Nasonia vitripennis as a parasitoid wasp model for pest-specific dsRNA safety assessment

Short title: Parasitoid wasp model for dsRNA safety assessment

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Abstract

RNA interference (RNAi) is a promising mode of action for pest control. It can play an important role in integrated pest management (IPM), where it could be used alongside beneficial insects (such as predators and parasitoids) for pest control. However, despite the possibility to design the active molecule double stranded RNA (dsRNA) to be pest-specific, potential adverse effects to beneficial insects cannot be ruled out a priori, thus prompting the need for safety assessment studies. In this study, the jewel wasp *Nasonia vitripennis* is presented as a parasitoid wasp model for the evaluation of possible unintended effects that could arise from oral exposure of a parasitoid to exogenously delivered pest-specific dsRNA. First, the susceptibility of *N. vitripennis* to ingested dsRNA was tested through a worst-case exposure scenario, where they were exposed to a high concentration (1 µg/µl) of *N. vitripennis*-specific dsRNA (dsNv_alphaCOP). Ingestion of dsNv_alphaCOP resulted in 97% wasps mortality by day 16 post treatment when compared to the control. Once risk of exposure was established, *N. vitripennis* were orally exposed to a pollen beetle pest (*Brassicogethes aeneus*)-specific dsRNA (dsBa_alphaCOP), after which both lethal and potential sub lethal effects (development time, fecundity and sex ratio) were evaluated. In this worst-case scenario exposure setup, dsBa_alphaCOP did not cause any adverse effects to *N. vitripennis*. This study confirms under laboratory conditions that RNAi-based pest control can be compatible with biological control, based on parasitoid wasps. Furthermore, this study can contribute in guiding the development and safety assessment of future RNAi-based pest control products.

Keywords: RNAi, IPM, Pest control, Biocontrol, Risk assessment

1. Introduction

Insects are considered to be responsible for about 20 to 40% of yield losses in agriculture (Oerke 2006). To prevent the damage caused by insects, farmers rely heavily on the use of conventional pesticides whose excessive use imposes significant costs and adverse effects on the environment. Accumulating data on the hazards of these pesticides over the decades has led to ever-increasing restrictions or even bans on their use, and in turn to a pressing need for effective, safe and sustainable alternatives. RNA interference (RNAi) is a new and very promising mode of action for pest control (Zotti et al. 2018; Taning et al. 2021). In RNAi-based pest control, the active molecule, double-stranded RNA (dsRNA), is delivered to the pest insect where it can suppress the expression of a gene that is essential for survival, thereby leading to reduced fitness or mortality of the pest insect (Rodrigues et al. 2021; Shen et al. 2021). Due to the sequence-dependent mode of action of RNAi, genes can be targeted very specifically, and this specificity is a huge advantage of RNAi-based pest control over conventional insecticides. RNAi-based pest control can also become an important component of integrated pest management (IPM), where it will be used alongside beneficial insects such as natural enemies (predators and parasitoids) for biological control (Sarmah et al. 2021; Castellanos et al. 2022). Despite the possibility to design dsRNA to be pest specific, adverse effects to non-target species cannot be ruled out a priori, thus prompting the need for proper safety assessment studies of pest-specific dsRNA on non-target species.

The jewel wasp, *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae), is one of the most widely studied parasitoid wasp species (Mair & Ruther 2019; Kalyanaraman & Lammers 2021). They are distributed across the whole world and can act as biological control agents by parasitizing insect pests that belong to the Diptera. *N. vitripennis* is a model organism for parasitoid research because it is easily maintained in the laboratory environment, due to its short

generation time (~2 weeks at 25°C) and can be easily reared in small vials with blowfly *Sarcophaga bullata* pupae (Dittmer & Brucker 2021). Moreover, its genome has been sequenced (Werren et al. 2010) and is publicly available for bioinformatics analysis to support empirical research. As objective in this study, *N. vitripennis* was used as a parasitoid wasp model for the evaluation of possible unintended effects that could arise from oral exposure of a parasitoid to exogenously delivered pest-specific dsRNA. First, the susceptibility of *N. vitripennis* to ingested dsRNA was tested through a worst-case exposure scenario, where *N. vitripennis* was exposed to a high concentration of *N. vitripennis*-specific dsRNA targeting its endogenous gene. Then, *N. vitripennis* was exposed to pest-specific dsRNA, after which lethal and sublethal effects were evaluated. The pest-specific dsRNA selected in this study targets an essential gene [alpha-coatomer protein, isoform A gene (*Ba_alphaCOP*)] in the pollen beetle, *Brassicogethes aeneus* (formerly *Meligethes aeneus*) Fabricius (Coleoptera: Nitidulidae). *Ba_alphaCOP* has been reported in our previous studies to be able to significantly knockdown *Ba*_alphaCOP transcripts in *B. aeneus*, following oral exposure (Willow et al. 2021a; 2021b). It encodes the alpha subunit of a non-clathrin-coated vesicular coat protein that is important in mediating protein transport between the endoplasmic reticulum and Golgi compartments (Gerich et al.1995). Altogether, the design and results from this study provide guidance to future Tier 1 safety assessments, of any exogenously delivered dsRNA-based product, that would use *N. vitripennis* as a model for parasitoid wasps.

2. Materials and methods

2.1. *N. vitripennis* culture maintenance

Adult *N. vitripennis* used in this study were obtained from a continuous culture at the Laboratory of Agrozoology (Ghent University, Belgium). They were maintained in 50 ml Eppendorf tubes with a Whatman filter paper (Merck, Darmstadt, Germany) soaked in BioGluc

sugar solution (Biobest, Westerlo, Belgium) (food source) and *S. bullata* pupae (for instar development) [25°C, 16:8 (light: dark) photoperiod and 65% relative humidity].

2.2. Evaluating *N. vitripennis* susceptibility to dsRNA following oral exposure

2.2.1. *N. vitripennis*-specific dsRNA design and synthesis

To verify whether *N. vitripennis* could be sensitive to dsRNA through oral exposure, dsRNA was designed and fed at a high dose to directly target a *N. vitripennis* essential gene, simulating a worst-case exposure scenario. A dsRNA sequence (dsNv_alphaCOP) targeting *Nv_alphaCOP* in *N. vitripennis* was designed and used in the bioassay. The *Nv_alphaCOP* coding sequence (CDS) was detected in the transcriptome of *N. vitripennis* available in the GenBank database (National Center for Biotechnology Information—NCBI) via BLAST analysis, using known *alphaCOP* sequences from other insect species. In order to avoid potential cross-silencing of other genes in *N. vitripennis*, a selected region from the *Nv_alphaCOP* CDS was screened for cross-homologies within the *N. vitripennis* transcriptome using BLAST analysis to ensure that there were no shared fragment similarities that could result to off-target transcript knockdown. The selected *Nv_alphaCOP* sequence, as well as a 455 bp sequence from the green fluorescent protein (*gfp*) gene as control, was used as the basis for the synthesis of the corresponding dsRNA products (dsNv_alphaCOP and dsGFP).

For *in vitro* dsRNA synthesis, total RNA was extracted from *N. vitripennis* adults, using an RNeasy Mini kit (Qiagen, Hilden, Germany). RNA was treated with Turbo DNA-free kit (Invitrogen, Carlsbad, CA, USA) to remove any genomic DNA remnants, and 1 µg was used to synthesize complementary DNA (cDNA) using a Superscript IV First-Strand Synthesis System kit (ThermoFisher, Waltham, MA, USA), as instructed by the manufacturer. DsRNA

templates were then produced by PCR using cDNA and gene-specific primers with a T7 promoter region (TAATACGACTCACTATAGGG) added to the 5′ end of each primer (Table 1). The following PCR program was used: 2 mins at 94°C, 5x (30 s at 94°C, 30 s at 59°C, 30 s at 72°C), 35 cycles of (30 s at 94°C, 30 s at 65°C, 30 s at 72°C), 3 min at 72°C. For the amplification of the GFP fragment, a plasmid with GFP insert (Genbank ID: NC_011521.1) was used as a template. The resulting PCR products were purified using the Wizard SV gel and PCR clean up system (Promega, Madison, WI, USA), and immediately used for *in vitro* dsRNA synthesis using MEGAscript RNAi kit (ThermoFisher, Vilnius, Lithuania) as instructed by the manufacturer. The synthesized dsRNA was verified by 1.5% agarose gel electrophoresis and quantified with a DS-11 FX spectrophotometer (DeNovix) at 260 nm. The dsRNA was stored at −20°C prior to further use.

2.2.2. Oral exposure bioassays with *N. vitripennis*-specific dsRNA

For the oral exposure bioassays, the newly emerged *N. vitripennis* adults were randomly divided into a group of six 50 ml Eppendorf tubes of 10-14 wasps (66-74 wasps in total), for each treatment. Each group was orally exposed to filter paper containing 20 µl of 50% sucrose solution diluted with either dsNv_alphaCOP (final concentration 1 µg/µL), dsGFP (final concentration 1 µg/µL), or nuclease-free water. A very high final concentration of 1 µg/µl was used in this bioassay for representing a worst-case exposure scenario, and also because the pest-specific dsRNA used in this study caused significant reduction in survival at that concentration in the pest insect (>75% at day 14) (Willow et al. 2021a). New sucrose solution diet containing either dsRNA or water was administered every 48 h, and the wasps were allowed to feed *ad libitum*. After an exposure period of 6 days, the sucrose solution diet was replaced by sucrose solution without the dsRNA, and survival was monitored daily for 16 days. An extra control was included in the bioassay where a total of 17 wasps were not given any sucrose solution diet

to see how long the wasps could survive without food, ensuring that all wasps that survived beyond a certain time point should be feeding on the treatment. A Kaplan–Meier survival curve was computed for the different treatment groups (alive = 0, dead = 1), and the Log-rank (Mantel-Cox) test was used to check for differences between the curves ($p < 0.05$), also using the GraphPad Prism v6.0 software. The Bonferroni method ($p < 0.05$) was used for multiple comparisons of survival curves.

To evaluate the transcript level of *Nv_alphaCOP* following exposure of *N. vitripennis* adults to dsNv_alphaCOP, 4 different time points (48 h, 72 h, 96 h, 120 h) post treatment were selected. In a separate setup, newly emerged *N. vitripennis* adults were randomly divided into a group of three 50 ml Eppendorf tubes of 15-20 wasps (45-60 wasps in total), for each time point, and for each treatment (dsNv_alphaCOP and dsGFP). Oral exposure to dsRNA took place in the same way as described in the paragraph above, and at the same dsRNA concentration of 1 µg/µl. At each time point, three tubes were taken from each treatment. All insects from 1 tube were pooled together and total RNA was extracted using the RNeasy Mini kit (Qiagen) as advised by the manufacturer. After RNA extraction, a DNase treatment was done using the Turbo DNA-free kit (Invitrogen, Carlsbad, CA, USA), the integrity, purity and concentration of the RNA were evaluated by agarose gel electrophoresis and spectrophotometry. Subsequently, cDNA was synthesized using the Superscript IV kit (Invitrogen, Carlsbad, California, USA) with 500 ng of template RNA. Real-time (RT)-qPCR was performed in a CFX96 real-time system, and the CFX Manager software (both from Bio-Rad). The reaction included 10 µl of SYBR green Supermix (Bio-Rad), 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM of reverse primer, 1 µl of nuclease-free water and 8 µl of cDNA, in a total volume of 20 µl. The amplification conditions were 5 min at 95 °C followed by 39 cycles of 30 s at 95 °C and 1 min at 60 °C. The reactions were set-up in 96-well format Microseal PCR plates (Bio-Rad) in triplicates.

Transcript levels of *Nv_alphaCOP* were normalized to three endogenous reference genes, *rpL32*, *ubi* and *pp1a*, by the equation ratio $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen 2001) (See primer sequences in Table 1). Appropriate controls, no-template control and no reverse transcriptase control, were also included in the assay. Significant differences in relative expression levels of *Nv_alphaCOP*, between treatments (dsNv_alphaCOP and dsGFP), were calculated by an unpaired t-test ($p < 0.05$) using the GraphPad Prism v8.4.3 software (San Diego, CA, USA).

2.3. Evaluating effects oral exposure to pest-specific dsRNA in *N. vitripennis*

Prior to bioassays investigating for potential unintended effects in *N. vitripennis* following oral exposure to pest-specific dsRNA, a dsRNA molecule (dsBa_alphaCOP) targeting *Ba_alphaCOP* transcripts in the pest pollen beetle *B. aeneus* was synthesized *in vitro*. Based on our previous study, a selected region on the *Ba_alphaCOP* sequence was used to synthesize dsRNA that could effectively target *Ba_alphaCOP* transcripts in *B. aeneus* (Willow et al. 2021a; 2021b). As such, the same region was amplified using the primers indicated in Table 1, and dsBa_alphaCOP was subsequently synthesized using the same commercial kits described in section 2.2.2 above for dsNv_alphaCOP synthesis.

To evaluate for potential lethal effects of pest-specific dsRNA to *N. vitripennis*, an oral exposure bioassay was set-up in the same format as described above (section 2.2.2), with the exception that an extra treatment, dsBa_alphaCOP, was included in the bioassay. The dsNv_alphaCOP treatment was maintained in the bioassay set up as a positive control. Survival was also monitored daily for 16 days, and generated Kaplan-Meier survival curves were statistically analysed as described above in section 2.2.2. At 120 h post treatment, *Nv_alphaCOP* transcript analyses was evaluated in the different treatment groups. In brief, *N. vitripennis* adults in each of the 3 tubes (about 15 -20 wasps per tube) per treatment were pooled

184 together into one tube for RNA extraction (3 tubes with each of 15-20 wasps per treatment).
185 RNA extraction, cDNA synthesis, and RT-qPCR were performed using the same protocols as
186 described in section 2.2.2 above.
187
188 To evaluate for potential sublethal effects on life history traits, another oral exposure bioassay
189 was set up, where a larger number of *N. vitripennis* adults (1200 insects in total) were exposed
190 to 20 µl of 50% sucrose containing either dsBa_alphaCOP (1 µg/µl) or nuclease-free water (as
191 control). In brief, newly emerged *N. vitripennis* adults were randomly divided into a group of
192 thirty 50 ml Eppendorf tubes, each with 20 wasps (600 wasps in total), for each treatment. New
193 sucrose solution diet containing either dsBa_alphaCOP or water was administered every 48 h.
194 After an exposure period of 6 days, five *N. vitripennis* adult females from each of the thirty
195 50 mL Eppendorf tubes were independently transferred to a new 50 mL Eppendorf tube
196 containing two blowfly (*S. bullata*) pupae for parasitization (in total 150 wasps), per treatment.
197 Each *N. vitripennis* adult female was provided with sucrose diet without dsRNA. After 48 h of
198 exposure time, to parasitize the *S. bullata* pupae, the *N. vitripennis* adult females were taken
199 out of the 50 ml Eppendorf tube. The *S. bullata* pupae (2 per tube) still present in the tubes were
200 monitored and the time taken for new *N. vitripennis* adults to emerge in each tube was recorded
201 as development time. The total number of newly emerged *N. vitripennis* adults per female was
202 recorded as fecundity while the total number of females and males per treated female was
203 recorded and used to determine the sex ratio. For data analysis, normality and equal variance
204 of the dataset were first analysed by the Shapiro–Wilk test and Brown–Forsythe test,
205 respectively. Based on the analysis, statistical differences among the different treatments were
206 determined by unpaired *t*-test ($p < 0.05$) using GraphPad Prism v8.4.3 software (San Diego,
207 CA, USA).
208

209 **3. Results**

210 **3.1. *N. vitripennis* is susceptible to ingested *Nasonia*-specific dsRNA**

211 The susceptibility of *N. vitripennis* to ingested insecticidal dsRNA was investigated by feeding
212 the insects with dsRNA targeting the *N. vitripennis alpha-COP* homolog (dsNv_alphaCOP) at
213 a high concentration of 1000 ng/µl, and survival was monitored daily for 16 days. Oral exposure
214 of the wasps to dsNv_alphaCOP resulted in 97% mortality by day 16 post treatment, which was
215 significantly ($p < 0.0001$) more than observed in the control groups, dsGFP (21%) and water
216 (37%) (Figure 1a, Supplementary file 1). In the absence of food, all wasps died by day 5,
217 indicating that all wasps that survived beyond the dsRNA exposure period (6 days) in this study
218 were orally exposed to the diet containing the different treatments (dsNv_alphaCOP, dsGFP
219 and water) (Figure 1a).

220

221 Furthermore, the transcript level of Nv_alphaCOP was evaluated in the dsNv_alphaCOP-
222 treated group to verify whether the significant decrease in survival in this group was due to
223 Nv_alphaCOP transcript knockdown. As such, the transcript level of Nv_alphaCOP was
224 evaluated at four time points, 48 h, 72 h, 96 h and 120 h, post exposure of the wasps to either
225 dsNv_alphaCOP or the dsGFP control. At 96 h and 120 h post treatment, a significant decrease
226 ($p = 0.0002$ and $p = 0.0028$, respectively), in the transcript level of Nv_alphaCOP (66% and
227 77%, respectively) was observed in dsNv_alphaCOP-treated wasps in comparison to the
228 dsGFP-treated controls (Figure 1b, Supplementary file 1). This confirmed that if *N. vitripennis*
229 is orally exposed to insecticidal dsRNA, with high sequence complementarity to its endogenous
230 mRNA, then transcript knockdown can occur, and this can subsequently lead to mortality.

231

232 **3.2. Ingested pest-specific dsRNA had no adverse effects *N. vitripennis***

Once established that *N. vitripennis* is susceptible to *Nasonia*-specific insecticidal dsRNA through the oral route, we proceeded to check whether a non-specific insecticidal dsRNA designed against a pest insect could have lethal and/or sublethal effects in *N. vitripennis*, following oral exposure. To evaluate potential lethal effects, *N. vitripennis* was orally exposed to dsBa_alphaCOP that targets the *alpha-COP* gene in the pest pollen beetle, *B. aeneus*. As positive control, dsNv_alphaCOP was included in the oral exposure assays where it caused 98% mortality to *N. vitripennis* that fed on it by day 16 post exposure (Figure 1c, Supplementary file 1). In contrast, no significant difference was observed in the survival of *N. vitripennis* treated with dsBa_alphaCOP (27%) when compared to the dsGFP (28%) and water (20%) controls at day 16 (Figure 1c). Furthermore, no significant difference ($p = 0.895$) was observed in the transcript level of Nv_alphaCOP in the dsBa_alphaCOP-treated wasps when compared to the dsGFP controls at 96 h post treatment (Figure 1d, Supplementary file 1). This confirmed that pest-specific dsRNA can be designed to avoid lethal effects in the parasitoid *N. vitripennis*.

Besides potential lethal effects to *N. vitripennis* following exposure to pest-specific dsRNA, the evaluation of potential sublethal effects is equally important, as it can hamper the biological control potential of the parasitoid wasp. In a separate bioassay, life history traits of *N. vitripennis* such as offspring development time, fecundity (offspring per female) and offspring sex ratio, were evaluated in wasps exposed to pest-specific dsRNA (dsBa_alphaCOP) in comparison to the water control (Figure 2a). No significant difference was observed in the development time, fecundity and in the sex ratio (female to male) of *N. vitripennis* offspring that emerged from fly pupae that were parasitized by dsBa_alphaCOP-treated female wasps when compared to offspring from the water-treated controls (Figure 2b, Supplementary file 2). An average of 18 days was required from parasitization of the fly pupae by adult female *N. vitripennis* to when adult *N. vitripennis* offspring emerged from the parasitized fly pupae. In

total, an average of 43 *N. vitripennis* adult offspring emerged from 2 parasitized fly pupae by a single female adult *N. vitripennis*. Of the 43 offspring that merged, 79% were female while 21% were male (Figure 2b).

4. Discussion

In practice, there is a reasonable chance that parasitoid wasps are exposed to insecticidal dsRNAs when they are used in an IPM strategy against pest insects, either as RNAi-based pest control spray products or transgenic plants producing dsRNA. Different exposure scenarios are possible depending on the dsRNA delivery strategy used for pest control. For example, most adult parasitoid wasps are known to feed on plant nectar (Godfray 1994; Wanner et al. 2006), implying that they can ingest dsRNA when transgenic dsRNA-expressing plants are used. The most likely exposure route for parasitoid wasps would be via the trophic chain, where the young parasitoid wasps would be exposed to dsRNA inside the parasitized host that they are feeding and developing on. Nevertheless, for any adverse effects to occur in parasitoid wasps that are exposed to dsRNA, they first have to be susceptible to RNAi induction through oral exposure to dsRNA. In this study, the risk of oral exposure of the parasitoid wasp *N. vitripennis* to dsRNA was evaluated. *N. vitripennis* has been reported to show strong systemic and parental RNAi upon injection (Lynch & Desplan 2006), however, there are no records of RNAi induction by ingested dsRNA (oral RNAi). Therefore, it was crucial to first determine whether *N. vitripennis* is susceptible to oral RNAi. Also, if ingested dsRNA specifically designed to target the expression of an endogenous gene in the parasitoid wasp shows no RNAi effects, this would imply that the risk of exposure to pest-specific dsRNA is low to null. For example, no RNAi effects were reported in the honeybee *Apis mellifera* Linnaeus (Hymenoptera: Apidae) following ingestion of its gene-specific dsRNA, indicating that the honeybee is simply not susceptible to environmental RNAi (Vélez et al. 2016). In another study, similar results were

283 reported for the monarch butterfly *Danaus plexippus* Linnaeus (Lepidoptera: Nymphalidae),
284 which was not affected by its gene-specific dsRNA, meaning that also non-specific dsRNA will
285 probably pose no risk to these insects (Pan et al. 2017). In this study, ingested *N. vitripennis*
286 gene-specific dsRNA (dsNv_alphaCOP) caused 97% mortality to exposed wasps within 16
287 days, and this was linked to the significant decrease in Nv_alphaCOP transcript levels. These
288 results confirm that oral RNAi is functional in *N. vitripennis* and that sufficiently homologous
289 dsRNA could lead to adverse effect upon exposure, making it relevant to always investigate for
290 potential unintended effects in representative non-target organisms.
291
292 By simulating a scenario where the parasitoid wasps are orally exposed to pest-specific dsRNA,
293 dsBa_alphaCOP targeting the *alphaCOP* gene in the pest pollen beetle *B. aeneus* was used.
294 Recent research within our group demonstrated that targeting *Ba_alphaCOP* can lead to
295 transcript knockdown and mortality in dsBa_alphaCOP-treated pollen beetles (Willow et al.
296 2021a; 2021b). This pollen beetle is an important pest in oilseed rape and problems are arising
297 with pesticide resistance (Thieme et al. 2010). Therefore, alternative strategies like the
298 combined use of RNAi-based pest control and natural enemies like parasitoid wasps could
299 provide a solution, fitting within an IPM strategy. Here, the pollen beetle-specific dsRNA was
300 tested for potential unintended effects in *N. vitripennis*, used as a parasitoid wasp model. In the
301 feeding assays, a dsRNA concentration of 1000 ng/µl in the sucrose diet was used. At these
302 high concentrations, a worst-case scenario was created, with exposure levels several times
303 higher than expected in the field. Experiments with other hymenopterans like the honeybee
304 showed that in general high levels of dsRNA are needed to elicit an RNAi response (Vélez et
305 al. 2016). Despite this high concentration of dsRNA, which could elicit an RNAi response in
306 the case of the *N. vitripennis*-specific dsRNA, the pest pollen beetle-specific dsRNA did not
307 cause a significant decrease in Nv_alphaCOP transcript levels or increased mortality in the

parasitoid wasps compared to the controls. This can be explained by the very low sequence homology between the pollen beetle-specific dsRNA (dsBa_alphaCOP) and the target gene (*alphaCOP*) homolog in *N. vitripennis*. The siRNA pathway is a sequence-dependent mechanism that relies on high sequence complementarity to cause target transcript knockdown, implying that a very low sequence homology between a dsRNA sequence and endogenous transcripts will likely not lead to transcript knockdown. However, when evaluating the effects of non-specific dsRNA in beneficial insects, only checking the transcript knockdown of the target gene homolog is not necessarily sufficient to rule out potential effects at the transcript level. Random genes in the genome can have more homology to the used non-specific dsRNA than the target gene homolog itself. Since the long dsRNA is cut into siRNAs before binding to complementary mRNA, it is the homology between the siRNA and regions in the transcriptome that is relevant to finding possible off-target gene transcripts. Nevertheless, the relevance of transcript knockdown is only important if it leads to adverse effects on the normal development and survival of the beneficial insect in question.

Not only mortality is relevant when evaluating potential adverse effects of pest-specific dsRNA on beneficial insects. There can also be other unwanted sublethal effects on important life history traits such as, reduction in fertility or fecundity, development time, changes in sex ratio, behaviour, feeding etc. In this study, potential adverse effects on important parasitoid wasp traits (offspring development time, fecundity and sex ratio) after exposure to pest-specific dsRNA were investigated. Adult male and female *N. vitripennis* were orally exposed to pollen beetle-specific dsRNA for 6 days, during which they ingested the dsRNA solution and also mated. The adult female parasitoid wasps were then allowed to individually parasitize their host pupae (2 pupae) for 2 days, after which the females were taken out and offspring developmental time to emergence, number of emerged offspring (fecundity) and offspring sex ratio were

monitored. No difference in any of the life trait parameters evaluated was observed between the dsBa_alphaCOP-treated group and the water-treated control group. These findings go in line with previous studies that also confirm that pest-specific dsRNA can be designed to be specific to a pest insect while leaving beneficial insects unharmed (Hollowell & Rieske 2022).

5. Conclusions

Parasitoid wasps can be an important part of IPM, as natural enemies of pest insects. In order for these parasitoid wasps to be compatible with the use of RNAi-based pest control, these beneficial insects cannot be harmed by dsRNA designed against pest insects. In this study, *N. vitripennis* was used as a model for parasitoid wasps, and the unintended effects of non-specific dsRNA, designed to target the pollen beetle were assessed. This study indicated that *N. vitripennis* is susceptible to oral RNAi, given that the dsRNA sequence complementarity to the endogenous gene transcript is high. However, although risk of exposure is possible, carefully designed pest-specific dsRNA can leave *N. vitripennis* unharmed following oral exposure. Altogether, this study confirms under laboratory conditions that RNAi-based pest control can be compatible with biological control, based on parasitoid wasps. Furthermore, the findings in this study can contribute to guiding the development and safety assessment of future RNAi-based pest control products.

Author contribution

CT, OC, and GS conceived and designed the research. JL and MD conducted the experiments. CT analyzed the data and wrote the first draft manuscript. JL, MD, OC, GS, and CT revised the draft manuscript. All authors read and approved the final version of the manuscript.

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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. OC has moved from a postdoctoral researcher position at Ghent University to a position in the private sector (Devgen N.V.). While the manuscript was submitted after this employment change, it had no influence on the content of the manuscript, as he was not involved anymore with the work and manuscript writing after his change of employment.

References

Castellanos, N. L., Smagghe, G., Taning, C. N. T., Oliveira, E. E., & Christiaens, O. (2022). Risk assessment of RNAi-based pesticides to non-target organisms: Evaluating the effects of sequence similarity in the parasitoid wasp *Telenomus podisi*. *Science of The Total Environment, 832*, 154746. https://doi.org/10.1016/j.scitotenv.2022.154746

Dittmer, J., & Brucker, R. M. (2021). When your host shuts down: larval diapause impacts host-microbiome interactions in *Nasonia vitripennis*. Microbiome, 9(1), 1-19. https://doi.org/10.1186/s40168-021-01037-6

Gerich, B., Orci, L., Tschochner, H., Lottspeich, F., Ravazzola, M., Amherdt, M., ... Harter, C. (1995). Non-clathrin-coat protein alpha is a conserved subunit of coatomer and in

Saccharomyces cerevisiae is essential for growth. *Proceedings of the National Academy of Sciences, 92*(8), 3229-3233. https://doi.org/10.1073/pnas.92.8.3229

Godfray, H. C. J. (1994). Parasitoids: behavioral and evolutionary ecology (Vol. 67). *Princeton University Press.* https://doi.org/10.1515/9780691207025

Hollowell, H., & Rieske, L. K. (2022). Southern pine beetle-specific RNA interference exhibits no effect on model nontarget insects. *Journal of Pest Science, 95*(3), 1429-1441. https://doi.org/10.1007/s10340-021-01473-1

Kalyanaraman, D., Gadau, J., & Lammers, M. (2021). The generalist parasitoid *Nasonia vitripennis* shows more behavioural plasticity in host preference than its three specialist sister species. *Ethology, 127*(11), 964-978. https://doi.org/10.1111/eth.13217

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *methods, 25*(4), 402-408. https://doi.org/10.1006/meth.2001.1262

Lynch, J. A., & Desplan, C. (2006). A method for parental RNA interference in the wasp *Nasonia vitripennis*. *Nature protocols, 1*(1), 486-494. https://doi.org/10.1038/nprot.2006.70

Mair, M. M., & Ruther, J. (2019). Chemical ecology of the parasitoid wasp genus *Nasonia* (Hymenoptera, Pteromalidae). *Frontiers in Ecology and Evolution, 7*, 184. https://doi.org/10.3389/fevo.2019.00184

Oerke, E. C. (2006). Crop losses to pests. *The Journal of Agricultural Science*, *144*(1), 31-43. https://doi.org/10.1017/S0021859605005708

Pan, H., Yang, X., Bidne, K., Hellmich, R. L., Siegfried, B. D., & Zhou, X. (2017). Dietary risk assessment of v-ATPase A dsRNAs on monarch butterfly larvae. *Frontiers in plant science, 8*, 242. https://doi.org/10.3389/fpls.2017.00242

Rodrigues, T. B., Mishra, S. K., Sridharan, K., Barnes, E. R., Alyokhin, A., Tuttle, R., ... Narva, K. E. (2021). First Sprayable double-stranded RNA-based biopesticide product targets *proteasome* subunit beta type-5 in Colorado potato beetle (*Leptinotarsa decemlineata*). *Frontiers in plant science, 12*, https://doi.org/10.3389/fpls.2021.728652

Sarmah, N., Kaldis, A., Taning, C. N. T., Perdikis, D., Smagghe, G., & Voloudakis, A. (2021). dsRNA-mediated pest management of *Tuta absoluta* is compatible with its biological control agent *Nesidiocoris tenuis*. *Insects, 12*(4), 274. https://doi.org/10.3390/insects12040274

Shen, Y., Chen, Y. Z., & Zhang, C. X. (2021). RNAi-mediated silencing of ferritin genes in the brown planthopper *Nilaparvata lugens* affects survival, growth and female fecundity. *Pest Management Science, 77*(1), 365-377. https://doi.org/10.1002/ps.6026

Taning, C. N. T., Mezzetti, B., Kleter, G., Smagghe, G., & Baraldi, E. (2021). Does RNAi-based technology fit within EU sustainability goals?. *Trends in Biotechnology, 39*(7), 644-647. https://doi.org/10.1016/j.tibtech.2020.11.008

Thieme, T., Heimbach, U., & Müller, A. (2010). Chemical control of insect pests and insecticide resistance in oilseed rape. *In Biocontrol-based integrated management of oilseed rape pests.* (pp. 313-335). Springer, Dordrecht. https://doi.org/10.1007/978-90-481-3983-5_12

Vélez, A. M., Jurzenski, J., Matz, N., Zhou, X., Wang, H., Ellis, M., & Siegfried, B. D. (2016). Developing an in vivo toxicity assay for RNAi risk assessment in honey bees, *Apis*

mellifera L. *Chemosphere, 144*, 1083-1090. https://doi.org/10.1016/j.chemosphere.2015.09.068

Wanner, H., Gu, H., & Dorn, S. (2006). Nutritional value of floral nectar sources for flight in the parasitoid wasp, *Cotesia glomerata*. *Physiological Entomology, 31*(2), 127-133. https://doi.org/10.1111/j.1365-3032.2006.00494.x

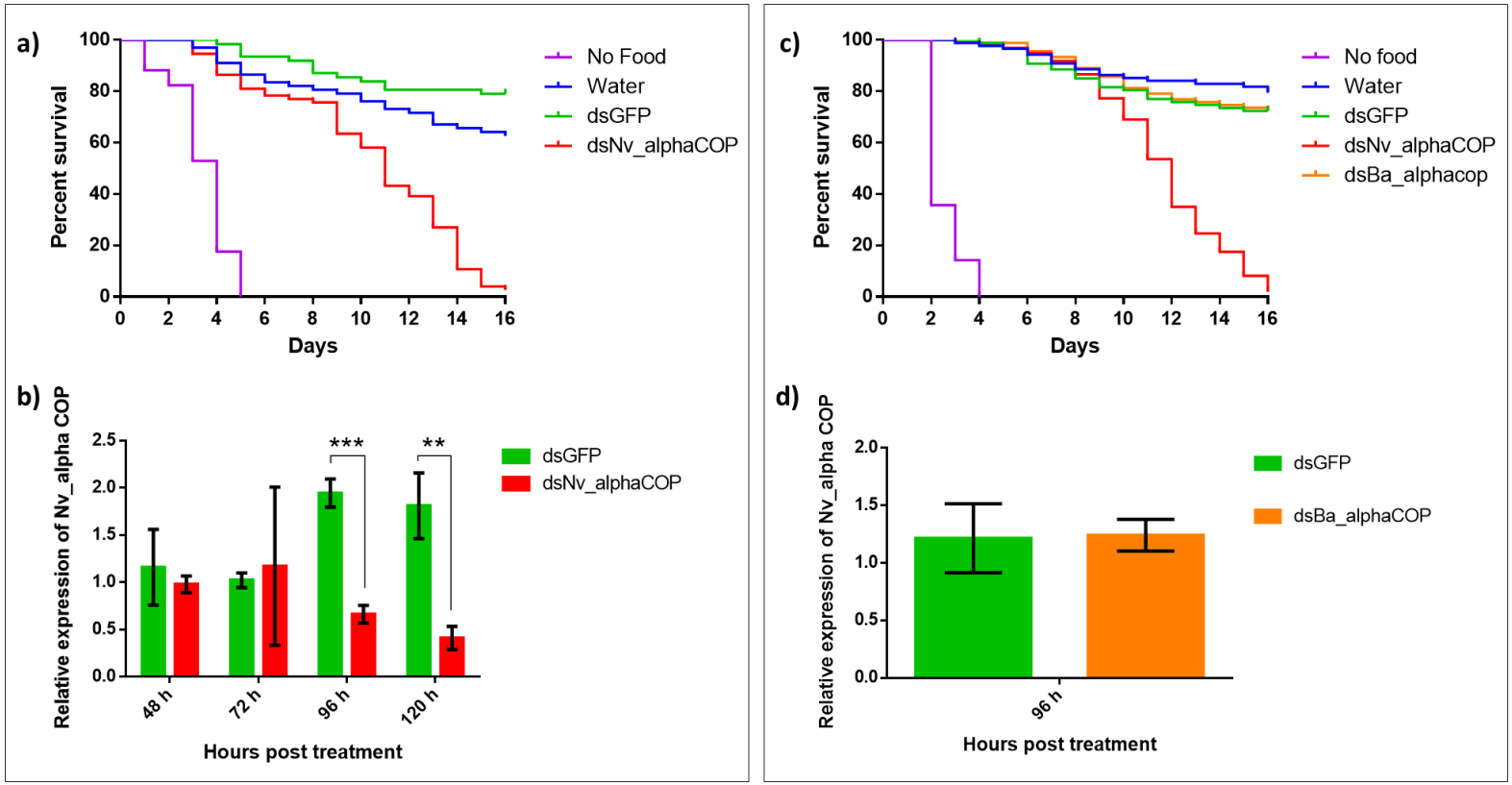
Werren, J. H., Richards, S., Desjardins, C. A., Niehuis, O., Gadau, J., Colbourne, J. K., ... Yamada, T. (2010). Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science, 327*(5963), 343-348. https://doi.org/10.1126/science.1178028

Willow, J., Sulg, S., Taning, C. N. T., Silva, A. I., Christiaens, O., Kaasik, R., ... & Veromann, E. (2021b). Targeting a coatomer protein complex-I gene via RNA interference results in effective lethality in the pollen beetle *Brassicogethes aeneus*. *Journal of Pest Science*, *94*(3), 703-712. https://doi.org/10.1007/s10340-020-01288-6

Willow, J., Soonvald, L., Sulg, S., Kaasik, R., Silva, A. I., Taning, C. N. T., ... Veromann, E. (2021b). RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle. *Communications biology, 4*(1), 1-8. https://doi.org/10.1038/s42003-021-01975-9Zotti, M., Dos Santos, E. A., Cagliari, D., Christiaens, O., Taning, C. N. T., & Smagghe, G. (2018). RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. *Pest management science, 74*(6), 1239-1250. https://doi.org/10.1002/ps.4813

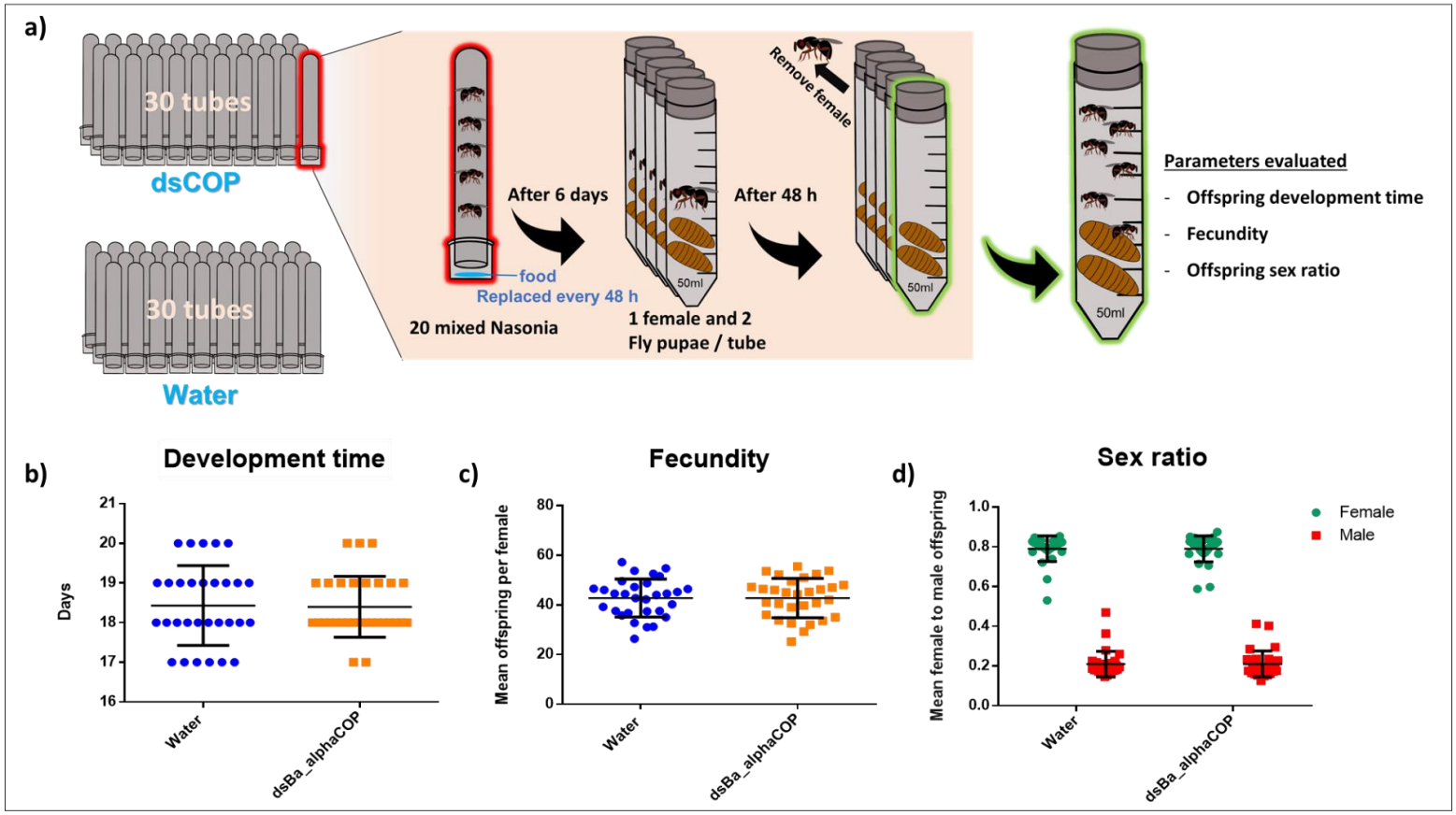
TABLE 1: Primers for dsRNA and RT-qPCR

Application of primers	Gene	Primer sequences (5′–3′)
DsRNA synthesis	*dsNv_alphaCOP-F*	taatacgactcactatagggGCAGTTGATGCGCTTCAGAA
	dsNv_alphaCOP-R	taatacgactcactatagggTCACAATTGGCTGCGGATCA
	dsBa_alphaCOP-F	taatacgactcactatagggAAGATGCACCACATAATG
	dsBa_alphaCOP-R	taatacgactcactatagggCGTCTTCGCTGGCCAAGA
	dsGFP-F	taatacgactcactatagggTACGGCGTGCAGTGCT
	dsGFP-R	taatacgactcactatagggTGATCGCGCTTCTCG
RT-qPCR	*qNv_alphaCOP-F*	CACTGTAACTTACAACCAGTTCACC
	qNv_alphaCOP-R	TCTGATGGATTATTGTCACACACCT
	UBI-F	TCACTCTGGAGGTTGAAGCA
	UBI-R	CCAGTTGCTTTCCTGCGAAAAT
	PP1A-F	ATGGGTCTGCCACGAGTATT
	PP1A-R	GAAGTTTTCAGCCGTCTTGG
	RPL32-F	GTTTCAAGGGACAATACCTTATGCC
	RPL32-R	ATTCCTTAACGTTGTGAACAAGGAC



449

450 **Figure 1:** Effects of ingesting either *Nasonia*-specific (dsNv_alphaCOP) or pest pollen beetle (*Brassicogethes aeneus*)-specific dsRNA
451 (dsBa_alphaCOP) by the parasitoid wasp *Nasonia vitripennis* on survival and target gene expression. (a) and (c) indicate the survival of *N.*
452 *vitripennis* over 16 days post oral exposure to either dsNv_alphaCOP, dsBa_alphaCOP, and the controls (dsGFP, water and No food). (b) and (d)
453 indicate the relative expression of *Nv_alphaCOP* in either dsNv_alphaCOP- or dsBa_alphaCOP-treated wasps compared to the control dsGFP-
454 treated wasps. Bars represent the mean ± standard error. Asterix (*) indicate statistically significant differences ($p < 0.05$)



455

456 **Figure 2:** Evaluation of unintended effects on important life history traits such as offspring development time, fecundity, and offspring sex ratio,
457 following ingestion of pest-specific dsRNA (dsBa_alphaCOP) by *N. vitripennis*. (a) indicates details of the experimental design used to collect the
458 necessary data to evaluate effects on the life history traits. (b), (c), and (d) indicate the effect of ingesting dsBa_alphaCOP by *N. vitripennis* on
459 offspring development time, fecundity and offspring sex ratio, compared to the control (water).