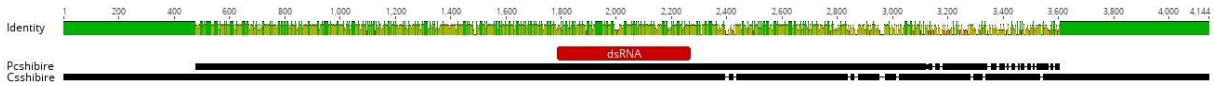
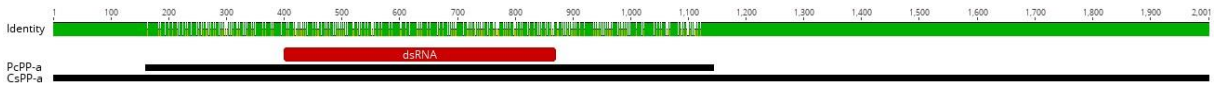
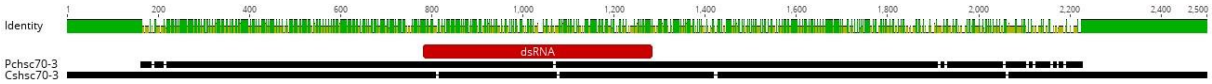


F



G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Identity
dsRNA
Pchsc70-3
Cshsc70-3

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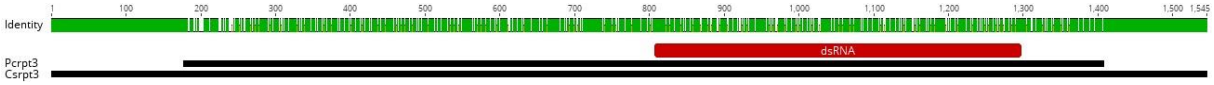
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Identity
dsRNA
Pcrpt3
Csrpt3

3. Manuscript 2: Profiling of RNAi sensitivity after foliar dsRNA exposure in different European populations of Colorado potato beetle reveals a robust response with minor variability

Authors:

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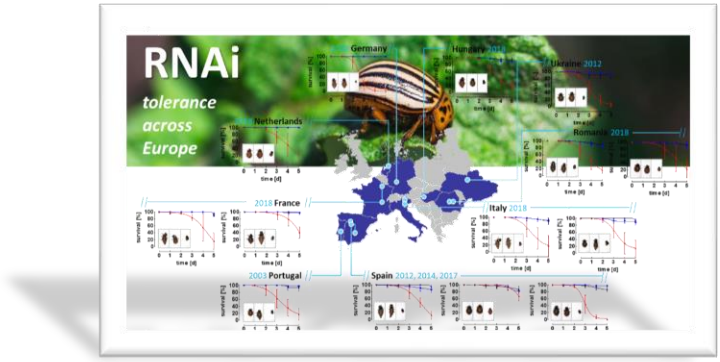
My Contributions:

- Performed all experiments
- Analyzed all experiments
- Drafted the manuscript
- Prepared the figures

Individual author contributions: see page 72

3.1 Abstract

In recent years, substantial effort was spent on the exploration and implementation of RNAi technology using double-stranded RNA (dsRNA) for pest management purposes. However, only few studies investigated the geographical variation in RNAi sensitivity present in field-collected populations of the targeted insect pest. In this baseline study, 2nd instar larvae of 14 different European populations of Colorado potato beetle (CPB), *Leptinotarsa decemlineata,* collected from nine different countries were exposed to a foliarly applied diagnostic dose of ds*actin* (ds*act*) to test for possible variations in RNAi response. Only minor variability in RNAi sensitivity was observed between populations. However, the time necessary to trigger a dsRNA-mediated phenotypic response varied significantly among populations, indicated by significant differences in mortality figures obtained five days after treatment. An inbred German laboratory reference strain D01 and a Spanish field strain E02 showed almost 100% mortality after foliar exposure to 30 ng ds*actin* (equal to 0.96 g/ha), whereas another Spanish strain E01 was least responsive and showed only 30% mortality. Calculated LD_{50}-values for foliarly applied ds*act* against strains D01 (most sensitive) and E01 (least sensitive) were 9.22 and 68.7 ng/leaf disc, respectively. The variability was not based on target gene sequence divergence or knock-down efficiency. Variability in expression of the core RNAi machinery genes *dicer (dcr2a)* and *argonaute (ago2a)* was observed but did not correlate with sensitivity. Interestingly, RT-qPCR data collected for all strains revealed a strong correlation between the expression level of *dcr2a* and *ago2a* (r 0.93) as well as *ago2a* and *stauC* (r 0.94), a recently described dsRNA binding protein in Coleopterans. Overall, this study demonstrates that sensitivity of CPB to sprayable RNAi slightly varies between strains but also shows that foliar RNAi as a control method works against all tested CPB populations collected across a broad geographic range in Europe. Thus, underpinning the potential of RNAi-based CPB control as a promising component in integrated pest management (IPM) and resistance management programs.



Graphical abstract

Keywords: RNA interference, Colorado potato beetle, *Leptinotarsa decemlineata*, population variability, resistance management, pest control

3.2. Introduction

RNA interference (RNAi) has been demonstrated more than 10 years ago to be a valuable tool to control coleopteran pests such as Western corn rootworm (WCR, *Diabrotica virgifera virgifera*) and Colorado potato beetle (CPB, *Leptinotarsa decemlineata*) upon ingestion of dsRNA (Baum et al., 2007). Since then, a lot of research was conducted to test the amenability of RNAi in a broad range of insect species (Andrade and Hunter, 2017; Mogilicherla et al., 2018; Rodrigues et al., 2018; Ulrich et al., 2015; Zotti et al., 2018), to improve dsRNA delivery (Christiaens et al., 2018; Lin et al., 2017; Thairu et al., 2017; Zhang et al., 2010) and to gain insights into the dsRNA machinery and uptake mechanism (Cooper et al., 2019; Saleh et al., 2006; Tomoyasu et al., 2008; Ulvila et al., 2006; Yoon et al., 2016).

So far, robust RNAi has been demonstrated particularly for Coleopteran species where cellular uptake of dsRNA appears to be strong (Bucher et al., 2002; Tomoyasu and Denell, 2004), and where oral uptake can lead to a strong phenotypic response (Baum et al., 2007). An exception are weevils, where several species such as the cotton boll weevil proved resilient to oral RNAi mediated control (Garcia et al., 2017; Prentice et al., 2017, 2019; Wu et al., 2019). In these cases, the lack of a pronounced RNAi response could often be attributed to the activity of dsRNA degrading nucleases present in the midgut rather than to a general malfunction of the RNAi mechanism. However, nucleases were also reported in other Coleopterans such as CPB but were not sufficient to abolish RNAi responses upon oral dsRNA exposure, although they do affect efficiency to some extent (Spit et al., 2017).

In sensitive insects, dsRNA is usually taken up by midgut cells and, depending on the species, further distributed within the insect body (Li et al., 2018; Ramaseshadri et al., 2013). The mechanism of dsRNA uptake is not yet entirely understood to rationally explain the limitations of the technology observed in a number of insect pests (Cooper et al., 2019). In CPB, clathrin-dependent endocytosis appears to be involved (Cappelle et al., 2016), but not SID (systemic RNA interference deficiency)-like proteins despite the relevance of SID proteins in dsRNA uptake in *Caenorhabditis elegans* (Feinberg and Hunter, 2003; Winston et al., 2002). Based on sequence comparison it was suggested that SID-1 might be a nematode-specific protein without a clear ortholog in beetles (Tomoyasu et al., 2008). Once taken up, subsequent endosomal release to the cytosol allows for processing of the dsRNA by Dicer2 (Dcr2) proteins (Bernstein et al., 2001; Saleh et al., 2006). The resulting small interfering RNAs (siRNAs) are then directed to complementary sequences by RISC (RNA-induced silencing complex) containing Argonaute2 (Ago2) proteins which are responsible for target sequence cleavage and thus gene silencing (Hammond et al., 2001; Song et al., 2004; Zamore et al., 2000). The resulting decrease in target protein expression leads to insect death when essential genes are targeted as for example demonstrated in *Tribolium castaneum* and WCR at larger scale (Knorr et al., 2018; Ulrich et al., 2015).

Recently however, a study on the RNAi sensitive chrysomelid beetle WCR revealed the ability of beetles to become resistant to dsRNA treatments if selection pressure is applied (Khajuria et al., 2018). Rather than the degradation of dsRNA, resistance appeared to be linked to a single gene tied into the dsRNA uptake mechanism and rendering any dsRNA treatment ineffective, i.e. irrespective of the targeted gene (Khajuria et al., 2018). This result raised awareness that potential resistance alleles are already present in unexposed populations and that populations of a single species may already exhibit different levels of susceptibility to oral dsRNA treatments upon foliar application. In a recent study examining variations and RNAi tolerance in *T. castaneum* populations from China, only minor differences in RNAi responsiveness between populations could be observed upon dsRNA injection (H. Wang et al., 2018).

CPB is a widespread destructive pest in potato and other crops of the Solanaceae family throughout the Northern Hemisphere and chemical insecticides are frequently applied to keep it below economic damage thresholds (Alyokhin et al., 2013). However, continuous selection pressure led to the development of field-relevant resistance levels to different chemical classes of insecticides (Alyokhin et al., 2008; Huseth et al., 2014), so alternative technologies for CPB control such as sprayable RNAi are under consideration for commercialization (Cingel et al., 2016; Palli, 2014). For dsRNA as an effective pest management tool, it is necessary to take the critical step of oral dsRNA uptake by pests upon foliar application into account (Kunte et al., 2020). CPB as a chewing, leaf eating pest insect susceptible to oral RNAi is well suited for this approach, and it has recently been shown that foliar application of dsRNA provided long-lasting control of CPB under greenhouse conditions (San Miguel and Scott, 2016).

Before new chemical classes of insecticides and/or modes of action are introduced to the market, baseline studies are conducted to investigate the natural variation in susceptibility among pest populations sampled in different geographies. In some regions such as Europe, baseline susceptibility studies are even mandatory regulatory requirements for plant protection product registration (EPPO/OEPP, 2012; Sparks and Nauen, 2015).

Such studies investigating RNAi baseline sensitivity at both biological and molecular level in CPB populations collected across different geographies are lacking. Therefore, the present study aims to examine the variability of the RNAi response at biological, developmental, phenotypical and molecular level after dsRNA uptake upon foliar exposure to second instar larvae of 14 different CPB populations collected across Europe.

3.3. Materials and Methods

3.3.1. Insects

Several *L. decemlineata* populations were sampled from nine different European countries (Figure 3.1). At least 50 individuals – either adults or larvae – were collected at different sites in single potato fields and shipped on potato foliage in plastic boxes to Monheim, Germany, by courier offering 24h delivery services. All populations were maintained in the greenhouse on potato plants (*Solanum tuberosum, var. Annabelle*) at 24°C under long day conditions (16h light, 8h dark) and 60% relative humidity. For experiments, egg masses were collected over several days and kept at 16°C for synchronization purposes before transfer to 24°C for hatching. The larvae were fed with eggplant leaves (*Solanum melongena*) and maintained under the same conditions as described above until further use in experiments. Strain D01 collected in Frankfurt, Germany, served as an inbred, insecticide susceptible laboratory reference strain maintained on potato plants under greenhouse conditions since 2002 (Tebbe et al., 2016).

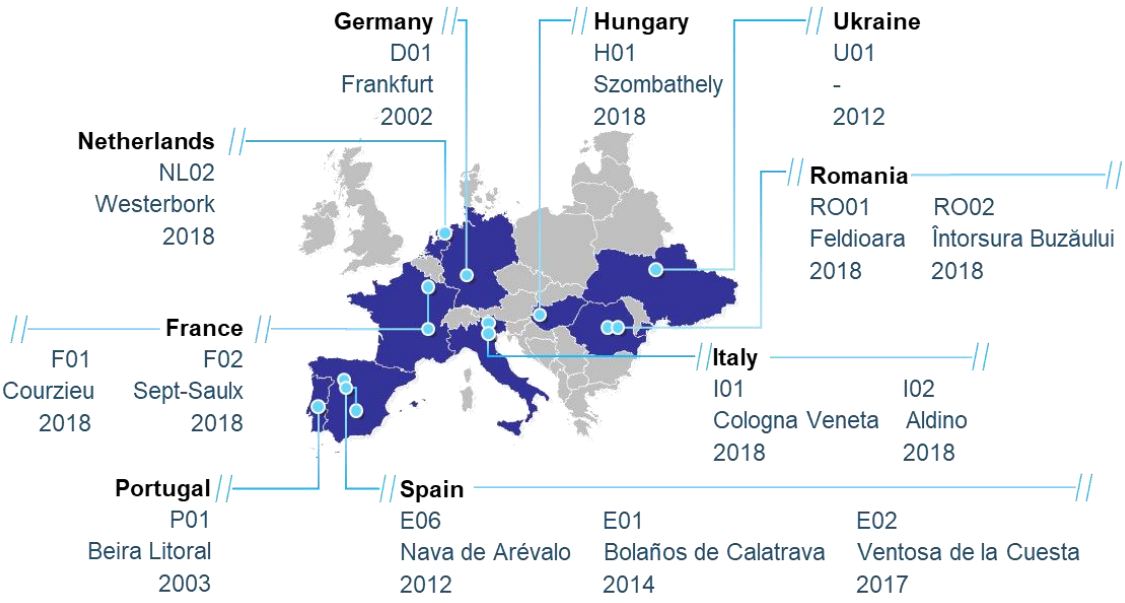


Figure 3.1: Schematic map indicating the origin of 14 different European CPB populations. Countries of origin are highlighted in dark blue and in written in bold. Additionally the population names, collection sites and years are indicated. The schematic map was created using EasyMap software (Lutum+Tappert DV-Beratung GmbH, Bonn, Germany).

3.3.2. Chemicals and double-stranded RNA (dsRNA)

All chemicals and solvents used were of analytical grade and purchased from Sigma (Munich, Germany) unless otherwise stated. Emulsifier W (EW; CAS No. 104376-72-9) used as a detergent was obtained from Lanxess (Leverkusen, Germany). dsRNA ds*act* and ds*GFP* targeting CPB *actin* (GenBank: EB761683.1) and *green fluorescent protein* (GFP) (GenBank U55761.1), respectively, was provided by GreenLight Biosciences (Medford, MA, USA). The 297 bp sequence of CPB ds*act* was originally published by Zhu et al. (2011) (Figure 3.S1). The dsRNA fragment of *GFP* served as a control and contained 524 bp (Figure 3.S1). All dsRNA samples were stored at -20°C before use.

3.3.3. Bioassay

3.3.3.1. Dose response feeding assay with dsactin for diagnostic dose determination

Second instar larvae of synchronized populations of strain D01 were foliarly exposed to six different application rates (100, 30, 10, 3, 1 and 0.3 ng ds*act*/leaf disc (Ø = 2 cm)). The foliar treatment was carried out using a purpose-built spraying device by applying 12 µL of different doses of ds*act* in aqueous 0.1% w/w EW to potato leaf discs placed onto 1.5 ml 1.2 % w/w agar in 12-well tissue culture plates (Greiner Bio-One). Control leaf discs were treated with 12 µL ds*GFP* (100 ng) and EW (0.1% w/w), respectively. A single 2nd instar larva was added per well and allowed to feed for three days. Afterwards, the plates were exchanged, and larvae supplied with fresh untreated leaf discs. The bioassay was replicated thrice, and each replicate consisted of nine larvae. Mortality was scored after five days. After correction for control mortality (Abbott, 1925) data were analysed by a four parameters non-linear regression model to calculate LC_{50}-values and 95 % confidence intervals by GraphPad Prism v8.0 (GraphPad Software, San Diego, CA). A second dose-response experiment was carried out with CPB strain E01 using the same method as described above, but different application rates, i.e. 200, 100, 50, 25, 12.5 and 6.25 ng ds*act*/leaf disc.

3.3.3.2. Diagnostic dose feeding bioassays

For all strains, second instar larvae with a weight of 3.5-4.5 mg were selected for ds*act* feeding experiments, because earlier studies indicated that this is the most sensitive life-stage (Zhu et al., 2011). Thirty ng dsRNA in 12 µL aqueous 0.1% w/w EW was applied to eggplant leaf discs (Ø = 2 cm) placed onto 1.5 ml 1.2 % w/w agar in 12-well tissue culture plates (Greiner Bio-One) using a custom-built spraying device. The foliarly applied dose is equivalent to an agricultural application rate of 0.96 g/ha. Aqueous EW (0.1 %) and 30 ng ds*GFP* served as detergent and dsRNA control, respectively. A single 2nd instar larva was added per well and allowed to feed for three days. Afterwards, the plates

were exchanged, and larvae supplied with fresh untreated leaf discs on days three and four. Survival and developmental stages were monitored every day for five days, and additionally the larval weight of surviving larvae was determined on day five. Larvae were considered alive when they still reacted to outward stimuli such as touch with forceps. Each replicate consisted of nine larvae for 0.1% EW and ds*GFP* treatment, respectively, and 18 larvae for ds*act* treatment. The bioassay was replicated 5 times unless otherwise stated. After two days, three and six larvae treated with EW/ds*GFP* and ds*act,* respectively, were individually flash frozen in liquid nitrogen for gene expression analysis by RT-qPCR. To phenotype growth (inhibition) and toxicity symptoms of larvae, representative specimens for each treatment and strain were photographed on day five using a Keyence VHX-5000 series digital microscope (Osaka, Japan) at 20x magnification.

3.3.4. Quantitative real time PCR (RT-qPCR)

Individual flash frozen larvae were homogenized using a MM300 laboratory bead mill (Retsch) set to 20 Hz for 2 x 10 s using 5mm stainless steel beads. RNA extraction was initiated by addition of 0.5 mL Trizol (QIAGEN) to each frozen sample. After 5 min of incubation at room temperature, 100 µL chloroform was added to each sample, inverted for 15 s and incubated for 3 min followed by centrifugation at 12,000 x g for 15 min at 4°C. Two-hundred µL of the resulting aqueous phase were used for further RNA purification using the Agencourt RNAdvance Tissue Kit (Beckman-Coulter) following the manufacturers´ protocol including the optional DNase I digestion on a CyBio® FeliX pipetting platform (Analytik Jena). RNA concentration was measured on a NanoQuant Plate™ with a Plate reader Infinite 200 PRO (Tecan Life Sciences) and RNA quality was assessed with a QIAxcel Quality Control Kit v2.0 (QIAGEN). gDNA contamination was evaluated on a QIAxcel DNA Screening Kit (QIAGEN) after a 20 µL PCR reaction with 2x JumpStart™ Taq Ready Mix (Thermo Fisher Scientific), 0.3 µM final concentration *ARF1* primers (Table 3.S1), 1 µL RNA template run in the following program: 98°C 3 min, (98°C 30 s, 60°C 30 s, 72°C 30 s) x 40, 72°C 3 min.

cDNA was generated from 500 ng RNA with the iScript™ cDNA Synthesis Kit (Bio-Rad) and used as a 1:20 dilution in all qPCRs. Expression levels were measured for CPB *actin*, *chc*, *vha16*, *stauC*, *dcr2* and *ago2* using *arf1*, *arf4* and *rps18* as reference genes for normalization (for GenBank accession numbers and respective primer sequences refer to Table 3.S1). RT-qPCR reactions were measured in triplicate in 10 µL reactions with 2.5 µL template, gene specific primers at a final concentration of 0.3 µM and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) according to manufacturer`s instructions in a CFX384™ cycler (Bio-Rad). Results were analyzed in qBase+ 3.2 software (Biogazelle) (Hellemans et al., 2007) and visualized using Prism 8 software (GraphPad).

3.3.5. Sequence identification

The dsRNA homologous fragment of six cDNA samples from each population from 0.1% EW and ds*GFP* control treatments was amplified in a 10 µL reaction mixture containing 2x Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 1 µM final ds*act* primer concentration (Table 3.S1), and 0.5 µL template. For subsequent cloning with the TOPO® TA Cloning® Kit for Sequencing (Thermo Fisher Scientific), the samples for each population were pooled and 3´ A-overhangs were added as described in the protocol. After cloning of the fragments into TOP10 chemically competent cells (Thermo Fisher Scientific), colonies were selected on kanamycin LB-plates. Three colonies were picked for each population and grown in 5 mL LB-medium complemented with kanamycin. Plasmids were purified using the Monarch Plasmid Miniprep Kit (New England Biolabs) and custom-sequenced by Eurofins Genomics (Ebersberg, Germany). Results were analyzed and visualized using Geneious 10.2.6 software (Biomatters).

3.4. Results

3.4.1. Dose response and diagnostic dose determination for ds*act*

In order to determine the diagnostic dose for baseline susceptibility testing, a dose response experiment using different concentrations of ds*act* was conducted with 2nd instar larvae of an inbred laboratory reference strain (D01) maintained for >15 years under greenhouse conditions (Figure 3.2). Based on the obtained toxicity data after 5d of foliar ds*act* exposure, a diagnostic dose of 30 ng was chosen to test the variability in RNAi response of field-collected CPB strains. Strain D01 exhibited an LD_{50}-value of 9.22 ng ds*act*/leaf disc (CI95%: 7.21 - 11.8) and the observed mortality at 30 ng was 82.7 % (CI95%: 65.9 – 99.5).

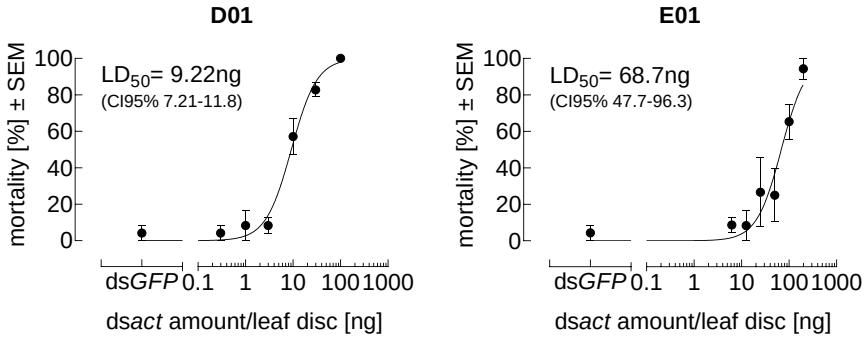


Figure 3.2: Dose reponse data for ds*act* against 2nd instar larvae of CPB strains D01 and E01 after foliar application (5d). Larvae were exposed to dsRNA for the first 3d and mortality was scored after 5d. Mortality figures obtained after foliar exposure to dsGFP served as a control. Data were subjected to four parameter non-linear regression analysis to calculate LD_{50}-values and 95% confidence intervals (CI95%).

3.4.2. Variability in mortality and growth of field-collected strains

Across Europe, 14 populations originating from nine countries were collected (Figure 3.1). The German population D01 has been kept as a laboratory inbred population since 2002 and therefore considered as a reference for comparison as described above. As expected from dose response data (Figure 3.2), 2nd instar larvae of population D01 were significantly affected by 30 ng of ds*act* after only two days of exposure followed by a sharp decline in survival. By day four >95% of the exposed larvae were dead (Figure 3.3). No mortality was observed over 5d for the included control treatments, i.e. foliar exposure to the aqueous detergent EW or ds*GFP*. Likewise, foliar ds*act* treatment lead to low survival rates for many of the other European populations after 4-5 days as well (Figure 3.3). However, we observed that the time of onset for a pronounced RNAi effect differed among CPB populations tested. The

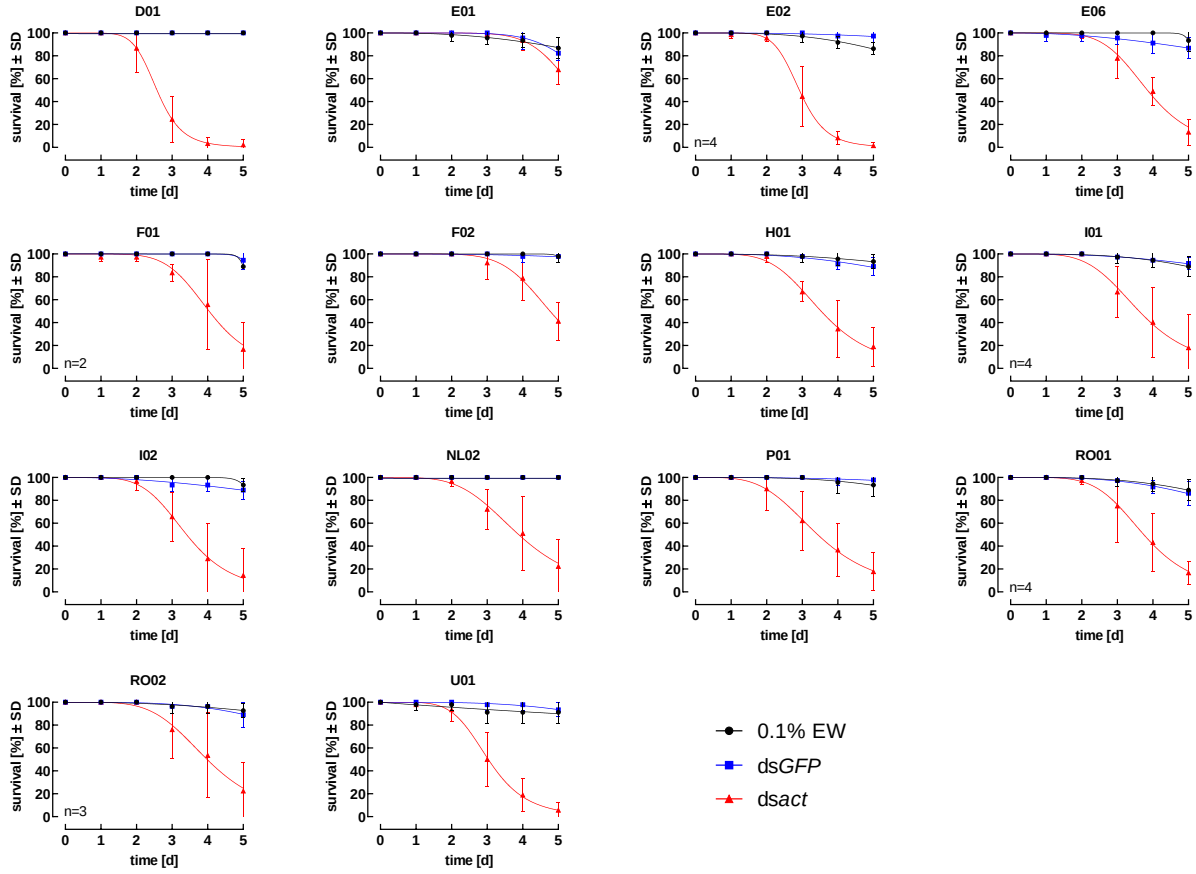


Figure 3.3: Percent survival at different elapsed time intervals of 2nd instar larvae of European CPB populations upon foliar exposure to 30ng ds*act* (triangle, red) for the first 3d. Emulsifier W 0.1% (dot, black) and ds*GFP* (square, blue) treatments served as controls. Data are mean values ± SD (n=5) unless otherwise stated. (For interpretation of the references to colour see web version of this article)

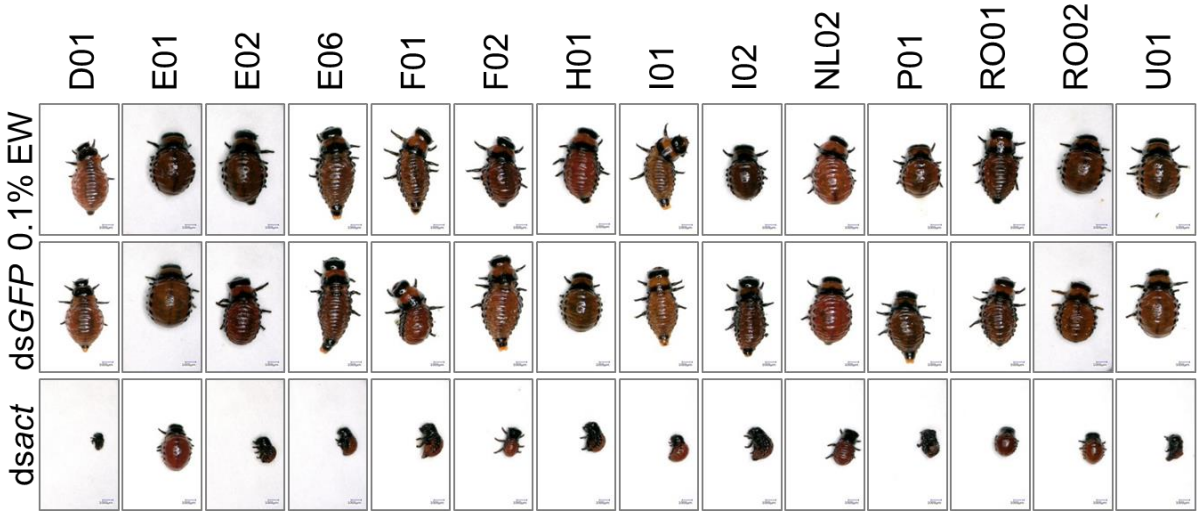


Figure 3.4: Phenotypic variation in different populations of CPB showing growth inhibition and toxicity five days after foliar application of 30ng ds*act.* EW (0.1% detergent) and ds*GFP* were used as control treatment. Scale bars in images represent 1mm.

temporal differences in the onset of the RNAi response were expressed as ET_{50}-values, i.e. the effective time necessary to kill 50% of the CPB larvae exposed to 30 ng ds*act* (Table 3.1). The calculated ET_{50}-values for all field-collected populations significantly differed from the inbred reference strain D01 (ET_{50} 2.6 days) based on non-overlapping 95% confidence intervals. The least affected population after ds*act* exposure was E01 collected in 2014 in Spain; it displayed high survival rates even after five days, resulting in an ET_{50}-value of >5 days. The surviving larvae of strain E01 were less severely affected by ds*act* treatment, as demonstrated by a healthy phenotype (Figure 3.4) and no or reduced feeding cessation (data not shown). However, some growth retardation was observed (Figures 3.4 and 3.5) when compared to D01. The markedly lower effect on growth and development is reflected by the observation that E01 was the only population reaching the fourth instar after ds*act* exposure (Figure 3.S2). In order to determine the overall tolerance to ds*act* of the least affected strain E01, we additionally conducted a dose-response experiment for a quantitative assessment of the difference in intrinsic toxicity compared to strain D01 (Figure 3.2). Strain E01 exhibited an LD_{50}-value to foliarly applied ds*act* of 68.7 ng/leaf disc (CI95%: 47.7 – 96.3), i.e. it is 7.5-fold less susceptible to ds*act* compared to D01. However, at 200 ng ds*act*/leaf disc the mean mortality of E01 was 94.3 ± 9.81%.

Table 3.1: Summary of ET_{50} values in days denoting 50 % survival of different European CPB population after foliar ds*act* treatment (30ng/leaf disc). Confidence intervals (CI95%) as well as the goodness of fit (R^2) are indicated. The ratio is calculated by dividing the ET_{50} of the field population by the ET_{50} of the inbred reference population D01.

Population	ET_{50} (d)	95% CI	R^2	Ratio
D01	2.575	2.410 - 2.748	0.9402	1.00
E01	>5	-	-	>1.94
E02	2.919	2.752 - 3.069	0.9502	1.13
E06	3.845	3.657 - 4.033	0.9180	1.49
F01	4.040	3.543 - 4.557	0.8256	1.57
F02	4.767	4.540 - 5.081	0.7907	1.85
H01	3.530	3.291 - 3.780	0.8841	1.37
I01	3.614	3.210 - 4.053	0.7781	1.40
I02	3.409	3.101 - 3.727	0.8186	1.32
NL02	3.887	3.516 - 4.296	0.7592	1.51
P01	3.435	3.087 - 3.799	0.8038	1.33
RO01	3.745	3.404 - 4.099	0.8218	1.45
RO02	3.984	3.465 - 4.610	0.7358	1.55
U01	3.032	2.837 - 3.227	0.9198	1.18

Other populations showing pronounced differences to D01 were F02 and NL02 collected in France and Netherlands in 2018, respectively. Survival of F02 remained at 41.1% after 5 d ds*act* exposure and the respective ET_{50}-value was 4.77 days. However, the intoxication symptoms coupled with strongly reduced growth when compared to control treatments (Figures 3.4 and 3.5) suggest that these larvae would not survive prolonged exposure periods exceeding 5 days. Though only 22.2% of NL02 larvae survived ds*act* treatment after five days, several of these larvae showed similar weight gain as the controls, indicating that at least some larvae are much less affected by ds*act* (Figure 3.S3).

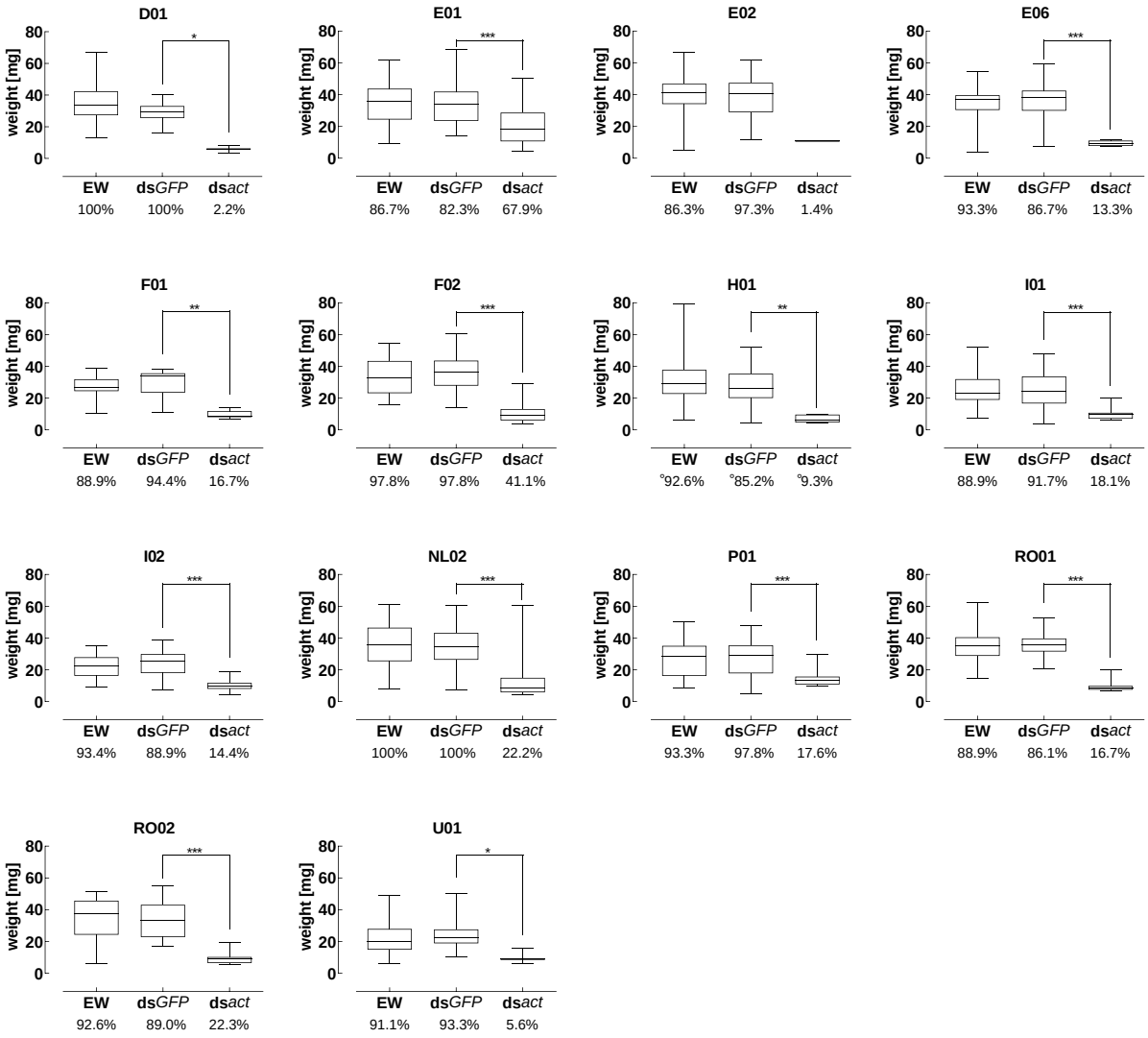


Figure 3.5: Weight of surviving CPB larvae after five days of ds*act* treatment (30 ng). EW (0.1% detergent) and ds*GFP* treatments served as negative controls. The percentages below treatments indicate the proportion of survivors used for weight analysis. Whiskers represent min and max values, boxes 25%-75% quartiles and lines the median. Significant differences are denoted by *p≤0.05, **p≤0.01 ***p≤0.001. The number of survivors after ds*act* treatment of strain E02 was too low to allow for statistical analyses.

In summary, we found significant variation in RNAi sensitivity between European CPB populations when applying a low diagnostic dose of 30 ng per leaf disc (equals 0.96 g/ha). However, even the population with the lowest sensitivity (E01) would be amenable to RNAi-based pest control when using higher concentrations of dsRNA (Figure 3.2).

3.4.3. Target gene conservation

Single nucleotide polymorphisms (SNPs) in the dsRNA targeted sequence may lead to reduced binding of siRNAs and thus a reduced pool of active siRNAs, which in turn may result in variation in RNAi sensitivity among populations. To investigate all strains for polymorphisms we amplified and sequenced the DNA 297 bp fragment targeted by the dsRNA from pooled larvae of all tested populations. Subsequent alignment of the obtained sequences revealed only two SNPs (Figure 3.S4). At position 148 of the targeted gene fragment, cytosine (C) was occasionally replaced by thymine (T), and the frequency varied between and within populations. The second SNP was found at position 295 in only one population (from Romania, RO01), where C was exchanged for G (Figure 3.S4). While the SNP at position 148 is a synonymous mutation, it is non-synonymous at position 295, i.e. resulting in an amino acid substitution of aspartic acid to glutamic acid, but without major biophysical consequences as both amino acids are negatively charged. Hence, the observed difference in sensitivity between populations is not based on target gene divergence.

3.4.4. Target gene knockdown and basal expression level

Next, we tested to what extent the natural expression level of the target gene and the efficiency of its knock-down differed between the strains. We conducted RT-qPCR using cDNA of the different strains to confirm the knock-down of *act* 48h after foliar dsRNA exposure. We detected a strong depletion of target transcript levels in all treated populations ranging from 70.5% (F02) to 92.6% (E02) reduction compared to the ds*GFP* control (Figure 3.6A-N). These levels did not correlate with the different phenotypes and/or calculated ET_{50}-values. For example, although strain E01 exhibited highest survival rates and rather mild phenotypes it showed target gene suppression by >80% (Figure 3.6B). Furthermore, we detected higher (e.g. U01, +53.8%) as well as lower (e.g. F01, -63.8%) basal *act* gene expression levels when compared to D01 in some populations (Figure 3.6O), but these did not correlate with the strength of target gene knock-down or lethality upon foliar ds*act* treatment (compare F01 and U01 in Figure 3.6E,N and O). Again, the most resistant strain E01 did not differ from D01 in terms of basal *act* gene expression levels (Figure 3.7O). The obtained data suggest that RNAi-induced

mortality in the tested populations is most likely not directly linked to the level of target gene knock-down and/or basal expression levels.

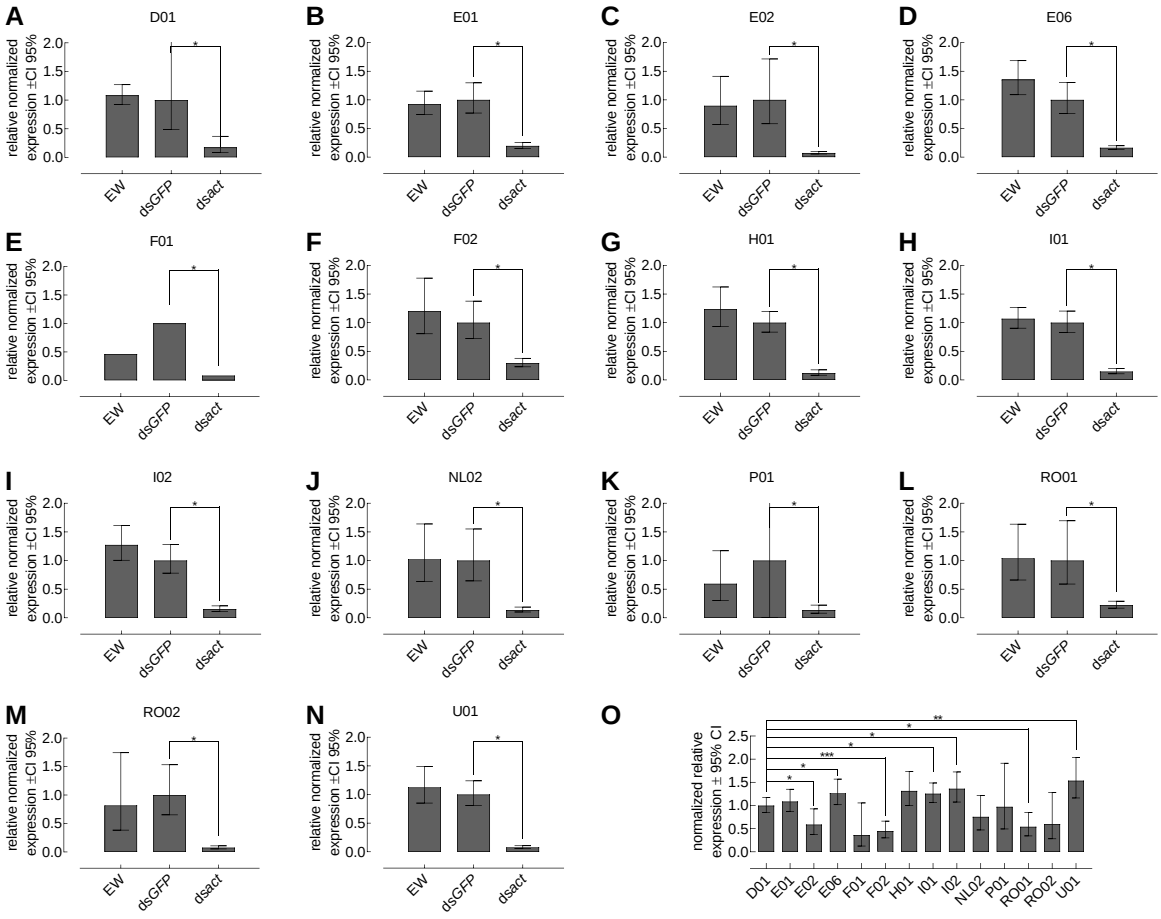


Figure 3.6: Target gene knock-down in larvae of different European CPB populations relative to ds*GFP* control (A-N). Expression levels of *act* for EW (0.1% detergent) and ds*GFP* control treatments did not differ significantly. Error bars represent 95% confidence intervals (CI). Significant differences are denoted by an asterisk (p≤0.05). Variation in *act* expression levels between European CPB populations in EW treatments relative to strain D01 (O). Significant differences are denoted by *p≤0.05, **p≤0.01, ***p≤0.001 .

3.4.5. Expression of core RNAi machinery and potential dsRNA uptake genes

Another potential factor resulting in differences of RNAi sensitivity between strains could be variations in the expression levels of two core RNAi machinery genes, i.e. *dcr2a* encoding an endoribonuclease (Dicer2a), and *ago2a* encoding an Argonaute protein forming part of the RISC. In addition, we analyzed the expression levels of *stauC* encoding a dsRNA binding protein (StaufenC) recently described to be a

major contributor to RNAi in CPB (Yoon et al., 2018). The expression levels of two more genes were compared since they are known to be important in the general dsRNA uptake mechanism in CPB, i.e. *chc* and *vha16*, both described to be involved in clathrin-dependent endocytosis (Cappelle et al., 2016; Saleh et al., 2006). The differences in expression level of these genes between strains 48h after foliar exposure to ds*act* are shown in Figure 3.7. For the proposed uptake genes *chc* and especially *vha16*, significant differences (based on non-overlapping CI95%) in expression level between strains were limited, e.g. lower expression of *chc* in I02 and U01 and higher expression of *vha16* in NL02 and RO01 when compared to D01 (Figure 3.8). For *dcr2a*, *ago2a* and *stauC*, the expression pattern between strains varied considerably and most field-collected populations displayed lower transcript levels than the inbred laboratory strain D01 (Figure 3.7). The lowest basal expression of RNAi machinery genes was measured in strains E01 and U01 (*dcr2a*: U01, -85.2%; *ago2a*: E01, -68.2%; *stauC*: E01, -71.2%).

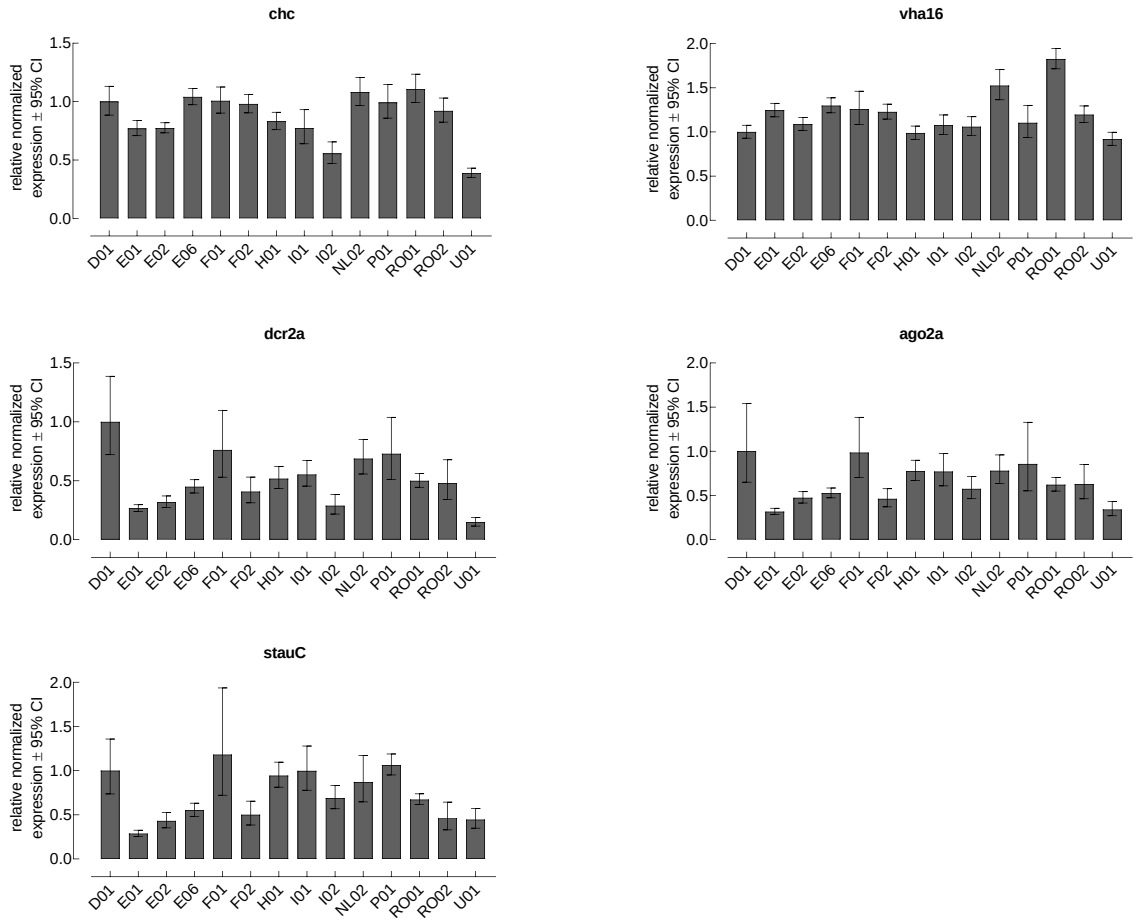


Figure 3.7: Patterns of expression level of dsRNA uptake (*chc*, *vha16*, *stauC*) and RNAi machinery genes (*dcr2a*, *ago2a*) after 2 days of ds*act* exposure (30ng) in different European CPB populations. Expression levels were normalfoliarized to the inbred D01 population. Error bars represent 95% confidence intervals (CI).

The strain with the strongest resistance against RNAi (E01) showed the lowest expression level of the basal RNAi components in our sample. However, besides this example there was no general correlation of mortality rates and the expression pattern of the genes analyzed (Figure 3.7). For instance, the highly susceptible strains D01, E02 and U01 showed both high and low expression levels. Likewise, the strains with comparably low susceptibility (E01, E06, F01, F02 and RO02) had both, low or high transcript levels. Noteworthy a strong correlation was observed between the expression levels of *dcr2a*, *ago2a* and *stauC* between CPB strains, suggesting that their expression is co-regulated (Figure 3.8).

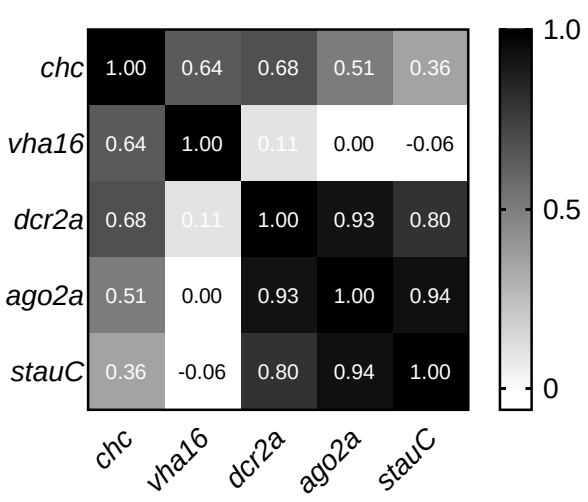


Figure 3.8: Heatmap Pearson correlation coefficient matrix of for the expresion levels of *chc*, *vha16*, *dcr2a*, *ago2a* and *stauC* in 14 different CPB populations after two days of foliar ds*act* exposure (30ng). Pearson correlation coefficients (r) are indicated in the respective squares.

3.5. Discussion

CPB is a highly destructive herbivorous coleopteran pest of agricultural importance in the Northern Hemisphere and has been one of the primary targets among researchers to explore the potential and limitations of foliar and transplastomic RNAi-based pest control in solanaceous plants such as potatoes (Baum et al., 2007; Zhu et al., 2011; Palli, 2014; Zhang et al., 2015; San Miguel and Scott, 2016; Yoon et al., 2016).

Our work focused strictly on foliar RNAi and we designed a broad baseline study to investigate the variation of RNAi sensitivity in 14 CPB populations collected across Europe. Such baseline data help to assess if the applied value of a pest control measure is possibly compromised by large variations in sensitivity in distant populations. Studies assessing for inter-/intra-populational variability towards RNAi are limited, and restricted to very few species such as *T. castaneum* and *L. migratoria* (Kitzmann et al., 2013; Sugahara et al., 2017; H. Wang et al., 2018). While studies following the dsRNA effects over time are more common, but typically consider a single population of the respective species as shown for *T. castaneum* (Ulrich et al., 2015), *Agrilus planipennis* (Rodrigues et al., 2018), WCR (Baum et al., 2007; Hu et al., 2016) and CPB (Zhu et al., 2011). The present study combined both approaches and resulted in the discovery of slight, but significant differences in sensitivity to RNAi responses in several CPB populations. Our study for the first time investigated inter-populational variations in RNAi response of a pest insect species collected from nine different countries across Europe. In order to diagnose possible inter-populational variations it is common practice to analyse the response of field-collected strains against discriminating insecticide rates obtained from dose-response data generated with insecticide susceptible reference strains (Cahill et al., 1996; Olson et al., 2000; Kramer and Nauen, 2011; Garrood et al., 2016). We have chosen a similar approach in our study by applying a diagnostic dose of 30 ng ds*act* which revealed the strongest RNAi response in an inbred laboratory CPB reference strain, D01, and known to be fully susceptible to chemical insecticides (Tebbe et al., 2016). However, based on the time necessary to evoke 50% mortality after foliar ds*act* exposure (ET_{50}) a Spanish field strain, E02, sampled in 2017 was almost as sensitive, whereas another inbred strain collected in 2003 in Portugal, P01, was slightly less responsive. On the other hand, several of the field strains collected in 2018 such as those from Italy, Netherlands and Romania were not significantly different from the inbred Portuguese strain collected 15 years earlier (Table 3.1). Thus, it seems fair to conclude that RNAi sensitivity in those strains included in our study is not primarily affected by collection date and/or higher genetic diversity as influenced by environmental factors as one would expect in field-collected populations (Grapputo et al., 2005).

In all populations tested, the question was not if, but rather how fast the larvae were affected after ds*act* exposure and whether the observed variability in sensitivity depends on the application. Here,

we found that the RNAi response differed significantly after different elapsed time intervals. Specifically, the calculated ET_{50}-values varied between 2.6 and >5 days (Table 3.1). However only in one strain, E01, the RNAi effect was rather weak after 5 days of incubation. However, the observed variability is facilitated by a rather low diagnostic dose of 30 ng ds*act*/leaf disc (equal to 0.96 g/ha). Higher doses would have obscured the variability as we found high levels of mortality in the least sensitive strain E01 when exposed to 200 ng dsact/leaf disc (equal to field rates of 6.4 g/ha). A significantly reduced weight gain and phenotypic symptoms of poisoning, even in those strains showing a delayed onset of the RNAi response (such as E01 and F02), suggest a strong effect beyond the incubation period of five days. Actually, our data underestimate the effect, since untreated leaf discs were offered after three days of dsRNA exposure. Hence, under an applied scenario, i.e. continuous exposure to dsRNA under field conditions, even the least sensitive populations in our study would have been strongly affected, particularly considering the recently shown 28d residual efficacy of higher rates of ds*act* against CPB (San Miguel and Scott, 2016). Principally a temporal shift in the onset of the RNAi effect between populations could also occur if pests exposed to dsRNA ingested varying amounts of treated plant material. This has been shown for WCR where 2 h and 24 h exposure to 50 ng dsRNA targeting *DvSnf7* resulted after 12 days in 0% and >90% mortality, respectively (Bolognesi et al., 2012). However, in the first two days of ds*act* exposure, CPB larvae appeared to feed normally and developed to the next instar (Figure 3.S2). A pronounced feeding cessation was only observed after 2-3 days for all CPB populations tested (based on the visual assessment of leaf area ingested), so one can assume that similar amounts of dsRNA were taken up by the larvae of the different strains.

Basically, we did not find much variability on the level of target sequence, expression level and gene knock-down efficiency. Polymorphisms in the targeted gene could be one determinant for different RNAi susceptibility. The fact that almost no nucleotide differences in the dsRNA-targeted CPB *actin* gene sequences between populations was detected, suggests that the number of possible siRNAs matching the target mRNA sequence was not a limiting factor. H. Wang et al. (2018) showed that the presence of even four polymorphic sites did not negatively impact dsRNA performance targeting a 185bp fragment of *vATPaseE* in seven geographically distinct populations of *T. castaneum.* In another study with *L. migratoria* targeting *CRZ* by dsRNA no differences in RNAi sensitivity between two strains were observed, despite the detection of 14 polymorphic sites (Sugahara et al., 2017).

Strong target gene downregulation is typically taken as an indicator for successful RNAi, whereas lack of target gene suppression is often associated with RNAi tolerance. The strong reduction of *actin* expression in all CPB population tested in this study therefore points towards similar RNAi sensitivities among the collected strains. This is reinforced by high levels of mortality in most CPB populations

exposed to low amounts of ds*actin*. However, it does not explain the observed shift in the onset of the RNAi response or the significantly increased tolerance of strain E01. Such variation is not necessarily directly linked to the downregulation of the target gene, such as demonstrated in *T. castaneum* laboratory strains where target gene suppression did not match the phenotypic response (Kitzmann et al., 2013). The authors ruled out off-target effects and sequence divergence, but described that the differences depended on the maternal genotype and different dynamics of mechanisms to compensate for the reduction of the targeted gene (Kitzmann et al., 2013).

Another factor we analyzed was the endogenous basal expression of *act* among the different CPB populations. Although we detected differences in basal expression levels between populations, no correlation with RNAi-mediated suppression of *act* expression levels was observed (Figure 3.6), suggesting that increased basal *act* levels did not influence the RNAi response by overloading the RNAi machinery. So far, we have not explored the possibility that there are different dynamics in upregulation of paralogs of the actin gene in order to compensate for the RNAi mediated knock-down. Likewise, different stability of the actin protein remains a potential reason for a higher resistance.

Next, we investigated if the observed variability is possibly linked to a decreased expression of genes involved in dsRNA uptake or processing, also known as the RNAi machinery. The expression pattern of *chc* and *vha16* varied not much and did not correlate with temporal differences in RNAi responsiveness among populations, indicating that the uptake of dsRNA – partly relying on these two genes – is rather unlikely to be a decisive factor (Figure 3.7). In fact, the constitutive expression of both genes in all populations supports their universal role in vesicular trafficking independent of the RNAi mechanism.

Contrarily, the expression pattern of the RNAi machinery genes *dcr2a*, *ago2a* and *stauC* varied to a greater extent between European CPB populations (Figure 3.7). Both *dcr2* and *ago2* expression levels have been associated with RNAi sensitivity in species and tissues. In mosquito salivary glands, reduced silencing efficiency coincided with low *dcr1*, *dcr2*, *ago2* and *ago3* expression, while other tissues with higher expression remained sensitive (Boisson et al., 2006). Similar observations upon *dcr2* and *ago2* knockdown were made in CPB (Yoon et al., 2016) and WCR (Miyata et al., 2014). Reduced expression of RNAi machinery genes was even proposed as a potential RNAi resistance mechanism in WCR (Vélez et al., 2016b), but more recent studies are less supportive (Davis-Vogel et al., 2018b; Wu et al., 2017). A dsRNA-binding protein, StaufenC, has been recently described in Coleopterans and discussed as a contributor to a robust RNAi response (Yoon et al., 2018). Reduction in expression of this gene resulted in deficiencies in dsRNA processing in a CPB cell line and in larvae (Yoon et al., 2018). The expression levels of *dcr2*, *ago2a* and *stauC* in different European CPB populations investigated here varied significantly but did not correlate with the observed differences in RNAi response. This observation is

for example supported by the fact that the expression level of RNAi machinery genes is rather low and not significantly different in strains U01 and E01 when compared to D01, but they significantly differ in RNAi responsiveness. This observation as well as the lack of differences between ds*act* and control treatments suggests that none of the three genes play a key role in the different RNAi responses observed in European CPB populations. However, earlier studies conducted with CPB larvae revealed that expression levels of *dcr2a* and *ago2a* were upregulated already 6h after dsRNA exposure and almost reduced to (*dcr2a*) or even below (*ago2a*) control expression levels by 48h (Guo et al., 2015), which corresponds to the sampling time point in the present study. Similarly, *dcr2*, *ago2* and *r2d2* expression in *Acyrthosiphon pisum* was demonstrated to be elevated 12h after dsRNA treatment but back to control levels by 36h, however high amounts of dsRNA were shown to prolong the induction of the RNAi machinery genes (Ye et al., 2019). Hence, an extensive time series following both, knock-down levels and RNAi machinery gene expression might reveal dynamics, which we could not cover by scoring one time point.

Data on *stauC* expression are limited, but intriguingly our study demonstrated that its expression level in different CPB populations is directly correlated with the expression level of *dcr2* and *ago2*, suggesting strong linkage (Figure 3.8). In different insect species such as WCR, *Spodoptera frugiperda*, *Nezara viridula* and *Drosophila melanogaster*, it was shown that at least expression of *dcr2* and *ago2* followed similar trends across developmental stages despite different basal levels (Davis-Vogel et al., 2018a). Contrastingly, expression levels of *dcr2a* and *ago2a* in larval stages of CPB did not appear to be related (Guo et al., 2015), something also shown in *Bactrocera dorsalis* (Xie et al., 2017). Generally, information on the transcriptional regulation of the RNAi machinery in insects is scarce. In *D. melanogaster*, transcription factor FOXO was found to trigger *dcr2* and *ago2* transcription and consequently affects RNAi efficiency (Spellberg and Marr, 2015). This result supports the co-expression of some RNAi machinery genes and it would be interesting to determine whether s*tauC*, which is not present in *D. melanogaster*, is a FOXO target in Coleopterans. Finally we would like to stress the point that only a few studies yet investigated the effect of RNAi on the actual reduction of respective protein levels (eg. Hu et al., 2019; Vallier et al., 2009; Vélez et al., 2019). Despite strong target gene suppression on mRNA level, varying half-lives of actin protein in analyzed CPB populations may contribute to a temporal shift in phenotypic RNAi responses. Future studies may also investigate possible differences between strains in the activity of dsRNA degrading enzymes such as endonucleases. However future research is necessary to investigate the implications of such effects on RNAi-mediated CPB control in more detail.

3.6. Conclusions

Here we investigated the phenotypical and molecular variation in RNAi responsiveness among 14 different CPB populations collected in nine European countries. RNAi baseline data across a broad geographical range were lacking so far but are required for an appropriate assessment of this technology as a pest management tool. After foliar treatment we observed low natural variation in RNAi tolerance in different European populations of CPB, except for one strain collected in Spain in 2014 exhibiting 7.5-fold lower sensitivity to ds*act* based on calculated LD_{50}-values when compared to an inbred laboratory reference strain. However, significant temporal differences (up to 2-fold) in the onset of the RNAi phenotype in field populations were found at a diagnostic dose. The slight variations in ds*act* RNAi responsiveness were not correlated with target gene knockdown or differences in expression levels of core RNAi machinery and uptake/transport genes. Our results support a rather low risk of CPB control failure for the new technology, because the application of higher rates under field conditions would supersede the variability observed with the a rather low diagnostic dose of 0.96 g/ha. Our data suggest that foliar (sprayable) RNAi as a control method works against diverse CPB populations across a broad geographic range in Europe, thus underpinning the potential of RNAi-based CPB control as a promising component in integrated pest management (IPM) and resistance management programs.

3.7. Acknowledgements

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3.8. Author contributions

RN, SG and GB conceived the study and RN supervised the technical realization. SM conducted the experiments and analyzed data. SM and RN wrote the paper. All authors reviewed and edited earlier drafts of the paper.

3.9. Additional material

Table 3.S1: Targeted genes and primer pairs used in qRT-PCR and cloning.

Target	Accession No.	Forward	Reverse	Origin
arf1	KC190026.1	CGGTGCTGGTAAAACGACAA	TGACCTCCCAAATCCCAAAC	Shi et al., 2013
arf4	KC190027.1	GTGCTCGTGAACCATGTGAA	AACCTCCAATCCCTCGTGAA	Shi et al., 2013
rp18	KC190034.1	TAGAATCCTCAAAGCAGGTGGCGA	AGCTGGACCAAAGTGTTTCACTGC	Shi et al., 2013
act	KC190031.1	TTCTGATTCCGTGAGGATTTTG	GTGAGGTGGATGTTCGTAGGG	Shi et al., 2013
stauC	SRP150964*	GGGTGTTTCATACCTCGTCTC	CCCTTACACTGGGAACAGAAA	Yoon et al., 2018
vha16	KP273189.1	TGCTGGGGTCAGAGGAATAG	GGCTACAATGAGACCATAAAGACC	Cappelle et al., 2016
chc	KP273191.1	CCTCACTTAGCGTGTGTAGCA	CGCCTACGCACCAAATAACG	Cappelle et al., 2016
dcr2a	KP230552.1	AAGGCCGCTGTATCTCACTT	CTTCATGCTGTCCTCCAGAA	Guo et al., 2015
ago2a	KP274882.1	TCGAAGCGATTACTGTGGAG	CTGAAACGCCATCTCTGAAA	Guo et al., 2015
act (cloning)	EB761683.1	GCACGAGGTTTTTCTGTCTAGTG	ATGTCATCCCAGTTGGTGATG	Zhu et al., 2011

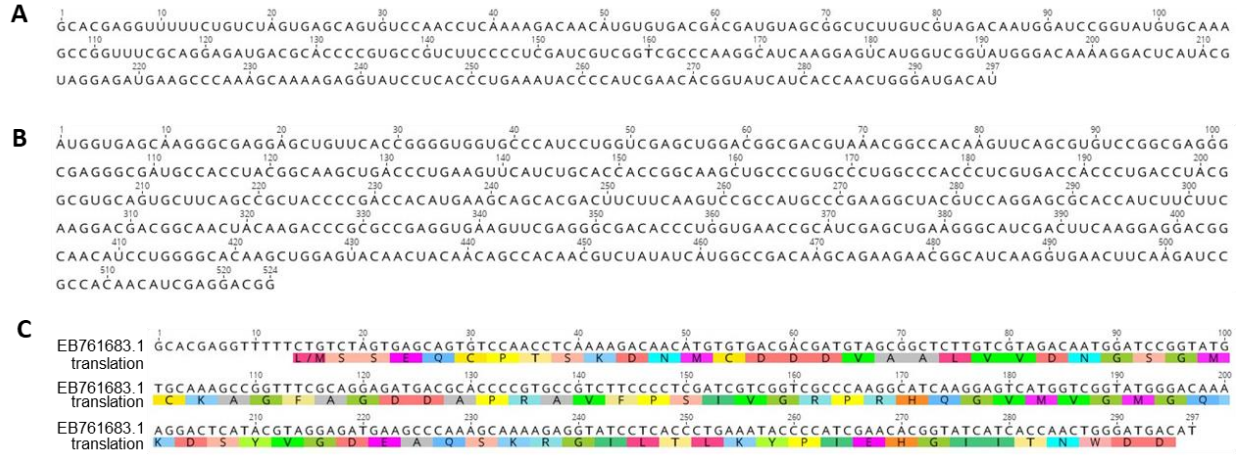

Figure 3.S1: dsRNA sequence of (A) ds*act* and (B) ds*GFP*. (C) cDNA and translated region covered by the ds*act* sequence.

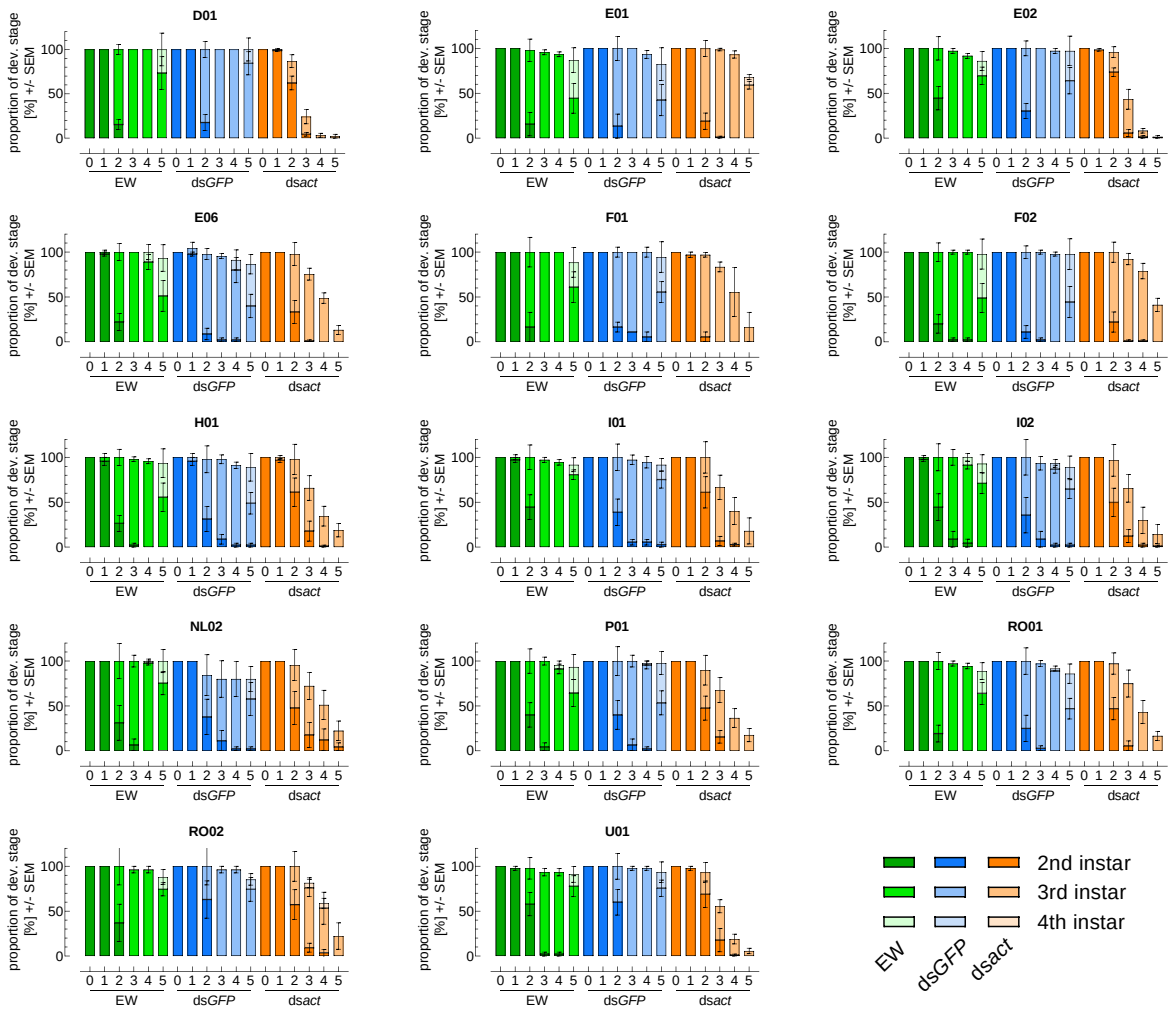

Figure 3.S2: Developmental stages of surviving larvae of different strains of CPB displayed as percentage of 2nd instar (dark), 3rd instar (light) and 4th instar (pale) larvae relative to the starting insect numbers. The larvae were treated with 0.1% emulsifier W (EW, green) and ds*GFP* (blue) as well as ds*act* (orange) and were monitored for development daily over five days.

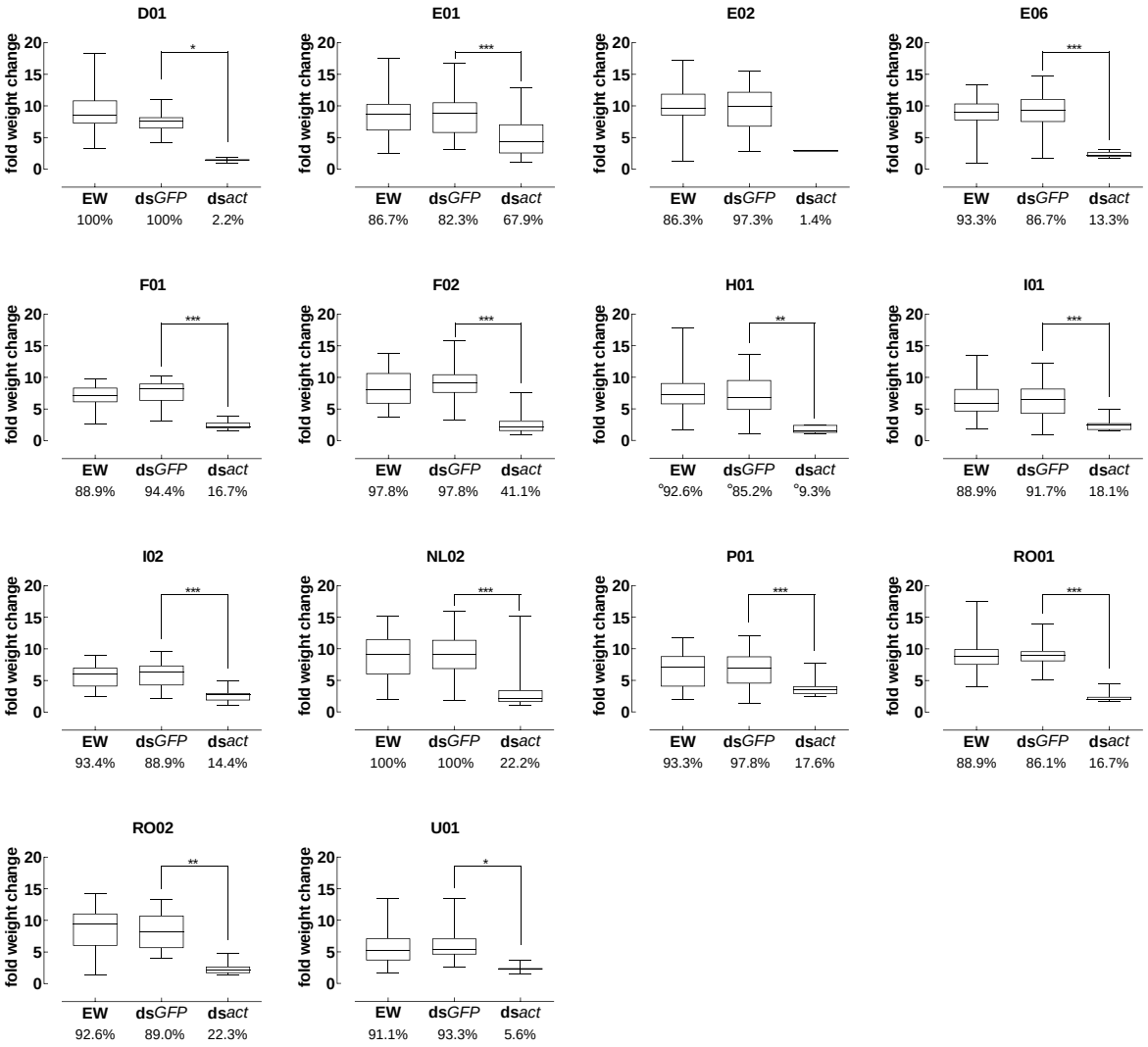

Figure 3.S3: Weight gain of surviving CPB larvae expressed as fold weight-change after five days of ds*act* treatment (30ng). EW (0.1% detergent) and ds*GFP* treatments served as negative controls. The percentages below treatments indicate the proportion of survivors used for weight analysis. Whiskers represent minimum and maximum values, boxes 25%-75% quartiles and lines the median. *p≤0.05, **p≤0.01 ***p≤0.001

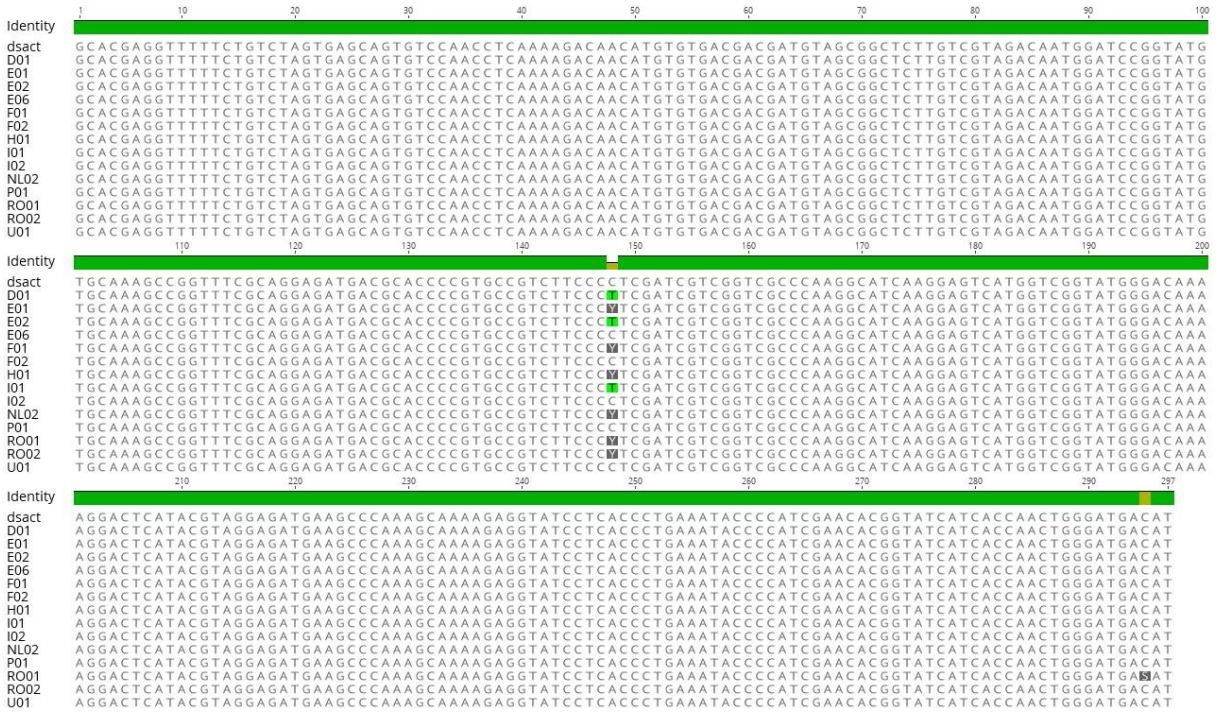

Fig. 3.S4: Target gene (*actin*) conservation among European CPB populations. The 297 bp ds*act* sequence used for silencing is based on GenBank entry EB761683.1 and served as a reference sequence displayed as DNA. Identity with this sequence is shown above in green, divergences in yellow with the individual differences highlighted within the sequences. Y = C or T, S = C or G. (For interpretation of the references to colour see web version of this article)

4. Discussion

4.1. Transfer of lethal target genes identified in *Tribolium castaneum* to other insect pest species

Many researchers have recognized the value of the recently identified highly lethal *T. castaneum* target genes and utilized them as starting points for their own research. So far and to the best of my knowledge, eight studies with other insect species using any of the lethal genes identified by Ulrich et al. (2015) were published during the period of my study. Six studies focused on other beetle species like emerald ash borer *A. planipennis* (Rodrigues et al., 2018), Western corn rootworm *D. v. virgifera* (Knorr et al., 2018), pollen beetle *B. aeneus* (Knorr et al., 2018), southern pine beetle *D. frontalis* (Kyre et al., 2019), willow leaf beetle *P. versicolora* (Xu et al., 2019b; Zhang et al., 2019) and Asian long horned beetle *A. glabripennis* (Dhandapani et al., 2020) and two studies examined Hemipteran species, i. e. brown marmorated stink bug *H. halys* (Mogilicherla et al., 2018) and Neotropical brown stink bug *E. heros* (Castellanos et al., 2019). However, in addition to the data presented for *P. cochleariae* in this work, only two other studies tested the full set of lethal genes (Dhandapani et al., 2020; Rodrigues et al., 2018), while the others investigated a selection of genes. Some of the lethal genes were tested more often such as *hsc70-3* in seven species, *P. cochleariae* included, whilst others such as *gw* were tested in just three species. Considering the variable experimental set-ups (delivery method, dsRNA concentration, time-point of examination etc.) partially attributable to the species tested, a comparison is rather complicated. The results and key parameters to each study are outlined in Table 4.1 which serves as an orientation for the following discussion.

4.1.1 Good transfer rate of lethal target genes in *P. cochleariae*

Selection of highly lethal target genes that are similarly efficacious in many insect pest species may allow for quick adaptation of the RNAi technology to new pest species. A species with a high transfer rate may be suited as a model organism since it implies a robust RNAi response and that it shares essential gene functions with other insects.

A high overall transfer rate of *T. castaneum* lethal genes to *P. cochleariae* was described in chapter 2. Compared to the other studies, it was only rivaled by *E. heros* where many *T. castaneum* orthologous genes were tested and also ended up being highly lethal after 14 days (highlighted in blue in Table 4.1), at least after injection (Castellanos et al., 2019). For several other species, either not as many genes were highly lethal resulting in low transfer rates or only a few genes were tested but proved to be efficient distorting an assessment of the transfer rate. For example *B. aeneus* was subjected to only

one of the genes (*rop*), resulting in mortality of approximately 90% both upon injection and feeding (Knorr et al., 2018). Therefore, *P. cochleariae* could be a good model insect for sprayable RNAi even in comparison with *E. heros* which would probably not be targeted by spray application because it is a piercing/sucking insect. Additionally, it shows that the target gene set described in *T. castaneum* is applicable to multiple pest insects.

4.1.2. Highly variable efficacy of individual target genes between species

When going into more detail from general transfer rates of the entire gene set to the performance of individual target genes, their efficiency varied greatly between the studies and the insect species.

The five genes *srp54k*, *α-SNAP*, *rop*, *rpn7* and *rpt3,* which elicited strong RNAi responses upon feeding in *P. cochleariae*, showed variable efficacy in other species (Table 4.1). Nevertheless, four of these genes lead to high mortality levels in at least one other species. For example, *srp54k*, next to the positive control *actin*, reached the highest overall mortality in *P. versicolora* and scored second-highest by only a small margin to *pros-α2* in *E. heros*. In *H. halys* and *A. planipennis*, *srp54k* at least elicits moderate levels of mortality and only in *A. glabripennis*, no mortality was observed. Already this first example covers the full range of efficacy levels demonstrating the discrepancy in transfer success of individual genes to other species. Comparison of responses to ds*α-SNAP* revealed similar results while for *rop* either none or high levels of mortality were observed. The only gene with good efficiency in *P. cochleariae* that did not elicit high mortality rates in other species, at least in those few species tested, was *rpt3*.

Contrariwise did the less efficient target genes for *P. cochleariae*, (*shi*, *PP-α*, *hsc70-3*, *cact*) not always behave similarly ineffective in other species, either, indicating that the use of *P. cochleariae* as a model for RNAi target gene screening may result in a loss of potentially highly lethal targets, despite the good transfer rate. This needs to be weighed against the benefits of pretesting or relying on results of target genes in *P. cochleariae,* such as the simple screening procedure for oral RNAi or easy maintenance. Both *hsc70-3* and *shi* were suitable targets in *A. planipennis* and *D. frontalis* with *hsc70-3* being the most active tested gene in these two species. This could have gone unnoticed when taking *P. cochleariae* data as a basis. *PP-α* was among the most lethal genes in two Hemipteran species, both of which belong to the Pentatomidae family, whereas it was less efficient in two tested beetle species in addition to *P. cochleariae*. It would be interesting to examine whether this is incidental or if *PP-α* is in fact a promising target for Pentatomidae in general. Testing of more members of this insect family could help to explore its inter-species efficacy, for example against *Nezara viridula* which was already shown to be amenable to RNAi (Riga et al., 2020).

Table 4.1: Comparison of studies using the eleven highly lethal genes found in *T. castaneum* by Ulrich et al. (2015). Key parameters of each study are indicated in the upper part of the table. Mortality rates [%] are shown for each of the eleven lethal genes, either estimated from figures (indicated with ~ before the number) or as stated in the publication. Mortalities of negative controls (GFP, YFP, IMPI) and positive controls (actin, IAP, V-ATPase) are included. Values of ≤ 40 % are marked in red, ≥ 80% in blue to highlight inefficient and highly lethal genes, respectively.

species	Phaedon cochleariae		Halyomorpha halys		Agrilus planipennis		Euschistus heros	Diabrotica virgifera	Brassicogethes aeneus		Dendroctonus frontalis	Anoplophora glabripennis		Plagiodera versicolora	
publication	this study, chapter 2		Mogilicherla et al. (2018)		Rodrigues et al. (2018)		Castellanos et al. (2019)	Knorr et al. (2018)	Knorr et al. (2018)		Kyre et al. (2019)	Dhandapani et al. (2020)		Zhang et al. (2019)	Xu et al. (2019)
developmental stage	2nd instar larvae		adults	2nd instar nymphs	neonates	adults	2nd instar nymphs	neonates	adults		adults	4th/5th instar larvae	adults	1st instar	2nd instar larvae
total no. of tested genes	9	9	13	5	13	2	16	50	4	4	3	48	18	6	4
delivery	injection	feeding	injection	feeding	feeding	feeding	injection	feeding	injection	feeding	feeding	injection	injection	feeding	feeding
dsRNA amount	150ng	0.3µg / 1µg / 3µg per leaf disc	1µg	20µg-soaked bean pole, exchanged for 3d	10µg/µl droplet for 4d	10µg/µl	27.5ng/mg body weight	500ng/cm² diet overlay	150nl 250ng/µl (=37.5ng)	initial: 5µl 1µg/µl, then diet with 500ng/cm2	10µg	8µg	10µg	? Heat-inactivated bacteria producing dsRNA	8ng/cm2, non-axenic
time point	d10	d10	d7	d7	d8	d10	d7 / d14	d9	d14	d14	d10	d15	d15	d7	d7
cactus	30.7	0 / 0 / 0	-	-	~43	-	~53 / ~67	-	-	-	-	0	-	~15	-
srp54k	100	100 / 100 / 100	~57	-	~48	-	~45 / ~98	-	-	-	-	0	-	75	100
rop	100	100 / 100 / 100	0	-	~40	-	-	82.8	~90	~90	-	0	-	-	-
α-SNAP	100	91.4 / 100/ 100	~43	-	~65	-	-	-	-	-	-	0	-	~60	100
shibire	63	11.4 / 0 / 0	~15	-	~80	~33	-	-	-	-	86.6	10	100	~10	?
PP-α	48	34.3/ 34.3 /45.7	100	~75	~67	-	~70 / ~96	-	-	-	-	0	-	-	-
inr-a	n.d.	n.d.	-	-	~72	-	~25 / ~30	51.89	-	-	-	0	-	-	-
hsc70-3	92.7	34.3 / 57.1 / 80	0	-	~92	~40	-	47.06	-	-	100	10	-	~38	-
rpn7	100	100 / 100 / 100	-	-	~62	-	~57 / ~95	59.2	-	-	-	0	-	-	-
gawky	n.d.	n.d.	-	-	~68	-	~50 / ~90	-	-	-	-	40	-	-	-
rpt3	100	100 / 100 / 100	-	-	~60	-	-	46.11	-	-	-	0	-	-	-
negative control	3.7 (GFP)	* / 2.3 (GFP)	*	*	~35 (GFP)	~10 (GFP)	~12 / ~18 (GFP)	11 (YFP)	~10 (IMPI)	~35 (IMPI)	~25	0 (GFP)	40 (GFP)	~15 (GFP)	*
actin	-	-	~43	-	~55	-	~60 / ~98	-	-	-	-	50	40	82	100
IAP	-	-	~70	~75	~55	-	-	-	-	-	~30	100	100	-	-
V-ATPase (various subunits)	-	-	100	~10	-	-	~55 / ~85	-	-	-	-	50	20	-	-

* Control was used for mortality calculation ? gene was tested in the study, but data were not available yet - gene was not tested in the study n.d. not detected

.

Interestingly, *cactus* did not elicit decent levels of mortality in most tested species, *P. cochleariae* included. The only species where moderate mortality levels were obtained was in *E. heros* (Castellanos et al., 2019). None of the other studies showed any transcript level data of *cactus* to indicate whether there were issues with gene suppression as in the case of *P. cochleariae* (Figures 2.6+2.7) or if there was (no) mortality despite knockdown. Nevertheless, the poor performance of *cactus* in most species indicates that this gene is of lesser interest as it lacks a good transferability.

For both *gw* and *inr-a*, only smaller fragments of the genes were found in the *P. cochleariae* transcriptome assembly and were thus not tested in *P. cochleariae*. Both targeted genes exceeded mortality rates of the positive controls in *A. planipennis*, although *inr-a* was not efficient in other species while *gw* was moderately to highly lethal in *A. glabripennis* and *E. heros*, respectively. Therefore, *inr-a* and *gw* should still be considered as putative target genes.

High identity to *T. castaneum* nucleotide or protein sequences was not predictive of the effectiveness of the individual target genes (see Tables 2.1+4.1, Castellanos et al., 2019; Knorr et al., 2018; Rodrigues et al., 2018). Neither appeared their involvement in important cellular processes like protein degradation (*rpn7*, *rpt3*) or vesicle trafficking (*α-SNAP*, *rop*) suggesting varying degrees of flexibility in regulatory networks including redundancy, expression patterns or in the overall RNAi response (depending on e. g. delivery strategy, tissue accessibility) between species. This somewhat contrasts with the study by Ulrich et al. (2015) in which the proteasome was proposed as a favorable target structure. It would be interesting to examine other components of the proteasome in other species in order test this hypothesis. Evidence in favor is already offered by the studies with *E. heros* and *A. glabripennis*. Five additional proteasome constituents were tested in *A. glabripennis* larvae, one of which (*pros-β5*) showed high mortality of 80 % (Dhandapani et al., 2020). In *E. heros*, *pros-α2* was the most efficient target gene. Therefore, the proteasome could still be a good target structure, but testing of more constituents than *rpn7* and *rpt3* may be necessary.

Similarly, considering the variable results on the efficiency of the individual genes, future studies could profit if they would not discard a (more or less) random subset of the eleven genes but testing the full set of genes instead, as it was done by Rodrigues et al. (2018) and Dhandapani et al. (2020), with the exclusion of *cactus*. This would also help to assess the value of the *T. castaneum* lethal genes in terms of transferability to other insect species, particularly those of importance as agricultural pests.

Nevertheless, it is fair to conclude that highly lethal genes identified in *T. castaneum* are a valuable starting point to explore effective RNAi in other species. In all tested species, at least one of the genes proved to be highly lethal, with the exception of *A. glabripennis* where none of the genes efficiently induced mortality in larvae despite the injection of high dsRNA amounts (Dhandapani et al., 2020).

4.1.3. Target genes identified in *T. castaneum* can outperform established target genes

One of the remarkable traits of the novel target genes emphasized upon identification in *T. castaneum* represented the increased efficiency in comparison to established target genes (Ulrich et al., 2015). Although no such gene was validated for *P. cochleariae* in chapter 2, some of the other studies included *actin*, *IAP* and/or various subunits of the *V-ATPase* as "positive controls" (Table 4.1). There, the trend from *T. castaneum* regarding higher efficacy of the novel target genes is continued, if in a somewhat attenuated form. For example, ds*srp54k* achieved the highest mortality together with ds*actin* in *E. heros* and *P. versicolora* (~98-100%). The same applies to ds*hsc70-3* in relation to ds*V-ATPase* in *H. halys*. In *A. planipennis*, eight of the eleven target genes demonstrated their higher efficacy by exceeding mortality rates of both *act* and *IAP*. In addition, the established target genes displayed quite variable efficacy among themselves, for example in *H. halys* and *A. glabripennis*. Therefore, good performance of such genes is not a given which questions their use as positive controls.

In summary, in each case in which established targets were tested apart from *A. glabripennis* larvae, one or more of the targets transferred from *T. castaneum* equaled or even exceeded other common target genes in terms of efficacy.

4.1.4. Effective transfer from dsRNA injection to feeding applications

When comparing injection and feeding experimental set-ups, oral dsRNA administration can be more complicated or requires more preparation and thus tends to be more time-consuming than injection (compare methods in e. g. Knorr et al., 2018; Luo et al., 2013; Mogilicherla et al., 2018; Wu et al., 2019). Therefore, injection of dsRNA is often used as a first means to validate functional RNAi responses in a species and to screen for and filter out suitable target genes before proceeding to feeding experiments (Dhandapani et al., 2020; Mogilicherla et al., 2018).

In my study, a direct comparison of application methods with all target genes was conducted which remains an exception amongst those studies summarized in Table 4.1, because many studies concentrated on a single treatment strategy or on few target genes. In general, only genes that performed well upon injection were effective upon feeding which is corroborated by results in *H. halys* and *B. aeneus*. In reverse, high efficacies upon feeding of dsRNA were always preceded by good results upon injection in the three species *P. cochleariae, H. halys* and *B. aeneus*. Still, validation through injection is not always predictive for good targets upon feeding, for example ds*hsc70-3* was highly lethal upon injection in *P. cochleariae* but not anymore upon oral administration of similar dsRNA amounts (Table 4.1). Weaker or no RNAi responses upon injection resulted in failure upon feeding as was seen for example for *shi* or *PP-α* in *P. cochleariae*.

In summary, injection is indeed a suitable method for preselection of target genes for insect pest control in feeding experiments. However, I would only recommend this detour if the experimental set-up for feeding is challenging or if many dsRNAs are screened. Otherwise, I suggest to directly test dsRNAs in a feeding situation, because injection does not save time with few target genes as the time needed for injection experiments and their analysis may be equivalent to directly testing all dsRNAs orally.

4.1.5 Synergism in dsRNA combinations could improve efficacy

Coadministration of *hsc70-3* and *shi* dsRNA in *A. planipennis* larvae and adults suggested a synergistic effect on mortality (Rodrigues et al., 2018) which was absent in *T. castaneum* where only additive effects were observed (Ulrich et al., 2015). In case of *hsc70-3* co-applied with the less potent *shi*, this even lead to lower efficacy compared to individual dsRNA treatment (Ulrich et al., 2015). Similarly, the feeding of two other dsRNAs targeting the genes *BiP* (binding immunoglobulin protein) and Armet (arginine rich, mutated in early stage of tumors) of the unfolded protein response protected by branched amphiphilic peptide capsule nanoparticles to *T. castaneum* lead to 40-50% mortality in single treatments but to 75% mortality in combination, indicating that only additive effects were at play in this case (Avila et al., 2018). In the mosquito *Aedes aegypti*, various combinations of five dsRNAs affecting spermatogenesis lead to higher levels of male sterility compared to individual dsRNA treatments (Whyard et al., 2015).

It would be interesting to investigate whether synergistic effects occur in other pest insects including *P. cochleariae* as well considering the advantageous effect of reducing the dsRNA amount that needs to be employed. When and why synergistic effects arise is not elucidated in the mentioned examples, if that is possible, considering that neither the same pair of genes worked synergistically equally well in different species nor are the targeted genes necessarily tied into the same pathways.

4.1.6. Developmental stages influence target gene efficacy

In *P. cochleariae*, both injection and feeding experiments were performed in the same developmental stage to ensure direct comparability (see section 4.1.4). However, also the adult life stage of a pest can infest and damage crops, therefore the examination of dsRNA efficacy in adults can be worthwhile. In *H. halys*, *A. planipennis* and *A. glabripennis*, dsRNA efficacies in different developmental stages were explored, typically comparing the adult against a juvenile life stage (Dhandapani et al., 2020; Mogilicherla et al., 2018; Rodrigues et al., 2018). *A. planipennis* adults displayed reduced mortality levels compared to neonates in a feeding scenario with ds*shi* and ds*hsc70-3* though the reasons were

not explored in the original study. This is in line with CPB where the RNAi sensitivity was reduced with each larval instar (Guo et al., 2015). One possible reason for this difference is the increased body size of older instars and adults compared to freshly hatched larvae which could result in a dilution effect of the dsRNA. Other reasons include the differential expression of midgut nucleases, target genes or the RNAi machinery.

In *H. halys*, the contrary was shown for *PP-α* which was a more efficient target gene in adults upon injection compared to nymphs upon feeding. A previous study showed that long dsRNA is relatively stable for at least one day in soaked bean poles (Ghosh et al., 2017) suggesting that sufficient amounts of long dsRNA is presented to the nymphs which makes dsRNA degradation by plant tissues as a contributing factor less likely. dsRNA was shown to be stable in *H. halys* saliva as well, though only in adults (Mogilicherla et al., 2018). Nevertheless, dsRNA injection was generally more efficient than feeding (see section 4.1.4), therefore the two different delivery strategies complicate the analysis of the influence of developmental stages in the *H. halys* RNAi response.

More prominently, when *shi* was tested via injection in *A. glabripennis* adults, it resulted in 100% mortality compared to 10% in larvae (Dhandapani et al., 2020). Considering this discrepancy, it would have been interesting to check the adulticidal efficacy of the other dsRNAs as well.

In summary, these results demonstrate the need to predefine the developmental stage that should be controlled by dsRNA treatment as differences in susceptibility independent from concentration / body weight ratios may exist.

4.2. Implications of RNAi based pest control for beneficial insects

One of the promoted advantages of RNAi compared to other strategies of insect control is its selectivity based on nucleotide sequence divergence resulting in single species specificity or at least a rather narrow species spectrum to be targeted. Therefore, non-target arthropods including beneficial insects such as bee pollinators (e. g. *Apis mellifera, Bombus terrestris*) and predatory insects such as lady beetles (e. g. *Coccinella septempuncata*, *Adalia bipunctata*, *Coleomegilla maculata*), green lacewing (*Chrysoperla carnea*) or parasitoid wasps (e. g. *Pediobius foveolatus*, *Trichogramma evanescens*) are generally considered unaffected by dsRNA treatments targeting specific pests when appropriate precautions are taken (Bachman et al., 2016; Grousset, 2019).

Such precautions to ensure the protection of non-target organisms include the bioinformatic screening for nucleotide sequence identities between dsRNA sequences designed for pest control and the respective sequences of orthologous genes of beneficials, i. e. checking the chosen sequence for the

absence of ≥21bp matches in beneficial insect sequences. Such an *in silico* approach was performed for *P. cochleariae*-based dsRNAs in comparison to *A. mellifera* and *C. septempunctata* orthologous sequences (Table 2.3). Ideally, the sequence is subjected to a genome wide scan to exclude potential off-target effects (targeting of unrelated genes leading to unintended effects). To this end, a public, web-based tool called DEQOR is available for human, mouse, fruit fly and other model organisms (Henschel et al., 2004). However, this tool is restricted to these few organisms making this otherwise helpful tool unavailable for comparison of dsRNAs to other non-target organisms. Instead, most studies focus on the affected region of the target gene. The potential for overlapping 21mers between gene sequences of pest and beneficial species is thought to be higher for more closely related species.

Insectivorous species of the Coccinellidae (lady beetles) family of Coleoptera are used in integrated pest management programs to control aphids or scale insects. They share the insect order of Coleoptera with the insects that will most likely be targeted by dsRNA for pest management purposes, including *P. cochleariae* and CPB, and may share the susceptibility towards RNAi commonly observed with beetles (see section 1.7). Therefore, dsRNA sequences should be checked early on for possible 21bp overlaps, ideally when designing the primers for dsRNA production and before dsRNA testing. This was overlooked in the *P. cochleariae* study so that the dsRNAs were only examined after already testing dsRNA efficacy.

Considering the insectivory of beneficial lady beetles, the question remains in how far they are really at risk for dsRNA exposure in sprayable RNAi instead of only considering the hazard-based risk assessment that is represented by the bioinformatical analysis. Together with their prey, lady beetles could potentially be present in the same fields as Coleopteran pests that are targeted by RNAi control methods, therefore it is important to consider adverse RNAi mediated effects on trophic interaction levels. In fact, in addition to the described potential tritrophic interaction, *Coleomegilla maculata* can for example extend its food spectrum to include CPB larvae (Groden et al., 1990) and thus possibly be exposed to applied dsRNA. *C. maculata* and related European species such as *C. septempunctata* and *A. bipunctata* were shown to be susceptible to dsRNA treatment, though dsRNA amounts exceeding reasonable field rates were provided to the larvae (Haller et al., 2019; Yang et al., 2015). The inclusion of these species in ecological risk assessment for RNAi appears sensible as was initiated for *C. septempunctata* in chapter 2 and done for ds*snf7* for WCR control in which dsRNA without 21nt overlaps did not affect *C. maculata* 1st instar larvae (Bachman et al., 2013, 2016). In fact, the chosen *snf7* dsRNA region did not show any overlap of any possible 21bp sequences in 23 non-target species and biotests revealed no adverse effects on survival in any of these species (Bachman et al., 2016).

These results emphasize that *P. cochleariae* lethal dsRNAs sequences should be adjusted to avoid possible 21nt-overlaps to *C. septempunctata* (chapter 2). Ideally, the improved dsRNAs should then be tested again by injection or oral feeding experiments in *P. cochleariae* to ensure that the new dsRNAs retained their efficacy and in *C. septempunctata* and *A. mellifera* to exclude off-target effects. In the future, more insect species may be included in the risk assessment and the design of appropriate dsRNA sequences, as more and more insect species are sequenced in integrated efforts such as the i5k initiative with its associated Ag100 pest initiative (i5k Consortium, 2013). Data are collected in public databases such as NCBI or "InsectBase", facilitating access and comparison of insect sequences (Sayers et al., 2019; Yin et al., 2016). In 2019, a total of 401 insect genomes were assembled, 155 of which were annotated though not all of these represent different species (Li et al., 2019).

Tests on bee pollinators are mandatory for registration of pesticides in Europe, particularly insecticides (EFSA, 2013b), therefore one can assume that they would be mandatory for insecticidal dsRNAs as well. Ecotoxicological effects on honey bees were evaluated as part the risk assessment for WCR ds*snf7* which is the active dsRNA species in the first GM-crop with insecticidal dsRNA-traits approved by USA authorities (Head et al., 2017; ISAAA website; Tan et al., 2016). However, accounts on RNAi efficiency in the honey bee *A. mellifera* are quite variable. It was shown that different dsRNA amounts were necessary to achieve knockdown of target genes with various effectiveness, an observation partially explained by differences in tissue susceptibility (Aronstein et al., 2006; Guo et al., 2018; Jarosch and Moritz, 2011). When choosing a worst case scenario with ds*vATPaseA* from WCR with the highest homology to honey bee, no adverse effects were found (Vélez et al., 2016a). In fact, even in case of full sequence identity with honey bee *vATPaseA*, survival of *A. mellifera* larvae and adults was not affected after dsRNA feeding (Vélez et al., 2016a). Contrastingly, off-target effects depend on dsRNA sequences but were even reported with ds*GFP* despite a lack of overlap with genomic sequences (Jarosch and Moritz, 2012). Taken together, both injection and oral delivery of dsRNA produced contradictory results which may reflect the high genetic variability of honey bees (Wallberg et al., 2014) and point towards population differences of the RNAi response.

An exclusion of 21 bp overlaps in dsRNA targeting *P. cochleariae* to *A. mellifera* as suggested in chapter 2 therefore is mandatory in order to mitigate lingering concerns. Additionally, all dsRNA targeting essential genes in pest species require careful experimental risk assessment to exclude both gene-specific and -unspecific effects and not just *in silico* comparison of target genes. Appropriate test methods were described by Vélez et al. (2016), however bee pollinator standard acute or chronic toxicity tests as mandatory for insecticides are available for honey bee adults and larvae as well as bumble bees which may receive higher acceptance from authorities (OECD, 2017a, 2017b, 2013, 1998).

So far, only safety concerns of potential dsRNA-based pest control products for beneficial insects were discussed. RNAi may hold promising health-care options for honey bees as well, which could profit from the highly lethal genes from *T. castaneum*. The small hive beetle *Aethina tumida* is an emerging invasive threat to bee colonies (Giangaspero and Turno, 2015; Neumann et al., 2016). A recent study revealed that treatments with dsRNA did not affect bee health, but decreased *A. tumida* survival both after injection and feeding though oral administration requires refinement of delivery strategies to overcome restrictions of putative gut nucleases (Powell et al., 2017). Considering that always at least one of the eleven target genes proved to be highly lethal when transferred to another species (see section 4.1.2), it is likely that suitable target genes for this pest can be identified fairly quickly.

RNAi using the *T. castaneum* target gene set could also help defend bee hives against the devastating honey bee ectoparasite *Varroa desctructor*, called Varroa mite. It can act as a virus vector, though these viruses such as Israeli acute paralysis virus (Chen et al., 2014; Hunter et al., 2010; Maori et al., 2009) or deformed wing virus (Desai et al., 2012) can be separately controlled by RNAi-mediated suppression. Even sequence-independent dsRNA treatments induced unspecific responses in bees leading to a decrease in viral infections (Flenniken and Andino, 2013). Incidence of the Varroa mite was significantly decreased by dsRNA feeding to honey bees and horizontal transfer to the mite via hemolymph (Garbian and Maori et al., 2012) while injection-based screening identified several promising candidate genes affecting survival and fecundity (Huang et al., 2019). It would be interesting to see how the highly lethal genes from *T. castaneum* would perform in *V. destructor* as a non-insect arthropod. The two-spotted spider mite *Tetranychus urticae* as another member of the order Acarida could serve as a surrogate species for the Varroa mite. RNAi responses can be elicited orally in this chelicerate by soaking in dsRNA solution or via leaf discs (Kwon et al., 2016; Suzuki et al., 2017), a well annotated genome allows for dsRNA target sequence selection (Grbić et al., 2011) and easy maintenance coupled with fast reproduction characterize *T. urticae* as a good model organism (Grbic et al., 2007). In fact, the highly lethal genes from *T. castaneum* were successfully tested in *T. urticae* with a similar efficacy profile of each target gene compared to *P. cochleariae* (Bensoussan et al., 2020). Apart from opening new lethal genes for the control of this devastating pest and other spider mites, this study offers starting points for dsRNA-based Varroa mite control. Additionally, the data from *T. urticae* demonstrate that the lethal target genes identified in *T. castaneum* are conserved and essential enough to even affect a different subphylum of arthropods in RNAi experiments.

Taken together, this shows that if the appropriate precautions are taken, RNAi can be both, a promising insect pest control measure as well as an insect health care agent.

4.3. Inter-population variability

4.3.1. RNAi sensitivity can vary within a species

Many studies examined the susceptibility of various species of different insect orders towards RNAi (see section 1.3.2), but only few studies so far have elaborated on possible susceptibility variation within a species (Kitzmann et al., 2013; Sugahara et al., 2017; H. Wang et al., 2018). Similar RNAi responses in the target species however are critical for successful implementation of RNAi as a pest control strategy to ensure consistent results across countries and climates. The few studies available pertaining intraspecific variation were introduced in section 1.5, and results of this study in CPB presented and discussed in chapter 3. Essentially, it was shown that differences in RNAi responses exist though the degree of intraspecific variability can vary greatly as well. In addition to these direct reports, contradictory results on susceptibility to oral dsRNA administration in literature may point towards variability within species as well.

One example can be found in *T. castaneum* where multiple reports exist in which oral delivery of naked dsRNA successfully induced mortality and target gene knockdown (Abd Halim et al., 2016; Cao et al., 2018; Whyard et al., 2009) while others were not successful (Miyata et al., 2014; Singh et al., 2017; Spit et al., 2017) and required nanoparticles for effective delivery (Avila et al., 2018). Without nanoparticles, uptake of dsRNA by midgut epithelial cells and further distribution to fat body and Malpighian tubules was not observed (Avila et al., 2018). Whether this was due to dsRNA degradation by midgut nucleases or a lack in uptake was not specified. Although no difference in susceptibility was found in *T. castaneum* populations from China, dsRNA was administered via injection, not through feeding (Wang et al., 2019) and injection in *T. castaneum* is considered to result in strong RNAi responses (Brown et al., 1999; Bucher et al., 2002; Tomoyasu and Denell, 2004). Taken together, an injection study reporting a lack of population variability of RNAi sensitivity in *T. castaneum* may not present the whole picture and a following generalization to an apparent homogenous RNAi response in *T. castaneum* could still be questioned by a study comparing populations from various locations and / or laboratories faced with environmental RNAi since the studies reporting ineffective RNAi used a feeding approach.

Other examples include hemipteran insects where the success of RNAi is more variable between species, and discrepancies within a species were published as well. Comparatively good mortality rates and disruption of feeding were observed upon feeding of *vATPase* or injection and feeding of *c002* dsRNA to *Acyrthosiphon pisum*, respectively (Mutti et al., 2008, 2006; Whyard et al., 2009), while another study targeted the same genes at the same rates but reported no phenotypic effects due to dsRNA degradation by nucleases (Christiaens et al., 2014). A similar approach in *Myzus persicae* failed

to reproduce RNAi responses previously shown to reduce fecundity which was again attributed to the presence of nucleases (Ghodke et al., 2019; Pitino et al., 2011).

The order of Lepidoptera is generally less susceptible towards RNAi though some species appear to be amenable at least to dsRNA injection (Terenius et al., 2011). In tissues of the lepidopteran insect *Spodoptera frugiperda*, the lack of processing of long dsRNA to siRNAs due to endosomal entrapment is considered a major reason for the RNAi insensibility of this species (Yoon et al., 2017). Nevertheless, other studies claim successful RNAi in larvae and Sf21 cells resulting in gene knockdown for various target genes or juvenile hormone modulation by the suppression of regulatory neuropeptides (Ghosh et al., 2016, 2014; Griebler et al., 2008; Rodríguez–Cabrera et al., 2010).

Together, these examples highlight the necessity to raise awareness and to investigate possible intraspecific variability and its molecular basis. My study is one of the first ones providing such data and I found some natural variability in the response, and although it might be too low to interfere with application in pest control in the beginning, it may be a starting point for the selection for resistance.

4.3.2. Possible reasons for population variability of the RNAi response

Several different mechanisms were described, which should be tested in CPB in future studies to identify the mechanistic basis of variability that I found. In numerous cases, the presence of midgut dsRNA nucleases appears to play a major role to block RNAi susceptibility in various insect species. Although some studies show that RNAi response variability can exist within a species, the reason for this is rarely explored and remains elusive, including studies on CPB carried out here (chapter 3).

In the migratory locust, environmental RNAi results in the degradation of dsRNA by midgut nucleases which were found to be the reason for the discrepancy of *L. migratoria* sensitivity towards orally delivered or injected dsRNA (Luo et al., 2013; Song et al., 2019, 2017). However, the study by Sugahara et al. (2017) confirmed population variability already upon injection excluding midgut nucleases as the only factor for RNAi tolerance. Instead, RNAses present in the hemolymph which normally degrade dsRNA at suboptimal pH conditions (hemolymph pH at 7, optimal activity at pH 5) (Song et al., 2019) could have adjusted to the environment or *vice versa* to more rapidly degrade dsRNA (Ren et al., 2014). Alternatively, the uptake of dsRNA may be hindered in the insensitive locusts, similarly to their ovary and follicle cells where dsRNA uptake is blocked (Ren et al., 2014). However, this remains to some extent speculative.

In the present CPB study, neither dsRNA uptake nor dsRNAses were directly tested as possible contributors to the sensitivity shift most prominently observed in the E01 population sampled in Spain.

The presence of nucleases in the midgut of *L. decemlineata* was already confirmed and appeared to somewhat influence RNAi efficiency (Spit et al., 2017). However, considering the good target gene knock-down also in the E01 population, this may not be relevant. Nevertheless, expression levels and activities of midgut nucleases could have been evaluated by qPCR and enzyme assays, respectively, to fully exclude their contribution. Midgut juice extracts are often used to assess dsRNA degradation in literature, though possible pH changes or the influence of enzymes of the destroyed surrounding tissue due to the preparation method complicate the analysis of such data.

Altered uptake efficiency in CPB populations was briefly discussed as another mechanism for RNAi response variability in chapter 3 and a time series study was suggested. In such an approach, the expression levels of the already tested genes (*act*, *dcr2*, *ago2a*, *stauC*, *chc*, *vha16*) could be monitored in shorter time intervals such as 3h or earlier to catch possible upregulation of uptake genes probably preceding RNAi machinery expression (dcr2, ago2a) modulation after 6h (Guo et al., 2015), and later time points at 12h, 16h and/or 24h to monitor possible regulation variability between the populations.

Another yet related mechanism could pertain upstream players of the RNAi machinery. The temporal expression of RNAi machinery regulator FOXO could be altered between populations as well which would most likely be visible at early time points. Additionally, direct uptake of fluorescently labeled dsRNA into midgut cells and distal tissue like fat body cells and its temporal dispersion within the insect could be observed by microscopy of dissected tissues at various time points. Such experiments would clarify the role of RNAi machinery and dsRNA uptake mechanisms in inter-population variability of the RNAi response.

An additional aspect for RNAi insensitivity discussed in the literature is represented by viral infections. Some viruses have evolved protein effectors that subdue the RNAi response of their host. Persistent viral infection caused for example by the flock house virus, Drosophila C virus or Nora virus inhibited RNAi machinery assembly or activity (Berry et al., 2009; Li et al., 2002; Nayak et al., 2010; van Mierlo et al., 2012). While these viral RNAi suppressors allow viral replication in their host (pest) insect, concomitant treatment with insecticidal dsRNA may not be efficient in eliciting the desired effect anymore. Alternatively, the RNAi machinery could be overloaded or diverted by the expression of excess viral RNA to undercut the RNAi response of insects (Flynt et al., 2009; Goic et al., 2013). However, despite recent additions only few viruses infecting beetles were identified, especially compared to their diversity in species (Käfer et al., 2019; Swevers et al., 2013). Merely one report mentions the possibility of viral infection in *L. decemlineata* (Kanyuka, 1984, referred to in Selman, 1988). Deep sequencing could reveal possible viral infections within CPB populations, though the following characterization of the virus(es), possible viral effectors and their effect on the RNAi response

would require exceptional efforts as part of a lengthy process. Despite the lack of research regarding beetle viruses, their low number in general may indicate that their contribution to possible interspecific variability in CPB could be low though it cannot be excluded completely.

Another factor possibly influencing population variability, at least when considering environmental RNAi only, could be the microbiome. In *P. versicolora*, RNAi efficiency was increased when bacteria inhabited their alimentary tract (Xu et al., 2019b). Especially *Pseudomonas putida* profited from the additional nutrition provided by dsRNA degradation products causing overgrowth and infection of the beetle (Xu et al., 2019b). The disruption of gut epithelia and translocation of bacteria accelerated the RNAi-induced mortality (Xu et al., 2019b). In *L. migratoria*, the gut microbiome was altered even upon dsRNA injection (Xie et al., 2019). Such a connection to gut bacteria was already found in other contexts. Mortality due to fungal disease in the pine beetle *Dendroctonus valens* is augmented by additional bacterial infection (Xu et al., 2019a). Gut bacteria can enhance the insecticidal effects of *Bacillus thuringiensis* (Bt) Cry toxins in *Lymantria dispar* and *Spodoptera littoralis* (Broderick et al., 2006; Caccia et al., 2016) though these results need to be treated with caution as rearing of insects on antibiotics can introduce artifacts by impeding Bt toxin activity (Johnston and Crickmore, 2009).

P. putida was also found among the bacteria in the gut of CPB and can act as an entomopathogen in larvae (Muratoglu et al., 2011). In other midgut isolates, this species was not present but other closely related bacteria instead (Blackburn et al., 2008), already demonstrating the variability of the microbiome composition in this beetle species. Consequently, the presence or rather the absence of certain bacterial strains could explain the temporal differences in RNAi onset. The composition of the bacterial community between CPB populations before and during dsRNA treatment and presence of bacteria in the haemocoel could be analyzed analogous to the approach used by Xu et al. (2019b) or Caccia et al. (2016), i. e. the amplification and sequencing of 16S rRNA sequences and histological sections of distal tissues.

The presented examples offer possible starting points for future experiments for the continued investigation of reason for the minor variability in RNAi responsiveness in CPB populations. The restriction to populations representing the extremes described in my work would facilitate and speed up data acquisition.

4.3.3. Implications for resistance evolution

Repeated exposure of insect pests to dsRNA in the field could promote the selection of resistant populations carrying resistance alleles at low frequency conferring low-level RNAi tolerance as

observed in one of the populations studied here, strain E01. Considering that the resistance mechanism of a laboratory selected WCR strain confers resistance to the entire RNAi mode of action and not only to the originally targeted dsRNA due to blocked dsRNA uptake (Khajuria et al., 2018), RNAi should only form part of an otherwise integrated insect control strategy utilizing several options including for example chemical control and transgenic crops expressing pore-forming proteins such as Cry toxins. One such effort is represented by stacked expression of three Cry toxins in combination with Dv*snf7* in maize (Head et al., 2017). Cross-resistance of the dsRNA-resistant WCR population towards Bt toxins or *vice versa* was not detected, indicating that pyramiding of these two independent modes of action may prolong the durability of both pest control strategies (Head et al., 2017; Khajuria et al., 2018; Moar et al., 2017). Control of *Helicoverpa armigera* on transgenic cotton expressing Bt toxin and RNAi traits produced similar results (Ni et al., 2017). Evidence suggests no cross-resistance of RNAi to synthetic insecticides either, demonstrated in emamectin benzoate-resistant *Frankliniella occidentalis* (Han et al., 2019) and considering the fact that many CPB populations used in this thesis showed resistance or tolerance towards deltamethrin and/or thiacloprid (Nauen, personal communication). Selection for dsRNA resistance in the Southern corn rootworm *Diabrotica undecimpunctata howardi* closely related to WCR did not considerably decrease RNAi sensitivity even after seven generations although applied selection pressure resulted in fitness costs (Pereira et al., 2019). Taking into account the recessive autosomal inheritance of the dsRNA resistance allele in WCR (Khajuria et al., 2018), RNAi as a pest control strategy is likely to spread rather slowly if appropriate resistance management tactics are implemented.

4.4. Concluding remarks on the future of RNAi in agriculture

Insect pest control by specific, insecticidal dsRNAs is generally still on the verge of commercialization. So far, only one product, the GM-maize SmartStax PRO expressing ds*snf7* amongst other traits (Head et al., 2017), gained approval and awaits its launch in the United States of America (ISAAA website).

However, RNAi-based products in agriculture in general have been on the market for more than twenty years. Initially in 1995, the squash cultivar Freedom II was the first crop with RNAi traits to be commercialized (Fuchs et al., 1998; Schultheis and Walters, 1998). Shortly afterwards in 1998, transgenic papaya targeting a coat protein of the papaya ringspot virus were commercialized in Hawaii to confer resistance against this devastating plant disease and are estimated to make up 77% of Hawaiian papaya plants in 2017 (Ferreira et al., 2002; ISAAA, 2017). More crops were genetically modified to utilize the RNAi mechanism to combat viral diseases, adjust nutritional value, improve crop quality or modulate secondary metabolite contents with subsequent variety approval and/or

commercialization such as bean (Bonfim et al., 2007), rice (Iida et al., 1993; Kusaba et al., 2003), potato (Waltz, 2015), tomato (Gupta et al., 2013), alfalfa, soybean, plum and apple (Baranski et al., 2019; ISAAA website; ISAAA, 2017). RNAi-based insect pest control can therefore arguably be considered an extension of this plant protection technology, with new benefits and risks.

The above-mentioned crop examples all used a transgenic approach. Sprayable RNAi for insect control as it was investigated in more detail in this thesis circumvents several problems of GM crops such as lacking public support of this technology especially in Europe, long registration processes or difficulties in the efficient generation of transgenic plants (Altpeter et al., 2016). With foliarly applied dsRNA, adjustments to target new or multiple pest species can be realized more quickly. Considering the fast degradation of dsRNA in the environment leaving no residues (only nucleotides), the possibility to create selective measures targeting individual pest species beneficial to non-target arthropods, inferred low health risks (Aliabadi et al., 2012; Petrick et al., 2015; Witwer and Hirschi, 2014) and low application rates (chapters 2 and 3), this control measure would be attractive not only for conventional agriculture, but particularly for organic farming – as dsRNA molecules are natural compounds and do not belong to synthetic chemical insecticides.

One drawback of RNAi as a control strategy is its rather slow action taking several days to elicit insecticidal effects (chapters 2 and 3), during which the insects can still directly damage the crop or transmit diseases. For this reason, dsRNA is not suitable for insect control in ornamental plants or cut flowers where pristine appearance is desired. Another drawback is the limited number of insect species that are efficiently targeted by RNAi. Stabilization of dsRNA with the focal aim to improve oral delivery to the respective insect was attempted by complexation with numerous nanoparticle types (Avila et al., 2018; Castellanos et al., 2019; Christiaens et al., 2018; He et al., 2013; Parsons et al., 2018; Zhang et al., 2010). While these formulations show promise, they may not be available to organic farming and need to be tested whether improved delivery also extends to mammals.

Additionally, prices for dsRNA products need to be comparable to current insecticides. For example, the “Decis forte” formulation with the pyrethroid deltamethrin as active ingredient costs approximately 50-60€/l depending on the vendor (e. g. Avagrar; myAGRAR). This corresponds to roughly 3-4€ per hectare at a field application rate of 5-7.5g/ha in potato or cereal crops (BVL, 2020a, 2020b, 2020c). Advances in the production of long dsRNA considerably dropped production costs below 0.5$/g (Maxwell et al. from GreenLight Biosciences). The results from this thesis indicates that field rates of 10g/ha may be enough to manage insect pests (depending on the species and target gene), as this rate was sufficient for *P. cochleariae* control with e. g. ds*rpn7* or ds*rpt3* (comparable to lowest rate used in chapter 2) and exceeded the rate necessary to control CPB larvae from different

locations (ten times higher than the rate used in chapter 3). Together, a production cost of at least 5$/ha anticipates much higher costs for the finished, marketable product. Prices of approximately 20-40€/ha for the Bt toxin-based product “Xentari” requiring application rates of at least 324g/ha in vegetable crops like cabbage, root vegetable or tomato raise hope that sprayable dsRNA products could be competitive (Avagrar; BVL, 2020d, 2020e, 2020f, 2020g)

Fast degradation of dsRNA is not just an advantage, but also represents a problem if a species has numerous generations per season making additional treatments necessary which are likely to be much more expensive than conventional insecticide sprays. Greenhouse-grown crops avoid one predominant source of degradation - UV light – deeming it the most favorable environment for foliarly applied dsRNA. Nevertheless, companies are pursuing field application of sprayable dsRNA as well with apparently encouraging results in field trials (GreenLight Biosciences; Syngenta).

Taken together, the future of sprayable RNAi in agriculture is difficult to predict and the balance can still tip either way. It continues to face many limitations and therefore might end up only as a niche product for specific pest control problems or as a putative resistance-breaking agent, despite its positive qualities. Instead, the focus of insecticidal RNAi could shift from agriculture to other insect nuisances such as ants and termites in domestics (Choi et al., 2012; Raje et al., 2018; Zhou et al., 2008) and mosquitoes as vectors of human diseases (Hapairai et al., 2017; Kumar et al., 2013; Mysore et al., 2019). Depending on the development of political and regulatory frameworks in Europe and other regions, RNAi may find its niche in some agricultural and horticultural production systems as a future alternative to chemical insecticides.

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