Research Article

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RNAi-mediated gene silencing in *Rhynchophorus ferrugineus* (Oliver) (Coleoptera: Curculionidae)

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Abstract: RNA interference (RNAi) is a powerful strategy for gene function analysis, and it is also widely studied in view of a promising use in pest control. The red palm weevil, *Rhynchophorus ferrugineus* (Oliver) (Coleoptera: Curculionidae), is one of the most devastating pests of palm in the world. Conventional pest management practices are not adequate to control this insect, thus the development of efficient approaches with minimal environmental impact are needed. In this work, the potential of RNAi in *R. ferrugineus* has been investigated through the silencing of three different genes (α-amylase, V-ATPase, Ecdysone receptor). For each gene we tested two different doses (1,500 and 5,500 ng) and two delivery techniques (injection and ingestion), evaluating both gene knockdown and mortality on insects. Results show that RNAi mediated gene silencing in *R. ferrugineus* varies from gene to gene, and that the response is dose-dependent, with stronger effects when dsRNA was administered by injection. In parallel, the same study was carried out with the model organism *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), with results showing a different pattern of response, although the two insects belong to the same order.

Keywords: α-amylase, Ecdysone receptor, dsRNA, injection, oral delivery, RNA interference, V-ATPase

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1 Introduction

RNA interference (RNAi) is a post-transcriptional process of gene expression inhibition, highly conserved in eukaryotes and naturally present in many other organisms, which is triggered by small interfering RNA (siRNA) mediating the silencing of a gene by the interception and destruction of the homologous mRNA fragment [1]. The use of RNAi for functional genomic studies has opened new avenues for the use of this technology in many fields of science, spanning from basic to applied research, supporting its potential employment in pest control [2, 3], and potentially contributing to efforts to develop alternative strategies to traditional chemical pesticides [4-6]. The efficacy of RNAi to control pests, and to protect beneficial insects from diseases, is widely accepted and documented [7, 8]. Recently, it has been demonstrated that it is possible to deliver dsRNA through foliar application for pest control like a conventional pesticide, further supporting that this method could be a working alternative strategy to control some pests [9]. Furthermore, a novel delivery method that uses genetically modified yeast expressing dsRNA has been proved to affect locomotor activity, decreased oviposition, and reduced larval survival in *Drosophila suzukii* Matsumura, introducing an innovative biopesticide that can deliver dsRNA to an insect pest without the need to genetically modify crops [10]. Unfortunately, results in this direction are not always predictable since dsRNA does not always produce suitable knockdown of gene transcripts, therefore not inducing the effects required for effective insect control [11]. Moreover, the slight differences in RNAi pathways provide a high level of variation in reactivity among different insect species, thus a comprehensive evaluation of RNAi for each target species and target gene results being crucial before proceeding in defining a RNAi-based pest management strategy [12, 13].

RNAi was first described in plants (petunias) in 1990 and called co-suppression [14], and subsequently explained in the nematode *Caenorhabditis elegans*

Maupas [15]. Afterwards, the technique has been widely tested among different insect lineages, and some evidence suggests that Coleoptera is the most sensitive order [16]. Among these beetles, *Tribolium castaneum* Herbst is considered an ideal genetic system in several fields of biology and in particular in RNAi studies for its versatility, easy handling and availability of the fully annotated genome sequence [17-20]. Relevant results have been obtained on *T. castaneum* [21-23] and *Diabrotica virgifera* LeConte [3]. Further studies highlighted that insects respond to both injection and ingestion of dsRNA [24]. Oral delivery of dsRNA was also effective on larvae of *T. castaneum* [18, 25]. It is noteworthy that despite the number of positive results, in the case of the cotton boll weevil, *Anthonomus grandis* Boheman, for a still unclear reason the method did not work [3, 26].

The unpredictability of the outcome, and the lack of genomic information for most insect pests, makes this technique harder to apply without a thorough pre-screening. On the other hand, the species-specificity of gene knockdown through RNAi makes this approach very attractive, in particular when dealing with invasive species. In this context, the red palm weevil, *Rhynchophorus ferrugineus* Olivier, is considered one of the most harmful insect pests of palm in the world [27]. Native to Asia and Melanesia, currently, *R. ferrugineus* has spread across the entire Mediterranean area, Asia and Oceania. In Italy, the insect was recorded for the first time in a nursery in Pistoia (Tuscany) during 2004 [28]. The larvae of red palm weevil feed and grow inside the plant, causing extensive damage to the apical meristem, and thus threatening plant survival and weakening the mechanical stability of the plant. The main symptoms are visible only at an advanced state of infestation, when larvae have already damaged the apical meristem and every control action is useless [27, 29]. All the conventional methods including chemical pesticides were not effective in controlling this pest [30]. As well, studies on biological control strategies provided promising results, mostly with microorganisms, but their application seems to be still a long term goal [31]. The potential of RNAi based knockdown in *R. ferrugineus* has been actually evaluated in a recent study, in which the *catalase* gene was investigated as a potential target [32]. The efficacy of dsRNA provided at different doses and with three different application methods caused different levels of silencing in various body parts, significant mortality and larval growth inhibition under laboratory conditions [32].

In this scenario, this work aimed at providing new insights on gene knockdown using RNAi on *R. ferrugineus*. Success in silencing and subsequent effects on *R. ferrugineus* mortality were evaluated through the delivery of different doses of dsRNA of three different genes coding for proteins with essential functions in insect development (i.e. α-amylase, V-ATPase and Ecdysone receptor). In parallel, we performed the same study using *T. castaneum*, for which RNAi is known to occur with both injection and ingestion delivery of dsRNA, allowing also a comparative analysis with the extensive information available in the literature.

2 Materials and Methods

2.1 Insects

Specimens of *R. ferrugineus* from 2nd to 4th larval instar were collected from plants of *Phoenix canariensis* and *Phoenix dactylifera* located in Locri (Italy, 38.236149 N, 16.264725 E) and reared as described by Montagna et al. [33]. *Tribolium castaneum* was obtained from the collection available at the School of Biology of the University of Newcastle Upon Tyne (Newcastle Upon Tyne, UK), and reared on artificial diet (90% wheat flour, 10% brewer's yeast). Larvae of *R. ferrugineus* and *T. castaneum* were maintained at 27±1°C and 60-70% R.H., with 12:12 light-dark photoperiod.

2.2 RNA extraction and dsRNA synthesis

Total RNA was extracted from the larvae of both *R. ferrugineus* and *T. castaneum* at the last larval stage using PerfectPure RNA Tissue Kit (5PRIME®, Hilden Deutschland), according to manufacturer's instructions. RNA concentration and quality was assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA), and then converted into cDNA using Precision nanoScript Reverse Transcription kit (Primer design®, Southampton, UK), as specified by manufacturer. The cDNA was used as a template to amplify a portion of the coding sequence of α-amylase, V-ATPase and Ecdysone receptor genes selected from the NCBI database, using the primers reported in Table S1. PCR reactions (total volume of 25 µl) were conducted using ≈50 µg of cDNA, 0.5 mM $MgCl_2$, 1 unit of Dream Taq PCR Master Mix (2X) (#K0171, Thermo Scientific™) and 0.5 µM of each primer. Amplifications were performed using a Mastercycler Ep Gradient S thermocycler (Eppendorf, Germany) with the following conditions: 5 cycles at 94°C for 2 min, then 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 45 s, with a final extension step of 10 min at 72°C. PCR products were analysed by electrophoresis in TBE buffer (1X) using

1.5% agarose gel stained with GelRed™ nucleic acid stain (Biotium, USA). In order to produce dsRNA a T7 promoter sequence (5´- TAATACGACTCACTATAGGG- 3´) was ligated to the amplicons, including this sequence at the 5› ends of each primer. The same process was employed to select a region (468bp) of the kanamycin resistance gene (*nptII*), (Genbank: JN638547, synthetic construct) from the cloning vector pSC-A-amp/kan (Stratagene) to be used as a negative control to assess the effect of injecting and feeding target-less dsRNA [17]. A non-template control in which target cDNA was replaced by nuclease-free water was also included in all PCR reactions.

PCR products were subsequently purified using MinElute Extraction Kit (QIAGEN® Venlo, Netherlands) and cloned using Strata cloning® kit (Agilent Technologies® Santa Clara, USA). Five insertion-positive *E. coli* colonies for each amplified gene were selected, the fragment was cut from the vector using *Eco*RI enzyme (Thermo Fisher Scientific, USA), and it was sequenced by Geneius Laboratories Ltd (UK) in order to check for the amplification of the correct target. dsRNAs for each target was synthesized starting from 1000 ng/µl cDNA, and through specific primers using the MEGAscript® RNAi Kit (Thermo Fisher Scientific®, USA) according to the manufacturer's instructions. The quality and integrity of dsRNA was also checked by electrophoresis on 1% agarose gel. Products were also quantified using a Nanodrop 2000 (Thermo Fisher Scientific®, USA), obtaining an average concentration ranging between 700-1000 ng/µl.

2.3 dsRNA delivery into larvae of *Rhynchophorus ferrugineus* and *Tribolium castaneum*

To evaluate the effects of the different delivery techniques on gene knockdown targeting α-amylase, V-ATPase and Ecdysone receptor genes, exogenous dsRNA was administered to *R. ferrugineus* larvae at the last larval instar by injection and feeding. Two different doses of dsRNA (1,500 ng and 5,500 ng) were tested on *R. ferrugineus*, chosen taking into account the data reported by Al-Ayedh et al. [32] and a preliminary set of experiments. A parallel study targeting the same genes was performed on *T. castaneum*, for which we tested a single dose of 150ng, known from previous studies to produce gene knockdown [19]. Molecular biology grade water was administered as negative control. The dsRNA molecule designed towards the gene *nptII* was administered as further negative control (150ng for each larva of *T. castaneum* and 1,500 ng for *R. ferrugineus*). Both injection and feeding procedures, described below, were carried out on both insects for all three genes, employing groups of 15 individuals and replicating each trial three times.

Furthermore, for each investigated gene, insects were also evaluated for mortality. Forty-five last larval instar from each gene/treatment, after the injection or the ingestion of dsRNA as described below (1,500 ng for *R. ferrugineus* and 150ng for *T. castaneum*), were individually placed in plastic boxes and fed with their diet. The rate of mortality was recorded every 24 hours, and observations were carried out up to one week.

2.4 Injection

Larvae of *T. castaneum* and *R. ferrugineus* were anesthetized by CO_2 (10-15 s) before the injection, then immobilized with forceps and positioned with dorsal side down so that the abdomen was easily accessible. The injection was performed between the III and IV abdominal segments using the microinjector Femtojet® (Eppendorf GmbH, Germany) coupled with the InjectMan NI 2 micromanipulator (Eppendorf GmbH, Germany). After injection, *R. ferrugineus* specimens were placed individually in small boxes and fed with their diet, while *T. castaneum* larvae were individually placed in 96-well tissue culture plates containing 10 mg of wheat flour. Plates were sealed with Parafilm "M" and the lid pricked with small holes to provide aeration.

2.5 Ingestion

Last instar larvae of *R. ferrugineus* and *T. castaneum* were collected and placed singly in plastic boxes without food for 48 hours. Starved larvae of *R. ferrugineus* were fed with a portion of apple (≈5g) wetted with a drop of dsRNA (i.e. 1,500 ng and 5,500 ng in molecular biology grade water). Specimens of *T. castaneum* were singularly placed inside wells of a 96-well tissue culture plate, provided with 150 ng of dsRNA and overlaid with 10 mg of diet. Plates were then sealed with Parafilm "M" and the lid pricked with small holes to provide some aeration. After 72h, both injection-treated and ingestion-treated larvae were collected and stored at -80°C until used for subsequent gene expression analyses. All experiments were replicated three times.

2.6 Gene expression analysis with quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted and processed into cDNA as described above. RT-qPCR was carried out using primers listed in Table S1, designed to detect a segment of each gene selected to be external to the segment targeted by the dsRNA. The ribosomal protein Rp6 was used as an internal reference gene (RP6F – 5'-GCAGATGCTTTAGGCGATGA-3'; RP6R – 5'-AAACGGACCCTGCTGTTAGT-3'). All primers were designed using Primer-BLAST software® and used in a final concentration of 0.5 µM. mRNA levels were measured by StepOnePlus™ Real-Time PCR System (Life Technologies®) using the 2X qPCR MasterMix (Primer Design Ltd, UK) according to the manufacturer's instructions. Reaction were performed using the following conditions: 95°C for 10 min, 50 cycles of 95 °C for 15 s and 60 °C for 1 min, and a final dissociation stage. The relative expression of the target gene was normalized using the $\Delta\Delta C_q$ method towards both the reference gene (Rp6) and negative control insects treated with molecular biology grade water.

2.7 Data analysis

Insect mortality data was $arcsin\sqrt{x}$ corrected and normalized against the negative control treatment using Abbott's formula [34]. Differences in gene knockdown and mortality among the targeted genes and different doses were assessed using the GLM procedure and Tukey's test for multiple comparisons. All statistical analyses were performed using Microsoft Excel® 2013 and IBM® SPSS® 20.

3 Results

3.1 RNAi in *Rhyncophorus ferrugineus*

The α-amylase gene expression appeared significantly down-regulated, being 0.052±0.28 and 0.182± 0.07 fold of the control in larvae injected with 1,500 ng ($F_{2, 42}$=54.05; P<0.001) and 5,500 ng ($F_{2, 42}$=27.29; P<0.001) of cognate dsRNA, respectively. Gene silencing of Ecdysone receptor by dsRNA injection also caused a reduction in gene expression, with values of 0.33±0.06 and 0.55±0.12 fold of the control in transcript level of the target mRNA in both doses tested, respectively ($F_{2, 42}$=14.21; P<0.01 – $F_{2, 42}$=10.39; P<0.01). On the contrary, injection of V-ATPase dsRNA did not affect the V-ATPase transcript levels at all doses considered (P>0.05). Differences were observed between α-amylase and Ecdysone receptor ($F_{1, 28}$=23.48; P<0.001 – $F_{1, 48}$=6.71; P<0.01, respectively using 1,500ng and 5,500 ng of dsRNA) and, just for α-amylase, between the different doses provided ($F_{1, 28}$=17.12; P<0.001 – Fig. 1).

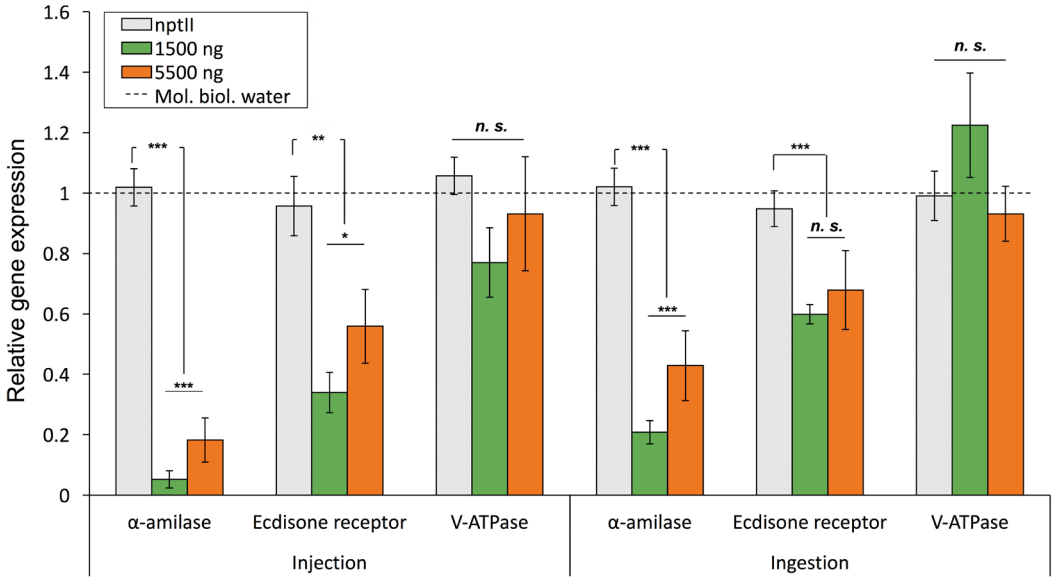

Fig. 1. Relative gene expression evaluated in control and treated specimens of *R. ferrugineus*. For each gene and administration technique differences are reported between control and treated insects, and between the two tested doses (1,500 ng and 5,500 ng). Grey dashed bar represents gene expression measured for each gene in insect administered with nptII dsRNA, and the dashed line represent the level of gene expression in samples administered with molecular biology grade water. Data were normalized with the reference gene (Rp6).

Oral delivery of α-amylase dsRNA resulted in a significant decrease in target mRNA expression in both larvae fed with 1,500 ng and 5,500 ng of dsRNA with values, respectively, of 0.20±0.03 fold ($F_{2, 42}$=54; P<0.001) and 0.42±0.05 fold ($F_{2, 42}$=16.06; P<0.001) compared to the control group. As well, a reduction in Ecdysone receptor transcripts was recorded to levels of 0.59±0.03 fold ($F_{2, 42}$=8.71; P<0.001) and 0.67±0.02 fold ($F_{2, 42}$=4.64; P<0.001) in insects treated, respectively, with doses of 1,500 ng at 5,500 ng of specific dsRNA. V-ATPase gene expression was not affected by the ingestion of dsRNA at all doses considered (P>0.05). Also in feeding trials, differences in gene expression between α-amylase and Ecdysone receptor were recorded using 1,500 ng of dsRNA ($F_{1, 28}$=15.87; P<0.001), and differences between doses provided were observed only for α-amylase ($F_{1, 28}$=22.73; P<0.001 – Fig.1). Furthermore, the knockdown recorded for α-amylase was different between the two delivery techniques used in our study ($F_{1, 28}$=16.01; P<0.001), while this effect was not recorded for Ecdysone receptor (P>0.05).

3.2 RNAi in *Tribolium castaneum*

To compare the effects observed in *R. ferrugineus*, we set up a comparison trial using the model insect *T. castaneum*, for which RNAi is known to occur. We targeted the same genes by both injection and feeding, using a single dsRNA dose of 150 ng. Results indicate that Ecdysone receptor gene expression appeared significantly down-regulated to 0.077±0.13 fold ($F_{1, 16}$=5.07; P<0.01) in larvae injected with cognate dsRNA. Similarly, a drastic reduction in gene expression to 0.091±0.02 fold ($F_{1, 16}$=9.21; P<0.01) was recorded in insects injected with V-ATPase gene dsRNA (Fig. 2). Feeding treatments in *T. castaneum* also resulted in a reduction of gene expression in larvae treated with Ecdysone receptor and V-ATPase dsRNA with a reduction, respectively, of 0.66±0.06 fold ($F_{1, 16}$=12.74; P<0.01) and 0.75±0.04 fold ($F_{1, 16}$=3.17; P<0.01) compared to control larvae (Fig. 2). Overall, injection delivery was more efficient than oral delivery ($F_{1, 16}$=97.33; P<0.001 – $F_{1, 16}$=16.8; P<0.01, respectively targeting Ecdysone receptor and V-ATPase genes), while no differences were highlighted between the two targets using the same delivery method (P>0.05).

Surprisingly, no PCR products were obtained for the α-amylase gene. Further amplifications were carried out using several sets of primers designed specifically for this gene. For all the primers used, resolving of PCR products on agarose gel, it has been evidenced the absence of bands in all samples, as well as in the negative control, while the use of reference gene primers (Rp6) led to successful amplification. This result supported the idea that probably it was not a random event linked to a wrong experimental procedure. This aspect is discussed in more detail in the discussion section.

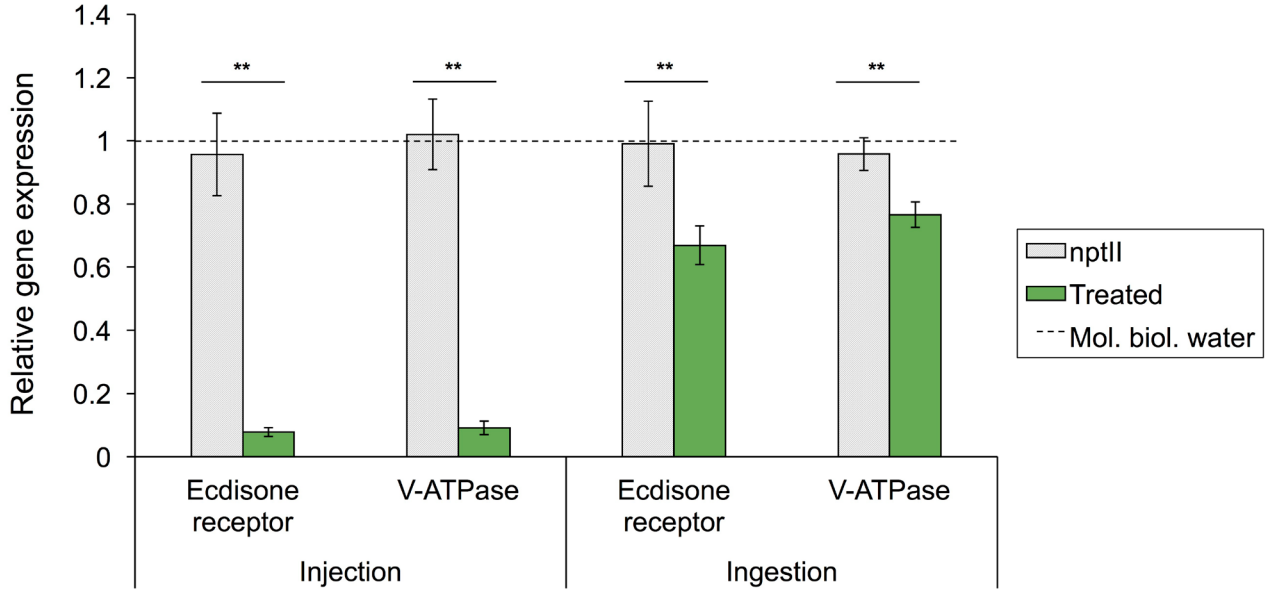

Fig. 2. Relative gene expression evaluated in control and treated specimens of *T. castaneum*. For each gene and administration technique differences are reported between control and treated insects. Grey dashed bar represents the gene expression measured for each gene in insect administered with nptII dsRNA, and the dashed line represents the level of gene expression in samples administered with molecular biology grade water. Data were normalized with the reference gene (Rp6).

3.3 Bioassays

Knockdown of Ecdysone receptor protein and α-amylase mRNA induced by injection of dsRNA in *R. ferrugineus* resulted in a percentage of mortality that never exceeded 34±5.56% in treated insects while, with the same delivery technique, the assay with the larvae treated with α-amylase resulted with a maximum mortality of 28±2.13% recorded after 120 h (Fig. 3). Ingestion of dsRNA, in *R. ferrugineus*, produced levels of efficacy comparable to injection for both Ecdysone receptor (32±2.93%) and α-amylase (25±2.35%). Silencing of V-ATPase and Ecdysone receptor protein in *T. castaneum* induced, as well, a daily increasing of the mortality of treated larvae (Fig. 4). Injection of dsRNA resulted in an observed mortality of 38±2.12% for Ecdysone receptor and 37±2.7% for V-ATPase. Mortality induced by ingestion of dsRNA daily increased, reaching a maximum after 72h (36±%2.24 for Ecdysone receptor protein; 32±2.36% for V-ATPase) (Fig. 4). In both cases, a remarkable mortality effect was observed after 72h from treatment. No differences were evidenced between the two genes tested ($P>0.05$) in both insects.

4 Discussion

In this study, we investigated the effectiveness of RNAi in the red palm weevil, targeting three genes coding for key proteins involved in different biological processes. Moreover, we compared our results to the widely used model organism *T. castaneum*. Since biological compartments are regulated by numerous proteins and enzymes, the identification of the most promising targets is one of the major challenges to exploit RNAi in pest control strategies [35]. Although in the recent past RNAi was successfully achieved using different delivery methods, the technique is still at an early stage of development

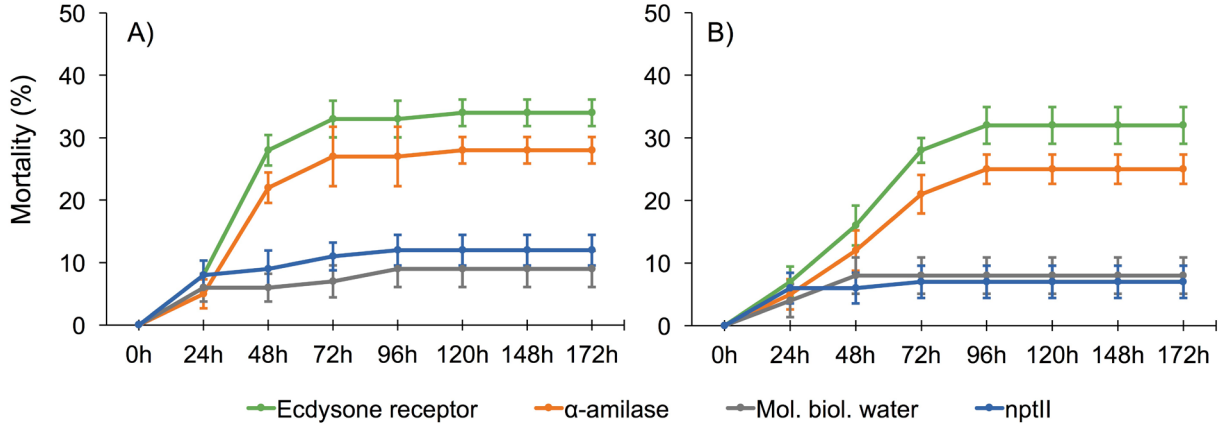

Fig. 3. Mortality curves resulting from bioassays targeting the α-amylase and Ecdysone receptor genes in *R. ferrugineus* (A – injection; B – feeding)

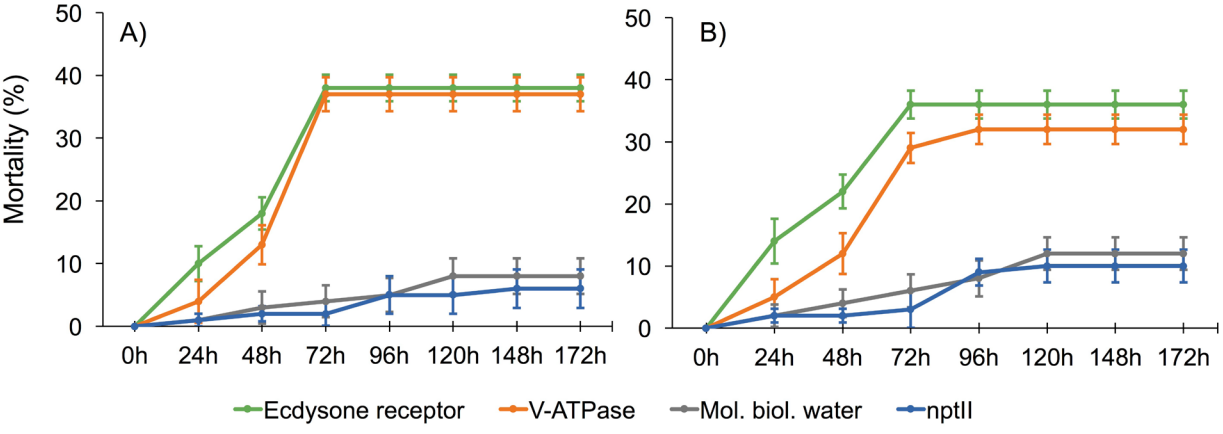

Fig. 4. Mortality curves resulting from bioassays targeting the α-amylase and Ecdysone receptor genes in *T. castaneum* (A – injection; B – feeding)

and it remains difficult to obtain consistent results with every insect, life stage or gene [35]. An example could be α-amylase, one of the most important digestive enzymes of many insects, which catalyses the hydrolysis of starch and other polysaccharides. Although α-amylases has been proposed as an ideal candidate for the developing RNAi strategy [35], to our knowledge no studies have been performed to date. The Ecdysone receptor controls development, insect moulting and metamorphosis and contributes to other processes such as reproduction [35, 36]. A recent study from Yu et al. [37] showed that the Ecdysone receptor gene might be a good potential target for *Nilaparvata lugens* (Stål). Feeding RNAi experiments demonstrated a significant reduction of the relative mRNA expression levels and a reduction in fecundity. Our experiments support the idea that α-amylase and the Ecdysone receptor genes might be promising target genes for *R. ferrugineus*. In fact, we observed that the interfering technique worked on both genes, with a greater efficiency on the α-amylase gene in most cases. Furthermore, knockdown of both genes induced mortality in larval stages as highlighted in bioassay tests.

RNAi has been widely investigated in *T. castaneum*, knocking down several genes and thus providing an extensive experience in RNAi and making this insect an exceptional resource for this kind of studies, thanks also to the robust systemic RNAi response observed in this insect [18, 19, 21, 25, 38]. The availability of *T. castaneum* genome sequence data [20] allows a fine investigation on new possible candidate genes. For example, the effectiveness of RNAi in silencing V-ATPase in *T. castaneum* was demonstrated in a previous study by Whyard et al. [25], which used specific dsRNA targeting the E-subunit of V-ATPase. The same authors demonstrated the species-specificity of this approach, reporting knockdown only in insects treated with specific dsRNA. Furthermore, Whyard et al. [25] supposed that, since the insects lived for several days after the uptake of dsRNA, the death of insects might be a consequence of a latent effect of dsRNA in the gut. Similarly, this theory could justify the increase of the mortality over the following four days after the injection in both *R. ferrugineus* and *T. castaneum* in our study. However, the theory does not explain the low percentage of mortality recorded in the treated larvae. Moreover, the present results concur with the experiment performed on *A. grandis* that showed no effects on mortality or body mass upon ingestion of dsRNAs [3].

To study RNAi in insects, different dsRNA delivery methods (feeding, microinjection, soaking, and transfection) have been developed to target key transcripts in insects [24]. The oral delivery and microinjection are the most common and efficient approaches of RNAi. Unfortunately, both methods show many disadvantages, like stability issues in the case of ingestion, and the need of technical expertise and specific equipment in the case of injection [24, 39]. Our results demonstrated that both approaches produce knockdown in *R. ferrugineus* and *T. castaneum* larvae, clearly showing that RNA interference mediated by dsRNA injection delivery is much more efficient than oral delivery, for both doses investigated. The higher efficiency of injection compared to other methods has been widely documented [24, 39-41].

Additional factors need to be considered to improve the RNAi method. An optimal concentration of dsRNA is required to induce efficient silencing of the targeted gene, and it also depends on the targeted gene, the target insect and the delivery method. In our study, comparing two different doses of dsRNA in larvae of *R. ferrugineus*, it was observed that a lower amount of dsRNA silenced both the α-amylase and Ecdysone receptor genes more efficiently. This is in contrast to the observations of Al-Ayedh et al. [32], who observed an increased gene knockdown at increasing doses of administered dsRNA in *R. ferrugineus*; however, previous studies highlighted that when higher doses of dsRNA are delivered it might not result in increased silencing [8, 42, 43].

The V-ATPase gene was chosen as a target for this study because a number of different studies demonstrated its vulnerability to RNAi silencing in different insect pests [3, 25, 44-46]. However, differences in mortality were observed for different species. The lack of silencing reported for V-ATPase gene in *R. ferrugineus,* although unexpected, should not be considered completely unusual. The absence of reduction of gene expression for this gene may result from the particular physiology of the insect or, as acknowledged in other studies [3], it might confirm that different lineages of Coleoptera are differently susceptible to delivery of dsRNA. Furthermore, many other factors can influence the efficiency of RNAi, such as concentration, length and sequence specificity of the dsRNA, as well as the tissue-dependence of the RNAi effect [47]. Certainly, targeting only one of numerous subunits of the V-ATPase protein might have contributed to insufficient efficacy.

Interestingly, no PCR products were obtained from the whole set of primers designed to target the α-amylase gene in *T. castaneum*. Although we primarily supposed that it might be due to a wrong experimental setup, the absence of amplicons in the samples and, in parallel, correct amplification of the reference gene, corroborated the hypothesis that it was not a random event. Unfortunately, to our knowledge, the lack of suitable literature reporting

experiences with α-amylase gene in other insects does not allow us to adequately explain this phenomenon. Thus, further research in order to figure out the mechanism involved in this unexpected result is needed.

Overall, this research allowed us to analyse some perspectives of RNAi mechanisms, upgrading the information about the efficacy of the method for target insects, for target genes and for different methods of delivery. Our study provides new important evidence that injections or oral intake of dsRNA can produce gene knockdown through RNAi in *R. ferrugineus*. This is particularly true for the α-amylase and Ecdysone receptor genes, which demonstrated good potential as a target for exploiting RNAi. Future studies can focus on these genes, improving the technique by studying more closely the knockdown and their effect on the insects' physiology. The collection of all this information allows a better understanding of the mechanisms behind RNAi in insects, and could contribute to outlining a new generation of species-specific, environmentally-friendly pest management techniques.

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Conflicts of interest: Authors state no conflict of interest

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