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# Ingestion of Species-Specific dsRNA Alters Gene Expression and Can Cause Mortality in the Forest Pest, *Ips calligraphus*

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**Abstract:** *Ips calligraphus* (Germar) is a conifer pest that causes economically and ecologically significant tree mortality, particularly when forests are stressed. As forests become increasingly vulnerable to pest outbreaks due to habitat fragmentation, invasive species, or climate change, innovative management strategies are needed to augment traditional approaches. Manipulating the RNA interference (RNAi) pathway is emerging as a novel pest management technology that could serve as a means of managing *I. calligraphus* while minimizing non-target effects. Demonstrating effectiveness of exogenous double-stranded RNA (dsRNA) in inducing changes in gene expression and causing mortality is an essential step. In this study, oral ingestion of dsRNA caused significant changes in gene expression and increased mortality for two of the three target dsRNAs tested. Additionally, we sequenced 5 mRNA libraries from adult beetles to assemble a transcriptome, from which we identified sequences of target genes for dsRNAs, and 10 genes in the *I. calligraphus* transcriptome putatively involved in the RNAi pathway. We demonstrate that oral ingestion of exogenous dsRNA can trigger the RNAi pathway. This is the first published study to artificially trigger the RNAi pathway in an *Ips* spp. and the first step in evaluating the potential for pest management strategies utilizing RNAi against this pest.

**Keywords:** RNA interference; six-spined *Ips*; bark beetle; forest pest management



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## 1. Introduction

Globally, forest ecosystems face unprecedented challenges from human disturbances, pathogens [1], climate extremes [2], and insect pests, both native and introduced [1,3,4]. These challenges have the potential to impact not only timber production [4], but vital ecosystem services, such as carbon sequestration, prevention of soil erosion, and provision of habitat that sustains biodiversity [5]. The effects of climate change have led to increasingly severe and prolonged droughts, intense storms, and temperature extremes [2,6,7]. Often, these changing abiotic conditions and disturbances pose a serious threat to forest health [8,9], and when combined with forest insect pests, the consequences can be especially devastating [4,10]. One of the most salient examples of this interaction between climate change and insect populations is that of bark beetles. While effects on individual bark beetle species may vary, overall projected warming temperatures and reduced vigor of host trees create an environment in which both native and exotic bark beetles are increasing in geographic range and propensity for eruptive outbreaks [11].

The six-spined ips, *Ips calligraphus* (Germar), is one such pest that has been elevated from a secondary pest to one of economic significance due to climate change and associated extreme disturbance events [12–14]. *Ips calligraphus* colonizes most *Pinus* species, including those of economic importance, such as loblolly (*Pinus taeda* L.), shortleaf (*P. echinata* Mill.), and slash pines (*P. elliottii* Engelm.) [15,16]. Upon emergence, male pioneer beetles locate an appropriate tree by utilizing host volatiles, particularly those pines that are in poor condition and thus have lowered resistance to bark beetle attack [17]. Once at a suitable host, the beetles use a combination of sex pheromones and aggregation pheromones to

overwhelm host defenses through mass attack [18] and simultaneously find a mate. After mating, female beetles excavate oviposition galleries and lay eggs, from which the larvae will create feeding galleries in the phloem as they develop [16]. In addition to introducing potentially harmful microorganisms, adult oviposition activity and larval feeding destroys the cambial tissue and prevents the flow of water and nutrients throughout the tree, leading to mortality [17].

Under normal conditions, *I. calligraphus* damage occurs sporadically, as previously weakened trees are primarily affected [12]. In these instances, traditional silvicultural techniques that promote healthy forests are sufficient for keeping populations at endemic levels [19]. However, these techniques fall short when abiotic extremes, such as storms and drought, lead to an excess of suitable breeding substrate and compromise host defenses, at which time beetle populations can grow rapidly, allowing mass attack on additional healthy trees [20–22]. Given the beetles quick generation time (mean 25 days), populations can grow rapidly, causing significant damage and economic losses [12,13,23,24]. Additionally, due to its cryptic nature, *I. calligraphus* is easily transported and thus has been intercepted at ports in multiple countries [25] and has established itself outside of its native range, becoming invasive in the Philippines [14]. Most recently, *I. calligraphus* has established populations in south mainland China, where it is being monitored to determine what risks it may pose to pine production in the country [26]. The ease of human transport, its extensive potential geographic range, and its wide host range have led to *I. calligraphus* being classified as a high-risk invader for the southern hemisphere [25].

As previously innocuous pests, such as *I. calligraphus*, enter new geographic ranges and begin to capitalize on disturbance conditions to cause increasing destruction, novel approaches must be considered to support traditional silvicultural management. Fortunately, recent advances in molecular genetic techniques have brought new opportunities for integrated pest management (IPM), and manipulation of the RNA interference (RNAi) pathway to induce mortality in target insects is one promising example. With the use of carefully designed species-specific double-stranded RNA (dsRNA), we can artificially induce a viral defense mechanism within a target insect, leading it to degrade critical messenger RNAs, resulting in mortality [27,28]. As dsRNA is recognized by the RNAi pathway, it is cleaved into 21-23 base pair pieces known as small-interfering RNA (siRNA) by the enzyme dicer [29], which are then used as a template in the RNAi-induced silencing complex (RISC) to bind to and then cleave complementary mRNA, disrupting the translational step and preventing the production of proteins [29]. To induce silencing, there must be a 16-24 nt match [30], or near match, allowing for dsRNAs to be designed with high specificity to the target species, minimizing non-target effects [31–33].

RNAi technology has already been deployed against agricultural pests [34–37] and more recently has become an area of investigation for forest pest management [38–41]. Oral ingestion of dsRNA is effective in inducing mortality and gene silencing in two other scolytines, the southern pine beetle *Dendroctonus frontalis* (Zimmermann) [39] and the mountain pine beetle *D. ponderosae* (Hopkins) [40], causing significant mortality and gene silencing. This success in other scolytines makes *I. calligraphus* a promising target for an RNAi-based management tool. Thus, this study aims to establish the basis for experimentally triggering the RNAi pathway in *I. calligraphus*. The main objectives of the present work are to demonstrate exogenous dsRNA-induced gene silencing and mortality and to identify key RNAi pathway genes in the *I. calligraphus* transcriptome. Demonstrating changes in gene expression following oral ingestion of dsRNAs, as well as linking these changes to a measurable effect on beetle mortality, is a critical step in evaluating the feasibility of RNAi technology as a potential management tool for *I. calligraphus*.

## 2. Materials and Methods

### 2.1. Insects

Insects were obtained by suspending *I. calligraphus* lures (Synergy Semiochemicals Corporation, Delta, BC, CA) on felled loblolly pine in Florida Forestry Service land in

Newnans Lake State Forest in central Florida for two days in September of 2021. Infested trees were collected and immediately transported to the University of Kentucky, Lexington, KY, where they were sectioned and transferred to rearing bins (55.6 × 62.7 × 81.3 cm) maintained at 20–22 °C with ambient light. Adult beetles were collected as they emerged and held in petri dishes (60 × 15 mm) oriented vertically and lined with moistened KimWipes (Kimtech, Neenah, WI, USA) until use in assays.

### 2.2. Gene Selection

Target genes of heat shock protein (*hsp*), inhibitor of apoptosis (*iap*), and shibire (*shi*) were selected based on their success in other scolytines [39,40]. Double-stranded RNA coding for green fluorescent protein (dsGFP) was selected as a negative control as it is not present in insects and, therefore, cannot be silenced [42]. Target gene sequences were obtained by using *D. frontalis hsp* (GAFI01018338.1), *iap* (GAFI01016651.1), and *shi* (GAFI01018708.1) sequences as query for a tBLASTx search using default parameters to the *I. calligraphus* transcriptome (described in Section 2.7). Identities were verified by then using the sequences for a BLASTx search to the NCBI non-redundant (nr) database (accessed 8 February 2022), ensuring top matches were the anticipated gene in other insects. After confirming the identities, the online tool Primer3Plus [43] was used to design primers for dsRNA synthesis and quantitative PCR. Primers for qPCR (Table 1) were restricted to amplicon lengths between 80 and 120 bp, and primers were selected that had a GC% of ~50% and the lowest self and any scores. The T7 promoter sequence (TAATACGACTCACTATAGGG) was included at the beginning of dsRNA primer sequences (Table 2) for compatibility with the MEGAscript RNAi Kit (Invitrogen, Waltham, MD, USA), and sequences were restricted to lengths between 200 and 450 bp, again selecting primers with a GC% of ~50% and low self and any scores.

**Table 1.** qPCR primer sequences, $R^2$ correlation coefficient, and percent primer efficiency.

| Gene Name | Sequence 5′3′ | $R^2$ | %E |
|---|---|---|---|
| *16s rRNA*—16s Ribosomal RNA | CAAACCTTTCATTCCAGCTTTC<br>AAAATACTGCGGCCGTTAAA | 0.99 | 103.8 |
| *28s rRNA*—28s Ribosomal RNA | TCGACCTCTGGTGACTGTTG<br>ACTTTCAGGACCCGTCTTGA | 0.99 | 105.4 |
| *hsp*—Heat Shock Protein | GTTAGAACGTCCCTCAGTTTC<br>TGGTTGCGGTTCGTTAAG | 0.99 | 96.2 |
| *iap*—Inhibitor of Apoptosis | AGCATCAGGCTGAGAATAAC<br>CTCTCACAGCGTTACAGATAG | 0.99 | 103.4 |
| *shi*—Shibire | CGAAGTGAGAACGAACCAATA<br>CCCTCGGCAATCAAGTAATC | 0.99 | 91.6 |

**Table 2.** Target gene dsRNA amplicon size and primer sequences, including the T7 promoter sequence (bolded).

| Gene name | Sequence 5′3′ | Amplicon Size |
|---|---|---|
| *hsp*—Heat Shock Protein | **TAATACGACTCACTATAGGG**CTTTGTCCGCAACCATAAATAC<br>**TAATACGACTCACTATAGGG**AGGATCGCCACTCGATTA | 429 |
| *iap*—Inhibitor of Apoptosis | **TAATACGACTCACTATAGGG**GAGAGCAACTTCTCCGTTTAG<br>**TAATACGACTCACTATAGGG**GCCAGAATATGGCACTGTAG | 373 |
| *shi*—Shibire | **TAATACGACTCACTATAGGG**CCCTGAGGATCAACTTCTTTAG<br>**TAATACGACTCACTATAGGG**CCTTCCTAGAGGATCTGGTATAG | 423 |
| *gfp*—Green Fluorescent Protein | **TAATACGACTCACTATAGGG**CGATGCCACCTACGGCAA<br>**TAATACGACTCACTATAGGG**TGTCGCCCTCGAACTTCA | 248 |

### 2.3. dsRNA Synthesis

Total RNA was extracted using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA), and RNA integrity was validated using gel electrophoresis and NanoDrop spectrom-

etry (Thermo Fisher Scientific Inc., Waltham, MA, USA). From each RNA sample, cDNA was synthesized (400 ng) using the SuperScript III Reverse Transcriptase protocol (Invitrogen, Waltham, MA, USA). PCR was performed (at 94 °C for 30 s, (94 °C 30 s, 60 °C 1 min, 68 °C 1 min) × 30, 68 °C 5 min, 4.0 °C ∞), and resulting PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germantown, MD, USA). This purified PCR product was then used to synthesize dsRNA using the MEGAscript RNAi Kit. NanoDrop spectrometry and gel electrophoresis were used to ensure generation of a single product, as well as the quality and quantity of the dsRNA.

### 2.4. Bioassays

Beetles were starved for 24 h, then fed by immersion in a 0.5 mL microcentrifuge tube containing 10 µg of dsRNA in 0.5% sucrose following methods described in Wallace and Rieske [44] for a total of 4 µL of the dsRNA solution. After their feeding period (~4 h), beetles were kept in petri dishes containing moistened KimWipes within a humidity chamber (~23 × 21 cm). Individuals evaluated for gene expression (n = 5 beetles per treatment) were maintained for 72 h before being crushed directly into TRIzol reagent to maximize RNA yield, while those evaluated for mortality (n = 29 per treatment) were monitored daily for 10 days. Mortality data were analyzed using a linear mixed-effects model, with treatment and day, as well as their interaction as the fixed effects, and replicate as a random effect (R statistical software Version 4.0.5, Vienna, Austria). A type III ANOVA was used to identify significant factors in the model. A Tukey's Honest Significant Difference (Tukey's HSD) post hoc test was then used to identify the effects of each treatment on beetle survival.

### 2.5. Gene Expression

Total RNA was extracted using the TRIzol reagent, then gel electrophoresis and NanoDrop spectrometry were used to ensure RNA integrity. cDNA was then synthesized using the SuperScript III Reverse Transcriptase following manufacturer's protocols. Quantitative PCR was then performed on a 5-fold dilution of cDNA (starting at a 1:25 dilution) to ensure a single melt curve, determine the concentration to evaluate treatments, and to ensure primers met parameters for efficiency percentage (90–110%) and linear regression coefficient ($R^2 > 0.99$). cDNA of each beetle treated with dsRNA (n = 5) was diluted to 1:125, and quantitative PCR was used to evaluate the expression of each target gene. Gene expression was normalized across individuals using *16s rRNA* and *28s rRNA* as reference genes [44] using the $2^{-\Delta\Delta Ct}$ method [45]. Interquartile range (IQR) analysis was used to remove extreme outliers (values above Q3 + 3 × IQR, or below Q1 − 3 × IQR). Control beetles were compared to the target gene dsRNA treated groups using a one-tailed *t*-test to test for significance.

### 2.6. mRNA Sequencing

Adult beetles were separated by sex using the number of protibial spurs and the presence or absence of the pars stridens [46] and then dissected to separate the heads from the abdomen and thorax. Two female head samples were generated by pooling 10 individual heads each, and two male head samples also containing 10 heads each were pooled (for a total of 40 heads). For the dissected abdomen and thorax samples, 5 insects were pooled. Total RNA was extracted using the TRIzol reagent, and RNA integrity, quantity, and purity were validated using gel electrophoresis and NanoDrop spectrometry. Total RNA was sent to Novogene Corporation Inc. (Sacramento, CA, USA) where RNA integrity was analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After passing quality control checks, RNA was then used for RNAseq library preparation with poly-A enrichment using poly-T oligo-attached magnetic beads and 150 bp paired-end sequencing using the NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA).

### 2.7. De Novo Transcriptome Assembly

High quality reads were achieved by trimming poly-A tails and by removing ambiguous or low-quality reads and those with erroneous kmers, using Rcorrector [47] (Version 1.0.4, Baltimore, MD, USA), Transcriptome Assembly Tools (https://github.com/harvardinformatics/TranscriptomeAssemblyTools (accessed on 20 July 2022)), and TrimGalore (Version 0.6.0, Cambridge, UK) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/ (accessed on 21 July 2022). Processed reads were combined and used for a de novo assembly using Trinity (Version 2.12.0, Cambridge, MA, USA), using in silico read normalization and default parameters [48,49]. TransDecoder (v5.5.0) (https://github.com/sghignone/TransDecoder#transde-coder-v550 (accessed on 22 July 2022) was used to extract long open reading frames (ORF) and predict likely coding regions in the transcriptome, and ORFs were queried using a BLASTp search with an e-value threshold of 1e-5 using the UniProt SWISS-PROT database (accessed on 22 July 2022). The BLASTp homology search was used as ORF retention criteria during the prediction of coding regions, and predicted coding sequences (CDS) were then processed using CD-hit (Version 4.6, La Jolla, CA, USA) to cluster redundant sequences, reducing duplication [50]. The resulting assembly was evaluated using the Benchmarking Universal Single-Copy Orthologs (BUSCO Version 5.1.2, Geneva, Switzerland) tool in transcriptome mode using the endopterygota_odb10 database to measure the completeness of the transcriptome [51]. Functional annotation of the assembled transcriptome was performed using eggNOG-mapper (Version 2.1.9, Madrid, Spain) [52,53] using default parameters.

### 2.8. Identification of RNAi Machinery

NCBI entries for insect RNAi genes were used to create BLAST databases, which were queried using tBLASTx with the predicted adult *I. calligraphus* CDS as the subject (e-value $< 1 \times 10^{-5}$). Resulting matching sequences were used to perform a BLASTx to the NCBI non-redundant (nr) protein database to corroborate that the RNAi gene query was the best match (e-value $< 1 \times 10^{-5}$), then InterProScan was used to evaluate these final matches for PANTHER subfamily annotations.

## 3. Results

### 3.1. Gene Expression

To evaluate gene expression, data were normalized using the reference genes *16s rRNA* and *28s rRNA* [44]. The one-tailed t-test indicated no significant change in *hsp* gene expression 72 h after exposure to the target dsRNA when compared to the negative control ($p = 0.32$) (Figure 1a). The *iap* gene showed a significant 41% reduction in relative mRNA levels ($p = 0.04$) (Figure 1b), while dsSHI treated beetles showed a significant 84% increase in gene expression ($p = 0.04$) (Figure 1c). Significant changes in gene expression for the *iap* and *shi* genes correspond to dsRNAs that also induce significant mortality.

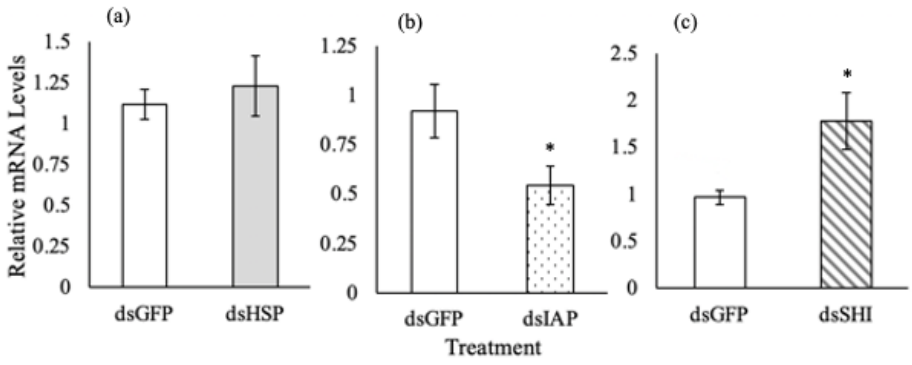


**Figure 1.** Relative mRNA levels of beetles treated with 10 µg target dsRNAs compared to the dsGFP negative control. (**a**) The *hsp* gene showed no changes in relative mRNA levels, (**b**) *iap* showed a significant decrease, and (**c**) *shi* showed a significant increase (one-tailed *t*-test). * Indicates significance.

### 3.2. Survival

There was a significant difference in the survival of adult beetles exposed to dsRNAs ($F_{3,82} = 9.61$, $p < 0.001$) (Figure 2). The post hoc Tukey's HSD test showed that survival of beetles treated with dsIAP ($p < 0.0001$) and dsSHI ($p < 0.01$) was lower than that of dsHSP- and dsGFP-treated beetles, and survival of dsHSP-treated beetles differed from beetles treated with dsIAP ($p < 0.01$) but not from the negative control (dsGFP). Temporal differences in survival became evident for dsIAP-treated beetles relative to control beetles beginning on day 4 and persisting through day 6. Differences in beetle survival for dsSHI treated beetles relative to dsGFP and dsHSP are evident on day 5 ($p < 0.01$).

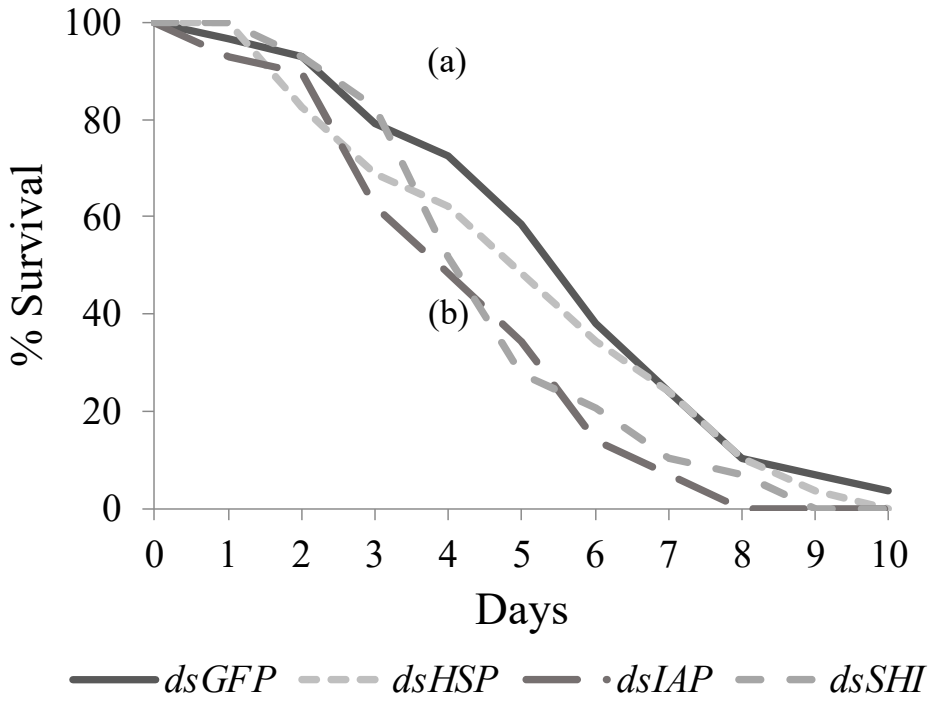


**Figure 2.** Beetle survival 10 days after exposure to 10 µg of target dsRNA (dsHSP, dsIAP, and dsSHI), and a negative control (dsGFP). The dsGFP treatment (**a**) differs significantly from dsIAP and dsSHI (**b**) but not from dsHSP.

### 3.3. Transcriptome Assembly and Identification of RNAi Pathway Homologs

NovaSeq6000 sequencing produced 430,208,758 raw reads, and after read correction and removal of low-quality reads, the 420,255,024 remaining high quality trimmed reads were used for the final assembly. The resulting BUSCO analysis identified 98.5% of complete orthologs from the endopterygota lineage (C: 98.5% (S: 83.7%, D: 14.8%), F: 0.5%, M: 1.0%, n: 2124). Only ten fragmented (0.5%) and 21 missing (1.0%) BUSCO orthologs were identified, indicating a highly complete and contiguous assembly. Data were deposited under a NCBI Bioproject (accession: PRJNA932788). Functional annotation using eggNOG-mapper assigned 19246 of 33224 unigenes (57.9%) to COG categories with known functions (Figure 3). Of these groups, the most represented were signal transduction (2381, 12.4%), post-translational modification, protein turnover, chaperone functions (2334, 12.1%), translation (1662, 8.6%), and transcription (1538, 8.0%).

Analyses for critical genes involved in the RNAi pathway present in the transcriptome resulted in identification of 10 homologs, including core RNAi machinery, such as *dicer*, *argonaute*, and *staufen*. All of the top BLASTx matches corresponded to other weevils, with 8 of the 10 genes sharing high sequence similarity to the scolytine *D. ponderosae* (Table 3). The importance of these genes in the RNAi pathway has been experimentally demonstrated in other insects [54,55].

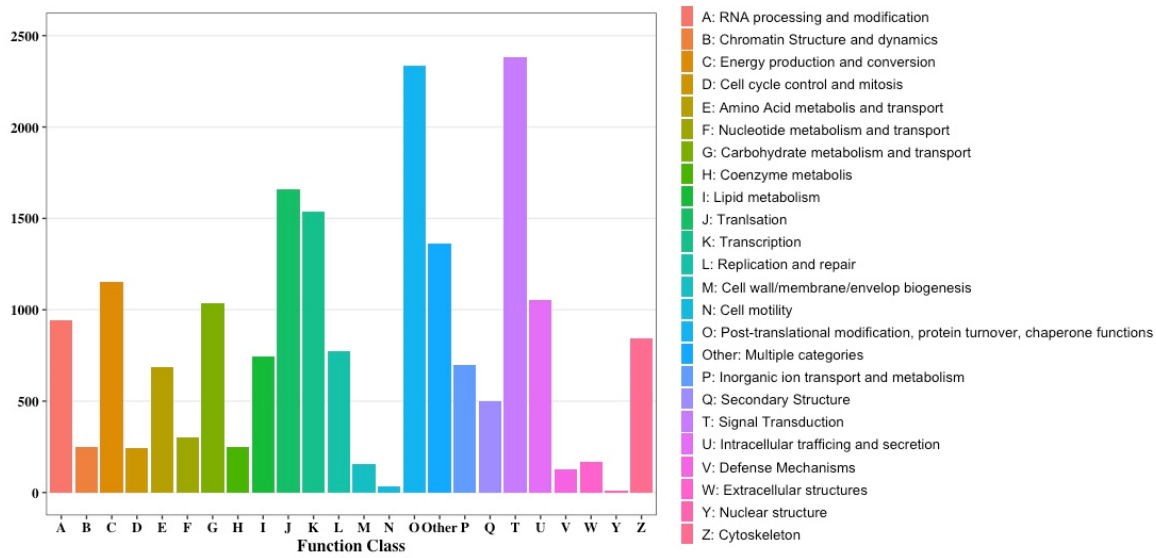


**Figure 3.** Histogram representing functional classification of adult *I. calligraphus* (Germar) unigenes into clusters of orthologous groups (COG).

**Table 3.** Overview of putative RNAi machinery sequences and their similarity (E-value, bit score, and percent identity) to the top hit sequence when used as query for a BLASTx search using NCBIs nr database and their PANTHER classification identified using InterProScan.

| RNAi-Related Gene | BLASTx Hit | Top Hit Accession | Comparison | PANTHER Classification | Accession |
|---|---|---|---|---|---|
| *Dicer-1* | Endoribonuclease Dcr-1 | XP_019765036.1 (*Dendroctonus ponderosae*) | E = 0.0; bits = 2863; %ID = 77.83% | Endoribonuclease dicer (PTHR14950: SF37) | OQ420293 |
| *Dicer-2* | Endoribonuclease Dicer isoform X2 | XP_048517572.1 (*Dendroctonus ponderosae*) | E = 0.0; bits = 2240; %ID = 69.03% | Dicer-2, isoform A (PTHR14950: SF36) | OQ420294 |
| *Ribonuclease 3* | Ribonuclease 3 | XP_030748887.1 (*Sitophilus oryzae*) | E = 0.0; bits = 1776; %ID = 83.43% | Ribonuclease 3 (PTHR11207: SF0) | OQ420295 |
| *Argonaute 2* | Protein argonaute-2 isoform X1 | XP_019754001.2 (*Dendroctonus ponderosae*) | E = 0.0; bits = 1796; %ID = 95.73% | Protein argonaute-2 (PTHR22891: SF59) | OQ420296 |
| *PIWI-like protein 1* | Piwi-like protein Siwi | XP_019768894.1 (*Dendroctonus ponderosae*) | E = 0.0; bits = 1073; %ID = 62.58% | PIWI-like protein 1 (PTHR22891: SF164) | OQ420297 |
| *PIWI-like protein 2* | Piwi-like protein Ago3 | XP_030752623.1 (*Sitophilus oryzae*) | E = 0.0; bits = 649; %ID = 70.34% | PIWI-like protein 2 (PTHR22891: SF111) | OQ420298 |
| *Staufen* | Double-stranded RNA-binding protein Staufen homolog 2 isoform X3 | XP_019760432.1 (*Dendroctonus ponderosae*) | E = 0.0; bits = 1126; %ID = 85.19% | Maternal effect protein staufen (PTHR46054: SF3) | OQ420299 |
| *SID-1* | SID1 transmembrane family member 1 isoform X2 | XP_019759106.1 (*Dendroctonus ponderosae*) | E = 0.0; bits = 1104; %ID = 76.21% | Cholesterol uptake associated (PTHR12185: SF14) | OQ420300 |
| *Loquacious* | Interferon-inducible dsRNA-dependent protein kinase activator A homolog isoform X2 | XP_048522468.1 (*Dendroctonus ponderosae*) | E = 0.0; bits = 598; %ID = 78.12% | Loquacious isoform B (PTHR46205: SF3) | OQ420301 |
| *Exportin-5* | Exportin-5 | XP_019754709.2 (*Dendroctonus ponderosae*) | E = 0.0; bits = 1885; %ID = 83.57% | Exportin-5 (PTHR11223: SF3) | OQ420302 |

## 4. Discussion

This is the first published study to demonstrate induction of RNAi-mediated gene silencing in an *Ips* species, a fundamental step in progressing toward novel technologies utilizing RNAi to suppress pest populations and additionally providing a key tool for reverse genetic studies. A 10 µg dose of dsIAP triggered changes in gene expression 72 h post-exposure, with a corresponding increase in beetle mortality. Given the key role *iap* plays in programmed cell death and the cell cycle, it has been found to be an effective target in a wide range of insect pests [56], including the bark beetle *D. ponderosae* [40]. Interestingly, while treatment with dsSHI causes gene silencing and subsequent mortality in *D. ponderosae* [40] and in *D. frontalis* [39], in *I. calligraphus* exposure to dsSHI resulted in a mortality rate similar to dsIAP but a significant increase in gene expression at 72 h, contrary to expectations. This increase may be attributable to factors regulating *shi* expression, the area of the gene targeted, the use of a high dose of dsRNA [57], or due to the role of *shi* in clathrin-dependent endocytosis [58], an important process in dsRNA uptake [59]. This clearly warrants further investigation, including a time series analysis to elucidate whether the effect is transient, and a result of the time point selected, or if other factors are at play. Additionally, a dose–response study evaluating gene expression for *I. calligraphus* could determine if the increase in expression is exacerbated by high dsRNA concentrations.

Integrating next generation approaches, such as RNAi, can prove difficult without foundational genomic information. Forest pests represent non-model organisms, and accessible genome and transcriptome datasets for these incredibly impactful insects are rare. Some, such as the congeneric Eurasian spruce bark beetle, *I. typographus* L. [60], or the equally devastating *D. ponderosae* [61], have draft genomes and a multitude of RNA-seq projects available, but most others have none. Prior to our work, minimal sequence data were publicly available for *I. calligraphus* with only a limited number of nucleotide sequences being present on NCBI, creating an obstacle to our efforts evaluating the RNAi pathway and associated molecular questions. Our study is the first to sequence the *I. calligraphus* transcriptome and only the second publicly available transcriptomic data of a North American *Ips* [62], and represents a highly complete assembly, validated by our BUSCO analysis. This high-quality genetic information is vital to more fully evaluating gene function in *I. calligraphus*, while also creating foundational information for a multitude of new avenues of investigation beyond the scope of our current work, including comparisons of *I. calligraphus* and other key bark beetle species.

Facilitated by this RNA-seq data, we investigated RNAi in *I. calligraphus in silico* by using homology to annotate key genes in the pathway. This in silico analysis uncovered complete coding regions with significant homology to important RNAi core genes, such as dicer and argonaute, as well as other genes involved in processes, such as dsRNA cellular uptake and transport, formation of the RISC complex, and cleavage of complementary target mRNA [63]. The identified sequences showed high similarity to the corresponding genes in other weevils with 8 of the 10 resulting in top BLAST hits from *D. ponderosae*, another Scolytine for which gene silencing has been experimentally induced using orally delivered dsRNA [40]. These canonical genes represent a subset of the many genes that can play a role in the RNAi pathway within insect cells. Further experimental work is needed to confirm the predicted function of each newly annotated sequence, as has been done for other insect species [54,64]; RNAi will be an invaluable tool in this validation and is now available for use in *I. calligraphus*. Expanding the analysis to include additional databases would identify RNAi pathway genes beyond those annotated in the present study.

The dsRNAs selected here were chosen to induce mortality in *I. calligraphus*, but screening additional genes could identify dsRNAs with greater insecticidal activity [65–67]. Additionally, RNAi technology is nimble. Genes could be targeted that disrupt aspects of beetle biology, such as mate or host finding behaviors, that compromise overall fitness; these genes are now more readily accessible due to the availability of our transcriptomic data. However, as a pest management strategy, RNAi technology must overcome additional barriers beyond identification of optimal target genes. Demonstration of specificity is

essential for risk assessment and is of international interest [68]. Before any RNAi-based product can be applied in the field, bioassays demonstrating the safety of these dsRNAs on non-target insects must be performed [31,32,68,69], and thus this will be an important next step in investigating the use of RNAi for management of *I. calligraphus*.

In addition to demonstrating specificity, practical delivery technology for forest systems is needed. Investigations into the behavior of dsRNA in plants have shown promising results for single tree protection in both deciduous [70,71] and coniferous seedlings [72], and success has been shown for soil drenches, foliar sprays, and trunk injections in citrus [73,74]. Scaling up application of dsRNA for forest protection poses additional challenges, but innovative techniques, such as those aiming to use microbial symbionts as a means for delivery [75,76] or developing transgenic trees that express pest-specific dsRNA [77], are examples of possible solutions to this barrier currently being investigated.

Forest pest outbreaks, coupled with catastrophic disturbance events and unprecedented abiotic extremes, are becoming increasingly devastating and threatening to forest ecosystem function. Traditional management techniques are proving inadequate and innovative approaches are greatly needed. While *I. calligraphus* is expanding its invaded ranges and threatening new ecosystems, it represents just one of a suite of *Ips* spp. that currently experience population outbreaks. The Eurasian *I. typographus* is devastating European forests, leading to the loss of millions of Norway spruce (*Picea abies* L.), nearly eliminating the species in some areas [78,79]. In the United States, *I. grandicollis* (Eichhoff) [12] and *I. avulsus* (Eichhoff) [22] cause tree mortality alongside *I. calligraphus* in the southeast, while *I. confusus* (LeConte) has experienced destructive regional epidemics in piñon pine (*P. edulis* Engelm.) in the west [80]. Our findings will facilitate future research into RNAi as a pest management strategy for *Ips* and other bark beetles. The alarming increases in tree mortality in conifer forests necessitates investigation into novel tools like RNAi, and the nimble nature of RNAi technology could prove advantageous in an IPM program. This research represents foundational knowledge needed to move forward evaluating *I. calligraphus* as a candidate for this tool.

## 5. Conclusions

This study demonstrated the use of ingested exogenous dsRNA to induce changes in gene expression and increased mortality in *I. calligraphus*. The ability to trigger this pathway artificially in an *Ips* species can serve as the basis for future work developing RNAi as a pest management tool as this technology continues to be developed in forest systems.

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