EXAMINING THIACLOPRID, ESSENTIAL OILS AND DOUBLE-STRANDED RNA FOR POTENTIAL USE IN BIOSAFE MANAGEMENT OF POLLEN BEETLE

TIAKLOPRIIDI, TAIMSETE EETERLIKE ÕLIDE JA KAHE-AHELALISE RNA RAKENDAMISE VÕIMALUSED HIILAMARDIKATE KESKKONNASÄÄSTLIKUS TÕRJES

ONDERZOEK OMTRENT THIACLOPRID, ESSENTIËLE OLIËN EN DUBBELSTRENGS RNA VOOR MOGELIJK GEBRUIK IN EEN VEILIG BEHEER VAN STUIFMEELKEVER

JONATHAN WILLOW

A Thesis
Submitted to Estonian University of Life Sciences (EMÜ) and Ghent University (UGent) in partial fulfilment of the requirements for the joint degree of Doctor of Philosophy in Agriculture (EMÜ) and Bioscience Engineering (UGent)

Väitekiri
Filosoofiadoktori kraadi taotlemiseks põllumajanduse (EMÜ) ja Biotehnoloogia erialal (UGent)

Tartu 2021

Eesti Maaülikooli doktoritööd

Doctoral Theses of the
Estonian University of Life Sciences
Doctoral Thesis of Ghent University

EXAMINING THIACLOPRID, ESSENTIAL OILS AND DOUBLE-STRANDED RNA FOR POTENTIAL USE IN BIOSAFE MANAGEMENT OF POLLEN BEETLE

TIAKLOPRIIDI, TAIMSETE EETERLIKE ÕLIDE JA KAHE-AHELALISE RNA RAKENDAMISE VÕIMALUSED HIILAMARDIKATE KESKKONNASÄÄSTLIKUS TÕRJES

ONDERZOEK OMTRENT THIACLOPRID, ESSENTIËLE OLIËN EN DUBBELSTRENGS RNA VOOR MOGELIJK GEBRUIK IN EEN VEILIG BEHEER VAN STUIFMEELKEVER

JONATHAN WILLOW

A Thesis
Submitted to Estonian University of Life Sciences (EMÜ) and Ghent University (UGent) in partial fulfilment of the requirements for the joint degree of Doctor of Philosophy in Agriculture (EMÜ) and Bioscience Engineering (UGent)

Väitekiri
Filosoofiadoktori kraadi taotlemiseks põllumajanduse (EMÜ) ja Biotehnoloogia erialal (UGent)

Tartu 2021

Institute of Agricultural and Environmental Sciences
Estonian University of Life Sciences

According to verdict No. 6-14/3-2, of March 16, 2021, the Doctoral Committee of Agricultural Sciences of the Estonian University of Life Sciences has accepted the thesis for the defence of the degree of Doctor of Philosophy in Agricultural Sciences.

Opponent: **Dr. Samantha Cook**
Rothamsted Research, United Kingdom

Supervisors: **Prof. Eve Veromann**
Estonian University of Life Sciences

Prof. dr. ir Guy Smagghe
Ghent University, Belgium

Defence of the thesis:
Estonian University of Life Sciences, room D239, Fr. R. Kreutzwaldi 5, Tartu; International video conference in the BigBlueButton web room on April 12, 2021, at 12:15

This research was supported by European Social Fund's Doctoral Studies and Internationalization Programme DoRa. The publication of this thesis was supported by the Estonian University of Life Sciences and the European Unions's European Regional Development Fund (Estonian University of Life Sciences ASTRA project 'Value-chain based bioeconomy')

© Jonathan Martin Willow, 2021
ISSN 2382-7076
ISBN 978-9949-698-70-7 (pdf)

CONTENTS

LIST OF ORIGINAL PUBLICATIONS 7
ABBREVIATIONS 9
1. INTRODUCTION 10
2. REVIEW OF THE LITERATURE 13
   2.1. Potential toxicity of thiacloprid, and synergistic toxicity when applied in combination with tebuconazole, in hymenopteran parasitoids 13
   2.2. Potential for using plant-based essential oils in pollen beetle management 14
   2.3. Potential for developing an RNA interference strategy towards pollen beetle management 15
3. AIMS AND HYPOTHESES 19
4. MATERIAL AND METHODS 21
   4.1. Insects and plants 21
   4.2. Experimental compounds and products 22
   4.3. Experimental setups 24
      4.3.1. Examining acute effect of thiacloprid alone and in combination with tebuconazole, in a model parasitoid (**I**)... 24
      4.3.2. Examining acute effect of EOs on pollen beetle (**II**) ... 27
      4.3.2.1. Screening EOs via topical dosing 27
      4.3.2.2. Acute effect of exposure to dry residues of cinnamon bark EO, on oilseed rape leaf and bud tissue 28
      4.3.3. Examining RNAi in pollen beetle 29
      4.3.3.1. Screening of potential RNAi targets via microinjection 29
      4.3.3.2. Confirming oral RNAi in pollen beetle 30
      4.3.3.3. RNAi via consumption of dsRNA-treated buds (**IV**) 33
      4.3.3.4. Larval RNAi via consumption of dsRNA-treated anthers (**V**) 36
      4.3.3.5. Short-term vs chronic dsRNA feeding (**VI**) 37
   4.4. Data analysis 40
      4.4.1. Analysing acute effect of thiacloprid alone and in combination with tebuconazole, in a model parasitoid (**I**)... 40
      4.4.2. Analysing acute effect of EOs on pollen beetle (**II**).... 40
      4.4.3. Analysing RNAi in pollen beetle 40

5. RESULTS 42
5.1. Acute effect of thiacloprid alone and in combination with tebuconazole, in a model parasitoid (**I**) 42
5.2. Acute effect of EOs on pollen beetle (**II**) 44
5.2.1. Screening EOs via topical dosing 44
5.2.2. Leaf and bud treatment 47
5.3. RNAi in pollen beetle 48
5.3.1. Microinjection screening 48
5.3.2. Confirmation of oral RNAi in pollen beetle 49
5.3.3. Bud feeding induced RNAi (**IV**) 52
5.3.4. Larval RNAi in pollen beetle (**V**) 54
5.3.5. RNAi is enhanced by chronic, compared to short-term, dsRNA feeding in pollen beetle 55
6. DISCUSSION 59
6.1. Potential non-biosafety of thiacloprid, alone and in mixture with tebuconazole, to nontarget organisms (**I**) 59
6.2. EO compounds and their potential in pollen beetle management (**II**) 60
6.3. dsRNA shows potential for use in biosafe management of pollen beetle 61
7. CONCLUSIONS 64
REFERENCES 66
SUMMARY 75
KOKKUVÕTE 78
SAMENVATTING 81
ACKNOWLEDGEMENTS 84
SUPPLEMENTARY INFORMATION 85
ORIGINAL PUBLICATIONS 93
CURRICULUM VITAE 164
LIST OF PUBLICATIONS 168

LIST OF ORIGINAL PUBLICATIONS

The present thesis is a review of the following papers, which are referred to by Roman numerals in the text.

I **Willow, J.**, Silva, A.I., Veromann, E., Smagghe, G. 2019. Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. PLOS ONE 14, e0212456. http://doi:10.1371/journal.pone.0212456

II **Willow, J.**, Sulg, S., Kaurilind, E., Silva, A.I., Kaasik, R., Smagghe, G., Veromann, E. 2020a. Evaluating the effect of seven plant essential oils on pollen beetle (*Brassicogethes aeneus*) survival and mobility. Crop Prot. 134, 105181. http://doi.org/10.1016/j.croppro.2020.105181

III **Willow, J.**, Sulg, S., Taning, C.N.T., Silva, A.I., Christiaens, O., Kaasik, R., Prentice, K., Lövei, G.L., Smagghe, G., Veromann, E. 2020b. Targeting a coatomer protein complex-I gene via RNA interference results in effective lethality in the pollen beetle *Brassicogethes aeneus*. J. Pest Sci. http://doi.org/10.1007/s10340-020-01288-6

IV **Willow, J.**, Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I., Taning, C.N.T., Christiaens, O., Smagghe, G., Veromann, E. 2020c. First evidence of bud feeding-induced RNAi in a crop pest via exogenous application of dsRNA. Insects 11, 769. http://doi.org/10.3390/insects11110769

V **Willow, J.**, Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I., Taning, C.N.T., Christiaens, O., Smagghe, G., Veromann, E. 2021. Anther feeding-induced RNAi in *Brassicogethes aeneus* larvae. (Submitted to Front. Agron.)

VI **Willow, J.**, Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I., Taning, C.N.T., Christiaens, O., Smagghe, G., Veromann, E. 2021. RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle. (Commun. Biol., In Press)

Authors' contributions to the papers was the following:

Paper	Conceptualisation	Method design	Investigation	Data analysis and visualisation	Writing – original draft	Writing – reviewing and editing
I	**JW**	**JW**, GS	**JW**	**JW**, AIS	**JW**	**JW**, AIS, EV, GS
II	**JW**	**JW**, SS, EK, EV	**JW**, EK	**JW**, AIS	**JW**	**JW**, AIS, RK, GS, EV
III	**JW**, CNTT, OC, GLL, GS, EV	**JW**, CNTT, KP, EV	**JW**, SS	**JW**, AIS	**JW**	**JW**, CNTT, AIS, OC, KP, GLL, GS, EV
IV	**JW**, GS, EV	**JW**, LS, SS, RK, AIS, CNTT, OC, GS, EV	**JW**, LS, SS, RK	**JW**, AIS	**JW**	**JW**, LS, SS, AIS, CNTT, OC, EV
V	**JW**, EV	**JW**, LS, SS, RK, EV	**JW**, LS, SS, RK	**JW**, AIS	**JW**	**JW**, LS, SS, RK, AIS, CNTT, OC, GS, EV
VI	**JW**, GS, EV	**JW**, CNTT, OC, EV	**JW**, LS, SS, RK	**JW**, AIS	**JW**	**JW**, LS, SS, AIS, CNTT, OC, GS, EV

JW – Jonathan Willow; EV – Eve Veromann; GS – Guy Smagghe; AIS – Ana Isabel Silva; LS – Liina Soonvald; SS – Silva Sulg; RK – Riina Kaasik; CNTT – Clauvis Nji Tizi Taning; OC – Olivier Christiaens; GLL – Gabor L. Lövei; EK – Eve Kaurilind; KP – Katterinne Prentice

ABBREVIATIONS

dsRNA	–	double-stranded RNA
mRNA	–	messenger RNA
siRNA	–	short interfering RNA
RISC	–	RNA-induced silencing complex
EU	–	European Union
RFC	–	recommended field concentration
EO	–	essential oil
RNAi	–	RNA interference
SIGS	–	spray-induced gene silencing
HIGS	–	host-induced gene silencing
COPI	–	coatomer protein complex-I
dH_2O	–	distilled water
GC–MS	–	gas chromatography–mass spectrometry
NCBI	–	National Center for Biotechnology Information
nfH_2O	–	nuclease-free water
qPCR	–	quantitative polymerase chain reaction

1. INTRODUCTION

Oilseed rape, or rapeseed (*Brassica napus* L.) is a brassicaceous crop cultivated in Europe, Asia, Canada, USA and Australia, primarily for the production of animal feed, vegetable oil and biodiesel, but also for some other uses (e.g. winter cover crop, green manure, forage for grazing livestock, and production of honey, lubricants and fertiliser). Across its growing range, oilseed rape production is threatened by both invertebrate pests and microbial pathogens.

Throughout Europe, one of the most severely damaging threats to oilseed rape cultivation is the pollen beetle *Brassicogethes aeneus* Fabricius (syn. *Meligethes aeneus*, Coleoptera: Nitidulidae). Being widespread and abundant throughout its European range, *B. aeneus*'s distribution also extends throughout Asia, as well as parts of northern Africa and North America. Adults overwinter both within their natal oilseed rape field as well as in surrounding habitats including forest edges, meadows, hedgerows, flower strips and forest interiors (Sutter et al., 2018). Upon spring emergence, overwintered adults feed on the pollen and nectar of a variety of blooming flowering plants. After females have completed the feeding required for egg production, males and females disperse to oilseed rape fields, as *B. aeneus* oviposition is exclusively associated with brassicaceous plants, and oilseed rape fields represent an abundant source of egg-laying habitat for this highly specialised species. While inhabiting oilseed rape fields, *B. aeneus* adults feed on pollen within developing reproductive buds, as well as the pollen and nectar of bloomed flowers. *B. aeneus* larvae feed on pollen within the buds in which they were oviposited, followed by feeding on the pollen and nectar of bloomed flowers, after their natal reproductive bud has bloomed, generally during their late first- or early second instar of development. During the late second instar of larval development, *B. aeneus* larvae cease feeding and pupate under the soil surface, below their associated oilseed rape plant (*B. aeneus* life history reviewed in Mauchline et al. (2018)). While oilseed rape has mechanisms that compensate the loss of reproductive buds necessary for seed development, the presence of dense *B. aeneus* populations can result in significant yield losses, thus creating a need for managing *B. aeneus* populations in oilseed rape agroecosystems.

Pyrethroid insecticides (IRAC class 3A of sodium channel modulators) have been the standard method for managing *B. aeneus* populations. However, as a result of the routine use of pyrethroids, *B. aeneus* populations have developed resistance to pyrethroids in several areas throughout Europe (Heimbach and Müller, 2013; Kaiser et al., 2018; Slater et al., 2011; Stará and Kocourek, 2018; Zamojska, 2017; Zimmer et al., 2014). Moreover, extensive research indicates that exposure to pyrethroids can be detrimental to many nontarget insect taxa that inhabit agroecosystems (Antwi and Reddy, 2015; Ceuppens et al., 2015; Delpuech and Delahaye, 2013; Desneux et al., 2007; Teder and Knapp, 2019; Wang et al., 2018).

Numerous parasitoids and other predators have co-evolved as natural biological control (biocontrol) agents against *B. aeneus*, and this co-evolution can be exploited for the benefit of ecologically biosafe management of *B. aeneus*. The most specialised arthropods involved in the biocontrol of *B. aeneus* populations include several species of hymenopteran parasitoids, while other predaceous arthropods (e.g. carabid beetles, staphylinid beetles, spiders) can also benefit biocontrol of *B. aeneus* populations. Several studies have demonstrated the potential these organisms represent for biocontrol of *B. aeneus* populations (Büchi, 2002; Kaasik et al., 2014; Riggi et al., 2017; Skellern and Cook, 2018; Thies and Tscharntke, 1999). Conservation biocontrol represents one ecologically biosafe measure for *B. aeneus* population management, whereby habitats and habitat characteristics required by biocontrol agents are preserved and/or restored within agroecosystems, with the intention of preserving and/or restoring species that contribute to biocontrol of one or more target pest species. Since conservation biocontrol does not rely on the input of agrochemicals, this crop pest management strategy is widely recommended by conservationists and other proponents of organic agriculture. However, the most effective *B. aeneus* management strategy may require the involvement of insecticide applications that, while showing efficacy against *B. aeneus*, minimise the impact on nontarget organisms, especially those performing agroecological services such as biocontrol and pollination.

As mentioned above, *B. aeneus* development begins inside the reproductive bud, where female *B. aeneus* adults oviposit; and larvae hatch and begin to feed on the anthers within their natal bud, followed by emergence of late first- and early second instar larvae that proceed to feed on the pollen and nectar of bloomed adjacent flowers. Reduction of pupating *B. aeneus* larvae and, in turn, overwintering next-generation *B. aeneus*

adults, certainly has potential to mitigate yield losses among oilseed rape crops within oilseed rape landscapes. While the adult phase of *B. aeneus* represents the typical focus of insecticide bioassays with respect to this pest species, *B. aeneus* larvae are also important to examine as an insecticide target, in the event that any biosafe insecticide examined in this work suggests promise via adult bioassays.

This thesis addresses the need for new and effective insecticides for use within a biosafe integrated *B. aeneus* management strategy. In brief, first we will look at one representative of a class of synthetic, non-species-specific insecticide, neonicotinoids (IRAC class 4A of nicotinic acetylcholine receptor (nAChR) competitive modulators). In particular, the compound thiacloprid has been the subject of research regarding its potential use in *B. aeneus* management. Neonicotinoids are applied in oilseed rape production (Kaiser et al., 2018; Seidenglanz et al., 2017); and recent field and greenhouse experiments using thiacloprid have demonstrated its efficacy in managing *B. aeneus* infestations (Brandes et al., 2018a, 2018b). We will examine thiacloprid's compatibility with a model hymenopteran parasitoid species, analysing thiacloprid's toxicity to this biocontrol species when applied alone, as well as in combination with tebuconazole (FRAC group 3, demethylation inhibitors, class 1 of sterol biosynthesis inhibitors), a representative of a group of compounds that are commonly tank-mixed with neonicotinoids for crop protection. Second, we will examine the effect of seven different plant-based essential oils, representing a non-species-specific biopesticide for potential use within *B. aeneus* management. Lastly, we will investigate the potential for applying *B. aeneus*-specific double-stranded ribonucleic acid (dsRNA), representing a potentially species-specific biopesticide option, within a *B. aeneus* management strategy.

2. REVIEW OF THE LITERATURE

2.1. Potential toxicity of thiacloprid, and synergistic toxicity when applied in combination with tebuconazole, in hymenopteran parasitoids

In 2013, after a mounting body of evidence had been collected regarding the adverse effects of neonicotinoids on economically beneficial insects, the European Commission banned the outdoor use of three neonicotinoids, namely imidacloprid, clothianidin and thiamethoxam, in all European Union (EU) countries (Gross, 2013). Another neonicotinoid compound, thiacloprid, and commercial formulations containing thiacloprid, have been demonstrated as representing a potentially effective *B. aeneus* control measure (Brandes et al., 2018a, 2018b; Kaiser et al., 2018; Seidenglanz et al., 2017). However, the non-taxon-specific mode of action of this class of insecticide makes necessary the examination of thiacloprid against different model hymenopteran parasitoid species, in order to provide an indication of whether thiacloprid can be used in conjunction with *B. aeneus* conservation biocontrol measures. Two previous studies have shown significant mortality in four different hymenopteran parasitoid species (three aphelinid wasps, namely *Eretmocerus eremicus* Rose and Zolnerowich, *Eretmocerus mundus* Mercet and *Encarsia formosa* Gahan; and the braconid wasp *Aphidius rhopalosiphi* De Stefani Perez) exposed to dry residues of thiacloprid in pure form or in commercial formulation (Jans, 2012; Sugiyama et al., 2011). Lacking in the scientific literature are data addressing the potential toxicity, in hymenopteran parasitoids, of combinations of pesticides that are simultaneously applied in agroecosystems. Indeed, insecticides are often tank-mixed with fungicides for simultaneous application to crops, for simultaneous management of insect pests and microbial pathogens (Põllumajandusamet, 2017; Thompson, 2012).

Toxicity of certain neonicotinoids can become synergised by the co-application of certain fungicides (e.g. triazoles) that are commonly tank-mixed with insecticides, and sprayed simultaneously for crop protection; and this has been demonstrated in three bee species, namely *Apis mellifera* L. (Apidae; Thompson et al., 2014; Zhu et al., 2017), *Osmia bicornis* L. (Megachilidae; Sgolastra et al., 2018, 2017) and *Bombus terrestris* L. (Apidae; Raimets et al., 2018). The suggested mechanism behind this

is that exposure to these fungicides, in certain organisms, results in the inhibition of production of cytochrome P450-dependent monooxygenases, enzymes necessary for oxidative metabolism of various xenobiotics including many insecticides (Johnson et al., 2006). Conservation biocontrol of many crop pests, including *B. aeneus*, relies on healthy hymenopteran parasitoid populations. Thus, it is imperative to examine the potentially synergistic effect of combining these two types of pesticide in a model hymenopteran parasitoid. Thiacloprid and tebuconazole are both applied, often in a tank-mixture, in a variety of agroecosystems, for the protection of various crops including but not limited to oilseed rape, wheat, orchard fruits and cotton. Thus, the combination of pesticide compounds examined here is highly field-relevant.

2.2. Potential for using plant-based essential oils in pollen beetle management

The use of plant-based essential oils (EOs) in managing crop pest insects is currently, as well as historically, a field of interest (Isman, 2006; Isman and Grieneisen, 2014); and this topic attracts much interest from researchers and industry alike. EOs can confer insecticidal, repellent and antifeedant effects on various crop pests. EOs derived from different sources contain different useful compounds, as well as different ratios of similar or equivalent compounds. As different compounds confer different effects, the modes of action of different EOs in insects will undoubtedly differ between biotic sources. Different insect taxa can show dramatically different phenotypes upon exposure to EO-derived compounds; and this is the basis for investigations into selectivity of EOs and EO-derived compounds that have potential to aid in the management of a given crop pest species, while simultaneously conferring minimal risk to nontarget taxa.

Regarding *B. aeneus*, the potential insecticidal and/or repellent efficacy of *Lavendula angustifolia* Mill. (lavender, Lamiaceae) EO has been examined in several studies (Dorn et al., 2014; Mauchline et al., 2013, 2005; Pavela, 2011). While the lack of chemical constituent information (i.e. via gas chromatography–mass spectrometry (GC–MS) analysis) in some of these studies makes comparisons between them difficult, an electroantennography experiment using *L. angustifolia* EO revealed the compounds linalool and linalyl acetate as being those most associated with *L. angustifolia*'s repellent effect on *B. aeneus* (Mauchline et al., 2008). Another study

indicated the EOs of *Carum carvi* L. (caraway, Apiaceae), *Thymus vulgaris* L. (thyme, Lamiaceae) and *Foeniculum vulgare* Mill. (fennel, Apiaceae) as having the greatest insecticidal effect on *B. aeneus*, out of the nine plant EOs examined (Pavela, 2011).

One additional study has investigated the potential of six plant EOs for managing *Carpophilus* spp., another genus of nitidulid beetle, for the benefit of walnut (*Juglans* spp.) protection (Comelli et al., 2018). Three EOs, particularly of the plant species *Pimpinella anisum* L. (anise, Apiaceae), *Cuminum cyminum* L. (cumin, Apiaceae) and *Aloysia polystachia* (Griseb.) Moldenke (bee-brush, Verbenaceae) were demonstrated as being the most insecticidal EOs examined against *Carpophilus* spp. The above-mentioned work with *B. aeneus*, as well as the EO work performed with *Carpophilus* spp., provide valuable insight into the potential usefulness of different plant EOs in *B. aeneus* management. Nevertheless, it remains critical to examine additional application methods (e.g. topical application, treated plant tissue), as well as both new and previously examined plant EOs, with intentions of both further exploration and corroboration of existing evidence. In addition to corroborating existing evidence, re-examining the EOs of previously examined plant species may reveal potential compounds of interest, as chemical constituents of a given plant species' EO may greatly vary between populations or cultivars.

2.3. Potential for developing an RNA interference strategy towards pollen beetle management

Post-transcriptional gene silencing, or RNA interference (RNAi), can be exploited for a biosafe approach to crop pest management (Bramlett et al., 2019; Huvenne and Smagghe, 2010; Mezzetti et al., 2020; Taning et al., 2019; Zhu and Palli, 2020). In brief, a target pest species is exposed to dsRNA (e.g. via feeding) designed as complementary to a specific region of a messenger RNA (mRNA) that encodes a vital gene in the target species. After this exogenous dsRNA has entered the cytoplasm of cells within the gut and/or other tissues, the ribonuclease III enzyme Dicer-2 cleaves this dsRNA into double-stranded segments approximately 21 nucleotides in length, called small interfering RNAs (siRNAs). A complex of proteins, with the endoribonuclease Argonaute2 as its catalytic centre, binds to one siRNA strand (guide strand); and this forms the RNA-induced silencing complex (RISC). This binding with the RISC results

in the degradation of the opposite siRNA strand (passenger strand). The guided RISC binds to complementary endogenous mRNA, cleaves this mRNA, and in turn inhibits the decoding of this mRNA in the ribosome, preventing synthesis of the respective protein (Bramlett et al., 2019). The nucleotide sequence-specific mode of action of dsRNA is the basis for its perceived biosafety to nontarget organisms.

One approach for inducing RNAi in crop pest populations is via spray-induced gene silencing (SIGS), whereby target-specific dsRNA is sprayed onto crops. The prospects of SIGS has been reviewed by Cagliari et al. (Cagliari et al., 2018); and indeed this approach has been demonstrated in both a greenhouse experiment (Miguel and Scott, 2015) and a field trial (Petek et al., 2020) examining SIGS in Colorado potato beetle (*Leptinotarsa decemlineata* Say; Chrysomelidae). This approach has the benefit of not requiring biotechnology resources needed for engineering an RNAi cultivar. One drawback to a SIGS approach, however, is the possibility that exogenously-applied dsRNA may not remain stable for long periods in given environmental conditions. Another drawback is that successive applications of dsRNA spray may become necessary during successive crop growth stages. This latter drawback is especially important to consider in the management of *B. aeneus* and other anthophilous species; these species acquire their nutrients from flowers, which are in constant development and senescence; rather than acquiring nutrients from leaves, which remain individually present on the crop for much longer periods.

Another approach, host-induced gene silencing (HIGS) via the use of RNAi cultivars, has been demonstrated as being effective in controlling western corn rootworm (*Diabrotica virgifera virgifera* LeConte; Chrysomelidae) via dietary exposure to transgenic maize (*Zea mays* L.) engineered to express dsRNA targeting mRNA that encodes *v-ATPase A* in *D. virgifera virgifera* (Baum et al., 2007). RNAi cultivars present a great benefit in that these transgenic crops constantly produce the target pest-specific dsRNA within the plant's tissues. This results in chronic exposure of the target pest population to this sequence-specific insecticide, so long as the population feeds on the transgenic plants. Indeed, the agritech company Monsanto's RNAi maize cultivar MON87411, which expresses dsRNA targeting the vacuolar sorting protein-encoding gene *Snf7* in *D. virgifera virgifera*, has been approved for outdoor cultivation in several countries (Arpaia et al., 2020; Papadopoulou et al., 2020). While current restrictions prevent the cultivation of transgenic crops in EU countries,

this legal barrier may very well be removed after a sufficient amount of risk assessment research has been performed, particularly after refinements are made to RNAi risk assessment methods (Arpaia et al., 2020).

Several crop pests in the order Coleoptera have demonstrated sensitivity to dsRNA via feeding. *L. decemlineata* and *D. virgifera virgifera* are two examples of highly RNAi-sensitive crop pests; and in turn, much RNAi work has been done with these pest species (Bachman et al., 2013; Baum et al., 2007; Knorr et al., 2018; Máximo et al., 2020; Mehlhorn et al., 2020; Miguel and Scott, 2015; Petek et al., 2020; Vélez et al., 2020). Some weevil (Curculionidae) species have been shown to be RNAi-sensitive via oral consumption of dsRNA. For example, the Sri Lanka weevil, *Myllocerus undecimpustulatus undatus* Marshall, has shown significant mortality following consumption of dsRNAs targeting *Snf7* and *proteasome subunit alpha type-2* (*Prosα2*; Pinheiro et al., 2020); and the sweetpotato weevils *Cylas brunneus* Fabricius and *Cylas puncticollis* Boheman have both shown significant mortality after consumption of dsRNAs targeting *Snf7*, *Prosα2* and *ribosomal protein S13* (*rps13*; Christiaens et al., 2016; Prentice et al., 2017). Finally, the lady beetle *Henosepilachna vigintioctopunctata* Fabricius (Coccinellidae) has demonstrated sensitivity to dsRNA targeting *Snf7* (Lü et al., 2020), as well as dsRNA targeting its *death-associated inhibitor of apoptosis protein 1* (*diap1*) gene (Chikami et al., 2020). Interestingly, silencing of *diap1* resulted in acute feeding cessation within 1–2 d after dsRNA consumption.

Recently, all genes involved in major RNAi pathways were identified from the transcriptome of *B. aeneus*, as well as genes and associated proteins that are believed to be critical for systemic RNAi (Knorr et al., 2018); systemic RNAi being the whole-body and persistent suppression of the target mRNA. The corresponding study was the first to report RNAi in *B. aeneus* via dsRNA feeding. The authors demonstrated the efficacy of oral consumption of dsRNAs targeting mRNAs encoding the protein-coding genes *dre4* (*dre4*), *nucampholin* (*ncm*), *Ras opposite* (*Rop*) and *RNA polymerase II 140kD subunit* (*RpII140*). These genes were chosen as RNAi targets in *B. aeneus* because they were orthologous to the four most sensitive RNAi targets in *D. virgifera virgifera* bioassays from the same study (Knorr et al., 2018). It remains necessary to carefully select additional potential RNAi targets in *B. aeneus*, and examine the effect (e.g. target species mortality rate) of downregulation per target gene. Furthermore, field relevant exposure routes must be examined in *B. aeneus*, in order

to determine the potential for applying this potentially species-specific biopesticide within a *B. aeneus* management framework.

One promising target gene for downregulation in *B. aeneus* via RNAi is *coatomer subunit alpha* (*αCOP*), which encodes the αCOP protein, a subunit of coatomer protein complex-I (COPI). COPI is involved in intracellular vesicular transport of proteins between the endoplasmic reticulum and Golgi apparatus (Beck et al., 2009), as well as maintaining lipid homeostasis (Beller et al., 2008), and possibly maintaining protein distribution within the Golgi stack (Beck et al., 2009). The COPI coat which adheres to intracellular vesicles interacts with cell division control protein 42 homolog (CDC42), a regulator of the cytoskeletal motor protein dyenin; and thus COPI is involved in the transport of various cellular cargo (Beck et al., 2009). Furthermore, knockdown of COPI subunits has been demonstrated to result in cytokinesis failure, by preventing the accumulation of essential proteins and lipid components at the dividing cells' cleavage furrow (Kitazawa et al., 2012).

3. AIMS AND HYPOTHESES

The overall objective of this thesis work was to examine the potential for using different insecticides within a biosafe- and integrated *B. aeneus* management framework. A relatively new synthetic insecticide with potential for use in *B. aeneus* management, thiacloprid, alone and in combination with a fungicide commonly tank-mixed with thiacloprid, was examined against a model hymenopteran parasitoid, in order to help determine potential biosafety to this group of nontarget biocontrol agents. Then, seven plant-based EOs, representing a biopesticide-based alternative to synthetic insecticides, were studied for their lethal and mobility effects on *B. aeneus*. Finally, over a series of several studies, we aimed to determine the potential for developing a target species-specific, and thus biosafe, approach to managing *B. aeneus* populations, via the use of dsRNA-based insecticides. Specific objectives within this thesis work included:

1. To examine the acute effect of exposure to dry thiacloprid residues, alone and in combination with dry tebuconazole residues, on survival and mobility of a model hymenopteran parasitoid. To demonstrate synergistic reductions in parasitoid survival and mobility, when exposed to both pesticides simultaneously; as well as demonstrate greater field relevance of insecticide risk assessment when pesticides are examined in combination, since these agrochemicals are tank-mixed and simultaneously sprayed on crops in agricultural practice (**I**).

   H1: Exposure to residues of thiacloprid, when applied at RFC, results in significant reductions in both survival and mobility in the model parasitoid wasp *Aphelinus abdominalis* Dalman (Aphelinidae).

   H2: Simultaneous exposure to residues of thiacloprid and tebuconazole results in synergistic reductions in *A. abdominalis* survival and mobility, and reveals a threshold-concentration (respective to RFC) that corresponds to the observed synergy.

2. To examine the acute effect, of seven different plant EOs, on *B. aeneus* survival and mobility (**II**).

   H1: Topical applications of different plant EOs result in different responses, regarding *B. aeneus* survival and mobility; and will suggest a particular plant EO as being the most promising, for use in biopes-

ticide-based management of *B. aeneus*, among each of the examined topically-applied EOs.

H2: In bioassays using treated leaf- and bud surfaces, we expected to find an effective concentration threshold for significant reductions in *B. aeneus* survival and mobility, using the most promising EO from the topical bioassays.

3. To detect one or more suitable RNAi targets via a microinjection-based screening of dsRNAs, each targeting mRNA that encodes a specific target gene; and confirming comparable RNAi activity via a dsRNA feeding bioassay. To examine the effect of target-specific dsRNA on *B. aeneus* survival via two additional field-relevant routes of exposure to *B. aeneus*-specific dsRNA. To investigate the potential for developing an RNAi strategy for integrated *B. aeneus* management.

   H1: The screening of six target mRNA-specific dsRNAs will show one or more of these as being significantly lethal, via microinjection, to *B. aeneus*.

   H2: The most promising dsRNA from the microinjection bioassays will be demonstrated as being effective in inducing oral RNAi in *B. aeneus* adults via consumption of dsRNA-treated honey water, confirming the presence of an effective oral RNAi machinery in *B. aeneus* (**III)**.

   H3: Consumption of dsRNA-treated oilseed rape bud epithelia, representing a highly field relevant route of exposure to sprayed dsRNA, results in significant RNAi-induced mortality in *B. aeneus* adults (**IV**).

   H4: Consumption of dsRNA-treated oilseed rape anthers, representing a highly field relevant route of exposure to target-specific dsRNA, results in significant RNAi-induced mortality in *B. aeneus* larvae (**V**) and adults (**VI**).

   H5: Chronic (daily) consumption of target-specific dsRNA-treated oilseed rape anthers will result in significantly greater RNAi-induced mortality in *B. aeneus* adults, compared to short-term (3 d) consumption of target-specific dsRNA-treated oilseed rape anthers (**VI**).

4. MATERIAL AND METHODS

4.1. Insects and plants

All parasitoids used in the bioassays with thiacloprid and tebuconazole belonged to the species *A. abdominalis*. Aphid mummies containing diapausing *A. abdominalis* adults were ordered from Biobest (Westerlo, Belgium), and upon arrival were maintained in a climate chamber (model MLR-352H-PE, Panasonic, Kadoma, Japan) at 22 °C, 60% relative humidity (RH) and a light : dark cycle of 16 : 8 h. Insect were used in experiments within 1–2 d after emergence from diapause (**I**).

For the microinjection-based screening of six potential target mRNAs in *B. aeneus*, in 2018, pollen beetles and oilseed rape flowers were collected from several untreated oilseed rape fields in Tartu County, Estonia; and subsequently kept in ventilated plastic containers, where they were allowed to feed *ad libitum* on pollen of oilseed rape. Only pollen beetles identified as *B. aeneus*, via Kirk-Spriggs (1996), were used in the all adult pollen beetle studies.

Pollen beetles used in the EO experiments (**II**), and the study where we confirm *B. aeneus*'s oral RNAi machinery (**III**), in 2019, were collected from an untreated oilseed rape field (58.37979°N, 26.66394°E) in the village of Kandiküla, Tartu County, Estonia; and subsequently kept in ventilated plastic containers, where they were allowed to feed *ad libitum* pollen of oilseed rape and dandelion (*Taraxacum* spp.). Oilseed rape flowers were picked from the same field where the pollen beetles were collected; and dandelion flowers were picked from wildflower areas surrounding the Estonian University of Life Sciences. We used laboratory-grown oilseed rape plants for *B. aeneus* bioassays using EO-treated leaf- and bud surfaces. Laboratory-grown oilseed rape plants were also used for flower production, in order to provide food to pollen beetles post microinjection- and feeding of dsRNA treatments.

For the 2020 RNAi studies, where we examined RNAi in *B. aeneus* adults via both bud- (**IV**) and anther feeding (**VI**), and in *B. aeneus* larvae via anther feeding (**V**), pollen beetles and oilseed rape flowers were collected from an untreated oilseed rape field (58.36377°N, 26.66145°E) in the village of Õssu, Tartu County, Estonia; and subsequently kept in venti-

lated plastic containers, where they were allowed to feed *ad libitum* pollen of oilseed rape. Larvae were collected in the lab, from the collected oilseed rape flowers; and only late first- and early second instar larvae identified as *B. aeneus*, via Osborne (1965), were used in the larval experiment. Oilseed rape plants used for dsRNA-treated bud feeding were collected from an untreated oilseed rape field (58.37389°N, 26.33114°E) in the village of Nasja, Tartu County, Estonia; and subsequently maintained in a 3 × 3 m climate room (Flohr Instruments, Utrecht, Netherlands) at 10 °C (70 ± 5% RH and a light : dark cycle of 16 : 8 h), in order to keep them at a low growth stage. Prior to beginning the experiment, the temperature in the climate room was increased to 18 °C.

4.2. Experimental compounds and products

The experiments examining thiacloprid and tebuconazole used commercial formulations, respectively Calypso (suspension concentrate, 480 g thiacloprid/L, Bayer CropScience) and Tebusip (emulsifiable concentrate, 250 g tebuconazole/L, OXON Italia). Treatments containing thiacloprid, tebuconazole, or both, were diluted in distilled water (dH_2O) to desired concentrations (**I**).

Pure EOs of seven plant species, namely *T. vulgaris*, *P. anisum*, *F. vulgare*, *C. cyminum*, *Cinnamomum verum* J. Presl. (cinnamon, Lauraceae), *C. carvi* and *Cannabis sativa* L. (hemp, Cannabaceae) were ordered from Talia (Rome, Italy), shipped at ambient temperature, and kept at 5 ± 1 °C once received. Table 1 (**II**) details the origin of plants, plant parts used, extraction method, and major compounds detected via GC–MS analysis, with respect to each EO. Each EO treatment consisted of 0.05% Tween80, and was diluted in acetone to the desired concentration (**II**).

For the microinjection screening in 2018, we used BLAST analysis and known sequences, from other insect species, of six potential target mRNAs. Coding sequences for *αCOP*, *coatomer subunit delta* (*δCOP*), *coatomer subunit gamma* (*γCOP*), *rps13*, *Snf7* and *vitellogenin* (*Vg*) were detected in the transcriptome of *B. aeneus* [available in the GenBank database (National Center for Biotechnology Information – NCBI); (Zimmer et al., 2014)]. SI Table 1 shows the primers for synthesis of dsRNAs designed to target respective mRNAs that code for these target genes. These dsRNAs are hereafter respectively referred to as dsαCOP,

Table 1. Plant species from which essential oil (EO) was used in *Brassicogethes aeneus* bioassays. Here we show country of cultivation, plant parts used for EO extraction, extraction method, and major compounds detected (**II**).

Plant species	Plant origin	Parts used	Extraction method	Major compounds detected
Thymus vulgaris	Portugal	leaf, flower	steam distilled	o-cymene (61%), α-pinene (15%), ç-terpinene (6%), camphene (4%)
Pimpinella anisum	Spain	seed	steam distilled	anethole (52%), D-limonene (21%), estragole (8%), o-cymene (5%)
Foeniculum vulgare	Hungary	seed	cold pressed	α-pinene (31%), anethole (22%), D-limonene (20%), L-fenchone (18%)
Cuminum cyminum	Morocco	seed	steam distilled	α-pinene (29%), o-cymene (26%), cuminaldehyde (25%), ç-terpinene (14%)
Cinnamomum verum	Sri Lanka	inner bark	steam distilled	cinnamaldehyde (46%), caryophyllene (15%), linalool (12%), D-limonene (8%)
Carum carvi	Hungary	seed	steam distilled	carvone (37%), D-limonene (37%), α-myrcene (6%), dihydrocarvone (5%)
Cannabis sativa	Italy	leaf, flower	steam distilled	α-myrcene (45%), α-pinene (38%), D-limonene (5%), α-ocimene (3%)

dsδCOP, dsγCOP, dsrps13, dsSnf7 and dsVg. These dsRNAs were produced using a MEGAscript Kit (Life Technologies, Carlsbad, USA), at Ghent University. The dsRNA products were shipped to Tartu, Estonia in nuclease-free water (nfH_2O) at ambient temperature, and kept at 5 ± 1 °C once received. For each nucleotide sequence, we screened for cross-homologies within the *B. aeneus* transcriptome via BLAST analysis; and thereby ensured the absence of any shared fragment identities exceeding 19 nucleotides in length, in order to avoid cross-silencing of any nontarget genes in *B. aeneus*.

To confirm oral RNAi in *B. aeneus*, we used *in vitro* synthesised (Genolution, Seoul, South Korea) dsαCOP and dsrps13, as well as dsRNA with a sequence complementary to the gene *gfp* (used as a control, as *gfp* is not present in insects). The *B. aeneus αCOP* and *rps13* regions, as well as the *gfp* region, for which the construction of complementary dsRNAs

was based, in shown in SI Table 1. The *gfp* region used to design the control dsRNA (hereafter referred to as dsGFP) was also screened for cross-homologies within the *B. aeneus* transcriptome via BLAST analysis, in order to avoid silencing of nontarget genes in *B. aeneus*. The dsRNA products were shipped to Tartu, Estonia in dH_2O at ambient temperature, and kept at 5 ± 1 °C once received. The absence of nucleic contaminants in dsRNA stock solutions was confirmed via gel electrophoresis (**III**).

For all experiments in 2020 (**IV**, **V**, **VI**), we used *in vitro* synthesised (Genolution) ds*α*COP and dsGFP, based on the same dsRNA construction indicated in SI Table 1. In addition to having been screen for cross-homologies within the *B. aeneus* transcriptome, the selected 222 bp *αCOP* region was screened against all bee species with available genome data in NCBI, via BLAST analysis. The dsRNA products were shipped to Tartu, Estonia in dH_2O at ambient temperature, and kept at 5 ± 1 °C once received. The absence of nucleic contaminants in dsRNA stock solutions was confirmed via gel electrophoresis.

4.3. Experimental setups

4.3.1. Examining acute effect of thiacloprid alone and in combination with tebuconazole, in a model parasitoid (I)

To expose groups of *A. abdominalis* to pesticide residues, we used a Cornelis spray tower (van de Veire et al., 1996; Fig. 1) to spray circular glass discs (9 cm diameter) with treatment solutions (thiacloprid- and/or tebuconazole formulation, diluted in dH_2O); each disc being sprayed once on a single side, with 1 mL of treatment solution, and 1 bar of air pressure. Concentrations of treatment solutions were prepared based on glass disc surface area, and the use of 1 mL of solution per disc, in order to apply treatments having active ingredient concentrations in g/ha based on RFC. Fresh treatments were prepared weekly, and kept in a refrigerator, in sterile 50 mL polypropylene centrifuge tubes (Nerbe Plus, Germany). Sprayed glass discs were left to air dry for approximately 2 h, to ensure that only dry treatment residues remained on the disc surfaces.

Enclosures (Fig. 2) were constructed from two treated glass discs, each bound to a plastic ring frame (14 mm height) with ventilation holes covered in fine mesh. For the construction of each enclosure, a ring frame

Figure 1. Cornelis spray tower, at Ghent University's Laboratory of Agrozoology (Ghent, Belgium).

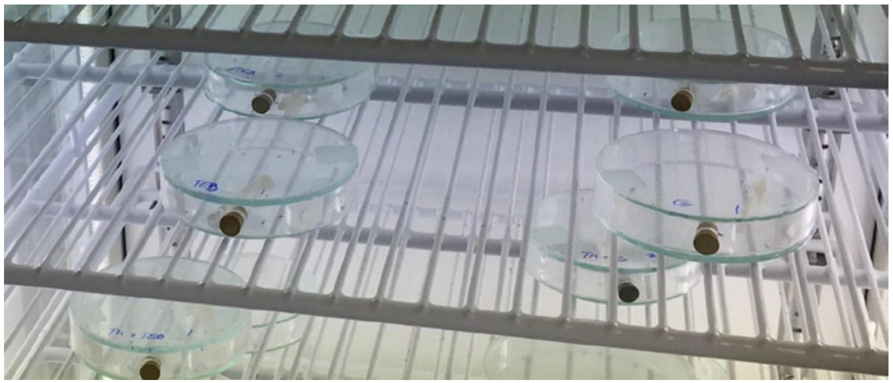


Figure 2. Ventilated glass enclosures used for *Aphelinus abdominalis* bioassays.

was placed over the perimeter of the bottom disc, which was then fastened to the ring frame; and 20 randomly chosen *A. abdominalis* adults were placed onto the base of the enclosure, using a fine paintbrush. The top disc was then placed over-, and fastened to, the ring frame. The treated side of each glass disc faced interiorly to its respective enclosure. A strip of filter paper soaked in 50% sucrose solution was added to each cage through a hole in the ring frame, which was then sealed using a rubber stopper. Each enclosure was then placed in the climate chamber at 22 °C, 60% RH and a light : dark cycle of 16 : 8 h.

The find a suitable concentration for examining synergy dynamics between dry residues of thiacloprid and tebuconazole, we first examined the effect of dry residues of thiacloprid applied at RFC (120 g/ha). A total of 100 *A. abdominalis* adults (five enclosures of 20 insects, n = 5) were exposed to this thiacloprid treatment, and an equal number were exposed to a dH_2O control treatment. The effect of each treatment on both survival and mobility was monitored at 2, 4, 6, 8 and 24 h post-exposure. The effectiveness of thiacloprid residues at this concentration (shown in section 5.1 of Results) suggested that this concentration would be unsuitable for examining synergy dynamics between thiacloprid and tebuconazole. Thus, for the experiment examining synergy dynamics, we reduced our experimental concentration of thiacloprid to one tenth RFC (12 g/ha).

Treatments for the experiment examining synergy dynamics between thiacloprid and tebuconazole are shown in SI Table 2 (**I**). Treatments here include a dH_2O control, tebuconazole at RFC (125 g/ha), thiacloprid at one tenth RFC, and five binary treatments containing both thiacloprid and tebuconazole. All binary treatments contained thiacloprid at one tenth RFC; while tebuconazole was co-applied in a series of ascending concentrations, including one one-hundredth RFC (1.25 g/ha), one twentieth RFC (6.25 g/ha), one tenth RFC (12.5 g/ha), one half RFC (62.5 g/ha) and RFC. This experiment was replicated six times, each replication allocating two enclosures per treatment. A total of 240 *A. abdominalis* adults (12 enclosures of 20 insects, n = 12) were exposed to each treatment. The effect of each treatment on both survival and mobility were monitored at 2, 4, 6, 8 and 24 h post-exposure.

Survival and mobility were both monitored by using a 14× magnification hand lens. Mortality was assumed when an individual showed no movement during 15 s of observation, even after gently prodding the insect with a fine paintbrush. Immobility was determined when erratic muscular activity (stumbling, convulsing), or a lack of muscular activity (partial or entire paralysis), inhibited an individual from moving in a stable manner or at all. Immobility furthermore included apparent mortality, and therefore represented total acute effect.

4.3.2. Examining acute effect of EOs on pollen beetle (II)

4.3.2.1. *Screening EOs via topical dosing*

Seven EOs, at five doses each (35 EO treatments), were topically applied to *B. aeneus* adults (four replicates of ten beetles; 40 beetles per EO treatment). Treatments consisted of a 0.05% Tween80 and varying amounts of EO and acetone together constituting 4 mL of solution per treatment. Each treatment was prepared in a glass vial immediately prior to application. The five concentrations of [acetone + Tween80 + EO] solution prepared for each EO were 1, 1.5, 2, 2.5 and 5% EO. Based on the average weight per individual *B. aeneus* adult (1.4 ± 0.1 mg), obtained via weighing (Sartorius Lab Instruments, Göttingen, Germany) twenty groups of ten (200 total) randomly chosen *B. aeneus* adults in plastic vials, these five EO concentrations per EO correspond to doses of 3.65, 5.48, 7.31, 9.14 and 18.27 nL/mg insect (after accounting for the 0.5 µL of treatment applied to each insect, described below). A control treatment of 0.05% Tween80 in 99.95% acetone was applied to 200 randomly chosen *B. aeneus* adults (four replicates of 50 beetles).

Randomly chosen, fast moving (used as a proxy for healthy specimens) individuals were anaesthetised via 2 min of exposure to diethyl ether-soaked cotton, in a covered glass Petri dish. After beetles had been anaesthetised, they were introduced to sterilised Petri dishes in groups of ten beetles per dish. Treatment solutions were applied at 0.5 µL per beetle, via micropipette (HTL Lab Solutions) with microloader (Eppendorf), over the dorsal pronotum. Treatments were administered under a stereomicroscope (Leica, Taiwan), in order to ensure both accuracy and precision during and across all treatment applications. Release of treatment solution from the microloader occurred via capillary action, requiring gentle touching- and removal of the microloader tip to and from the pronotum of the anaesthetised beetle several times. Treatment solutions diffused over surrounding body surfaces. Care was taken to allow acetone to completely evaporate from the body surface between capillary actions, ensuring the administration of the entire dose, without loss of treatment solution onto the surrounding Petri dish. Treated beetles were placed into transparent, polystyrene, ventilated insect breeding dishes (10 cm diameter × 4 cm height) (SPL Life Sciences, Gyeonggi-do, South Korea), hereafter referred to as cages, in groups of ten beetles per cage.

Cages of treated beetled were subsequently kept in a climate chamber (Sanyo MLR-351H, Osaka, Japan) at 20 ± 2 °C, 60% RH and a light : dark cycle of 16 : 8 h. Treated *B. aeneus* adults were allowed to feed *ad libitum* on pollen of dandelion flowers, and were provisioned with cotton soaked in dH_2O for access to drinking water. Fresh food and water were provided to each cage after 24 h; and the previous day's food and water were removed. Treated beetles were monitored for survival and mobility at 6, 24 and 48 h post-treatment. Mortality of an individual was determined through observing no movement, even when placed in the warm palm of hand and having warm air exhaled over the individual (a way to determine whether a pollen beetle is playing dead). Immobility was determined through observing erratic walking behaviour, inability to stay dorsal-side-up, or an inability to walk at all. Immobility furthermore included apparent mortality, and therefore represented total acute effect.

4.3.2.2. *Acute effect of exposure to dry residues of cinnamon bark EO, on oilseed rape leaf and bud tissue*

We examined the effect of treating oilseed rape leaf and bud surfaces with six concentrations of *C. verum* EO. Here, we examined only *C. verum* EO, as this EO showed the most promising insecticidal effect on *B. aeneus*, out of the seven EOs examined in topical application assays. The *C. verum* EO treatments were diluted in acetone, and contained 0.05% Tween80. The six *C. verum* EO concentrations examined here included 1.3, 1.4, 1.5, 1.6, 1.7 and 1.8% *C. verum* EO. A control treatment consisted of 0.05% Tween80 in acetone. All treatments were prepared in 15 mL quantities immediately prior to application. Application was performed via spraying treatment as a fine mist over five oilseed rape leaves (each approximately 14.5 × 14.5 cm) per treatment. In addition, four oilseed rape buds, per treatment, were dipped in the respective [acetone + Tween80 + EO] concentrations, and inserted by the petiole into small cotton balls soaked in dH_2O (hereafter referred to as buds). Treated leaves and buds were allowed to air dry for 1 h, and were placed into cages. In each cage, one leaf was pressed to the interior bottom of the cage; and four buds corresponding to the same treatment were added to the cage. Four untreated oilseed rape flowers were added to each cage, allowing the *B. aeneus* adults to feed *ad libitum* on pollen of oilseed rape. Eight *B. aeneus* adults were introduced to each cage, and cages were kept in the climate chamber at 20 ± 2 °C, 60% RH and a light : dark cycle of 16 : 8 h. Survival and mobility were both recorded at 24 h post-exposure, and both

end-points were determined as described in the previous section. The experiment was replicated three times, altogether constituting 15 cages (n = 15) per treatment, with 120 beetles per treatment.

4.3.3. Examining RNAi in pollen beetle

4.3.3.1. *Screening of potential RNAi targets via microinjection*

To screen potential RNAi targets in *B. aeneus*, we injected dsαCOP, dsδCOP, dsγCOP, dsrps13, dsSnf7, dsVg and an nfH_2O control, into approximately 90 *B. aeneus* adults per treatment. Prior to microinjection, randomly chosen, fast moving individuals were anaesthetised via 2 min of exposure to diethyl ether-soaked cotton, in a covered glass Petri dish. Delivery of dsRNAs into the haemolymph was performed under a stereomicroscope (Leica), using a microinjector (FemtoJet 4i, Eppendorf, Hamburg, Germany) and micromanipulator (Narishige, Tokyo, Japan) equipped with injection needles. Injection needles were prepared from glass capillary tubes. Between the administering of different dsRNAs, sterile needles were equipped to the apparatus.

Each beetle was injected with 0.2 µL of dsRNA solution at 1 µg dsRNA/µL. To inject *B. aeneus* adults, each specimen was laid on their dorsal surface upon a glass slide, and held in place via gently pressing a glass cover slip over their ventral abdominal surface. This gentle pressing resulted in the extension and visualisation of at least one of two arthrodial membranes (intersegmental areas of unsclerotised, soft and flexible cuticle); one of these being the cervix (membrane separating head from thorax, i.e. neck); the second being the arthrodial membrane separating thorax from abdomen.

At 24 ± 1 h post-injection, 70 randomly chosen and fast moving individuals, for each treatment, were chosen for survival monitoring. These beetles were placed in plastic vials (35 mm diameter × 75 mm height), in groups of five beetles per vial, and allowed to feed *ad libitum* on pollen of oilseed rape flowers. Vials were capped with a ventilated lid, and kept in a climate chamber (Sanyo MLR-351H) at 20 ± 2 °C, 60% RH and a light : dark cycle of 16 : 8 h. Fresh food was replaced every 48 h, and survival monitoring occurred every 24 h over ten days.

4.3.3.2. *Confirming oral RNAi in pollen beetle*

As an overall positive control for RNAi in this first feeding study, we injected *B. aeneus* adults with dsαCOP (**III**), dsrps13 and a dsGFP negative control. Prior to microinjection, randomly chosen, fast moving individuals were anaesthetised via 2 min of exposure to diethyl ether-soaked cotton, in a covered glass Petri dish. Delivery of dsRNAs into the haemolymph was performed under a stereomicroscope (Leica), using a microinjector (FemtoJet 4i) and micromanipulator (Narishige) equipped with injection needles. Injection needles were prepared from glass capillary tubes. Between the administering of different dsRNAs, sterile needles were equipped to the apparatus. Twenty groups of ten (200 total) randomly chosen *B. aeneus* adults were weighed (Sartorius Lab Instruments) in plastic vials in order to obtain an average weight per individual beetle (1.4 ± 0.1 mg). Each beetle was injected with 0.2 µL of dsRNA solution at 1 µg dsRNA/µL (approximately 0.14 µg dsRNA/mg insect). To inject *B. aeneus* adults, each specimen was laid on their dorsal surface upon a glass slide, and held in place via gently pressing a glass cover slip over their ventral abdominal surface. This gentle pressing resulted in the extension and visualisation of at least one of two arthrodial membranes (intersegmental areas of unsclerotised, soft and flexible cuticle); one of these being the cervix (membrane separating head from thorax, i.e. neck); the second being the arthrodial membrane separating thorax from abdomen (**III**).

For survival analysis, approximately 15 to 20 beetles were injected per treatment; and this was replicated four times. Injected beetles were introduced to cages, and allowed to feed *ad libitum* on pollen of oilseed rape flowers, as well as provided dH_2O-soaked cotton. At 24 ± 1 h after each replication was performed, ten randomly chosen and fast moving individuals, per treatment, were relocated to new cages, in their respective group of ten beetles, and kept in a climate chamber (Sanyo MLR-351H) at 20 ± 2 °C, 60% RH and a light : dark cycle of 16 : 8 h. In total, 40 beetles per injection treatment were monitored for survival and mobility. Monitoring occurred every 24 ± 1 h, until 10 d post-injection; and treated beetles were provisioned with fresh food and water daily (**III**).

Another group of approximately 55 to 60 were injected for each dsRNA treatment, placed in cages in groups of 15 to 20 beetles per cage, and kept in the climate chamber. At 24 ± 1 h post-injection, 36 randomly chosen, fast moving beetles per treatment were placed into new cages, kept in

the climate chamber, and used later for analysis of mRNA expression via quantitative polymerase chain reaction (qPCR). For each treatment, relative mRNA expression was analysed at both 2 and 5 d post-injection. Each analysis was allocated three replicates of six beetles (qPCR method for this study is described below). Experimental setup for dsRNA micro-injection is illustrated in Figure 3a (**III**).

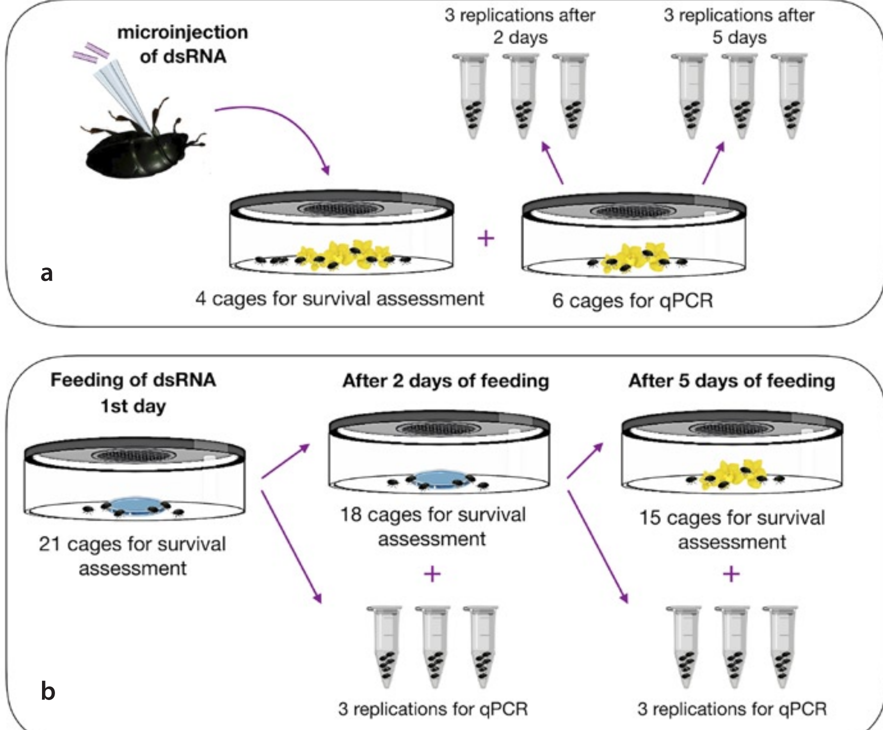


Figure 3. Experimental setup for microinjection (a) and feeding (b) experiments, for each treatment. **Microinjection experiment**: n=40 (4 replicates of 10 adult *Brassicogethes aeneus*) per treatment for survival assessment; n=3 (3 replicates of 6 adult *B. aeneus*) for each time-point of analysis within each dsαCOP- and dsGFP treatment, for qPCR analysis. Microinjected beetles fed *ad libitum* on anthers of oilseed rape flowers post-injection. **Feeding experiment**: n=21 (21 cages of 6 adult *B. aeneus*; days 0–2), 18 (18 cages of 6 adult *B. aeneus*; days 3–5) and 15 (15 cages of 6 adult *B. aeneus*; days 6–19) per treatment for survival assessment; n=3 (3 replicates of 6 adult *B. aeneus*) for each time-point of analysis within each dsαCOP- and dsGFP treatment, for qPCR analysis. In feeding experiment, beetles were fed dsRNA for 5 d, followed by *ad libitum* feeding on anthers of oilseed rape flowers for the remaining duration of survival assessment (**III**; Willow et al., 2020b, J. Pest Sci.).

For confirmation of oral RNAi, *B. aeneus* adults were identified and placed into cages, in groups of six randomly chosen and fast moving beetles per cage. We tested six treatments, including dsGFP, dsαCOP (**III**) and dsrps13, each at both 1 and 3 µg/µL. Treatment solutions consisted of 25% organic honey, and nfH_2O was used to obtain desired dsRNA concentrations. Bromophenol blue was added to allow confirmation of feeding via the presence of blue faeces in cages. Each treatment was allocated 21 cages, each cage containing six beetles and one modified Eppendorf cap (removed from 1.5 mL Eppendorf tubes; hereafter referred to as cap) forming a drinking basin. The height of the cap was reduced via razorblade, to allow beetles to stand up and drink from the cap. Into each cap was pipetted 100 µL of treatment solution. Prior to pipetting dsRNA treatments, treatment stocks were vortexed (Vortex-Genie 2, Scientific Industries, Bohemia, USA). Cages were then kept in the climate chamber, where beetles were allowed to feed *ad libitum* on treatments. A sterilised cap with freshly-prepared treatment was provided to each cage every 24 ± 1 h, and the previous day's cap was removed. Survival and mobility were assessed every 24 ± 1 h for 19 d; and dead beetles were removed from cages daily (**III**).

After 2 d of dsRNA feeding, three cages of six live beetles, per treatment, were removed for qPCR analysis (qPCR method for this study is described below). After 5 d of dsRNA feeding, three more cages, per treatment, were removed for qPCR analysis. This left 18 and 15 cages, respectively, for survival/mobility analysis after these time points. Experiment setup for dsRNA feeding is illustrated in Figure 3b. For the remaining two weeks of survival/mobility monitoring, beetles were allowed to feed *ad libitum* on pollen of laboratory-grown oilseed rape flowers, as well as provisioned with cotton soaked in dH_2O. Fresh food and water were provided to each cage every 24 ± 1 h; and the previous day's food and water were removed from the cages (**III**).

B. aeneus adults used for qPCR analysis were removed from their cages at 2 and 5 d after first exposure to dsRNA treatments (three replicates of six beetles, per time point, per treatment; see above). There were six total treatment groups for qPCR analysis. These included beetles injected with dsGFP and dsαCOP, each at 1 µg/µL; and those fed with dsGFP and dsαCOP, each at both 1 and 3 µg/µL. Beetles allocated for qPCR were placed in RNA*later* RNA Stabilization Solution (Thermo Fisher Scientific, Waltham, USA) until qPCR analysis. RNA extraction was per-

formed via RNeasy Mini Kit (Qiagen, Venlo, Netherlands); and 200 ng of RNA were used to determine relative mRNA expression (SOLIScript 1-step kit, Solis BioDyne, Tartu, Estonia). Cycle conditions were 50 °C for 15 min, 40 cycles at 95 °C for 15 s, 58 °C for 60 s, ending with a melting curve analysis (QuantStudio 5 Real-Time PCR System, Applied Biosystems, Foster City, USA). Normalisation of data was performed using the two housekeeping genes *actin* (*act*) and *ribosomal protein S3* (*rps3*). Primer amplification efficiencies were determined through an RNA serial dilution (SI Table 3, **III**). Relative mRNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) (**III**).

4.3.3.3. *RNAi via consumption of dsRNA-treated buds* (**IV**)

Leading racemes (range 18–24 cm in length) were cut from oilseed rape plants during the green bud stage (BBCH 53–55). There were three treatment solutions in this experiment, into which bud clusters were swirled. Treatments included dsGFP at 5 μg/μL, and dsαCOP at 2.5 and 5 μg/μL. Treatments were prepared from dsRNA, dH_2O and the surfactant Triton X-100 (Fisher Bioreagents, Leicestershire, UK). A concentration of 180 ppm of Triton X-100 was present in each treatment. Treatment solutions were vortexed prior to treating bud clusters. After swirling buds clusters in their respective treatment solutions for 1 min (this duration of swirling was required in order to reliably break the surface tension caused by the bud epithelial wax), bud clusters were allowed to air dry for 1 h. The bottom end (cut tip) of each raceme was then kept underwater in modified plastic labware (height 12 cm). The raceme was held in place, at the top of the labware, using aluminium foil. For each sample, six *B. aeneus* adults were placed into a distal corner of a transparent-white organza fabric bag (20 × 30 cm), and kept in the corner by pinching the bag near that corner. During this time, the bag was fastened with string to the neck of the labware. Once fastened, the six beetles were released from the corner of the bag, and allowed to feed *ad libitum* on the treated buds for 3 d. The 3 d exposure to treatments took place in a climate room (Flohr Instruments, Utrecht, Netherlands) at 18 °C, 70 ± 5% RH and 16 : 8 h light : dark cycle. Figure 4 shows bioassay setup. The experiment was replicated three times.

In each replication of the experiment, each treatment was allocated five samples. Beetles remained undisturbed during their 3 d period of exposure to dsRNA treatments; thus, survival monitoring started after the

dsRNA feeding period, and thereafter occurred every 24 ± 1 h. After 3 d of feeding on dsRNA-treated buds, bud-feeding setups were dismantled; and the beetles were transferred to the laboratory, and kept in cages with their respective samples. Upon relocation, the beetles were maintained in a climate chamber (Sanyo MLR-351H) at 20 °C, 60% RH and 16 : 8 h light : dark cycle; and provisioned daily with fresh untreated oilseed rape flowers dH_2O-soaked cotton. For each replication of the experiment, survival was monitored until 15 d post treatment. Beetles that escaped from bud feeding setups were accounted for in the statistical analysis. Any samples where more than two beetles escaped from bud feeding setups

Figure 4. Setup for *ad libitum* feeding of dsRNA-treated oilseed rape buds to adult *Brassicogethes aeneus*. Clockwise from top-left: soaking a bud cluster in dsRNA treatment; dsRNA-soaked bud cluster; dsRNA-treated, dried bud cluster setup; pollen beetles feeding on dsRNA-treated oilseed rape buds.

were removed from the study. Total sample size for each treatment was n = 14 (83 beetles), n = 14 (80 beetles) and n = 15 (87 beetles), for dsGFP 5 µg/µL, dsαCOP at 2.5 µg/µL and dsαCOP at 5 µg/µL, respectively.

Relative *αCOP* expression analysis was performed via qPCR for all treatments. For each experimental replicate, one random cage of six live beetles (qPCR sample size n = 3), per treatment, was removed at 3 d (upon dismantling the bud feeding setups), and again at 6 d. Beetles removed for qPCR were accounted for in the statistical analysis regarding survival. Beetles removed for qPCR were immediately placed in Eppendorf tubes, and kept with individuals from their respective cages. Beetles were homogenised, using a sterile plastic pestle designed for Eppendorf tubes, in 600 µL of RTL buffer (with 10 µL of β-mercaptoethanol added), and stored at −80 °C until analysis. Total RNA was extracted via RNeasy Mini Kit. RNA concentration, and absence of nucleic contaminants, were assessed via NanoDrop spectrophotometer (Thermo Fisher Scientific). Absence of nucleic contaminants was further verified via gel electrophoresis. Genomic DNA was removed via Turbo DNA-Free Kit (Invitrogen, Carlsbad, USA), following manufacturer's protocol. The cDNA was reverse transcribed from 1 µg of RNA via FIREScript RT cDNA Synthesis Kit (Solis BioDyne); and qPCR was performed via QuantStudio 5 Real-Time PCR System. The reaction mix included 4 µL of 5xHOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne), 0.5 µL of both 10 µM forward and reverse primers (Microsynth, Balgach, Switzerland; SI Table 4, **IV**), 14 µL of nfH_2O and 500 ng of cDNA, in a total volume of 20 µL. Amplification consisted of 15 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 58 °C, and ending with a melting curve analysis (range 60–95 °C). Reactions were performed in 384-well PCR plates, in triplicate. Normalisation of the data was performed using the two housekeeping genes *act* and *rps3*. Primer amplification efficiencies were determined via cDNA dilution series; and primer sequences and amplification efficiencies are shown in SI Table 4 (**IV**). Relative *αCOP* expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A no-template- and no-reverse-transcriptase control were both included in the assay.

To confirm the presence and stability of applied dsRNA treatments on buds, over the 3 d duration of the bud feeding, we performed RT-PCR at 1 h, and 1, 2 and 3 d post dsRNA-application. We used dsGFP as the model dsRNA for this analysis, and applied this dsRNA at both 2.5 and

5 µg/µL. Bud clusters for dsRNA-stability analysis were treated and maintained in the same manner as those used for bud feeding. At each of the four time points of interest, total RNA was extracted from 0.1 g of buds via RNeasy Plant Mini Kit (Qiagen), following manufacturer's protocol. RNA concentration was quantified, and absence of nucleic contaminants was assessed, via NanoDrop spectrophotometer. Absence of nucleic contaminants was further verified via gel electrophoresis. Detection of dsGFP was performed using 500 ng of RNA, via SuperScript III One-Step RT-PCR System (Invitrogen) with *gfp*-specific primers at 10 pmol (SI Table 4, **IV**). Both 200 ng and undiluted dsGFP were included as positive controls. Samples were ran in an Eppendorf Mastercycler (Hamburg, Germany) under the following conditions: 10 min at 75 °C, 30 min at 55 °C, 2 min at 94 °C, 40 cycles of 15 s at 94 °C, 30 s at 55 °C, 1 min at 68 °C and 5 min at 68 °C. The denaturing step of 10 min at 75 °C was added to the protocol, in order to denature the secondary structure of the dsGFP. Amplified fragments were analysed via gel electrophoresis.

4.3.3.4. *Larval RNAi via consumption of dsRNA-treated anthers* (**V**)

B. aeneus larvae were gently placed in cages, using a fine paintbrush, in groups of eight randomly chosen larvae per cage. The four treatments included dsGFP at 5 µg/µL, and dsαCOP at 0.5, 2.5 and 5 µg/µL. Each treatment was allocated ten cages (n = 10; 80 larvae per treatment). Petals were removed from oilseed rape flowers, and the anthers were soaked in respective treatment solutions for 15 s and allowed to air dry. Treatment solutions contained dsRNA, dH_2O and 180 ppm of the surfactant Triton X-100, and were vortexed prior to dipping anthers. Larvae were allowed to feed *ad libitum* on treated anthers, and were maintained in a climate chamber (Sanyo MLR-351H) at 20 °C, 70% RH and 16 : 8 h light : dark cycle. Freshly-treated anthers were replaced every 24 ± 1 h for 3 d, followed by provision of fresh untreated anthers every 24 ± 1 h for an additional 4 d. Dead larvae were removed from cages daily. After the first 24 h of the bioassay, any dead larvae removed from the study, since these deaths could not be the result of RNAi, but rather stress from manipulations and changing conditions. Mortality after 24 h was minimal, and these removals were accounted for in the statistical analysis. Minimal deaths of *B. aeneus* larvae also resulted from inadvertent predation events by dipteran larvae; and these RNAi-unrelated losses were also accounted for in the statistical analysis.

At both 3 and 6 d after the start of the experiment, 15 larvae per treatment were randomly removed from cages for relative *αCOP* expression analysis via qPCR (n = 3 per time point, per treatment; 5 larvae pooled per sample). Removals of larvae for qPCR were accounted for in the statistical analysis. Larvae used for qPCR were immediately placed in Eppendorf tubes, homogenised in 600 µL of RTL buffer (with 10 µL of *β*-mercaptoethanol added) using sterile plastic pestles designed for Eppendorf tubes, and stored at −80 °C until analysis. Total RNA was extracted from samples via RNeasy Mini Kit. RNA concentration was quantified, and absence of nucleic contaminants was assessed, via NanoDrop spectrophotometer. Absence of nucleic contaminants was further verified via gel electrophoresis. Genomic DNA was removed via Turbo DNA-Free Kit (Invitrogen). Reverse transcription of cDNA was performed via FIREScript RT cDNA Synthesis Kit, using 1 µg of total RNA. The qPCR was performed in QuantStudio 5 Real-Time PCR System. The reaction mix included 4 µL of 5xHOT FIREPol EvaGreen qPCR Supermix, 0.5 µL of both 10 µM forward and reverse primers (SI Table 5, **V**), 14 µL of nfH_2O and 1 µg of cDNA, in a total volume of 20 µL. Amplification consisted of 15 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 58 °C, and ending with a melting curve analysis (range 60–95 °C). Reactions were performed in 384-well PCR plates, in triplicate. Normalisation of the data was performed using the two housekeeping genes *act* and *rps3*. Primer amplification efficiencies were determined via cDNA dilution series; and primer sequences and amplification efficiencies are shown in SI Table 5 (**V**). Relative mRNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A no-template- and no-reverse-transcriptase control were both included in the assay.

4.3.3.5. *Short-term vs chronic dsRNA feeding* (**VI**)

B. aeneus adults were transferred to cages, in groups of eight randomly chosen and fast moving beetles per cage. Treatment provision included *ad libitum* access to dsRNA-treated anthers of oilseed rape flowers. Petals were removed from flowers, and anthers were soaked in respective treatment solutions for 15, and allowed to air dry. Treatment solutions contained dsRNA, dH_2O and 180 ppm of the surfactant Triton X-100. Treatments were vortexed prior to treating anthers. The eight treatments included dsGFP at 5 µg/µL, and dsαCOP at 0.5, 2.5 and 5 µg/µL, each provided for 3 d to one group (receiving untreated anthers after 3 d), another group receiving daily treatment (hereafter respectively referred

to as short-term- and chronic dsRNA feeding). Beetles were provisioned with fresh anthers every 24 ± 1 h. Cages of beetles were maintained in a climate chamber (Sanyo MLR-351H) at 20 °C, 60% RH and 16 : 8 h light : dark cycle. Each cage was additionally provisioned with a piece of cotton soaked in dH_2O. The experiment was replicated three times, each replication allocating five cages per treatment (starting n = 15; 120 insects per treatment). Experimental setup is illustrated in Figure 5 (**VI**).

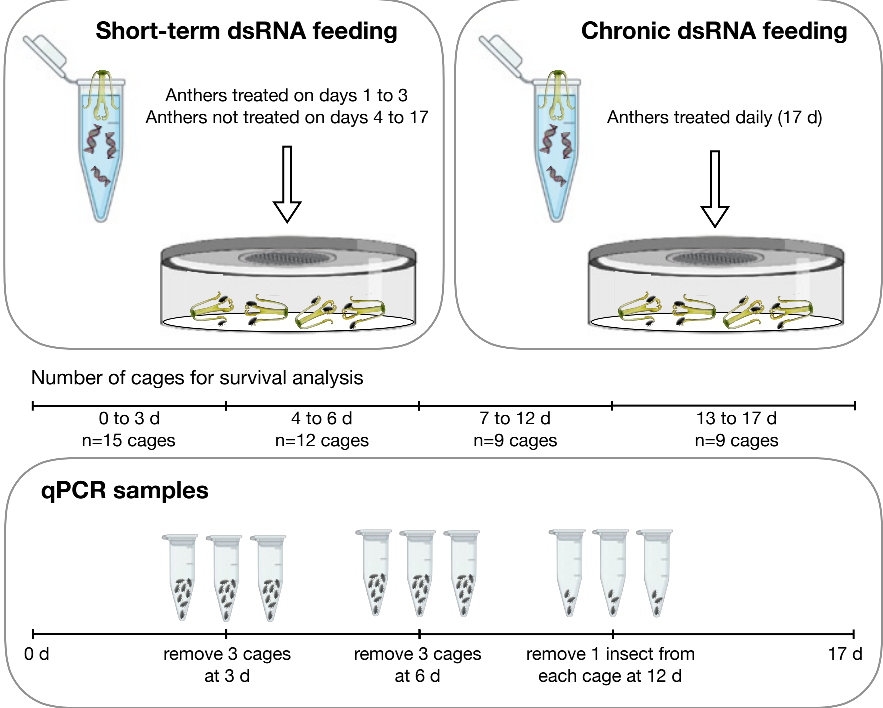


Figure 5. Experimental setup for each treatment (dsαCOP at 0.5, 2.5 and 5 µg/µL, and dsGFP at 5 µg/µL), for both short-term (3 d) and chronic (17 d) dsRNA feeding, in *Brassicogethes aeneus* adult RNAi assays. Here we monitored *B. aeneus* survival and corresponding *αCOP* expression in specimens. For survival analysis, starting n=15 cages per treatment, each cage with eight insects. For both short-term- and chronic dsRNA feeding, three cages were removed for qPCR analysis at both 3 and 6 d, and one insect was removed from each of the nine remaining cages at 12 d, for each treatment (**VI**; Willow et al., In Press, Commun. Biol.).

Each experimental replicate lasted 17 d. After 1 d, any dead beetles were removed from the study, since these deaths could not be due to RNAi, but likely stress resulting from manipulations and changing conditions. These mortalities after 1 d were minimal, and removal of these insects was accounted for in the statistical analysis. Survival was monitored every 24 ± 1 h, and dead insects were removed from cages.

Relative *αCOP* expression analysis was performed for all treatments via qPCR at 3, 6 and 12 d after the start of each replicate. At both 3 and 6 d, one cage was randomly removed from each treatment (min 6, max 8 beetles per sample; qPCR sample n = 3 cages; leaving n = 12 and n = 9 after 3 and 6 d, respectively, for survival monitoring). At 12 d, one beetle was removed from each remaining cage and used for qPCR analysis (3 beetles were pooled per experimental replicate; qPCR sample n = 3). Removals of beetles for qPCR were accounted for in the statistical analysis. Samples used for qPCR were immediately transferred into respective Eppendorf tubes and homogenised, via sterile plastic pestles designed for Eppendorf tubes, in 600 µL of RTL buffer (with 10 µL of *β*-mercaptoethanol added), and stored at −80 °C until analysis. Total RNA was extracted from samples via RNeasy Mini Kit. RNA concentration was quantified, and absence of nucleic contaminants was assessed, via NanoDrop spectrophotometer. Absence of nucleic contaminants was further verified via gel electrophoresis. Genomic DNA was removed via Turbo DNA-Free Kit (Invitrogen). Reverse transcription of cDNA was performed via FIREScript RT cDNA Synthesis Kit, using 1 µg of total RNA. The qPCR was performed in QuantStudio 5 Real-Time PCR System. The reaction mix included 4 µL of 5xHOT FIREPol EvaGreen qPCR Supermix, 0.5 µL of both 10 µM forward and reverse primers (SI Table 6, **VI**), 14 µL of nfH_2O and 500 ng of cDNA, in a total volume of 20 µL. Amplification consisted of 15 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 58 °C, and ending with a melting curve analysis (range 60–95 °C). Reactions were performed in 384-well PCR plates, in triplicate. Normalisation of the data was performed using the two housekeeping genes *act* and *rps3*. Primer amplification efficiencies were determined via cDNA dilution series; and primer sequences and amplification efficiencies are shown in SI Table 6 (**VI**). Relative mRNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A no-template- and no-reverse-transcriptase control were both included in the assay.

4.4. Data analysis

4.4.1. Analysing acute effect of thiacloprid alone and in combination with tebuconazole, in a model parasitoid (I)

Statistical analyses comparing pesticide treatments to the control treatment were performed in R v1.0.136 (R foundation for Statistical Computing, Vienna, Austria). We used a one-way ANOVA followed by *post hoc* pairwise comparisons via two-tailed unpaired t-tests, correcting for multiple comparisons via Bonferroni correction. Since residuals of the linear model were normally distributed, we used non-transformed data. Synergistic toxicities of each pesticide mixture treatment was determined by subtracting single-pesticide effects, of both thiacloprid ($Effect_{TH}$) and tebuconazole ($Effect_{TEB}$), from the effect of the respective combinatory treatment ($Effect_{TH+TEB}$). An $Effect_{TH+TEB}$ greater than the sum of $Effect_{TH}$ and $Effect_{TEB}$ constituted synergistic toxicity.

4.4.2. Analysing acute effect of EOs on pollen beetle (II)

Statistical analyses were performed in R v1.1.463. After performing topical dosing of EOs, differences between EO treatments and the control treatment, regarding both mobility and survival, were assessed via Fisher's exact test followed by Bonferroni correction for multiple comparisons. After exposing *B. aeneus* adults to dry residues of cinnamon bark EO, on oilseed rape leaf and bud tissue, homogeneity of variance and normality of data distributions were determined via Levene- and Shapiro-Wilk tests, respectively. Since only higher concentrations were normally distributed, we used the Kruskal-Wallis test as a nonparametric alternative to ANOVA, followed by Bonferroni-Dunn's test for *post hoc* pairwise comparisons.

4.4.3. Analysing RNAi in pollen beetle

After performing the microinjection-based screening of potential RNAi targets in *B. aeneus* adults, we compared survival for each target treatment to the control treatment in R via Fisher's exact test followed by Bonferroni correction for multiple comparisons. In the study confirming dietary RNAi response in *B. aeneus* adults (**III**), statistical analyses were performed in R v1.1.463. Survival and mobility after microinjection of dsRNAs targeting dsαCOP and dsrps13 treatments was compared to

that of the dsGFP control via Fisher's exact test. In addition, survival and mobility after feeding of dsαCOP and dsrps13 treatments were compared to respective dsGFP controls. Homogeneity of variance and normality of data distributions were determined via Levene- and Shapiro-Wilk tests, respectively. Since data were overall not normally distributed, we used the Kruskal-Wallis test as a nonparametric alternative to ANOVA, followed by Wilcoxon rank-sums test for *post hoc* pairwise comparisons. Comparisons between dsGFP- and respective dsαCOP treatments, regarding relative *αCOP* expression, were determined via Welch's t-test for both microinjected and dsRNA-fed beetles.

For the bud feeding experiment, the experiment where we fed dsRNA-treated oilseed rape anthers to *B. aeneus* larvae, and the experiment where we provided short-term and chronic dsRNA treatments via anther feeding to *B. aeneus* adults, all statistical analyses were performed in R v3.6.3. In the bud feeding experiment (**IV**), for both survival and *αCOP* expression analysis, we compared the dsGFP control to both dsαCOP concentrations. In the larval experiment (**V**), for both survival and *αCOP* expression analysis, we compared the dsGFP control to dsαCOP at all three concentrations. For the short-term vs chronic dsRNA feeding experiment, treatment comparisons taken into consideration are listed in SI Table 7 (**VI**). Regarding survival analysis, for dsGFP and all three dsαCOP concentrations, comparisons between short-term- and chronic exposure were statistically assessed. In addition, comparisons in survival were made between dsGFP and all three dsαCOP concentrations, as well as between dsαCOP concentrations, within both short-term- and chronic exposure groups. When comparing different dsRNAs or concentrations, comparisons in survival were only made between treatment groups that were given the same duration of exposure to dsRNA. Regarding gene expression analysis, comparisons were made between dsGFP and all three dsαCOP concentrations, within both short-term- and chronic exposure groups. For survival analysis, homogeneity of variance and normality of data distributions were determined via Levene- and Shapiro–Wilk tests, respectively (**III**, **IV**, **V**, **VI**). As the data were overall not normally distributed, the Kruskal–Wallis test was used as a nonparametric alternative to ANOVA, followed by the Wilcoxon rank-sums test with Bonferroni correction for *post hoc* pairwise comparisons. Comparisons in *αCOP* expression were made via Welch's t-test (**III**, **IV**, **V**, **VI**).

5. RESULTS

5.1. Acute effect of thiacloprid alone and in combination with tebuconazole, in a model parasitoid (I)

After 24 h of exposing *A. abdominalis* to dry residues of thiacloprid applied at RFC, we observed 52% mortality ($p = 0.009$, Fig. 6, **I**) and 79% immobility ($p = 0.0002$), indicating that this concentration of thiacloprid was too effective on immobility to reliably use RFC of thiacloprid for our experiment.

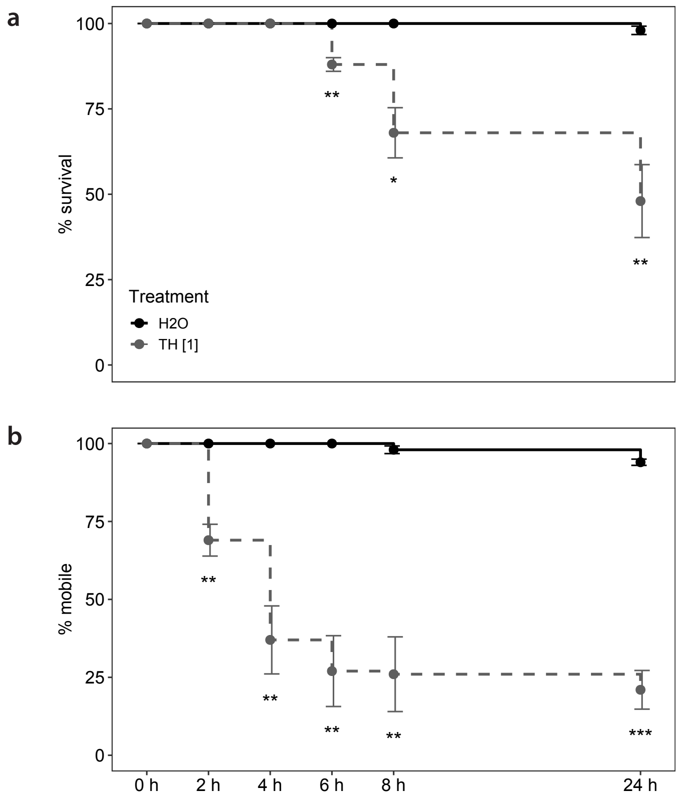


Figure 6. Effect of thiacloprid [Calypso (suspension concentrate, 480 g thiacloprid/L, Bayer CropScience)] at recommended field concentration (RFC, 120 g/ha) on survival (a) and mobility (b) of the parasitoid wasp *Aphelinus abdominalis* (n=5 cages, each with 20 insects) at different hours post exposure to dry residues of thiacloprid on glass surface (error bars: ±SEM). H_2O=control, TH=thiacloprid. Asterisk (*) indicates statistical significance compared to control treatment. Welch's two-tailed unpaired t-test: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ (**I**; Willow et al., 2019, PLoS One).

Exposure to dry residues of thiacloprid–tebuconazole combinations demonstrated tebuconazole's synergising effect, after 24 h, on thiacloprid toxicity when tebuconazole was co-applied at one twentieth RFC, one tenth RFC, one half RFC and RFC (Fig. 7a, **I**). *Post hoc* pairwise comparisons showed that thiacloprid at one tenth RFC by itself did not significantly affect survival at 24 h ($p = 0.44$) compared to the dH_2O control treatment. However, a significant effect on survival resulted from combining thiacloprid at one tenth RFC with tebuconazole at one tenth RFC ($p = 0.03$), one half RFC (0.02) and RFC ($p = 0.009$). Combining

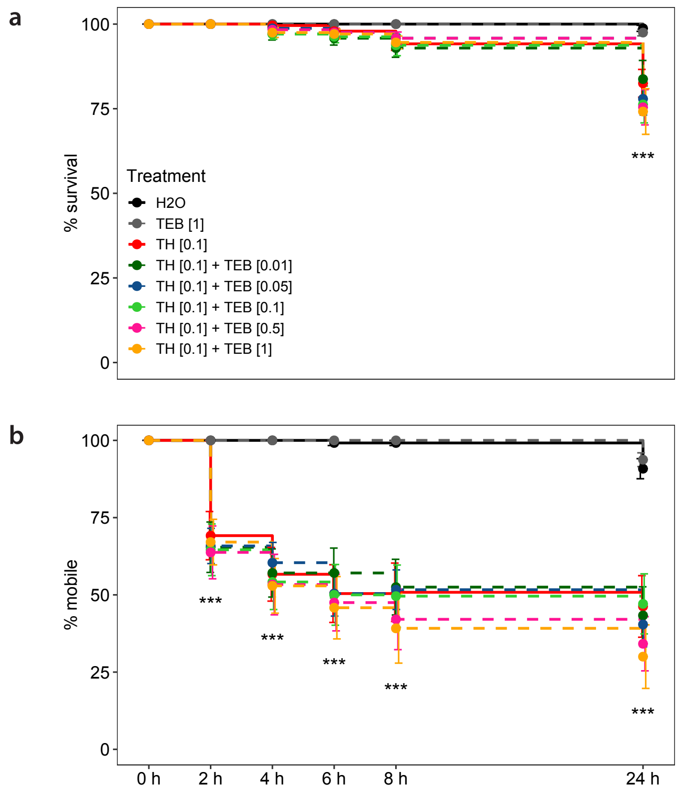


Figure 7. Effect of treatments, containing thiacloprid [Calypso (suspension concentrate, 480 g thiacloprid/L, Bayer CropScience)] and/or tebuconazole [Tebusip (emulsifiable concentrate, 250 g tebuconazole/L, OXON Italia)], on survival (a) and mobility (b) of the parasitoid wasp *Aphelinus abdominalis* (n=12 cages, each with 20 insects) at different hours post exposure to dry residues of thiacloprid and/or tebuconazole on glass surface (error bars: ±SEM). H_2O=control, TH=thiacloprid, TEB=tebuconazole, [0.01]=one one-hundredth recommended field concentration (RFC), [0.05]=one twentieth RFC, [0.1]=one tenth RFC, [0.5]=one half RFC, [1]=RFC. Asterisk (*) indicates statistical significance. One-way ANOVA: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ (**I**; Willow et al., 2019, PLoS One).

thiacloprid at one tenth RFC with tebuconazole at one twentieth RFC resulted in a marginally significant ($p = 0.06$) effect on survival.

Tebuconazole synergised the immobilising effect of thiacloprid at 2, 4, 6, 8 and 24 h in combinatory treatments containing tebuconazole at one half RFC (Fig. 7b, **I**). F statistics, assessed via one-way ANOVA, showed significant effect of different treatments on mobility at 2, 4, 6, 8 and 24 h (Fig. 7b, **I**). *Post hoc* pairwise comparisons showed that, starting at 2 h post exposure, thiacloprid by itself at one tenth RFC significantly affected mobility compared to the dH_2O control treatment (2 h $p = 0.04$, 24 h $p = 0.006$) as did combinatory treatments containing tebuconazole at one one-hundredth RFC (2 h $p = 0.01$, 24 h $p = 0.002$), one twentieth RFC (2 h $p = 0.01$, 24 h $p = 0.0009$), one tenth RFC (2 h $p = 0.009$, 24 h $p = 0.007$), one half RFC (2 h $p = 0.007$, 24 h $p = 0.0001$) and RFC (2 h $p = 0.02$, 24 h $p < 0.0001$).

5.2. Acute effect of EOs on pollen beetle (II)

5.2.1. Screening EOs via topical dosing

We observed that at 5.48 nL/mg, *C. verum* was the only EO that significantly lowered both survival ($p < 0.0001$ at 24 and 48 h) and mobility ($p < 0.0001$ at 6, 24 and 48 h) of *B. aeneus* (Fig. 8, **II**). Topical application of *C. verum* EO at 5.48 nL/mg resulted in 90%, 67.5% and 60% survival at 6, 24 and 48 h post application, respectively; the only difference between survival and mobility was an initial drop in mobility to 67.5% at 6 h. With each increase in dose of this EO, a decrease was observed for both *B. aeneus* survival (at 48 h: 35%, 12.5% and 2.5%, for 7.31 nL/mg, 9.14 nL/mg and 18.27 nL/mg, respectively) and mobility (at 48 h: 35% and 10%, for 7.31 nL/mg and 9.14 nL/mg, respectively).

Three EOs began to show a significant effect on *B. aeneus* survival at 7.31 nL/mg, this effect generally increasing with increased doses. These include EOs of *T. vulgaris* (60% survival, $p < 0.0001$ at 48 h), *P. anisum* (60% and 55%, $p < 0.0001$ at 24 and 48 h, respectively) and *C. cyminum* (72.5%, 55% and 52.5%, $p < 0.0001$ at 6, 24 and 48 h, respectively). These same three EOs also began to show a significant effect on *B. aeneus* mobility at this dose, during each time point (67.5%, 72.5% and 60% mobile, $p < 0.0001$ for *T. vulgaris* at 6, 24 and 48 h, respectively; 65%,

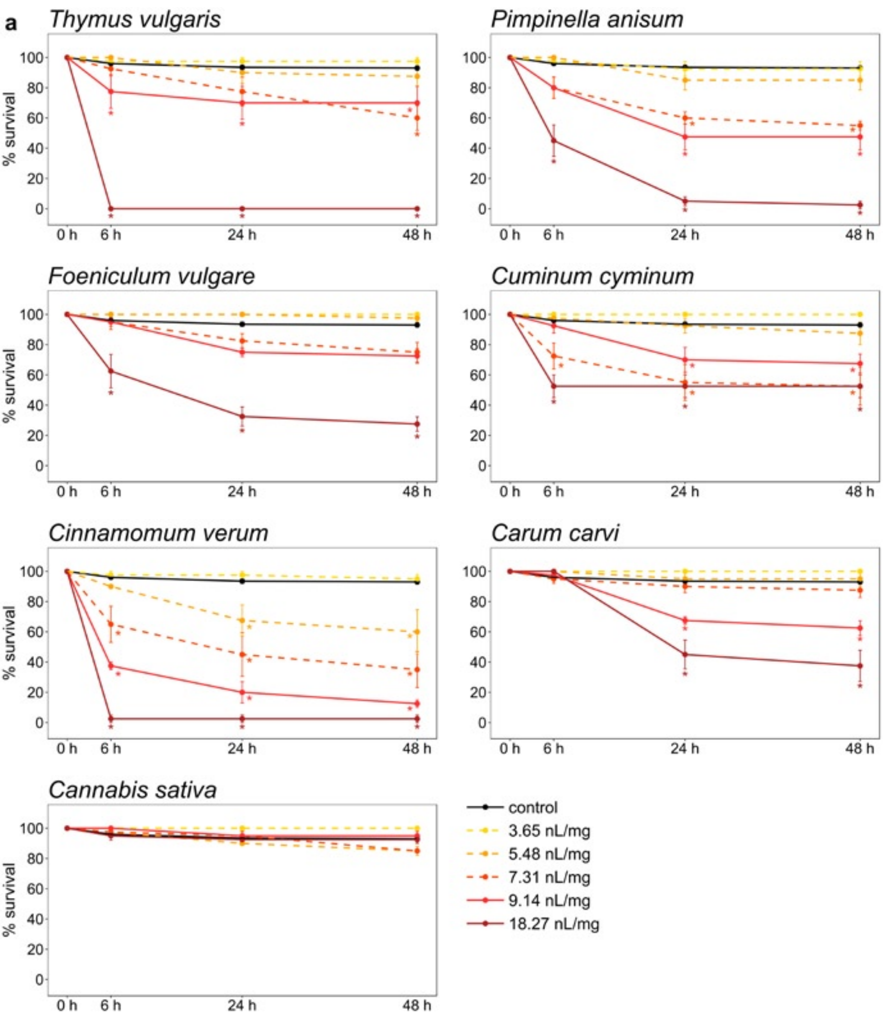


Figure 8. Effect of seven plant essential oils at different doses on *Brassicogethes aeneus* survival (a) and mobility (b), via direct dosing of individual beetles. n=40 (four replications of ten beetles) per essential oil treatment; n=200 (four replications of 50 beetles) for the control treatment. Asterisk (*) indicates statistical significance compared to the control treatment. Fisher's exact test (error bars: ±SE): * = $p < 0.00046$ (Bonferroni correction threshold) (**II**; Willow et al., 2020a, Crop Prot.).

57.5% and 55%, $p < 0.0001$ for *P. anisum* at 6, 24 and 48 h, respectively; and 60%, 52.5% and 50%, $p < 0.0001$ for *C. cyminum* at 6, 24 and 48 h, respectively). At this dose, *F. vulgare* EO significantly affected *B. aeneus* mobility at 6 h (72.5% mobile, $p < 0.0001$ at 6 h).

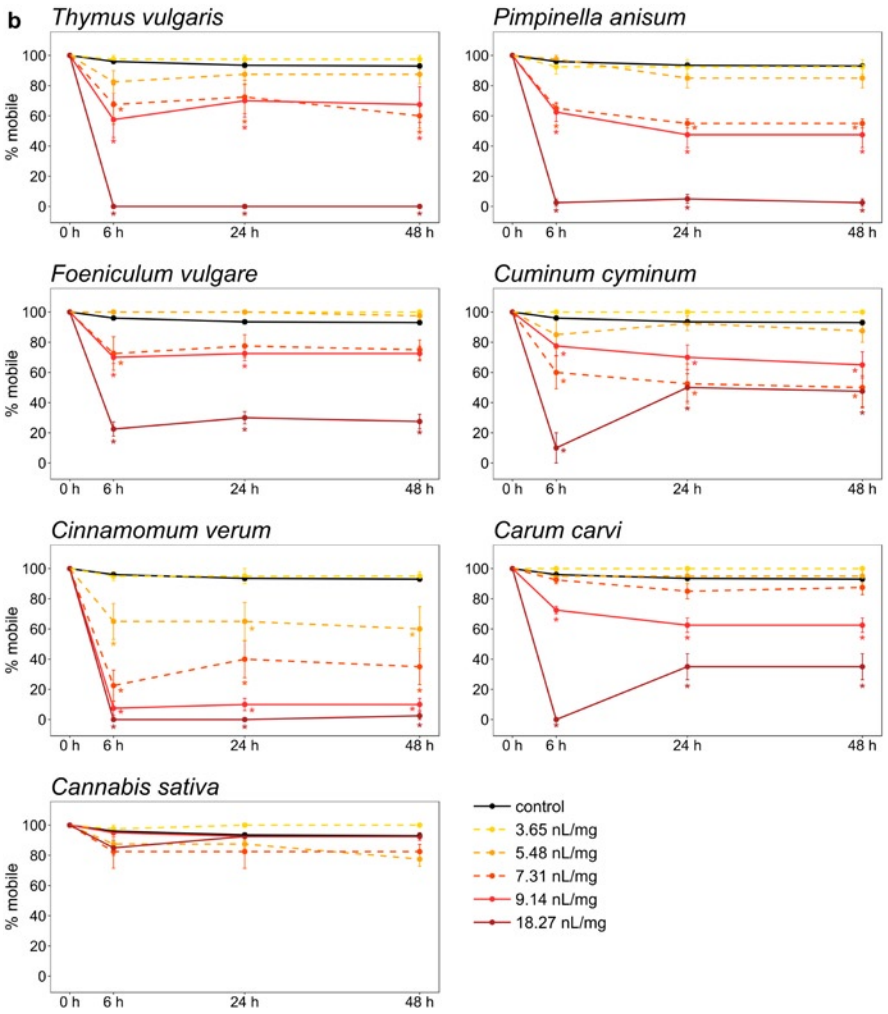


Figure 8. (*cont.*)

At 9.14 nL/mg, *C. carvi* began to significantly affect *B. aeneus* survival (67.5% and 62.5% survival, $p < 0.0001$ at 24 and 48 h, respectively) and mobility (72.5%, 62.5% and 62.5% mobile, $p < 0.0001$ at 6, 24 and 48 h, respectively), the effect increasing with the highest dose. No significant effect of *C. sativa* EO on either survival or mobility of *B. aeneus* was observed at any time point.

5.2.2. Leaf and bud treatment

Compared to the control treatment, significant effects on both survival ($X^2 = 72.15$, df = 6, $p < 0.0001$) and mobility ($X^2 = 76.48$, df = 6, $p < 0.0001$) when oilseed rape leaf and bud surfaces were sprayed with *C. verum* EO. *Post hoc* tests showed a significant effect of exposure to dry *C. verum* EO residues on leaf and bud surfaces sprayed with *C. verum* EO concentrations of 1.5% (86.7% survival, $p = 0.04$; 82.5% mobile, $p = 0.01$), and higher (71.7% survival, $p = 0.0001$, and 68.3% mobile, $p < 0.0001$, for 1.6% concentration; 53.3% survival and 49.1% mobile for 1.7% concentration, $p < 0.0001$; and 46.7% survival and 40.8% mobile for 1.8% concentration, $p < 0.0001$; Fig. 9, **II**). At 1.4% concentration, 97.5% survival and mobility ($p > 0.05$) was observed. At 1.3% concentration, and in the control treatment, there was 100% *B. aeneus* survival and mobility.

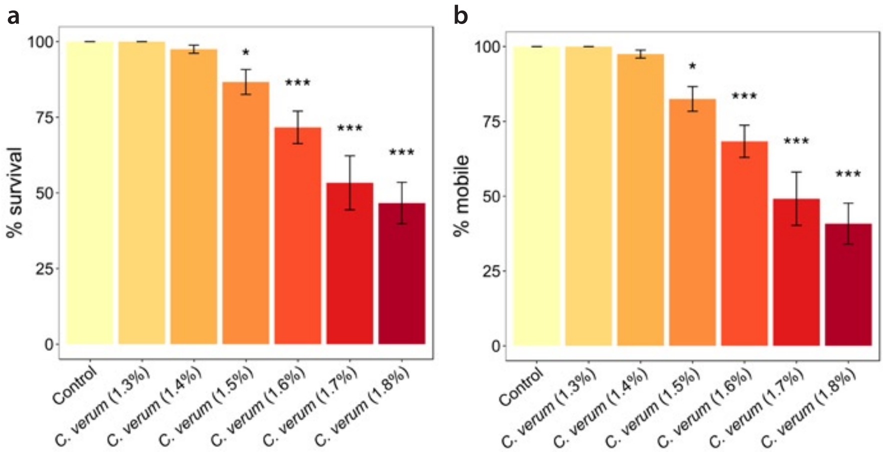


Figure 9. Effect of *Cinnamomum verum* inner bark essential oil at different concentrations on *Brassicogethes aeneus* survival (a) and mobility (b), at 24 h post exposure, via exposure to treated (sprayed) oilseed rape leaves and buds in ventilated cage bioassays. n=15 cages (eight beetles per cage) per treatment. Effect of *C. verum* EO treatments were compared to that of the control treatment, via Kruskal-Wallis test followed by Bonferroni-Dunn's test for *post hoc* pairwise comparisons (error bars: ±SEM). Asterisk (*) indicates statistical significance compared to the control treatment. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (**II**; Willow et al., 2020a, Crop Prot.).

5.3. RNAi in pollen beetle

5.3.1. Microinjection screening

All dsRNAs examined significantly reduced *B. aeneus* survival, compared to the nfH_2O control treatment (Fig. 10). At 3 d post microinjection, *B. aeneus* survival significantly decreased from microinjection of dsαCOP (54.6% survival, $p < 0.0001$), dsγCOP (50%, $p < 0.0001$), dsrps13 (61.8%, $p = 0.002$) and dsSnf7 (67.2%, $p = 0.0109$). At 4 d, microinjection of dsδCOP-injected beetles showed 67.2% survival ($p = 0.0256$), and dsVg-injected beetles showed 61.3% survival ($p = 0.006$). At 8 d, survival of dsαCOP-, dsSnf7- and dsrps13-injected beetles reached their lowest at 1.5%, 1.6% and 5.5% survival, respectively. At 10 d, survival of dsγCOP-injected beetles reached 6%, followed by dsδCOP (12.1%). Survival of dsVg-injected beetles reached its lowest (33.9%) at 9 d post injection. Survival of nfH_2O-injected beetles reached its lowest (85.7%) at 7 d post injection.

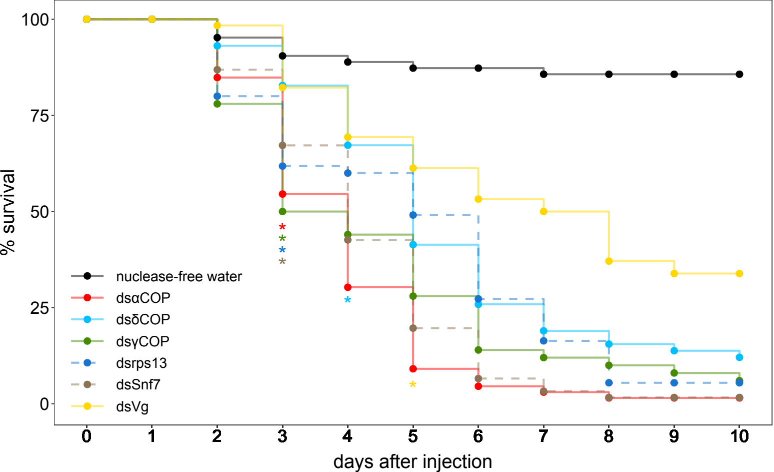


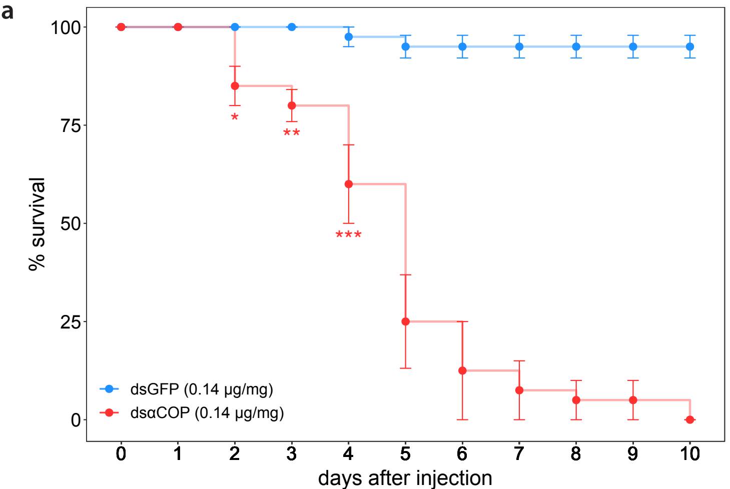
Figure 10. *Brassicogethes aeneus* survival curves, comparing lethal effect of six dsRNA treatments (1 µg dsRNA/µL; in total 0.2 µL of solution injected per beetle) to that of the nuclease-free water control. n=70 beetles per treatment. Asterisk (*) indicates statistical significance compared to the control treatment, via Fisher's exact test.

5.3.2. Confirmation of oral RNAi in pollen beetle

Direct microinjection of dsαCOP (Fig. 11a; **III**) and dsrps13 (SI Fig. 1a) both resulted in significant reduction in *B. aeneus* survival. At 10 d post injection, dsGFP-injected beetles showed 95% survival. In contrast, survival of dsαCOP-injected beetles fell to 85% ($p = 0.03$) at 2 d, 60% ($p < 0.0001$) at 4 d, 12.5% at 6 d, and 0% at 10 d post injection (**III**). For dsrps13-injected beetles, survival fell to 85% ($p = 0.03$) at 2 d, 77.5% ($p = 0.002$) at 4 d, 67.5% ($p = 0.003$) at 8 d, 60% ($p = 0.0003$) at 9 d, and 40% ($p < 0.0001$) at 10 d post injection. Mortality of dsαCOP- and dsrps13-injected beetles was often preceded by a loss of mobility (SI Fig. 2a, **III**; SI Fig. 3a).

Dietary exposure to dsαCOP (Fig. 11b; **III**) and dsrps13 (SI Fig. 1b), each at both concentrations examined, resulted in significant reductions in *B. aeneus* survival. At 19 d, we observed 95% survival in the dsGFP 1 µg/µL treatment, and 87% survival in the dsGFP 3 µg/µL treatment. Beetles fed dsαCOP at 1 µg/µL showed significant mortality (78% survival, $df = 3$, $p = 0.003$) 6 d after first exposure, followed by a steady decrease to 61% (8 d, $p < 0.0001$), 54% (10 d), 44% (12 d), 29% (14 d), 18% (16 d), and 10% survival (18 d). Beetles fed dsαCOP at 3 µg/µL showed significant mortality (77% survival, $df = 3$, $p = 0.0007$) 4 d after first exposure, survival here falling more rapidly, to 43% ($p < 0.0001$) at 6 d, 20% (8 d) 11% (10 d) and 1% (17 d, **III**). Beetles fed dsrps13 at 1 µg/µL showed significant mortality (82.2% survival, $df = 3$, $p = 0.02$) 12 d after first exposure, followed by a steady decrease to 54.4% ($p < 0.0001$, 15 d) and 27.8% survival (19 d). Beetles fed dsrps13 at 3 µg/µL showed significant mortality (86.1% survival, $df = 3$, $p = 0.0007$) 3 d after first exposure, survival here also falling more rapidly compared to the dsrps13 1 µg/µL treatment, to 67.4% ($p < 0.0001$, 7 d), % (9 d), 51.7% (10 d), 9% (14 d) and 4.5% (18 d).

From 2 d after first exposure to dsαCOP, we observed significantly lower survival in the 3 µg/µL treatment compared to the 1 µg/µL treatment (2 d $p = 0.014$, 3 d $p = 0.0085$, 4–12 d $p < 0.0001$, 13–14 d $p = 0.0002$, 15 d $p = 0.0033$, 16 d $p = 0.009$, 17 d $p = 0.002$, 18–19 d $p = 0.015$, $df = 3$, **III**). From 1 d after first exposure to dsrps13, we observed significantly lower survival in the 3 µg/µL treatment compared to the 1 µg/µL treatment (1 d $p = 0.005$, 2 d $p = 0.0002$, 3–15 d $p < 0.0001$, 16 d $p = 0.0001$, 17 d $p = 0.0002$, 18 d $p < 0.0001$, 19 d $p = 0.0004$, $df = 3$).



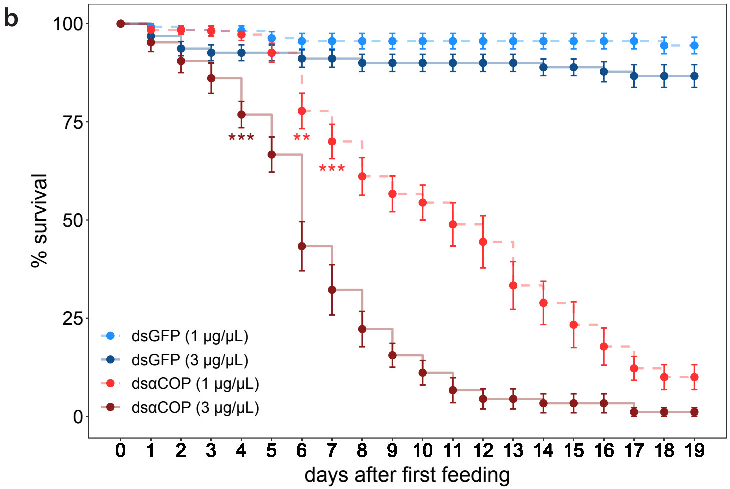


Figure 11. Survival curves, comparing dsαCOP treatments with their respective dsGFP controls, in microinjected (a) and dsRNA-fed (b) pollen beetles. Microinjection experiment: n=40 (four replicates of ten beetles) per treatment. Feeding experiment: n=21 (21 cages of six beetles; days 0–2), 18 (18 cages of six beetles; days 3–5) and 15 (15 cages of six beetles; days 6–19) per treatment. Microinjection data were analysed via Fisher's exact test (error bars: ±SE). Feeding data were analysed via Kruskal–Wallis test with *post hoc* Wilcoxon rank-sum test for pairwise comparisons (error bars: ±SEM). Asterisk (*) indicates significant differences between dsαCOP and respective dsGFP control treatments. df=3, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (**III**; Willow et al., 2020b, J. Pest Sci.).

Blue faeces were observed extensively throughout all cages from each treatment, providing further indication that pollen beetles fed on their respective treatments. Similar to dsαCOP- and dsrps13-injected beetles, mortality of dsαCOP- and dsrps13-fed beetles was often preceded by a loss of mobility (SI Fig. 2b, **III**; SI Fig. 3b).

Our qPCR results indicated that *αCOP* was downregulated by the dsRNA targeting this gene when delivered by microinjection and feeding (Fig. 12, **III**). The dsαCOP-injected beetles ($t = 7.56$, $df = 3.19$, $p = 0.0038$) and the beetles that fed on dsαCOP at 1 μg/μL ($t = 2.38$, $df = 2.65$, $p = 0.109$ showed a respective mean reduction in expression of the target gene of 82% and 52% at 5 d compared to respective dsGFP controls. At 2 d, there was no apparent reduction in relative expression of *αCOP* (microinjection: $p = 0.67$; feeding 1 μg/μL: $p = 0.44$). Beetles fed dsαCOP at 3 μg/μL showed minor reduction in relative expression of *αCOP*, with only 36% mean reduction at 2 d ($t = 1.87$, $df = 2.88$, $p = 0.16$), and 15% at 5 d ($t = 1.24$, $df = 3.60$, $p = 0.29$).

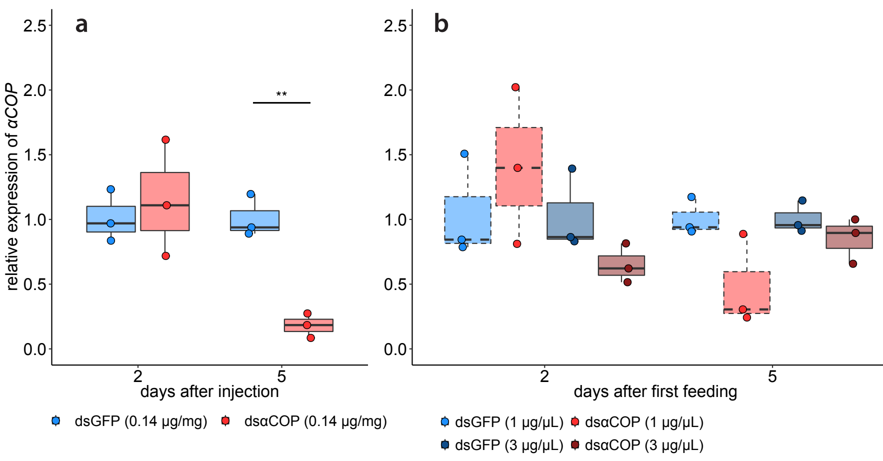


Figure 12. Relative *αCOP* expression in microinjected (a) and dsRNA-fed (b) pollen beetles, at 2 and 5 d after treatment. Data were normalised via the housekeeping genes *act* and *rps3*. n=3 (three replicates of six beetles) for each time point of analysis within each treatment. Asterisks (*) indicate significant differences between dsαCOP- and respective dsGFP treatments. Welch's t-test: ** = $p < 0.01$ (**III**; Willow et al., 2020b, J. Pest Sci.).

5.3.3. Bud feeding induced RNAi (IV)

RT-PCR results confirmed the presence and stability of dsRNA on oilseed rape buds, over the entire 3 d of exposure to dsRNA treatments, for both dsGFP concentrations examined (Fig. 13, **IV**). In the *B. aeneus* adults that fed upon the treated buds, our qPCR results showed a trend of reduced *αCOP* expression with increasing concentrations of applied dsαCOP, at both 3 and 6 d (Fig. 14, **IV**). At 3 d, we observed a 49% mean decrease in *αCOP* expression in the dsαCOP 2.5 μg/μL treatment ($t = 1.25$, $df = 2.87$, $p = 0.3$), and a 72% mean decrease in *αCOP* expression in the dsαCOP 5 μg/μL treatment ($t = 3.09$, $df = 3.99$, $p = 0.037$). At 6 d, we observed a 19% mean decrease in *αCOP* expression in the dsαCOP 2.5 μg/μL treatment ($t = 0.79$, $df = 3.13$, $p = 0.49$), and a 48% mean decrease in *αCOP* expression in the dsαCOP 5 μg/μL treatment ($t = 2.11$, $df = 2.88$, $p = 0.13$).

Regarding *B. aeneus* survival, we began observing a significant effect of treatment at 10 d (10–14 d: $X^2 = 7.8$, $df = 2$, $p = 0.02$; 15 d: $X^2 = 10.38$, $df = 2$, $p = 0.006$; Fig. 15, **IV**). After correcting for pairwise comparisons, mortality in the dsαCOP 5 μg/μL treatment was marginally significant at 10–14 d ($p = 0.056$), becoming significant at 15 d ($p = 0.021$). Survival for this treatment slowly fell from 100% (4 d) to 88% (10 d), reach its lowest at 84% (15 d). No significant effect on survival occurred in the dsαCOP 2.5 μg/μL treatment, survival falling from 100% (7 d) to 98%

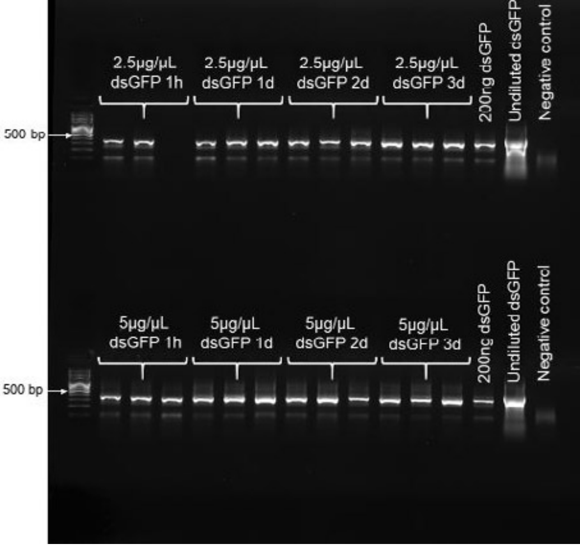


Figure 13. RT-PCR results showing presence of dsRNA (dsGFP applied at both 2.5 and 5 μg/μL) on oilseed rape bud tissue at 1 h, and 1, 2 and 3 d post dsRNA-application (**IV**; Willow et al., 2020c, Insects).

(8 d), where it settled. No mortality occurred in the dsGFP treatment. After the 3 d treatment–exposure period, all bud clusters had numerous buds incised, with both anthers and bud epithelia consumed. Together with the fact that all caged beetles survived over the entire 3 d treatment–exposure period, this indicates that all beetles fed on dsRNA-treated bud tissue.

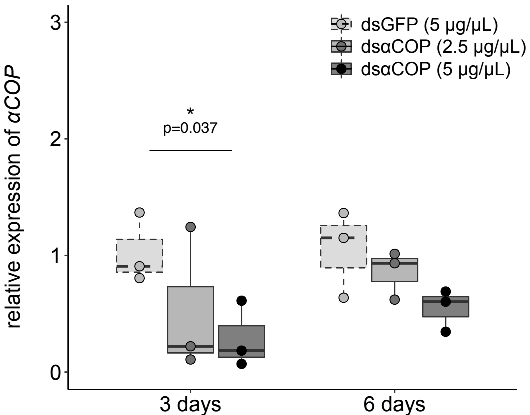


Figure 14. Results of qPCR, showing relative expression of *αCOP* in *Brassicogethes aeneus* at 3 and 6 d, comparing target treatments (dsαCOP at 2.5 and 5 µg/µL) to the dsGFP control. Asterisk (*) indicates significant difference between treatments (analysed via Welch's t-test) (**IV**; Willow et al., 2020c, Insects).

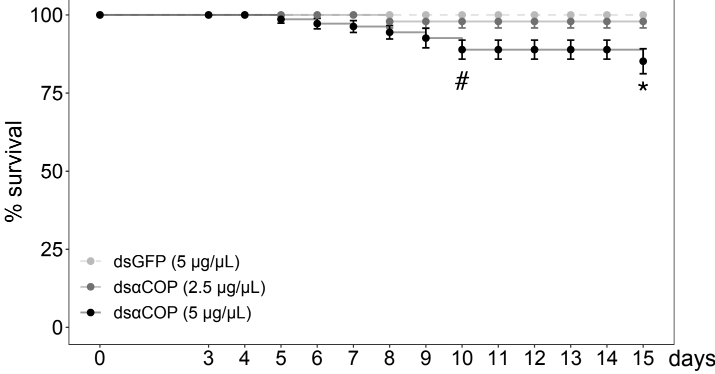
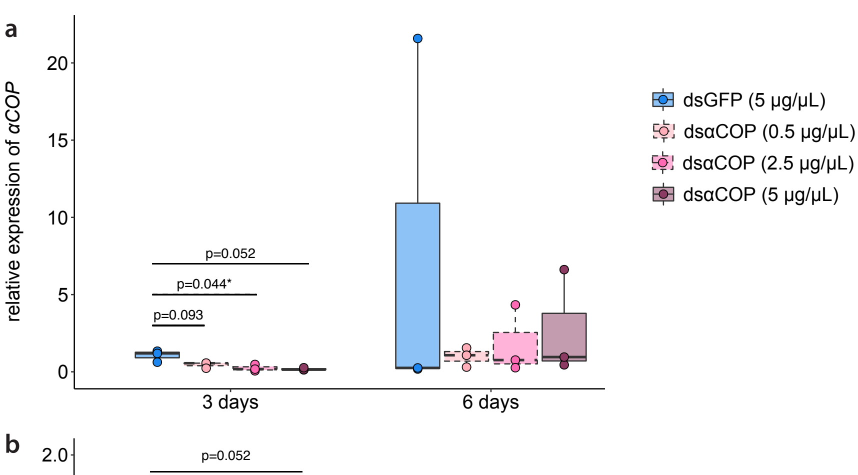


Figure 15. Survival (%) of *Brassicogethes aeneus* in each treatment, accounting for all three experimental replicates. Hash symbol (#) indicates significant effect of treatment (X^2). Asterisk (*) indicates significant difference between dsαCOP treatment and the dsGFP control ($p < 0.05$; Kruskal–Wallis test, followed by Wilcoxon rank-sums test with Bonferroni correction) (**IV**; Willow et al., 2020c, Insects).

5.3.4. Larval RNAi in pollen beetle (V)

After 3 d of feeding on dsαCOP-treated oilseed rape anthers, *B. aeneus* larvae showed 57% ($t = 2.46$, $df = 2.94$, $p = 0.093$), 77% ($t = 3.25$, $df = 3.16$, $p = 0.044$) and 83% ($t = 3.93$, $df = 2.17$, $p = 0.052$) mean reductions in *αCOP* expression, respectively for dsαCOP 0.5, 2.5 and 5 µg/µL treatments, compared to the dsGFP 5 µg/µL treatment (Fig. 16, **V**). At 6 d after the start of the experiment, *B. aeneus* larvae showed no reduction in *αCOP* expression, and more variability within treatments.

Survival monitoring showed significant reductions in survival of larvae fed dsαCOP at 2.5 µg/µL, at 4 d (79% survival, $df = 3$, $p = 0.041$) and 5 d (63%, $p = 0.02$) after the start of the experiment, followed by marginal significance (47% survival, $p = 0.066$) at 6 d, compared to the dsGFP 5 µg/µL control treatment (Fig. 17, **V**). At 7 d, survival of dsGFP control larvae dropped to 49% (dsαCOP at 0.5 µg/µL, 39%; dsαCOP at 2.5 µg/µL, 21%; dsαCOP at 5 µg/µL, 62%).



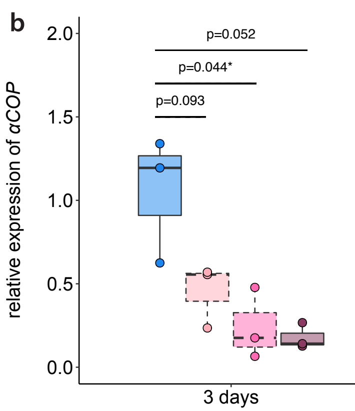


Figure 16. Results of qPCR, showing relative expression of *αCOP* in *Brassicogethes aeneus* larvae at 3 and 6 d (a); reduced y-axis value-limits for better visualisation of 3 d qPCR data (b). Target treatments (dsαCOP at 0.5, 2.5 and 5 µg/µL) were compared to the dsGFP control. n=3 (three replicates of five larvae) for each time point of analysis within each treatment. Asterisk (*) indicates significant difference between treatments. Welch's t-test: * = $p < 0.05$ (**V**; Willow et al., Submitted to Front. Agron.).

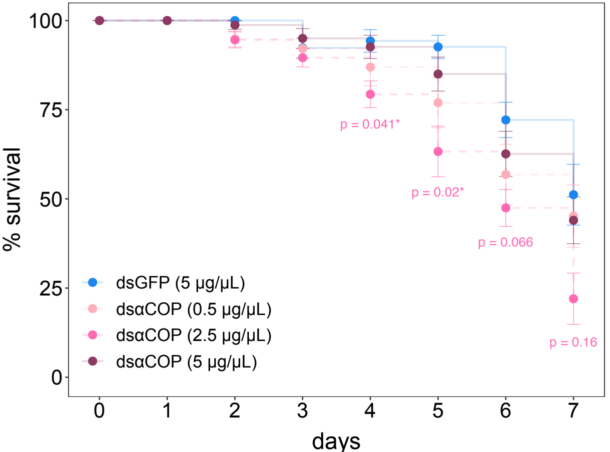


Figure 17. Survival curves comparing mortality effect of dsαCOP treatments to the dsGFP control treatment in *Brassicogethes aeneus* larvae. Starting n=10 (ten cages of eight larvae) per treatment. Data were analysed via Kruskal–Wallis test followed by Wilcoxon rank-sums test with *post hoc* Bonferroni correction for multiple comparisons (error bars: ±SEM). Asterisk (*) indicates significant difference between treatments. * = $p < 0.05$ (**V**; Willow et al., Submitted to Front. Agron.).

5.3.5. RNAi is enhanced by chronic, compared to short-term, dsRNA feeding in pollen beetle

We observed significant reductions in *B. aeneus* survival as a result of feeding on dsαCOP-treated anthers of oilseed rape, for both short-term (3 d) and chronic (daily) dsRNA feeding (Fig. 18, **VI**). With short-term dsRNA feeding, significant reductions in survival were observed starting at 8 d (64% survival) in the dsαCOP 5 μg/μL treatment, compared to the dsGFP control treatment (p = 0.007) and the dsαCOP 0.5 μg/μL treatment (p = 0.006). Survival for short-term dsαCOP feeding at 5 μg/μL fell from 64% (p = 0.007, 8 d) to 39% (p = 0.0096, 13 d), afterwards settling at 38% (p = 0.005). Similarly, significant reductions in survival (65% survival, p = 0.027) were observed starting at 9 d in short-term dsαCOP feeding at 2.5 μg/μL compared to the dsGFP control, though this difference became statistically insignificant (p = 0.08) at 15 d; here, survival largely reached its lowest at 13 d (53% survival, p = 0.04), afterwards settling at 52% (p = 0.08). When comparing the dsαCOP 2.5 μg/μL- to the dsαCOP 0.5 μg/μL treatment, reductions in survival were marginally significant starting at 8 d (p = 0.054). Similar to the dsGFP control treatment, short-term

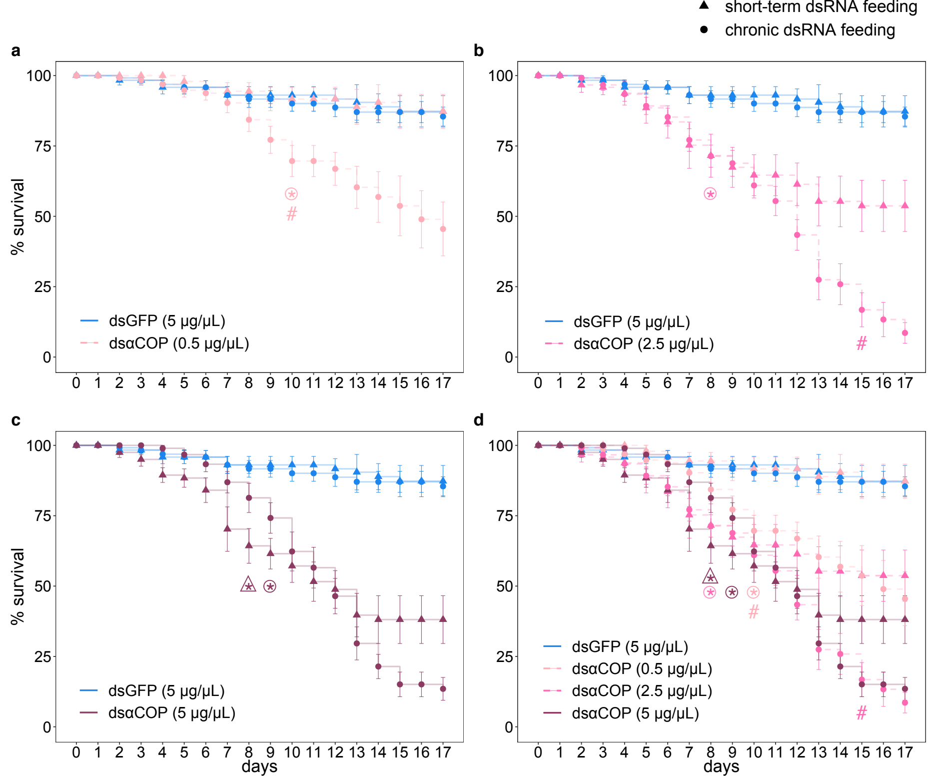


Figure 18. Survival (%) of *Brassicogethes aeneus* adults in each treatment in RNAi assay, totalled over all three experimental replicates. Survival curves show *B. aeneus* survival rates for short-term (3 d) and chronic (17 d) exposure to dsRNA treatments: dsαCOP 0.5 µg/µL (a); dsαCOP 2.5 µg/µL (b); dsαCOP 5 µg/µL (c); plot showing all three dsαCOP concentrations (d). Asterisk (*) indicates significant difference ($p < 0.05$) in survival compared to dsGFP 5 µg/µL (control) treatment. Colour of asterisk indicates the corresponding dsRNA and concentration. Asterisk in triangle indicates that the significant difference corresponds to short-term dsRNA feeding. Asterisk in circle indicates that the significant difference corresponds to chronic dsRNA feeding. Hash symbol (#) indicates significant difference ($p < 0.05$) in survival, between short-term- and chronic dsRNA feeding groups. Colour of hash symbol indicates the corresponding dsRNA and concentration. Asterisks and hash symbols are only used where values become- and remain significant. Analysed via Kruskal–Wallis test followed by Wilcoxon rank-sums test with Bonferroni correction for multiple comparisons (error bars: ±SEM) (**VI**; Willow et al., In Press, Commun. Biol.).

dsαCOP feeding at 0.5 μg/μL resulted in 87% survival at 17 d; and thus no difference in survival was observed between the short-term dsαCOP 0.5 μg/μL treatment and the short-term dsGFP control treatment.

With chronic dsRNA feeding, significant reductions in *B. aeneus* survival were observed starting at 8, 9 and 10 d, for dsαCOP 2.5 μg/μL (72% survival, $p = 0.02$), dsαCOP 5 μg/μL (74% survival, $p = 0.03$) and dsαCOP 0.5 μg/μL (70% survival, $p = 0.036$) treatments, respectively. Survival for chronic dsαCOP feeding at 0.5 μg/μL continued to steadily fall to 46% ($p = 0.018$, 17 d), whereas survival from chronic dsαCOP feeding at both 2.5 μg/μL and 5 μg/μL fell more rapidly, respectively reaching 26% ($p = 0.003$) and 30% (0.003) at 13 d, and reaching their lowest at 8% ($p = 0.002$, 17 d) and 13% ($p = 0.002$, 17 d).

We also observed significant differences in *B. aeneus* survival when comparing short-term- to chronic dsαCOP feeding. Starting at 10 d, chronic dsαCOP feeding at 0.5 μg/μL showed significantly reduced ($p = 0.04$) survival of *B. aeneus* compared to short-term feeding of the same concentration, this difference becoming more significant further into the study (17 d $p = 0.01$). Similarly, chronic dsαCOP feeding at 2.5 μg/μL showed significantly reduced ($p = 0.027$) *B. aeneus* survival compared to short-term feeding of the same concentration, starting at 15 d; this difference also became more significant further into the study (17 d $p = 0.004$).

We observed contrasting results of relative *αCOP* expression, between short-term- and chronic dsRNA feeding groups (Fig. 19, **VI**). With short-term dsαCOP feeding, we observed a trend of reduced *αCOP* expression at 3 d. Here we detected a 39% mean decrease in the dsαCOP 0.5 μg/μL treatment ($t = 1.15$, $df = 2.79$, $p = 0.34$), a 60% mean decrease in the dsαCOP 2.5 μg/μL treatment ($t = 1.95$, $df = 2.01$, $p = 0.19$) and a 64% mean decrease in the dsαCOP 5 μg/μL treatment ($t = 1.85$, $df = 3.02$, $p = 0.16$), compared to the dsGFP control treatment. At 6 d, qPCR data showed no *αCOP* silencing (dsαCOP 0.5 μg/μL: $t = -0.8$, $df = 3.17$, $p = 0.48$); dsαCOP 2.5 μg/μL: $t = 0.18$, $df = 4$, $p = 0.87$; dsαCOP 5 μg/μL: $t = -0.06$, $df = 3.71$, $p = 0.95$). At 12 d, we again observed no *αCOP* silencing (dsαCOP 0.5 μg/μL: $t = 0.25$, $df = 3.99$, $p = 0.82$); dsαCOP 2.5 μg/μL: $t = -0.59$, $df = 3.18$, $p = 0.6$; dsαCOP 5 μg/μL: $t = -1.14$, $df = 2.04$, $p = 0.37$; SI Fig. 4, **VI**).

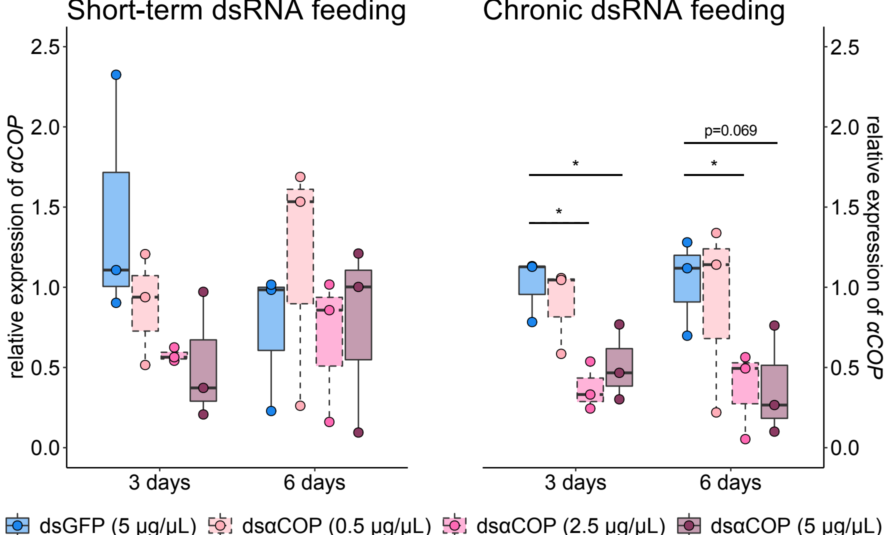


Figure 19. Results of qPCR analysis of relative *αCOP* expression in *Brassicogethes aeneus* at 3 d and 6 d after the start of the experiment. Target treatments (dsαCOP at 0.5, 2.5 and 5 µg/µL) are statistically compared to the dsGFP 5 µg/µL (control) treatment. Asterisk (*) indicates significant difference ($p \leq 0.05$) between treatments. Analysed using Welch's t-test (**VI**; Willow et al., In Press, Commun. Biol.).

With chronic dsαCOP feeding, differences in *αCOP* expression were more pronounced, and statistically significant in some treatments compared to the dsGFP control treatment. At 3, 6 and 12 d, *αCOP* silencing was not observed in the dsαCOP 0.5 µg/µL treatment (3 d: $t = 0.61$, $df = 3.69$, $p = 0.58$; 6 d: $t = 0.34$, $df = 2.95$, $p = 0.75$; 12 d: $t = -0.33$, $df = 2.46$, $p = 0.69$). Chronic dsαCOP feeding resulted in *αCOP* silencing in both the 2.5 µg/µL and 5 µg/µL treatments, at both 3 and 6 d. At 3 d, we observed a 63% mean decrease in *αCOP* expression in the dsαCOP 2.5 µg/µL treatment ($t = 4.45$, $df = 3.71$, $p = 0.01$), and a 50% mean decrease in the dsαCOP 5 µg/µL treatment ($t = 2.81$, $df = 3.98$, $p = 0.05$). At 6 d, we observed a 64% mean decrease in the dsαCOP 2.5 µg/µL treatment ($t = 2.9$, $df = 3.97$, $p = 0.049$), and a 64% mean decrease in the dsαCOP 5 µg/µL treatment ($t = 2.49$, $df = 3.93$, $p = 0.069$). At 12 d after chronic dsαCOP feeding, no *αCOP* silencing was observed in either the dsαCOP 2.5 µg/µL treatment ($t = -0.18$, $df = 3.92$, $p = 0.87$) or the dsαCOP 5 µg/µL treatment ($t = 0.81$, $df = 2.96$, $p = 0.48$).

6. DISCUSSION

6.1. Potential non-biosafety of thiacloprid, alone and in mixture with tebuconazole, to nontarget organisms (I)

Our results regarding the effects of thiacloprid, alone and in combination with tebuconazole, on the model parasitoid wasp *A. abdominalis* suggest that thiacloprid is unlikely to be a biosafe option for use in cases where the benefits of biocontrol agents are desirable. Thiacloprid applied at both RFC and one tenth RFC was detrimental to *A. abdominalis*. Furthermore, the toxic effects of thiacloprid in *A. abdominalis* were synergised by co-application of tebuconazole when tebuconazole was co-applied at concentrations of one twentieth RFC or higher. These results suggest that even when field-applied thiacloprid residues are degraded to a potency one order of magnitude less than application rate, significant reductions in parasitoid populations could occur, especially when thiacloprid is tank-mixed with tebuconazole or other related fungicides. As our results suggest that the degree of tebuconazole's synergising effect on thiacloprid depends on the concentration of co-applied tebuconazole, it is important that we better our understanding of how these types of compounds act together. This can occur via enzyme assays confirming cytochrome P450-dependent monooxygenase inhibition, and its response to various co-applied concentrations/doses of tebuconazole or other related fungicides.

We examined the effect of exposure to dried pesticide residues. However, under field conditions, multiple routes of exposure to systemic compounds like thiacloprid and tebuconazole are likely to occur simultaneously in parasitoid wasps (e.g. direct topical exposure to sprayed droplets; contact with dried residues on plant- and soil surfaces; larval feeding on contaminated host/prey; and adult feeding on contaminated nectar, honeydew, guttation and pollen. Accounting for these multiple routes of pesticide exposure is encouraged for further studies. Furthermore, our understanding of pesticide effects would benefit from experiments that simulate realistic exposure; and mesocosm experiments simulating exposure to pesticide combinations, within natural communities (Barmentlo et al., 2018), should be encouraged.

6.2. EO compounds and their potential in pollen beetle management (II)

The results of our EOs study showed that, of all EOs topically applied to *B. aeneus*, that of *C. verum* led to the greatest decrease in *B. aeneus* survival and mobility at lower doses. Therefore, this EO was regarded as potentially the most effective EO tested here, for targeting *B. aeneus*. GC–MS results suggest that the primary active compounds in our *C. verum* EO were (E)-cinnamaldehyde (representing 46% of the EO content), followed by caryophyllene (15%), linalool (12%) and D-limonene (8%). Similarly, Saad et al. (2018) showed *trans*-cinnamaldehyde as being among the most potent compounds, examined for acetylcholinesterase inhibition, against the rice weevil *Sitophilus oryzae* L. While our *C. verum* EO showed the greatest toxicity, in topical assays, of all seven EOs examined against *B. aeneus*, results of our assays using dried residues of *C. verum* EO on oilseed rape leaf/bud surfaces showed that these spray treatment significantly affected *B. aeneus* survival and mobility when applied at concentrations of 1.6% and higher. This threshold is likely rather high for practical field application of an EO. However, future studies with *B. aeneus* could use technical/analytical grade cinnamaldehyde, in order to examine potential for using this isolated compound in *B. aeneus* management.

Intraspecific differences in EO composition makes comparisons between studies difficult. Therefore, other than cinnamaldehyde, future studies with *B. aeneus* should examine the effects of other individual compounds that have been indicated, via GC–MS, as being associated with low survival rates of *B. aeneus* (e.g. carvone, thymol; Pavela, 2011) or related insects such as nitidulids of the genus *Carpophilus* (anethole; Comelli et al., 2018). Compounds showing promise for *B. aeneus* management should be examined against model taxa that are relevant to the context of biocontrol and pollination in oilseed rape agroecosystems. Using two non-model parasitoid wasp species, directly relevant to biocontrol of *B. aeneus*, Cook et al. (2007) showed that two primary parasitoids of *B. aeneus* are not repelled by compounds in *L. angustifolia* EO (this EO being repellent against *B. aeneus*); and the compounds linalool and linalyl acetate were subsequently indicated, via electroantennography, as the two compounds primarily involved in *L. angustifolia*'s repellent effect on *B. aeneus* (Mauchline et al., 2008). It is still unknown whether are optimal ratios of these two compounds that might optimise this repellent effect; and this topic would represent of valuable contribution to integrated *B. aeneus* management research.

6.3. dsRNA shows potential for use in biosafe management of pollen beetle

Using *αCOP* as a model RNAi target in *B. aeneus*, we provide evidence suggesting potential for using dsRNA in *B. aeneus* management, as both gene silencing and gene silencing-induced mortality were observed in *B. aeneus* via feeding of dsRNA-treated honey water (simulating nectar; **III**), buds (**IV**) and anthers (**V**, **VI**). While similar dsαCOP concentrations were used in these experiments, we observed differences in *B. aeneus* survival depending on the type of dsRNA-treated food source. RNAi efficacy was shown to be greatest via feeding on dsRNA-treated honey water, likely because the experimental dsRNA concentration was present throughout the entire food source, rather than merely coating the food source as in our oilseed rape bud- and anther feeding experiments. Feeding on buds exogenously treated with dsRNA also resulted in less mortality compared to feeding on exogenously treated anthers, likely because *B. aeneus* adults chew through- and consume bud epithelial tissue mostly to acquire nutrients from the anthers within the bud; and thus these individuals are orally exposed to a smaller amount of exogenously-applied dsRNA compared to when feeding on treated anthers. Developing a dsRNA formulation exhibiting properties that allow dsRNA to absorb past the bud epithelium, and into the anthers within, would be valuable to furthering our understanding of the potential for a SIGS approach to *B. aeneus* management via bud feeding.

We observed quicker RNAi-induced mortality in *B. aeneus* larvae (**V**) fed dsαCOP 2.5 µg/µL for 3 d compared to what we observed in *B. aeneus* adults fed the same dsαCOP treatment (**VI**). This result raises the question of whether *B. aeneus* larva are more susceptible to RNAi than adults. While the control mortality in our larval study considerably decreased at 6 and 7 d, the significant larval mortality at 4 and 5 d in the dsαCOP 2.5 µg/µL treatment, together with the significant *αCOP* silencing observed at 3 d in the same treatment, demonstrate that mortality effect observed in this dsαCOP treatment was indeed a result of RNAi. More robust experiments on this topic are required if we aim to understand the comparative potential for RNAi-based management between *B. aeneus* larvae and adults. However, refinements must be made to *B. aeneus* larval bioassay setups to ensure optimal conditions for keeping larvae alive in a controlled environment, as it is likely that *B. aeneus* larvae are highly sensitive under unnatural conditions, and that consequent high mortal-

ity can occur, as evidenced by both Melander et al. (2003) and our larval RNAi experiment (**V**). The aim of these refinements should be to mimic conditions to which *B. aeneus* larvae are subjected under natural conditions. For example, under natural conditions, *B. aeneus* larvae are able to seek refuge within oilseed rape flower petals, providing them a microhabitat that facilitates greater retention of moisture and less direct exposure to sunlight. We removed this microhabitat from the feeding setup, for ease of both dsRNA application to anthers and monitoring of larvae. Future *B. aeneus* studies examining the effect of larval feeding on exogenously treated anthers should consider spraying a highly surface-active dsRNA formulation on oilseed rape flower clusters, and allow cohorts of larvae to feed *ad libitum* in this semi-field-realistic setup. Other potential future studies relating to a SIGS approach to managing *B. aeneus* larvae include administering submicron quantities of dsRNA formulations directly onto the larval body, as well as a miniature-scale dsRNA soil drench experiment examining the potential impact on soil-inhabiting second instar larval- and pupating *B. aeneus*.

We demonstrated significantly greater reductions in *B. aeneus* survival via chronic dsαCOP feeding compared to short-term dsαCOP feeding (**VI**); and our data suggest that, with chronic dsRNA feeding, reduced dsRNA concentrations can be applied to achieve a similar effect compared to that achieved via short-term dietary exposure to higher dsRNA concentrations. These observations have important implications for optimal practice and economics of a SIGS approach to managing *B. aeneus* populations. Specifically, our results suggest that, while *B. aeneus* management would likely benefit from successive dsRNA spray treatments, this may still benefit the economics of spraying dsRNA, as lower concentrations may be suitable for an effective outcome. While we provide clear evidence to support this idea, semi- or small field studies are required for further exploring and confirming the RNAi approach. It has also yet to be determined the total length of time that exogenously applied dsRNA-based insecticides remain present and stable on- and in oilseed rape reproductive structures (i.e. buds, flowers) under field conditions. This will undoubtedly depend on both environmental conditions and the dsRNA formulation sprayed.

While we used exogenously applied dsRNA to bring about gene silencing-induced mortality, the results of our experiment comparing RNAi efficacy via chronic- vs short-term dsαCOP feeding (**VI**) raise the ques-

tion of whether HIGS or SIGS represents the most optimal approach to RNAi-based management of *B. aeneus*. The development of RNAi oilseed rape cultivars for use in experiments is necessary for examining the potential for RNAi-based management of *B. aeneus* via HIGS, and for simulating this against different SIGS approaches, in order to increase our understanding of the practical differences between these approaches. For *B. aeneus* in particular, it is critical to consider the constant development and senescence of reproductive structures within the crop, and the implications this has for a SIGS approach, specifically the potential requirement of successive dsRNA spray applications over the growing season. While current restrictions prevent the field use of RNAi cultivars within EU countries, these restrictions could become voided with increases in both our experience with this technology and our understanding of its impacts. The basic concept- and practice of RNAi risk assessment is still under refinement, and RNAi risk assessments are expected to provide evidence supporting the biosafety of RNAi cultivars (Arpaia et al., 2020). This predicted biosafety of RNAi technology results from dsRNA's nucleotide sequence-specific mode of action, together with the accelerating use of genome- and transcriptome sequencing technologies that can allow precise predictions of potential gene silencing effects in nontarget organisms, given a corresponding acceleration in the sequencing of relevant species.

Finally, while our RNAi experiments with *B. aeneus* suggest that *B. aeneus*'s sensitivity to oral RNAi, via field relevant routes of exposure, is relatively moderate compared to some other coleopteran pest species (Bachman et al., 2013; Baum et al., 2007; Chikami et al., 2020; Christiaens et al., 2016; Knorr et al., 2018; Lü et al., 2020; Máximo et al., 2020; Mehlhorn et al., 2020; Miguel and Scott, 2015; Petek et al., 2020; Pinheiro et al., 2020; Prentice et al., 2017), there remains potential for enhancing efficacy and speed-to-effect of dsRNA via co-formulants (e.g. nanoparticles) that may improve efficiency of dsRNA uptake and RNAi (Christiaens et al., 2020; Yan et al., 2020). Improving the efficiency of dsRNA uptake and RNAi will allow us to more fully realise the potential for using a SIGS approach within *B. aeneus* management.

7. CONCLUSIONS

We examined several types of insecticide, namely thiacloprid, plant EOs and dsRNA, for their potential use in ecologically biosafe management of the pollen beetle *B. aeneus*. We exposed a model parasitoid wasp, *A. abdominalis*, to dry pesticide residues of thiacloprid alone and in combination with the fungicide tebuconazole (the two compounds being commonly tank-mixed for field use). We subsequently observed the detrimental effects of a low concentration of thiacloprid, both when applied alone as well as when applied in combination with tebuconazole. Our results supported our hypotheses that exposure to residues of thiacloprid, when applied at RFC, results in significant reductions in *A. abdominalis* survival and mobility; and that simultaneous exposure to residues of thiacloprid and tebuconazole results in synergistic reductions in *A. abdominalis* survival and mobility, revealing a threshold-concentration (respective to RFC) that corresponded to the observed synergy (**I**).

The most effective EO observed against survival and mobility of *B. aeneus*, via topical application, was that of *C. verum* (**II**) Topical applications of seven different plant EOs resulted in different responses, regarding *B. aeneus* survival and mobility; which supported our hypothesis that a particular plant EO would be demonstrated as the most promising for against *B. aeneus*, among each of the examined topically-applied EOs (**II**). Our results also supported our hypothesis that bioassays using treated leaf- and bud surfaces would allow us to detect an effective concentration threshold for significant reductions in *B. aeneus* survival and mobility, using the most promising EO from the topical bioassays (i.e. *C. verum*, **II**). However, when sprayed onto oilseed rape leaf and bud surfaces, exposure to dry residues of *C. verum* EO demonstrated efficacy only at concentrations of 1.5% and higher, which is rather high for practical field application of an EO. Therefore, individual compounds dominant in EOs showing promise for *B. aeneus* management should be examined against *B. aeneus* and model taxa that are relevant to the context of biocontrol and pollination in oilseed rape agroecosystems.

Field relevant dietary routes of exposure to dsRNA targeting *B. aeneus* *αCOP* showed significant gene silencing and gene silencing-induced mortality (**III**, **IV**, **V**, **VI**), confirming this hypothesis for each *B. aeneus* RNAi study conducted. Refinements to *B. aeneus* larval bioassay setups,

as well as developing dsRNA formulations that enhance transport past the oilseed rape bud epithelium, and/or enhance dsRNA uptake in *B. aeneus*, represent critical steps to understanding the potential for RNAi-based management of *B. aeneus*. Most notably, we observed that RNAi efficacy is enhanced by chronic, compared to short-term, dsRNA feeding in *B. aeneus* (**VI**), confirming our hypothesis that this would be the case. This result has implications for the economics and development of a potential dsRNA-spray approach for managing *B. aeneus*, as well as highlighting the need for research into the development and potential future use of RNAi oilseed rape cultivars.

REFERENCES

Antwi, F.B., Reddy, G.V.P., 2015. Toxicological effects of pyrethroids on non-target aquatic insects. Environ. Toxicol. Pharmacol. 40, 915–923. https://doi.org/10.1016/j.etap.2015.09.023

Arpaia, S., Christiaens, O., Giddings, K., Jones, H., Mezzetti, B., Moronta-Barrios, F., Perry, J.N., Sweet, J.B., Taning, C.N.T., Smagghe, G., Dietz-Pfeilstetter, A., 2020. Biosafety of GM crop plants expressing dsRNA: Data requirements and EU regulatory considerations. Front. Plant Sci. 11. https://doi.org/10.3389/fpls.2020.00940

Bachman, P.M., Bolognesi, R., Moar, W.J., Mueller, G.M., Paradise, M.S., Ramaseshadri, P., Tan, J., Uffman, J.P., Warren, J., Wiggins, B.E., Levine, S.L., 2013. Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). Transgenic Res. 22, 1207–1222. https://doi.org/10.1007/s11248-013-9716-5

Barmentlo, S.H., Schrama, M., Hunting, E.R., Heutink, R., van Bodegom, P.M., de Snoo, G.R., Vijver, M.G., 2018. Assessing combined impacts of agrochemicals: Aquatic macroinvertebrate population responses in outdoor mesocosms. Sci. Total. Environ. 631–632, 341–347. https://doi.org/10.1016/j.scitotenv.2018.03.021

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T., Roberts, J., 2007. Control of coleopteran insect pests through RNA interference. Nat. Biotechnol. 25, 1322–1326. https://doi.org/10.1038/nbt1359

Beck, R., Ravet, M., Wieland, F.T., Cassel, D., 2009. The COPI system: Molecular mechanisms and function. FEBS Letters 583, 2701–2709. https://doi.org/10.1016/j.febslet.2009.07.032

Beller, M., Sztalryd, C., Southall, N., Bell, M., Jäckle, H., Auld, D.S., Oliver, B., 2008. COPI Complex Is a Regulator of Lipid Homeostasis. PLoS Biol. https://doi.org/10.1371/journal.pbio.0060292

Bramlett, M., Plaetinck, G., Maienfisch, P., 2019. RNA-based biocontrols—A new paradigm in crop protection. Engineering. https://doi.org/10.1016/j.eng.2019.09.008

Brandes, M., Heimbach, U., Ulber, B., 2018a. Effects of thiacloprid, tau-fluvalinate and lambda-cyhalothrin on overwintered pollen beetles (*Brassicogethes aeneus* (Fabricius)) and their offspring in oilseed rape.

Arthropod Plant Interact. 12, 823–833. https://doi.org/10.1007/s11829-018-9621-1
Brandes, M., Heimbach, U., Ulber, B., 2018b. Impact of insecticides on oilseed rape bud infestation with eggs and larvae of pollen beetle (*Brassicogethes aeneus* (Fabricius)). Arthropod Plant Interact. 12, 811–821. https://doi.org/10.1007/s11829-018-9616-y
Büchi, R., 2002. Mortality of pollen beetle (*Meligethes* spp.) larvae due to predators and parasitoids in rape fields and the effect of conservation strips. Agric. Ecosyst. Environ. 90, 255–263. https://doi.org/10.1016/S0167-8809(01)00213-4
Cagliari, D., Santos, E.A. dos, Dias, N., Zotti, G.S. and M., 2018. Non-transformative Strategies for RNAi in Crop Protection. Modulating Gene Expression - Abridging the RNAi and CRISPR-Cas9 Technologies. https://doi.org/10.5772/intechopen.80874
Ceuppens, B., Eeraerts, M., Vleugels, T., Cnops, G., Roldan-Ruiz, I., Smagghe, G., 2015. Effects of dietary lambda-cyhalothrin exposure on bumblebee survival, reproduction, and foraging behavior in laboratory and greenhouse. J. Pest Sci. 88, 777–783. https://doi.org/10.1007/s10340-015-0676-9
Chikami, Y., Kawaguchi, H., Suzuki, T., Yoshioka, H., Sato, Y., Yaginuma, T., Niimi, T., 2020. Oral RNAi of diap1 results in rapid reduction of damage to potatoes in *Henosepilachna vigintioctopunctata*. J. Pest Sci. https://doi.org/10.1007/s10340-020-01276-w
Christiaens, O., Petek, M., Smagghe, G., Taning, C.N.T., 2020. The Use of Nanocarriers to Improve the Efficiency of RNAi-Based Pesticides in Agriculture, in: Fraceto, L.F., S.S. de Castro, V.L., Grillo, R., Ávila, D., Caixeta Oliveira, H., Lima, R. (Eds.), Nanopesticides. Springer International Publishing, Cham, pp. 49–68. https://doi.org/10.1007/978-3-030-44873-8_3
Christiaens, O., Prentice, K., Pertry, I., Ghislain, M., Bailey, A., Niblett, C., Gheysen, G., Smagghe, G., 2016. RNA interference: a promising biopesticide strategy against the African Sweetpotato Weevil *Cylas brunneus*. Sci. Rep. 6, 1–11. https://doi.org/10.1038/srep38836
Comelli, N.C., Romero, O.E., Diez, P.A., Marinho, C.F., Schliserman, P., Carrizo, A., Ortiz, E.V., Duchowicz, P.R., 2018. QSAR study of biologically active essential oils against beetles infesting the walnut in Catamarca, Argentina. J. Agric. Food Chem. 66, 12855–12865. https://doi.org/10.1021/acs.jafc.8b04161
Cook, S.M., Jönsson, M., Skellern, M.P., Murray, D.A., Anderson, P., Powell, W., 2007. Responses of *Phradis* parasitoids to volatiles of

lavender, *Lavendula angustifolia*—a possible repellent for their host, *Meligethes aeneus*. BioControl 52, 591–598. https://doi.org/10.1007/s10526-006-9057-x

Delpuech, J.-M., Delahaye, M., 2013. The sublethal effects of deltamethrin on *Trichogramma* behaviors during the exploitation of host patches. Sci. Total Environ. 447, 274–279. https://doi.org/10.1016/j.scitotenv.2012.12.096

Desneux, N., Decourtye, A., Delpuech, J.-M., 2007. The sublethal effects of pesticides on beneficial arthropods. Ann. Rev. Entomol. 52, 81–106. https://doi.org/10.1146/annurev.ento.52.110405.091440

Dorn, B., Jossi, W., Humphrys, C., Hiltbrunner, J., 2014. Screening of natural products in the laboratory and the field for control of pollen beetles. J Appl. Ecol. 138, 109–119. https://doi.org/10.1111/jen.12086

Gross, M., 2013. EU ban puts spotlight on complex effects of neonicotinoids. Curr. Biol. 23, R462–R464. https://doi.org/10.1016/j.cub.2013.05.030

Heimbach, U., Müller, A., 2013. Incidence of pyrethroid-resistant oilseed rape pests in Germany. Pest Manag. Sci. 69, 209–216. https://doi.org/10.1002/ps.3351

Huvenne, H., Smagghe, G., 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. J. Insect Physiol. 56, 227–235. https://doi.org/10.1016/j.jinsphys.2009.10.004

Isman, M.B., 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu. Rev. Entomol. 51, 45–66. https://doi.org/10.1146/annurev.ento.51.110104.151146

Isman, M.B., Grieneisen, M.L., 2014. Botanical insecticide research: many publications, limited useful data. Trends Plant Sci. 19, 140–145. http://dx.doi.org/10.1016/j.tplants.2013.11.005

Jans, D., 2012. Toxicity to the parasitoid wasp *Aphidius rhopalosiphi* (DeStephani-Perez) (Hymenoptera: Braconidae) using an extended laboratory test with aged residues on maize - thiacloprid OD 240 g/L - final report. European Commission. Renewal Assessment Report prepared according to the Commission Regulation (EU) N° 1107/2009. Vol 3 - B.9 (PPP)-Thiacloprid OD 240(240 g/L). 2017:262-266. http://www.efsa.europa.eu/en/consultations/call/180123

Johnson, R.M., Wen, Z., Schuler, M.A., Berenbaum, M.R., 2006. Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. J. Econ. Entomol. 99, 1046–1050. https://doi.org/10.1603/0022-0493-99.4.1046

Kaasik, R., Kovács, G., Kaart, T., Metspalu, L., Williams, I.H., Veromann, E., 2014. *Meligethes aeneus* oviposition preferences, larval parasitism rate and species composition of parasitoids on *Brassica nigra*, *Raphanus sativus* and *Eruca sativa* compared with on *Brassica napus*. BioControl 69, 65–71. https://doi.org/10.1016/j.biocontrol.2013.11.002

Kaiser, C., Jensen, K.-M.V., Nauen, R., Kristensen, M., 2018. Susceptibility of Danish pollen beetle populations against λ-cyhalothrin and thiacloprid. J. Pest Sci. 91, 447–458. https://doi.org/10.1007/s10340-017-0856-x

Kirk-Spriggs, A.H., 1996. Pollen Beetles - Coleoptera: Kateretidae and Nitidulidae: Meligethinae, Handbooks for the Identification of British Insects. Royal Entomological Society.

Kitazawa, D., Yamaguchi, M., Mori, H., Inoue, Y.H., 2012. COPI-mediated membrane trafficking is required for cytokinesis in *Drosophila* male meiotic divisions. J. Cell Sci. 125, 3649–3660. https://doi.org/10.1242/jcs.103317

Knorr, E., Fishilevich, E., Tenbusch, L., Frey, M.L.F., Rangasamy, M., Billion, A., Worden, S.E., Gandra, P., Arora, K., Lo, W., Schulenberg, G., Valverde-Garcia, P., Vilcinskas, A., Narva, K.E., 2018. Gene silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi targets in crop pests. Sci. Rep. 8, 1–15. https://doi.org/10.1038/s41598-018-20416-y

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-deltadeltaCT method. Methods 25, 402–408. https://doi.org/doi:10.1006/meth.2001.1262

Lü, J., Liu, Z., Guo, W., Guo, M., Chen, S., Li, H., Yang, C., Zhang, Y., Pan, H., 2020. Feeding Delivery of dsHvSnf7 Is a Promising Method for Management of the Pest *Henosepilachna vigintioctopunctata* (Coleoptera: Coccinellidae). Insects 11, 34. https://doi.org/10.3390/insects11010034

Mauchline, A.L., Birkett, M.A., Woodcock, C.M., Pickett, J.A., Osborne, J.L., Powell, W., 2008. Electrophysiological and behavioural responses of the pollen beetle, *Meligethes aeneus*, to volatiles from a non-host plant, lavender, *Lavandula angustifolia* (Lamiaceae). Arthropod Plant Interact. 2, 109–115. https://doi.org/10.1007/s11829-008-9038-3

Mauchline, A.L., Cook, S.M., Powell, W., Osborne, J.L., 2013. Effects of non-host plant odour on *Meligethes aeneus* during immigration to oilseed rape. Entomol. Exp. Appl. 146, 313–320. https://doi.org/10.1111/eea.12030

Mauchline, A.L., Hervé, M.R., Cook, S.M., 2018. Semiochemical-based alternatives to synthetic toxicant insecticides for pollen beetle management. Arthropod Plant Interact. 12, 835–847. https://doi.org/10.1007/s11829-017-9569-6

Mauchline, A.L., Osborne, J.L., Martin, A.P., Poppy, G.M., Powell, W., 2005. The effects of non-host plant essential oil volatiles on the behaviour of the pollen beetle *Meligethes aeneus*. Entomol. Exp. Appl. 114, 181–188. https://doi.org/10.1111/j.1570-7458.2005.00237.x

Máximo, W.P.F., Howell, J.L., Mogilicherla, K., Basij, M., Chereddy, S.C.R.R., Palli, S.R., 2020. Inhibitor of apoptosis is an effective target gene for RNAi-mediated control of Colorado potato beetle, *Leptinotarsa decemlineata*. Arch. Insect Biochem. Physiol. 104, e21685. https://doi.org/10.1002/arch.21685

Mehlhorn, S.G., Geibel, S., Bucher, G., Nauen, R., 2020. Profiling of RNAi sensitivity after foliar dsRNA exposure in different European populations of Colorado potato beetle reveals a robust response with minor variability. Pestic. Biochem. Physiol. 166, 104569. https://doi.org/10.1016/j.pestbp.2020.104569

Melander, M., Åhman, I., Kamnert, I., Strömdahl, A.-C., 2003. Pea Lectin Expressed Transgenically in Oilseed Rape Reduces Growth Rate of Pollen Beetle Larvae. Transgenic Res. 12, 555–567. https://doi.org/10.1023/A:1025813526283

Mezzetti, B., Smagghe, G., Arpaia, S., Christiaens, O., Dietz-Pfeilstetter, A., Jones, H., Kostov, K., Sabbadini, S., Opsahl-Sorteberg, H.-G., Ventura, V., Taning, C.N.T., Sweet, J., 2020. RNAi: What is its position in agriculture? J. Pest Sci. https://doi.org/10.1007/s10340-020-01238-2

Miguel, K.S., Scott, J.G., 2015. The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. Pest Manag. Sci. 72, 801–809. https://doi.org/10.1002/ps.4056

Osborne, P., 1965. Morphology of the immature stages of *Meligethes aeneus* (F.) and *M. viridescens* (F.) (Coleoptera, Nitidulidae). Bull. Entomol. Res. 55, 747–759. https://doi.org/10.1017/S0007485300049853

Papadopoulou, N., Devos, Y., Álvarez-Alfageme, F., Lanzoni, A., Waigmann, E., 2020. Risk Assessment Considerations for Genetically Modified RNAi Plants: EFSA's Activities and Perspective. Front. Plant Sci. 11. https://doi.org/10.3389/fpls.2020.00445

Pavela, R., 2011. Insecticidal and repellent activity of selected essential oils against of the pollen beetle, *Meligethes aeneus* (Fabricius) adults. Ind. Crops Prod. 34, 888–892. https://doi.org/10.1016/j.indcrop.2011.02.014

Petek, M., Coll, A., Ferenc, R., Razinger, J., Gruden, K., 2020. Validating the potential of double-stranded RNA targeting Colorado potato beetle *mesh* gene in laboratory and field trials. Front. Plant Sci. 11. https://doi.org/10.3389/fpls.2020.01250

Pinheiro, D.H., Taylor, C.E., Wu, K., Siegfried, B.D., 2020. Delivery of gene-specific dsRNA by microinjection and feeding induces RNAi response in Sri Lanka weevil, *Myllocerus undecimpustulatus undatus* Marshall. Pest Manag. Sci. 76, 936–943. https://doi.org/10.1002/ps.5601

Põllumajandusamet, 2017. PMAIS Avalik Vaade. Plant protection products authorized in Estonia. https://portaal.agri.ee/avalik/#/taimekaitse/taimekaitsevahendid-otsing/en (accessed 12.4.18).

Prentice, K., Christiaens, O., Pertry, I., Bailey, A., Niblett, C., Ghislain, M., Gheysen, G., Smagghe, G., 2017. RNAi-based gene silencing through dsRNA injection or ingestion against the African sweet potato weevil *Cylas puncticollis* (Coleoptera: Brentidae). Pest Manag. Sci. 73, 44–52. https://doi.org/10.1002/ps.4337

Raimets, R., Karise, R., Mänd, M., Kaart, T., Ponting, S., Song, J., Cresswell, J.E., 2018. Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.). Pest Manag. Sci. 74, 541–546. https://doi.org/10.1002/ps.4756

Riggi, L.G., Gagic, V., Rusch, A., Malsher, G., Ekbom, B., Bommarco, R., 2017. Pollen beetle mortality is increased by ground-dwelling generalist predators but not landscape complexity. Agric. Ecosyst Environ. 250, 133–142. https://doi.org/10.1016/j.agee.2017.06.039

Saad, M.M.G., Abou-Taleb, H.K., Abdelgaleil, S.A.M., 2018. Insecticidal activities of monoterpenes and phenylpropenes against *Sitophilus oryzae* and their inhibitory effects on acetylcholinesterase and adenosine triphosphatases. Appl. Entomol. Zool. 53, 173–181. https://doi.org/10.1007/s13355-017-0532-x

Seidenglanz, M., Poslušná, J., Kolařík, P., Rotrekl, J., Eva, E., Tóth, P., Havel, J., Plachká, E., Táncik, J., Hudec, K., 2017. Negative correlations between the susceptibilities of Czech and Slovak pollen beetle populations to lambda-cyhalothrin and chlorpyrifos-ethyl in 2014 and 2015. Plant Prot. Sci. 53, 108–117. https://doi.org/10.17221/187/2015-PPS

Sgolastra, F., Arnan, X., Cabbri, R., Isani, G., Medrzycki, P., Teper, D., Bosch, J., 2018. Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and

longevity in a solitary bee. Proc. R. Soc. B 285, 20180887. https://doi.org/10.1098/rspb.2018.0887

Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M.T., Tosi, S., Bogo, G., Teper, D., Porrini, C., Molowny-Horas, R., Bosch, J., 2017. Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. Pest Manag. Sci. 73, 1236–1243. https://doi.org/10.1002/ps.4449

Skellern, M.P., Cook, S.M., 2018. Prospects for improved off-crop habitat management for pollen beetle control in oilseed rape. Arthropod Plant Interact. 12, 849–866. https://doi.org/10.1007/s11829-018-9598-9

Slater, R., Ellis, S., Genay, J.-P., Heimbach, U., Huart, G., Sarazin, M., Longhurst, C., Müller, A., Nauen, R., Rison, J.L., Robin, F., 2011. Pyrethroid resistance monitoring in European populations of pollen beetle (*Meligethes* spp.): a coordinated approach through the Insecticide Resistance Action Committee (IRAC). Pest Manag. Sci. 67, 633–638. https://doi.org/10.1002/ps.2101

Stará, J., Kocourek, F., 2018. Seven-year monitoring of pyrethroid resistance in the pollen beetle (*Brassicogethes aeneus* F.) during implementation of insect resistance management. Pest Manag. Sci. 74, 200–209. https://doi.org/10.1002/ps.4695

Sugiyama, K., Katayama, H., Saito, T., 2011. Effect of insecticides on the mortalities of three whitefly parasitoid species, *Eretmocerus mundus*, *Eretmocerus eremicus* and *Encarsia formosa* (Hymenoptera: Aphelinidae). Appl. Entomol. Zool. 46, 311–317. https://doi.org/10.1007/s13355-011-0044-z

Sutter, L., Amato, M., Jeanneret, P., Albrecht, M., 2018. Overwintering of pollen beetles and their predators in oilseed rape and seminatural habitats. Agric. Ecosyst. Environ. 265, 275–281. https://doi.org/10.1016/j.agee.2018.06.030

Taning, C.N., Arpaia, S., Christiaens, O., Dietz-Pfeilstetter, A., Jones, H., Mezzetti, B., Sabbadini, S., Sorteberg, H.-G., Sweet, J., Ventura, V., Smagghe, G., 2019. RNA-based biocontrol compounds: current status and perspectives to reach the market. Pest Manag. Sci. https://doi.org/10.1002/ps.5686

Teder, T., Knapp, M., 2019. Sublethal effects enhance detrimental impact of insecticides on non-target organisms: A quantitative synthesis in parasitoids. Chemosphere. 214, 371–378. https://doi.org/10.1016/j.chemosphere.2018.09.132

Thies, C., Tscharntke, T., 1999. Landscape structure and biological control in agroecosystems. Science 285, 893–895. https://doi.org/10.1126/science.285.5429.893

Thompson, H.M., 2012. Interaction between pesticides and other factors in effects on bees. EFSA Supporting Publications 9, 340E. https://doi.org/10.2903/sp.efsa.2012.EN-340

Thompson, H.M., Fryday, S.L., Harkin, S., Milner, S., 2014. Potential impacts of synergism in honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops. Apidologie 45, 545–553. https://doi.org/10.1007/s13592-014-0273-6

van de Veire, M., Smagghe, G., Degheele, D., 1996. Laboratory test method to evaluate the effect of 31 pesticides on the predatory bug *Orius laevigatus* (Het: Anthocoridae). Entomophaga 41, 235–243. https://doi.org/10.1007/BF02764249

Vélez, A.M., Fishilevich, E., Rangasamy, M., Khajuria, C., McCaskill, D.G., Pereira, A.E., Gandra, P., Frey, M.L., Worden, S.E., Whitlock, S.L., Lo, W., Schnelle, K.D., Lutz, J.R., Narva, K.E., Siegfried, B.D., 2020. Control of western corn rootworm via RNAi traits in maize: lethal and sublethal effects of Sec23 dsRNA. Pest Manag. Sci. 76, 1500–1512. https://doi.org/10.1002/ps.5666

Wang, D., Lü, L., He, Y., 2018. Effects of insecticides on sex pheromone communication and mating behavior in *Trichogramma chilonis*. J. Pest Sci. 91, 65–78. https://doi.org/10.1007/s10340-017-0864-x

Yan, S., Ren, B.-Y., Shen, J., 2020. Nanoparticle-mediated double-stranded RNA delivery system: A promising approach for sustainable pest management. Insect Sci. 0, 1–14. https://doi.org/10.1111/1744-7917.12822

Zamojska, J., 2017. Differences in susceptibility of the cabbage seed weevil (*Ceutorchynhus assimilis* Payk.) (Coleoptera: Curculionidae) and the pollen beetle (*Meligethes aeneus* F.) (Coleoptera: Nitidulidae) to indoxacarb and deltamethrin and resistance mechanisms of the cabbage seed weevil to indoxacarb. Phytoparasitica 45, 407–418. https://doi.org/10.1007/s12600-017-0588-8

Zhu, K.Y., Palli, S.R., 2020. Mechanisms, applications, and challenges of insect RNA interference. Annu. Rev. Entomol. 65, 293–311. https://doi.org/10.1146/annurev-ento-011019-025224

Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). PLOS ONE 12, e0176837. https://doi.org/10.1371/journal.pone.0176837

Zimmer, Christoph T., Köhler, H., Nauen, R., 2014. Baseline susceptibility and insecticide resistance monitoring in European populations of *Meligethes aeneus* and *Ceutorhynchus assimilis* collected in winter oilseed rape. Entomol. Exp. Appl. 150, 279–288. https://doi.org/10.1111/eea.12162

Zimmer, C. T., Maiwald, F., Schorn, C., Bass, C., Ott, M.-C., Nauen, R., 2014. A de novo transcriptome of European pollen beetle populations and its analysis, with special reference to insecticide action and resistance. Insect Mol. Biol. 23, 511–526. https://doi.org/10.1111/imb.12099

SUMMARY

Throughout Europe, one of the most severely damaging threats to oilseed rape (*Brassica napus*) cultivation is the pollen beetle *Brassicogethes aeneus* (syn. *Meligethes aeneus*, Coleoptera: Nitidulidae). While inhabiting oilseed rape fields, *B. aeneus* adults feed on pollen within developing reproductive buds, as well as the pollen and nectar of bloomed flowers. *B. aeneus* larvae feed on pollen within the buds in which they were oviposited, followed by feeding on the pollen and nectar of bloomed flowers, after their natal reproductive bud has bloomed, generally during their late first- or early second instar of development. While oilseed rape has mechanisms that compensate the loss of reproductive buds necessary for seed development, the presence of dense *B. aeneus* populations can result in significant yield losses, thus creating a need for managing *B. aeneus* populations in oilseed rape agroecosystems. Pyrethroid insecticides (IRAC class 3A of sodium channel modulators) have been the standard method for managing *B. aeneus* populations. However, as a result of the routine use of pyrethroids, *B. aeneus* populations have developed resistance to pyrethroids in several areas throughout Europe. Moreover, extensive research indicates that exposure to pyrethroids can be detrimental to many nontarget insect taxa that inhabit agroecosystems. For effective and sustainable management of *B. aeneus* populations, it is particularly important to safeguard organisms that contribute to biological control (biocontrol) of *B. aeneus*. The most specialised arthropods involved in biocontrol of *B. aeneus* populations include several species of hymenopteran parasitoids, while other predaceous arthropods (e.g. carabid beetles, staphylinid beetles, spiders) can also benefit biocontrol of *B. aeneus* populations. Several studies have demonstrated the potential these organisms represent for biocontrol of *B. aeneus* populations. However, the most effective *B. aeneus* management strategy may require the involvement of insecticide applications that, while showing efficacy against *B. aeneus*, minimise the impact on nontarget organisms, especially those performing agroecological services such as biocontrol.

This thesis addresses the need for new and effective insecticides for use within a biosafe integrated *B. aeneus* management strategy. In brief, first we will look at one representative of a class of synthetic, non-species-specific insecticide, neonicotinoids (IRAC class 4A of nicotinic acetylcholine receptor (nAChR) competitive modulators). In particular, the compound

thiacloprid has been the subject of research regarding its potential use in *B. aeneus* management. Neonicotinoids are applied in oilseed rape production; and recent field and greenhouse experiments using thiacloprid have demonstrated its efficacy in managing *B. aeneus* infestations. We examined thiacloprid's compatibility with a model hymenopteran parasitoid species, *Aphelinus abdominalis*; and analysed thiacloprid's toxicity to this biocontrol species when applied alone, as well as in combination with tebuconazole (FRAC group 3, demethylation inhibitors, class 1 of sterol biosynthesis inhibitors), a representative of a group of compounds that are commonly tank-mixed with neonicotinoids for crop protection. Second, we will examine the effect of seven different plant-based essential oils (EOs), representing a non-species-specific biopesticide for potential use within *B. aeneus* management. Last, we will investigate the potential for applying *B. aeneus*-specific double-stranded ribonucleic acid (dsRNA), representing a potentially species-specific biopesticide option, within a *B. aeneus* management strategy.

After exposing *A. abdominalis* to dry pesticide residues of thiacloprid alone and in combination with the tebuconazole, we observed the detrimental effects of a low concentration of thiacloprid, both when applied alone as well as when applied in combination with tebuconazole. Indeed, our results supported our hypotheses that exposure to residues of thiacloprid, when applied at recommended field concentration (RFC), results in significant reductions in *A. abdominalis* survival and mobility; and that simultaneous exposure to residues of thiacloprid and tebuconazole results in synergistic reductions in *A. abdominalis* survival and mobility, revealing a threshold-concentration (respective to RFC) that corresponded to the observed synergy (**I**).

The most effective EO observed against survival and mobility of *B. aeneus*, via topical application, was that of cinnamon (*Cinnamomum verum*) bark (**II**). Topical applications of seven different plant EOs resulted in different responses, regarding *B. aeneus* survival and mobility; which supported our hypothesis that a particular plant EO would be demonstrated as the most promising for against *B. aeneus*, among each of the examined topically-applied EOs (**II**). Our results also supported our hypothesis that bioassays using treated leaf- and bud surfaces would allow us to detect an effective concentration threshold for significant reductions in *B. aeneus* survival and mobility, using the most promising EO from the topical bioassays (i.e. *C. verum*, **II**). However, when sprayed onto oilseed rape leaf

and bud surfaces, exposure to dry residues of *C. verum* EO demonstrated efficacy only at concentrations rather high for practical field application of an EO. Therefore, individual compounds dominant in EOs showing promise for *B. aeneus* management should be examined against *B. aeneus* and model taxa that are relevant to the context of biocontrol and pollination in oilseed rape agroecosystems.

Field relevant dietary routes of exposure to dsRNA targeting *B. aeneus coatomer subunit alpha* (*αCOP*) showed significant gene silencing and gene silencing-induced mortality (**III**, **IV**, **V**, **VI**), confirming this hypothesis for each *B. aeneus* RNAi study conducted. Refinements to *B. aeneus* larval bioassay setups, as well as developing dsRNA formulations that enhance transport past the oilseed rape bud epithelium, and/or enhance dsRNA uptake in *B. aeneus*, represent critical steps to understanding the potential for RNAi-based management of *B. aeneus*. Most notably, we observed that RNAi efficacy is enhanced by chronic, compared to short-term, dsRNA feeding in *B. aeneus* (**VI**), confirming our hypothesis that this would be the case. This result has implications for the economics and development of a potential dsRNA-spray approach for managing *B. aeneus*; as well as it highlights the need for research into the development and potential future use of RNAi oilseed rape cultivars, given the enhanced RNAi efficacy resulting from chronic dsRNA feeding in *B. aeneus*.

KOKKUVÕTE

Rapsi (*Brassica napus*) kõige olulisemaks kahjuriks kogu Euroopas on naerihiilamardikas (*Brassicogethes aeneus* (syn. *Meligethes aeneus*, Coleoptera: Nitidulidae)). Kuigi rapsitaimedel on suur regeneratsioonivõime ja vastuseks kahjurite rünnakule produtseerivad taimed uusi võrseid ja õisi, et kompenseerida kahurite poolt tekitatud kahjustusi, suudavad hiilamardikad suure arvukuse korral siiski tekitada olulist kahju saagile. Taimekasvatajad kasutavad hiilamardikate arvukuse vähendamiseks rapsipõldudel peamiselt püretroididel põhinevaid insektitsiide, mida sageli kasutatakse rutiinselt kahjurite arvukust kontrollimata ja tõrjekriteeriumitest lähtumata. Selle tulemusel on väga paljude Euroopa riikide hiilamardika populatsioonidel kujunenud resistentsus püretroididel põhinevate insktitsiidide suhtes. Lisaks sellele, massiliste uuringute tulemusena on tõestanud, et püretroidid on lisaks kahjuritele ka väga ohtlikud paljudele neutraalsetele ja kasulikele liikidele, kelle elupaigad on samuti agroökosüsteemides ja kes seetõttu võivad insektitsiididega kokku puutuda. Samas selleks, et tagada efektiivne ja jätkusuutlik hiilamardikate tõrjestrateegia, on eriti olulisel kohal kasulike lülijalgsete soodustamine põllumajanduskooslustes, et loodusliku foonina oleks tagatud bioloogiline tõrje. Kõige efektiivsemalt panustavad hiilamardikate populatsioonide suuruse looduslikku reguleerimisse kiletiivalised parasitoidid ja röövtoidulised lülijalgsed (nagu näiteks jooksiklased, lühitiiblased, ämblikulaadsed). Paljud uurimistööd on tõestanud nende suurt potentsiaali *B. aeneus*'e arvukuse looduslike reguleerijatena. Samas peaks efektiivne *B. aeneus*'e tõrjestrateegia sisaldama nii looduslikku bioloogilist tõrjet kui ka otsest insektitsiididega sekkumist, kui olukord seda nõuab. Ning sellisel juhul peaksid vastavad insektitsiidid olema võimalikult ohutud mitte-sihtrühma lülijalgsetele ja eriti nendele, kellel baseerub bioloogiline tõrje.

Käeolev töö ongi suunatud vajadusele leida uusi ja efektiivseid tõrjevahendeid, mis oleks bioloogiliselt ohutud ja mida saaks sobitada täiendava lülina hiilamardika integreeritud tõrjestrateegiasse. Lühidalt, esiteks me uurisime, kas ja kuidas sünteetilised, neonikotinoidide klassi (IRAC klass 4A, nikotiin-atsetüülkoliin retseptori (nAChR) radade konkureerivad moodulid) kuuluvad putukamürgid, mida kasutatakse rapsipõldudel hiilamardikate tõrjes, mõjutavad parasitoide, kui looduslikke biotõrje agente. Me uurisime tiaklopriidi mõju kiletiivalise parasitoidi mudelliigile (*Aphelinus abdominalis*) ja analüüsisime tema toksilisust nii eraldi kui ka

koosmõjus fungitsiidi tebukonasooliga (FRAC grupp 3, demetülatsiooni inhibiitor, klass 1 sterooli biosünteesi inhibiitor). Need kaks pestitsiidi viiakse tavaliselt rapsi põllule koos paagisegus ja seega on nende koosmõju uurimine äärmiselt relevantne. Teiseks, me uurisime seitsme erineva taimse eeterliku õli, kui potentsiaalsete mitte-liigispetsiifiliste looduslike taimekaitsevahendite mõju hiilamardikatele. Viimasena uurisime potentsiaalselt liigispetsiifilise kahe-ahelalise ribonukleiinhappe (dsRNA) geenivaigistavat mõju *B. aeneus*'e suremusele, et arendada potentsiaalselt liigispetsiifiline biopestitsiid.

Sünteetiliste pestitsiidide katses kiletiivalise parasitoidiga (*Aphelinus abdominalis*) selgus, et tiaklopriidi erinevad kontsentratsioonid eraldi ja kombinatsioonis tebukonasooliga olid surmava neile efektiga (**I**). Tõepoolest, katsetulemused toetasid hüpoteesi, et tiaklopriidi kontsentratsioonid, mida võib rapsitaimedel leida, kui kasutatakse preparaadile soovitatud põllukoguseid, vähendasid oluliselt *A. abdominalis*'e ellujäävust ja mobiilsust ning paagisegu (tiaklopriid ja tebukonasool) toime osutus sünergiliselt toksiliseks (**I**). Looduslikes tingimustes on ka mobiilsuse oluline vähenemine tavaliselt letaalne, sest ollakse haavatavamad erinevatele ohtudele.

Taimsetest eeterlikest õlidest osutus kõige efektiivsemaks kaneelikooreõli (*Cinnamomum verum*), mis hiilamardikate välispidisel töötlusel vähendas oluliselt nende ellujäävust ja mobiilsust (**II**). Seitsme taimse eeterliku õli välispidine toime hiilamardikate ellujäävusele ja mobiilsusele oli erinev, mis toetas püsitatud hüpoteesi, et vaid mõni kindel taimne õli toimib hiilamardikat tõrjuvana. Katse tulemused toetasid ka hüpoteesi, et taimsete õlidega töödeldud rapsi lehepinnad ja pungad aitavad tuvastada efektiivse kontsentratsiooni lävendit, millest alates hiilamardikate ellujäävus ja mobiilsus oluliselt väheneb (meie katsete tulemusel *C. verum*, **II**). Samas leidsime, et rapsilehtede ja –pungade pindmisel töötlemisel eeterlike õlidega hiilamardika efektiivseks tõrjeks tuleks kasutada selliseid kontsentratsioone, mis siiski praktilistes põllutingimustes ei ole relevantsed kasutada. Seega peaks neid taimseid eeterlike õlisid, mis mõjutasid oluliselt hiilamardika suremust, edaspidi täiendavalt uurima nii neutraalsete kui kasulike organismide osas.

Katsed, kus kasutati põllukontsentratsioonidele relevantseid dsRNA koguseid, näitasid, et hiilamardikatele nii mikrosüstimisel kui suukaudsel dsRNA (mis oli suunatud hiilamardika coatomer alfa alam-üksuse

(*αCOP*) proteiini vaigistamisele) manustamisel saavutasime olulise geenivaigistuse ja sellest põhjustatud suremuse (**III, IV, V, VI**) ning seega katsete tulemused kinnitasid püsitatud hüpoteese. Tulemused on küll paljulubavad, kuid katseid tuleb kindlasti jätkata, et leida võimalused dsRNA stabiliseerimiseks, selle mõju tugevdamiseks, mõju uurimiseks nii hiilamardika füsioloogiale, vastsetele kui ka mittesihtrühma organismidele, uurima peaks nii ühekordse kui kroonilise manustamise mõju nii vastsetele kui valmikutele jne.

SAMENVATTING

In heel Europa is de stuifmeelkever *Brassicogethes aeneus* (syn. *Meligethes aeneus*, Coleoptera: Nitidulidae) één van de meest schadelijke bedreigingen voor de teelt van koolzaad (*Brassica napus*). Terwijl ze in koolzaadvelden wonen, voeden *B. aeneus*-volwassenen zich met stuifmeel in de zich ontwikkelende reproductieve knoppen, evenals met het stuifmeel en de nectar van bloeiende bloemen. Larven van *B. aeneus* voeden zich met stuifmeel in de knoppen waarin ze werden afgezet als eitje door de moeder. Daarna voeden ze zich met het stuifmeel en de nectar van bloeiende bloemen, nadat de knop tot bloei is gekomen, meestal tijdens hun late-eerste of vroege-tweede ontwikkelingsstadium. Hoewel koolzaad mechanismen heeft die het verlies van reproductieve toppen, die nodig zijn voor zaadontwikkeling, compenseren, kan de aanwezigheid van dense populaties van *B. aeneus* resulteren in aanzienlijke opbrengstverliezen, waardoor de noodzaak ontstaat om de B. aeneus populaties in agro-ecosystemen van koolzaad te beheren, te bestrijden. Pyrethroïde insecticiden (IRAC-klasse 3A van natriumkanaalmodulatoren) waren de standaardmethode voor het beheer van *B. aeneus* populaties. Als gevolg van het routinematige gebruik van pyrethroïden hebben *B. aeneus* populaties echter insecticideresistentie tegen pyrethroïden ontwikkeld in verschillende gebieden in Europa. Bovendien geeft uitgebreid onderzoek aan dat blootstelling aan pyrethroïden schadelijk kan zijn voor veel niet-doelinsectentaxa die in agro-ecosystemen leven. Voor een effectief en duurzaam beheer van *B. aeneus* populaties is het bijzonder belangrijk om organismen die bijdragen aan de biologische bestrijding (biocontrole) van *B. aeneus* te beschermen. De meest gespecialiseerde geleedpotigen die betrokken zijn bij de biocontrole van *B. aeneus* omvatten verschillende soorten Hymenoptera parasitoïden zoals parasitaire wespen, terwijl andere predatoren geleedpotigen (b.v. Carabide en Staphylinide kevers, spinnen) ook kunnen bijdragen aan de biocontrole van *B. aeneus*. Verschillende onderzoekingen hebben het potentieel aangetoond van deze organismen in de biologische bestrijding van *B. aeneus*. De meest efficiënte beheerstrategie van *B. aeneus* kan echter ook het inzetten vereisen van insecticidetoepassingen die enerzijds werkzaam zijn tegen *B. aeneus* maar anderzijds geen of een minimale impact hebben op niet-doelorganismen, vooral diegenen die agro-ecologische diensten verlenen, zoals biologische bestrijding.

Dit doctoraal proefschrift behandelt de noodzaak aan nieuwe en effectieve insecticiden voor gebruik binnen een veilige geïntegreerde managementstrategie van *B. aeneus*. In het kort, er werd eerst onderzoek (**I**) gedaan met een vertegenwoordiger van de neonicotinoïden; dit is een klasse van synthetische, niet-soortspecifieke insecticiden (IRAC-klasse 4A van nicotine-acetylcholinereceptor (nAChR) competitieve modulatoren). In het bijzonder is de verbinding thiacloprid onderwerp van onderzoek geweest met betrekking tot het mogelijke gebruik ervan bij het beheer van *B. aeneus*. Neonicotinoïden worden toegepast bij de productie van koolzaad, en recente veld- en kasexperimenten met thiacloprid hebben de doeltreffendheid ervan aangetoond bij het beheersen van *B. aeneus*. De compatibiliteit van thiacloprid werd onderzocht met een model Hymenoptera parasitaire wesp, *Aphelinus abdominalis*. De toxiciteit van thiacloprid werd bepaald wanneer alleen toegepast, evenals in combinatie met tebuconazole (FRAC-groep 3, demethyleringsremmers, klasse 1 van sterolbiosyntheseremmers), een vertegenwoordiger van een groep verbindingen die gewoonlijk in tanks worden gemengd met neonicotinoiden voor gewasbeschermingsdoeleinden. Ten tweede (**II**) werd het effect onderzocht van zeven verschillende plantaardige essentiële oliën (EO's), die een niet-soortspecifiek biopesticide vertegenwoordigen voor mogelijk gebruik binnen het beheer van *B. aeneus*. Ten slotte werd het potentieel onderzocht van *B. aeneus*-specifiek dubbelstrengs ribonucleïnezuur (dsRNA) (**III-VI**). Dit dsRNA kan dan worden ingezet als een veilig, soortspecifiek biopesticide binnen een *B. aeneus* beheerstrategie.

Na blootstelling van parasitaire wespen van *A. abdominalis* aan droge pesticideresiduen van thiacloprid alleen en in combinatie met tebuconazole, werden nadelige effecten waargenomen van een lage concentratie aan thiacloprid, zowel alleen als toegepast in combinatie met tebuconazole. Onze resultaten ondersteunden inderdaad onze hypothesen dat blootstelling aan residuen van thiacloprid, indien toegepast bij de aanbevolen veldconcentratie (RFC), resulteert in een significante vermindering van de overleving en mobiliteit van *A. abdominalis*; en dat gelijktijdige blootstelling aan residuen van thiacloprid en tebuconazole resulteert in synergetische reducties in de overleving en mobiliteit van A. abdominalis. Dit onthult een drempelconcentratie (in overeenstemming met RFC) die overeenkwam met de waargenomen synergie (**I**).

De topische toepassing van zeven verschillende plant-EO's resulteerden in verschillende reacties met betrekking tot de overleving en mobiliteit van

B. aeneus. De EO met de sterkste werking tegen de overleving en mobiliteit van *B. aeneus* was die van kaneelschors (*Cinnamomum verum*) (**II**). Dit ondersteunde onze hypothese dat een bepaald plant-EO zou worden aangetoond als de meest veelbelovende tegen *B. aeneus*, onder elk van de onderzochte en topisch aangebrachte EO's (**II**). Onze resultaten ondersteunden ook onze hypothese dat biotoetsen met behandelde blad- en knopoppervlakken ons in staat zouden stellen om een effectieve concentratiedrempel te detecteren voor een significante reductie in de overleving en mobiliteit van *B. aeneus*, met behulp van de meest veelbelovende EO uit de actuele biotoetsen (dit was het geval met *C. verum,* **II**). Wanneer echter de EO's werden gesproeid op oppervlakken van koolzaad en toppen van oliehoudende zaden, toonde blootstelling aan droge residuen van *C. verum* EO alleen werkzaamheid aan bij concentraties die vrij hoog zijn voor een praktische veldtoepassing van een EO. Daarom wordt voorgesteld om het onderzoek te richten op de individuele verbindingen die dominant zijn in die EO's die veelbelovend zijn voor *B. aeneus* beheer, en deze dan te testen met *B. aeneus* en ook met modeltaxa die relevant zijn in de context van biologische bestrijding en bestuiving in agro-ecosystemen van koolzaad.

In het derde deel van dit doctoraat veroorzaakte de veld-relevante blootstelling via de voeding aan *B. aeneus*-specifiek dsRNA (gericht tegen het coatomer subunit alpha, αCOP) een significante daling in de expressie van het doelgen en ook mortaliteit bij de doelkevers (**III, IV, V, VI**). De hypothese van doelgenexpressiereductie-geïnduceerde toxiciteit werd voor elke uitgevoerde *B. aeneus* RNAi-studie bevestigd. Andere cruciale stappen voor het begrijpen van het potentieel van een op RNAi-gebaseerd beheer van *B. aeneus* werden onderzocht: namelijk verfijningen aan de opstelling van de biotoets met larven van *B. aeneus*, en de ontwikkeling van dsRNA-formuleringen die het transport langs het epitheel van de koolzaadknoppen verbeteren en/of de opname van dsRNA in het insect *B. aeneus* verhogen. Met name hebben we waargenomen dat de RNAi-werkzaamheid wordt verbeterd door een chronische, in vergelijking met een kortdurende dsRNA-voeding in *B. aeneus* (**VI**), wat onze hypothese bevestigt dat dit het geval zou zijn. Dit resultaat heeft gevolgen voor de ontwikkeling en het praktische gebruik van een potentiële dsRNA-spray-benadering voor het beheer van *B. aeneus*. Evenals het benadrukt de noodzaak van onderzoek naar de ontwikkeling en het mogelijke toekomstige gebruik van RNAi-koolzaadcultivars, gezien de verbeterde RNAi-werkzaamheid als gevolg van chronische dsRNA-voeding in *B. aeneus*.

ACKNOWLEDGEMENTS

I am very grateful to my supervisors Eve Veromann and Guy Smagghe, for their unending support, and for recognising my ability to demonstrate a reasonable level of independence in managing our work together. That being said, it could not have been done without their expertise, nor the help and expertise of each co-author, especially Silva Sulg, Riina Kaasik, Clauvis Nji Tizi Taning, Olivier Christiaens, Liina Soonvald and Ana Isabel Silva, who all showed consistent dedication to our RNAi work together. Big thanks to Gabor L. Lövei for being a voice of reason and continuing to provide unbiased advice on important matters when I require it; and to both the Estonian University of Life Sciences and Ghent University for the opportunity to achieve this joint PhD degree. Finally, Ana Isabel Silva deserves additional special recognition, not because of her impeccable statistics expertise, nor her excellent contributions in research planning and presentation overall; but rather because of her persistence in encouraging me to always make the most ethical decisions regarding research integrity, even when upholding an ethical standard brings disquiet to some.

This work was supported by the institutional Research Funding project no IUT36-2 of the Estonian Research Council, the personal Research Funding project no PRG1056 of the Estonian Research Council, the European ERA-NET C-IPM project "IPM4Meligethes" (project no 3G0H0416), the European Union's European Regional Development Fund (Estonian University of Life Sciences ASTRA project "Value-chain based bioeconomy"), the Special Research Fund (BOF) of Ghent University and the Research Foundation − Flanders (FWO-Vlaanderen).

SUPPLEMENTARY INFORMATION

SI Table 1. Primers or dsRNA synthesis.

Gene	Name	Target region length (bp) for dsRNA synthesis	Primers for dsRNA synthesis
αCOP	*coatomer subunit alpha*	222	Fw: AAGATGCACCACATAATGGACA Rv: ACAAAACTGCGTCTTCGCTG
δCOP	*coatomer subunit delta*	467	Fw: CAGAGGCACCAAACTCTCGT Rv: AGAAACCGAGGGGCATGAAG
γCOP	*coatomer subunit gamma*	434	Fw: TGCTTGCCCGTTGTCAAATG Rv: CTTGATGCAGCGCACAAAGT
rps13	*ribosomal protein S13*	401	Fw: ACATCTTAACTTAAGCCACTAAAGCA Rv: ACCAACATGGCTTAAAGTAACCC
Snf7	*sucrose non-fermenting protein 7*	431	Fw: CAAAGAAAAAGCGGCCCCAA Rv: CGTCACCAAACCCAACTGGA
Vg	*vitellogenin*	355	Fw: GGGGTAGGGCAATTTGCATCAACTT Rv: TCCATCCAAGACCGCCAACA

SI Table 2. List of treatments in pesticide mixture experiment. dH_2O = distilled water (control); TH = thiacloprid; TEB = tebuconazole; [0.01] = one one-hundredth manufacturer's recommended field concentration (RFC); [0.05] = one twentieth RFC; [0.1] = one tenth RFC; [0.5] = one half RFC; [1] = RFC (**I**; Willow et al., 2019, PLoS One).

Treatment	Concentration of TH (g/ha)	Concentration of TEB (g/ha)
dH_2O	0	0
TEB [1]	0	125
TH [0.1]	12	0
TH [0.1] + TEB [0.01]	12	1.25
TH [0.1] + TEB [0.05]	12	6.25
TH [0.1] + TEB [0.1]	12	12.5
TH [0.1] + TEB [0.5]	12	62.5
TH [0.1] + TEB [1]	12	125

SI Table 3. Primer amplification efficiencies of *αCOP* and the housekeeping genes *rps3* and *act* (**III**; Willow et al., 2020b, J. Pest Sci.).

Gene	Name	Direction	Primer sequence	Efficiency (%)
αCOP	*coatomer subunit alpha*	Fw Rv	CCAACTTGGTCATTTAACAATCTGG CCCTTCCTTCATCATGGACA	182
rps3	*ribosomal protein S3*	Fw Rv	CCAACGCGTACCGAAATTAT GAGTTTTCGGGGAAGTTGAA	220
act	*actin*	Fw Rv	TCACGGACAATTTCCCTTTC TATCCTCCGTTTGGACTTGG	188

SI Table 4. Primer amplification efficiencies of *αCOP* and the housekeeping genes *rps3* and *act*; and primer information regarding RT-PCR for detecting *gfp* on bud tissue (**IV**; Willow et al., 2020c, Insects).

Gene	Name	Direction	Primer sequence	PCR type and efficiency (%)
gfp	*green fluorescent protein*	Fw Rv	CACATGAAGCAGCACGACTT TGCTCAGGTAGTGGTTGTCG	RT-PCR
αCOP	*coatomer subunit alpha*	Fw Rv	CCAACTTGGTCATTTAACAATCTGG CCCTTCCTTCATCATGGACA	qPCR, 107.8
rps3	*ribosomal protein S3*	Fw Rv	CCAACGCGTACCGAAATTAT GAGTTTTCGGGGAAGTTGAA	qPCR, 100
act	*actin*	Fw Rv	TCACGGACAATTTCCCTTTC TATCCTCCGTTTGGACTTGG	qPCR, 90.8

SI Table 5. Primer amplification efficiencies of *αCOP* and the housekeeping genes *rps3* and *act* (**V**; Willow et al., Submitted to Front. Agron.).

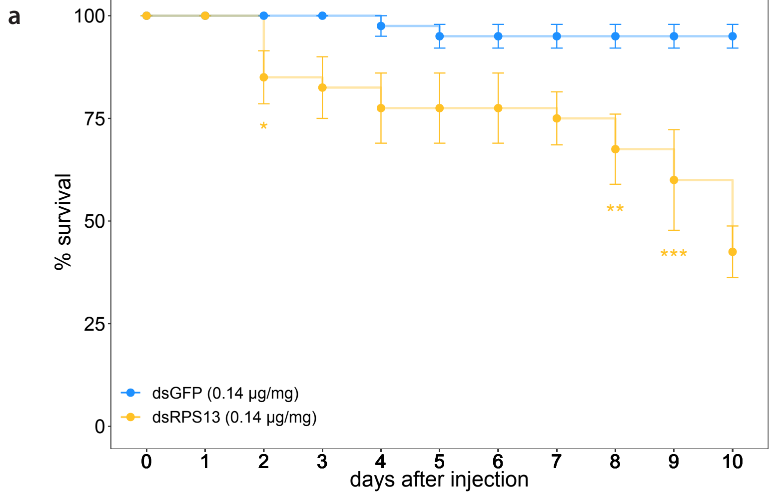
Gene	Name	Direction	Primer sequence	Efficiency (%)
αCOP	*coatomer subunit alpha*	Fw Rv	CCAACTTGGTCATTTAACAATCTGG CCCTTCCTTCATCATGGACA	88.69
rps3	*ribosomal protein S3*	Fw Rv	CCAACGCGTACCGAAATTAT GAGTTTTCGGGGAAGTTGAA	98.1
act	*actin*	Fw Rv	TCACGGACAATTTCCCTTTC TATCCTCCGTTTGGACTTGG	106.9

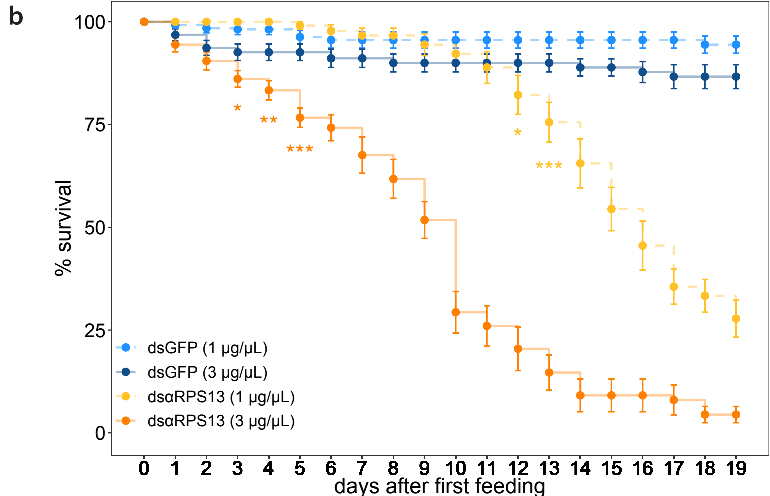
SI Table 6. Primer amplification efficiencies of *αCOP* and the housekeeping genes *rps3* and *act* (**VI**; Willow et al., In Press, Commun. Biol.).

Gene	Name	Direc-tion	Primer sequence	Efficiency (%)
αCOP	*coatomer subunit alpha*	Fw	CCAACTTGGTCATTTAACAATCTGG	105.2
		Rv	CCCTTCCTTCATCATGGACA	
rps3	*ribosomal protein S3*	Fw	CCAACGCGTACCGAAATTAT	90.3
		Rv	GAGTTTTCGGGGAAGTTGAA	
act	*actin*	Fw	TCACGGACAATTTCCCTTTC	105.6
		Rv	TATCCTCCGTTTGGACTTGG	

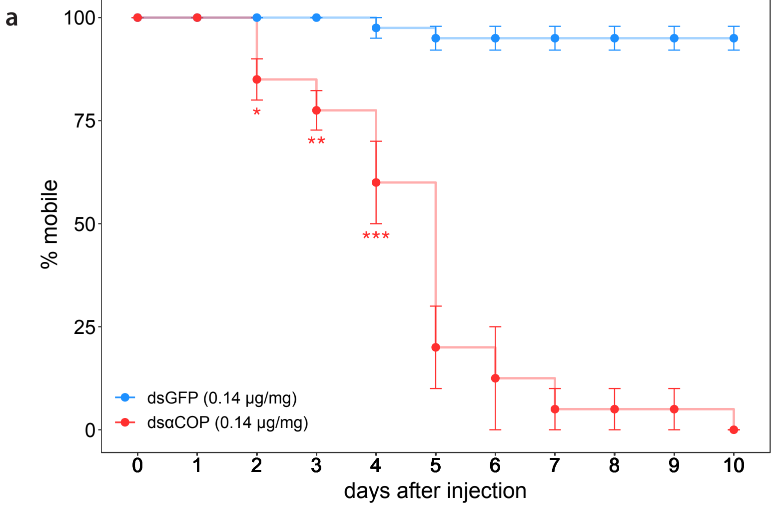
SI Table 7. List of treatment comparisons considered in survival- and gene expression analyses. ST = short-term dsRNA feeding; C = chronic dsRNA feeding (**VI**; Willow et al., In Press, Commun. Biol.).

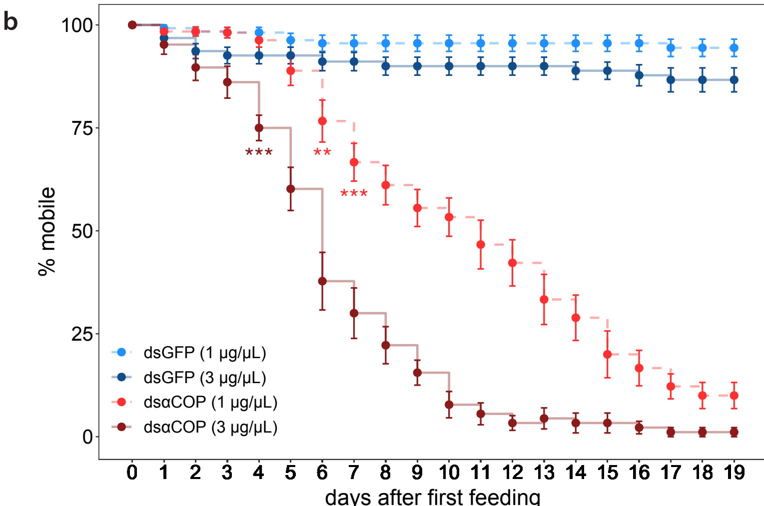
Treatment comparison	
Survival	Gene expression
Short-term dsRNA feeding	Short-term dsRNA feeding
dsGFP 5 μg/μL × dsαCOP 0.5 μg/μL	dsGFP 5 μg/μL × dsαCOP 0.5 μg/μL
dsGFP 5 μg/μL × dsαCOP 2.5 μg/μL	dsGFP 5 μg/μL × dsαCOP 2.5 μg/μL
dsGFP 5 μg/μL × dsαCOP 5 μg/μL	dsGFP 5 μg/μL × dsαCOP 5 μg/μL
dsαCOP 0.5 μg/μL × dsαCOP 2.5 μg/μL	dsαCOP 0.5 μg/μL × dsαCOP 2.5 μg/μL
dsαCOP 0.5 μg/μL × dsαCOP 5 μg/μL	dsαCOP 0.5 μg/μL × dsαCOP 5 μg/μL
dsαCOP 2.5 μg/μL × dsαCOP 5 μg/μL	dsαCOP 2.5 μg/μL × dsαCOP 5 μg/μL
Chronic dsRNA feeding	Chronic dsRNA feeding
dsGFP 5 μg/μL × dsαCOP 0.5 μg/μL	dsGFP 5 μg/μL × dsαCOP 0.5 μg/μL
dsGFP 5 μg/μL × dsαCOP 2.5 μg/μL	dsGFP 5 μg/μL × dsαCOP 2.5 μg/μL
dsGFP 5 μg/μL × dsαCOP 5 μg/μL	dsGFP 5 μg/μL × dsαCOP 5 μg/μL
dsαCOP 0.5 μg/μL × dsαCOP 2.5 μg/μL	dsαCOP 0.5 μg/μL × dsαCOP 2.5 μg/μL
dsαCOP 0.5 μg/μL × dsαCOP 5 μg/μL	dsαCOP 0.5 μg/μL × dsαCOP 5 μg/μL
dsαCOP 2.5 μg/μL × dsαCOP 5 μg/μL	dsαCOP 2.5 μg/μL × dsαCOP 5 μg/μL
Short-term- vs chronic dsRNA feeding	
dsGFP 5 μg/μL (ST) × dsGFP 5 μg/μL (C)	
dsαCOP 0.5 μg/μL (ST) × dsαCOP 0.5 μg/μL (C)	
dsαCOP 2.5 μg/μL (ST) × dsαCOP 2.5 μg/μL (C)	
dsαCOP 5 μg/μL (ST) × dsαCOP 5 μg/μL (C)	



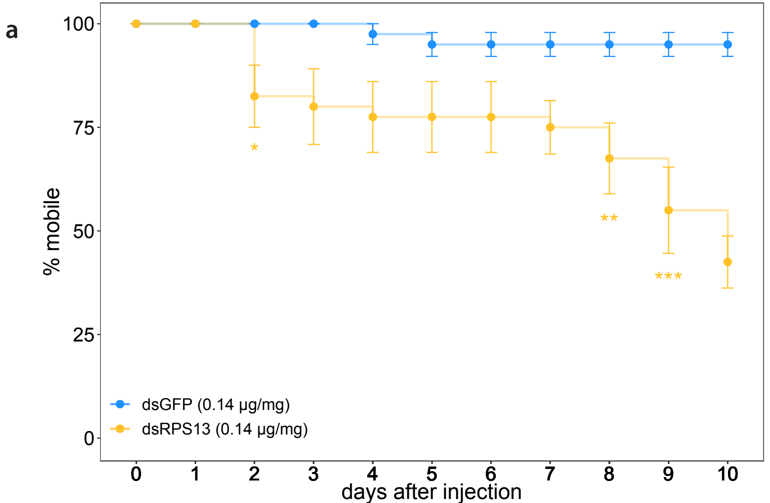


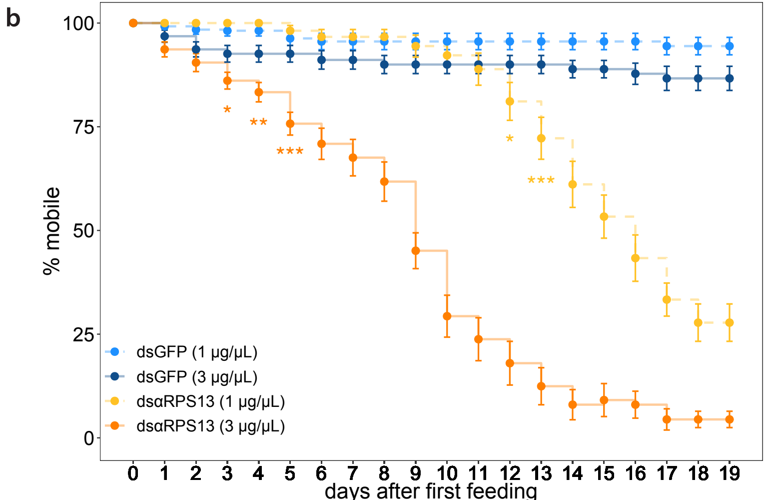
SI Figure 1. Survival curves, comparing dsrps13 treatments with their respective dsGFP controls, in microinjected (a) and dsRNA-fed (b) pollen beetles.
Microinjection experiment: n=40 (four replicates of ten beetles) per treatment. Feeding experiment: n=21 (21 cages of six beetles; days 0–2), 18 (18 cages of six beetles; days 3–5) and 15 (15 cages of six beetles; days 6–19) per treatment. Microinjection data were analysed via Fisher's exact test (error bars: ±SE). Feeding data were analysed via Kruskal–Wallis test with *post hoc* Wilcoxon rank-sum test for pairwise comparisons (error bars: ±SEM). Asterisk (*) indicates significant differences between dsrps13 and respective dsGFP control treatments. df=3, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.



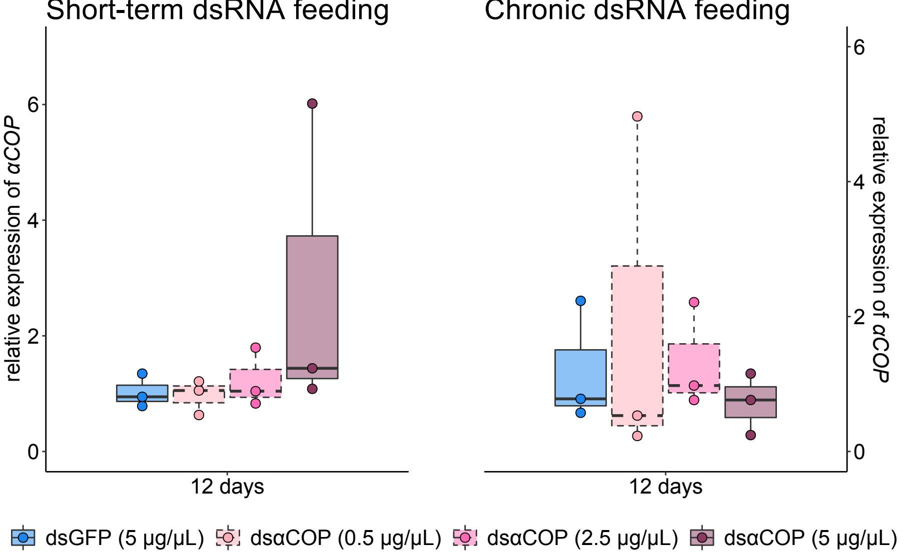


SI Figure 2. Mobility curves, comparing dsαCOP treatments with their respective dsGFP controls, in microinjected (a) and dsRNA-fed (b) pollen beetles. Microinjection experiment: n=40 (four replicates of ten beetles) per treatment. Feeding experiment: n=21 (21 cages of six beetles; days 0–2), 18 (18 cages of six beetles; days 3–5) and 15 (15 cages of six beetles; days 6–19) per treatment. Microinjection data were analysed via Fisher's exact test (error bars: ±SE). Feeding data were analysed via Kruskal–Wallis test with *post hoc* Wilcoxon rank-sum test for pairwise comparisons (error bars: ±SEM). Asterisk (*) indicates significant differences between dsαCOP and respective dsGFP control treatments. df=3, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ (**III**; Willow et al., 2020b, J. Pest Sci.).





SI Figure 3. Mobility curves, comparing dsrps13 treatments with their respective dsGFP controls, in microinjected (a) and dsRNA-fed (b) pollen beetles.
Microinjection experiment: n=40 (four replicates of ten beetles) per treatment. Feeding experiment: n=21 (21 cages of six beetles; days 0–2), 18 (18 cages of six beetles; days 3–5) and 15 (15 cages of six beetles; days 6–19) per treatment. Microinjection data were analysed via Fisher's exact test (error bars: ±SE). Feeding data were analysed via Kruskal–Wallis test with *post hoc* Wilcoxon rank-sum test for pairwise comparisons (error bars: ±SEM). Asterisk (*) indicates significant differences between dsrps13 and respective dsGFP control treatments. df=3, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.



SI Figure 4. Results of qPCR, showing relative expression of *αCOP* in *Brassicogethes aeneus* at 12 d, comparing target treatments (dsαCOP at 0.5, 2.5 and 5 µg/µL) to the dsGFP control. Analysed using Welch's t-test (**VI**; Willow et al., In Press, Commun. Biol.).

I

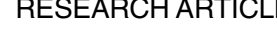
ORIGINAL PUBLICATIONS

I **Willow, J.**, Silva, A.I., Veromann, E., Smagghe, G. 2019. Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. PLOS ONE 14, e0212456. http://doi:10.1371/journal.pone.0212456

RESEARCH ARTICLE

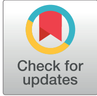
Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp

Jonathan Willow[1,2¤]*, **Ana Silva**[3], **Eve Veromann**[2☯], **Guy Smagghe**[1☯]



1 Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium, **2** Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu, Estonia, **3** Cardiff University Brain Research Imaging Centre (CUBRIC), School of Psychology, Cardiff University, Cardiff, United Kingdom

☯ These authors contributed equally to this work.
¤ Current address: Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu, Estonia
* jonathan.willow@student.emu.ee



OPEN ACCESS

Citation: Willow J, Silva A, Veromann E, Smagghe G (2019) Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. PLoS ONE 14(2): e0212456. https://doi.org/10.1371/journal.pone.0212456

Editor: James C. Nieh, University of California San Diego, UNITED STATES

Received: November 14, 2018

Accepted: February 1, 2019

Published: February 22, 2019

Copyright: © 2019 Willow et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This study was partially funded by European ERA-NET C-IPM project no 33: IPM4Meligethes, the Institutional Research Funding project no IUT36-2 of Estonian Research Council, and Research Foundation-Flanders (FWO-Vlaanderen). JW received a PhD grant from European Social Fund Doctoral Students, as well as the Internalisation Programme DoRa (carried out by Archimedes Foundation). The funders had no

Abstract

Agricultural practices often involve tank-mixing and co-application of insecticides with fungicides to control crop pests. However, natural methods relying on biological control agents such as hymenopteran parasitoids have been shown to be highly effective in suppressing crop pest populations. The current body of insecticide risk assessment data accounting for fungicide co-application is very small, the present study being the first to examine this in a parasitoid wasp. Through low-dose exposure to dry residues of the neonicotinoid insecticide thiacloprid, we examined its mortal and knockdown effect on *Aphelinus abdominalis* when co-applied with increasing doses of the fungicide tebuconazole. Both of these acute effects of thiacloprid were synergised (toxicity increased to a greater-than-additive effect) by tebuconazole, resulting in significant mortality from low-dose co-applications of tebuconazole, and significant knockdown even without co-applied tebuconazole, the effect increasing as tebuconazole concentration increased. We show the highly toxic effect that a low dose of thiacloprid imposes on *A. abdominalis* populations, and a synergistic toxicity when co-applied with low doses of tebuconazole. Our work suggests a need for updating pesticide risk assessment methods, accounting for pesticide mixtures, in order to make these risk assessments more field relevant.

Introduction

Insects contribute to several ecosystem services that are indispensable to agriculture [1], one of which is biological control of crop pests. Parasitoid wasps in particular can be very effective at suppressing insect pest populations in agroecosystems [2–10]. However, in conventional agriculture, farmers apply pesticides to manage crop pests, often routinely without regard for pest

role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There was no additional external funding received for this study.

Competing interests: The authors have declared that no competing interests exist.

incidence and abundance, even though research has indicated lethal and sublethal effects of both botanical and synthetic pesticides on numerous parasitoid wasp species of ecological and economic importance [11–24]. Among insecticide classes, chloronicotinyls (neonicotinoids, IRAC class 4A of nicotinic acetylcholine receptor (nAChR) competitive modulators) are especially hazardous for insect populations due to their systemic action in plants, resulting in not only surface contamination from spray residues, but potential contamination of all plant tissues and floral/extrafloral rewards (e.g. nectar, pollen, guttation). Recently in April 2018, after considerable evidence had been gathered regarding adverse effects of these systemic insecticides on beneficial insects [25], all member states of the European Union agreed to ban outdoor use of three neonicotinoid insecticides, namely imidacloprid, clothianidin and thiamethoxam. However, there are 13 neonicotinoid active ingredients patented for use as insecticides [26].

In practice, insecticides are often tank-mixed with fungicides for simultaneous application to agricultural fields [27,28]. The ability of a fungicide to synergise the toxicity of an insecticide has been clearly demonstrated in the honeybee *Apis mellifera* (L.) [29–32], the mason bee *Osmia bicornis* (L.) [33,34] and the bumblebee *Bombus terrestris* (L.) [35]. This means the effect of pyrethroid and neonicotinoid insecticides combined with ergosterol biosynthesis inhibitor fungicides is greater than the sum of each one's effect when applied individually. The suggested mechanism behind this is that exposure these fungicides inhibits production of cytochrome P450-dependent monooxygenases, enzymes necessary for oxidative metabolism of a variety of xenobiotics including insecticides [36]. The available data demonstrating this phenomenon in non-target insects are currently limited to the above-mentioned three species in the bee superfamily Apoidea. Parasitoid wasps represent another relevant group of hymenopteran insects for examining this phenomenon, their populations being essential for self-sustaining pest control processes and integrated pest management (IPM). In addition to their role as biocontrol agents, their size and behavioural differences compared to the above-mentioned bee species suggests the need for insecticide risk assessment data accounting for fungicide co-application in a parasitoid model.

The neonicotinoid insecticide thiacloprid and the fungicide tebuconazole (FRAC code 3, demethylation inhibitors, class 1 of sterol biosynthesis inhibitors) are both applied, sometimes as tank-mixture [28], for crop protection in a variety of agroecosystems, including but not limited to oilseed rape, wheat, orchards and cotton. The parasitoid wasp family Aphelinidae is an important taxon of parasitoids (primarily of aphids and other Homoptera) distributed across the world, inhabiting almost all habitat types. This diverse family contains approximately 1160 species in 33 genera and 7 subfamilies [37].

Here we exposed the aphelinid wasp *Aphelinus abdominalis* (Dalman), an important biological control agent for suppressing aphid populations, to a low concentration of dry residues of thiacloprid, with and without co-applications of tebuconazole at various concentrations at or below manufacturer's recommended dose (MRD). After exposure via dry residues on glass surface, we monitored the effect of each treatment on *A. abdominalis* mortality (lethal effect) and knockdown (loss of motor control, sublethal effect) over a 24 h period. Based on the findings of above-mentioned analogous research on bees, we expected tebuconazole to synergise these acute effects of thiacloprid in *A. abdominalis*, and to observe a threshold-dose of tebuconazole corresponding to this synergy. This work is intended to show the increased lethality and sublethality imposed on *A. abdominalis* populations when exposed to multiple pesticides simultaneously, as well as the increased field-relevance of insecticide risk assessment when examining pesticides in combination, since these compounds are tank-mixed and simultaneously applied in agricultural practice.

Materials and methods

Prior to this study, we explored the use of three different parasitoid wasp species, namely *Aphidius matricariae* (Haliday) (Braconidae), *Diglyphus iseae* (Walker) (Eulophidae) and *Aphelinus abdominalis*. These preliminary tests showed a similar mortality effect of thiacloprid on each species examined (data not shown). We decided to focus on *A. abdominalis* due to the ease of handling this species without the need to slow down their activity using cold temperatures.

The two active ingredients examined, and their respective formulations, were thiacloprid [Calypso, active ingredient 480 g/l, suspension concentrate (Bayer CropScience)] and tebuconazole [Tebusip, active ingredient 250 g/l, emulsifiable concentrate (OXON Italia)]. Aphid mummies containing diapausing *A. abdominalis* adults, were ordered from Biobest (Westerlo, Belgium), and subsequently maintained in an incubator (22 ˚C, 60% relative humidity, 16:8 h light:dark; model MLR-352H-PE Climate Chamber, Panasonic, Kadoma, Japan). Insects were used in experiments shortly (1–2 days) after emergence from diapause.

In search of a suitable dose of thiacloprid for examining synergy dynamics, 100 individuals (5 cages of 20 insects) of *A. abdominalis* were exposed to dried residues of thiacloprid at MRD (120 g/ha), as well as a control treatment (de-ionized water). The effectiveness of thiacloprid at MRD on knockdown (shown in Results) suggested that this dose was unsuitable for examining synergy dynamics, and thus we subsequently reduced our experimental dose of thiacloprid to one tenth MRD (12 g/ha).

Treatments for the experiment are shown in Table 1. They include an untreated control (de-ionized water), tebuconazole at MRD (125 g/ha), thiacloprid at one tenth MRD, and five treatments containing both thiacloprid and tebuconazole. In combinatory treatments, we applied thiacloprid always at one tenth MRD, while tebuconazole was co-applied at one one-hundredth MRD (1.25 g/ha), one twentieth MRD (6.25 g/ha), one tenth MRD (12.5 g/ha), one half MRD (62.5 g/ha) and MRD (125 g/ha). We used 240 insects (12 cages of 20 insects) per treatment.

For each treatment, circular glass discs (9 cm diameter) were individually loaded into a Cornelis spray tower [38], and sprayed with 1 ml of treatment solution (formulation and de-ionized water) using 1 bar of air pressure. Prior to experiments, treatment solutions were diluted and mixed based on the surface area of the glass plates and the use of 1 ml of solution per spray per disc, in order to apply solutions representing a series of active ingredient concentrations in g/ha based on MRD. To ensure the use of fresh treatments for each experimental repeat, new treatment solutions were prepared weekly, kept in sterile 50 ml polypropylene centrifuge tubes (Nerbe Plus, Germany) and stored in a refrigerator. The treated glass discs were

Table 1. List of treatments in pesticide mixture experiment. H_2O = control, TH = thiacloprid, TEB = tebuconazole, [0.01] = one one-hundredth manufacturer's recommended dose (MRD), [0.05] = one twentieth MRD, [0.1] = one tenth MRD, [0.5] = one half MRD, [1] = MRD.

Treatment	Dose of TH (g/ha)	Dose of TEB (g/ha)
H_2O	0	0
TEB [1]	0	125
TH [0.1]	12	0
TH [0.1] + TEB [0.01]	12	1.25
TH [0.1] + TEB [0.05]	12	6.25
TH [0.1] + TEB [0.1]	12	12.5
TH [0.1] + TEB [0.5]	12	62.5
TH [0.1] + TEB [1]	12	125

https://doi.org/10.1371/journal.pone.0212456.t001

left to air-dry for approximately 2 h, ensuring that only dried treatment residues remained on the plates.

Cages were constructed from two treated glass discs and a plastic ring frame (height of 14 mm) with small holes covered in fine mesh to provide ventilation and prevent internal condensation. For each cage, the plastic ring frame was placed over the perimeter of the bottom disc, which was then fastened to the ring frame, and 20 randomly chosen individuals were introduced to the base of the cage using a fine paintbrush. Then the top disc was placed over the ring frame to complete the cage, and all materials were fastened together. The treated side of each glass disc faced interiorly in each constructed cage. The sex of each wasp was not taken into account, in order to prevent any damage to the insects from too much handling. Finally, as a food source, a strip of filter paper soaked in 50% sucrose solution (blotted with a paper towel to reduce wetness, further preventing internal condensation) was added to the cage through a larger hole in the plastic frame, which was then sealed with a rubber stopper. Cages with wasps were then placed in the incubator.

Using a 14x magnification hand lens, both mortality and knockdown were recorded at 2, 4, 6, 8 and 24 h post-exposure. Mortality was assumed when an individual showed no movement (14x magnification) during 15 s of observation, even after gently prodding and stroking the insect with a fine paintbrush. Knockdown was determined when erratic muscular activity (stumbling, convulsing) or a lack of muscular activity (partial or entire paralysis) inhibited an individual from moving about in a stable manner or at all, taking into account all situations where an individual was incapable of performing biological control services (including apparent mortality, therefore representing total acute effect). Knockdown is an important endpoint to examine, as a very small insect like *A. abdominalis* may likely die shortly after knockdown in natural situations due to various factors (e.g. dehydration, predation, inability to forage).

Statistical analyses were performed with R software (version 1.0.136) [39]. We used a one-way ANOVA followed by *post-hoc* pairwise comparisons using two-tailed unpaired t-tests and corrected for multiple comparisons using the Bonferroni correction method. Since the residuals of the linear model were normal, we used non-transformed data. Synergistic toxicity of pesticide mixture treatments was determined by subtracting single-compound effects, of both thiacloprid ($Effect_{TH}$) and tebuconazole ($Effect_{TEB}$), from the effect of a given combinatory treatment ($Effect_{TH+TEB}$). An $Effect_{TH+TEB}$ greater than the combined sum of $Effect_{TH}$ and $Effect_{TEB}$ indicates synergistic toxicity.

Results

When examining the effect of thiacloprid at MRD on *A. abdominalis*, after 24 h we observed 52% mortality (p = 0.009, Welch two-tailed unpaired t-test) and 79% knockdown (p = 0.0002, Welch two-tailed unpaired t-test) from thiacloprid at MRD (Table 2, Fig 1, see S1 and S2 Tables for raw data), showing that this concentration of thiacloprid was too effective on knockdown to reliably use MRD of thiacloprid for our experiment.

When exposing *A. abdominalis* to all 8 treatments in our examination of thiacloprid at one tenth MRD combined with increasing doses of tebuconazole (N = 240 per treatment), we observed tebuconazole's synergising effect on thiacloprid (effect of combinatory treatments were greater than the summed effect of both thiacloprid and tebuconazole by themselves), with regard to mortality at 24 h for combinatory treatments containing tebuconazole at one twentieth MRD, one tenth MRD, one half MRD and MRD (Fig 2a). F statistics, assessed using one-way ANOVA, showed significant lethal effects, at 24 h, of using different treatments (Table 3, Fig 2a, see S3 Table for raw data).

Table 2. Effect of thiacloprid at manufacturer's recommended dose (MRD, 120 g(ha)$^{-1}$) on mortality and knock-down of the parasitoid wasp *Aphelinus abdominalis* at different hours after treatment. N = 100 (5 cages of 20 insects) per treatment. H_2O = control, TH = thiacloprid, [1] = manufacturer's recommended dose (MRD).

Mortality			
t-test (H2O vs TH [1]	t	df	p-value
2 h			
4 h			
6 h	6	4	0.004
8 h	4.35	4	0.01
24 h	4.65	4.11	0.009
Knockdown			
t-test (H2O vs TH [1]	t	df	p-value
2 h	6.08	4	0.004
4 h	5.78	4	0.004
6 h	6.43	4	0.003
8 h	5.98	4.08	0.004
24 h	11.62	4.21	0.0002

https://doi.org/10.1371/journal.pone.0212456.t002

Post-hoc pairwise comparisons revealed that thiacloprid at one tenth MRD by itself did not significantly affect mortality at 24 h ($p = 0.44$) (Table 4) compared to the control (H_2O). However, a significant effect on mortality resulted from combining thiacloprid at one tenth MRD with tebuconazole at one tenth MRD ($p = 0.03$), one half MRD ($p = 0.02$) and MRD ($p<0.01$), as well as a marginally significant effect when combined with tebuconazole at one twentieth MRD ($p = 0.06$). We observed a trend of increased mortality as the concentration of tebuconazole in the binary mixture increased.

Regarding knockdown, we observed that tebuconazole synergised the effect of thiacloprid at 2, 4, 6, 8 and 24 h in combinatory treatments containing tebuconazole at one half MRD and MRD (Fig 2b, see S4 Table for raw data). F statistics, assessed using one-way ANOVA, showed significant knockdown effects, at 2, 4, 6 8 and 24 h, of using different treatments (Table 3, Fig 2b). *Post-hoc* pairwise comparisons revealed a significant difference in knockdown at 2 h for all treatments containing thiacloprid (Table 4). At 24 h, we observed increased knockdown in all treatments. Knockdown was significant for the treatment containing only thiacloprid at one tenth MRD ($p<0.01$), as well as the combinatory treatments containing tebuconazole at one one-hundredth MRD ($p<0.005$), one twentieth MRD ($p<0.001$), one tenth MRD ($p<0.01$), one half MRD ($p = 0.0001$) and MRD ($p<0.0001$) (Table 4). As with mortality, we observed a trend of increased knockdown as the concentration of tebuconazole in combinatory treatments increased.

Discussion

Using the parasitoid *wasp A. abdominalis* exposed to field-realistic doses of dry pesticide residues, our study provides evidence of an insecticide's acute lethal and sublethal effect synergised by co-application of a fungicide, the two compounds being commonly tank-mixed for simultaneous use in a variety of agroecosystems [28]. To our knowledge, these are the first data showing this type of agrochemical synergy occurring in a biological control insect.

We observed a synergising of thiacloprid's effect, by co-exposure to tebuconazole, on mortality for all combinatory treatments containing tebuconazole at one twentieth MRD or higher. We did not observe significant mortality at 24 h when exposing *A. abdominalis* to dried residues of thiacloprid at one tenth MRD by itself. However, when thiacloprid at one tenth MRD

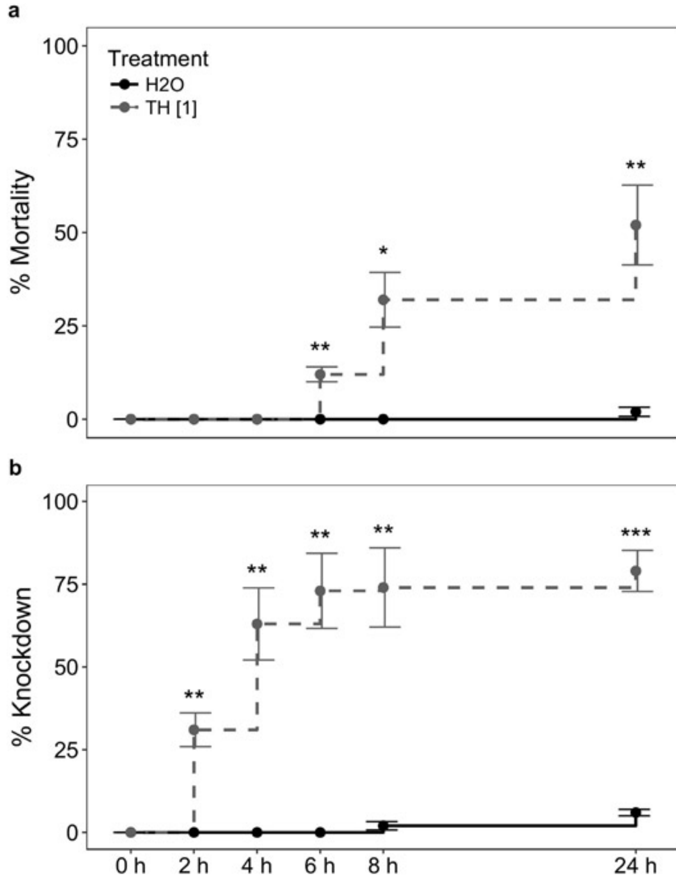


Fig 1. Effect of thiacloprid at manufacturer's recommended dose (MRD, 120 g/ha) on a) mortality, and b) knockdown of the parasitoid wasp *Aphelinus abdominalis* at different hours after treatment (error bars: ±SEM). N = 100 (5 cages of 20 insects). H_2O = control, TH = thiacloprid. Welch two-tailed unpaired t-test: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

https://doi.org/10.1371/journal.pone.0212456.g001

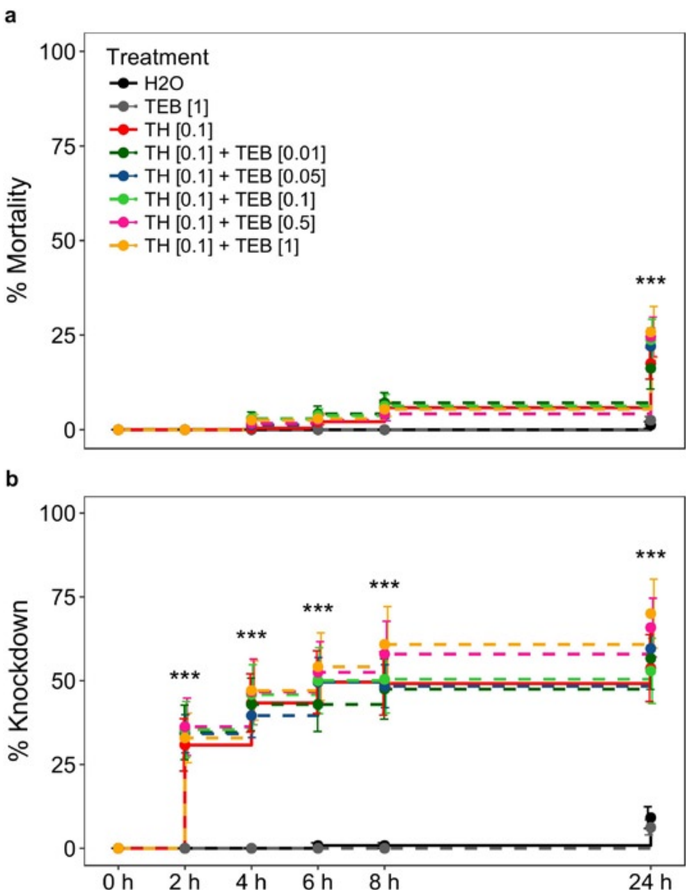


Fig 2. Effect of each treatment on a) mortality and b) knockdown of the parasitoid wasp *Aphelinus abdominalis* at different hours after treatment (error bars: ±SEM). N = 240 (12 cages of 20 insects) per treatment. H_2O = control, TH = thiacloprid, TEB = tebuconazole, [0.01] = one one-hundredth manufacturer's recommended dose (MRD), [0.05] = one twentieth MRD, [0.1] = one tenth MRD, [0.5] = one half MRD, [1] = MRD. One-way ANOVA: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

https://doi.org/10.1371/journal.pone.0212456.g002

Table 3. Results from analysis of variance (ANOVA) for the effect of the different treatments on mortality and knockdown of the parasitoid wasp *Aphelinus abdominalis* in each cage (12 cages, each with 20 insects) at different hours after treatment. dfn = degrees of freedom numerator, dfd = degrees of freedom denominator.

Time	dfn, dfd	F-statistics	Pr (>F)
		Mortality	
2 h	(7,88)	1	0.44
4 h	(7,88)	1.28	0.27
6 h	(7,88)	1.53	0.17
8 h	(7,88)	2.05	0.06
24 h	(7,88)	4.37	<0.001
		Knockdown	
2 h	(7,88)	5.62	<0.0001
4 h	(7.88)	7.86	<0.0001
6 h	(7.88)	8.73	<0.0001
8 h	(7.88)	8.95	<0.0001
24 h	(7,88)	9.28	<0.0001

https://doi.org/10.1371/journal.pone.0212456.t003

was co-applied with tebuconazole at one tenth MRD or higher, significant mortality was observed. Our observations on knockdown further revealed the potential harm that tank-mixing thiacloprid and tebuconazole can impose on *A*. *abdominalis* populations. We observed a trend towards increased knockdown as the concentration of tebuconazole increased in combinatory treatments. When thiacloprid at one tenth MRD was co-applied with tebuconazole at one half MRD or higher, a greater-than-additive knockdown effect of combining these two pesticides was consistently observed over 24 h. Thiacloprid at one tenth MRD by itself resulted in significant knockdown of *A*. *abdominalis*, and this effect increased as the co-applied dose of

Table 4. Results from *post-hoc* pairwise comparisons (two-tailed unpaired t-test) between the control and each other treatment, for both mortality and knockdown. H_2O = control, TH = thiacloprid, TEB = tebuconazole, [0.01] = one one-hundredth manufacturer's recommended dose (MRD), [0.05] = one twentieth MRD, [0.1] = one tenth MRD, [0.5] = one half MRD, [1] = MRD. All p-values shown have been adjusted for multiple comparisons using Bonferroni correction method.

Treatment vs H_2O	p-value (Bonferroni)	
Mortality		24 h
TEB [1]		1
TH [0.1]		0.44
TH [0.1] + TEB [0.01]		0.71
TH [0.1] + TEB [0.05]		0.06
TH [0.1] + TEB [0.1]		0.03
TH [0.1] + TEB [0.5]		0.02
TH [0.1] + TEB [1]		0.009
Knockdown	2h	24 h
TEB [1]	1	1
TH [0.1]	0.04	0.006
TH [0.1] + TEB [0.01]	0.01	0.002
TH [0.1] + TEB [0.05]	0.01	0.0009
TH [0.1] + TEB [0.1]	0.009	0.007
TH [0.1] + TEB [0.5]	0.007	0.0001
TH [0.1] + TEB [1]	0.02	< 0.0001

https://doi.org/10.1371/journal.pone.0212456.t004

tebuconazole increased. Our results suggest that even when field-applied thiacloprid residues are degraded to a potency one order of magnitude less than application rate, or even if thiacloprid is applied at lower doses such as one tenth MRD, this may cause significant reductions in populations of *A*. *abdominalis* or similar species, especially when thiacloprid is tank-mixed with tebuconazole or perhaps other fungicides. Fungicide application to crops containing aged residues of thiacloprid may represent an equal or greater issue, as 14 day old thiacloprid residues on leaves were shown to cause significant mortality in the braconid wasp *Aphidius rhopalosiphi* (De Stefani Perez) [40].

As endpoints, the present study examined mortality and knockdown, both being appropriate and relevant. Indeed, knockdown may be a more accurate estimation, compared to mortality, of the damage these agrochemicals can inflict on a population, since insects that lack or simply cannot control their motor activity are highly unlikely to successfully perform the ecological services for which they are revered. Moreover, very small insects like *A*. *abdominalis* may indeed be likely to die not long after knockdown, simply from environmental exposure (e.g. dehydration, predation) and inability to forage. Adding to the concern is a body of evidence indicating that key processes involved in achieving their ecological function (e.g. mate and host recognition, maximising fecundity, optimising offspring sex ratio) can be significantly hindered by exposure to both botanical and synthetic pesticides [11,12,14–18,20,21,25]. It would be beneficial for future studies to investigate how these processes are affected by exposure to pesticide combinations used in agricultural practice.

Our study comes at an appropriate time, as three neonicotinoid insecticides were recently banned for outdoor use in all European Union member states. Thiacloprid was not one of these, and therefore it will be increasingly important to study the lethal and sublethal effects of the neonicotinoids that remain in use. With the new ban on imidacloprid, clothianidin and thiamethoxam, remaining neonicotinoids such as thiacloprid are indeed likely to increase in use.

Results of our study are consistent with the results of Sugiyama and colleagues [13] who exposed three other species of the parasitoid wasp family Aphelinidae to thiacloprid residues. They observed very high mortality rates in *Eretmocerus eremicus* (Rose and Zolnerowich) (98%) and *Encarsia formosa* (Gahan) (86%), and 37% mortality in *Eretmocerus mundus* (Mercet), while in our preliminary test with thiacloprid at MRD we observed 52% mortality and 79% knockdown for *A*. *abdominalis*. Furthermore, a potentially detrimental knockdown effect (an effect which typically precedes death) of thiacloprid, when applied at doses as low as one tenth MRD, is indicated by our results. Sugiyama et al. [13] also examined the lethal effect of five other neonicotinoids (acetamiprid, clothianidin, dinotefuran, imidacloprid and nitenpyram) in the same study, and observed 100% mortality in all three aphelinid species for all five of these insecticides. Our work, combined with that of Sugiyama et al. [13], suggests that exposure to thiacloprid residues may be detrimental to numerous parasitoid wasp species of the family Aphelinidae. To confirm this, however, would require exhaustive research within the context of this species-rich family.

The present study examined the effect of contact with dried pesticide residues. Under field conditions, multiple routes of exposure to these systemic compounds are likely to occur simultaneously (e.g. direct exposure to spray droplets; contact with dried surface residues; larval feeding on contaminated prey; and adult feeding on contaminated nectar, pollen, honeydew and guttation). Accounting for these various routes of pesticide exposure is encouraged for further studies. Furthermore, pesticide exposure is poorly understood for many non-target invertebrate taxa, and it is needed that experiments simulate realistic exposure. Mesocosm experiments simulating natural community exposure to relevant pesticide combinations [41] are also encouraged.

Our results suggest that the degree of tebuconazole's synergising effect on thiacloprid depends on the dose of co-applied tebuconazole. Enzyme assays confirming cytochrome P450-dependent monooxygenase inhibition, and its response to increasing or decreasing co-applied doses of tebuconazole, could increase our understanding of how these compounds act together, a prerequisite to informed use of these agrochemicals.

We examined the effect on mortality and knockdown, accounting for any instance of clear incapability to perform biological control services. Examining other sublethal effects (e.g. on reproduction, microbiome, feeding, longevity, etc.) could add much to our understanding of how these field-applied pesticide mixtures may affect population sustenance, and should be incorporated into further studies. In order to increase field realism, further studies should also incorporate the use of treated live plants as the contaminated experimental surface, as unlike glass these living tissues are absorptive to the liquid treatments examined here. In addition, although our treatments were prepared weekly and kept refrigerated in the dark, pesticide formulations are best used immediately after preparation/dilution.

The synergising of thiacloprid's acute effect, via co-application of tebuconazole, as shown in our study, suggests the necessity for updating the standards by which we perform insecticide risk assessments on non-target organisms, by including other pesticides with which these insecticides are commonly tank-mixed and co-applied, promoting increased field relevance in risk assessments.

Supporting information

S1 Table. Raw data showing numbers of dead *Aphelinus abdominalis* per cage (thiacloprid vs H_2O).
(DOCX)

S2 Table. Raw data showing numbers of knocked down *Aphelinus abdominalis* per cage (thiacloprid vs H_2O).
(DOCX)

S3 Table. Raw data showing numbers of dead *Aphelinus abdominalis* per cage for all treatments.
(DOCX)

S4 Table. Raw data showing numbers of knocked down *Aphelinus abdominalis* per cage for all treatments.
(DOCX)

Acknowledgments

We thank members of Ghent University's Laboratory of Agrozoology for providing spray tower training and swift insect deliveries for our time-sensitive experiments. We also thank both reviewers for their valuable comments and suggestions.

Author Contributions

Conceptualization: Jonathan Willow.

Data curation: Jonathan Willow.

Formal analysis: Jonathan Willow, Ana Silva.

Funding acquisition: Jonathan Willow, Eve Veromann, Guy Smagghe.

Investigation: Jonathan Willow.

Methodology: Jonathan Willow.

Resources: Guy Smagghe.

Supervision: Eve Veromann, Guy Smagghe.

Validation: Jonathan Willow, Ana Silva, Eve Veromann, Guy Smagghe.

Visualization: Jonathan Willow.

Writing – original draft: Jonathan Willow.

Writing – review & editing: Jonathan Willow, Ana Silva, Eve Veromann, Guy Smagghe.

References

1. Schowalter TD, Noriega JA, Tscharntke T. Insect effects on ecosystem services—Introduction. Basic Appl Ecol. 2018; 26: 1–7.
2. Lashomb J, Ng YS, Jansson RK, Bullock R. *Edovum puttleri* (Hymenoptera: Eulophidae), an Egg Parasitoid of Colorado Potato Beetle (Coleoptera: Chrysomelidae): Development and Parasitism on Eggplant. J Econ Entomol. 1987; 80: 65–8.
3. Zhao JZ, Ayers GS, Grafius EJ, Stehr FW. Effects of Neighboring Nectar-Producing Plants on Populations of Pest Lepidoptera and Their Parasitoids in Broccoli Plantings. 1992; 25: 253–8.
4. Thies C, Tscharntke T. Landscape Structure and Biological Control in Agroecosystems. Science. 1999; 285: 893–5. PMID: 10436158
5. Kruess A, Tscharntke T. Species richness and parasitism in a fragmented landscape: experiments and field studies with insects on *Vicia sepium*. Oecologia. 2000; 122: 129–37. https://doi.org/10.1007/PL00008829 PMID: 28307950
6. Garcia-Marí F, Vercher R, Costa-Comelles J, Marzal C, Villalba M. Establishment of *Citrostichus phyllocnistoides* (Hymenoptera: Eulophidae) as a biological control agent for the citrus leafminer *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) in Spain. Biol Control. 2004; 29:215–26.
7. Veromann E, Williams IH, Kaasik R, Luik A. Potential of parasitoids to control populations of the weevil *Ceutorhynchus obstrictus* (Marsham) on winter oilseed rape. Int J Pest Manag. 2011; 57: 85–92.
8. Kovács G, Kaasik R, Kaart T, Metspalu L, Luik A, Veromann E. In search of secondary plants to enhance the efficiency of cabbage seed weevil management. BioControl. 2017; 62: 29–38.
9. Zumoffen L, Signorini M, Salvo A. Bidirectional movement of aphid parasitoids (Braconidae: Aphidiinae) between crops and non-crop plants in agroecosystems of central Argentina. Appl Entomol Zool. 2018; 53: 1–9.
10. Kovács G, Kaasik R, Lof ME, van der Werf W, Kaart T, Holland JM, et al. Effects of land use on infestation and parasitism rates of cabbage seed weevil in oilseed rape. Pest Manag Sci. 2018. https://doi.org/10.1002/ps.5161 PMID: 30070017
11. Simmonds MSJ, Manlove JD, Blaney WM, Khambay BPS. Effects of selected botanical insecticides on the behaviour and mortality of the glasshouse whitefly *Trialeurodes vaporariorum* and the parasitoid *Encarsia formosa*. Entomol Exp Appl. 2002; 102: 39–47.
12. Desneux N, Decourtye A, Delpuech J-M. The Sublethal Effects of Pesticides on Beneficial Arthropods. Annu Rev Entomol. 2007; 52: 81–106. https://doi.org/10.1146/annurev.ento.52.110405.091440 PMID: 16842032
13. Sugiyama K, Katayama H, Saito T. Effect of insecticides on the mortalities of three whitefly parasitoid species, *Eretmocerus mundus*, *Eretmocerus eremicus* and *Encarsia formosa* (Hymenoptera: Aphelinidae). Appl Entomol Zool. 2011; 46: 311–7.
14. Biondi A, Zappalà L, Stark JD, Desneux N. Do Biopesticides Affect the Demographic Traits of a Parasitoid Wasp and Its Biocontrol Services through Sublethal Effects? PLoS ONE. 2013; 8: e76548. https://doi.org/10.1371/journal.pone.0076548 PMID: 24098793
15. Whitehorn PR, Cook N, Blackburn CV, Gill SM, Green J, Shuker DM. Sex allocation theory reveals a hidden cost of neonicotinoid exposure in a parasitoid wasp. Proc R Soc B Biol Sci. 2015. https://doi.org/10.1098/rspb.2015.0389 PMID: 25925105
16. Cook N, Green J, Shuker DM, Whitehorn PR. Exposure to the neonicotinoid imidacloprid disrupts sex allocation cue use during superparasitism in the parasitoid wasp *Nasonia vitripennis*. Ecol Entomol. 2016; 41: 693–7.

17. Tappert L, Pokorny T, Hofferberth J, Ruther J. Sublethal doses of imidacloprid disrupt sexual communication and host finding in a parasitoid wasp. Sci Rep. 2017. https://doi.org/10.1038/srep42756 PMID: 28198464
18. Francesena N, Desneux N, de Campos MR, Schneider MI. Side effects of spirotetramat on pupae and adults of a Neotropical strain of *Eretmocerus mundus* (Hymenoptera: Aphelinidae): Effects on the life parameters and demography. Environ Sci Pollut Res. 2017; 24: 17719–30.
19. Khan MA, Ruberson JR. Lethal effects of selected novel pesticides on immature stages of *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae). Pest Manag Sci. 2017; 73: 2465–72. https://doi.org/10.1002/ps.4639 PMID: 28600808
20. Wang D, Lü L, He Y. Effects of insecticides on sex pheromone communication and mating behavior in *Trichogramma chilonis*. J Pest Sci. 2018; 91: 65–78.
21. Drobnjaković T, Marčić D, Prijović M, Perić P, Milenković S, Bošković J. Sublethal effects of NeemAzal-T/S botanical insecticide on Dutch and Serbian populations of *Encarsia formosa* (Hymenoptera: Aphelinidae). Biocontrol Sci Technol. 2018; 28: 1–19.
22. Schneider MI, Smagghe G, Gobbi A, Vinuela E. Toxicity and pharmacokinetics of insect growth regulators and other novel insecticides on pupae of *Hyposoter didymator* (Hymenoptera: Ichneumonidae), a parasitoid of early larval instars of lepidopteran pests. J Econ Entomol. 2003; 96: 1054–65. https://doi.org/10.1603/0022-0493-96.4.1054 PMID: 14503575
23. Delpuech J-M, Delahaye M. The sublethal effects of deltamethrin on *Trichogramma* behaviors during the exploitation of host patches. Sci Total Environ. 2013; 447: 274–9. https://doi.org/10.1016/j.scitotenv.2012.12.096 PMID: 23391894
24. Teder T, Knapp M. Sublethal effects enhance detrimental impact of insecticides on non-target organisms: A quantitative synthesis in parasitoids. Chemosphere. 2019; 214: 371–8. https://doi.org/10.1016/j.chemosphere.2018.09.132 PMID: 30267910
25. Pisa L, Goulson D, Yang E-C, Gibbons D, Sánchez-Bayo F, Mitchell E, et al. An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. Part 2: impacts on organisms and ecosystems. Environ Sci Pollut Res. 2017. https://doi.org/10.1007/s11356-017-0341-3 PMID: 29124633
26. Giorio C, Safer A, Sánchez-Bayo F, Tapparo A, Lentola A, Girolami V, et al. An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. Part 1: new molecules, metabolism, fate, and transport. Environ Sci Pollut Res. 2017. https://doi.org/10.1007/s11356-017-0394-3 PMID: 29105037
27. Thompson HM. Interaction between pesticides and other factors in effects on bees. EFSA Support Publ. 2012:EN-340.
28. Põllumajandusamet. PMAIS Avalik Vaade [Internet]. Plant protection products authorized in Estonia. 2017 [cited 2018 Dec 4]. https://portaal.agri.ee/avalik/#/taimekaitse/taimekaitsevahendid-otsing/en
29. Vandame R, Belzunces LP. Joint actions of deltamethrin and azole fungicides on honey bee thermoregulation. Neurosci Lett. 1998; 251: 57–60. PMID: 9714464
30. Zhu W, Schmehl DR, Mullin CA, Frazier JL. Four Common Pesticides, Their Mixtures and a Formulation Solvent in the Hive Environment Have High Oral Toxicity to Honey Bee Larvae. PLoS ONE. 2014; 9: e77547. https://doi.org/10.1371/journal.pone.0077547 PMID: 24416121
31. Zhu YC, Yao J, Adamczyk J, Luttrell R. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). PLOS ONE. 2017; 12: e0176837. https://doi.org/10.1371/journal.pone.0176837 PMID: 28467462
32. Thompson HM, Fryday SL, Harkin S, Milner S. Potential impacts of synergism in honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops. Apidologie. 2014; 45: 545–53.
33. Sgolastra F, Medrzycki P, Bortolotti L, Renzi MT, Tosi S, Bogo G, et al. Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. Pest Manag Sci. 2017; 73: 1236–43. https://doi.org/10.1002/ps.4449 PMID: 27685544
34. Sgolastra F, Arnan X, Cabbri R, Isani G, Medrzycki P, Teper D, et al. Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee. Proc R Soc B. 2018. https://doi.org/10.1098/rspb.2018.0887 PMID: 30135154
35. Raimets R, Karise R, Mänd M, Kaart T, Ponting S, Song J, et al. Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.). Pest Manag Sci. 2018; 74: 541–6. https://doi.org/10.1002/ps.4756 PMID: 28991419
36. Johnson RM, Wen Z, Schuler MA, Berenbaum MR. Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. J Econ Entomol. 2006; 99: 1046–50. https://doi.org/10.1603/0022-0493-99.4.1046 PMID: 16937654
37. Noyes J. Universal Chalcidoidea Database; 2018 [cited 2018 Oct 9]. http://www.nhm.ac.uk/our-science/data/chalcidoids/database

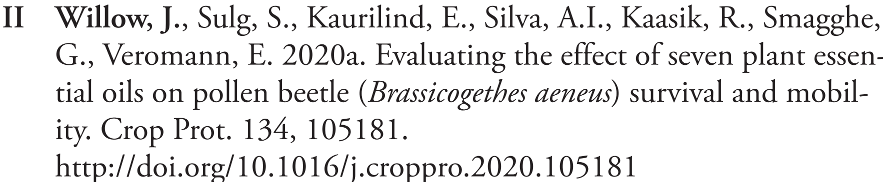
38. van de Veire M, Smagghe G, Degheele D. Laboratory test method to evaluate the effect of 31 pesticides on the predatory bug, *Orius laevigatus* (Het: Anthocoridae). Entomophaga. 1996 Jun 1; 41(2):235–43.

39. R: The R Project for Statistical Computing; 2018 [cited 2018 Nov 5]. https://www.r-project.org

40. Jans D. Toxicity to the parasitoid wasp Aphidius rhopalosiphi (DeStephani-Perez) (Hymenoptera: Braconidae) using an extended laboratory test with aged residues on maize—thiacloprid OD 240 g/L—final report. European Commission. Renewal Assessment Report prepared according to the Commission Regulation (EU) N˚ 1107/2009. Vol 3 – B.9 (PPP)–Thiacloprid OD 240 (240 g/L. 2017: 262–6. https://www.efsa.europa.eu/en/consultations/call/180123

41. Barmentlo SH, Schrama M, Hunting ER, Heutink R, van Bodegom PM, de Snoo GR, et al. Assessing combined impacts of agrochemicals: Aquatic macroinvertebrate population responses in outdoor mesocosms. Sci Total Environ. 2018; 631–632: 341–7. https://doi.org/10.1016/j.scitotenv.2018.03.021 PMID: 29525713

II



II **Willow, J.**, Sulg, S., Kaurilind, E., Silva, A.I., Kaasik, R., Smagghe, G., Veromann, E. 2020a. Evaluating the effect of seven plant essential oils on pollen beetle (*Brassicogethes aeneus*) survival and mobility. Crop Prot. 134, 105181.
http://doi.org/10.1016/j.croppro.2020.105181

Crop Protection 134 (2020) 105181

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Evaluating the effect of seven plant essential oils on pollen beetle (*Brassicogethes aeneus*) survival and mobility

Jonathan Willow[a,f,*], Silva Sulg[a], Eve Kaurilind[b], Ana Isabel Silva[c,d,e], Riina Kaasik[a], Guy Smagghe[f,1], Eve Veromann[a,**,1]

[a] *Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu, Estonia*
[b] *Chair of Crop Science and Plant Biology, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu, Estonia*
[c] *School of Mental Health and Neuroscience, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, Netherlands*
[d] *Cardiff University Brain Imaging Research Centre (CUBRIC), School of Psychology, Cardiff University, Cardiff, United Kingdom*
[e] *Neuroscience and Mental Health Research Institute, MRC Centre for Neuropsychiatric Genetics and Genomics, Hadyn Ellis Building, Cathays, Cardiff, United Kingdom*
[f] *Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium*

ARTICLE INFO

Keywords:
Insect
Coleoptera
Meligethes
Biopesticide
Brassica napus
Bioassay

ABSTRACT

Plant essential oils (EOs) represent an area of interest for managing agricultural pests. We examined the insecticidal efficacy of seven plant EOs on the survival and mobility of the pollen beetle *Brassicogethes aeneus*, a primary pest of oilseed rape (*Brassica napus*) in Europe. Topical dosing tests showed the EO of the inner bark of *Cinnamomum verum* (cinnamon) to be the most effective EO examined in our study. Subsequent bioassays, exposing *B. aeneus* to oilseed rape plant surfaces sprayed with various concentrations of *C. verum* EO, additionally suggested a concentration threshold at which this EO may significantly control *B. aeneus* populations. We suggest that further studies on *B. aeneus* examine the effect of certain pure compounds associated with the most promising EOs (individually as well as in binary combinations) in order to reveal optimal chemical composition and ratios to exploit within a *B. aeneus* management framework.

1. Introduction

In Europe, the pollen beetle *Brassicogethes aeneus* Fabricus (Coleoptera: Nitidulidae; syn. *Meligethes aeneus*) is a primary pest of oilseed rape (*Brassica napus* L.), an important crop cultivated for food products, animal feed and biodiesel. Overwintered *B. aeneus* adults feed on the pollen of blooming plants belonging to a variety of families (e.g. Roseaceae, Asteraceae, Laminaceae, etc.). After maturation feeding (feeding required for egg production), they colonise cruciferous (Brassicaceae) plants, where they feed on pollen within buds and flowers, mate, and deposit eggs into buds. Upon hatching, larvae feed on pollen within buds, and eventually within open flowers, followed by pupation under the soil surface below the plant (see review by Mauchline et al. (2018)).

B. aeneus has evolved natural enemies, primarily hymenopteran parasitoids and coleopteran predators, that have been shown to be effective suppressors of *B. aeneus* populations (Thies and Tscharntke, 1999; Büchi, 2002; Riggi et al., 2017; Kaasik et al., 2014; Skellern and Cook, 2018). However, large-scale destruction and fragmentation of natural and seminatural habitats associated with large-scale conventional agriculture have resulted in a scenario where natural enemies of *B. aeneus* are often unable to deliver the biocontrol service effectively enough to control *B. aeneus* populations. Thus, synthetic insecticides are the standard method of *B. aeneus* control. Due to the routine use of pyrethroid insecticides, *B. aeneus* has developed resistance to pyrethroids in several areas throughout Europe (Heimbach and Müller, 2013; Zamojska, 2017; Kaiser et al., 2018; Stará and Kocourek, 2018; Zimmer et al., 2014). In addition to pyrethroids, neonicotinoid insecticides are also being applied in oilseed rape production (Kaiser et al., 2018; Seidenglanz et al., 2017). Recent field and greenhouse experiments using the neonicotinoid thiacloprid demonstrated the efficacy of its use in managing *B. aeneus* infestations (Brandes et al., 2018a, 2018b). However, exposure to several compounds within this class of insecticides has shown negative effects on a broad spectrum of nontarget organisms, from vertebrates such as fishes, amphibians and mammals

* Corresponding author. Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu, Estonia.
** Corresponding author.
E-mail addresses: jonathan@emu.ee (J. Willow), eve.veromann@emu.ee (E. Veromann).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.cropro.2020.105181
Received 8 January 2020; Received in revised form 8 April 2020; Accepted 12 April 2020
Available online 16 April 2020
0261-2194/© 2020 Elsevier Ltd. All rights reserved.

(Gibbons et al., 2015; Berheim et al., 2019; Wu et al., 2019), to economically beneficial pollinators and biological control agents (Pisa et al., 2017; Calvo-Agudo et al., 2019; Willow et al., 2019). Resistance of *B. aeneus* to certain insecticides, along with the widespread call for developing effective pest control strategies that minimise the impact on nontarget organisms, suggests a severe need for effective and ecologically-sustainable techniques for controlling *B. aeneus* populations.

The use of botanical essential oils (EOs) for the management of agricultural pest insects is both historically and currently an avenue of great interest (Isman, 2006; Isman and Grieneisen, 2014). Several studies have examined the potential of EOs as an insecticide or repellent against *B. aeneus*. EO of *Lavendula angustifolia* Mill. (lavender, Lamiaceae) has shown variable repellent- and lethal efficacies against *B. aeneus* (Pavela, 2011; Mauchline et al., 2005, 2013; Dorn et al., 2014). The lack of information regarding chemical constituents of products used in some studies with EOs makes comparisons among studies difficult. However, an electroantennography experiment examining the EO of *L. angustifolia* revealed that the compounds linalool and linalyl acetate were those primarily involved the repellent effect on *B. aeneus* (Mauchline et al., 2008). Regarding lethal effects of EOs on *B. aeneus*, the work of Pavela (2011) suggested *Carum* carvi L. (caraway, Apiaceae), *Thymus vulgaris* L. (thyme, Lamiaceae) and *Foeniculum vulgare* Mill. (fennel, Apiaceae) as being the most insecticidal EOs so far examined against *B. aeneus*. An additional study has looked at the potential of using different EOs against another genus of nitidulid beetles, *Carpophilus* spp (Comelli et al., 2018). These authors observed *Pimpinella anisum* L. (anise, Apiaceae), *Cuminum cyminum* L. (cumin, Apiaceae) and *Aloysia polystachya* (Griseb.) Moldenke (bee-brush, Verbenaceae) as being the most insecticidal EOs examined against this genus of nitidulids. Overall, these previous works provide valuable insight into the insecticidal potential of certain plant EOs against *B. aeneus*. However, further studies using additional application methods, as well as examining both new and previously examined EOs, for corroborating evidence, are necessary in order to confirm the best-choice EOs for potential application in a *B. aeneus* management framework.

The aim of the present study was to examine the insecticidal effect of seven different plant EOs against *B. aeneus*. We included three EOs previously shown to be effective against *B. aeneus* (*C. carvi*, *T. vulgaris* and *F. vulgare*) (Pavela, 2011), and four EOs not previously examined for their insecticidal effect on *B. aeneus*, two of which were shown to be effective against *Carpophilus* spp (*P. anisum* and *C. cyminum*) (Comelli et al., 2018), the other two being *Cinnamomum verum* J. Presl. (cinnamon, Lauraceae) and *Cannabis sativa* L. (hemp, Cannabaceae), both of which have not been studied for their effects on any nitidulid. Specifically, we first applied each EO topically, expecting insecticidal activity in *B. aeneus* to respond differently for the different EOs examined. We then chose the most effective EO to continue with further tests using exposure to the treated oilseed rape plant surface as the method of exposure, expecting to find an effective concentration threshold for significant insecticidal efficacy.

2. Materials and methods

2.1. Essential oil products

Pure EOs of *T. vulgaris*, *P. anisum*, *F. vulgare*, *C. cyminum*, *C. verum*, *C. carvi* and *C. sativa* were ordered from the company Talia (Rome, Italy; www.taliaessenze.com), shipped at ambient temperature, and subsequently kept at 5 ± 1 °C once received. Details regarding origin of plants, plant parts used and extraction method are shown in Table 1. To identify the major relevant compounds present in each EO, gas chromatography—mass spectrometry (GC-MS) was performed on each EO (Shimadzu, 2010 Plus GC-MS system, Kyoto, Japan). For that, 1 μL of each EO was pipetted into an empty 500 mL glass flask, and headspace was collected using multibed adsorbent cartridges filled with Carbopack (PerkinElmer) for 10 s at 200 mL/min. Volatile compounds were identified based on mass spectrum (OpenChrom), using NIST 14 Mass Spectral Library. An overview of major compounds detected in each EO is shown in Table 1.

2.2. Insects

Pollen beetles were collected from an untreated oilseed rape field in the village of Kandiküla, Tartu County, Estonia. The beetles were kept in ventilated plastic containers and allowed to feed *ad libitum* on pollen of oilseed rape- and dandelion (*Taraxacum* spp.) flowers. Oilseed rape flowers were picked from the same untreated field where pollen beetles were collected, and dandelion flowers were picked from wildflower areas surrounding the Estonian University of Life Sciences. All individual pollen beetles used in the study were identified as *B. aeneus*, using an identification guide by Kirk-Spriggs (1996), prior to their addition to the study.

2.3. Screening EOs via topical dosing

Twenty groups of ten randomly chosen *B. aeneus* were weighed (Sartorius Lab Instruments, Göttingen, Germany) in plastic vials in order to obtain an average weight per individual beetle. Based on their average weight (1.4 ± 0.1 mg), all seven EOs at five doses each (3.65, 5.48, 7.31, 9.14 and 18.27 nL/mg insect; 7 EOs x 5 doses = 35 EO treatments) were topically applied to 40 random individuals per EO treatment (ten beetles per treatment, replicated four times). These treatments consisted of 0.05% Tween80 and varying percentages of EO and acetone together constituting 4 mL of solution per treatment, each prepared in a glass vial immediately before application. Corresponding to the five doses applied, the five concentrations of [acetone + Tween80 + EO] solution prepared for each EO were 1, 1.5, 2, 2.5 and 5% EO. The control treatment consisted of 0.05% Tween80 and 99.95% acetone, and was applied to 200 randomly chosen individuals (50 beetles replicated four times). Overview of dates and cages corresponding to each experimental replication, as well as the corresponding raw data, is shown in SI Table 1.

Randomly chosen and fast moving (used as a proxy for healthy

Table 1
Plant species from which essential oils (EO) were used in *Brassicogethes aeneus* bioassays; includes country of cultivation, plant parts used in EO extraction, extraction method, and major relevant compounds detected.

Plant species	Plant origin	Parts used	Extraction method	Major compounds detected
Thymus vulgaris	Portugal	leaf, flower	steam distilled	o-cymene (61%), α-pinene (15%), ç-terpinene (6%), camphene (4%).
Pimpinella anisum	Spain	seed	steam distilled	anethole (52%), D-limonene (21%), estragole (8%), o-cymene (5%).
Foeniculum vulgare	Hungary	seed	cold pressed	α-pinene (31%), anethole (22%), D-limonene (20%), L-fenchone (18%).
Cuminum cyminum	Morocco	seed	steam distilled	α-pinene (29%), o-cymene (26%), cuminaldehyde (25%), ç-terpinene (14%).
Cinnamomum verum	Sri Lanka	inner bark	steam distilled	(E)- cinnamaldehyde (46%), caryophyllene (15%), linalool (12%), D-limonene (8%).
Carum carvi	Hungary	seed	steam distilled	carvone (37%), D-limonene (37%), α-myrcene (6%), dihydrocarvone (5%).
Cannabis sativa	Italy	leaf, flower	steam distilled	α-myrcene (45%), α-pinene (38%), D-limonene (5%), α-ocimene (3%).

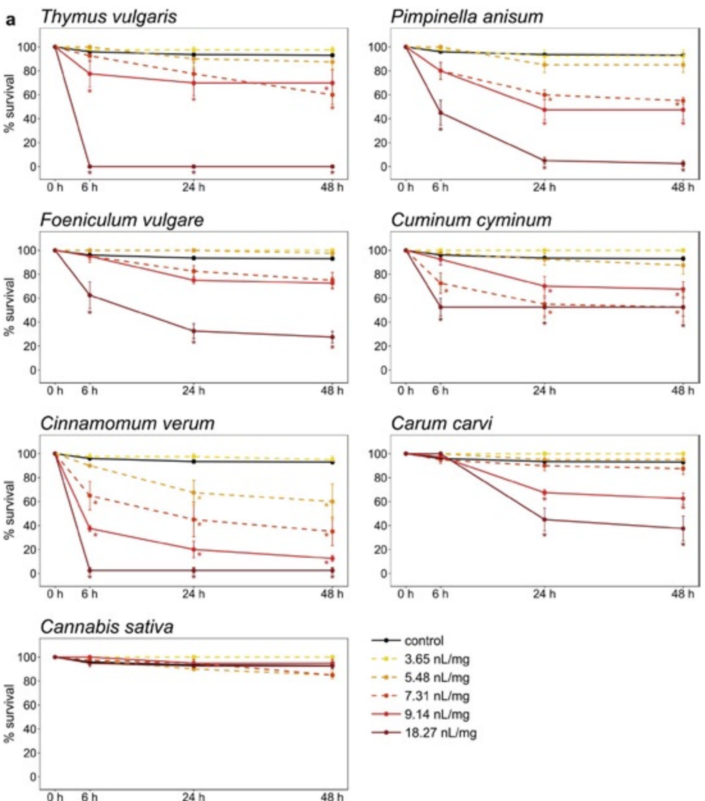


Fig. 1. Effect of seven essential oils at different doses on *Brassicogethes aeneus* survival (a) and mobility (b), via direct dosing of individual beetles. n = 40 (four replications of ten beetles) per essential oil treatment; n = 200 (four replications of 50 beetles) for control treatment. Each treatment was compared to the control treatment using Fisher's exact test (error bars: ±SE). * = $p < 0.00046$ (Bonferroni correction threshold).

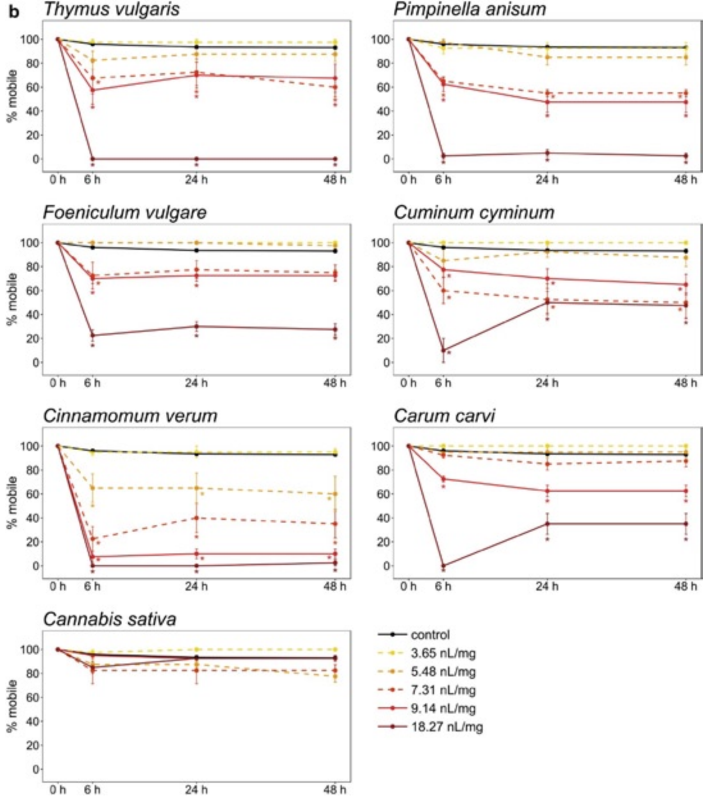


Fig. 1. (*continued*).

specimens) beetles were anaesthetised with diethyl ether for 2 min. Treatment solutions were applied at 0.5 µL, via micropipette (HTL Lab Solutions) with microloader (Eppendorf), over the dorsal pronotum of individual beetles. Treatments were applied under a stereomicroscope (Leica, Taiwan) to ensure precise application. Release of treatment from the microloader occurred via capillary action, and required gently touching- and removing the microloader tip to and from the surface of the anaesthetised beetle several times. Treatment solutions diffused over surrounding body surfaces, but care was taken to allow acetone to completely evaporate from the body surface between applications, ensuring application of the entire dose, without loss of treatment onto the surrounding Petri dish.

Treated beetles were placed into transparent, polystyrene, ventilated insect breeding dishes (diameter 10 cm x height 4 cm) (SPL Life Sciences, Gyeonggi-do, South Korea), hereafter referred to as cages, in groups of 10 beetles per cage. Cages of treated beetles were kept in a growth chamber (Sanyo MLR-351H, Japan) at 20 ± 2 °C, 60% RH and 16:8 h light:dark regime. They were allowed to feed *ad libitum* on pollen of dandelion flowers, and were provisioned with a moist cotton ball for access to drinking water. Fresh food and water were provided after 24 h, and treated beetles were monitored for mobility and survival at 6, 24 and 48 h. Immobility was determined through observing erratic walking behaviour, inability to stay dorsal-side-up, or an inability to walk at all, and included apparent mortality. Mortality was determined through observing no movement of the insect, even when placed in the warm palm of hand and having warm air exhaled over the insect (a way to examine whether a pollen beetle is playing dead or not). Differences between EO treatments ($n = 40$) and control ($n = 200$), regarding both mobility and survival, were statistically assessed using Fisher's exact test in R software v1.1.463. In order to account for multiple comparisons, we used Bonferroni corrections, where different concentrations of essential oils (with control, total of 36 treatments) as well as different time points (3 time points) were taken into account, setting the significance threshold at $p = 0.00046$ (0.05/(3*36)).

2.4. Leaf and bud treatment

Based on our results from topical dosing of EOs (*C. verum* showing

the most promising insecticidal effect on *B. aeneus*), we examined the effect of treating oilseed rape leaf and bud surfaces with EO of *C. verum*. Six concentrations were examined: 1.3, 1.4, 1.5, 1.6, 1.7 and 1.8% EO of *C. verum*. Treatments were diluted in acetone and contained 0.05% Tween80. A control treatment consisted of acetone and 0.05% Tween80. For each treatment, five oilseed rape leaves, each approximately 14.5 × 14.5 cm, were laid down next to each other, and 15 mL of treatment solution was sprayed as mist over the five leaves. Treated leaves were allowed to sit in open air for 1 h before they were added to the cages (described above). One leaf was pressed against the interior bottom and sides of each cage, and used as a treated surface. In addition, four oilseed rape flower buds dipped in the respective [acetone + Tween80 + EO] concentration were inserted by the petiole into small moist cotton balls, allowed to air dry for 1 h, and added to each cage, on top of the leaf, along with four uncontaminated oilseed rape flowers as a food source. Eight beetles were introduced to each cage, and cages were placed in the growth chamber at 20 °C, 60% RH and 16:8 h light:dark. Mobility and survival were monitored at 24 h, and determined as described in the previous section. The experiment was replicated two more times, making 15 cages per treatment (in total 120 specimens per treatment). Overview of cages corresponding to each experimental replication, as well as the corresponding raw data, is shown in SI Table 2. Homogeneity of variance and normality of data distributions were examined using the Levene- and Shapiro-Wilk tests, respectively. Given that only higher concentrations were normally distributed, we used the nonparametric Kruskal-Wallis test as an alternative to ANOVA, followed by Bonferroni-Dunn's test for *post-hoc* pairwise comparisons. All statistical analyses were performed in R software v1.1.463.

3. Results

3.1. Screening EOs via topical dosing

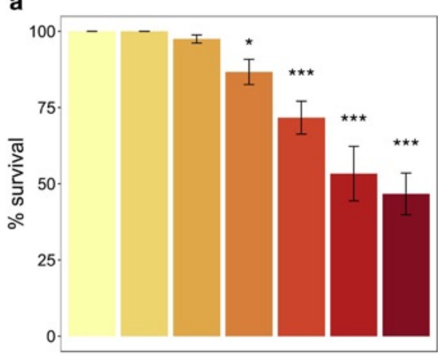
Our study revealed that at 5.48 nL/mg, *C. verum* was the only EO that significantly lowered both survival ($p < 0.0001$ at 24 and 48 h) and mobility ($p < 0.0001$ at 6, 24 and 48 h) of *B. aeneus* (Fig. 1a and b; SI Table 3). Application of *C. verum* at 5.48 nL/mg resulted in 90%, 67.5% and 60% survival at 6, 24 and 48 h post-application, respectively; the only difference between survival and mobility was an initial drop to 67.5% mobility after 6 h. An increase in the effect of *C. verum* was observed, regarding both survival (at 48 h: 35%, 12.5% and 2.5%, for 7.31 nL/mg, 9.14 nL/mg and 18.27 nL/mg respectively) and mobility (at 48 h: 35% and 10%, for 7.31 nL/mg and 9.14 nL/mg, respectively) of *B. aeneus*, with each increase in dose of this EO.

Four EOs began to show a significant effect on survival at 7.31 nL/mg, with this effect generally increasing with increased doses. These include *T. vulgaris* (60% survival, $p < 0.0001$ at 48 h), *P. anisum* (60% and 55%, $p < 0.0001$ at 24 and 48 h, respectively) and *C. cyminum* (72.5%, 55% and 52.5%, $p < 0.0001$ at 6, 24 and 48 h, respectively). These same EOs also began to show a significant effect on mobility during each time point at this dose (67.5%, 72.5% and 60% mobile, $p < 0.0001$ for *T. vulgaris* at 6, 24 and 48 h, respectively; 65%, 57.5% and 55%, $p < 0.0001$ for *P. anisum* at 6, 24 and 48 h, respectively; 60%, 52.5% and 50%, $p < 0.0001$ for *C. cyminum* at 6, 24 and 48 h, respectively; and 72.5%, $p < 0.0001$ for *F. vulgare* at 6 h).

C. carvi began to significantly affect ($p < 0.0001$) survival (67.5% and 62.5% at 24 and 48 h, respectively) and mobility (72.5%, 62.5% and 62.5% at 6, 24 and 48 h, respectively) at the 9.14 nL/mg dose, the effect increasing with the highest dose. No significant effect on survival or mobility, at any time point, was observed with *C. sativa*.

3.2. Leaf and bud treatment

Significant effects on both survival ($X^2 = 72.146$, df = 6, $p < 0.0001$) and mobility ($X^2 = 76.48$, $p < 0.0001$), compared to the control treatment, were observed when the oilseed rape leaf and bud surfaces were treated with *C. verum* EO. *Post-hoc* tests for each concentration showed a significant effect at concentrations of 1.5% (86.7% survival, $p = 0.04$; 82.5% mobile, $p = 0.01$) and higher (71.7% survival, $p = 0.0001$, and 68.3% mobile, $p < 0.0001$, for 1.6% concentration treatment; 53.3% survival and 49.1% mobile for 1.7% concentration treatment, $p < 0.0001$; and 46.7% survival and 40.8% mobile for 1.8% treatment, $p < 0.0001$) (Fig. 2a and b). At 1.4% concentration, 97.5% survival and mobility ($p > 0.05$) was observed. In both the 1.3% concentration and



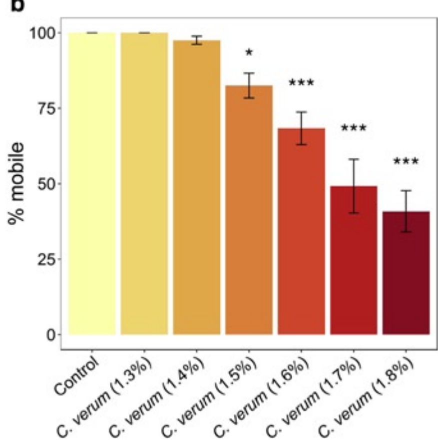


Fig. 2. Effect of *Cinnamomum verum* inner bark essential oil at different doses on *Brassicogethes aeneus* survival (a) and mobility (b), at 24 h post-exposure, via exposure to treated oilseed rape leaves and buds in cage bioassays. n = 15 (eight beetles per cage) per treatment. Effects of treatments were compared to that of the control, using Kruskal-Wallis test, followed by Bonferroni-Dunn's test for *post-hoc* pairwise comparisons. (Error bars: ±SEM). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

the control treatment, there was 100% survival and mobility.

4. Discussion

The present study showed that, of the EOs examined for topical contact toxicity, application of *C. verum* resulted in the greatest reduction of survival and mobility at lower doses, and therefore represented the most effective EO tested here for targeting *B. aeneus*. GC-MS showed that the primary active compounds in our *C. verum* EO were 46% (E)-cinnamaldehyde, followed by caryophyllene (15%), linalool (12%) and D-limonene (8%). A study using the rice weevil *Sitophilus oryzae* L. (Coleoptera: Curculionidae) showed that, from all the compounds examined for acetylcholinesterase (AChE) inhibition, *trans*-cinnamaldehyde was among the most potent (Saad et al., 2018). Regarding *B. aeneus*, Pavela (2011) observed a relatively intermediate insecticidal effect of the EO derived from the leaves of *Cinnamomum osmophloeum* Kaneh., whereas we used EO derived from the inner bark of *C. verum*. GC-MS of *C. osmophloeum* leaf EO in Pavela (2011) showed 89.7% cinnamaldehyde, followed by β-cubebine (5.7%) and linalool (4.2%). Another study examining the effect of *C. verum* (though referred to by its synonym *C. zeylanicum*) EO on the cowpea weevil *Callosobruchus maculatus* Fabricus (Coleoptera: Chrysomelidae) showed significant impacts on rate of population increase, percent crop loss, adult emergence, and number of eggs laid (Jumbo et al., 2018). However, their GC-MS results showed only 2.3% cinnamaldehyde, where the primary compound present was eugenol (73.1%), followed by caryophyllene (7.7%), acetyleugenol (3.6%), benzyl benzoate (3.4%), linalool (2.6%) and cinnamyl acetate (2.5%). *C. verum* showed the greatest toxicity of all the EOs examined, via direct dosing of *B. aeneus*, in the present study, and results of leaf/bud treatment assays showed that *C. verum* EO significantly affected *B. aeneus* survival and mobility at sprayed concentrations as low as 1.6%. This threshold is rather high for practical field application; however, the threshold would likely vary with different *C. verum* EO products.

Differences in EO composition, between products derived from the same plant species or genera used across *B. aeneus* (and related nitidulid) toxicology studies, make it difficult to compare findings. For example, of nine EOs examined, Pavela (2011) observed *C. carvi* to be the most effective, yet this EO was among the least effective in our study. Pavela's GC-MS results showed a carvone:limonene ratio of almost 2:1, while our carvone:limonene ratio was 1:1. This difference in compound ratio may in part explain the difference in efficacy of *C. carvi* between these two studies if carvone acts as a highly insecticidal compound against *B. aeneus*.

There are other examples where EO composition confounds comparisons between *B. aeneus* studies. EOs of *T. vulgaris* and *F. vulgare* were the second and third most effective in the Pavela (2011) study (LC_{50} of *T. vulgaris* and *F. vulgare* EOs at 24 h was 250 μg/cm and 346 μg/cm, respectively), yet in the present study these were only effective at high doses. Regarding *T. vulgaris*, our GC-MS results strongly differed from those of Pavela (2011), in that Pavela detected 78.5% thymol content in his *T. vulgaris* EO, followed by ρ-cymene (12.7%) and γ-terpinene (5.3%). We detected no thymol in our *T. vulgaris* EO, but rather 61% o-cymene as the primary compound in our *T. vulgaris* EO, followed by α-pinene (15%), ç-terpinene (6%) and camphene (4%). Similarly, Pavela (2011) detected *trans*-anethole (65.8%) as the primary compound in his *F. vulgare* EO, followed by fenchone (20.3%), methyl cavicol (5.8%) and limonene (4.3%). While our *F. vulgare* EO contained a comparable percentage of fenchone, only 22% was anethole, and the most abundant compound in our *F. vulgare* EO was α-pinene (31%); D-limonene (20%) was also detected in our sample. Altogether, this represents a major difference between the products tested in these two studies, concerning both *T. vulgaris* and *F. vulgare*, which likely resulted in different relative toxicity levels between the two studies.

Comelli et al. (2018) observed that the EOs of *P. anisum* and *C. cyminum* were both highly toxic to *Carpophilus* spp, a member of the same family as *B. aeneus* (Nitidulidae), at concentrations as low as 0.2 μL/cm. Low doses of *P. anisum* and *C. cyminum* EO directly applied to *B. aeneus* did not show comparable effects in our study. This may again be due to differences in chemical composition between the *P. anisum* and *C. cyminum* EOs used in these two studies, or due to differences between these species regarding their biological responses to the EOs examined. The *P. anisum* EO used by Comelli et al. (2018) consisted of 94.17% *trans*-anethole, whereas the *P. anisum* EO used in the present study consisted of 52% anethole, followed by D-limonene (21%), estragole (8%) and o-cymene (5%). The *C. cyminum* EO used by Comelli et al. (2018) consisted of 28.91% cuminaldehyde, followed by γ-terpinene (21.64%), p-cymene (18.12%) and α-terpinen-7-al (18.01%), whereas the *C. cyminum* EO used in our study consisted of 29% α-pinene, followed by o-cymene (26%), cuminaldehyde (25%) and ç-terpinene (14%). As with the *T. vulgaris* and *F. vulgare* EO compositional differences mentioned above, between our study and Pavela (2011), the *P. anisum* and *C. cyminum* EOs examined in the present study had major differences in chemical composition compared to the EOs examined by Comelli et al. (2018).

Due to these intraspecific differences in EO composition making comparisons between studies difficult, future studies should examine the effects of individual compounds, with various modes of action, associated with observations of low survival rates (i.e. carvone, thymol, anethole, cinnamaldehyde), as well as binary combinations of these compounds. Indeed, studies on the noctuid lepidopterans *Spodoptera* spp. (Hummelbrunner and Isman, 2001; Pavela, 2014) and *Trichoplusia ni* Hübner (Tak and Isman, 2015, 2017), as well as *Culex* spp. (Diptera: Culicidae) (Pavela, 2015; Benelli et al., 2017; Youssefi et al., 2019), have shown synergistic toxicity of compounds found in plant EOs.

It also remains important to examine the EOs of species currently unexamined against *B. aeneus*. Studies on insects closely-related to *B. aeneus* (Comelli et al., 2018) may also help guide choices of plant species to examine. The choice of cultivar clearly matters; and if EOs, rather than pure compounds, are to be used in future studies, cultivars should be chosen based on optimum chemical composition regarding insecticidal action against *B. aeneus*. Furthermore, when possible, insecticidal action should be examined using additional endpoints relevant to pest management, rather than survival only.

We analysed mobility of *B. aeneus*, a simple and highly relevant endpoint to examine, as immobility of an insect under natural conditions will dramatically reduce its chances of survival. Furthermore, there may be potential strategies of integrated *B. aeneus* management in exploiting compounds of such toxicities that significantly immobilise *B. aeneus* yet show an insignificant effect on its natural enemies. Indeed, behavioural tests by Cook et al. (2007) suggest that two primary parasitoids of *B. aeneus* are not repelled by compounds in EO of *L. angustifolia*, and thus this EO is not expected to interrupt host-finding habitat for these parasitoids. However, intraspecific differences in the chemical makeup of *L. angustifolia* EO likely exist between cultivars, and while the two compounds linalool and linalyl acetate have been indicated as the primary compounds involved in *L. angustifolia*'s repellent effect on *B. aeneus* (Mauchline et al., 2008), it is still unknown as to whether there are optimal ratios or blends of active compounds that could optimise this repellent effect. This avenue of inquiry could be of great benefit to the development of a sustainable framework for integrated *B. aeneus* management.

Future studies examining the potential for implementing EO applications within integrated *B. aeneus* management should consider the potential phytotoxic effect of insecticidal compounds, and thus should examine the phytotoxicity that any promising compound imparts on the development and physiology of oilseed rape plants. Indeed, EOs and their chemical constituents can be phytotoxic, depending on the EO or chemical compound being used, as well as the plant species being treated (e.g. (Smeriglio et al., 2019; Rolli et al., 2014; Ibáñez and Blázquez, 2018)). Finally, nontarget organisms should be included in biosafety tests (e.g. (Toledo et al., 2020)), especially representatives of

taxa that are directly involved in *B. aeneus* control. This can reveal which EOs or compounds are more selective for *B. aeneus*.

5. Conclusions

C. verum EO was the most promising EO examined in this study when compared with six other plant EOs via topical application to *B. aeneus* adults. Leaf/bud treatment assays suggest a rather high concentration threshold for practical application against *B. aeneus* populations. However, our work also suggests the presence of a compound, of potential relevance for *B. aeneus* control, found in *C. verum* EO. We suggest that future work should examine pure compounds that have been implicated in, or otherwise show potential for, insecticidal or repellent activity against *B. aeneus* and/or other nitidulids. Examining binary combinations of compounds in various ratios may allow the isolation of the most promising active ingredient composition for application within a *B. aeneus* management framework.

Funding

This study was partially funded by the Institutional Research Funding [project no. IUT36-2 of Estonian Research Council], the Special Research Fund (BOF) of Ghent University, Research Foundation-Flanders (FWO-Vlaanderen), the ERA-Net C-IPM project IPM4Meligethes (project no. 33), and the European Regional Development Fund (Center of Excellence EcolChange [project 2014-2020.4.01.15–0002]). JW received a PhD grant from European Social Fund Doctoral Students, as well as the Internalisation Programme DoRa (carried out by Archimedes Foundation). Eve Kaurilind is a recipient of a research grant from the European Regional Development Fund (Center of Excellence EcolChange). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Jonathan Willow: Methodology, Formal analysis, Visualization, Writing - original draft. **Silva Sulg:** Methodology. **Eve Kaurilind:** Methodology. **Ana Isabel Silva:** Formal analysis, Visualization. **Riina Kaasik:** Methodology. **Guy Smagghe:** Formal analysis. **Eve Veromann:** Methodology, Formal analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2020.105181.

References

Benelli, G., Pavela, R., Iannarelli, R., Petrelli, R., Cappellacci, L., Cianfaglione, K., et al., 2017. Synergized mixtures of Apiaceae essential oils and related plant-borne compounds: larvicidal effectiveness on the filariasis vector *Culex quinquefasciatus* Say. Ind. Crop. Prod. 96, 186–195. https://doi.org/10.1016/j.indcrop.2016.11.059.

Berheim, E.H., Jenks, J.A., Lundgren, J.G., Michel, E.S., Grove, D., Jensen, W.F., 2019. Effects of neonicotinoid insecticides on physiology and reproductive characteristics of captive female and fawn white-tailed deer. Sci. Rep. 9, 1–10. https://doi.org/10.1038/s41598-019-40994-9.

Brandes, M., Heimbach, U., Ulber, B., 2018a. Effects of thiacloprid, tau-fluvalinate and lambda-cyhalothrin on overwintered pollen beetles (*Brassicogethes aeneus* (Fabricius)) and their offspring in oilseed rape. Arthropod-Plant Interact. 12, 823–833. https://doi.org/10.1007/s11829-018-9621-1.

Brandes, M., Heimbach, U., Ulber, B., 2018b. Impact of insecticides on oilseed rape bud infestation with eggs and larvae of pollen beetle (*Brassicogethes aeneus* (Fabricius)). Arthropod-Plant Interact. 12, 811–821. https://doi.org/10.1007/s11829-018-9616-y.

Büchi, R., 2002. Mortality of pollen beetle (*Meligethes* spp.) larvae due to predators and parasitoids in rape fields and the effect of conservation strips. Agric. Ecosyst. Environ. 90, 255–263. https://doi.org/10.1016/S0167-8809(01)00213-4.

Calvo-Agudo, M., González-Cabrera, J., Picó, Y., Calatayud-Vernich, P., Urbaneja, A., Dicke, M., et al., 2019. Neonicotinoids in excretion product of phloem-feeding insects kill beneficial insects. Proc. Natl. Acad. Sci. Unit. States Am. 116, 16817–16822. https://doi.org/10.1073/pnas.1904298116.

Comelli, N.C., Romero, O.E., Diez, P.A., Marinho, C.F., Schliserman, P., Carrizo, A., et al., 2018. QSAR study of biologically active essential oils against beetles infesting the walnut in catamarca, Argentina. J. Agric. Food Chem. 66, 12855–12865. https://doi.org/10.1021/acs.jafc.8b04161.

Cook, S.M., Jönsson, M., Skellern, M.P., Murray, D.A., Anderson, P., Powell, W., 2007. Responses of *Phradis* parasitoids to volatiles of lavender, *Lavendula angustifolia*—a possible repellent for their host. *Meligethes aeneus*. BioControl. 52, 591–598. https://doi.org/10.1007/s10526-006-9057-x.

Dorn, B., Jossi, W., Humphrys, C., Hiltbrunner, J., 2014. Screening of natural products in the laboratory and the field for control of pollen beetles. J. Appl. Entomol. 138, 109–119. https://doi.org/10.1111/jen.12086.

Gibbons, D., Morrissey, C., Mineau, P., 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environ. Sci. Pollut. Res. 22, 103–118. https://doi.org/10.1007/s11356-014-3180-5.

Heimbach, U., Müller, A., 2013. Incidence of pyrethroid-resistant oilseed rape pests in Germany. Pest Manag. Sci. 69, 209–216. https://doi.org/10.1002/ps.3351.

Hummelbrunner, L.A., Isman, M.B., 2001. Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (lep., noctuidae). J. Agric. Food Chem. 49, 715–720. https://doi.org/10.1021/jf000749t.

Ibáñez, M.D., Blázquez, M.A., 2018. Phytotoxicity of essential oils on selected weeds: potential hazard on food crops. Plants 7, 79. https://doi.org/10.3390/plants7040079.

Isman, M.B., 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu. Rev. Entomol. 51, 45–66. https://doi.org/10.1146/annurev.ento.51.110104.151146.

Isman, M.B., Grieneisen, M.L., 2014. Botanical insecticide research: many publications, limited useful data. Trends Plant Sci. 19, 140–145. https://doi.org/10.1016/j.tplants.2013.11.005.

Jumbo, L.O.V., Haddi, K., Faroni, L.R.D., Heleno, F.F., Pinto, F.G., Oliveira, E.E., 2018. Toxicity to, oviposition and population growth impairments of *Callosobruchus maculatus* exposed to clove and cinnamon essential oils. PloS One 13, e0207618. http://10.1371/journal.pone.0207618.

Kaasik, R., Kovács, G., Kaart, T., Metspalu, L., Williams, I.H., Veromann, E., 2014. *Meligethes aeneus* oviposition preferences, larval parasitism rate and species composition of parasitoids on *Brassica nigra, Raphanus sativus* and *Eruca sativa* compared with on *Brassica napus*. Biol. Contr. 69, 65–71. https://doi.org/10.1016/j.biocontrol.2013.11.002.

Kaiser, C., Jensen, K.-M.V., Nauen, R., Kristensen, M., 2018. Susceptibility of Danish pollen beetle populations against λ-cyhalothrin and thiacloprid. J. Pest. Sci. 91, 447–458. https://doi.org/10.1007/s10340-017-0856-x.

Kirk-Spriggs, A.H., 1996. Pollen Beetles - Coleoptera: Kateretidae and Nitidulidae: Meligethinae. Royal Entomological Society.

Mauchline, A.L., Osborne, J.L., Martin, A.P., Poppy, G.M., Powell, W., 2005. The effects of non-host plant essential oil volatiles on the behaviour of the pollen beetle *Meligethes aeneus*. Entomol. Exp. Appl. 114, 181–188. https://doi.org/10.1111/j.1570-7458.2005.00237.x.

Mauchline, A.L., Birkett, M.A., Woodcock, C.M., Pickett, J.A., Osborne, J.L., Powell, W., 2008. Electrophysiological and behavioural responses of the pollen beetle, *Meligethes aeneus*, to volatiles from a non-host plant, lavender, *Lavandula angustifolia* (Lamiaceae). Arthropod-Plant Interact. 2, 109–115. https://doi.org/10.1007/s11829-008-9038-3.

Mauchline, A.L., Cook, S.M., Powell, W., Osborne, J.L., 2013. Effects of non-host plant odour on *Meligethes aeneus* during immigration to oilseed rape. Entomol. Exp. Appl. 146, 313–320. https://doi.org/10.1111/eea.12030.

Mauchline, A.L., Hervé, M.R., Cook, S.M., 2018. Semiochemical-based alternatives to synthetic toxicant insecticides for pollen beetle management. Arthropod-Plant Interact. 12, 835–847. https://doi.org/10.1007/s11829-017-9569-6.

Pavela, R., 2011. Insecticidal and repellent activity of selected essential oils against of the pollen beetle, *Meligethes aeneus* (Fabricius) adults. Ind. Crop. Prod. 34, 888–892. https://doi.org/10.1016/j.indcrop.2011.02.014.

Pavela, R., 2014. Acute, synergistic and antagonistic effects of some aromatic compounds on the *Spodoptera littoralis* Boisd. (Lep., Noctuidae) larvae. Ind. Crop. Prod. 60, 247–258. https://doi.org/10.1016/j.indcrop.2014.06.030.

Pavela, R., 2015. Acute toxicity and synergistic and antagonistic effects of the aromatic compounds of some essential oils against *Culex quinquefasciatus* Say larvae. Parasitol. Res. 114, 3835–3853. https://doi.org/10.1007/s00436-015-4614-9.

Pisa, L., Goulson, D., Yang, E.-C., Gibbons, D., Sánchez-Bayo, F., Mitchell, E., et al., 2017. An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. Part 2: impacts on organisms and ecosystems. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-017-0341-3.

Riggi, L.G., Gagic, V., Rusch, A., Malsher, G., Ekbom, B., Bommarco, R., 2017. Pollen beetle mortality is increased by ground-dwelling generalist predators but not landscape complexity. Agric. Ecosyst. Environ. 250, 133–142. https://doi.org/10.1016/j.agee.2017.06.039.

Rolli, E., Marieschi, M., Maietti, S., Sacchetti, G., Bruni, R., 2014. Comparative phytotoxicity of 25 essential oils on pre- and post-emergence development of

Solanum lycopersicum L.: a multivariate approach. Ind. Crop. Prod. 60, 280–290. https://doi.org/10.1016/j.indcrop.2014.06.021.
Saad, M.M.G., Abou-Taleb, H.K., Abdelgaleil, S.A.M., 2018. Insecticidal activities of monoterpenes and phenylpropenes against *Sitophilus oryzae* and their inhibitory effects on acetylcholinesterase and adenosine triphosphatases. Appl Entomol Zool 53, 173–181. https://doi.org/10.1007/s13355-017-0532-x.
Seidenglanz, M., Poslušná, J., Kolařík, P., Rotrekl, J., Eva, E., Tóth, P., et al., 2017. Negative correlations between the susceptibilities of Czech and Slovak pollen beetle populations to lambda-cyhalothrin and chlorpyrifos-ethyl in 2014 and 2015. Plant Protect. Sci. 53, 108–117. https://doi.org/10.17221/187/2015-PPS.
Skellern, M.P., Cook, S.M., 2018. Prospects for improved off-crop habitat management for pollen beetle control in oilseed rape. Arthropod-Plant Interact. 12, 849–866. https://doi.org/10.1007/s11829-018-9598-9.
Smeriglio, A., Trombetta, D., Cornara, L., Valussi, M., De Feo, V., Caputo, L., 2019. Characterization and phytotoxicity assessment of essential oils from plant byproducts. Molecules 24, 2941. https://doi.org/10.3390/molecules24162941.
Stará, J., Kocourek, F., 2018. Seven-year monitoring of pyrethroid resistance in the pollen beetle (*Brassicogethes aeneus* F.) during implementation of insect resistance management. Pest Manag. Sci. 74, 200–209. https://doi.org/10.1002/ps.4695.
Tak, J.-H., Isman, M.B., 2015. Enhanced cuticular penetration as the mechanism for synergy of insecticidal constituents of rosemary essential oil in *Trichoplusia ni*. Sci. Rep. 5, 12690. https://doi.org/10.1038/srep12690.
Tak, J.-H., Isman, M.B., 2017. Penetration-enhancement underlies synergy of plant essential oil terpenoids as insecticides in the cabbage looper, *Trichoplusia ni*. Sci. Rep. 7, 42432. https://doi.org/10.1038/srep42432.
Thies, C., Tscharntke, T., 1999. Landscape structure and biological control in agroecosystems. Science 285, 893–895. https://doi.org/10.1126/science.285.5429.893.
Toledo, P.F., Viteri Jumbo, L.O., Rezende, S., Haddi, K., Silva, B., Mello, T., et al., 2020. Disentangling the ecotoxicological selectivity of clove essential oil against aphids and non-target ladybeetles. Sci. Total Environ. 718, 137382. https://doi.org/10.1016/j.scitotenv.2020.137328.
Willow, J., Silva, A., Veromann, E., Smagghe, G., 2019. Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. PloS One, e0212456. https://doi.org/10.1371/journal.pone.0212456.
Wu, C.-H., Lin, C.-L., Wang, S.-E., Lu, C.-W., 2019. Effects of imidacloprid, a neonicotinoid insecticide, on the echolocation system of insectivorous bats. Pestic. Biochem. Physiol. 163, 94–101. https://doi.org/10.1016/j.pestbp.2019.10.010.
Youssefi, M.R., Tabari, M.A., Esfandiari, A., Kazemi, S., Moghadamnia, A.A., Sut, S., et al., 2019. Efficacy of two monoterpenoids, carvacrol and thymol, and their combinations against eggs and larvae of the west nile vector *Culex pipiens*. Molecules 24, 1867. https://doi.org/10.3390/molecules24101867.
Zamojska, J., 2017. Differences in susceptibility of the cabbage seed weevil (*Ceutorchynhus assimilis* Payk.) (Coleoptera: Curculionidae) and the pollen beetle (*Meligethes aeneus* F.) (Coleoptera: Nitidulidae) to indoxacarb and deltamethrin and resistance mechanisms of the cabbage seed weevil to indoxacarb. Phytoparasitica 45, 407–418. https://doi.org/10.1007/s12600-017-0588-8.
Zimmer, C.T., Köhler, H., Nauen, R., 2014. Baseline susceptibility and insecticide resistance monitoring in European populations of *Meligethes aeneus* and *Ceutorhynchus assimilis* collected in winter oilseed rape. Entomol. Exp. Appl. 150, 279–288. https://doi.org/10.1111/eea.12162.

III

III **Willow, J.**, Sulg, S., Taning, C.N.T., Silva, A.I., Christiaens, O., Kaasik, R., Prentice, K., Lövei, G.L., Smagghe, G., Veromann, E. 2020b. Targeting a coatomer protein complex-I gene via RNA interference results in effective lethality in the pollen beetle *Brassicogethes aeneus*. J. Pest Sci. http://doi.org/10.1007/s10340-020-01288-6

Journal of Pest Science
https://doi.org/10.1007/s10340-020-01288-6

ORIGINAL PAPER

Targeting a coatomer protein complex-I gene via RNA interference results in effective lethality in the pollen beetle *Brassicogethes aeneus*

Jonathan Willow[1,2] · Silva Sulg[1] · Clauvis Nji Tizi Taning[2] · Ana Isabel Silva[3] · Olivier Christiaens[2] · Riina Kaasik[1] · Katterinne Prentice[2] · Gabor L. Lövei[4] · Guy Smagghe[2] · Eve Veromann[1]

Received: 4 March 2020 / Revised: 6 July 2020 / Accepted: 24 September 2020
© The Author(s) 2020

Abstract
The pollen beetle *Brassicogethes aeneus* is a serious pest of oilseed rape (*Brassica napus*) in Europe. Management of this pest has grown difficult due to *B. aeneus*'s development of resistance to pyrethroid insecticides, as well as the pressure to establish control strategies that minimise the impact on nontarget organisms. RNA interference represents a nucleotide sequence-based, and thus potentially species-specific, approach to agricultural pest control. The present study examined the efficacy of targeting the coatomer gene *coatomer subunit alpha* (*αCOP*), via both microinjection and dietary exposure to exogenous complementary dsRNA, on *αCOP*-silencing and subsequent mortality in *B. aeneus*. Beetles injected with dsRNA targeting *αCOP* (at 0.14 µg/mg) showed 88% and 100% mortality at 6 and 10 days post-injection, respectively; where by the same time after dietary exposure, 43%–89% mortality was observed in the 3 µg dsRNA/µL treatment, though the effect was concentration-dependent. Thus, the effect was significant for both delivery routes. In working towards RNA-based management of *B. aeneus*, future studies should include *αCOP* as a target of interest.

Keywords RNAi · *Brassicogethes aeneus* · *Meligethes aeneus* · Oilseed rape · Rapeseed · COPI · Biopesticide · Coleoptera

Communicated by M. Traugott .

Guy Smagghe and Eve Veromann have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10340-020-01288-6) contains supplementary material, which is available to authorised users.

✉ Jonathan Willow
jonathan@emu.ee

✉ Eve Veromann
eve.veromann@emu.ee

[1] Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu, Estonia

[2] Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

[3] Faculty of Health, Medicine and Life Sciences, School of Mental Health and Neuroscience, Maastricht University, Maastricht, The Netherlands

[4] Department of Agroecology, Flakkebjerg Research Centre, Aarhus University, Slagelse, Denmark

Key message

- We examined RNAi efficacy, targeting the coatomer gene *αCOP* in *Brassicogethes aeneus*, via both microinjection and feeding of dsRNA.
- Our work represents the first demonstration of highly significant mortality in a pest insect through targeting a coatomer protein complex-I gene via feeding.
- Our work further indicates *B. aeneus*'s sensitivity to naked dsRNA.
- Future RNAi studies in this important pest species and other pest insects should include *αCOP* as a potential target of interest.

Introduction

The pollen beetle *Brassicogethes aeneus* Fabricius (Coleoptera: Nitidulidae; formerly *Meligethes aeneus*) is Europe's primary pest of oilseed rape (*Brassica napus* L.). After overwintering, adult *B. aeneus* feed on pollen and nectar of a variety of blooming plants (e.g., Rosaceae, Asteraceae,

Published online: 16 November 2020

Lamiaceae, etc.), and subsequently colonise cruciferous (Brassicaceae) plants, where they acquire nutrients from buds and open flowers, mate, and oviposit into buds. Upon emergence from eggs, larvae feed on pollen within buds, eventually obtaining their nutrients from open flowers, followed by pupation in the soil under their host plant (see review by Mauchline et al. (2018)).

Synthetic insecticides are currently the standard method for *B. aeneus* control (Zhang et al. 2017; Zamojska 2017; Raimets et al. 2020). Consequently, *B. aeneus* has developed high levels of resistance to pyrethroid insecticides in several areas across Europe (Slater et al. 2011; Heimbach and Müller 2013; Zamojska 2017; Stará and Kocourek 2018; Kaiser et al. 2018). While neonicotinoid insecticides are also being applied in *B. aeneus* management (Seidenglanz et al. 2017; Kaiser et al. 2018), exposure to neonicotinoids has shown negative effects on a wide range of nontarget organisms (Gibbons et al. 2015; Pisa et al. 2017; Willow et al. 2019; Berheim et al. 2019; Calvo-Agudo et al. 2019; Wu et al. 2019). For example, in oilseed rape agroecosystems, nontarget organisms negatively affected by thiacloprid applications could include economically important parasitoids of *B. aeneus*, given that laboratory studies have demonstrated the detrimental effect of thiacloprid on other parasitoid wasps (Sugiyama et al. 2011; Jans 2012; Willow et al. 2019). Notably, among a European Union (EU)-wide ban on the outdoor use of three other neonicotinoids, thiacloprid is currently banned from both outdoor and greenhouse use in France, with an EU-wide ban on the outdoor use of thiacloprid tentative for implementation by 3 August 2020, with maximum grace period up to 3 February 2021 (European Commission 2018), based on the European Food Safety Authority's (EFSA) peer review of the risk assessment of thiacloprid (Abdourahime et al. 2019). Thus, there is urgent need for developing pest control strategies that minimise the impact on nontarget organisms, for effective and ecologically sustainable *B. aeneus* management.

The process of double-stranded ribonucleic acid (dsRNA)-mediated gene silencing, also known as RNA interference (RNAi), represents a potentially species-specific approach to agricultural pest control (Huvenne and Smagghe 2010; Taning et al. 2019; Bramlett et al. 2019; Zhu and Palli 2020; Mezzetti et al. 2020). In brief, exogenous dsRNA is taken up by the target species. Once the dsRNA enters the cell cytoplasm, the ribonuclease III enzyme Dicer-2 cleaves this dsRNA into double-stranded segments approximately 21 nucleotides in length, called small interfering RNAs (siRNAs). A multiprotein complex, with the endoribonuclease Argonaute2 as its catalytic centre, binds to one strand (the guide strand) of an siRNA, forming the RNA-induced silencing complex (RISC), and results in the degradation of the opposite (the passenger strand) siRNA strand. The guided RISC becomes bound to complementary endogenous messenger RNA (mRNA), and the RISC cleaves this mRNA, thus inhibiting its decoding in the ribosome and, in turn, subsequent protein synthesis (Bramlett et al. 2019).

Recently, all major RNAi pathway genes were identified in the *B. aeneus* transcriptome (Knorr et al. 2018). Genes and associated proteins thought to be necessary for systemic RNAi were also identified by the same authors. These authors were the first to report RNAi in *B. aeneus* via dietary exposure to exogenous dsRNA, and showed the efficacy of dsRNAs targeting the protein-coding genes *nucampholin* (*ncm*), *Ras opposite* (*Rop*), *RNA polymerase II 140kD subunit* (*RpII140*), and *dre4* (*dre4*). These target genes were chosen because they were orthologous to the four most RNAi-sensitive target genes from western corn rootworm (*Diabrotica virgifera* LeConte) diet bioassays conducted by the same authors (Knorr et al. 2018). However, before considering field use of dsRNA-based crop protection products in oilseed rape agroecosystems, it remains critical to carefully select additional RNAi target genes in *B. aeneus* and determine the effect of their inactivation, especially during this very early stage of developing an RNAi technique for this species.

One target gene of interest, *coatomer subunit alpha* (*αCOP*), encodes the coatomer subunit alpha (αCOP) protein. The αCOP protein is a subunit of coatomer protein complex-I (COPI), which is involved in vesicular transport of proteins between the endoplasmic reticulum and Golgi apparatus, as well as possibly maintaining distribution of proteins within the Golgi stack (Beck et al. 2009). The COPI coat adhering to intracellular vesicles also interacts with cell division control protein 42 homolog (CDC42), a regulator of the cytoskeletal motor protein dyenin, which transports various cellular cargo (Beck et al. 2009). A large-scale RNAi screening revealed COPI's additional role in maintaining lipid homeostasis (Beller et al. 2008). Finally, knockdown of COPI subunits results in failure of cytokinesis, via preventing the accumulation of vital proteins and lipid components at the cleavage furrow, as well as reducing the number of overlapping microtubules at the central spindle, a key regulating centre for cytokinesis (Kitazawa et al. 2012). Thus, *αCOP* was chosen as the RNAi target of interest in the present study, based on the expectation that its downregulation can be lethal. Indeed, previous dsRNA-microinjection experiments suggest the potential efficacy of targeting *αCOP* in larvae of the African sweetpotato weevils *Cylas brunneus* Fabricius (Christiaens et al. 2016) and *C. puncticollis* Boheman (Prentice et al. 2017). However, we are not aware of any study to date that has thoroughly examined, via dietary exposure, the effect of targeting *αCOP* in a coleopteran pest.

The present study examined the efficacy, via both microinjection and dietary exposure, of dsRNA targeting *αCOP* in *B. aeneus*. We also examined its potential dsRNA-concentration-dependent RNAi effect through dietary exposure.

Due to *αCOP*'s various biological functions, we expected this gene to be an effective RNAi target in *B. aeneus*, and furthermore expected to observe dsRNA-concentration-dependent reductions in survival of *B. aeneus* when targeting *αCOP*.

Materials and methods

dsRNA products

The gene *αCOP* was detected in the transcriptome of *B. aeneus* (Zimmer et al. 2014) [available in the GenBank database (National Centre for Biotechnology Information—NCBI)] via BLAST analysis, using known *αCOP* sequences from other insect species. In order to avoid cross-silencing of other genes in *B. aeneus*, a selected region (222 bp; Online Resource 1) from the *B. aeneus αCOP* coding sequence was screened for cross-homologies within the *B. aeneus* transcriptome using BLAST analysis to ensure that there were no shared fragment identities greater than 19 nucleotides in length. The chosen *αCOP* region was additionally screened against all bee species with available genome data in NCBI.

The chosen *αCOP* region, as well as a 455 bp sequence from the *green fluorescent protein* (*gfp*) gene (Online Resource 1), was used as the basis for the in vitro synthesis of corresponding dsRNA products by AgroRNA (Genolution, Seoul, South Korea). The synthesised dsRNA products were shipped in distilled water at ambient temperature, and kept at 5 ± 1 °C once received. Products used in this study included dsRNAs with sequences complementary to specific genes: *gfp*, which represented our control treatment, as it is not present in insects; and the target gene *αCOP*. Treatments targeting the genes *gfp* and *αCOP* are hereafter respectively referred to as dsGFP and dsαCOP. The length and purity of the dsRNA products used were confirmed via gel electrophoresis (Online Resource 2).

Insects

Pollen beetles were collected from an untreated organic oilseed rape field (58.37979°N, 26.66394°E) in the village of Kandiküla, Tartu County, Estonia. Beetles were kept in ventilated plastic containers and allowed to feed ad libitum on pollen of oilseed rape and dandelion (*Taraxacum* spp.) flowers. Oilseed rape flowers were collected from the same field where pollen beetles were collected, and dandelion flowers were collected from wildflower areas within and around the campus of the Estonian University of Life Sciences. All pollen beetles used in the study were identified as *B. aeneus*, using an identification guide by Kirk-Spriggs (1996), prior to their addition to the study.

Experimental set-up: microinjection

Ensured delivery of dsRNA into the haemolymph of *B. aeneus* was performed under a stereomicroscope using a microinjector (FemtoJet 4i, Eppendorf, Hamburg, Germany) and micromanipulator (Narishige, Tokyo, Japan) equipped with an injection needle prepared from glass capillary tubes. Twenty groups of ten randomly chosen *B. aeneus* adults were weighed (Sartorius Lab Instruments, Göttingen, Germany) in plastic vials in order to obtain an average weight per individual beetle (1.4 ± 0.1 mg). Prior to microinjection, beetles were anaesthetised with diethyl ether for 2 min. Subsequently, they were individually placed on their dorsal surface upon a glass slide, and held in place by gently pressing a glass cover slip over their ventral abdominal surface. This gentle pressing resulted in the extension, and subsequent visualisation, of at least one of two intersegmental areas composed of arthrodial membrane (unsclerotised, soft and flexible cuticular surface), including the cervix (membrane separating head from thorax, i.e. neck) and a similar area separating thorax from abdomen.

For both treatments (dsGFP (control) and dsαCOP), beetles were microinjected with 0.2 µL of dsRNA solution at 1 µg dsRNA/µL (approximately 0.14 µg dsRNA/mg). Approximately 15–20 beetles were injected per treatment, and after 24 ± 1 h, ten randomly chosen and fast moving (used as a proxy for health) individuals from both treatments were removed and placed into transparent, polystyrene, ventilated insect breeding dishes (diameter 10 cm x height 4 cm) (SPL Life Sciences, Gyeonggi-do, South Korea), hereafter referred to as cages. This was replicated 4 times to obtain a total of 40 dsRNA-injected beetles per treatment. Another group of approximately 55–60 beetles was injected for each treatment, and after 24 ± 1 h post-injection, 36 fast moving beetles per treatment were removed and used for analysis of gene expression via quantitative polymerase chain reaction (qPCR; three replicates of six beetles were analysed at 2 and 5 d post-injection; see *Analysis of gene expression*). Experimental set-up is illustrated in Fig. 1a.

Post-injection, beetles were placed in a growth chamber (Sanyo MLR-351H, Osaka, Japan) at 20 ± 2 °C, 60% RH and 16:8 h L:D cycle, allowed to feed ad libitum on oilseed rape pollen via laboratory-grown oilseed rape flowers, and were provisioned with a moist piece of cotton for access to drinking water. Fresh food and water were provided every 24 ± 1 h, when the previous day's food and water were removed from the cages. Survival and mobility were assessed every 24 ± 1 h for 10 d post-injection, and comparisons between the dsGFP and dsαCOP treatment were statistically assessed using Fisher's exact test in R software v1.1.463 (R Foundation for Statistical Computing, Vienna, Austria).

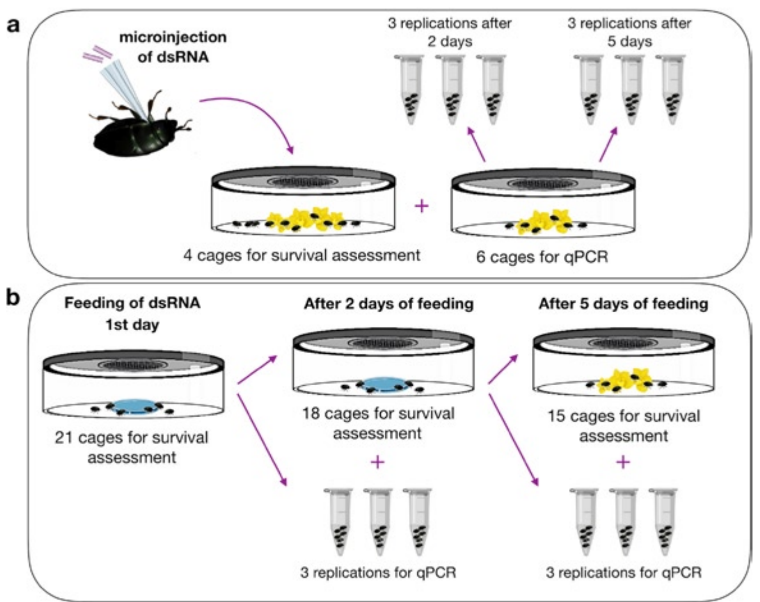


Fig. 1 Experimental set-up for microinjection **(a)** and feeding **(b)** experiments, for each treatment. Microinjection experiment: $n=40$ (four replicates of ten beetles) per treatment for survival assessment; $n=3$ (3 replicates of 6 beetles) for each time-point of analysis within each treatment. Microinjected beetles fed ad libitum on pollen of oilseed rape flowers post-injection. Feeding experiment: $n=21$ (21 cages of 6 beetles; days 0–2), 18 (18 cages of 6 beetles; days 3–5) and 15 (15 cages of 6 beetles; days 6–19) per treatment; $n=3$ (3 replicates of 6 beetles) for each time-point of analysis within each treatment. In feeding experiment, beetles were fed dsRNA for 5 d, followed by ad libitum feeding on pollen of oilseed rape flowers for the remaining during of survival assessment

Experimental set-up: feeding

Brassicogethes aeneus were identified and placed into cages (described above), in groups of six randomly chosen and fast moving (used as a proxy for health) beetles per cage. We tested four treatments, including dsGFP and dsαCOP each at both 1 and 3 µg dsRNA/µL. Treatment solutions were 25% organic honey for nutrition; nuclease-free water was used to obtain the desired dsRNA concentrations; and bromophenol blue was added to allow confirmation of feeding (i.e. the presence of blue faeces in cages).

Each treatment was allocated 21 cages, each cage containing six beetles and one treatment source. The treatment source was a modified Eppendorf cap (removed from a 1.5 mL Eppendorf tube; hereafter referred to as cap) forming a basin, the height of which was reduced using a razorblade in order to allow the beetles to stand up and drink from the cap without having to sit upon the lip of the basin (which, according to preliminary observations, increased the chance of beetles falling into the treatment solution). Each cap held 100 µL of treatment solution. Prior to pipetting dsRNA treatment solutions into the caps, treatment stocks were homogenised for approximately 5 s at 3200 rpm (Vortex-Genie 2, Scientific Industries, Bohemia, New York, USA).

Once exposed to treatments, beetles were kept in the growth chamber at 20 °C, 60% RH and 16:8 h L:D cycle. A new cap with freshly prepared treatment was provided to each cage every 24 ± 1 h, when the previous day's cap was removed. Survival and mobility were assessed every 24 ± 1 h for 19 d. Dead beetles were removed from cages daily.

After 2 d of allowing the beetles to feed on their respective treatments, three cages containing six live beetles, per treatment, were removed for analysis of relative gene expression via qPCR (see *Analysis of gene expression*), and thus 18 cages per treatment remained for survival/mobility assessment. A second removal for gene expression analysis

occurred after 5 d of feeding on treatments, leaving 15 cages for mortality/mobility assessment per treatment. Experimental set-up is illustrated in Fig. 1b.

For the remainder of the experiment, beetles were allowed to feed ad libitum on oilseed rape pollen, via laboratory-grown oilseed rape flowers, and were provisioned with a moist piece of cotton for access to drinking water. Fresh food and water were provided every 24 ± 1 h, when the previous day's food and water were removed from the cages. Reductions in survival and mobility between dsαCOP treatments and their respective dsGFP controls, as well as between the two dsαCOP treatments, were then statistically assessed in R software v1.1.463. Homogeneity of variance and normality of data distributions were examined using the Levene- and Shapiro–Wilk tests, respectively. Given that the data were overall not normally distributed, the nonparametric Kruskal–Wallis test was used as an alternative to ANOVA, followed by *post hoc* pairwise comparisons using the Wilcoxon rank–sums test.

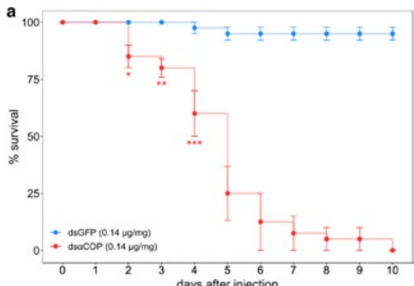
Analysis of relative gene expression

Relative gene expression was measured using qPCR. Beetles analysed were removed from their cages at 2 and 5 d after first exposure to dsRNA treatments (see above). Treatment groups analysed for relative gene expression were six in total, including: those microinjected with dsGFP and dsαCOP; as well as those fed with dsGFP and dsαCOP, both at 1 and 3 µg dsRNA/µL. For each treatment group, three replicates of six beetles were used for gene expression analysis at 2 d post-treatment, and another three replicates of six beetles at 5 d post-treatment. Beetles to be analysed were placed in RNA*later* RNA Stabilisation Solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA), at their respective time point of interest, until analysis. RNA extraction was performed using an RNeasy mini kit (Qiagen, Hilden, Germany), and 200 ng of RNA was used for quantifying relative gene expression (SOLIScript 1-step kit, Solis BioDyne, Tartu, Estonia). Cycle conditions, using QuantiStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, California, USA), were: 50 °C for 15 min, 40 cycles at 95 °C for 15 s, 58 °C for 60 s, and ending with a melting curve analysis. Normalisation of the data was performed using the two reference genes *ribosomal protein S3* (*rps3*) and *actin* (*act*). Primer amplification efficiencies were determined via RNA dilution series (Online Resource 3). Relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Comparisons between dsGFP and dsαCOP treatments, regarding relative gene expression, were statistically assessed for both microinjected and dsRNA-fed beetles using Welch's t-test in R software v1.1.463.

Results

Survival of dsRNA-microinjected pollen beetles

Direct microinjection of dsαCOP resulted in a significant reduction in survival of *B. aeneus* (Fig. 2a, Online Resource 4). At 10 d post-injection, dsGFP-injected beetles showed 95% survival. In contrast, survival of dsαCOP-injected beetles fell to 85% ($p = 0.03$) after 2 d, 60% ($p < 0.0001$) after 4 d, 12.5% after 6 d, and 0% after 10 d post-injection. Moreover, mortality of the dsαCOP-injected beetles was often preceded by a loss of mobility (Online Resources 5, 6a).



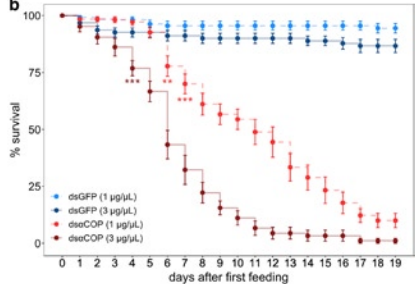


Fig. 2 Survival curves, comparing dsαCOP treatments with their respective dsGFP controls, in microinjected **(a)** and dsRNA-fed **(b)** pollen beetles. Microinjection experiment: $n = 40$ (4 replicates of 10 beetles) per treatment. Feeding experiment: $n = 21$ (21 cages of 6 beetles; days 0–2), 18 (18 cages of 6 beetles; days 3–5) and 15 (15 cages of 6 beetles; days 6–19) per treatment. Microinjection data were analysed using Fisher's exact test (error bars: ± SE). Feeding data were analysed using the Kruskal–Wallis test with *post hoc* Wilcoxon rank-sum test for pairwise comparisons (error bars: ± SEM). Asterisks indicate significant differences between dsαCOP and respective dsGFP treatments. $df = 3$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Survival of dsRNA-fed pollen beetles

Dietary exposure to dsαCOP, at both concentrations examined, resulted in significant reductions in *B. aeneus* survival (Fig. 2b, Online Resource 4). At 19 d after the start of the feeding experiment, we observed 95% survival in the dsGFP at 1 µg/µL treatment, and 87% survival in the dsGFP at 3 µg/µL treatment. Beetles fed dsαCOP at 1 µg/µL showed significant mortality (78% survival, $df=3$, $p=0.003$) 6 d after first exposure, followed by a steady decrease to 61% (8 d, $df=3$, $p<0.0001$), 54% (10 d), 44% (12 d), 29% (14 d), 18% (16 d), and 10% survival (18 d). Beetles fed dsαCOP at 3 µg/µL showed significant mortality (77% survival; $df=3$, $p=0.0007$) 4 d after first exposure, survival here falling more rapidly, to 43% ($df=3$, $p<0.0001$) after 6 d, 20% (8 d), 11% (10 d), and 1% (17 d).

From 2 d after first exposure to dsαCOP, we observed significantly lower survival in the 3 µg/µL than in the 1 µg/µL treatment (2 d $p=0.014$, 3 d $p=0.0085$, 4–12 d $p<0.0001$, 13–14 d $p=0.0002$, 15 d $p=0.0033$, 16 d $p=0.009$, 17 d $p=0.002$, 18–19 d $p=0.015$, $df=3$).

Blue faeces were observed extensively throughout all cages from each treatment, providing further indication that the beetles fed on their respective treatments. Similar to dsαCOP-injected beetles, mortality of the dsαCOP-fed beetles was often preceded by a loss of mobility (Online Resources 5, 6b).

Effect on *αCOP* expression

As shown in Fig. 3, the obtained qPCR results indicated that *αCOP* was downregulated by the dsRNA targeting this gene when delivered by microinjection and feeding. The dsαCOP-injected beetles ($t=7.56$, $df=3.19$, $p=0.0038$) and the beetles that fed on the dsαCOP at 1 µg/µL ($t=2.38$, $df=2.65$, $p=0.109$) showed a respective mean reduction in expression of the target gene of 82% and 52% after 5 d, compared to respective dsGFP controls. In contrast, at the shorter time point of 2 d, there was no apparent reduction in relative expression of *αCOP* (microinjection: $p=0.67$; feeding 1 µg/µL: $p=0.44$). In addition, with the higher dsαCOP concentration of 3 µg/µL by feeding, the reduction in relative expression of *αCOP* was minor, with only 36% mean reduction at 2 d ($t=1.87$, $df=2.88$, $p=0.16$), and 15% at 5 d ($t=1.24$, $df=3.60$, $p=0.29$).

Discussion

αCOP is an effective RNAi target in *Brassicogethes aeneus*

We provide laboratory evidence suggesting that *αCOP* is an effective RNAi target in *B. aeneus*, as mortality in *B. aeneus* was highly significant after dietary exposure to both concentrations of dsαCOP, confirming *B. aeneus*'s sensitivity to RNAi via dietary exposure to dsαCOP, which is in agreement with Knorr et al. (2018). We believe that the high mortality in our *B. aeneus* RNAi assays, especially for microinjection, was caused by gene silencing of the target gene, *αCOP*. Indeed, in dsαCOP-injected beetles, we observed a significant reduction in *αCOP* mRNA 5 d after treatment.

While we observed high *B. aeneus* mortality rates, yet did not detect corresponding significant *αCOP* silencing in beetles that fed upon dsαCOP, previous research has shown that relative gene expression does not always

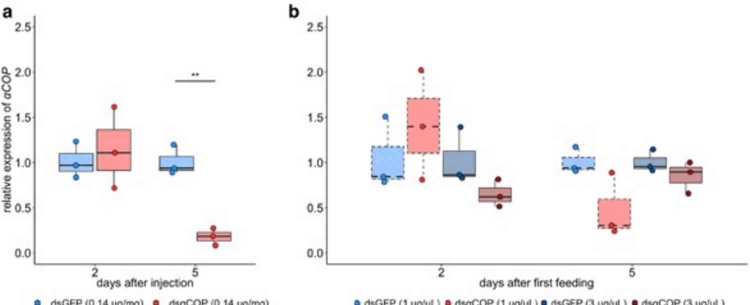


Fig. 3 Relative gene expression of *αCOP* in microinjected **(a)** and dsRNA-fed **(b)** pollen beetles, at 2 and 5 d after treatment. Data were normalised using the reference genes *rps3* and *act*. $n=3$ (3 replicates of 6 beetles) for each time point of analysis within each treatment. Relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical comparisons were made using Welch's *t*-test. Asterisks indicate significant differences between dsαCOP and respective dsGFP treatments. ** $=p<0.01$

reflect protein levels (Michel et al. 2005; Scott et al. 2013). There were indeed samples indicating downregulation of *αCOP* in dsαCOP-fed beetles; however, high variability resulted in non-significant qPCR results for these treatments. Several factors may play a role in the variability and differences we observed. A decrease in *αCOP* mRNA and protein levels could, through a feedback mechanism, stimulate the overexpression of *αCOP*, making it difficult to detect significant changes at the transcript level while the protein level is decreasing during *B. aeneus*'s exposure to dsαCOP; the decreased αCOP protein level ultimately leading to *B. aeneus* mortality. Furthermore, an inferior gene silencing effect was observed with respect to feeding, compared to microinjection. This could be related to the requirement for crossing an additional physiological barrier, for example, that of the midgut epithelium; and/or maintaining dsRNA stability in the midgut lumen, where a much higher concentration of dsRNA-degrading nucleases are present, compared to that which is found in the haemolymph (Peng et al. 2018). In addition, we performed qPCR on whole insects; and microinjection may have spread the exogenous dsRNA to more tissues than via dietary exposure to dsRNA. Future studies should consider extracting RNA from gut tissue for the evaluation of target gene silencing following dsRNA feeding treatments, in contrast to whole body samples where the overall gene silencing effect could be diluted.

Targeting COPI genes via RNAi has been performed in several agricultural pest insect studies. The results of nearly 100% mortality, post-microinjection of dsRNA targeting *αCOP*, has suggested the potential efficacy of targeting this gene in both *C. brunneus* (Christiaens et al. 2016) and *C. puncticollis* (Prentice et al. 2017). Taning et al. (2016) observed significant gene silencing and subsequent mortality (46 ± 9%) when targeting *αCOP* via microinjection of dsRNA in spotted wing drosophila (*Drosophila suzukii* Matsumura) adults, though less effective gene silencing and mortality effects through dietary exposure to dsRNA targeting this gene in both larvae and adults. Lastly, another COPI subunit, *coatomer subunit beta* (*βCOP*), is a suitable target in several agricultural pests (Baum et al. 2007; Kwon et al. 2013; Mao et al. 2015; Rodrigues et al. 2017; Shin et al. 2020).

The present study further indicates a concentration-dependent effect of dsαCOP on *B. aeneus* survival, where the higher feeding concentration dsαCOP treatment resulted in significant mortality 2 d earlier than the lower concentration, as well as a steeper mortality rate compared to that of the lower concentration. Rodrigues et al. (2017) also observed a concentration-dependent effect of exogenous dsRNA on mortality when targeting *βCOP*.

Future steps towards application

While a 6 d time-to-effect is less than ideal for field use as an insecticide, an RNAi approach cannot be expected to cause mortality as quickly as some other (e.g. neurotoxic) insecticides; turnover time of the target protein will remain a limiting factor. However, there remains the possibility to improve efficacy and speed via co-formulants (e.g. nanoparticles) that enhance efficiency of both dsRNA uptake and subsequent RNAi (Christiaens et al. 2020; Yan et al. 2020). The benefits of this technology lie in the associated biosafety aspects, due to its mode of action. Ideally, an RNAi approach should be used in combination with other ecologically sustainable approaches (e.g. conservation biocontrol), in an integrated pest management context, for maximum benefit.

There are various application methods for effective dsRNA-based control of agricultural pest insects, and the most suitable method is always species-dependent. It is possible that the most effective method of *B. aeneus* control in oilseed rape crops is via the use of an RNAi cultivar expressing dsRNA in nectar and pollen. This method would allow the crop to continuously produce dsRNA, preferably in the plant parts on which *B. aeneus* feeds. While current restrictions prevent the implementation of this technology within EU countries, this could change with further experience with the technology, and understanding of its impacts (e.g. after refinements are made to RNAi risk assessments) (Arpaia et al. 2020). There may also be the possibility to apply appropriately timed dsRNA-based spray treatments to effectively manage *B. aeneus*. The exploitation of exogenous dsRNA-based biocontrol compounds, for application within a wide variety of crop—pest systems, is an expanding and momentous field of interest, and likely has both a prominent and practicable place in the nearing future's crop protection market (Taning et al. 2019; Mezzetti et al. 2020). Field-realistic experiments simulating dietary exposure to dsRNAs are required with regard to *B. aeneus*, and should include examining the effect of spraying dsRNA-based treatments onto both bud and flower clusters of oilseed rape. The bud stage is oilseed rape's most vulnerable period, as adult bud feeding and oviposition by *B. aeneus*, as well as larval bud feeding, can result in considerable yield losses. At the same time, targeting both adults and larvae that feed on nectar and pollen of open flowers could reduce the abundance of overwintering *B. aeneus*, potentially reducing yield losses in the following growing season. Thus far, only *B. aeneus* adults have been examined for efficacy of RNAi; future dietary exposure studies should include *B. aeneus* larvae. Furthermore, as *B. aeneus* is one of several major pests of oilseed rape in Europe, future studies should explore the potential for RNAi-based management of other oilseed rape pests as well, and the prospect of targeting multiple

jointly-present oilseed rape pests simultaneously via stacked dsRNA treatments.

Dietary exposure to dsRNA is not the only potential dsRNA uptake method for effective control of agricultural pest insects. RNAi via topical exposure to dsRNA has been observed in some hemipteran insects, including the pea aphid (*Acyrthosiphon pisum* Harris), green peach aphid (*Myzus persicae* Sulzer) and brown citrus aphid (*Toxoptera citricida*, formerly *Toxoptera citricidus* and *Aphis citricidus* Kirkaldy) (Niu et al. 2019). *B. aeneus* is a good candidate for testing RNAi via topical exposure to dsRNA, especially in larvae, which have a soft unsclerotised cuticle, although the body of adult *B. aeneus* also has soft regions that are potentially vulnerable to topical exposure to dsRNAs. Candidate methods for testing the effect of topical exposure to dsRNAs on *B. aeneus* include administering submicron amounts of dsRNA-based treatments directly onto the bodies of larval and adult *B. aeneus*, and miniature-scale dsRNA soil drench experiments examining the potential impact on soil-inhabiting second instar larval- and pupating *B. aeneus*.

Conclusion

The requirement of nucleotide sequence complementarity makes dsRNA-based biopesticides likely the most selective pesticides known to date, since they potentially affect only the target pest, and no other organisms, resulting in a more ecologically sustainable method of control; though this method of control would require the application of multiple dsRNAs in the event of managing multiple pest insect species. We showed that *αCOP* represents an effective RNAi target in the oilseed rape pest *B. aeneus*. We observed significant gene silencing-induced mortality via both microinjection and feeding of dsαCOP, confirming *B. aeneus*'s sensitivity to dsαCOP via both routes of exposure. This work represents the first study to demonstrate highly significant gene silencing-induced mortality in an agricultural pest through dietary exposure to dsRNA targeting a COPI gene. Thus, future studies towards the application of RNAi in *B. aeneus* management should examine *αCOP* alongside other RNAi targets previously shown to be associated with high levels of gene silencing and subsequent mortality in *B. aeneus*. Next steps include examining additional routes of exposure to dsRNAs, particularly within a field-realistic context.

Author contributions

JW, CNTT, OC, GLL, GS, and EV conceived the study. JW, CNTT, KP, and EV designed the method. JW and SS performed the study. JW and AIS analysed data and prepared figures. JW wrote the original draft. JW, CNTT, AIS, OC, KP, GLL, GS, and EV contributed revisions to subsequent drafts. All authors read and approved the manuscript.

Acknowledgements We thank the owners of the land on which pollen beetles were collected for this study, and a member of the Estonian University of Life Sciences' Chair of Plant Breeding and Plant Biology for molecular support. Jonathan Willow is the recipient of a PhD grant from the European Social Fund for Doctoral Students, as well as the Internationalisation Programme DoRa (carried out by the Archimedes Foundation). Olivier Christiaens is a recipient of a postdoctoral fellowship from the Research Foundation—Flanders (FWO-Vlaanderen). The authors acknowledge financial support from the institutional Research Funding project no IUT36-2 of the Estonian Research Council, the European ERA-NET C-IPM project "IPM4Meligethes" (Project No 3G0H0416), the Special Research Fund (BOF) of Ghent University and the Research Foundation—Flanders (FWO-Vlaanderen).

Funding This study was partially funded by the institutional Research Funding project no IUT36-2 of the Estonian Research Council, the European ERA-NET C-IPM project "IPM4Meligethes" (Project No 3G0H0416), the Special Research Fund (BOF) of Ghent University and the Research Foundation—Flanders (FWO-Vlaanderen). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and material Datasets related to the present study are available upon reasonable request from the corresponding author.

Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

Consent to participate Consent was given by all participants included in the study.

Consent for publication All authors consent to the publication of this manuscript in Journal of Pest Science.

Ethical approval This study does not contain any experiments using any animal species that requires ethical approval.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Abdourahime H, Anastassiadou M, Arena M et al (2019) Peer review of the pesticide risk assessment of the active substance thiacloprid. EFSA J 17:e05595. https://doi.org/10.2903/j.efsa.2019.5595

Arpaia S, Christiaens O, Giddings K et al (2020) Biosafety of GM crop plants expressing dsRNA: data requirements and EU regulatory considerations. Front Plant Sci. https://doi.org/10.3389/fpls.2020.00940
Baum JA, Bogaert T, Clinton W et al (2007) Control of coleopteran insect pests through RNA interference. Nat Biotechnol 25:1322–1326. https://doi.org/10.1038/nbt1359
Beck R, Ravet M, Wieland FT, Cassel D (2009) The COPI system: molecular mechanisms and function. FEBS Lett 583:2701–2709. https://doi.org/10.1016/j.febslet.2009.07.032
Beller M, Sztalryd C, Southall N et al (2008) COPI complex is a regulator of lipid homeostasis. PLOS Biol. https://doi.org/10.1371/journal.pbio.0060292
Berheim EH, Jenks JA, Lundgren JG et al (2019) Effects of neonicotinoid insecticides on physiology and reproductive characteristics of captive female and fawn white-tailed deer. Sci Rep 9:1–10. https://doi.org/10.1038/s41598-019-40994-9
Bramlett M, Plaetinck G, Maienfisch P (2019) RNA-based biocontrols—a new paradigm in crop protection. Engineering. https://doi.org/10.1016/j.eng.2019.09.008
Calvo-Agudo M, González-Cabrera J, Picó Y et al (2019) Neonicotinoids in excretion product of phloem-feeding insects kill beneficial insects. Proc Natl Acad Sci 116:16817–16822. https://doi.org/10.1073/pnas.1904298116
Christiaens O, Prentice K, Pertry I et al (2016) RNA interference: a promising biopesticide strategy against the African sweetpotato weevil *Cylas brunneus*. Sci Rep 6:1–11. https://doi.org/10.1038/srep38836
Christiaens O, Petek M, Smagghe G, Taning CNT (2020) The use of nanocarriers to improve the efficiency of RNAi-based pesticides in agriculture. In: Fraceto LF, SS de Castro VL, Grillo R, et al. (eds) Nanopesticides. Springer International Publishing, Berlin
European Commission (2018) Public consultation on the active substance thiacloprid. In: Eur. Food Saf. Auth. https://www.efsa.europa.eu/en/consultations/call/180123. Accessed 28 Jun 2020
Gibbons D, Morrissey C, Mineau P (2015) A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environ Sci Pollut Res 22:103–118. https://doi.org/10.1007/s11356-014-3180-5
Heimbach U, Müller A (2013) Incidence of pyrethroid-resistant oilseed rape pests in Germany. Pest Manag Sci 69:209–216. https://doi.org/10.1002/ps.3351
Huvenne H, Smagghe G (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol 56:227–235. https://doi.org/10.1016/j.jinsphys.2009.10.004
Jans D (2012) Toxicity to the parasitoid wasp *Aphidius rhopalosiphi* (DeStephani-Perez) (Hymenoptera: Braconidae) using an extended laboratory test with aged residues on maize-thiacloprid OD 240 g/L-final report. European Commission. Renewal Assessment Report prepared according to the Commission Regulation (EU) N° 1107/2009. Vol 3–B.9 (PPP)–Thiacloprid OD 240 (240 g/L. 2017: 262–6. https://www.efsa.europa.eu/en/consultations/call/180123
Kaiser C, Jensen K-MV, Nauen R, Kristensen M (2018) Susceptibility of Danish pollen beetle populations against λ-cyhalothrin and thiacloprid. J Pest Sci 91:447–458. https://doi.org/10.1007/s10340-017-0856-x
Kirk-Spriggs AH (1996) Pollen Beetles - Coleoptera: Kateretidae and Nitidulidae: Meligethinae. Royal Entomological Society, London
Kitazawa D, Yamaguchi M, Mori H, Inoue YH (2012) COPI-mediated membrane trafficking is required for cytokinesis in *Drosophila* male meiotic divisions. J Cell Sci 125:3649–3660. https://doi.org/10.1242/jcs.103317
Knorr E, Fishilevich E, Tenbusch L et al (2018) Gene silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi targets in crop pests. Sci Rep 8:1–15. https://doi.org/10.1038/s41598-018-20416-y
Kwon DH, Park JH, Lee SH (2013) Screening of lethal genes for feeding RNAi by leaf disc-mediated systematic delivery of dsRNA in *Tetranychus urticae*. Pestic Biochem Physiol 105:69–75. https://doi.org/10.1016/j.pestbp.2012.12.001
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262
Mao J, Zhang P, Liu C, Zeng F (2015) Co-silence of the *coatomer β* and *v-ATPase A* genes by siRNA feeding reduces larval survival rate and weight gain of cotton bollworm, *Helicoverpa armigera*. Pestic Biochem Physiol 118:71–76. https://doi.org/10.1016/j.pestbp.2014.11.013
Mauchline AL, Hervé MR, Cook SM (2018) Semiochemical-based alternatives to synthetic toxicant insecticides for pollen beetle management. Arthropod-Plant Interact 12:835–847. https://doi.org/10.1007/s11829-017-9569-6
Mezzetti B, Smagghe G, Arpaia S et al (2020) RNAi: what is its position in agriculture? J Pest Sci. https://doi.org/10.1007/s10340-020-01238-2
Michel K, Budd A, Pinto S et al (2005) *Anopheles gambiae SRPN2* facilitates midgut invasion by the malaria parasite *Plasmodium berghei*. EMBO Rep 6:891–897. https://doi.org/10.1038/sj.embor.7400478
Niu J, Yang W-J, Tian Y et al (2019) Topical dsRNA delivery induces gene silencing and mortality in the pea aphid. Pest Manag Sci 75:2873–2881. https://doi.org/10.1002/ps.5457
Peng Y, Wang K, Fu W et al (2018) Biochemical comparison of dsRNA degrading nucleases in four different insects. Front Physiol 9:624. https://doi.org/10.3389/fphys.2018.00624
Pisa L, Goulson D, Yang E-C et al (2017) An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. part 2: impacts on organisms and ecosystems. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-017-0341-3
Prentice K, Christiaens O, Pertry I et al (2017) RNAi-based gene silencing through dsRNA injection or ingestion against the African sweet potato weevil *Cylas puncticollis* (Coleoptera: Brentidae). Pest Manag Sci 73:44–52. https://doi.org/10.1002/ps.4337
Raimets R, Bontšutšnaja A, Bartkevics V et al (2020) Pesticide residues in beehive matrices are dependent on collection time and matrix type but independent of proportion of foraged oilseed rape and agricultural land in foraging territory. Chemosphere 238:124555. https://doi.org/10.1016/j.chemosphere.2019.124555
Rodrigues TB, Rieske LKJ, Duan et al (2017) Development of RNAi method for screening candidate genes to control emerald ash borer *Agrilus planipennis*. Sci Rep 7:7379. https://doi.org/10.1038/s41598-017-07605-x
Scott JG, Michel K, Bartholomay LC et al (2013) Towards the elements of successful insect RNAi. J Insect Physiol 59:1212–1221. https://doi.org/10.1016/j.jinsphys.2013.08.014
Seidenglanz M, Poslušná J, Kolařík P et al (2017) Negative correlations between the susceptibilities of Czech and Slovak pollen beetle populations to lambda-cyhalothrin and chlorpyrifos-ethyl in 2014 and 2015. Plant Prot Sci 53:108–117. https://doi.org/10.17221/187/2015-PPS
Shin YH, Lee SH, Park Y-D (2020) Development of mite (*Tetranychus urticae*)-resistant transgenic Chinese cabbage using plant-mediated RNA interference. Hortic Environ Biotechnol 61:305–315. https://doi.org/10.1007/s13580-019-00203-y
Slater R, Ellis S, Genay J-P et al (2011) Pyrethroid resistance monitoring in European populations of pollen beetle (*Meligethes* spp.): a coordinated approach through the Insecticide Resistance Action

Committee (IRAC). Pest Manag Sci 67:633–638. https://doi.org/10.1002/ps.2101

Stará J, Kocourek F (2018) Seven-year monitoring of pyrethroid resistance in the pollen beetle (*Brassicogethes aeneus* F.) during implementation of insect resistance management. Pest Manag Sci 74:200–209. https://doi.org/10.1002/ps.4695

Sugiyama K, Katayama H, Saito T (2011) Effect of insecticides on the mortalities of three whitefly parasitoid species, *Eretmocerus mundus*, *Eretmocerus eremicus* and *Encarsia formosa* (Hymenoptera: Aphelinidae). Appl Entomol Zool 46:311–317. https://doi.org/10.1007/s13355-011-0044-z

Taning CNT, Christiaens O, Berkvens N et al (2016) Oral RNAi to control *Drosophila suzukii*: laboratory testing against larval and adult stages. J Pest Sci 89:803–814. https://doi.org/10.1007/s10340-016-0736-9

Taning CN, Arpaia S, Christiaens O et al (2019) RNA-based biocontrol compounds: current status and perspectives to reach the market. Pest Manag Sci. https://doi.org/10.1002/ps.5686

Willow J, Silva A, Veromann E, Smagghe G (2019) Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. PLoS ONE. https://doi.org/10.1371/journal.pone.0212456

Wu C-H, Lin C-L, Wang S-E, Lu C-W (2019) Effects of imidacloprid, a neonicotinoid insecticide, on the echolocation system of insectivorous bats. Pestic Biochem Physiol. https://doi.org/10.1016/j.pestbp.2019.10.010

Yan S, Ren B-Y, Shen J (2020) Nanoparticle-mediated double-stranded RNA delivery system: a promising approach for sustainable pest management. Insect Sci. https://doi.org/10.1111/1744-7917.12822

Zamojska J (2017) Differences in susceptibility of the cabbage seed weevil (*Ceutorchynhus assimilis* Payk.) (Coleoptera: Curculionidae) and the pollen beetle (*Meligethes aeneus* F.) (Coleoptera: Nitidulidae) to indoxacarb and deltamethrin and resistance mechanisms of the cabbage seed weevil to indoxacarb. Phytoparasitica 45:407–418. https://doi.org/10.1007/s12600-017-0588-8

Zhang H, Breeze T, Bailey A et al (2017) Arthropod pest control for UK oilseed rape–comparing insecticide efficacies, side effects and alternatives. PLoS ONE 12:e0169475. https://doi.org/10.1371/journal.pone.0169475

Zhu KY, Palli SR (2020) Mechanisms, applications, and challenges of insect RNA interference. Annu Rev Entomol 65:293–311. https://doi.org/10.1146/annurev-ento-011019-025224

Zimmer CT, Maiwald F, Schorn C et al (2014) A de novo transcriptome of European pollen beetle populations and its analysis, with special reference to insecticide action and resistance. Insect Mol Biol 23:511–526. https://doi.org/10.1111/imb.12099

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

IV

IV **Willow, J.**, Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I., Taning, C.N.T., Christiaens, O., Smagghe, G., Veromann, E. 2020c. First evidence of bud feeding-induced RNAi in a crop pest via exogenous application of dsRNA. Insects 11, 769. http://doi.org/10.3390/insects11110769

Communication

First Evidence of Bud Feeding-Induced RNAi in a Crop Pest via Exogenous Application of dsRNA

Jonathan Willow [1,*], Liina Soonvald [1], Silva Sulg [1], Riina Kaasik [1], Ana Isabel Silva [2], Clauvis Nji Tizi Taning [3], Olivier Christiaens [3], Guy Smagghe [3] and Eve Veromann [1,*]

[1] Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, 51006 Tartu, Estonia; liina.soonvald@emu.ee (L.S.); silva.sulg@emu.ee (S.S.); riina.kaasik@emu.ee (R.K.)

[2] School of Mental Health and Neuroscience, Faculty of Health, Medicine and Life Sciences, Maastricht University, 6229 ER Maastricht, The Netherlands; silvaai@cardiff.ac.uk

[3] Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, 9000 Ghent, Belgium; tiziclauvis.taningnji@ugent.be (C.N.T.T.); olchrist.christiaens@ugent.be (O.C.); guy.smagghe@ugent.be (G.S.)

* Correspondence: jonathan@emu.ee (J.W.); eve.veromann@emu.ee (E.V.)

Received: 29 September 2020; Accepted: 6 November 2020; Published: 7 November 2020

Simple Summary: An ecologically sustainable strategy for managing the pollen beetle *Brassicogethes aeneus*, a key pest of oilseed rape (*Brassica napus*) in Europe, is greatly needed. Gene silencing via RNA interference, through sprayed applications of target-specific double-stranded RNA, represents a potential alternative to conventional insecticides. We used dsRNA designed to target a vital gene in this pollen beetle species and allowed the beetles to feed on dsRNA-coated oilseed rape buds. We observed a significant silencing of the target gene; and this was followed by a significant, albeit delayed, reduction in pollen beetle survival rate. Further experiments are necessary in order to better understand the potential for developing a dsRNA-spray approach to pollen beetle management.

Abstract: Spray-induced gene silencing (SIGS) is a potential strategy for agricultural pest management, whereby nucleotide sequence-specific double-stranded RNA (dsRNA) can be sprayed onto a crop; the desired effect being a consumption of dsRNA by the target pest, and subsequent gene silencing-induced mortality. Nucleotide sequence-specificity is the basis for dsRNA's perceived biosafety. A biosafe approach to pollen beetle (*Brassicogethes aeneus*) management in oilseed rape (*Brassica napus*) agroecosystems is needed. We examined the potential for SIGS in *B. aeneus*, via bud feeding, a field-relevant dsRNA exposure route. Oilseed rape buds were uniformly treated with dsRNA designed to target *αCOP* in *B. aeneus*. Our model control dsRNA (dsGFP) remained detectable on buds throughout the entire 3 d exposure period. When applied at 5 μg/μL, dsαCOP induced significant *αCOP* silencing 3 d after dietary exposure to buds treated with this dsαCOP concentration. We also observed a trend of increased *αCOP* silencing with increasing concentrations of dsαCOP at both 3 and 6 d. Furthermore, we observed a marginally significant and significant reduction in *B. aeneus* survival at 10 and 15 d, respectively. Our results suggest potential for developing a SIGS approach to *B. aeneus* management—though further experiments are needed to more fully understand this potential.

Keywords: RNA interference; *Meligethes aeneus*; rapeseed; biopesticide; insecticide; Nitidulidae; Coleoptera

1. Introduction

The pollen beetle *Brassicogethes aeneus* Fab. (syn. *Meligethes aeneus*) is a key pest of oilseed rape (*Brassica napus* L.) in Europe. Adult *B. aeneus* overwinter in soil, under vegetation and leaf litter; they emerge in early spring to feed on the pollen and nectar of a variety of blooming plants, and subsequently colonize brassicaceous plants, where they obtain nutrients from reproductive buds and open flowers. After mating, females oviposit into buds, and upon hatching, larvae feed on anthers within buds, eat their way out of the buds, and feed in open flowers, eventually pupating under the soil surrounding the host plant (reviewed in Mauchline et al. [1]). Oilseed rape crops are most susceptible to *B. aeneus* during the green bud stage. Model predictions demonstrate that the extensive bud feeding by *B. aeneus* can result in great economic losses, depending on different factors such as the number of pollen beetles and immigration time [2,3]. Current *B. aeneus* control measures usually occur via the application of synthetic agrochemicals, for example the neonicotinoid insecticide thiacloprid [4,5]. These, however, have shown detrimental effects on nontarget organisms, including hymenopteran parasitoids [6–8], a functional group of critical importance for the biocontrol of *B. aeneus* populations [9,10].

To achieve ecologically sustainable oilseed rape production, an integrated and biosafe scheme for *B. aeneus* management is needed. One biosafe approach to *B. aeneus* management is via conservation biocontrol, where habitats and habitat features required by the parasitoids of *B. aeneus* are preserved or restored in oilseed rape agroecosystems, ideally at both local and regional scales [9–14]. Insecticide use represents another measure for preventing steep yield losses in oilseed rape production. However, to contribute to a biosafe management design, the insecticidal compounds used must be as specific to the target pest as possible.

Gene silencing via RNA interference (RNAi) represents a potential approach to utilize within integrated pest management [15]. As RNAi occurs via double-stranded RNA's (dsRNA) nucleotide sequence-specific mode of action, this control measure has potential species-specificity. RNAi efficacy via sprayable dsRNA, known as a spray-induced gene silencing (SIGS) approach, represents a potential strategy for insect pest management in agriculture, the prospects of which are reviewed in Cagliari et al. [16] and Taning et al. [17]; and this approach has indeed been demonstrated, in both a greenhouse experiment [18] and a field trial [19], against the Colorado potato beetle (*Leptinotarsa decemlineata* Say). In contrast to host-induced gene silencing (HIGS) via the use of an RNAi cultivar, a SIGS approach has the benefit of not requiring the biotechnology or time required for engineering an RNAi cultivar.

We recently targeted the vital gene coatomer subunit alpha (*αCOP*), encoding the αCOP protein, and showed RNAi efficacy in *B. aeneus* via honeywater feeding (Willow et al. In Press), indicating potential for RNAi-based control of *B. aeneus* via dsRNA-contaminated nectar. However, *B. aeneus* also requires the lipid and protein constituents of pollen, which they consume from both buds and open flowers. As mentioned above, the most vulnerable stage of oilseed rape growth, with respect to *B. aeneus*, is the green bud stage; as this is the time when *B. aeneus* females oviposit within buds, and both male and female adult *B. aeneus* feed on pollen within buds in order to acquire lipid and protein constituents. Therefore, it is critical to examine RNAi efficacy via bud feeding in *B. aeneus*.

The aim of the present study was to examine RNAi efficacy via a field-relevant and thus far unexamined dietary exposure route, bud feeding, simulating a SIGS approach by uniformly treating bud epithelia. We expected that, by consuming dsRNA-treated bud epithelial tissue, *B. aeneus* individuals would undergo gene silencing and subsequent gene silencing-induced mortality.

2. Materials and Methods

A selected 222 bp region from *B. aeneus*'s *αCOP* sequence, and a 455 bp region from the *gene green fluorescent protein* (*gfp*) (Table S1), were the basis for in vitro synthesis of dsRNA by AgroRNA (Genolution, Seoul, South Korea). Both dsRNAs were shipped in distilled water (dH2O) at ambient temperature and kept at 5 ± 1 °C once received. The nucleotide sequences of these dsRNAs were complementary to the genes *gfp* (our control, as *gfp* is not present in insects) and *αCOP* (our target gene).

The dsRNAs are hereafter referred to as dsGFP and dsαCOP. The absence of nucleic contaminants in dsRNA products was determined via gel electrophoresis.

Pollen beetles and oilseed rape plants (BBCH 31−32) were both collected from untreated organic oilseed rape fields (beetles: 58.36377°N, 26.66145°E; plants: 58.37389°N, 26.33114°E) in the respective villages of Õssu and Nasja, Tartu County, Estonia. Beetles were kept in ventilated plastic containers, allowed to feed ad libitum on the pollen of oilseed rape flowers, and identified as *B. aeneus* prior to their use in this study. Winter oilseed rape plants were kept in a 3 × 3 m climate room (Flohr Instruments, Utrecht, Netherlands) at 10 °C (70 ± 5% relative humidity and 16:8 h light:dark cycle), in order to maintain them at a low growth stage. Before starting the experiment, the temperature in the climate room was increased to 18 °C.

Leading racemes, ranging 18-24 cm in length, were removed from oilseed rape plants during the green bud stage (BBCH 53-55). Treatments were prepared from dsRNA, dH2O and a constant concentration (180 ppm) of the surfactant Triton X-100 (Fisher Bioreagents) and were vortexed prior to soaking bud clusters. There were three treatments in total, including dsGFP at 5 µg/µL, and dsαCOP at 2.5 and 5 µg/µL. Bud clusters were swirled in treatment solutions for 1 min (this action and duration were both required in order to reliably break the surface tension caused by the waxiness of the bud epithelium), and subsequently allowed to air dry for 1 h. The cut tip of each raceme was then kept underwater, individually, in modified plastic labware (height 12 cm). For each sample, six *B. aeneus* were released within a transparent-white organza fabric bag (20 × 30 cm) that was fastened with string to the neck of the labware. The beetles were allowed to feed ad libitum on the treated buds for 3 d. The 3 d exposure to the dsRNA-treated bud took place in the climate room at 18 °C, 70 ± 5% relative humidity and 16:8 h light:dark cycle.

For each experimental replicate, each treatment was initially allocated five samples; and the experiment was replicated three times. Beetles were not disturbed during their 3 d exposure to dsRNA treatments; thus, survival monitoring began after the 3 d exposure period, and thereafter occurred every 24 ± 1 h. After 3 d of feeding on treated buds, bud-feeding setups were dismantled, and the beetles were transferred to transparent, polystyrene, ventilated insect breeding dishes (diameter 10 cm × height 4 cm) (SPL Life Sciences, Gyeonggi-do, South Korea), hereafter referred to as cages; and the beetles were kept in their respective samples. After this relocation to the laboratory, the beetles were maintained in an incubator (Sanyo MLR-351H, Osaka, Japan) and provisioned daily with fresh untreated oilseed rape flowers, and a dental cotton roll soaked with dH2O. Survival monitoring for each experimental replicate lasted 15 d post-exposure to dsRNA. Escaped beetles were accounted for in the statistical analysis, and any sample where more than two beetles escaped were removed from the analysis at the start of survival monitoring (n = 14 (83 beetles), 14 (80 beetles) 15 (87 beetles), for dsGFP at 5 µg/µL, dsαCOP at 2.5 µg/µL and dsαCOP at 5 µg/µL, respectively).

Relative gene expression analysis was performed for all treatments via quantitative polymerase chain reaction (qPCR). For each experimental replicate, at 3 d (upon dismantling the bud-feeding setups), and again 6 d after the start of bud feeding, one cage of six live beetles was randomly removed from each treatment (qPCR sample n = 3 per treatment). The removal of beetles for qPCR was accounted for in the statistical analysis. Beetles used for qPCR were immediately placed in their respective Eppendorf tubes and homogenized using a sterilized plastic pestle designed for Eppendorf tubes, in 600 µL of RLT buffer (with added 10 µL of β-mercaptoethanol), and stored at −80 °C until analysis. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands); and RNA concentration and purity were assessed using a nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA), with purity further verified via gel electrophoresis. Genomic DNA was removed using a Turbo DNA-Free Kit (Invitrogen, Carlsbad, USA), following manufacturer's instructions. The cDNA was reverse transcribed from 1 µg of total RNA using a FIREScript RT cDNA Synthesis Kit (Solis BioDyne, Tartu, Estonia); and qPCR was performed in the Quantistudio 5 Real-Time PCR System (Applied Biosystems, Foster City, USA). The reaction included 4 µL of 5xHOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia), 0.5 µL of both 10 µM forward and reverse primers

(Microsynth, Balgach, Switzerland; Table S2), 14 µL of PCR-grade water and 500 ng of cDNA, in a total volume of 20 µL. Amplification conditions were 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 58 °C, and ending with a melting curve analysis with a temperature range of 60-95 °C. The reactions were set up in 384-well PCR plates, in triplicate. The two housekeeping genes *ribosomal protein S3* (*rps3*) and *actin* (*act*) were used to normalize the data. Primer amplification efficiencies were determined via a cDNA dilution series. Primer sequences and amplification efficiencies are shown in Table S2. Relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. A no-template control and a no reverse transcriptase control were included in the assay.

To confirm that dsRNA remained stable over the chosen experimental duration of 3 d, RT-PCR was performed to confirm the presence of dsRNA on buds at the four time points of 1 h, and 1, 2 and 3 d post dsRNA-application. For this, we applied dsGFP at both 2.5 and 5 µg/µL, both treating- and maintaining these bud clusters in the same manner as was performed for bud feeding. At each time point of interest, total RNA was extracted from 0.1 g of buds, using an RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's protocol; and RNA concentration was quantified, and purity assessed, using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), with purity further verified via gel electrophoresis. The detection of dsGFP was performed from 500 ng of RNA, using a SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) with gfp-specific primers at 10 pmol (Table S2). Both 200 ng and undiluted dsGFP were used as positive controls. Samples were run on an Eppendorf Mastercycler (Hamburg, Germany) under the following conditions: 10 min at 75 °C, 30 min at 55 °C, 2 min at 94 °C, 40 cycles of 15 s at 94 °C, 30 s at 55 °C, 1 min at 68 °C, and 5 min at 68 °C. In order to denature the secondary structure of the dsGFP, a denaturing step of 10 min at 75 °C was added to the protocol. The amplified fragments were analyzed via gel electrophoresis.

Regarding both survival- and gene expression analysis, comparisons were made between dsGFP and both concentrations of dsαCOP, and between the two concentrations of dsαCOP. For survival analysis, the homogeneity of variance and normality of data distributions were determined using the Levene and Shapiro–Wilk tests, respectively. Since the data were overall not normally distributed, the Kruskal–Wallis test was used as a nonparametric alternative to ANOVA; this was followed by the Wilcoxon rank-sums test, with Bonferroni correction, for post hoc pairwise comparisons. For gene expression analysis, comparisons were made using Welch's t-test. All statistical analyses were done in R v3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

RT-PCR results confirmed the presence and stability of dsRNA on buds, over the entire 3 d of exposure to treatments, for both dsGFP concentrations examined (Figure 1). In the insects that fed upon the buds, our obtained qPCR results showed a trend of reduced αCOP expression, with an increasing concentration of dsαCOP application, at both 3 and 6 d (Figure 2). At 3 d, we observed a 49% mean decrease in αCOP expression in the dsαCOP 2.5 µg/µL treatment ($t = 1.25$, $df = 2.87$, $p = 0.3$), and a 72% mean decrease in the dsαCOP 5 µg/µL treatment ($t = 3.09$, $df = 3.99$, $p = 0.037$). At 6 d, we observed a 19% mean decrease in αCOP expression in the dsαCOP 2.5 µg/µL treatment ($t = 0.79$, $df = 3.13$, $p = 0.49$), and a 48% mean decrease in the dsαCOP 5 µg/µL treatment ($t = 2.11$, $df = 2.88$, $p = 0.13$).

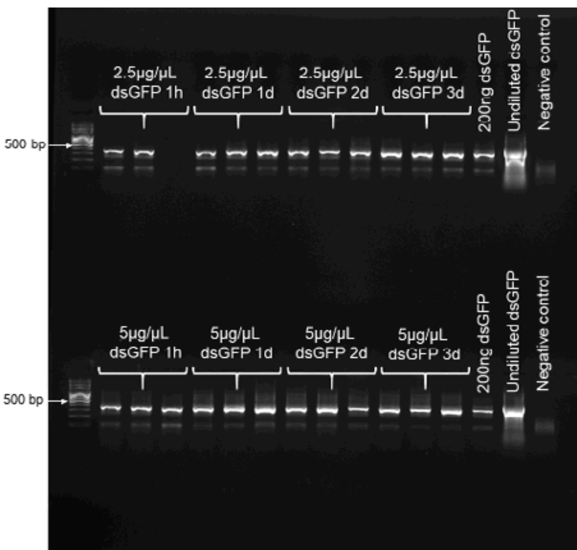


Figure 1. RT-PCR results showing presence of dsRNA (dsGFP applied at both 2.5 and 5 µg/µL) on bud tissue at 1 h, and 1, 2 and 3 d post dsRNA-application.

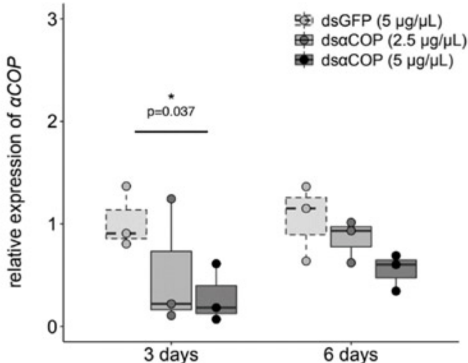


Figure 2. Results of qPCR, showing the relative expression of *αCOP* in *Brassicogethes aeneus* at 3 and 6 d, comparing target treatments (dsαCOP at 2.5 and 5 µg/µL) to dsGFP control. Asterisk (*) indicates significant difference between treatments (analyzed using Welch's *t*-test).

Regarding survival, we began observing a significant effect of treatment at 10 d (10-14 d: chi-square = 7.8, df = 2, $p = 0.02$; 15 d: chi-square = 10.38, df = 2, $p = 0.006$; Figure 3). After correcting for pairwise comparisons, mortality in the dsαCOP 5 µg/µL treatment was marginally significant at 10-14 d ($p = 0.056$), becoming significant at 15 d ($p = 0.021$). Survival in this treatment slowly fell from 100% (4 d) to 99 (5 d), 97 (6 d), 96 (7 d), 94 (8 d), 92 (9 d), 88 (10 d) and 84% (15 d). No significant effect on survival was observed for the dsαCOP 2.5 µg/µL treatment, survival falling from 100% (7 d) to 98% (8 d), where it settled. No mortality was observed in the dsGFP treatment. After the 3 d treatment–exposure period, all bud clusters had numerous buds incised, with both anthers and bud epithelium consumed.

Together with the fact that all caged beetles survived over the entire 3 d treatment–exposure period, which indicates that all beetles fed on dsRNA-treated bud tissue.

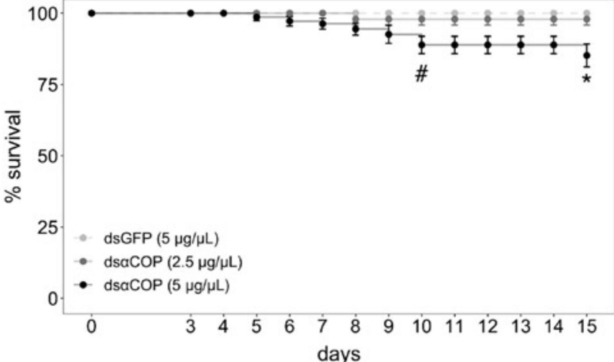


Figure 3. Survival (%) of *Brassicogethes aeneus* in each treatment, accounting for all three experimental replicates. The hash symbol (#) indicates a significant effect of treatment (chi-square). Asterisk (*) indicates a statistically significant difference between dsαCOP treatment and dsGFP control ($p < 0.05$; Kruskal–Wallis test, followed by Wilcoxon rank-sums test with Bonferroni correction).

4. Discussion

We provide laboratory evidence suggesting some potential for incorporating a SIGS approach within integrated *B. aeneus* management. We observed marginally significant and significant reductions in survival at 10 and 15 d, respectively, as well as a trend of lower relative expression of αCOP with increasing concentrations of dsαCOP, indicating that the mortality observed in our *B. aeneus* RNAi assays were a result of silencing the target gene αCOP. However, while we suggest some potential for SIGS in *B. aeneus* management via dsRNA-treated buds, there is certainly more to be explored here before this idea can be further developed.

We treated the oilseed rape bud epithelia, where *B. aeneus* chews through and consumes this tissue mostly to obtain nutrients from the anthers within. If *B. aeneus*-specific dsRNA formulations were to exhibit properties that allow the dsRNA to absorb past the bud epithelium, and into the anthers within, a SIGS approach utilizing such formulations would likely show greater RNAi efficacy. Furthermore, as *B. aeneus* development begins within the reproductive bud, and larvae are in their late first- or early second instar when oilseed rape buds blossom, it is plausible that such an approach could target both larval and adult *B. aeneus* simultaneously. Studies examining the potential for RNAi in *B. aeneus* larvae, via the use of co-formulants to enhance the transport of dsRNA past the bud epithelium, would be of great value to our understanding of the potential for *B. aeneus* management via SIGS. Moreover, as adults of *B. aeneus* appear to show modest RNAi-sensitivity, it would be of great value to investigate whether *B. aeneus* larvae are more RNAi-sensitive than adults, as this would further guide research endeavors to target this larval life-stage of this species.

With regard to adult bud feeding, it is possible that a duration of dsRNA exposure greater than 3 d is necessary for inducing RNAi at a quicker rate and in a higher percent of the sample. While a longer exposure duration is likely to be especially crucial, this would be limited by the duration of oilseed rape's bud stage, as well as the total length of time that the applied dsRNA-based insecticide remains present and stable on- and in the oilseed rape bud under field conditions. Both the duration of bud stage and the duration of dsRNA stability will undoubtedly vary depending on environmental conditions. However, the results of a small-scale field trial near Ljubljana, Slovenia, using sprayed naked dsRNA for the control of *L. decemlineata*, showed that the sprayed dsRNA remained stable

long enough to have the desired effect under natural environmental conditions [19]. As oilseed rape's flowering structures (i.e., reproductive buds, bloomed flowers) are in constant development and senescence, the possibility of requiring successive dsRNA spray applications must be considered.

While RNAi will likely never result in target pest mortality as quickly as seen in some other (e.g., neurotoxic) insecticides, there are great benefits to using dsRNA-based insecticides due to the associated biosafety to nontarget organisms, stemming from its unique mode of action. Moreover, there remains potential for increasing speed-to-effect via co-formulants (e.g., nanoparticles) that may improve dsRNA-uptake and RNAi efficiency [20,21]. Improving the efficacy of this technology, with regard to *B. aeneus* control via bud feeding, will be a critical aspect to explore if we are to more fully realize the potential for using a SIGS approach in *B. aeneus* management.

5. Conclusions

Ecologically sustainable control measures are greatly needed in oilseed rape production; and dsRNA-based insecticides, due to their mode of action, represent a potentially species-specific complement to other biosafe measures (e.g., conservation biocontrol) for managing *B. aeneus*. While our work suggests potential for developing a SIGS approach for implementation in *B. aeneus* management, further experiments are needed to more fully explore the potential for incorporating this approach. Focal points necessary for progress here include determining the potential for enhancing adult *B. aeneus* control efficacy, and that of both larval and adult *B. aeneus* simultaneously, via the use of co-formulants to enhance the transport of dsRNA to anthers within oilseed rape buds. Other important focus points include determining the total duration at which exogenously-applied dsRNA remains viable both on- and within the reproductive bud; and determining the optimal duration of exposure to dsRNA-treated buds, taking into account the time-to-flowering of buds. Finally, it will be critical to determine the overall feasibility of using a SIGS approach in the context of a potential requirement for successive dsRNA spray applications.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/11/769/s1, Table S1: *Green fluorescent protein* (*gfp*) and *Brassicogethes aeneus coatomer subunit alpha* (*αCOP*) regions for in vitro dsRNA synthesis. Table S2: Primer amplification efficiencies of genes used in the present study.

Author Contributions: Conceptualization, J.W., E.V., G.S.; methodology, J.W., L.S., S.S., R.K., A.I.S., C.N.T.T., O.C., G.S., E.V.; investigation, J.W., L.S., S.S., R.K.; formal analysis, A.I.S., J.W.; validation, A.I.S., J.W., G.S., E.V., C.N.T.T., O.C.; data curation, J.W., A.I.S.; visualization, A.I.S., J.W.; resources, E.V.; funding acquisition, E.V.; supervision, J.W., E.V., G.S.; writing—original draft, J.W.; writing—review and editing, J.W., E.V., C.N.T.T., O.C., A.I.S., S.S., L.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the institutional Research Funding project no IUT36-2 of the Estonian Research Council, the European ERA-NET C-IPM project "IPM4Meligethes" (project no 3G0H0416), the Special Research Fund (BOF) of Ghent University and the Research Foundation – Flanders (FWO-Vlaanderen).

Acknowledgments: We thank the farm-owner whose land was used for collecting pollen beetles and oilseed rape plants. Jonathan Willow is the recipient of a Ph.D. grant from the European Social Fund for Doctoral Students, as well as the Internationalisation Programme DoRa (carried out by the Archimedes Foundation). Olivier Christiaens is a recipient of a postdoctoral fellowship from the Research Foundation – Flanders (FWO-Vlaanderen).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mauchline, A.L.; Hervé, M.R.; Cook, S.M. Semiochemical-based alternatives to synthetic toxicant insecticides for pollen beetle management. *Arthropod Plant Interact.* **2017**, *12*, 835–847. [CrossRef]
2. Hansen, L.M. Economic damage threshold model for pollen beetles (Meligethes aeneus F.) in spring oilseed rape (Brassica napus L.) crops. *Crop. Prot.* **2004**, *23*, 43–46. [CrossRef]
3. Ga, R.L.; Riggi, L.G.; Ekbom, B.; Malsher, G.; Rusch, A.; Bommarco, R. Interactive effects of pests increase seed yield. *Ecol. Evol.* **2016**, *6*, 2149–2157. [CrossRef]

4. Seidanglanz, M.; Poslušná, J.; Kolařík, P.; Rotrekl, J.; Hrudová, E.; Tóth, P. Negative Correlations between the susceptibilities of Czech and Slovak pollen beetle populations to lamba-cyhalothrin and chlorpyrifos-ethyl in 2014 and 2015. *Plant Prot. Sci.* **2017**, *53*, 108–117.
5. Kaiser, C.; Jensen, K.-M.V.; Nauen, R.; Kristensen, M. Susceptibility of Danish pollen beetle populations against λ-cyhalothrin and thiacloprid. *J. Pest Sci.* **2017**, *91*, 447–458. [CrossRef]
6. Sugiyama, K.; Katayama, H.; Saito, T. Effect of insecticides on the mortalities of three whitefly parasitoid species, Eretmocerus mundus, Eretmocerus eremicus and Encarsia formosa (Hymenoptera: Aphelinidae). *Appl. Èntomol. Zool.* **2011**, *46*, 311–317. [CrossRef]
7. Jans, D. Toxicity to the Parasitoid Wasp Aphidius Rhopalosiphi (DeStephani-Perez) (Hymenoptera: Braconidae) Using an Extended Laboratory Test with Aged Residues on Maize–Thiacloprid OD 240 g/L–Final Report. 2012, pp. 262–266. Available online: https://www.efsa.europa.eu/en/consultations/call/180123 (accessed on 24 September 2020).
8. Willow, J.; Silva, A.I.; Veromann, E.; Smagghe, G. Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. *PLoS ONE* **2019**, *14*, e0212456. [CrossRef]
9. Thies, C. Landscape Structure and Biological Control in Agroecosystems. *Science* **1999**, *285*, 893–895. [CrossRef]
10. Kaasik, R.; Kovacs, G.; Toome, M.; Metspalu, L.; Veromann, E. The relative attractiveness of Brassica napus, B. rapa, B. juncea and Sinapis alba to pollen beetles. *BioControl* **2013**, *59*, 19–28. [CrossRef]
11. Scheid, B.E.; Thies, C.; Tscharntke, T. Enhancing rape pollen beetle parasitism within sown flower fields along a landscape complexity gradient. *Agric. For. Èntomol.* **2011**, *13*, 173–179. [CrossRef]
12. Rusch, A.; Suchail, S.; Valantin-Morison, M.; Sarthou, J.-P.; Roger-Estrade, J. Nutritional state of the pollen beetle parasitoid Tersilochus heterocerus foraging in the field. *BioControl* **2012**, *58*, 17–26. [CrossRef]
13. Berger, J.S.; Birkhofer, K.; Hanson, H.I.; Hedlund, K. Landscape configuration affects herbivore–parasitoid communities in oilseed rape. *J. Pest Sci.* **2018**, *91*, 1093–1105. [CrossRef] [PubMed]
14. Hatt, S.; Uyttenbroeck, R.; Lopes, T.; Chen, J.L.; Piqueray, J.; Monty, A.; Francis, F. Effect of flower traits and hosts on the abundance of parasitoids in perennial multiple species wildflower strips sown within oilseed rape (Brassica napus) crops. *Arthropod Plant Interact.* **2017**, *12*, 787–797. [CrossRef]
15. Mezzetti, B.; Smagghe, G.; Arpaia, S.; Christiaens, O.; Dietz-Pfeilstetter, A.; Jones, H.D.; Kostov, K.; Sabbadini, S.; Opsahl-Sorteberg, H.-G.; Ventura, V.; et al. RNAi: What is its position in agriculture? *J. Pest Sci.* **2020**, *93*, 1125–1130. [CrossRef]
16. Cagliari, D.; dos Santos, E.A.; Dias, N.; Zotti, G.S. Nontransformative Strategies for RNAi in Crop Protection. Modulating Gene Expression-Abridging the RNAi and CRISPR-Cas9 Technologies. 2018. Available online: https://www.intechopen.com/books/modulating-gene-expression-abridging-the-rnai-and-crispr-cas9-technologies/nontransformative-strategies-for-rnai-in-crop-protection (accessed on 19 August 2020).
17. Taning, C.N.T.; Arpaia, S.; Christiaens, O.; Dietz-Pfeilstetter, A.; Jones, H.; Mezzetti, B.; Sabbadini, S.; Sorteberg, H.; Sweet, J.; Ventura, V.; et al. RNA-based biocontrol compounds: current status and perspectives to reach the market. *Pest Manag. Sci.* **2019**, *76*, 841–845. [CrossRef] [PubMed]
18. Miguel, K.S.; Scott, J.G. The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Manag. Sci.* **2015**, *72*, 801–809. [CrossRef] [PubMed]
19. Petek, M.; Coll, A.; Ferenc, R.; Razinger, J.; Gruden, K. Validating the Potential of Double-Stranded RNA Targeting Colorado Potato Beetle Mesh Gene in Laboratory and Field Trials. *Front. Plant Sci.* **2020**, *11*, 11. [CrossRef] [PubMed]
20. Christiaens, O.; Petek, M.; Smagghe, G.; Taning, C.N.T. The Use of Nanocarriers to Improve the Efficiency of RNAi-Based Pesticides in Agriculture. *Nanopesticides* **2020**, 49–68. [CrossRef]
21. Yan, S.; Ren, B.; Shen, J. Nanoparticle-mediated double-stranded RNA delivery system: A promising approach for sustainable pest management. *Insect Sci.* **2020**, 1–14. [CrossRef] [PubMed]

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

V

V **Willow, J.**, Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I., Taning, C.N.T., Christiaens, O., Smagghe, G., Veromann, E. 2021. Anther feeding-induced RNAi in *Brassicogethes aeneus* larvae. (Submitted to Front. Agron.)

Anther feeding-induced RNAi in Brassicogethes aeneus larvae

Jonathan Willow[1*], Liina Soonvald[1], Silva Sulg[1], Riina Kaasik[1], Ana I. Silva[2], Clauvis N. Taning[3], Olivier Christiaens[3], Guy Smagghe[3], Eve Veromann[1]

[1]Chair of Plant Health, Estonian University of Life Sciences, Estonia, [2]School for Mental Health and Neuroscience, Maastricht University, Netherlands, [3]Department of Plants and Crops, Ghent University, Belgium

Submitted to Journal:
Frontiers in Agronomy

Specialty Section:
Pest Management

Article type:
Brief Research Report Article

Manuscript ID:
633120

Received on:
24 Nov 2020

Frontiers website link:
www.frontiersin.org

In review

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

JW and EV conceived the study. JW, LS, SS, RK and EV designed the methods. JW, LS, SS and RK performed the investigation. JW and AIS analyzed and visualized the data. JW wrote the original draft. All authors contributed to revisions. All authors approved the final manuscript.

Keywords

RNA Interference, RNAi, Brassicogethes aeneus, Meligethes aeneus, Brassica napus, Biopesticide, insecticide, coleoptera

In review

Abstract

Word count: 244

The biosafety aspect of applying double-stranded RNA (dsRNA) in crop pest management is rooted in dsRNA's mode of action, that being nucleotide sequence-specificity to a particular region of a messenger RNA (mRNA), against which the insecticidal dsRNA is designed. This prominent and promising class of insecticide therefore has the potential to target a single pest species while conferring negligible effect on nontarget organisms. Recent studies examining the effect of target-specific dsRNA in adults of the pollen beetle Brassicogethes aeneus, a major pest of oilseed rape (Brassica napus) crops in Europe, suggest potential for developing a gene silencing approach within integrated B. aeneus management. The present study examines the efficacy of target-specific dsRNA on target-mRNA silencing, and subsequent gene silencing-induced mortality, in B. aeneus larvae, as this life stage represents a critical target for achieving optimal integrated B. aeneus control. Treatment applications occurred via feeding on dsRNA-treated anthers for 3 d. We observed variable gene silencing efficacy, all target treatments having a significant or marginally significant effect after 3 d of dsRNA feeding, with greater variability at 6 d. These results further validated significant gene silencing-induced mortality observed for one of the target treatments. Moreover, gene silencing-induced mortality occurred at a quicker rate in B. aeneus larvae, compared to what has been previously observed in B. aeneus adults. Finally, we consider refinements that must be made to B. aeneus larval bioassay setups, to promote and strengthen future larval studies regarding this important crop pest species.

Contribution to the field

The pollen beetle Brassicogethes aeneus is a major oilseed rape pests throughout Europe. Widespread cultivation of oilseed rape, together with high demand for sustainable alternatives to synthetic insecticides, requires investigations into the potential for RNAi-based management of B. aeneus. Recent studies on B. aeneus adults suggest potential for developing RNAi strategies targeting this species. However, the larval stage represents a crucial target, since controlling B. aeneus larvae could reduce the numbers of both pupating larvae and emerging next-generation adults. Here we examined the potential for developing an RNAi-based approach targeting B. aeneus larvae. Our investigation was performed by allowing B. aeneus larvae to feed on dsRNA-treated oilseed rape anthers for 3 days; and subsequently examining target gene silencing and larval survival. We provide the first evidence that B. aeneus larvae represent a promising RNAi target in oilseed rape protection, via a highly field relevant exposure route. We observed target gene silencing across all target treatments at 3 days, as well as corresponding significant mortality at 4 and 5 days in one treatment. We also provide several suggestions regarding how future bioassays with B. aeneus larvae can reduce control mortality by using setups that minimize potential stressors to B. aeneus larvae.

Funding statement

The authors acknowledge financial support from the institutional Research Funding project no IUT36-2 of the Estonian Research Council, the ERA-NET C-IPM project "IPM4Meligethes" (project no 3G0H0416 and 8T160091), the Special Research Fund (BOF) of Ghent University and the Research Foundation – Flanders (FWO-Vlaanderen).

Ethics statements

Studies involving animal subjects

Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects

Generated Statement: No human studies are presented in this manuscript.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.

Data availability statement

Generated Statement: The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

Anther feeding-induced RNAi in *Brassicogethes aeneus* larvae

Jonathan Willow[1]*, Liina Soonvald[1], Silva Sulg[1], Riina Kaasik[1], Ana Isabel Silva[2], Clauvis Nji Tizi Taning[3], Olivier Christiaens[3], Guy Smagghe[3], Eve Veromann[1]*

[1]Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu, Estonia

[2]School for Mental Health and Neuroscience, Department of Psychiatry and Neuropsychology, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, Netherlands

[3]Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

*** Correspondence:**
Corresponding Authors
jonathan@emu.ee; eve.veromann@emu.ee

Keywords: RNA interference, RNAi, *Brassicogethes aeneus*, *Meligethes aeneus*, *Brassica napus*, biopesticide, insecticide, Coleoptera

In review

Abstract

The biosafety aspect of applying double-stranded RNA (dsRNA) in crop pest management is rooted in dsRNA's mode of action, that being nucleotide sequence-specificity to a particular region of a messenger RNA (mRNA), against which the insecticidal dsRNA is designed. This prominent and promising class of insecticide therefore has the potential to target a single pest species while conferring negligible effect on nontarget organisms. Recent studies examining the effect of target-specific dsRNA in adults of the pollen beetle *Brassicogethes aeneus*, a major pest of oilseed rape (*Brassica napus*) crops in Europe, suggest potential for developing a gene silencing approach within integrated *B. aeneus* management. The present study examines the efficacy of target-specific dsRNA on target-mRNA silencing, and subsequent gene silencing-induced mortality, in *B. aeneus* larvae, as this life stage represents a critical target for achieving optimal integrated *B. aeneus* control. Treatment applications occurred via feeding on dsRNA-treated anthers for 3 d. We observed variable gene silencing efficacy, all target treatments having a significant or marginally significant effect after 3 d of dsRNA feeding, with greater variability at 6 d. These results further validated significant gene silencing-induced mortality observed for one of the target treatments. Moreover, gene silencing-induced mortality occurred at a quicker rate in *B. aeneus* larvae, compared to what has been previously observed in *B. aeneus* adults. Finally, we consider refinements that must be made to *B. aeneus* larval bioassay setups, to promote and strengthen future larval studies regarding this important crop pest species.

Introduction

Double-stranded RNA (dsRNA) is a prominent class of insecticide, and can be applied to crops, with potentially no impact on nontarget taxa. Cagliari et al. (2018) and Taning et al. (2019) review the prospects of an approach in which dsRNA can be sprayed onto crops in order to manage target pests.

37 Indeed, this approach has been demonstrated in both greenhouse (Miguel and Scott, 2015) and field
38 (Petek et al., 2020), for the control of Colorado potato beetle (*Leptinotarsa decemlineata* Say). The
39 biosafety of dsRNA-based insecticides lies in their mode of action against the target pest species, that
40 being nucleotide sequence-specificity. Indeed, the structure of a dsRNA for use in crop protection
41 can be designed to target a specific region of a messenger RNA (mRNA), potentially resulting in
42 RNA interference (RNAi)-induced gene silencing and subsequent mortality in the target species.

43 Recent studies suggest potential for developing an RNAi approach for use in integrated management
44 of a major oilseed rape (*Brassica napus* L.) pest, the pollen beetle *Brassicogethes aeneus* Fab. (syn.
45 *Meligethes aeneus*), via field relevant routes of exposure, including consumption of dsRNA-treated
46 oilseed rape buds (Willow et al., 2020a) and anthers (Willow et al., Under Review); as well as
47 dsRNA-treated honey water, representing dsRNA-contaminated nectar (Willow et al., 2020b). The
48 above-mentioned studies were performed on *B. aeneus* adults, the adult stage being the typical focus
49 of insecticide bioassays regarding this pest species (but see Melander et al., 2003).

50 *B. aeneus* development starts inside the reproductive bud, where hatched larvae feed on the anthers
51 within, followed by emergence of late first- and early second instar larvae that proceed to feed on the
52 pollen and nectar of open flowers. Therefore, it is plausible that dsRNA application during oilseed
53 rape's flowering stage could additionally target larval *B. aeneus*, and in turn reduce not only
54 abundances of pupating larvae and overwintering next-generation adults, but oilseed rape yield losses
55 as well. The present study examines RNAi in *B. aeneus* larvae, via the consumption of dsRNA-
56 treated anthers. We expected consumption of *B. aeneus*-specific dsRNA to result in reduced target
57 mRNA expression, followed by gene silencing-induced mortality.

In review

58 **Methods**

59 A 222 bp region of *B. aeneus*'s *coatomer subunit alpha* (*αCOP*) sequence, and a 455 bp region of
60 *green fluorescent protein* (*gfp*; **Supplementary Table 1**; Willow et al., 2020a, 2020b), were the basis
61 for *in vitro* synthesis of two corresponding dsRNAs (AgroRNA, Seoul, South Korea). Both dsRNAs
62 were shipped in distilled water (dH_2O) and kept at 5 ± 1 °C upon reception. The absence of nucleic
63 contaminants in these dsRNAs, hereafter respectively called dsαCOP and dsGFP (control, since *gfp*
64 is not present in insects) was verified via gel electrophoresis.

65 Pollen beetle larvae were collected via collection of oilseed rape flowers from an untreated field
66 (58.36377°N, 26.66145°E) in the village of Õssu, Tartu County, Estonia. Flowers were transported to
67 the lab and examined for presence of pollen beetle larvae. Only late first- and early second instar
68 larvae identified as *B. aeneus* via Osborne (1965) were used in the study. *B. aeneus* larvae were
69 immediately transferred to transparent, polystyrene, ventilated insect breeding dishes (diameter 10
70 cm x height 4 cm; SPL Life Sciences, Gyeonggi-do, South Korea), hereafter referred to as cages.
71 Eight randomly chosen *B. aeneus* larvae were gently placed in each cage, using a fine paintbrush to
72 avoid any mechanical damage to larvae, and were immediately provisioned with their respective
73 dietary treatment.

74 There were four treatments, including dsαCOP at 0.5, 2.5 and 5 µg/µL, and dsGFP at 5 µg/µL. Each
75 treatment was allocated ten cages (n = 10; 80 larvae per treatment). Treatments were provided as *ad*
76 *libitum* access to dsRNA-treated anthers of oilseed rape flowers. Petals were removed from flowers,
77 and anthers were soaked in treatment solution for 15 s and allowed to air dry. Treatment solutions
78 consisted of dsRNA, dH_2O and the surfactant Triton X-100 (always at 180 ppm; Fisher Bioreagents,
79 Leicestershire, UK), and were vortexed prior to treating anthers. After treatment provision, cages

This is a provisional file, not the final typeset article

were placed in a climate chamber (Sanyo MLR-351H, Osaka, Japan) at 20 °C, 70% relative humidity and 16:8 h light:dark cycle. Freshly-treated anthers were replaced every 24 ± 1 h for 3 d. After 3 d, *B. aeneus* larvae were allowed to feed *ad libitum* on untreated oilseed rape anthers, which were replaced every 24 ± 1 h for a subsequent 4 d. Survival monitoring occurred over a total of 7 d, and dead larvae were removed from cages daily. After the first 24 h of the experiment, any dead larvae were removed from the experiment, since at this time no mortality could be attributed to RNAi, but rather stress from manipulations and changing conditions. These mortalities after 1 d were few, and were accounted for in the statistical analysis. One *B. aeneus* larva was also removed from the study (dsαCOP 0.5 µg/µL treatment, at 5 d) due to predation by a dipteran larva that was inadvertently introduced to a cage when providing untreated oilseed rape flowers *B. aeneus* larvae. This RNAi-unrelated loss was also accounted for in the statistical analysis.

At 3 and 6 d after the start of the experiment, 15 larvae per treatment were randomly removed from cages, and relative mRNA expression was analyzed via quantitative polymerase chain reaction (qPCR; $n = 3$ per time point, per treatment; 5 larvae pooled per sample). Removal of larvae for qPCR was accounted for in the statistical analysis of survival. Larvae used for qPCR were immediately placed in Eppendorf tubes. Samples were homogenized in 600 µL of RTL buffer (with 10 µL of β-mercaptoethanol added), using a sterile plastic pestle designed for Eppendorf tubes, and stored at −80 °C until analysis. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA concentration and purity was assessed via NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA); and absence of nucleic contaminants was further verified via gel electrophoresis. Genomic DNA was removed via Turbo DNA-Free Kit (Invitrogen, Carlsbad, USA). Reverse transcription of cDNA was performed via FIREScript RT cDNA Synthesis Kit (Solis BioDyne, Tartu, Estonia), using 1 µg of total RNA. The qPCR was performed in Quantistudio 5 Real-Time PCR System (Applied BioSciences, Foster City, USA); and the reaction mixture consisted of 4 µL of 5xHOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne), 0.5 µL of 10 µM forward and reverse primers (**Supplementary Table 2**; Willow et al., 2020a, 2020b; Microsynth, Balgach, Switzerland), 14 µL of nuclease-free water and 1 µg of cDNA, in a total volume 20 µL. Amplification conditions were 15 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 58 °C and ending with a melting curve analysis (range 60−95 °C). Reactions were organized, in triplicate, in a 384-well PCR plate. The housekeeping genes *actin* (*act*) and *ribosomal protein S3* (*rps3*) were used to normalize target gene levels. Primer amplification efficiencies were calculated via cDNA dilution series (**Supplementary Table 2**). Relative *αCOP* expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. A no-template- and no-reverse-transcriptase control were both included in the assay.

For both survival- and gene expression analysis, comparisons were made between the dsGFP control and dsαCOP at 0.5, 2.5 and 5 µg/µL. For survival analysis, homogeneity of variance and normality of data distributions were respectively determined via Levene- and Shapiro−Wilk tests. As the data were overall not normally distributed, we used the Kruskal−Wallis test as a nonparametric alternative to ANOVA, followed by the Wilcoxon rank-sums test with *post hoc* Bonferroni correction for multiple comparisons. Comparisons regarding gene expression were made via Welch's t-test. All statistical analyses were performed in R v3.6.3 (R Foundation for Statistical Computing, Vienna, Austria.

Results

After 3 d of feeding on dsRNA-treated oilseed rape anthers, *B. aeneus* larvae showed 57% ($t = 2.46$, $df = 2.94$, $p = 0.093$), 77% ($t = 3.25$, $df = 3.16$, $p = 0.044$) and 83% ($t = 3.93$, $df = 2.17$, $p = 0.052$) mean reductions in *αCOP* expression, respectively for dsαCOP 0.5, 2.5 and 5 µg/µL treatments,

125 compared to the dsGFP 5 µg/µL control treatment (**Figure 1**). At 6 d after the start of the experiment,
126 *B. aeneus* larvae showed no reduction in *αCOP* expression, and more variability within treatments.

127 Survival monitoring showed significant reductions in survival, in larvae fed dsαCOP at 2.5 µg/µL, at
128 4 d (79% survival, $df = 3$, $p = 0.041$) and 5 d (63% survival, $p = 0.02$) after the start of the
129 experiment, followed by marginal significance (47% survival, $p = 0.07$) at 6 d, compared to the
130 dsGFP 5 µg/µL control treatment (**Figure 2**). At 7 d, survival of dsGFP control larvae dropped to
131 49% (dsαCOP 0.5 µg/µL = 39% survival; dsαCOP 2.5 µg/µL = 21% survival; dsαCOP 5 µg/µL =
132 62% survival).

133 **Discussion**

134 In a previous experiment comparing short-term- to chronic dsRNA feeding, *B. aeneus* adults that fed
135 for 3 d on dsαCOP 2.5 µg/µL-treated anthers did not show significantly reduced survival (Willow et
136 al., Under Review). A significant reduction in *B. aeneus* adult survival was observed rather after 8 d
137 of chronic feeding on dsαCOP 2.5 µg/µL-treated anthers, compared to the present study which
138 showed significantly reduced survival at 4 d in larvae fed dsαCOP 2.5 µg/µL-treated anthers for 3 d.
139 Thus, we here observed quicker RNAi-induced mortality in *B. aeneus* larvae, compared to what has
140 been previously observed in *B. aeneus* adults that were fed comparable dsαCOP treatments. In the
141 present study, significant *αCOP* silencing at 3 d, in *B. aeneus* larvae fed dsαCOP at 2.5 µg/µL,
142 substantiates the interpretation that this significant mortality was indeed the result of *αCOP* silencing.
143 The above-mentioned *B. aeneus* adult study also showed significantly greater mortality in beetles
144 chronically fed oilseed rape anthers treated with dsαCOP (at both 0.5 and 2.5 µg/µL concentrations),
145 compared to that which was observed for short-term (3 d) treatments. In another previous study, *B.*
146 *aeneus* adults that fed on dsαCOP 2.5 µg/µL-treated buds for 3 d did not show significant mortality
147 nor significant reduction (albeit 49%) in *αCOP* expression; yet did show significant *αCOP* silencing
148 after 3 d of feeding on dsαCOP 5 µg/µL-treated buds, with corresponding significant (albeit delayed,
149 15 d) mortality (Willow et al., 2020a). Similar to the effect we observed in the present study
150 regarding *B. aeneus* larvae fed dsαCOP 2.5 µg/µL-treated anthers, *B. aeneus* adults fed honey water
151 at 1 and 3 µg dsαCOP/µL showed significant mortality at 6 d and 4 d, respectively (Willow et al.,
152 2020b). Knorr et al. (2018) reported significant *B. aeneus* adult mortality at 6 d, 6 d and 8 d,
153 respectively, after continuous uptake of 500 ng dsRNA/cm^2 gelatin diets targeting *dre4* (*dre4*),
154 *nucampholin* (*ncm*) and *RNA polymerase II 140kD subunit* (*RpII140*). Regarding *B. aeneus* adults,
155 the quicker effect observed via honey water feeding, compared to anther or bud feeding, may be
156 attributed to the potentially lower total amount of dsRNA being consumed when coating edible plant
157 parts with dsRNA, compared to feeding on a liquid solution consisting entirely of a near-equivalent
158 concentration of dsRNA. The design of future RNAi studies with *B. aeneus* larvae should consider
159 the potential difference in RNAi efficacy between short-term and chronic feeding on dsRNA-treated
160 anthers. In addition, other field relevant routes of oral exposure to *B. aeneus*-specific dsRNA should
161 be examined in larvae, including oilseed rape buds sprayed with highly surface-active dsRNA
162 formulations prior to larval emergence from buds; as well as bioengineered, *B. aeneus*-specific,
163 RNAi cultivars of oilseed rape.

164 While larvae that fed on dsαCOP at both 0.5 and 5 µg/µL showed marginally significant *αCOP*
165 silencing at 3 d, significant reductions in survival were never observed for these treatments. One
166 reason for this could be the sudden increases in control mortality at 6 and 7 d. This potential
167 confound coincides with 6 d qPCR results that suggest greater variability in *αCOP* expression at this
168 time, compared to the somewhat expected results observed at 3 d. Nevertheless, a trend of increased
169 *αCOP* silencing, with respect to dsαCOP treatments at 3 d, suggests potential for developing a RNAi

This is a provisional file, not the final typeset article

170 approach targeting *B. aeneus* larvae. Regarding the sudden increases in control mortality at 6 and 7 d,
171 similar results were observed by Melander et al. (2003), which is to our knowledge the only other
172 study performing insecticide bioassays with *B. aeneus* larvae. Previous experiments using the same
173 *gfp*-specific control dsRNA showed no effect of this control dsRNA on *B. aeneus* adult mortality
174 (Willow et al., 2020a, 2020b, Under Review). It is instead likely that *B. aeneus* larvae are very
175 sensitive under unnatural conditions, and consequently high mortality can occur, as evidenced by
176 both Melander et al. (2003) and the present study.

177 Refinements must be made to *B. aeneus* larval bioassay setups, in order to ensure optimal conditions
178 for keeping *B. aeneus* larvae alive in a controlled environment. These refinements should strive to
179 mimic conditions to which *B. aeneus* larvae are subjected under natural conditions. For example, in
180 the field, *B. aeneus* larvae are able to seek refuge within flower petals, providing them a microhabitat
181 that facilitates greater retention of moisture and less direct light. We removed this microhabitat from
182 the feeding setup, for ease of dsRNA application and monitoring of larvae. If petals are removed in
183 future studies, comparable microhabitats should be provided in the setup, or climate chamber
184 conditions (e.g. relative humidity, light intensity) should be adjusted accordingly, in order to reduce
185 potential stressors. Future studies aiming to examine, via anther feeding, the effect of dsRNA-based
186 insecticides on *B. aeneus* larvae under more natural conditions, should consider spraying highly
187 surface-active dsRNA formulations on oilseed rape raceme tips consisting of whole flower clusters,
188 and allowing cohorts of larvae to feed *ad libitum* in this type of semi-field-realistic bioassay setup.

189 **Conclusion**

In review

190 In *B. aeneus* larvae, we observed significant *αCOP* silencing and corresponding *αCOP* silencing-
191 induced mortality, via 3 d of feeding on dsαCOP 2.5 µg/µL-treated anthers of oilseed rape. These
192 results suggest that, compared to what has previously been observed in *B. aeneus* adults, *B. aeneus*
193 larvae may represent a more RNAi-sensitive life stage of this pest species. While larval mortality
194 rates were variable between treatments, our observed trend of *αCOP* silencing via field relevant
195 dsRNA feeding suggests potential for further research in RNAi targeting *B. aeneus* larvae. Further
196 experiments examining the potential for RNAi-based oilseed rape protection against *B. aeneus* larvae
197 should include semi-field-realistic bioassay setups; not only through spraying dsRNA formulations
198 on open flowers and closed buds containing *B. aeneus* larvae, but also via the use of bioengineered
199 RNAi cultivars for targeting both larval and adult *B. aeneus*.

200 **Conflict of Interest**

201 The authors declare that the research was conducted in the absence of any commercial or financial
202 relationships that could be construed as a potential conflict of interest.

203 **Author Contributions**

204 JW and EV conceived the study. JW, LS, SS, RK and EV designed the methods. JW, LS, SS and RK
205 performed the investigation. JW and AIS analyzed and visualized the data. JW wrote the original
206 draft. All authors contributed to revisions. All authors approved the final manuscript.

207 **Funding**

208 The authors acknowledge financial support from the institutional Research Funding project no
209 IUT36-2 of the Estonian Research Council, the ERA-NET C-IPM project "IPM4Meligethes" (project

210 no 3G0H0416 and 8T160091), the Special Research Fund (BOF) of Ghent University and the
211 Research Foundation – Flanders (FWO-Vlaanderen).

212 **Acknowledgments**

213 We thank the farm-owner whose land was used for collecting pollen beetle larvae and oilseed rape
214 flowers. Jonathan Willow is the recipient of a PhD grant from the European Social Fund for Doctoral
215 Students, as well as the Internationalisation Programme DoRa (carried out by the Archimedes
216 Foundation). Olivier Christiaens is a recipient of a postdoctoral fellowship from the Research
217 Foundation – Flanders (FWO-Vlaanderen).

218 **References**

219 Cagliari D, dos Santos EA, Dias N, Smagghe G, and Zotti M. "Nontransformative Strategies for
220 RNAi in Crop Protection". In: Khan MW and Singh A, editors. Modulating Gene Expression
221 - Abridging the RNAi and CRISPR-Cas9 Technologies. (2018). doi:
222 10.5772/intechopen.80874

223 Knorr E, Fishilevich E, Tenbusch L, Frey MLF, Rangasamy M, Billion A, et al. (2018). Gene
224 silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi
225 targets in crop pests. Sci Rep (2018) 8:1–15. doi: 10.1038/s41598-018-20416-y

226 Melander M, Åhman I, Kamnert I, Strömdahl A-C. Pea Lectin Expressed Transgenically in Oilseed
227 Rape Reduces Growth Rate of Pollen Beetle Larvae. Transgenic Res (2003) 12, 555–567. doi:
228 10.1023/A:1025813526283

229 Miguel KS, Scott JG. The next generation of insecticides: dsRNA is stable as a foliar-applied
230 insecticide. Pest Manag Sci (2015) 72:801–809. doi: 10.1002/ps.4056

231 Osborne P. Morphology of the immature stages of *Meligethes aeneus* (F.) and *M. viridescens* (F.)
232 (Coleoptera, Nitidulidae). Bulletin of Entomological Research (1965) 55:747–759. doi:
233 10.1017/S0007485300049853

234 Petek M, Coll A, Ferenc R, Razinger J, Gruden K. Validating the Potential of Double-Stranded RNA
235 Targeting Colorado Potato Beetle Mesh Gene in Laboratory and Field Trials. Front Plant Sci
236 (2020) 11. doi: 10.3389/fpls.2020.01250

237 Taning CNT, Arpaia S, Christiaens O, Dietz-Pfeilstetter A, Jones H, Mezzetti B, et al. RNA-based
238 biocontrol compounds: current status and perspectives to reach the market. Pest Manag Sci.
239 (2019) 76:841–845. doi: 10.1002/ps.5686

240 Willow J, Soonvald L, Sulg S, Kaasik R, Silva AI, Taning CNT, et al. First Evidence of Bud
241 Feeding-Induced RNAi in a Crop Pest via Exogenous Application of dsRNA. Insects (2020a)
242 11:769. doi: 10.3390/insects11110769

243 Willow J, Sulg S, Taning CNT, Silva AI, Christiaens O, Kaasik R. Targeting a coatomer protein
244 complex-I gene via RNA interference results in effective lethality in the pollen beetle
245 *Brassicogethes aeneus*. J Pest Sci (2020b) doi: 10.1007/s10340-020-01288-6

246

247 **Data Availability Statement**

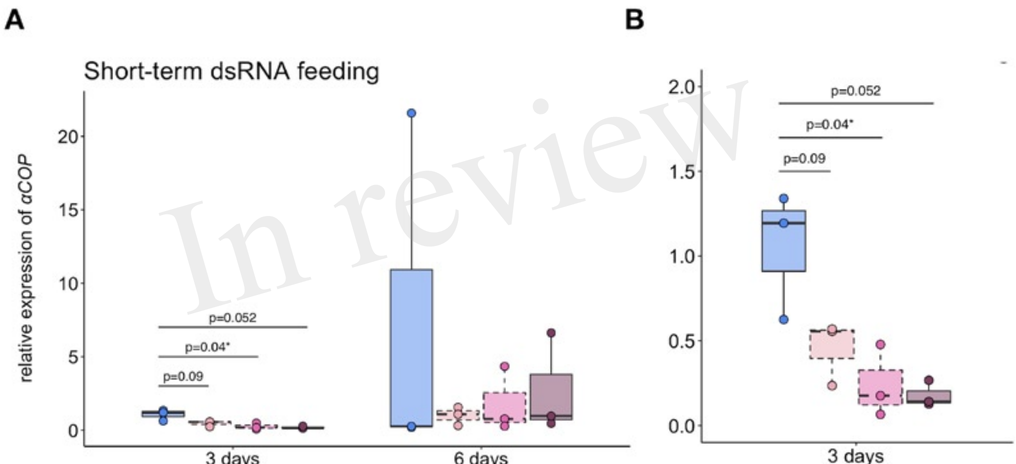
248 All data generated or analyzed for this study are included in this manuscript and its supplementary
249 material.

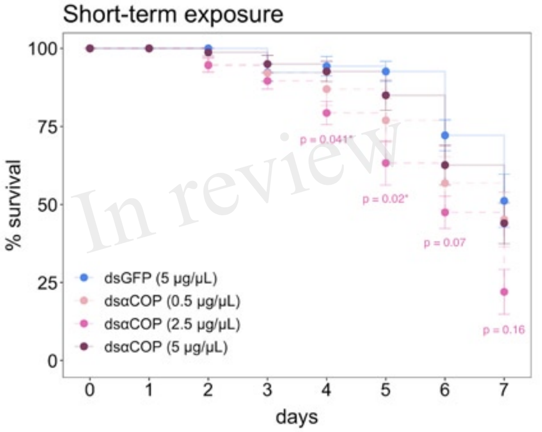
This is a provisional file, not the final typeset article

250 **Figure captions**

251 **Figure 1.** Relative expression of *Brassicogethes aeneus coatomer subunit alpha* (*αCOP*) in *B. aeneus*
252 larvae at **(a)** 3 and 6 d after start of experiment; **(b)** reduced y-axis value-limits, for better
253 visualization of 3 d qPCR data. Data were normalized using the housekeeping genes *actin* (*act*) and
254 *ribosomal protein S3* (*rps3*). Relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$
255 method. $n = 3$ (3 replicates of 5 larvae) for each time point of analysis within each treatment.
256 Statistical comparisons were made via Welch's t-test. Asterisk indicates significant difference
257 between dsαCOP and dsGFP treatment. * = $p < 0.05$.

258 **Figure 2.** Survival curves, comparing mortality effect of dsαCOP treatments to the dsGFP control
259 treatment in *Brassicogethes aeneus* larvae. Starting $n = 10$ (10 cages of 8 larvae) per treatment. Data
260 were analyzed via Kruskal−Wallis test, followed by the Wilcoxon rank-sums test with *post hoc*
261 Bonferroni correction for multiple comparisons (error bars: ± SEM). Asterisk indicates significant
262 difference between dsαCOP and dsGFP treatment. * = $p < 0.05$.





In review

7

VI

VI **Willow, J.**, Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I., Taning, C.N.T., Christiaens, O., Smagghe, G., Veromann, E. 2021. RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle. (Commun. Biol., In Press)

Title: RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle

Authors: Jonathan Willow[1,2]*, Liina Soonvald[1], Silva Sulg[1], Riina Kaasik[1], Ana Isabel Silva[3], Clauvis Nji Tizi Taning[2], Olivier Christiaens[2], Guy Smagghe[2], Eve Veromann[1]*

Affiliations:
[1]Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, 51006 Tartu, Estonia
[2]Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, 9000 Ghent, Belgium
[3]School of Mental Health and Neuroscience, Faculty of Health, Medicine and Life Sciences, Maastricht University, 6229 ER Maastricht, Netherlands

*Corresponding author email:
jonathan@emu.ee
eve.veromann@emu.ee

ORCIDs:
Jonathan Willow: 0000-0003-2377-4917
Liina Soonvald: 0000-0002-0370-7947
Silva Sulg: 0000-0002-4060-9596
Riina Kaasik: 0000-0002-8090-2199
Ana Isabel Silva: 0000-0002-1184-4909
Clauvis Nji Tizi Taning: 0000-0002-7597-1261
Olivier Christiaens: 0000-0001-5850-340X
Guy Smagghe: 0000-0001-8334-3313
Eve Veromann: 0000-0002-0977-5270

Abstract
Double-stranded RNAs (dsRNAs) represent a promising class of biosafe insecticidal compounds. We examined the ability to induce RNA interference (RNAi) in the pollen beetle *Brassicogethes aeneus* via anther feeding, and compared short-term- (3 d) to chronic (17 d) feeding of various concentrations of dsRNA targeting *αCOP* (dsαCOP). In short-term dsαCOP feeding, only the highest concentration resulted in significant reductions in *B. aeneus* survival; whereas in chronic dsαCOP feeding, all three concentrations resulted in significant mortality. Chronic dsαCOP feeding also resulted in significantly greater mortality compared to short-term feeding of equivalent dsαCOP concentrations. Our results have implications for the economics and development of dsRNA spray approaches for managing crop pests, in that multiple lower-concentration dsRNA spray treatments across crop growth stages may result in greater pest management efficacy, compared to single treatments using higher dsRNA concentrations. Furthermore, our results highlight the need for research into the development of RNAi cultivars for oilseed rape protection, given the enhanced RNAi efficacy resulting from chronic, compared to short-term, dsRNA feeding in *B. aeneus*.

Keywords: RNA interference, *Brassicogethes aeneus*, *Brassica*, biopesticide, insecticide, Nitidulidae, Coleoptera, insect

Introduction

The pollen beetle *Brassicogethes aeneus* Fabricius (Coleoptera: Nitidulidae; synonym *Meligethes aeneus*) is a major pest of oilseed rape (*Brassica napus* L.) in Europe. Overwintered adult *B. aeneus* feed on pollen and nectar of blooming plants of various taxonomic families; and later become monophagous on brassicaceous plants, where they obtain nutrients from reproductive buds and open flowers; followed by mating and subsequent oviposition into buds (usually 2−3 mm in length). Upon hatching from eggs, larvae feed on anthers within buds, followed by late first- and early second instar feeding in open flowers, the late second instar larvae eventually pupating in the soil under their host plant (reviewed in Mauchline et al. [1]).

As both the area of land used for- and the number of people relying on crop production increases exponentially, the importance of achieving ecologically sustainable crop production also continues to grow. In order to achieve this, biologically safe strategies for managing crop pest populations are needed. A crop pest management strategy should consist of multiple approaches, together constituting one integrated pest management design. One widely suggested sustainable pest management approach is to enhance conservation biological control, based on the preservation or restoration of habitats and habitat features that provide food, alternative prey, shelter, overwintering sites, and other natural resources to relevant natural enemies (i.e. predators, parasitoids) of crop pests; and these conservation measures are ideally implemented at both local and

61 regional scales [2–9]. The use of trap crops represents an additional pest management strategy, whereby an
62 attractive companion crop diverts pests from the main crop, with the aim to reduce damage inflicted to the main
63 crop; and this strategy has shown promise for use in integrated *B. aeneus* management (reviewed in Skellern &
64 Cook [10]). Another area of interest in oilseed rape protection, against *B. aeneus* and other insect pests, is the use of
65 plant breeding techniques such as the exploitation of oilseed rape's natural variation, introgressive hybridization
66 with other brassicaceous species, and the introduction of transgenes in the oilseed rape genome (reviewed by
67 Hervé [11]. The application of insecticides can dramatically reduce yield losses in oilseed rape production.
68 However, these compounds often kill nontarget organisms, including economically beneficial insects that
69 contribute to pest management [12–14]. In order to contribute to a biosafe outcome for nontarget organisms,
70 insecticidal compounds used should be as specific to the target pest as possible.
71 Post-transcriptional gene silencing via RNA interference (RNAi) represents a potential tool for use in
72 an integrated and biosafe crop pest management design [15]. As RNAi occurs via the nucleotide sequence-specific
73 mode of action of double-stranded RNA (dsRNA), this control measure may affect a desirably-narrow range of
74 species, taken on a case by case basis, depending on the design of target-specific dsRNAs. In brief, when a
75 sequence homology exists between a small interfering (si)RNA (20−24 nucleotides) processed *in vivo* from
76 exogenous (e.g. ingested) dsRNA, and an endogenous messenger (m)RNA, this homology allows the
77 complementary region of endogenous mRNA to base-pair to the siRNA and become cleaved by the RNA-
78 induced silencing complex (RISC) ribonucleoprotein, preventing translation of the target mRNA. Two
79 overarching strategies for inducing RNAi in crop pest populations have recently made significant progress in
80 their development. One of these is host-induced gene silencing (HIGS) via the use of an RNAi cultivar; the other
81 is spray-induced gene silencing (SIGS) via sprayable dsRNA (reviewed in Christiaens et al. [16]). The prospects of
82 the latter are further reviewed in Cagliari et al. [17]. HIGS has been shown to be an effective approach, for
83 example, in controlling western corn rootworm (*Diabrotica virgifera virgifera* LeConte) via dietary exposure to
84 transgenic maize (*Zea mays* L.) engineered to produce dsRNA targeting the gene *v-ATPase A* in *D. virgifera*
85 *virgifera* [18]. This approach has the benefit in that an RNAi cultivar constantly produces the target pest-specific
86 dsRNA within the plant's tissues, chronically exposing the target pest to the sequence-specific insecticide, so
87 long as the insect feeds on the transgenic crop. Indeed, the RNAi maize cultivar MON87411, expressing dsRNA
88 targeting *Snf7* in *D. virgifera virgifera*, has been approved in several countries [19,20]. RNAi efficacy via a SIGS
89 approach has been demonstrated in a greenhouse experiment where the foliar surface of four-week-old potato
90 (*Solanum tuberosum* L.) plants were treated with dsRNA targeting the gene *act* in Colorado potato beetle
91 (*Leptinotarsa decemlineata* Say) [21]; as well as in a field trial, where spraying of dsRNA targeting the *mesh* gene
92 in the same species resulted in less leaf damage, and greater *L. decemlineata* mortality, compared to the
93 mortality observed on untreated plants [22]. This approach has the benefit of not requiring the biotechnology or
94 time required for engineering an RNAi cultivar. However, two potential drawbacks include the possibility that
95 exogenously-applied dsRNA may not remain stable for long periods under natural outdoor conditions, and that
96 successive applications may become necessary across stages of plant growth. The latter potential drawback is
97 especially important to consider in the management of *B. aeneus* and other anthophilous species, as these acquire
98 nutrients from flowering structures, which are in constant development and senescence, rather than leaves, which
99 remain individually established on the growing plant for much longer periods. Thus, if a SIGS approach was to
100 be put into practice within an integrated *B. aeneus* management framework for oilseed rape protection, the
101 potential requirement of successive dsRNA spray applications must be considered.
102 Knorr et al. [23] first demonstrated oral RNAi and subsequent RNAi-induced mortality in *B. aeneus*,
103 targeting several genes (e.g. *ncm*, *Rop*, *RpII140*, *dre4*) that were orthologous to RNAi-sensitive genes targeted in
104 *D. virgifera virgifera* bioassays performed in the same study. An additional vital gene, *αCOP*, encodes the αCOP
105 protein, a subunit of coatomer protein complex-I (COPI). COPI is involved in intracellular vesicular transport of
106 proteins between the endoplasmic reticulum and Golgi apparatus; the transport of various other cellular cargo,
107 via its indirect interaction with the cytoskeletal motor protein dyenin; and possibly the maintenance of protein
108 distribution within the Golgi stack [24]. Furthermore, COPI is active in maintaining lipid homeostasis [25]; and
109 knockdown of COPI subunits inhibits protein- and lipid accumulation at the cleavage furrow, and reduces the
110 number of microtubules at the central spindle, together resulting in cytokinesis failure [26]. Targeting *αCOP*
111 expression, we recently demonstrated, under laboratory conditions, significant RNAi-induced mortality in *B.*
112 *aeneus* via honey-solution feeding, simulating dsRNA-contaminated nectar [27]; as well as significant *αCOP*
113 silencing via bud feeding, suggesting potential for developing an RNAi technique exploiting dsRNA-
114 contaminated buds [28]. Besides carbohydrates from nectar, and the lipid and protein constituents of buds, pollen
115 beetles such as *B. aeneus* also consume pollen to acquire lipids and proteins, which helps *B. aeneus* in
116 maintaining fitness at both the individual- (e.g. energy storage) and population (e.g. gametogenesis) scale.
117 Studies have suggested that, while consumption of pollen positively influences *B. aeneus* survival and
118 reproductive fitness, pollen is not critical for *B. aeneus* survival [29,30]. Nevertheless, *B. aeneus*'s consumption of
119 pollen, together with the potential for SIGS or HIGS of *B. aeneus* populations via early-flowering trap crops

120 respectively treated with- or bioengineered to produce dsRNA, makes it critical to examine RNAi efficacy via
121 anther feeding in *B. aeneus*.
122 The aims of the present study were to confirm the ability to induce the RNAi effect via anther-based
123 feeding of dsRNA, a field-relevant and thus far unexamined dietary exposure route; and to compare RNAi
124 efficacy between short-term and chronic feeding of dsRNA targeting *B. aeneus αCOP* (hereafter dsαCOP),
125 simulating two approaches to SIGS. We show that, for our low and medium dsαCOP concentrations, chronic
126 dsαCOP feeding results in significantly greater mortality compared to short-term dsαCOP feeding; and that
127 *αCOP* silencing was only significant in *B. aeneus* adults chronically fed dsαCOP-treated anthers. Considering
128 the economics and development of a SIGS approach, our results highlight the potential for enhancing pest
129 management efficacy via successive low-concentration treatments, compared to a single high-concentration
130 treatment.
131
132 **Results**
133 **Survival**. We observed significant reductions in *B. aeneus* survival as a result of consumption of dsαCOP-
134 treated anthers, for both short-term (3 d)- and chronic (daily for 17 d) dsRNA feeding (Fig. 1). With short-term
135 dsRNA feeding, significant reductions in survival were observed starting at 8 d (64% survival) in the dsαCOP 5
136 µg/µL treatment (Fig. 1c), compared to the dsGFP control (p=0.007) and dsαCOP 0.5 µg/µL (p=0.006). Survival
137 for short-term dsαCOP feeding at 5 µg/µL fell from 64% (p=0.007, 8 d) to 39% (p=0.0096, 13 d), afterwards
138 remaining at 38% (p=0.005) until the end of the experiment. Similarly, significant reductions in survival (65%
139 survival, p=0.027) were observed starting at 9 d in short-term dsαCOP feeding at 2.5 µg/µL (Fig. 1b), compared
140 to dsGFP control, though this difference became statistically insignificant (p=0.08) at 15 d; here, survival largely
141 reached its lowest level at 13 d (53% survival, p=0.04), afterwards remaining at 52% (p=0.08) until the end of
142 the experiment. When comparing the dsαCOP 2.5 µg/µL- to the dsαCOP 0.5 µg/µL treatment (Fig. 1d),
143 reductions in survival were marginally significant starting at 8 d (p=0.054). Similar to the dsGFP control, short-
144 term dsαCOP feeding at 0.5 µg/µL resulting in a total of 87% survival at 17 d (Fig. 1a). Thus, no difference in
145 survival was observed between the dsαCOP 0.5 µg/µL and dsGFP treatment, regarding short-term dsRNA
146 feeding.
147 With chronic dsRNA feeding, significant reductions in *B. aeneus* survival were observed starting at 8, 9
148 and 10 d, for dsαCOP 2.5 µg/µL (72% survival, p=0.02; Fig. 1b), dsαCOP 5 µg/µL (74% survival, p=0.03; Fig
149 1c) and dsαCOP 0.5 µg/µL (70% survival, p=0.036; Fig. 1a) treatments, respectively. Survival for chronic
150 dsαCOP feeding at 0.5 µg/µL continued to steadily fall to 46% (p=0.018, 17 d), whereas survival for chronic
151 dsαCOP feeding at both 2.5 µg/µL and 5 µg/µL fell more rapidly, respectively reaching 26% (p=0.003) and 30%
152 (p=0.003) at 13 d, and reaching their lowest levels at 8% (p=0.002) and 13% (p=0.002).
153 We also observed significant differences in *B. aeneus* survival when comparing short-term- to chronic
154 dsαCOP feeding. Starting at 10 d, chronic dsαCOP feeding at 0.5 µg/µL showed significantly reduced (p=0.04)
155 survival of *B. aeneus*, compared to short-term feeding of the same concentration (Fig. 1a), this difference
156 becoming more significant further into the experiment (17 d p=0.01). Similarly, chronic dsαCOP feeding at 2.5
157 µg/µL showed significantly reduced (p=0.027) survival of *B. aeneus*, compared to short-term feeding of the
158 same concentration (Fig. 1b), starting at 15 d; this difference also became more significant further into the
159 experiment (17 d p=0.004).
160
161 **Gene expression**. We observed contrasting results, with respect to relative expression of *αCOP*, between short-
162 term- and chronic dsRNA feeding groups (Fig. 2). Regarding short-term dsαCOP feeding, we observed a trend
163 of reduced *αCOP* expression at 3 d. Here, we detected a 39% mean decrease in *αCOP* expression in the dsαCOP
164 0.5 µg/µL treatment (t=1.15, df=2.79, p=0.34), a 60% mean decrease in the dsαCOP 2.5 µg/µL treatment
165 (t=1.95, df=2.01, p=0.19), and a 64% mean decrease in the dsαCOP 5 µg/µL treatment (t=1.85, df=3.02,
166 p=0.16), compared to the dsGFP control. At 6 d, our gene expression data showed no *αCOP* silencing (dsαCOP
167 0.5 µg/µL: t=-0.8, df=3.17, p=0.48; dsαCOP 2.5 µg/µL: t=0.18, df=4, p=0.87; dsαCOP 5 µg/µL: t=-0.06,
168 df=3.71, p=0.95). At 12 d, we again observed no *αCOP* silencing (dsαCOP 0.5 µg/µL: t=0.25, df=3.99, p=0.82;
169 dsαCOP 2.5 µg/µL: t=-0.59, df=3.18, p=0.6; dsαCOP 5 µg/µL: t=-1.14, df=2.04, p=0.37; Supplementary Figure
170 1).
171 Regarding chronic dsαCOP feeding, differences in *αCOP* expression were more pronounced, and
172 statistically significant in some treatments, compared to the dsGFP control. At 3, 6 and 12 d, *αCOP* silencing
173 was not observed in the dsαCOP 0.5 µg/µL treatment (3 d: t=0.61, df=3.69, p=0.58; 6 d: t=0.34, df=2.95,
174 p=0.75; 12 d: t=-0.33, df=2.46, p=0.69). Chronic dsαCOP feeding resulted in *αCOP* silencing in both the 2.5
175 µg/µL and 5 µg/µL treatments, at both 3 and 6 d. At 3 d, we observed a 63% mean decrease in *αCOP* expression
176 in the dsαCOP 2.5 µg/µL treatment (t=4.45, df=3.71, p=0.01), and a 50% mean decrease in the dsαCOP 5 µg/µL
177 treatment (t=2.81, df=3.98, p=0.05). At 6 d, we observed a 64% mean decrease in *αCOP* expression in the
178 dsαCOP 2.5 µg/µL treatment (t=2.9, df=3.97, p=0.049), and a 64% mean decrease in the dsαCOP 5 µg/µL
179 treatment (t=2.49, df=3.93, p=0.069). At 12 d after chronic dsαCOP feeding, no *αCOP* silencing was observed in

180 either the dsαCOP 2.5 µg/µL treatment (t=-0.18, df=3.92, p=0.87) or the dsαCOP 5 µg/µL treatment (t=0.81,
181 df=2.96, p=0.48).
182
183 **Discussion**
184 We aimed to induce RNAi via anther-based feeding of dsαCOP, and to compare RNAi efficacy between short-
185 term and chronic dsαCOP feeding, simulating two SIGS approaches. Our data suggest that, with chronic dsRNA
186 feeding, reduced dsRNA concentrations can be applied in order to achieve a similar effect compared to that
187 achieved from short-term exposure to higher dsRNA concentrations. Overall, these observations have important
188 implications for the potential optimal practice and economics of a SIGS approach to managing crop pest
189 populations. Specifically, our results suggest that, while the management of *B. aeneus*, or of other crop pests,
190 would likely benefit greatly from successive dsRNA spray treatments, this may also benefit the economics of
191 dsRNA application, as lower concentrations may be suitable for an effective outcome. While we provide clear
192 evidence to support this idea, semi- or small field experiments are necessary to further explore and confirm this
193 RNAi approach. Furthermore, if SIGS was to be incorporated within a trap cropping approach, whereby adjacent
194 trap crops were the target site for dsRNA spray treatments, this may represent a strategy for even greater
195 efficiency of dsRNA use, as less total crop area would be treated.
196 We recently demonstrated significant *B. aeneus* mortality via 5 d of dsαCOP-treated honey-solution
197 feeding, where significant mortality was first observed at 4 and 6 d, in 3 µg/µL and 1 µg/µL treatments,
198 respectively, with survival continuing to steadily fall in the 1 µg/µL treatment, and falling more rapidly in the 3
199 µg/µL treatment [27]. The present study, which shows significant gene silencing-induced mortality in *B. aeneus* via
200 consumption of anthers of dsRNA-treated flowers, provides evidence that suggests potential for using an RNAi-
201 based approach within *B. aeneus* management. Treating anthers is indeed highly field-relevant when the target
202 organism is an anthophilous species. However, compared to honey-solution containing similar concentrations of
203 dsRNA, the amount of dsRNA being consumed when applied to anthers is likely far lower. As *B. aeneus*
204 consumes large amounts of pollen via anther-feeding, this difference in total dsRNA concentration between *B.*
205 *aeneus*'s nutrient sources is of vital consideration for any potential SIGS approach to *B. aeneus* management.
206 Similarly, we recently observed only 12% and 16% *B. aeneus* adult mortality at 10 and 15 d, respectively, after
207 short-term (3 d) feeding on oilseed rape buds exogenously treated with dsαCOP at 5 µg/µL [28]; compared to the
208 present study in which we observed 43% (10 d) and 66% (15 d) mortality after short-term feeding of anthers
209 treated with dsαCOP at 5 µg/µL. This difference in RNAi efficacy between bud- and anther-based dsRNA
210 feeding is similar to that between honey-solution- and anther-based dsRNA feeding, in that *B. aeneus* adults
211 chew through- and consume bud epithelial tissue mostly to acquire nutrients from the anthers within the bud;
212 thereby being orally exposed to a smaller amount of exogenously-applied dsRNA compared to when feeding on
213 dsRNA-treated anthers.
214 While dsRNA applications are unlikely to result in target species mortality as quickly as with some
215 other (e.g. neurotoxic) insecticides, the benefit to using a dsRNA-based insecticide is its associated biosafety to
216 nontarget species, due to the unique mode of action of dsRNA. There indeed also remains potential for
217 enhancing efficacy and speed-to-effect of dsRNA via co-formulants (e.g. nanoparticles) that can improve
218 efficiency of dsRNA-uptake and RNAi [31,32]. Improving the efficacy of this technology will be a critical step to
219 more fully realising the potential for using a SIGS approach in managing *B. aeneus* and other anthophilous pest
220 species. The present study, together with our previous RNAi studies on *B. aeneus* [27,28], suggest that *B. aeneus*'s
221 sensitivity to oral RNAi, via field relevant routes of exposure, is relatively moderate compared to some other
222 crop pests. Indeed, several coleopteran taxa have demonstrated sensitivity to dsRNA via feeding. For example, in
223 addition to *L. decemlineata* and *D. virgifera virgifera*, on which much RNAi work has been done [18,21–23,33–36],
224 robust sensitivity to oral RNAi has been observed in the Sri Lanka weevil (*Myllocerus undecimpustulatus*
225 *undatus* Marshall) [37], the sweetpotato weevils *Cylas brunneus* Fabricius and *Cylas puncticollis* Boheman [38,39],
226 and the lady beetle *Henosepilachna vigintioctopunctata* Fabricius [40].
227 Pollen beetle larvae begin development within the flower bud, feeding on anthers, and are typically in
228 their late first- or early second instar when oilseed rape flowers bloom; at this point, *B. aeneus* larvae proceed to
229 feed upon the anthers of open flowers of oilseed rape (reviewed in Mauchline et al. [1]). Therefore, it is plausible
230 that dsRNA applications during flowering could target both larval and adult *B. aeneus* stages simultaneously.
231 Studies examining the potential for anther feeding-induced RNAi in *B. aeneus* larvae would greatly enhance our
232 understanding of the potential for using an RNAi approach in *B. aeneus* management.
233 While the present study used exogenously applied dsRNA to bring about gene silencing-induced
234 mortality, our results raise the question of whether HIGS or SIGS represents the most optimal and effective
235 RNAi approach to agricultural pest management. Current restrictions prevent the agricultural use of RNAi
236 cultivars within European Union countries. However, this could change as our experience with this technology,
237 and our understanding of its impacts, increases. RNAi risk assessment is a concept and practice that is under
238 constant refinement, and is expected to provide evidence of the biosafety of RNAi cultivars, naturally on a case
239 by case basis [20]. Based on our results, it is conceivable that a HIGS approach, exploiting the continuous

240 production of target-specific dsRNA, could represent the optimal approach to RNAi-based management of *B.*
241 *aeneus*, and possibly other crop pest species. However, development of RNAi cultivars, and experiments
242 simulating both HIGS and SIGS approaches, are necessary steps to more fully understanding the practical
243 differences between these approaches to RNAi-based crop pest management. In the context of *B. aeneus* and
244 other anthophilous species, it remains critical to consider the constant development and senescence of flowers
245 within the crop, and the implications this may have for a SIGS approach, specifically with respect to the
246 potential requirement of successive dsRNA spray applications over the flowering season.
247 Ecologically sustainable agricultural pest management is required in order to attain ecologically
248 sustainable crop production. Insecticides based on dsRNA represent a potentially species-specific complement to
249 other biosafe measures (e.g. conservation biocontrol) for managing agricultural pests, due to the unique mode of
250 action of dsRNA. Our work demonstrates major differences between short-term and chronic feeding of target-
251 specific dsRNA, regarding both gene silencing and gene silencing-induced mortality in *B. aeneus*; and suggests
252 similar differences may be important factors in other crop pest species. Our results also provide further evidence
253 of the potential for RNAi-based management of *B. aeneus*, particularly via a SIGS approach utilising
254 appropriately-timed spray applications during the oilseed rape flowering period; but also applies to the ongoing
255 conversation regarding the use of HIGS vs SIGS in crop pest management. Focal points critical for progress here
256 include determining the duration at which exogenously-applied dsRNA remains viable on the anthers of
257 flowering crops; as well as the optimal duration of exposure to dsRNA-treated anthers, taking into account the
258 potential length of time between blooming- and senescence of flowers within a given crop. It is also critical to
259 determine the potential for management of *B. aeneus* larvae, via consumption of dsRNA-treated anthers; as well
260 as the overall feasibility of adopting a SIGS approach for controlling anthophilous pest species, in the context of
261 potential requirements for successive dsRNA spray applications. Lastly, the development of RNAi cultivars for
262 use in experiments must be considered, in order to examine the potential for RNAi-based management of crop
263 pests via HIGS, and simulate this against different SIGS approaches.
264
265 **Methods**
266 **dsRNAs**. A selected 222 base pair (bp) region from the *B. aeneus αCOP* coding sequence, and a 455 bp
267 sequence from the gene *gfp* (Supplementary Table 1), were used as the basis for *in vitro* synthesis of
268 corresponding dsRNA products by AgroRNA (Genolution, Seoul, South Korea). These products contained
269 dsRNAs with sequences complementary to the genes *gfp* and *αCOP*, and are respectively referred to as dsGFP
270 (control) and dsαCOP. Both dsRNA products were shipped in distilled water (dH_2O) at ambient temperature, and
271 kept at 5±1 °C once received. Absence of nucleic contaminants in both the dsGFP and dsαCOP stocks was
272 confirmed via gel electrophoresis.
273
274 **Insects and flowers**. Pollen beetles and oilseed rape flowers were both collected fresh from an untreated organic
275 oilseed rape crop (58.36377°N, 26.66145°E) in the village of Õssu, Tartu County, Estonia. Beetles were kept in
276 ventilated plastic containers and allowed to feed *ad libitum* on oilseed rape flowers. All pollen beetles were
277 identified via Kirk-Spriggs [41], and only *B. aeneus* were used in experiments.
278
279 **Experimental setup**. *B. aeneus* were placed into transparent, polystyrene, ventilated insect breeding dishes
280 (diameter 10 cm x height 4 cm) (SPL Life Sciences, Gyeonggi-do, South Korea), hereafter referred to as cages.
281 Eight fast moving beetles (used as a proxy for good health) were selected at random and introduced to each cage.
282 Treatments were provided as *ad libitum* access to dsRNA-treated anthers of oilseed rape flowers, where petals
283 were removed from flowers, and anthers were soaked in the treatment solution for 15 s, and subsequently
284 allowed to air dry. All treated anthers were treated on the day of their provision. All anthers provided were
285 dehisced, and thus pollen grains were freely-available to the insects. Treatment solutions contained a given
286 amount of dsRNA diluted in dH_2O, and a constant concentration (180 ppm) of the surfactant Triton X-100
287 (Fisher Bioreagents), and were vortexed for 10 s prior to soaking anthers. There were eight treatments, including
288 dsαCOP at 0.5, 2.5 and 5 µg/µL, and dsGFP at 5 µg/µL, each provided for 3 d to one group (receiving untreated
289 anthers after 3 d), with another group receiving daily (17 d) treatment (hereafter respectively referred to as short-
290 term- and chronic dsRNA feeding). Fresh anthers were provided every 24±1 h. Once provisioned with dsRNA-
291 treated- or untreated anthers, beetles were maintained in a climate chamber (Sanyo MLR-351H, Osaka, Japan) at
292 20 °C, 60% RH and 16:8 h light:dark cycle. Each cage was additionally provisioned with a small piece of dental
293 cotton roll saturated with dH_2O, to provide drinking water for beetles. The experiment was replicated three
294 times, over three consecutive days, each time allocating five cages per treatment (starting n=15 cages; 120
295 insects per treatment). Experimental setup is illustrated in Figure 3.
296 Each experimental replicate lasted for 17 d. For each replicate, after 1 d, any dead beetles were removed
297 from the experiment, as at this time the mortality could not have been due to RNAi, but rather likely stress
298 resulting from manipulations and changing conditions; these mortalities after 1 d were few, and were accounted

for in the statistical analysis. Survival was monitored every 24±1 h, and dead insects were removed from cages daily.

Relative gene expression analysis was performed for all treatments via quantitative polymerase chain reaction (qPCR), represented by the time points 3, 6 and 12 d. At 3 and 6 d after the start of each experimental replicate, one cage was randomly removed from each treatment (min 6, max 8 beetles per sample; qPCR sample n=3 cages; leaving n=12 and n=9 after 3 and 6 d, respectively, for survival analysis). At 12 d, for each experimental replicate, one beetle was removed from each remaining cage, and used for qPCR analysis (3 beetles were pooled per replicate; qPCR sample n=3). Removal of beetles for qPCR was accounted for in the statistical analysis. Beetles used for qPCR were immediately placed in their respective Eppendorf tubes and homogenised using a sterilised plastic pestle designed for Eppendorf tubes, in 600 µL of RLT buffer (with added 10 µL of β-mercaptoethanol), and stored at −80 °C until analysis. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands); and RNA concentration was quantified, and purity assessed, using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA); purity was further verified via gel electrophoresis. Genomic DNA was removed using the Turbo DNA-Free Kit (Invitrogen, Carlsbad, USA), following the manufacturer's protocol. The cDNA was reverse transcribed from 1 µg of total RNA using the FIREScript RT cDNA Synthesis Kit (Solis BioDyne, Tartu, Estonia); and qPCR was performed in the QuantStudio 5 Real-Time PCR System (Applied Biosciences, Foster City, USA). The reaction mixture included 4 µL of 5xHOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne), 0.5 µL of 10 µM forward primer (Microsynth, Balgach, Switzerland; Supplementary Table 2), 0.5 µL of 10 µM reverse primer (Microsynth; Supplementary Table 2), 14 µL of PCR-grade water and 500 ng of cDNA, to a total volume of 20 µL. Amplification conditions were as follows: 15 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 58 °C, followed by a melting curve analysis with a temperature range of 60–95 °C. The reactions were set up in 384-well PCR plates, in triplicate. Normalisation of data was performed using the two housekeeping genes *rps3* and *act*. Primer amplification efficiencies were determined from a cDNA dilution series. Primer sequences and amplification efficiencies are shown in Supplementary Table 2. Relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. A no-template control as well as a no reverse transcriptase control were both included in the assay.

Statistics and reproducibility. Treatment comparisons taken into consideration are listed in Supplementary Table 3. Regarding survival analysis, for dsGFP and all three dsαCOP concentrations, comparisons between short-term- and chronic exposure were statistically assessed. In addition, comparisons in survival were made between dsGFP and all three dsαCOP concentrations, as well as between dsαCOP concentrations, within both short-term- and chronic exposure groups. When comparing different dsRNAs or concentrations, comparisons in survival were only made between treatment groups that were given the same duration of exposure to dsRNA. Regarding gene expression analysis, comparisons were made between dsGFP and all three dsαCOP concentrations, within both short-term- and chronic exposure groups. For survival analysis, homogeneity of variance and normality of data distributions were determined using the Levene- and Shapiro-Wilk tests, respectively. Since the data were overall not normally distributed, the Kruskal-Wallis test was used as a nonparametric alternative to ANOVA; this was followed by the Wilcoxon rank-sums test, with Bonferroni correction, for *post-hoc* pairwise comparisons. For gene expression analysis, comparisons were made using Welch's t-test. All statistical analyses were performed in R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

The experiment was replicated three times, each experiment consisting of five cages (total n=15 cages) per treatment. At both 3 and 6 d after each experiment, one cage of beetles was removed from the bioassay and used to analyse relative gene expression (total n= 3 qPCR samples), for each treatment. Thus, for survival analysis, sample size per treatment was reduced from n=15 to n=12 at 3 d, and from n=12 to n=9 at 6 d.

Data availability

All data are available upon request from corresponding author JW.

Acknowledgements

We thank the farm-owner whose land was used for collecting pollen beetles and oilseed rape flowers. We also thank Samantha M. Cook and one anonymous reviewer for their constructive comments on the manuscript. Jonathan Willow is the recipient of a PhD grant from the European Social Fund for Doctoral Students, as well as the Internationalisation Programme DoRa (carried out by the Archimedes Foundation). Olivier Christiaens is a recipient of a postdoctoral fellowship from the Research Foundation – Flanders (FWO-Vlaanderen). The authors acknowledge financial support from the institutional Research Funding project no IUT36-2 of the Estonian Research Council, the institutional Research Funding project no PRG1056 of the Estonian research council, the European ERA-NET C-IPM project "IPM4Meligethes" (project no 3G0H0416), the European Union's European Regional Development Fund (Estonian University of Life Sciences ASTRA project "Value-chain based

bioeconomy"), the Special Research Fund (BOF) of Ghent University and the Research Foundation – Flanders (FWO-Vlaanderen).

References

1. Mauchline, A. L., Hervé, M. R. & Cook, S. M. Semiochemical-based alternatives to synthetic toxicant insecticides for pollen beetle management. *Arthropod-Plant Interact.* **12**, 835–847 (2018).
2. Hatt, S. *et al.* Effect of flower traits and hosts on the abundance of parasitoids in perennial multiple species wildflower strips sown within oilseed rape (*Brassica napus*) crops. *Arthropod-Plant Interact.* **12**, 787–797 (2018).
3. Berger, J. S., Birkhofer, K., Hanson, H. I. & Hedlund, K. Landscape configuration affects herbivore–parasitoid communities in oilseed rape. *J. Pest Sci.* **91**, 1093–1105 (2018).
4. Riggi, L. G. *et al.* Pollen beetle mortality is increased by ground-dwelling generalist predators but not landscape complexity. *Agric. Ecosyst. Environ.* **250**, 133–142 (2017).
5. Scheid, B. E., Thies, C. & Tscharntke, T. Enhancing rape pollen beetle parasitism within sown flower fields along a landscape complexity gradient. *Agric. For. Entomol.* **13**, 173–179 (2011).
6. Thies, C. & Tscharntke, T. Landscape Structure and Biological Control in Agroecosystems. *Science* **285**, 893–895 (1999).
7. Kovács, G. *et al.* Effects of land use on infestation and parasitism rates of cabbage seed weevil in oilseed rape. *Pest Manag. Sci.* **75**, doi: 10.1002/ps.5161 (2018).
8. Albrecht, M. *et al.* The effectiveness of flower strips and hedgerows on pest control, pollination services and crop yield: a quantitative synthesis. *Ecol. Lett.* **23**, 1488–1498 (2020).
9. Bartual, A. M. *et al.* The potential of different semi-natural habitats to sustain pollinators and natural enemies in European agricultural landscapes. *Agric. Ecosyst. Environ.* **279**, 43–52 (2019).
10. Skellern, M. P. & Cook, S. M. Prospects for improved off-crop habitat management for pollen beetle control in oilseed rape. *Arthropod-Plant Interact.* **12**, 849–866 (2018).
11. Hervé, M. R. Breeding for insect resistance in oilseed rape: Challenges, current knowledge and perspectives. *Plant Breed.* **137**, 27–34 (2018).
12. Willow, J., Silva, A., Veromann, E. & Smagghe, G. Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. *PLoS One* **14**, doi: 10.1371/journal.pone.0212456 (2019).
13. Calvo-Agudo, M. *et al.* Neonicotinoids in excretion product of phloem-feeding insects kill beneficial insects. *Proc. Natl. Acad. Sci.* **116**, 16817–16822 (2019).
14. Pisa, L. *et al.* An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. Part 2: impacts on organisms and ecosystems. *Environ. Sci. Pollut. Res.* doi: 10.1007/s11356-017-0341-3 (2017).
15. Mezzetti, B. *et al.* RNAi: What is its position in agriculture? *J. Pest Sci.* **93**, 1125–1130 (2020).
16. Christiaens, O., Whyard, S., Vélez, A. M. & Smagghe, G. Double-stranded RNA technology to control insect pests: Current status and challenges. *Front. Plant Sci.* **11**, doi: 10.3389/fpls.2020.00451 (2020).
17. Cagliari, D., Santos, E. A. dos, Dias, N., Smagghe, G. & Zotti, M. Nontransformative strategies for RNAi in crop protection. *Modul. Gene Expr. - Abridging RNAi CRISPR-Cas9 Technol.* doi: 10.5772/intechopen.80874 (2018).
18. Baum, J. A. *et al.* Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* **25**, 1322–1326 (2007).
19. Papadopoulou, N., Devos, Y., Álvarez-Alfageme, F., Lanzoni, A. & Waigmann, E. Risk assessment considerations for genetically modified RNAi plants: EFSA's activities and perspective. *Front. Plant Sci.* **11**, doi: 10.3389/fpls.2020.00445 (2020).
20. Arpaia, S. *et al.* Biosafety of GM crop plants expressing dsRNA: Data requirements and EU regulatory considerations. *Front. Plant Sci.* **11**, doi: 10.3389/fpls.2020.00940 (2020).
21. Miguel, K. S. & Scott, J. G. The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Manag. Sci.* **72**, 801–809 (2015).
22. Petek, M., Coll, A., Ferenc, R., Razinger, J. & Gruden, K. Validating the potential of double-stranded RNA targeting Colorado potato beetle *mesh* gene in laboratory and field trials. *Front. Plant Sci.* **11**, doi: 10.3389/fpls.2020.01250 (2020).
23. Knorr, E. *et al.* Gene silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi targets in crop pests. *Sci. Rep.* **8**, 1–15 (2018).
24. Beck, R., Ravet, M., Wieland, F. T. & Cassel, D. The COPI system: Molecular mechanisms and function. *FEBS Lett.* **583**, 2701–2709 (2009).
25. Beller, M. *et al.* COPI complex is a regulator of lipid homeostasis. *PLoS Biol.* doi: 10.1371/journal.pbio.0060292 (2008).
26. Kitazawa, D., Yamaguchi, M., Mori, H. & Inoue, Y. H. COPI-mediated membrane trafficking is required for cytokinesis in *Drosophila male* meiotic divisions. *J. Cell Sci.* **125**, 3649–3660 (2012).

418 27. Willow, J. *et al.* Targeting a coatomer protein complex-I gene via RNA interference results in effective
419 lethality in the pollen beetle *Brassicogethes aeneus*. *J. Pest Sci.* doi: 10.1007/s10340-020-01288-6 (2020).
420 28. Willow, J. *et al.* First evidence of bud feeding-induced RNAi in a crop pest via exogenous application of
421 dsRNA. *Insects* **11**, 769 (2020).
422 29. Cook, S. M., Murray, D. A. & Williams, I. H. Do pollen beetles need pollen? The effect of pollen on
423 oviposition, survival, and development of a flower-feeding herbivore. *Ecol. Entomol.* **29**, 164–173 (2004).
424 30. Seimandi Corda, G., Leblanc, M., Faure, S. & Cortesero, A. M. Impact of flower rewards on phytophagous
425 insects: importance of pollen and nectar for the development of the pollen beetle (*Brassicogethes aeneus*).
426 *Arthropod-Plant Interact.* **12**, 779–785 (2018).
427 31. Christiaens, O., Petek, M., Smagghe, G. & Taning, C. N. T. The use of nanocarriers to improve the
428 efficiency of RNAi-based pesticides in agriculture. in *Nanopesticides* (eds. Fraceto, L. F. et al.) 49–68
429 (Springer International Publishing, 2020).
430 32. Yan, S., Ren, B.-Y. & Shen, J. Nanoparticle-mediated double-stranded RNA delivery system: A promising
431 approach for sustainable pest management. *Insect Sci.* **0**, 1–14 (2020).
432 33. Bachman, P. M. *et al.* Characterization of the spectrum of insecticidal activity of a double-stranded RNA
433 with targeted activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte).
434 *Transgenic Res.* **22**, 1207–1222 (2013).
435 34. Máximo, W. P. F. *et al.* Inhibitor of apoptosis is an effective target gene for RNAi-mediated control of
436 Colorado potato beetle, *Leptinotarsa decemlineata*. *Arch. Insect Biochem. Physiol.* **104**, doi:
437 10.1002/arch.21685 (2020).
438 35. Mehlhorn, S. G., Geibel, S., Bucher, G. & Nauen, R. Profiling of RNAi sensitivity after foliar dsRNA
439 exposure in different European populations of Colorado potato beetle reveals a robust response with minor
440 variability. *Pestic. Biochem. Physiol.* **166**, 104569 (2020).
441 36. Vélez, A. M. *et al.* Control of western corn rootworm via RNAi traits in maize: lethal and sublethal effects
442 of Sec23 dsRNA. *Pest Manag. Sci.* **76**, 1500–1512 (2020).
443 37. Pinheiro, D. H., Taylor, C. E., Wu, K. & Siegfried, B. D. Delivery of gene-specific dsRNA by
444 microinjection and feeding induces RNAi response in Sri Lanka weevil, *Myllocerus undecimpustulatus*
445 *undatus* Marshall. *Pest Manag. Sci.* **76**, 936–943 (2020).
446 38. Christiaens, O. *et al.* RNA interference: a promising biopesticide strategy against the African Sweetpotato
447 Weevil *Cylas brunneus*. *Sci. Rep.* **6**, 1–11 (2016).
448 39. Prentice, K. *et al.* RNAi-based gene silencing through dsRNA injection or ingestion against the African
449 sweet potato weevil *Cylas puncticollis* (Coleoptera: Brentidae). *Pest Manag. Sci.* **73**, 44–52 (2017).
450 40. Chikami, Y. *et al.* Oral RNAi of diap1 results in rapid reduction of damage to potatoes in *Henosepilachna*
451 *vigintioctopunctata*. *J. Pest Sci.* **94**, 505–515 (2020).
452 41. Kirk-Spriggs, A. H. *Pollen Beetles - Coleoptera: Kateretidae and Nitidulidae: Meligethinae*. (Royal
453 Entomological Society, 1996).
454

455 **Author contributions**

456 JW conceived experiment, designed methods, performed experiment, supervised experiment, analysed and
457 visualised data, validated analyses, wrote original draft and suggested edits to revised manuscript versions. LS
458 performed experiment and suggested edits to revised manuscript versions. SS performed experiment and
459 suggested edits to revised manuscript versions. RK performed experiment. AIS analysed and visualised data,
460 validated analyses and suggested edits to revised manuscript versions. CNTT designed methods, validated
461 analyses and suggested edits to revised manuscript versions. OC designed methods, validated analyses and
462 suggested edits to revised manuscript versions. GS conceived experiment, validated analyses and suggested edits
463 to revised manuscript versions. EV conceived experiment, designed methods, provided resources, supervised
464 experiment, validated analyses and suggested edits to revised manuscript versions. All authors read and
465 approved the final manuscript.

466

467 **Competing interests**

468 The authors declare no competing interests.

469

470 **Figure captions**

471

472 Figure 1. Survival (%) of *Brassicogethes aeneus* in each treatment in RNAi assay, totalled over all three
473 experimental replicates (starting n=15 biologically independent cages of insects per treatment). Survival curves
474 show *B. aeneus* survival rates for short-term (3 d) and chronic (17 d) exposure to dsRNA treatments: dsαCOP at
475 0.5 µg/µL (a); dsαCOP at 2.5 µg/µL (b); dsαCOP at 5 µg/µL (c); and all dsαCOP treatments shown together (d).
476 Asterisk (*****) indicates significant difference ($p<0.05$) in survival, compared to dsGFP 5 µg/µL (control)
477 treatment. Colour of asterisk indicates the corresponding dsRNA and concentration. Asterisk in a triangle

478 indicates that the significance corresponds to short-term dsRNA feeding. Asterisk in a circle indicates that the
479 significance corresponds to chronic dsRNA feeding. Hash symbol (#) indicates significant difference ($p<0.05$) in
480 survival, between short-term- and chronic dsRNA feeding groups. Colour of hash symbol indicates the
481 corresponding dsRNA and concentration. Asterisks and hash symbols are only used where values become- and
482 remain significant. Analysed using Kruskal-Wallis test, followed by Wilcoxon rank-sums test with Bonferroni
483 correction. Error bars: ±SEM.
484
485 Figure 2. Results of gene expression analysis via quantitative polymerase chain reaction (qPCR), showing
486 relative expression of *αCOP* in *Brassicogethes aeneus* at 3 d and 6 d after the start of the experiment (n=3
487 biologically independent qPCR samples per treatment and time point). Target treatments (dsαCOP at 0.5, 2.5 and
488 5 µg/µL) are statistically compared to the dsGFP 5 µg/µL (control) treatment. Asterisk (*****) indicates significant
489 difference ($p \leq 0.05$) between treatments. Analysed using Welch's t-test.
490
491 Figure 3. Experimental setup for each treatment (dsαCOP at 0.5, 2.5 and 5 µg/µL, and dsGFP at 5 µg/µL), for
492 both short-term (3 d) and chronic (17 d) dsRNA feeding, in *Brassicogethes aeneus* RNAi assays. Here we
493 monitored *B. aeneus* survival and corresponding *αCOP* expression in specimens. For survival analysis, starting
494 n=15 cages per treatment, each cage with eight insects. For both short-term- and chronic dsRNA feeding, three
495 cages (n=3) were removed for qPCR analysis at both 3 and 6 d, and one insect was removed from each of the
496 nine remaining cages at 12 d, for each treatment.

CURRICULUM VITAE

Jonathan Willow
Date of birth: 28.03.1985
E-mail: jonwillowtree@gmail.com; jonathan@emu.ee
ORCID: 0000-0003-2377-4917

Higher education

2017–2021: PhD studies, Ghent University, Ghent, Belgium
2017–2021: PhD studies, Estonian University of Life Sciences, Tartu, Estonia
2014–2016: MS studies, University of Iceland, Reykjavík, Iceland
2009–2013: BS studies, State University of New York College of Environmental Science and Forestry (SUNY-ESF), Syracuse, New York, USA

Relevant work experience

January 2021 – present: **Specialist**, Chair of Plant Health, Estonian University of Life Sciences**,** Tartu, Estonia
Work description: Publication- and preparation of RNAi research on pollen beetle. Teaching Applied Entomology

February 2017 – December 2020: **Junior Researcher**, Chair of Plant Health, Estonian University of Life Sciences, Tartu, Estonia
Work description: Collecting and identifying pollen beetles and their parasitoids; preparation, performance and publication of biopesticide studies (e.g. dsRNAs, essential oils) on pollen beetle, and agrochemical tests on a model parasitoid wasp; teaching Applied Entomology
Supervisors: Eve Veromann (Professor, Estonian University of Life Sciences); Guy Smagghe (Professor, Ghent University)

June – October 2016: **Research Fellow**, Palmi Jónsson's Nature Conservation Fund, Reykjavík, Iceland
Title of study: Case study on forage plants of the heath bumblebee (*Bombus jonellus*) in southwest Iceland
Work description: Collecting data on forage plants used by *Bombus jonellus* in semi-natural areas on the outskirts of Reykjavík; project write-up

Summer 2012: **Cranberry Lake Biological Station Undergraduate Research Fellow**, Cranberry Lake, New York, USA
Title of study: Investigating richness of pond insects (Coleoptera, Hemiptera, Odonata) in the Cranberry Lake region, New York
Work description: Collecting and identifying aquatic insects from wetlands/ponds of the Cranberry Lake region in the Adirondack Park; mapping distributions using ArcGIS; statistical analyses; project write-up
Research mentor: Neil H. Ringler, Vice Provost for Research (SUNY-ESF)

Summer 2011: **Academic Assistant**, Cranberry Lake Biological Station, Cranberry Lake, New York, USA
Work description: Maintaining laboratories; maintaining laboratory- and field equipment for aquatic- and forest biology research; assisting students and faculty; general office duties
Supervisor: Alexander Weir, Professor (SUNY-ESF), Director (Cranberry Lake Biological Station)

Publications

Willow J, Soonvald L, Sulg S, Kaasik R, Silva AI, Taning CNT, Christiaens O, Smagghe G, Veromann E (In Press) RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle. *Communications Biology*

Willow J, Sulg S, Taning CNT, Silva AI, Christiaens O, Kaasik R, Prentice K, Lövei GL, Smagghe G, Veromann E (2020) Targeting a coatomer protein complex-I gene via RNA interference results in effective lethality in the pollen beetle *Brassicogethes aeneus*. *Journal of Pest Science*, DOI: 10.1007/s10340-020-01288-6

Willow J, Soonvald L, Sulg S, Kaasik R, Silva AI, Taning CNT, Christiaens O, Smagghe G, Veromann E (2020) First evidence of bud feeding-induced RNAi in a crop pest via exogenous application of dsRNA. *Insects* **11**, 769

Willow J, Sulg S, Kaurilind E, Silva AI, Kaasik R, Smagghe G, Veromann E (2020) Evaluating the effect of seven plant essential oils on pollen beetle (*Brassicogethes aeneus*) survival and mobility. *Crop Protection* **134**, 105181

Sulg S, Kassik R, **Willow J**, Veromann E (2020) Damage rate of cabbage stem weevil in winter oilseed rape. *Agronomy 2020*, https://pk.emu.ee/userfiles/instituudid/pk/file/PKI/agronoomia/Agronoomia_2020_veebi.pdf

Willow J, Silva AI, Veromann E, Smagghe G (2019) Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. *PLOS ONE* **14**, e0212456

Willow J, Tamayo M, Jóhansson MH (2017) Potential impact of Nootka lupine (*Lupinus nootkatensis*) invasion on pollinator communities in Iceland. *Icelandic Agricultural Sciences* **30**, 51−54

Willow J (2017) Case study on forage plants of the heath bumblebee (*Bombus jonellus*) in southwest Iceland. *Icelandic Agricultural Sciences* **30**, 39−42

Conference speakings

II Plant Pests and Diseases Forum: Redefining Concepts, Mechanisms & Management Tools, 2021; Porto, Portugal (virtual)
Title of talk: Developing an RNAi approach to pollen beetle management

The Annual Conference of PhD Students of the Institute of Agricultural and Environmental Sciences, 2019; Tartu, Estonia
Title of talk: Evaluating two target genes for RNA-based management of pollen beetle

Cruciferous Plant Production Conference, 2018; Tartu, Estonia
Title of talk: Acute effect of low-dose thiacloprid exposure synergized by tebuconazole in a parasitoid wasp

12th meeting of the IOBC-WPRS Working Group "Pesticides and Beneficial Organisms", 2018; Zadar, Croatia
Title of talk: Acute effect of low-dose thiacloprid exposure synergized by tebuconazole in a parasitoid wasp

17th meeting of the IOBC-WPRS Working Group "Integrated Control in Oilseed Crops", 2018; Zagreb, Croatia
Title of talk: Does distance matter? An investigation into the effect of distance from previous oilseed rape crops on abundance of pollen beetle parasitoids and parasitism

XI European Congress of Entomology, 2018; Naples, Italy.
Title of talk: Revealing an agrochemical synergy and its effect on a biocontrol insect

Ecological Society of Iceland (Vistfræðifélag Íslands) conference, 2016; Reykjavík, Iceland

Title of talk: Pollinator diversity in native heath and alien Nootka lupine stands in Iceland

Icelandic Biological Society (Líffræðifélag Íslands) conference, 2015; Reykjavík, Iceland

Title of talk: Pollinator diversity in native heath and alien Nootka lupine stands in Iceland

Awards and special training

2020: Best Scientific Publication by a Young Researcher, 2020 Scholarship Competition to Reward Research on Organic Farming, Tartu, Estonia
Title of publication: Evaluating the effect of seven plant essential oils on pollen beetle (*Brassicogethes aeneus*) survival and mobility

2018: Training in taxonomy and identification of European wild bees, Bologna, Italy
Coordinator: Marino Quaranta, Researcher, Council for Agricultural Research and Agricultural Economy Analysis, Rome, Italy

2016: Research Fellowship Award, Palmi Jónsson's Nature Conservation Fund, Reykjavík, Iceland
Title of study: Case study on forage plants of the heath bumblebee (Bombus jonellus) in southwest Iceland

2012: Cranberry Lake Biological Station Undergraduate Research Fellowship Award, Cranberry Lake, New York, USA
Title of study: Investigating richness of pond insects (Coleoptera, Hemiptera, Odonata) in the Cranberry Lake region, New York

LIST OF PUBLICATIONS

1.1 Publications indexed in the ISI Web of Science database

Willow J, Soonvald L, Sulg S, Kaasik R, Silva AI, Taning CNT, Christiaens O, Smagghe G, Veromann E (In Press) RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle. *Communications Biology*

Willow J, Sulg S, Taning CNT, Silva AI, Christiaens O, Kaasik R, Prentice K, Lövei GL, Smagghe G, Veromann E (2020) Targeting a coatomer protein complex-I gene via RNA interference results in effective lethality in the pollen beetle *Brassicogethes aeneus*. *Journal of Pest Science*, DOI: 10.1007/s10340-020-01288-6

Willow J, Soonvald L, Sulg S, Kaasik R, Silva AI, Taning CNT, Christiaens O, Smagghe G, Veromann E (2020) First evidence of bud feeding-induced RNAi in a crop pest via exogenous application of dsRNA. *Insects* **11**, 769

Willow J, Sulg S, Kaurilind E, Silva AI, Kaasik R, Smagghe G, Veromann E (2020) Evaluating the effect of seven plant essential oils on pollen beetle (*Brassicogethes aeneus*) survival and mobility. *Crop Protection* **134**, 105181

Willow J, Silva AI, Veromann E, Smagghe G (2019) Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. *PLOS ONE* **14**, e0212456

Willow J, Tamayo M, Jóhansson MH (2017) Potential impact of Nootka lupine (*Lupinus nootkatensis*) invasion on pollinator communities in Iceland. *Icelandic Agricultural Sciences* **30**, 51–54

Willow J (2017) Case study on forage plants of the heath bumblebee (*Bombus jonellus*) in southwest Iceland. *Icelandic Agricultural Sciences* **30**, 39–42

3.2 Publications published in books by Estonian or foreign publishers not listed in the ISI Web of Proceedings

Sulg S, Kassik R, **Willow J**, Veromann E (2020) Damage rate of cabbage stem weevil in winter oilseed rape. *Agronomy 2020*, https://pk.emu.ee/userfiles/instituudid/pk/file/PKI/agronoomia/Agronoomia_2020_veebi.pdf

5.2 Conference abstracts

Willow J, Smagghe G, Veromann E (2018) Detrimental synergistic effects of pesticides on the survival of parasitoids. ECE2018, XI European Congress of Entomology, 2−6 July 2018, Naples. PAGEpress Publications, 60−60

Willow J, Tamayo M, Jóhansson MH (2016) Pollinator diversity in native heath and alien Nootka lupine stands in Iceland. Ecological Society of Iceland (Vistfræðifélag Íslands) conference 2016. WordPress, 6−6

Willow J, Tamayo M, Jóhansson MH (2015) Pollinator diversity in native heath and alien Nootka lupine stands in Iceland. Biologia.is

VIIS VIIMAST KAITSMIST

OTTAR TAMM

THE EFFECTS OF LAND USE AND CLIMATE CHANGE
ON THE HYDROPOWER POTENTIAL IN ESTONIAN RIVERS

MAAKASUTUSE JA KLIIMAMUUTUSE MÕJU EESTI JÕGEDE
HÜDROENERGEETILISELE POTENTSIAALILE

Dotsent **Toomas Tamm**
11. detsember 2020

ALEKSANDR SEMJONOV

EVALUATION OF A FIXED-DOSE COMBINATION OF BUTORPHANOL-AZAPERONE-MEDETOMIDINE (BAM) FOR CHEMICAL IMMOBILISATION OF AFRICAN LION, BLESBOK, AND CHEETAH

BUTORFANOOLI-ASAPEROONI-MEDETOMIDIINI (BAM) FIKSEERITUD ANNUSTE KOMBINATSIOONI KASUTAMINE AAFRIKA LÕVI, BLESBOKI JA GEPARDI KEEMILISEKS IMMOBILISEERIMISEKS

Doktor **Vladimir Andrianov**, konsultant doktor **Jacobus Phillippus Raath**
(Wildlife Pharmaceuticals South Africa)
17. detsember 2020

HELIS LUIK-LINDSAAR

EESTI PÕLLUMAJANDUSETTEVÕTETE JA MAAPIIRKONNA KOHALIKE OMAVALIT-SUSTE TEHNILISE EFEKTIIVSUSE HINDAMINE ANDMERAJA ANALÜÜSI MEETODIGA

EVALUATION OF TECHNICAL EFFICIENCY IN FARMS AND RURAL MUNICIPALI-TIES IN ESTONIA USING DATA ENVELOPMENT ANALYSIS

Professor **Rando Värnik** ja vanemteadur **Ants-Hannes Viira**
21. detsember 2020

KAIRI MAILEHT

FÜTOPLANKTON JÄRVEDE SEISUNDI INDIKAATORINA

PHYTOPLANKTON AS ECOLOGICAL QUALITY INDICATOR OF LAKES

Professor Emeritus **Ingmar Ott**, juhtivteadur **Peeter Nõges**
19. veebruar 2021

PAUL FRIDTJOF MÕTSKÜLA

ELEKTRO- JA PILTDIAGNOSTIKA TÄIENDAVAD RAKENDUSED KOERTE SÜDAMEHAIGUSTE DIAGNOOSIMISEL NING PROGNOOSIMISEL

CONTRIBUTION TO THE DIAGNOSIS AND PROGNOSIS OF CANINE CARDIAC DISEASE THROUGH ELECTRODIAGNOSTICS AND DIAGNOSTIC IMAGING

Professor **Toomas Orro**, Professor **Virginia Luis Fuentes** (The Royal Veterinary College, UK),
Professor **David Connolly** (The Royal Veterinary College, UK) ja doktor **Ranno Viitmaa**
8. märts 2021

ISSN 2382-7076
ISBN 978-9949-698-70-7 (pdf)