Review

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RNA meets toxicology: efficacy indicators from the experimental design of RNAi studies for insect pest management

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Abstract

RNA interference (RNAi) selectively targets genes and silences their expression *in vivo*, causing developmental defects, mortality and altered behavior. Consequently, RNAi has emerged as a promising research area for insect pest management. However, it is not yet a viable alternative over conventional pesticides despite several theoretical advantages in safety and specificity. As a first step toward a more standardized approach, a machine learning algorithm was used to identify factors that predict trial efficacy. Current research on RNAi for pest management is highly variable and relatively unstandardized. The applied random forest model was able to reliably predict mortality ranges based on bioassay parameters with 72.6% accuracy. Response time and target gene were the most important variables in the model, followed by applied dose, double-stranded RNA (dsRNA) construct size and target species, further supported by generalized linear mixed effect modeling. Our results identified informative trends, supporting the idea that basic principles of toxicology apply to RNAi bioassays and provide initial guidelines standardizing future research similar to studies of traditional insecticides. We advocate for training that integrates genetic, organismal, and toxicological approaches to accelerate the development of RNAi as an effective tool for pest management.

Supporting information may be found in the online version of this article.

Keywords: dsRNA; pest management; mortality; gene ontology; toxicology; random forest

1 INTRODUCTION

First observed in 1990[1] in petunias (*Petunia spec.*; Solanales) and described 8 years later[2] in *Caenorhabditis elegans* (Rhabditia: Rhabditidae) Maupas, RNA interference (RNAi) has become a widespread and powerful tool for molecular biologists. Thus, it is natural that applications from the technology are actively pursued in a variety of areas, including pest control. The potential effect of a pesticide treatment that only harms the target species and effectively controls its populations without affecting other organisms, makes RNAi especially attractive for pest management applications. The topic has been subjected to numerous reviews in the last 15 years[3–20] and insects have become the most relevant organism for studies in applying RNAi for pest management.[7] RNAi uses the double-stranded RNA (dsRNA) system, an intrinsic regulatory and defense system against viral infection and transposons, that is present in most organisms.

RNAi can effectively silence target genes in a species-specific manner and cause phenotypic changes that result in mortality,[21] or disrupt development.[22,23] Its function, scope, and possible effects are still not fully understood, and many integral steps of the pathway are shrouded in mystery, especially in non-model organisms.[5,6,19,24,25] The basic physiological sequence of an RNAi response, however, is widespread. In nature dsRNA molecules can enter the organism by means of viral infection or ingestion of dsRNA viruses.[6] Both modes of RNA introduction occur naturally and are used as modes to administer dsRNA in RNAi research, with the addition of topical dsRNA spray application,[26] soaking the organism in a dsRNA solution and the direct injection of dsRNA.[9] A single dsRNA molecule can deactivate multiple messenger RNAs (mRNAs) and therefore even a small, single dose of dsRNA can lead to significant gene knockdown.[27] With increased understanding of the RNAi pathway, researchers have begun to build large screening libraries of tested target genes for RNAi to investigate gene function, including in the common fruit fly (*Drosophila melanogaster*; Diptera: Drosophilidae) Meigen[28] and the red flour beetle (*Tribolium castaneum*; Coleoptera: Tenebrionidae) Herbst.[29–33] Additional screening on a smaller scale has been conducted for the eastern subterranean termite (*Reticulitermes flavipes*; Blattodea: Rhinotermitidae) Kollar[34] and the Asian corn borer (*Ostrinia furnacalis*; Lepidoptera: Crambidae) Guenée.[35]

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Despite its promise as a future pest management tool, RNAi still faces many roadblocks. Some notable challenges include a lack of understanding of the dsRNA uptake, inter-cellular transport, and retention pathways of RNAi, how these can vary among insect orders, and the environmental fate of dsRNA when used in the field.[36] Ideally, assessment of RNAi efficacy requires that researchers standardize many factors that can influence RNAi efficacy (Fig. 1), including the effects of dsRNA construct size, identification of a dose–response, use of negative and positive controls, and conduct assays over a long enough timeframe to observe the effects of interest. With possible use of RNAi in the field in mind, the application method of choice could be through feeding[37] (including transgenic plants[38]), or through alternative methods that can be easily scaled to field application levels such as topical sprays[39] or infection with transgenic pathogens.[40]

There are many unresolved questions regarding research to develop RNAi for pest management, there is no consensus regarding the method of application of dsRNA (e.g. feeding, or topical spray), nor is there agreement on the ideal length for dsRNA constructs. It is also unclear if there are species differences in the time it takes to exhibit a phenotypic response after RNAi treatment. While there are initial studies that enable basic comparisons of different doses,[31,41,42] these are exceptions that provided no clear guidelines for dose effects. Even though the state of the field is promising, there is marked heterogeneity in approaches to identifying ideal control strategies. This situation is clearly at odds with standards for insect control via traditional chemical analyses, where the field has established expectations about the impact of a chemical on an insect as a function mostly of dose and response. Thus, although the field of RNAi-based insect control research is relatively new and variable for understandable reasons, it may be the case that a major impediment to application is the failure of the field to adopt a proven uniform strategy of testing RNAi efficacy. This review aims to provide new insights into the research on RNAi for pest management over the last decade by evaluating the available research. Through enrichment analyses and machine learning applied to the relevant literature, biases in target organism, gene choice, applied dose became evident and some support for improved construct size was found. Machine learning identified response time and target gene as the most important factors underlying efficacy, and a generalized linear model indicates interaction of dose with at least one of these factors as a likely reason that dose effects are not clear unless controlling for the other factors. Taken together our results indicate the importance of incorporating genetic, organismal, and toxicological knowledge to improve RNAi control of pest insects.

2 MATERIAL AND METHODS

Peer reviewed publications focusing on pest management RNAi were collected using PubMed, Google Scholar, and Web of Science databases, using Insects, Pest, dsRNA, RNAi, 'Pest control' and 'RNA interference' as well as combinations of these terms in a keyword search through December 2021. Only primary literature aiming to use RNAi as a means of pest management was used in the data survey to avoid duplication and additional noise within the dataset. Duplicates and manuscripts not dealing with RNAi, insects and pest management were removed and only studies that reported significant silencing of their target genes were considered. From the remaining manuscripts the following information was extracted when available: (i) species used, (ii) dsRNA construct size, (iii) application method, (iv) time until maximum effect occurred (response time), (v) maximum mortality observed, (vi) target genes, and (vii) dose applied. Due to the large variation in reported data among studies surveyed, the following parameters were standardized. Targeted genes, based on their flyBase ortholog name where available to make comparisons possible. Isoforms and subunits of the same protein product were simplified by merging into the common name. Applied dose was

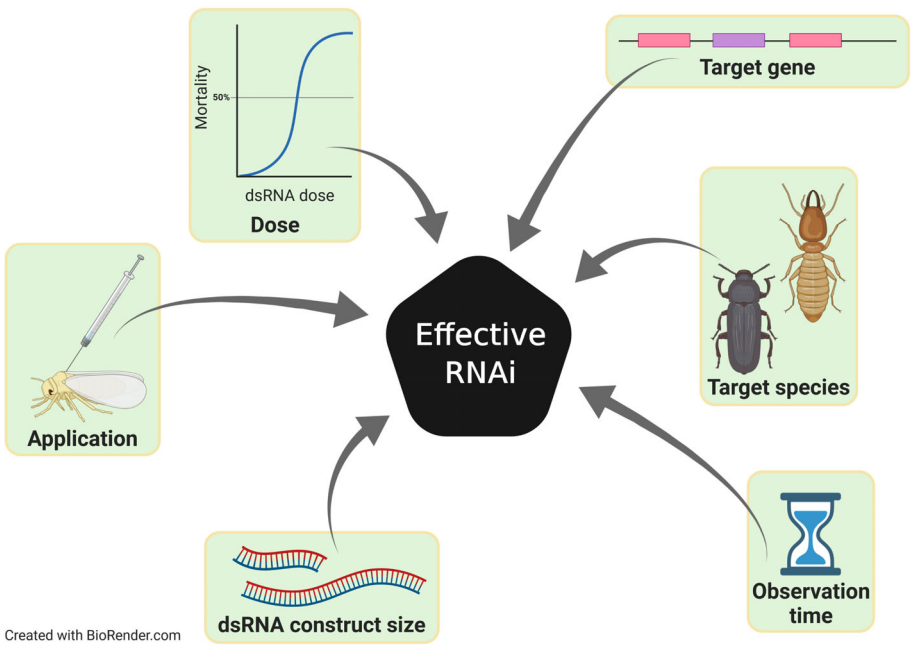

Figure 1. Experimental factors influencing efficacy of RNAi based on findings in the survey, variable factors include application method (e.g. injection or feeding), the applied dose of dsRNA, the size of the introduced dsRNA (e.g. 450 bp) as the basic parts of any bioassay for RNAi. Additionally, the target species determines the available range of target gene candidates and the minimum time a RNAi treatment takes to show a response in the bioassay.

calculated to total µg injected/available for feeding, when possible, from given concentrations and volumes applied in trials.

In order to identify trends and biases present in the literature, the surveyed data were plotted. A random forest model (RFM)[43] was applied for effects on mortality to identify parameters that influence mortality. The RFM used all target genes that had been reported at least five times (genes = 45, total n = 558), response time, dose applied, order, species, and size. Additionally, method of administration and origin of publication were added to test for potential bias in the dataset. Mortality was ranked for use in the RFM using the ranks high (100–67%), medium (66–34%), low (33–1%) and no mortality (0%). Training of the model was performed using 65% of the total dataset, setting 35% aside for *post hoc* validation. Multiple variants of the RFM were executed and showed best predictive power using two factors per node and 2000 trees. Furthermore, a possible relationship between mortality, dose, and construct size was evaluated through generalized linear mixed effect model (GLMM), using the binomial error distribution and logit link function. The GLMM allows one to assess the effect of fixed factors (those of interest based on specific differences between treatments and interactions[44]) and random-effects variables (factors of interest based on variations among them[44]). The GLMM is flexible enough to be applied to compare studies, as random-effect variables can accommodate violations of statistical independence among observations (e.g. spatial or temporal autocorrelation[45]) and without losing as many degrees of freedom (as would be the case if one were to force a random-effect variable into a fixed effect variable[44,46]). In this study, taxonomic order and application method were used as random factors in order to deal with the lack of independence among sampling units caused by phylogenetic relatedness (e.g. species of the same genus tend to have similar RNAi reaction) and application method (e.g. different methods might need different doses). Data were checked for overdispersion and $\log_{10}$-transformed (dose and size) to reduce undue influence of extreme values and to confirm GLMM assumptions. GLMM parameters were estimated by restricted maximum likelihood.[44] The fit of the model was estimated with marginal R^2 (R^2_m; variance explained by fixed effects variables) and conditional R^2 values (R^2_c; variance explained by fixed and random effects variables) following Nakagawa and Schielzeth.[47]

All tests were performed in the R free software environment for statistical computing and graphics (R Core Team 2017) using the 'randomForest',[43] 'lme4'[48] packages. Additionally, all identified flyBase gene names were used to build a gene ontology network and tested for enrichment in specific terms using DAVID 6.8[49,50] to assess possible biases in target gene choices. The functional annotation clustering tool was used adopting high stringency parameters and an EASE score of 0.05 to accommodate high variance in target genes using GOTERM_BP_FAT, an annotation based on biological processes.

3 RESULTS

The data survey resulted in 350 papers which, after filtering, totaled 82 studies that presented experimental results using RNAi for pest management in insects. These papers resulted in 979 data points, with good representation of the two main administration methods: feeding (n = 491) and injection (n = 475). Additionally, two lesser represented (n < 20) administration methods – topical and infection with transgenic pathogens – were observed in the survey. The search results produced studies on eight insect orders (Fig. 2) and 54 species. All compiled data can be found in Supporting Information (Table S1), with the original published data and the standardized data used in the analyses.

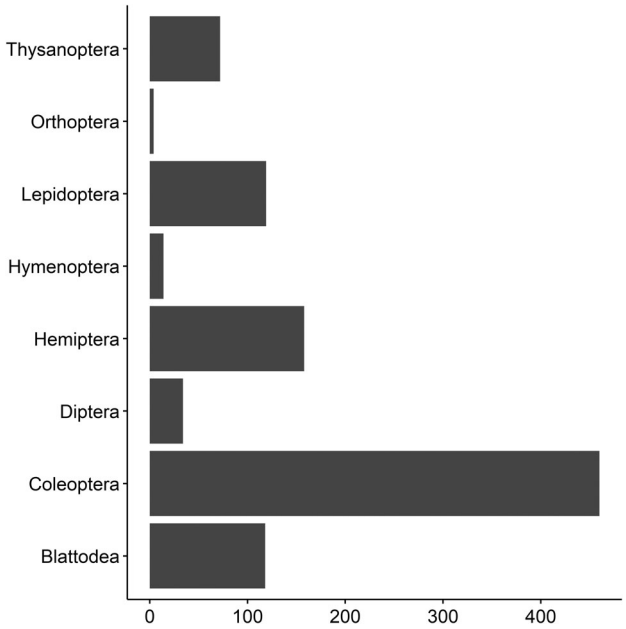

Figure 2. Total number of occurrences (dark grey bars) for each insect order from 979 data points gathered from 82 manuscripts from the data survey performed for pest control in insects using RNAi in the databases until December 2021, showing a strong bias in publications toward Coleoptera.

3.1 Model predictions

The RFM reported response time and target gene as the strongest factors to correctly predict mortality class based on all given parameters, with species and dose following in decreasing importance (Fig. 3). Administration method and the origin of publication had the least impact on the performance of the RFM. With an Out of Bag (OOB) error rate of 24.35% and a *post hoc* validation accuracy of 72.63% (P = 1.742E-15) the model has very good accuracy in predictions of new data (Table 1). This is observable in the confusion matrix, where the model performs well in predicting most classes of mortality (12–33% class errors). However, the RFM is less predictive in the medium lethality range (i.e. it is worst at predicting 33–67% toxicity with a class error of > 55%) (Table 1). The additional validation with the small, partial dataset confirmed this performance (Table 1), but while the error in predicting medium lethality was similar (64%), the error in predicting the other classes ranged from 13 to 33%. With the GLMM, mortality rate was influenced by both dose and dsRNA construct size and their interactions (GLMM: Estimate = −6.31, z = −4.92, P < 0.001). The GLMM had a low level of predictive power by the fixed values only (R^2_m = 0.12). The addition of the random factors increased the viability of the model (R^2_c = 0.93), since most of the variability can be attributed to differences in dose and taxonomical order, as indicated by a higher R^2_c than R^2_m. Both models confirmed the importance of other factors apart from target gene. Based on the available data and these predictions; (i) response time, (ii) target gene, (iii) taxonomic order, (iv) dose, (v) construct

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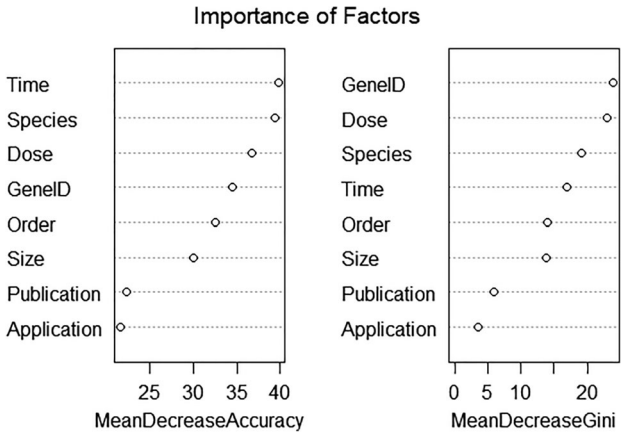

Figure 3. Importance parameter plots from the random forest model, showing which factor was given highest importance as a predictor, using 65% of the dataset as training set and considering two factors per node for 2000 trees. MeanDecreaseAccuracy = Higher accuracy decrease gives estimation on how much accuracy is lost when feature is omitted from analysis; MeanDecreaseGini = higher Gini decrease gives estimation of loss in purity (ability to cleanly split data) when feature is omitted from analysis. Time = response time in days, Species = targeted species, Dose = amount of dsRNA applied by injection or maximum amount by feeding, GeneID = target gene, Order = taxonomic order, Size = dsRNA construct size in base pairs, Publication = origin of data, Application = mode of application (injection, feeding).

size, and (vi) administration method were identified as important factors that influence the efficacy of RNAi treatment. The statistical significance of dose in the GLMM, including its interaction with dsRNA size, along with its relatively low rank in the RFM, implies that interactions with dose cloud the ability to observe its effect without controlling for the other factors.

3.2 Target genes

Target gene was a major factor driving RFM predictions. Some curation was required as gene names differed widely in the surveyed literature. In some cases, the *Drosophila melanogaster* ortholog name was used, but most publications used a different nomenclature and a total of 493 unique gene identifiers were reported. Once gene names were standardized to *Drosophila melanogaster* orthologs, the total unique target gene number was reduced to 192 (Table S1). Among all reported genes (n = 688), *Vha* and its subunits had the highest count throughout the dataset (n = 51) and represented 7.4% of all target genes used. The random forest algorithm was able to identify target gene as a significant factor determining RNAi efficacy from the available data. Representation of the ten most common target genes in relation to the complete dataset are shown in Fig. 4. The gene ontology network analysis identified 55 annotation clusters that had a significant level of enrichment (full table in Table S1). The clustering revealed a strong enrichment of genes involved in positive and negative regulation (Table 2, Clusters 1, 3 and 4). In addition to the 55 clusters of gene function overrepresented in the literature.

In the majority of the surveyed publications, only one target gene was used per bioassay, but multiple studies used two (for examples see the literature[41,51,52]) and up to four target genes in a single RNAi assay.[53] The top five target genes by number observed, *Vha*, *Ace*, chitinase (*Cht*), COPI coatomer (*COP*), and cellulase (*GHF*) represent 24.5% of the data. If compared within these five genes only, *Vha* has been reported to effectively induce a minimum of 30% mortality in Diptera,[54] Coleoptera,[52] Hemiptera,[55–57] and Lepidoptera,[58] with a mean mortality of 70% ± 26.7% through feeding with 125–580 bp of *Vha* dsRNA at doses ranging from 0.027 to 24 µg. In *R. flavipes*[34] *Vha* dsRNA applied at a dose of 10 µg resulted in only up to 18% mortality, whereas the silencing of *GHF* was more effective (up to 35% mortality at 10 µg). In Thysanoptera a 15 µg dose of *Vha* dsRNA failed to produce any mortality.[59]

Table 1. Confusion matrices for the random forest model, using the training dataset (complete data*0.65) with the resulting predictions of the model including error rate per class and validation matrix using the remainder of the dataset, but excluding the mortality data to test predictive power

Training dataset	High	Medium	Low	None	Class.error
High	65	1	5	4	0.133
Medium	4	16	9	7	0.556
Low	5	3	56	0	0.125
None	7	1	10	37	0.328

Validation matrix	High	Medium	Low	None
High	23	1	1	2
Medium	2	6	5	0
Low	2	4	25	6
None	1	2	0	15

3.3 Response time

Reported times until the maximum mortality response varied greatly among orders (Fig. 5(B)). Very short response times were observed in some cases, e.g. feeding of a vacuolar ATPase (*Vha*) construct to the silverleaf whitefly (*Bemisia tabaci*; Hemiptera: Aleyrodidae) Gennadius, achieved 70% mortality within just 48 h.[60] However, much slower responses were observed by injecting bed bugs (*Cimex lectularius*; Hemiptera: Cimicidae) Linnaeus, with 0.5–0.05 µg *Vha*, which took 28 days to reach 80–85% mortality.[61] Similarly, feeding German cockroaches (*Blattella germanica*; Blattodea: Ectobiidae) Linnaeus, with lipovesicle coated *α-Tubulin* (*tub*) resulted in > 60% mortality after 16 days.[62] In Lepidoptera high mortality (70%) was achieved after 20 days by feeding corn earworm (*Helicoverpa zea*; Lepidoptera: Noctuidae) Boddie, larvae with a construct targeting the *pheromone biosynthesis activating neuropeptide* (*PBAN*).[37] There appears to be a clear effect of the duration of an experiment on mortality (Fig. 5(B)).

3.4 Construct size

The size of dsRNA constructs also varied within the surveyed data and no functional justification for any specific dsRNA size was given in any of the studies. Construct size ranges from short pieces of around 100 bp,[34,63,64] to long pieces above 800 bp[65,66] and even full gene coding sequences of up to 1872 bp.[67,68] Limiting the range of construct sizes by their effectiveness and removing any results of less than 67% mortality revealed an effective range of 125 to 628 bp (Fig. 5(A)) corresponding with findings in *Drosophila melanogaster* cells expressing *Caenorhabditis elegans Sid1* gene, where a single application of a 100-bp construct needed a dose of 100 to 1000 times greater than a 500 bp construct to achieve equal silencing effect.[69] Current research points toward an optimal size of dsRNA constructs. Outside of insects, optimal outcomes (highest mortality) were obtained with 500 bp in *Caenorhabditis elegans*[69] and 400 bp in the two-spotted

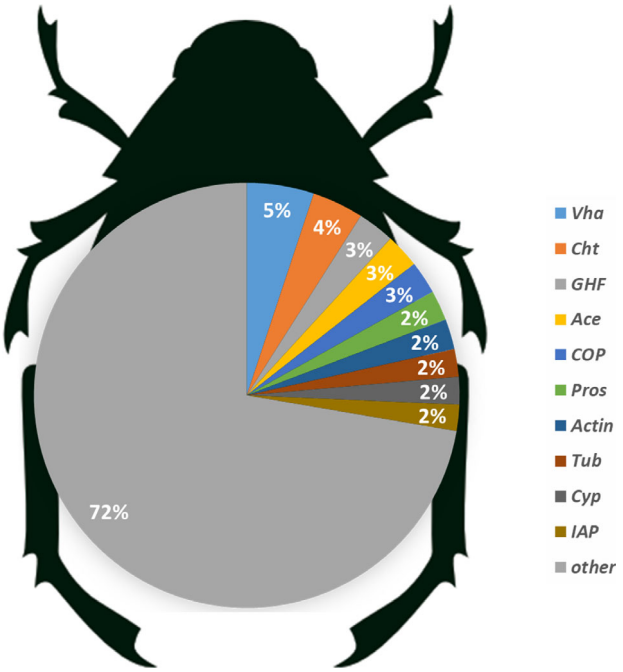

Figure 4. Visualization of target gene distribution throughout the survey data, colored bands show percentage of observations made per gene with the ten most represented genes singled out. The genes are (from top to bottom) vacuolar ATPases (*Vha*), $n = 49$; chitinases (*Cht*), $n = 32$; GHF cellulase (*GHF*), $n = 26$; acetylcholinesterase (*Ace*), $n = 23$; coatomer proteins (*COP*), $n = 23$; proteasomes (*Pros*), $n = 22$; actins (*Actin*), $n = 21$; tubulins (*Tub*), $n = 20$; cytochrome P450s (*Cyp*), $n = 19$; inhibitor of apoptosis (*IAP*), $n = 18$ and all other genes, $n = 635$.

spider mite (*Tetranychus urticae*; Trombidiformes: Tetranychidae) Koch.[70] Within insects, only data on *Tribolium castaneum* are available comparing multiple construct sizes, showing increased efficacy of a 480 bp construct over a 30 or 60 bp construct.[71] Taken as a whole, present results on invertebrates indicate the optimal dsRNA construct size is likely within 100 to 600 bp.

3.5 Insect order

Orders with a high number of tested species and strong RNAi reaction were more commonly studied than orders with fewer numbers of pest species or weaker RNAi effects. Foremost, Coleoptera was the order with the highest representation in the current literature, followed by Lepidoptera and Hemiptera. All three orders exhibit functional response to RNAi treatment through feeding and injection of dsRNA constructs. Other orders are less represented in the literature; Blattodea as an outlier owes its large representation to a single large-scale screening study,[34] while Orthoptera, Thysanoptera, Diptera, and Hymenoptera are less commonly represented in the dataset. However, the data show varying levels of mortality in all orders surveyed. It is important to note, that for all surveyed orders the presence of core RNAi genes has been verified.[21,24,72–77] Multiple orders however, especially Orthoptera and Diptera, show differential expression of the core machinery or lack a systemic response that limit the possible efficacy of RNAi.[78]

3.6 Applied dose

Dose of dsRNA applied to insects covered a wide range within the data set and no obvious evidence of a dose effect on mortality could be found in the surveyed data set without accounting for other variables simultaneously (Fig. 5(C)). However, there were individual reports that showed a positive linear relationship between dose and mortality.[34,42] The lack of a clear picture in dose effects is, in turn, underlined by results that fail to find a relationship between dose and mortality.[37] Overall, application by injection used lower doses. The lowest reported dose was 1.5E-08 µg of a 485 bp cactus (*cact*) dsRNA construct, injected into the *Tribolium castaneum*. This dose resulted in 75% mortality within 16 days and 100 times higher dose increased mortality to > 90% but had no effect on the response time.[79] On the other side of the scale, injections of up to 20 µg Dicer (*Dcr*) RNAi were applied to fourth instar migratory locusts (*Locusta migratoria*; Orthoptera: Acrididae) Linnaeus. At over 1.3 billion times the smallest dose reported, this high dose of *Dcr* caused mortality rates of 64%.[80] In contrast, similarly high doses have been applied to adult tobacco cutworm (*Spodoptera litura*; Lepidoptera: Noctuidae) Fabricius, where injections of 10 µg *PBAN* dsRNA caused 0% mortality.[81]

Feeding dose could not be quantified like injection, as it is impossible to determine how much of the available dsRNA was ingested by the target organism. It can be assumed that the total available dsRNA was never fully consumed, leading to smaller actual doses for each individual. The variation of available dsRNA doses that induce mortality were also narrower than those of injection trials, span a range of 800-fold between the largest and smallest dose (minimum = 0.5 µg, maximum = 400 µg). As with injection, the dose of RNAi applied by feeding did not seem to impact mortality outcomes. Just 0.5 µg of *Laccase2* and *Vha* dsRNA per pellet of artificial diet was the lowest dose reported, causing up to 60% mortality in adults of Western corn rootworm (*Diabrotica virgifera virgifera*; Coleoptera: Chrysomelidae) LeConte.[82] The highest reported available dose of dsRNA used for a feeding assay was 400 µg *ribosomal protein* (*RpL*) dsRNA. This high dose was used in a research approach for adult oriental fruit fly (*Bactrocera dorsalis*; Diptera: Tephritidae) Hendel and did not induce mortality.[68] Note however that Diptera lack a systemic RNAi response, which may explain this result.[83]

3.7 Application methods

Feeding of dsRNA is the most common method in the scope of the survey ($n = 441$), with injecting dsRNA being second ($n = 431$). Thus, these strategies were similarly represented in the dataset. The effective application by these two common approaches was supported by the comparison of induced mortality by dsRNA between injection and feeding, where feeding appeared to show similar induction of mortality compared to injection (Fig. 5(A–C)). The feeding approach included the use of artificial diet,[84] transgenic host plants,[58] soaked leaves,[85] transgenic bacteria,[52] or simple dsRNA solution[42] and all were able to cause significant mortality (up to 100%). Two additional methods were found but are underrepresented in the survey ($n < 20$ for each) – infection with a transgenic entomopathogenic vector expressing dsRNA[40] and soaking in a dsRNA solution.[86]

4 DISCUSSION

4.1 General findings

Since its discovery, RNAi has been hailed as ushering in a new era of genetics[17,19] and this is reflected in the numbers of total publications on RNAi in recent years. Searching for publications using 'RNAi' as keyword, Google Scholar returns over 38 100 results since 2016, 15 800 of these since 2019 (retrieved March

Table 2. Extract from the GOTERM cluster analysis using DAVID 6.8, presenting the ten strongest enriched processes in the dataset, using options for high classification stringency and an enrichment threshold (EASE) score of 0.05

	GOTERM biological process	Enrichment score	Average count	Percentage	*P* Value	Benjamini
Annotation Cluster 1	Positive regulation of genetic, biosynthetic, metabolic processes	6.04	22.9	13%	0.000167	0.00165
Annotation Cluster 2	Biosynthetic process	5.84	44.4	26%	2.69E-06	0.00013
Annotation Cluster 3	Negative regulation of genetic, biosynthetic, metabolic processes	5.6	25.6	15%	5.44E-06	0.00020
Annotation Cluster 4	Regulation of genetic, biosynthetic, metabolic processes	5.55	40.9	24%	2.22E-05	0.00054
Annotation Cluster 5	RNA mechanism and processes	5.46	8.2	5%	0.004116	0.02096
Annotation Cluster 6	Regulation of translation and posttranscriptional expression	4.82	11.3	6%	2.54E-05	0.00069
Annotation Cluster 7	Reproductive process	4.73	41.2	24%	5.39E-05	0.00110
Annotation Cluster 8	Female reproductive process	4.57	31.2	18%	4.84E-05	0.00110
Annotation Cluster 9	Neuronal development and cell differentiation	4.18	25.9	15%	0.000434	0.00528
Annotation Cluster 10	Negative regulation of protein metabolic process	4.17	16.0	9%	0.001872	0.01452

Average count is the number of genes per GOTERM in each cluster. The enrichment score of each annotation cluster reflects the $\log_{10}$ geometric mean of the *P*-values within the cluster, higher values express a stronger enrichment. GOTERM_BP_FAT uses annotations based on biological processes (see Supporting Information Table S1 for full table of all 55 annotation clusters).

31, 2020, no citations, no patents), revealing the large amount of interest this topic has sparked in the scientific community overall. This raises the question: What has slowed the progress of the development of RNAi as a pest management tool? Surveying the results of many insect management studies with machine learning and enrichment analyses, uncovered valuable information about the factors that can affect progress in this area of research. RNAi works in a variety of insect orders and in many cases a functional if not always optimal target gene can be identified. One thing is clear: there are almost as many experimental designs as there are published studies, and this makes comparison between individual results difficult. Such a state of affairs calls for more standards in experiments using RNAi for pest management and the standards for dose–response analysis of potential insecticides can serve as a roadmap to guide future research. Specifically, following quantal bioassay standards[87] while using a single standard size (or defined range of sizes) for dsRNAs, would be a valuable addition to current RNAi studies, as identifying optimal dose ranges can help identify effective candidate target genes that might be missed by testing them at a suboptimal dose. However, all signs indicate that a viable strategy should be within reach of the scientific community. More uniform approaches of RNAi studies hold the potential to increase efficacy and reproducibility while decreasing current variability in results and overall cost of experiments.

4.2 Target genes

Observed results indicate that RNAi for pest management is technically possible, as studies on RNAi cover most insect orders of interest for pest management and show some efficacy. The next step is to identify a general guideline for choosing promising target genes without the need to conduct expensive large-scale screening of candidate genes. Currently, there is no clear justification in the target gene choice. Genes chosen for study are often genes of essential function, like *Ace*[42,88] or *Vha*,[31,56,58,60] and other genes that have shown initial potential for high mortality in large-scale screenings like the proteasome (*Pros*).[30,31,55] The proteasome was identified as the most promising biological process to find effective target genes for killing *Tribolium castaneum* by Ulrich *et al.*,[31] highlighting an important concept: there is a clear functional bias in the genes studied for killing insects. On some level, bias is expected as lethal genes (a subset of any genome)[89] should be targeted by RNAi studies for insect control. This pattern of gene choice could exist due to publication biases related to the report of positive results only.[90] However, another explanation for a bias in the literature is that researchers gravitate toward what appears to have already worked. In this instance, these targets may be valuable positive controls as they are observed to work in many taxa and across application methods. It might be that there is a set of genes that are perfectly viable control options, but which have not been targeted due to no other reason than a lack of inquiry (e.g. a gene in a target taxon that is not found in common model organisms).

Future studies should ideally investigate multiple genes from an existing screening study[31,34] and use a known target gene for their species of interest as a positive control to compare with the efficacy of the tested candidates. If no such control gene is available, the use of a widespread target gene like *Vha* may serve as a good estimate of baseline RNAi efficacy in the target species. The ideal bioassay requires the use of at least five concentration levels in ten-fold increments to calculate dose–response regression curves and build more reliable datasets on target genes. This should be done for any individual target sequence within a gene. While positive control of RNAi treatments is still in its infancy, the use of a negative control treatment has found almost complete consensus. Green fluorescent protein (*GFP*) is widespread and functional in use as negative control for RNAi experiments and based on the literature surveyed no evidence of negative impacts of *GFP* on the survival of the tested organisms was found throughout the survey.

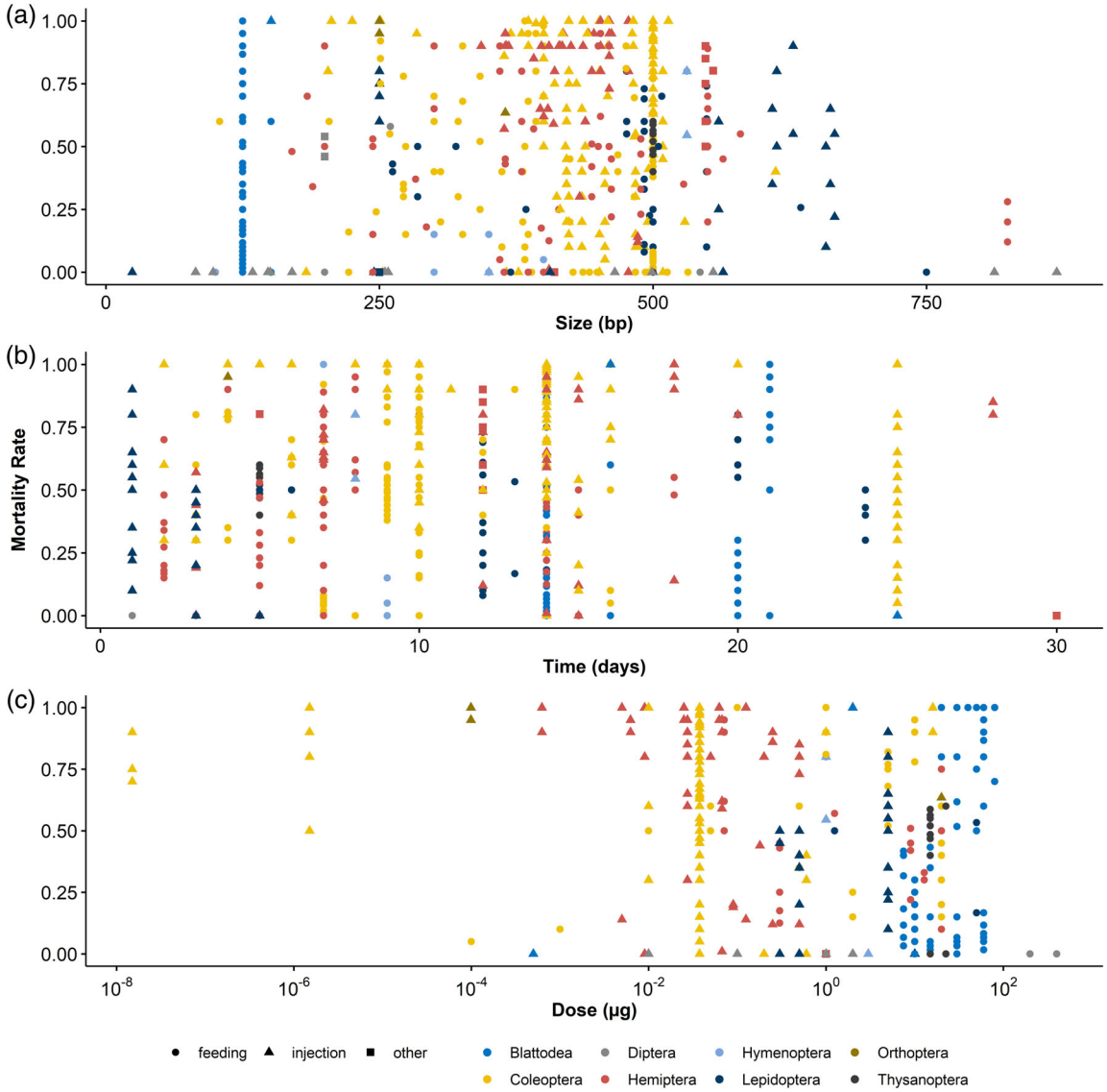

Figure 5. Plotted data on three factors identified in the dataset, datapoints are color coded by order (Blattodea, blue; Coleoptera, yellow; Diptera, grey; Hemiptera, red; Hymenoptera, light blue; Lepidoptera, purple; Orthoptera, orange; Thysanoptera, maroon) and point format indicating mode of dsRNA application (circles = feeding, triangle = injection, square = other). All data is plotted against reported mortality rate and shows (A) size of ds RNA construct applied in base pairs (bp), (B) response time until mortality was recorded in days and (C) applied dose of RNA in total micrograms available. Data shows an immense range of variability, effectively reflecting on the prevalent lack of standardization in RNAi experimental design.

4.3 Response time

One factor that is critical to interpreting the ability of a treatment to kill an insect is how long it takes for that organism to die after exposure. In chemical assays, these response times can be short. However, there is no reason to expect equally similar times to kill in RNAi applications, and one potential problem with RNAi is that experiments may not last long enough to observe death, indicating that longer bioassays are required for RNAi than most chemical insecticides.

4.4 Construct size

This aspect of RNAi research is distinct from chemical pesticide research, as the same gene can be targeted, but with different regions of that gene applied. Thus, research on a single gene with RNAi, where different dsRNAs from the same gene are investigated (e.g. 5′ *versus* 3′ ends of the gene) may be analogous to chemical classes of insecticide that affect the same target gene but do not kill at the same rates.[34] No information about why any specific construct size had been chosen in a study has been found. Moreover, there is still no study in insects that confirmed or refuted equal effects between an exemplary range between 125 bp construct and a 600 bp construct of any single target gene, nor in any single species, nor between species. Future studies are needed to better understand the possible effects of construct size. The available results from non-insect species, *Caenorhabditis elegans* and *Tetranychus urticae*,[69,70] show that with improvement of construct size, a reduction in dose is possible; reducing the amount of dsRNA needed will decrease the cost of RNAi research.

4.5 Applied dose

Dose is arguably the most variable factor in current RNAi research. In the RFM, this factor was relatively unimportant compared to

other factors. For instance, taxon-specific issues like size (resulting in differences between dose applied and dose per milligram) are logical factors that may be in play when considering dose. Should 1 µg of any dsRNA be expected to produce the same effect in a 50 mm long, 3000 µg locust as in a 0.9 mm long, 39 µg whitefly? That means that assessing dose effects may require controlling for the other factors noted here, especially those with a higher importance than dose in the RFM. The use of not a single, but a range of different doses following toxicological standard procedures may also be beneficial in identifying functional RNAi responses. This approach was again supported by examples from *Caenorhabditis elegans*, *Tribolium castaneum* and *Tetranychus urticae*[69–71] where different sizes of dsRNA at equal dose varied in efficacy. Therefore, while dose only had medium predictive power on efficacy in the RFM, the identification of functional median lethal dose (LD_{50}) or similar classifications following common toxicological practices, will help identify effects of RNAi and help validate the findings of the GLMM. Our results indicate that there is significant room to optimize dose for RNAi applications. To achieve this, the collaboration between geneticists and toxicologists is highly desirable in future studies. The reduction in cost of RNAi and other genetic studies over the last 15 years has made comparative studies more affordable and should encourage researchers to standardize dsRNA size and build functional dose–response models in future studies, similar to Velez *et al.* in a recent study on *Diabrotica virgifera virgifera*.[91]

4.6 Is the only good pest a dead pest?

While many surveyed studies aimed to induce high mortality in the target organism, it has become clear that simply killing the pest might not be the only viable strategy of RNAi pest management. Sublethal effects can alter behavior, negatively affect fitness and fertility, and consequently decrease damage caused by the pest population. This has been known for far longer than RNAi research has been conducted[92] and can be observed in multiple RNAi studies.[54,93,94] For example, the feeding or injection with *Dcr* 1 or *Dcr* 2 reduced emergence numbers in *Diabrotica virgifera virgifera* and surviving adults had malformed wings and were therefore unable to fly.[82,93] Such sublethal effects can reduce populations and limit the spread of pests from infested areas. Silencing *chitin synthase* caused severe deformities in trachea and cuticle development in beet armyworm (*Spodoptera exigua*; Lepidoptera: Noctuidae) Hübner[95] and similar effects were observed in Orthoptera.[80] A combination of mortality and additional sublethal effects was shown in *Tuta absoluta*.[58] In addition to a significant induction of mortality, a dose dependent reduction of damage to host plant leaves was reported. An approach that does not solely target mortality, but aims to limit development, reproduction, spread and impact of target species could offer great value in agricultural pest control. Therefore, sublethal control targets could serve as a complementary approach to control pests over extended time periods, as has been done with insect growth regulators in termite and ant control for decades.[96,97] The use of a sublethal target gene is of special interest in orders that exhibit low or slow reaction to RNAi treatments and in such cases the length of time effects can be measured at the population level. Effective management of population size with RNAi can become an important component of an integrated pest management program.

4.7 Combinatorial effects

Studies of multiple gene targets or combinations of gene targets and/or stressors are relatively rare and understudied but have the potential promise of synergistic effects. Using multiple genes can potentially increase mortality from the silencing of a combination of essential genes, like *COP* and *inhibitor of apoptosis* (*IAP*).[41] This kind of strategy can be useful for pest species that show weak RNAi responses, and this would not require a synergistic effect to be feasible if the additive potentiation of mortality effects can achieve the desired mortality levels. However, it is still unclear how the target organism will react to treatment with multiple dsRNA constructs, as was observed by Singh *et al.*[98] in their experiments with *Thrips tabaci* (Thysanoptera: Thripidae) Lindeman, where feeding an *AQP* construct alone resulted in 15% higher mortality than a mix of *AQP* + *GFP*. Such a result may be due to an unappreciated aspect of experimental design. Theoretically, this kind of phenomenon could also be caused by overloading of the RNAi machinery of the organism. However, to our knowledge no research into the biological capacity of the RNAi machinery has been published yet. A broader use of control treatments that return a predictable baseline efficacy will help identify effects of single target genes compared with mixed target genes.

Pairing dsRNA treatment with environmental stressors like an insecticide, heat, or a pathogen is a different approach of combinatorial RNAi. Evidence on the efficacy of pairing a stressor with a corresponding dsRNA treatment have been shown by at least two independent studies.[40,99] However, the true potential of any multifactorial target gene approach remains to be seen. The use of multiple target genes simultaneously is even less understood than the 'simpler' single target gene approaches and additional studies will be needed to evaluate their efficacy.

4.8 The need for multidisciplinary RNAi for pest management

Observed results from the machine learning algorithms indicate that RNAi insect management is in many ways similar to classic toxicological bioassays, with identified effects of the effective compound (dsRNA sequence and size), applied dose and response time. Potential considerations should additionally be given to traditional pharmacokinetics for RNAi. Where in chemical insecticides the pharmacophore (structural/molecular traits influencing specificity) and dianophore (uptake, distribution, metabolism) are both determined by their chemical structure, dsRNA in contrast has its pharmacophore determined by its nucleic sequence while the dianophore consists of the construct size and other structural properties.[100] This split between the two opens additional pathways to optimize the dianophore of dsRNA, by altering construct size, 5′ or 3′ modifications, or secondary structure for example, without impacting its pharmacophore. This is also supported by indications that construct size, while not directly affecting the resulting small interfering RNA (siRNA) of 20 to 24 bp as part of the gene silencing process, has clear effects on the uptake efficiency for dsRNA and therefore ultimately mortality.

Thus, it appears that RNAi research would benefit greatly from a multidisciplinary approach. Application of RNAi requires genetic knowledge; thus, it is not surprising that the studies initially approaching this problem take such a perspective. However, the high variability among projects, in aggregate, reiterate the basic toxicological principles of dose–response and can be demonstrated in the reduced accuracy of the RFM for the 'medium' range (33–66%). These patterns coincide with similar observations in toxicological trials, as 0% and 100% mortality are finite points on the scale and variability decreases with proximity to these extremes. Therefore, the medium range experiences larger variability in mortality than the low-end and high-end points, which

is another parallel to chemical toxicology that needs to be considered.

The use of RNAi-based pest management is arguably more complex and species dependent than traditional chemical approaches, making the adoption of a multidisciplinary approach seem logical. Genetic knowledge is essential to identify potential target gene candidates, while training in insect toxicology is essential for development and interpretation of functional bioassays. Furthermore, taxon-specific, and organismal knowledge is necessary. This need is highlighted most clearly in the response time results. A well-trained geneticist/toxicologist may not appreciate the amount of time necessary to kill an insect with RNAi if their perspective on insect control was solely informed by fast-acting chemicals. Therefore, future training programs that integrate knowledge of RNAi machinery, taxon-specific organismal biology, and toxicology are a key to advancing RNAi as a pest management tool.

5 CONCLUDING REMARKS

Research into RNAi for pest management is sufficiently mature enough to begin to glean lessons from the growing literature in order to guide the field forward. The field is highly heterogeneous in its approaches, which made direct comparisons difficult. However, it was possible to identify important features of RNAi as an insect control strategy. Important factors identified are choice of target gene and response time. These factors appear analogous to the use of specific chemicals used to kill insects (active ingredients) and their exposure times. Thus, these results highlight the need to integrate toxicological, genetic, and organismal research, and we advocate that these should be incorporated into training programs. Researchers should make the effort to design their experiments in a standardized manner to facilitate comparisons and reproducibility. If such an approach is taken, we argue that RNAi applications for insect control will be accelerated.

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CONFLICT OF INTEREST

All authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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