



Anther-Feeding-Induced RNAi in *Brassicogethes aeneus* Larvae

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OPEN ACCESS

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Specialty section:

This article was submitted to
Pest Management,
a section of the journal
Frontiers in Agronomy

Received: 24 November 2020

Accepted: 30 April 2021

Published: 21 June 2021

Citation:

Willow J, Soonvald L, Sulg S, Kaasik R, Silva AI, Tanning CNT, Christiaens O, Smaghe G and Veromann E (2021) Anther-Feeding-Induced RNAi in *Brassicogethes aeneus* Larvae. *Front. Agron.* 3:633120. doi: 10.3389/fagro.2021.633120

The biosafety aspect of applying double-stranded RNA (dsRNA) in crop pest management is rooted in dsRNA's mode of action, which displays 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 insecticides therefore has the potential to target a single pest species while conferring negligible effect on non-target 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 the 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.

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

INTRODUCTION

Double-stranded RNA (dsRNA) is a prominent class of insecticides and can be applied to crops with potentially no impact on non-target taxa. Cagliari et al. (2018) and Tanning et al. (2019) review the prospects of an approach in which dsRNA can be sprayed onto crops in order to manage target pests. Indeed, this approach has been demonstrated in both the greenhouse (Miguel and Scott, 2015) and field (Petek et al., 2020) for the control of Colorado potato beetle (*Leptinotarsa decemlineata* Say). The biosafety of dsRNA-based insecticides lies in their mode of action against the target pest species, that being nucleotide sequence-specificity. Indeed, the structure of a dsRNA for

use in crop protection can be designed to target a specific region of a messenger RNA (mRNA), potentially resulting in RNA interference (RNAi)-induced gene silencing and subsequent mortality in the target species.

Recent studies suggest potential for developing an RNAi approach for use in integrated management of a major oilseed rape (*Brassica napus* L.) pest, the pollen beetle *B. aeneus* Fab. (syn. *Meligethes aeneus*), via field relevant routes of exposure, including consumption of dsRNA-treated oilseed rape buds (Willow et al., 2020a) and anthers (Willow et al., 2021) as well as dsRNA-treated honey water, representing dsRNA-contaminated nectar (Willow et al., 2020b). The above-mentioned studies were performed on *B. aeneus* adults, the adult stage being the typical focus of insecticide bioassays regarding this pest species (but see Melander et al., 2003).

Brassicogethes aeneus development starts inside the reproductive bud, where hatched larvae feed on the anthers within, followed by the emergence of late first- and early second instar larvae that proceed to feed on the pollen and nectar of open flowers. Therefore, it is plausible that dsRNA application during oilseed rape's flowering stage could additionally target larval *B. aeneus* and, in turn, reduce not only the abundances of pupating larvae and overwintering next-generation adults but oilseed rape yield losses as well. Thus, it is critical that *B. aeneus* larvae are examined as a potential RNAi target for oilseed rape protection. Indeed, if the potential for implementing RNAi-based management of *B. aeneus* is ever examined under natural outdoor conditions, larvae will undoubtedly represent the life stage monitored in such a study. This is because adults can fly from plot to plot, confounding monitoring; whereas larvae will remain on their natal plant until pupation. The present study represents the first attempt to examine RNAi in *B. aeneus* larvae. We expected consumption of *B. aeneus*-specific dsRNA, via treated anthers, to result in reduced target mRNA expression followed by gene silencing-induced mortality.

METHODS

A 222 bp region of *B. aeneus*'s coatamer subunit alpha (α COP) sequence, and a 455 bp region of green fluorescent protein [*gfp*; **Supplementary Table 1**; (Willow et al., 2020a,b)], were the basis for *in vitro* synthesis of two corresponding dsRNAs (AgroRNA, Seoul, South Korea). Both dsRNAs were shipped in distilled water (dH₂O) and kept at 5 ± 1°C upon reception. The absence of nucleic contaminants in these dsRNAs, hereafter respectively called ds α COP and dsGFP (control since *gfp* is not present in insects), was verified via gel electrophoresis.

Pollen beetle larvae were collected via the collection of oilseed rape flowers from an untreated field (58.36377°N, 26.66145°E) in the village of Össu, Tartu County, Estonia. Flowers were transported to the lab and examined for the presence of pollen beetle larvae. Only late first and early second instar larvae identified as *B. aeneus* via Osborne (1965) were used in the study. *B. aeneus* larvae were immediately transferred to polystyrene insect-breeding dishes (diameter 10 x height 4 cm; SPL Life Sciences, Gyeonggi-do, South Korea) that were ventilated and

transparent, hereafter referred to as cages. Eight randomly chosen *B. aeneus* larvae were gently placed in each cage, using a fine paintbrush to avoid any mechanical damage to larvae, and were immediately provisioned with their respective dietary treatment.

There were four treatments, including ds α COP at 0.5, 2.5 and 5 μ g/ μ L, and dsGFP at 5 μ g/ μ L. Previous studies on *B. aeneus* adults showed no difference in control dsGFP concentration used (Willow et al., 2020b), and therefore we continued to use only the highest corresponding concentration to the ds α COP concentrations examined, as in, for example, Willow et al. (2020a). Each treatment was allocated ten cages ($n = 10$; 80 larvae per treatment). Treatments were provided as *ad libitum* access to dsRNA-treated anthers of oilseed rape flowers. Petals were removed from flowers, and anthers were soaked in treatment solution for 15 s and allowed to air dry. Treatment solutions consisted of dsRNA, dH₂O, and the surfactant Triton X-100 (always at 180 ppm; Fisher Bioreagents, Leicestershire, UK), and were vortexed prior to treating anthers. After treatment provision, cages 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 (ds α COP 0.5 μ g/ μ L 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 μ L of RTL buffer (with 10 μ L 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 were assessed via NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA); the 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 μ g 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 μ L of 5xHOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne), 0.5 μ L of 10 μ M

forward and reverse primers (**Supplementary Table 2**; Willow et al., 2020a,b; 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 $2^{-\Delta\Delta\text{Ct}}$ 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 ds αCOP at 0.5, 2.5, and 5 $\mu\text{g}/\mu\text{L}$. 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 non-parametric 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 ds αCOP 0.5, 2.5 and 5 $\mu\text{g}/\mu\text{L}$ treatments, compared to the dsGFP 5 $\mu\text{g}/\mu\text{L}$ control treatment (**Figure 1**). At 6 d after the start of the experiment, *B. aeneus* larvae showed no reduction in αCOP expression, and more variability within treatments.

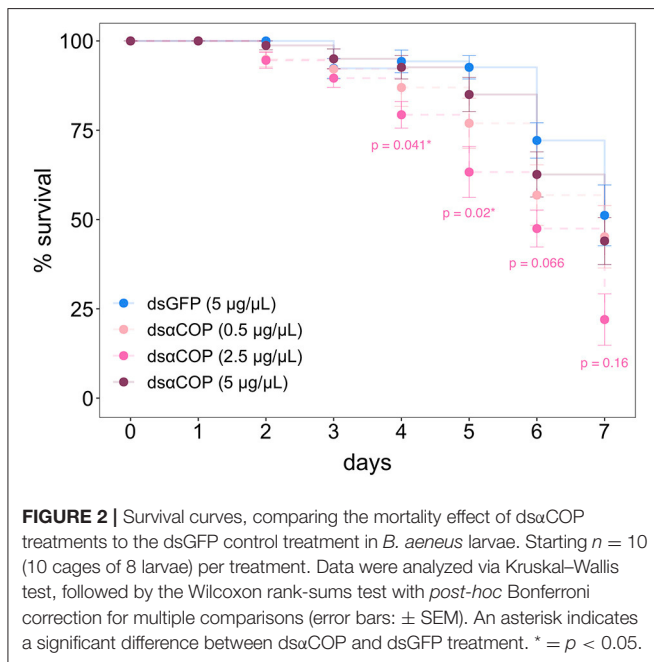
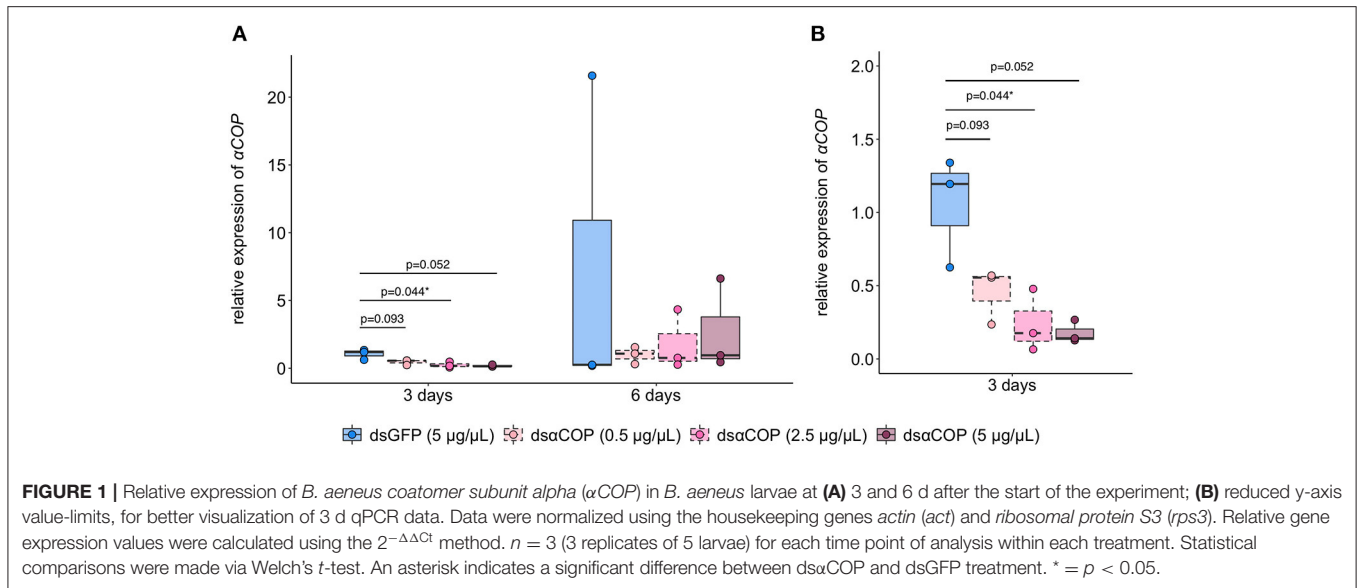
Survival monitoring showed significant reductions in survival, in larvae fed ds αCOP at 2.5 $\mu\text{g}/\mu\text{L}$, at 4 d (79% survival, $df = 3$, $p = 0.041$) and 5 d (63% survival, $p = 0.02$) after the start of the experiment, followed by marginal significance (47% survival, $p = 0.07$) at 6 d, compared to the dsGFP 5 $\mu\text{g}/\mu\text{L}$ control treatment (**Figure 2**). At 7 d, survival of dsGFP control larvae dropped to 49% (ds αCOP 0.5 $\mu\text{g}/\mu\text{L} = 39\%$ survival; ds αCOP 2.5 $\mu\text{g}/\mu\text{L} = 21\%$ survival; ds αCOP 5 $\mu\text{g}/\mu\text{L} = 62\%$ survival). At no time point did mortality significantly differ between dsGFP control larvae and those fed with ds αCOP at either 0.5 or 5 $\mu\text{g}/\mu\text{L}$, even though marginally significant αCOP silencing was observed for both of the ds αCOP concentrations at 3 d.

DISCUSSION

In a previous experiment comparing short-term- to chronic dsRNA feeding, *B. aeneus* adults that fed for 3 d on ds αCOP 2.5 $\mu\text{g}/\mu\text{L}$ -treated anthers did not show significantly reduced survival (Willow et al., 2021). A significant reduction in *B. aeneus* adult survival was observed rather after 8 d of chronic

feeding on ds αCOP 2.5 $\mu\text{g}/\mu\text{L}$ -treated anthers, compared to the present study which showed significantly reduced survival at 4 d in larvae fed ds αCOP 2.5 $\mu\text{g}/\mu\text{L}$ -treated anthers for 3 d. Thus, we here observed quicker RNAi-induced mortality in *B. aeneus* larvae, compared to what has been previously observed in *B. aeneus* adults that were fed comparable ds αCOP treatments. Quicker RNAi-induced mortality in earlier life stages has been observed in both the spotted wing drosophila (*Drosophila suzukii*; Taning et al., 2016) and emerald ash borer (*Agrilus planipennis*; Rodrigues et al., 2018). In the present study, significant αCOP silencing at 3 d, in *B. aeneus* larvae fed ds αCOP at 2.5 $\mu\text{g}/\mu\text{L}$, substantiates the interpretation that this significant mortality was indeed the result of αCOP silencing. Willow et al. (2021) also showed significantly greater mortality in *B. aeneus* adults chronically fed ds αCOP , compared to the mortality observed in short-term (3 d) treatments. Similar to the effect we observed in the present study regarding *B. aeneus* larvae fed ds αCOP 2.5 $\mu\text{g}/\mu\text{L}$ -treated anthers, *B. aeneus* adults fed honey water at 1 and 3 μg ds $\alpha\text{COP}/\mu\text{L}$ showed significant mortality at 6 d and 4 d, respectively (Willow et al., 2020b). Regarding *B. aeneus* adults, the quicker effect observed via honey water feeding, compared to anther feeding, may be attributed to the potentially lower total amount of dsRNA being consumed when coating edible plant parts with dsRNA, compared to feeding on a liquid solution consisting entirely of a near-equivalent concentration of dsRNA. The design of future RNAi studies with *B. aeneus* larvae should consider the potential difference in RNAi efficacy between short-term and chronic feeding on dsRNA-treated anthers. In addition, other field-relevant routes of oral exposure to *B. aeneus*-specific dsRNA should be examined in larvae, including oilseed rape buds sprayed with highly surface-active dsRNA formulations prior to larval emergence from buds; as well as bioengineered, *B. aeneus*-specific, RNAi cultivars of oilseed rape.

While larvae that fed on ds αCOP at both 0.5 and 5 $\mu\text{g}/\mu\text{L}$ showed marginally significant αCOP silencing at 3 d, significant reductions in survival were never observed for these treatments. One reason for this could be the sudden increases in control mortality at 6 and 7 d. This potential confound coincides with 6 d qPCR results that suggest greater variability in αCOP expression at this time, compared to the somewhat expected results observed at 3 d. Nevertheless, a trend of increased αCOP silencing, with respect to ds αCOP treatments at 3 d, suggests the potential for developing an RNAi approach targeting *B. aeneus* larvae. We unexpectedly observed less mortality in larvae fed ds αCOP at 5 $\mu\text{g}/\mu\text{L}$ compared to those fed ds αCOP at 2.5 $\mu\text{g}/\mu\text{L}$. As saturation of the RNAi machinery has yet to be shown to exist in insects, this cannot represent a possible explanation for our observation. In addition, previous studies on adult *B. aeneus* show clear negative relationships between survival and ds αCOP concentration (Willow et al., 2020a,b, 2021). Therefore, we cannot be certain as to what resulted in this observation in *B. aeneus* larvae; and further work must be done in order to address these uncertainties. Regarding the sudden increases in control mortality at 6 and 7 d, similar results were observed by Melander et al. (2003), which is to our knowledge the only other study performing insecticide bioassays with *B. aeneus* larvae.



Previous experiments using the same *gfp*-specific control dsRNA showed no effect of this control dsRNA on *B. aeneus* adult mortality (Willow et al., 2020a,b, 2021). It is instead likely that *B. aeneus* larvae are very sensitive under unnatural conditions, and, consequently, high mortality can occur, as evidenced by both Melander et al. (2003) and the present study.

Refinements must be made to *B. aeneus* larval bioassay setups, in order to ensure optimal conditions for keeping *B. aeneus* larvae alive in a controlled environment. These refinements should strive to mimic conditions to which *B. aeneus* larvae are subjected under natural conditions. For example, in the field,

B. aeneus larvae are able to seek refuge within flower petals, providing them a microhabitat that facilitates greater retention of moisture and less direct light. We removed this microhabitat from the feeding setup, for ease of dsRNA application and monitoring of larvae. If petals are removed in future studies, comparable microhabitats should be provided in the setup, or climate chamber conditions (e.g., relative humidity and light intensity) should be adjusted accordingly, in order to reduce potential stressors. Future studies aiming to examine, via anther feeding, the effect of dsRNA-based insecticides on *B. aeneus* larvae under more natural conditions, should consider spraying highly surface-active dsRNA formulations on oilseed rape raceme tips consisting of whole flower clusters and allowing cohorts of larvae to feed *ad libitum* in this type of semi-field-realistic bioassay setup.

CONCLUSION

In *B. aeneus* larvae, we observed significant α COP silencing and corresponding α COP silencing-induced mortality, via 3 d of feeding on ds α COP 2.5 μ g/ μ L-treated anthers of oilseed rape. These results suggest that, compared to what has previously been observed in *B. aeneus* adults, *B. aeneus* larvae may represent a more RNAi-sensitive life stage of this pest species. While larval mortality rates were variable between treatments, our observed trend of α COP silencing via field-relevant dsRNA feeding suggests the potential for further research in RNAi targeting *B. aeneus* larvae. Further experiments examining the potential for RNAi-based oilseed rape protection against *B. aeneus* larvae should include semi-field-realistic bioassay setups not only through spraying dsRNA formulations on open flowers and closed buds containing *B. aeneus* larvae but also via the use of bioengineered RNAi cultivars for targeting both larval and adult *B. aeneus*. Finally, other target genes should be examined for potentially greater RNAi efficacy against *B. aeneus*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

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 AS analyzed and visualized the data. JW wrote the original draft. All authors contributed to revisions and approved the final manuscript.

FUNDING

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 institutional Research

Funding project no. PRG1056 of the Estonian Research Council, 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 beetle larvae and oilseed rape flowers. JW 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). OC is a recipient of a postdoctoral fellowship from the Research Foundation – Flanders (FWO-Vlaanderen).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fagro.2021.633120/full#supplementary-material>

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Conflict of Interest: 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.

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