Journal of Basic Microbiology

Journal of Basic Microbiology

RESEARCH PAPER OPEN ACCESS

Small Interfering RNA Mediated Messenger RNA Knockdown in the Amphibian Pathogen *Batrachochytrium dendrobatidis*

Rebecca J. Webb[1,2] | Alexandra A. Roberts[2] | Catherine Rush[2] | Lee F. Skerratt[1] | Mark L. Tizard[3] | Lee Berger[1]

[1]One Health Research Group, Melbourne Veterinary School, University of Melbourne, Werribee, Victoria, Australia | [2]Australian Institute of Tropical Health and Medicine, James Cook Univiersity, Townsville, Queensland, Australia | [3]Australian Centre for Disease Preparedness, CSIRO Health and Biosecurity, Geelong, Victoria, Australia

Correspondence: Rebecca J. Webb (Rebecca.webb@unimelb.edu.au)

Received: 14 February 2024 | **Revised:** 3 May 2024 | **Accepted:** 19 May 2024

Funding: Australian Research Council, Grant/Award Numbers: DP220101361, FT190100462; Ecological Society of Australia; Holsworth Wildlife Research Endowment

Keywords: *Batrachochytrium dendrobatidis* | glutathione | RNAi | siRNA

ABSTRACT

RNA interference (RNAi) has not been tested in the pandemic amphibian pathogen, *Batrachochytrium dendrobatidis*, but developing this technology could be useful to elucidate virulence mechanisms, identify therapeutic targets, and may present a novel antifungal treatment option for chytridiomycosis. To manipulate and decipher gene function, rationally designed small interfering RNA (siRNA) can initiate the destruction of homologous messenger RNA (mRNA), resulting in the "knockdown" of target gene expression. Here, we investigate whether siRNA can be used to manipulate gene expression in *B. dendrobatidis* via RNAi using differing siRNA strategies to target genes involved in glutathione and ornithine synthesis. To determine the extent and duration of mRNA knockdown, target mRNA levels were monitored for 24–48 h after delivery of siRNA targeting glutamate–cysteine ligase, with a maximum of ~56% reduction in target transcripts occurring at 36 h. A second siRNA design targeting glutamate-cysteine ligase also resulted in ~53% knockdown at this time point. siRNA directed toward a different gene target, ornithine decarboxylase, achieved 17% reduction in target transcripts. Although no phenotypic effects were observed, these results suggest that RNAi is possible in *B. dendrobatidis*, and that gene expression can be manipulated in this pathogen. We outline ideas for further optimization steps to increase knockdown efficiency to better harness RNAi techniques for control of *B. dendrobatidis*.

1 | Introduction

RNA interference (RNAi) is a powerful gene silencing tool, harnessing a widespread natural phenomenon that regulates both host and pathogen gene expression [1]. This pathway has been exploited for a variety of applications, from characterizing gene function [2–5] to targeted pest [6, 7] and disease [8, 9] control. Gene silencing via RNAi has proved a particularly useful treatment to suppress the virulence of fungal pathogens such as *Talaromyces marneffei* in mice, and *Fusarium graminearum*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea* in plants leading to increased host survival [10–12]. RNAi has not

Abbreviations: cDNA, complementary DNA; dsRNA, double stranded RNA; GCL, glutamate cysteine ligase; mRNA, messenger RNA; ODC, ornithine decarboxylase; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time reverse-transcription PCR; RdRp, RNA-dependent RNA polymerase; RNA, ribonucleic acid; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; TGhL, tryptone, gelatin hydrolysate and lactose media.

yet been developed for mitigating diseases threatening wildlife, but its specificity and lack of off-target effects are advantages for environmental applications. Hence there is high potential to adapt this technology for understanding and controlling important wildlife dieases such as amphibian chytridiomycosis.

The RNAi process is initiated by the introduction of double-stranded RNA (dsRNA) sequences into the cytoplasm, resulting in transient reduction or "knockdown" of corresponding messenger RNA (mRNA). The presence of exogenous dsRNA in the cytoplasm triggers the enzyme Dicer to cleave the dsRNA into smaller fragments, termed "small interfering RNA" (siRNA) [13, 14], which are loaded onto an Argonaute protein [15], forming an RNA-induced silencing complex (RISC) [16]. The siRNA then guides RISC to complementary mRNA, which are then cleaved by Argonaute [17], preventing translation [13]. RNA-dependent RNA polymerase (RdRp) creates additional siRNA from the cleaved mRNA, amplifying the RNAi process [18]. The RNAi pathway can be manipulated to knockdown target genes by introducing either dsRNA or siRNA that are complementary to a gene of interest.

The RNAi pathway is widespread in fungi. However, RNAi has not yet been observed in the fungal phylum Chytridiomycota [19, 20]. This phylum contains the devastating amphibian pathogen, *Batrachochytrium dendrobatidis*, an introduced species responsible for the extinction of at least 90 amphibian species worldwide [21]. The amphibian chytrid fungus infects the host epidermis, impairing epidermal function leading to electrolyte imbalance [22]. The resulting disease, chytridiomycosis, often progresses to host cardiac failure and death [23]. Efforts to understand the virulence of *B. dendrobatidis* have included comparative genomics [24, 25] and transcriptomics [26, 27], but without genetic modification protocols [28] it has been difficult to confirm putative virulence factors. Developing a RNAi reverse genetics approach would provide a valuable tool to study gene function in this ecologically important pathogen. The genome of *B. dendrobatidis* was thought to lack one of the core RNAi components, RdRp [29, 30], however, RdRp was recently reported by one study [31]. Therefore, it is still unclear whether the RNAi pathway is functional in this species. Establishing if RNAi-mediated knockdown can be manipulated in *B. dendrobatidis* will facilitate functional genomic studies and may lead to novel antifungal strategies.

Here, we explore whether siRNA can induce gene silencing in *B. dendrobatidis* as evidence of a functional RNAi pathway. Initially, we designed siRNA to target the first enzyme required for the biosynthesis of glutathione; glutamate cysteine ligase (GCL). A time course experiment determined the extent and duration of mRNA knockdown via qRT-PCR. There are many algorithms and modifications available for siRNA design, therefore, to confirm consistency of the knockdown results, mRNA quantification experiments were repeated using a second GCL siRNA molecule. The efficacy of siRNA-mediated knockdown was also assessed against a second gene target, ornithine decarboxylase (*ODC*). This approach represents a promising step towards developing RNAi techniques to understand and reduce the virulence of this devastating wildlife pathogen.

2 | Materials and Methods

2.1 | Culturing *B. dendrobatidis*

Isolates of *B. dendrobatidis* were collected from naturally infected Australian frogs [32, 33], and maintained in a tryptone, gelatin hydrolysate, and lactose media (TGhL) at 20°C in tissue culture flasks, as per standard protocols [34]. The isolates used were Yanchep-L.moorei-2019-RW, Frenchmanscreek_torrrenttadpole-2020-RW and NarielValley-L.spenceri-2020-LB. Pure zoospore suspensions were obtained by removing the TGhL from mature culture flasks and incubating the zoosporangia monolayer with 50% TGhL for 2 h, after which the solutions were filtered with a sterile isopore PC 10 μM filter (Millipore). Zoospores were concentrated by centrifugation (2500*g* for 5 min), washed once with sterile SM buffer (5 mM KCl, 15 mM sodium phosphate buffer [pH 7.2], 15 mM $MgCl_2$, 25 mM sodium succinate dibasic hexahydrate, 25 mM D-mannitol) [35], centrifuged at 2500*g* for 5 min and resuspended in SM buffer to a final concentration of 5×10^6 zoospores/mL.

2.2 | Gene Targets

Initial experiments were designed to knockdown expression of glutamate cysteine ligase (GCL, BATDEDRAFT_35498), the enzyme that catalyses the first, rate-limiting step in glutathione biosynthesis. Glutathione is important for growth and cadmium tolerance in *B. dendrobatidis* [36]. Additional experiments were designed to knockdown ornithine decarboxylase (ODC, BATDEDRAFT_35584), the enzyme responsible for spermidine synthesis. Spermidine is important for *B. dendrobatidis* growth and is a likely virulence factor that can suppress amphibian lymphocytes [37].

2.3 | siRNA Design

GCL expression was targeted using two siRNA strategies. The first strategy, "siRNA #1" was a 19 bp construct designed using the siDesign Center (Dharmacon), with a 3′-UU sequence overhang. The second strategy, "siRNA #2" was a pool of three different 19 bp siRNAs designed by the Rosetta algorithm (Sigma), all of which contained 3′-dTdT overhangs. The siRNA was reconstituted to 100 μM using Horizon siRNA buffer (siRNA #1) or water (siRNA #2). Each siRNA had a corresponding negative control siRNA with no homology to *B. dendrobatidis* genes. For siRNA #1, a 19 bp scrambled sequence with UU sequence overhangs was used as a negative control, and the proprietary universal negative control #1 (Sigma) was used for siRNA #2 experiments. Ornithine decarboxylase was targeted with siRNA #3, a pool of three different 19 bp siRNAs designed by the Rosetta algorithm (Sigma), all of which contained 3′-dTdT overhangs. As per siRNA #2, the proprietary universal negative control #1 (Sigma) was used as the control. The siRNA sequences are listed in Supporting Information S1: Table S1.

2.4 | Electroporation Optimization

A series of single-replicate experiments were conducted to establish the optimal electroporation voltage and siRNA concentration for efficient siRNA delivery into *B. dendrobatidis*. Zoospores were electroporated in the presence of siRNA #1 (with a FITC modification on the 5′ sense strand terminus) using protocols adapted from Swafford et al. [35]. One million zoospores in 200 μL SM media were transferred to a 2 mm cuvette (BioRad) with siRNA ranging from 0.5 to 12.5 μM. The cuvettes were chilled on ice, electroporated with 2 × 3 ms square wave pulses ranging from 750 to 1500 V. Immediately following electroporation, cuvettes were incubated on ice for 10 min, after which 200 μL ice cold TGhL was gently added before a final 10 min incubation on ice to allow the zoospores to recover. Cells were fixed in a PFA buffer, then analyzed by flow cytometry using a BD LSR Fortessa 3 Laser cytometer in conjunction with FlowJo analysis software. Experiments included controls without siRNA and/or without electroporation to assess cell survival and background staining, as well as a dextran positive control to allow comparison to a previously optimized delivery method in *B. dendrobatidis* [35].

2.5 | siRNA Delivery

One million zoospores in 200 μL SM buffer were in chilled in a 2 mm cuvette and then electroporated at 1000 V with either 3 μM siRNA (siRNA #1 + siRNA #2) or 7.5 μM siRNA (siRNA #3). After 10 min recovery on ice, 200 μL ice-cold TGhL broth was added to each cuvette and the zoospores gently pipetted to a 24-well culture plate (Nunc). The plate was sealed with parafilm and incubated at 20°C until mRNA quantification.

2.6 | Relative mRNA Quantification

Initial time course experiments were conducted with siRNA #1 over a 24–48 h period to identify if mRNA knockdown occurred. Subsequent experiments tested siRNA #2 and siRNA #3 at the peak knockdown timepoint determined for siRNA #1. After the appropriate incubation, *B. dendrobatidis* zoosporangia were carefully detached from the well surface using a cell scraper and total RNA extracted using the Quick RNA Micro extraction kit (Zymo). The cells were pelleted, treated with 90 μL of RNA/DNA shield (Zymo), and subjected to 1 min of bead beating with 0.05 g of 0.1 mm and 0.5 mm silica beads (Daintree Scientific) to disrupt the cell walls. RNA was extracted from the homogenized solution following manufacturer's instructions, including DNase treatment. RNA concentration and purity was measured using a Nanodrop spectrophotometer (Thermo Scientific), and complementary DNA (cDNA) synthesis was performed using the QuantiTect reverse transcription kit (Qiagen). qRT-PCR was performed in a 20 μL reaction containing 1 ng cDNA template, 1× Rotor-Gene SYBR green master mix, and 1 μM of each forward and reverse primer. The reactions were run in triplicate on a Rotorgene 6000 (Qiagen) with an initial activation step of 95°C for 5 min, followed by 35 cycles of a 5 s 95°C denaturation step and a 10 s 60°C annealing/extension step. Relative target mRNA was calculated by normalizing the C_T value to the reference genes *α-centractin* [29], and *APRT* [38] using the Pfaffl method. Relative quantity (RQ) was calculated using the equation $RQ = E^{\Delta CT}$, and the geometric mean of the two reference genes was used to determine the fold change in target mRNA in the treatment compared to the control [39] for each individual experiment. For each time point, a *T* test was used to determine if the fold change of the target siRNA-treated cells was significantly different to that of the control siRNA (GraphPad Prism).

To confirm the qRT-PCR results for siRNA #1 and siRNA #2 experiments, a subsample of cDNA was retested using semi-quantitative agarose gel PCR using a different primer set (Table S1) and *α-centractin* for normalization. The PCR was performed as a 25 μL reaction containing 15 ng of template, 2.5 μL 10× ThermoPol Reaction Buffer, 10 mM dNTP's, 0.625 U Taq polymerase (NEB), with 0.2 μM of each forward and reverse primer. The reactions were performed on a Bio-Rad S1000 thermocycler with an initial activation step of 95°C for 30 s, followed by cycles of 30 s 95°C denaturation, 30 s 55°C annealing, and 40 s 68°C extension. With these PCR conditions, 28 cycles were optimal for *α centractin* amplification and 35 cycles for *GCL*. The PCR product (10 μL) was run on a 1.2% agarose gel with gel red (Biotium) and visualized under UV light.

3 | Results

3.1 | siRNA Delivery

Both voltage and siRNA concentration affected overall siRNA delivery success (Figure 1). Higher voltages delivered siRNA to more cells, but also resulted in increased cell death (Supporting Information S1: Figure S1). Higher siRNA concentrations slightly increased siRNA delivery efficacy, but the increased siRNA usage is financially costly. Therefore, to maximize siRNA delivery in an economical manner, 3 μM siRNA and 1000 V were chosen for the remaining experiments.

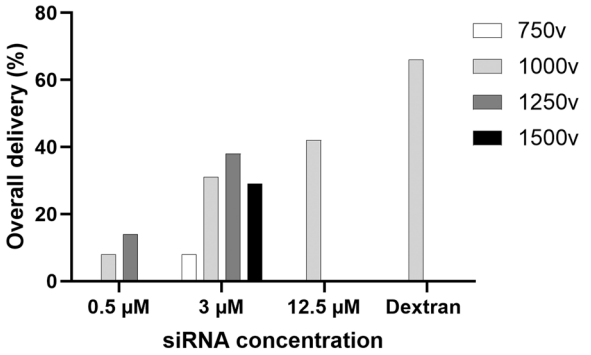

FIGURE 1 | Comparing overall success of siRNA delivery into *Batrachochytrium dendrobatidis* zoospores using various voltages and siRNA concentrations. FITC-tagged siRNA was used to track siRNA delivery via flow cytometry. Each bar represents success (overall delivery %), which was calculated as zoospore survival multiplied by zoospore fluorescence. Higher voltage and siRNA concentration increased siRNA delivery per cell, but higher voltages decreased zoospore survival resulting in overall lower delivery. Fluorescent dextran (1 mg/mL) was included as a positive control. Note that not all voltages were tested per siRNA concentration.

3.2 | mRNA Knockdown

qRT-PCR analysis indicated that zoospores electroporated with siRNA #1 developed into zoosporangia displaying a significant reduction in GCL expression from 36 h postelectroporation and persisting until at least 48 h (Figure 2). Maximum knockdown occurred at 36 h post electroporation, with an average of 56% less GCL mRNA in cells treated with siRNA #1 compared to scrambled siRNA.

siRNA #2 was a pool of three different siRNA molecules targeting GCL, each with dTdT overhangs. This siRNA also resulted in significant reduction of mRNA levels at 36 and 42 h compared to negative control siRNA. Overall, there was a 36%–51% decrease in GCL mRNA at these time points (Figure 3).

To confirm the GCL qRT-PCR results, a subset of cDNA was also analyzed via semiquantitative PCR and gel electrophoresis using a different set of *GCL* primers. The GCL band was visibly less intense in the target siRNA-treated samples compared to the controls (Figure 4) in agreement with the qRT-PCR results.

To confirm that siRNA can act against a different gene target, we tested 7.5 μM siRNA designed to target ODC (siRNA #3). Cells treated with siRNA #3 had a slight but significant reduction in mRNA ($p = 0.0353$), with a 17% decrease in ODC mRNA at 39 h.

No phenotypic changes associated with mRNA knockdown were observed for either GCL or ODC knockdown.

4 | Discussion

These results suggest that siRNA could be used to manipulate gene expression in *B. dendrobatidis* and provide evidence of a functional RNAi pathway in this species. We have performed preliminary optimization of siRNA delivery methods by comparing various siRNA concentrations and electroporation voltages. Consistent

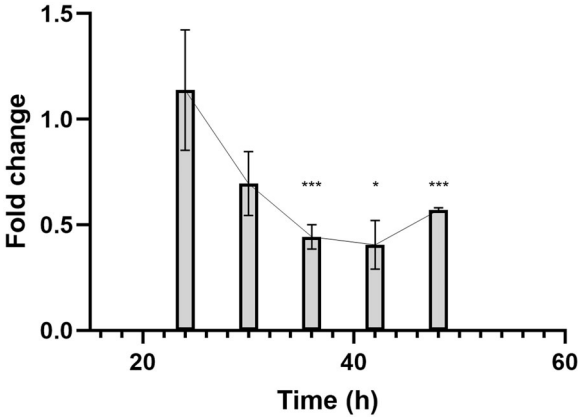

FIGURE 2 | Time course of fold change in GCL mRNA levels in *Batrachochytrium dendrobatidis* cells treated with siRNA #1 compared to control scrambled siRNA, normalized to both α centractin and APRT. A significant reduction in GCL mRNA was detected in zoosporangia previously treated with siRNA #1 at 36 ($p = 0.0007$), 42 ($p = 0.0354$) and 48 h ($p = 0.0005$) post-siRNA delivery. Bars represent the average of two to five experimental replicates with SEM error bars.

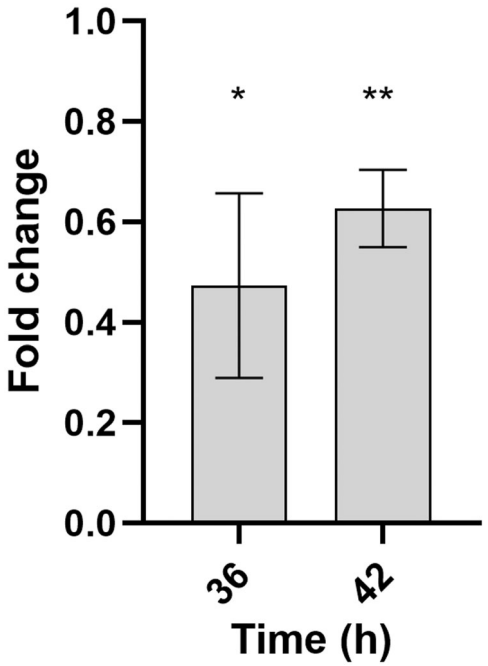

FIGURE 3 | Mean fold change in GCL mRNA levels in *Batrachochytrium dendrobatidis* cells treated with siRNA #2 compared to control siRNA, normalized to α centractin and APRT. A significant reduction in GCL mRNA was detected in zoosporangia previously treated with siRNA #2 at both 36 h ($p = 0.045$) and 42 h ($p = 0.008$). Data from three experimental replicates showing SEM error bars.

mRNA knockdown was observed across different siRNA designs and validated with two PCR techniques and two gene targets involved in glutathione and ornithine synthesis. However, the extent and duration of knockdown was insufficient to produce an observable phenotypic response such as reduced growth.

Peak mRNA knockdown occurred at 36–42 h, and persisted for at least 48 h. This onset of mRNA knockdown is late compared to other fungal systems, where knockdown can occur as early as 18 h [40]. However, as the ΔC_T method relies on stable reference gene expression, failure to detect mRNA knockdown via PCR at early timepoints may be due to the dynamic nature of gene expression at this stage in the *B. dendrobatidis* life cycle due to the large metabolic shifts associated with encystation [26]. By 48 h, mRNA knockdown appears to wane, although extended time course studies are required to confirm the precise duration of effect. While the heterogeneous effect of cell cycle was moderated in our experiments by synchronization of zoospores, longer time course studies are complicated in *B. dendrobatidis* due to the cyclical release of zoospores from mature zoosporangia. Overall, the duration of RNAi activity in *B. dendrobatidis* appears relatively short compared to effects in other fungi, such as *S. sclerotiorum*, where knockdown persisted for at least 96 h [12]. There are conflicting reports whether *B. dendrobatidis* possess the RdRp gene [29–31]. If *B. dendrobatidis* indeed lacks RdRp may explain this subdued RNAi response, as this key RNAi component amplifies and sustains mRNA knockdown [18].

Traditionally, effective knockdown is defined by at least a 70% decrease in mRNA [41]. We observed no phenotypic effects resulting from 50% GCL mRNA knockdown. Increasing

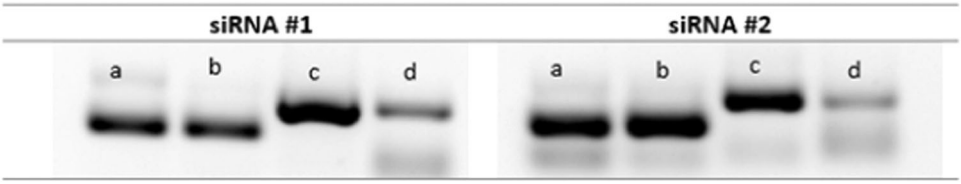

FIGURE 4 | Semiquantitative PCR of GCL mRNA at 36 h in *Batrachochytrium dendrobatidis* cells treated with either siRNA #1 or siRNA #2 compared to control siRNA, with α centractin expression serving as a reference gene. GCL expression is reduced in zoosporangia previously treated with ether GCL targeting siRNA (siRNA #1 and siRNA #2), whereas reference gene expression remains stable. Lane a = reference gene (α centractin) expression in control siRNA treated cells; b = reference gene (α centractin) expression in GCL siRNA treated cells; c = target gene (GCL) expression in control siRNA treated cells; d = target gene (GCL) expression in GCL siRNA treated cells.

knockdown efficiency is essential for the further development of RNAi techniques in *B. dendrobatidis*, which is especially important given the apparent brief duration of knockdown in this species. Increased optimization of electroporation protocols for siRNA delivery is a logical first step to increase mRNA knockdown. We found voltage to be positively correlated with uptake of siRNA, but inversely correlated with cell survival necessitating a compromise to achieve the highest transfection efficiency [42]. Waveform (exponential decay or square), duration and capacitance, and buffer constituents [43, 44], could all be further optimized for increased siRNA delivery *to B. dendrobatidis*.

Another variable for consideration is the siRNA design. Although there is vast literature on the rational design of siRNA, a comparison of different designs has not been explicitly tested in fungi. Synthetic siRNA duplexes can be designed to include two extra nucleotide overhangs to increase efficiency [45], and several types of overhangs have been shown to initiate RNAi in fungi, including dTdT [40, 46, 47] UU [47] and asymmetrical dsiRNA overhangs [48]. We found similar results targeting GCL mRNA using siRNA with UU and dTdT overhangs, suggesting that either approach may be acceptable for use in *B. dendrobatidis*. Further optimization studies could incorporate other designs to extend the half-life of the siRNA molecules, including phosphothioate or locked nucleic acid modifications [49], or diced siRNA pools [48]. Even with optimized siRNA delivery and longevity, knockdown is expected to be inherently transient due to dilution of the finite siRNA as the zoosporangia divide into zoospores. Development of RNAi constructs to produce interfering RNA (e.g., shRNA) could provide a solution to extending knockdown. These could be transformed into *B. dendrobatidis* to produce knock-down strains, or into host cells as a form of host-induced gene silencing [50].

Some genes are inherently difficult to target with siRNA, whether it is because of the low abundance of transcripts [51], or other unknown gene features [41]. Furthermore, knockdown of certain genes does not necessarily produce a phenotypic response, despite successful reduction in transcripts [52–54]. We achieved moderate mRNA knockdown of GCL, but only slight knockdown targeting ODC despite increasing the siRNA concentration. Further testing of additional gene targets is required, ideally those with low redundancy, short protein half-life, and obvious phenotypes. Testing the effect of other forms of interfering RNAs (such as dsRNA) is also warranted.

Although moderate, the reduction in mRNA we detected via PCR suggests that siRNA can be delivered and recognized by *B. dendrobatidis*. Our results are a promising first step in the development of RNAi as a tool to manipulate gene expression in *B. dendrobatidis,* providing a platform for future studies to harness RNAi for understanding and controlling fungal virulence in this threat to biodiversity.

Author Contributions

Rebecca J. Webb: conceptualization, formal analysis, funding acquisition, methodology, writing–original draft. **Alexandra A. Roberts:** conceptualization, funding acquisition, methodology, writing–review and editing. **Catherine Rush:** resources, supervision. **Lee F. Skerratt:** formal analysis, funding acquisition, supervision. **Mark L. Tizard:** funding acquisition, methodology. **Lee Berger:** funding acquisition, supervision, writing–review and editing.

Acknowledgments

This study was funded by Australian Research Council grants DP220101361 and FT190100462, and the Ecological Society of Australia; Holsworth Wildlife Research Endowment. We greatly appreciate the assistance and advice from Karl-Heinz Kogel at Justus Liebig University Giessen, and Andrew Swafford and Lillian Fritz-Laylin at UMass Amherst. We are grateful for the support from staff and students at JCU and UoM. Open access publishing facilitated by The University of Melbourne, as part of the Wiley - The University of Melbourne agreement via the Council of Australian University Librarians.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. G. J. Hannon, "RNA Interference," *Nature* 418 (2002): 244–251.

2. F. S. Barreto, S. D. Schoville, and R. S. Burton, "Reverse Genetics in the Tide Pool: Knock-Down of Target Gene Expression Via RNA Interference in the Copepod *Tigriopus californicus*," *Molecular Ecology Resources* 15 (2015): 868–879.

3. R. S. Kamath, A. G. Fraser, Y. Dong, et al., "Systematic Functional Analysis of the *Caenorhabditis elegans* genome using RNAi," *Nature* 421 (2003): 231–237.

4. D. Prawitt, L. Brixel, C. Spangenberg, et al., "RNAi Knock-Down Mice: An Emerging Technology for Post-Genomic Functional Genetics," *Cytogenetic and Genome Research* 105 (2004): 412–421.

5. S. Travella, T. E. Klimm, and B. Keller, "RNA Interference-Based Gene Silencing as an Efficient Tool for Functional Genomics in Hexaploid Bread Wheat," *Plant Physiology* 142 (2006): 6–20.

6. K. Tariq, A. Ali, T. G. E. Davies, et al., "RNA Interference-Mediated Knockdown of Voltage-Gated Sodium Channel (MpNav) Gene Causes Mortality in Peach-Potato Aphid, *Myzus persicae*," *Scientific Reports* 9 (2019): 5291.

7. J. A. Baum, T. Bogaert, W. Clinton, et al., "Control of Coleopteran Insect Pests Through RNA Interference," *Nature Biotechnology* 25 (2007): 1322–1326.

8. P. C. Lima, J. O. Harris, and M. Cook, "Exploring RNAi as a Therapeutic Strategy for Controlling Disease in Aquaculture," *Fish & Shellfish Immunology* 34 (2013): 729–743.

9. W. Hunter, J. Ellis, D. van Engelsdorp, et al., "Large-Scale Field Application of RNAi Technology Reducing Israeli Acute Paralysis Virus Disease in Honey Bees (*Apis mellifera*, Hymenoptera: Apidae)," *PLoS Pathogens* 6 (2010): e1001160.

10. J. Sun, X. Li, P. Feng, et al., "RNAi-Mediated Silencing of Fungal acuD Gene Attenuates the Virulence of *Penicillium marneffei*," *Medical Mycology* 52 (2014): 167–178.

11. A. Koch, D. Biedenkopf, A. Furch, et al., "An RNAi-Based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs Involves a Plant Passage and is Controlled by the Fungal Silencing Machinery," *PLoS Pathogens* 12 (2016): e1005901.

12. A. G. McLoughlin, N. Wytinck, P. L. Walker, et al., "Identification and Application of Exogenous dsRNA Confers Plant Protection Against *Sclerotinia sclerotiorum* and *Botrytis cinerea*," *Scientific Reports* 8 (2018): 7320.

13. P. D. Zamore, T. Tuschl, P. A. Sharp, and D. P. Bartel, "RNAi," *Cell* 101 (2000): 25–33.

14. E. Bernstein, A. A. Caudy, S. M. Hammond, and G. J. Hannon, "Role for a Bidentate Ribonuclease in the Initiation Step of RNA Interference," *Nature* 409 (2001): 363–366.

15. S. M. Hammond, S. Boettcher, A. A. Caudy, R. Kobayashi, and G. J. Hannon, "Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi," *Science* 293 (2001): 1146–1150.

16. S. M. Hammond, E. Bernstein, D. Beach, and G. J. Hannon, "An RNA-Directed Nuclease Mediates Post-Transcriptional Gene Silencing in *Drosophila* Cells," *Nature* 404 (2000): 293–296.

17. J. Liu, M. A. Carmell, F. V. Rivas, et al., "Argonaute2 is the Catalytic Engine of Mammalian RNAi," *Science* 305 (2004): 1437–1441.

18. K. Nishikura, "A Short Primer on RNAi: RNA Directed RNA Polymerase Acts as a Key Catalyst," *Cell* 107 (2001): 415–418.

19. M. M. Moore, "Genetic Engineering of Fungal Cells," *Biotechnology, Fundamentals in Biotechnology* 3 (2009): 36–66.

20. T. C. Cairns, D. J. Studholme, N. J. Talbot, and K. Haynes, "New and Improved Techniques for the Study of Pathogenic Fungi," *Trends in Microbiology* 24 (2016): 35–50.

21. B. C. Scheele, F. Pasmans, L. F. Skerratt, et al., "Amphibian Fungal Panzootic Causes Catastrophic and Ongoing Loss of Biodiversity," *Science* 363 (2019): 1459–1463.

22. J. Voyles, L. Berger, S. Young, et al., "Electrolyte Depletion and Osmotic Imbalance in Amphibians With Chytridiomycosis," *Diseases of Aquatic Organisms* 77 (2007): 113–118.

23. J. Voyles, S. Young, L. Berger, et al., "Pathogenesis of Chytridiomycosis, a Cause of Catastrophic Amphibian Declines," *Science* 326 (2009): 582–585.

24. S. Joneson, J. E. Stajich, S. H. Shiu, and E. B. Rosenblum, "Genomic Transition to Pathogenicity in Chytrid Fungi," *PLoS Pathogens* 7 (2011): e1002338.

25. M. S. Greener, E. Verbrugghe, M. Kelly, et al., "Presence of Low Virulence Chytrid Fungi Could Protect European Amphibians from More Deadly Strains," *Nature Communications* 11 (2020): 5393.

26. E. B. Rosenblum, J. E. Stajich, N. Maddox, and M. B. Eisen, "Global Gene Expression Profiles for Life Stages of the Deadly Amphibian Pathogen *Batrachochytrium dendrobatidis*," *Proceedings of the National Academy of Sciences of the United States of America* 105 (2008): 17034–17039.

27. E. B. Rosenblum, T. J. Poorten, S. Joneson, and M. Settles, "Substrate-Specific Gene Expression in *Batrachochytrium dendrobatidis*, The Chytrid Pathogen of Amphibians," *PLoS One* 7 (2012): e49924.

28. E. B. Rosenblum, M. Fisher, T. James, et al., "A Molecular Perspective: Biology of the Emerging Pathogen *Batrachochytrium dendrobatidis*," *Diseases of Aquatic Organisms* 92 (2010): 131–147.

29. R. A. Farrer, A. Martel, E. Verbrugghe, et al., "Genomic Innovations Linked to Infection Strategies Across Emerging Pathogenic Chytrid Fungi," *Nature Communications* 8 (2017): 14742.

30. T. Wacker, N. Helmstetter, D. Wilson, M. C. Fisher, D. J. Studholme, and R. A. Farrer, "Two-Speed Genome Evolution Drives Pathogenicity in Fungal Pathogens of Animals," *Proceedings of the National Academy of Sciences of the United States of America* 120 (2023): e2212633120.

31. M. Torres-Sánchez, J. Villate, S. McGrath-Blaser, and A. V. Longo, "Panzootic Chytrid Fungus Exploits Diverse Amphibian Host Environments Through Plastic Infection Strategies," *Molecular Ecology* 31 (2022): 4558–4570.

32. A. Waddle, M. Sai, J. E. Levy, G. Rezaei, F. van Breukelen, and J. R. Jaeger, "Systematic Approach to Isolating *Batrachochytrium dendrobatidis*," *Diseases of Aquatic Organisms* 127 (2018): 243–247.

33. M. C. Fisher, P. Ghosh, J. M. G. Shelton, et al., "Development and Worldwide Use of Non-Lethal, and Minimal Population-Level Impact, Protocols for the Isolation of Amphibian Chytrid Fungi," *Scientific Reports* 8 (2018): 7772.

34. S. M. Prostak and L. K. Fritz-Laylin, "Laboratory Maintenance of the Chytrid Fungus *Batrachochytrium dendrobatidis*," *Current Protocols* 1 (2021): e309.

35. A. J. M. Swafford, S. P. Hussey, and L. K. Fritz-Laylin, "High-Efficiency Electroporation of Chytrid Fungi," *Scientific Reports* 10 (2020): 15145.

36. R. J. Webb, C. Rush, L. Berger, L. F. Skerratt, and A. A. Roberts, "Glutathione is Required for Growth and Cadmium Tolerance in the Amphibian Chytrid Fungus, *Batrachochytrium dendrobatidis*," *Biochimie* 220 (2024): 22–30.

37. L. A. Rollins-Smith, A. C. Ruzzini, J. S. Fites, et al., "Metabolites Involved in Immune Evasion by *Batrachochytrium dendrobatidis* Include the Polyamine Spermidine," *Infection and Immunity* 87 (2019): PMC6479046.

38. E. Verbrugghe, F. Pasmans, and A. Martel, "Reference Gene Screening of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* for Quantitative Real-Time PCR Studies," *Scientific Reports* 9 (2019): 18534.

39. J. Vandesompele, K. de Preter, F. Pattyn, et al., "Accurate Normalization of Real-Time Quantitative RT-PCR Data by Geometric Averaging of Multiple Internal Control Genes," *Genome Biology* 3 (2002): RESEARCH0034.

40. M. Khatri and M. V. Rajam, "Targeting Polyamines of *Aspergillus nidulans* by siRNA Specific to Fungal Ornithine Decarboxylase Gene," *Medical Mycology* 45 (2007): 211–220.

41. U. Krueger, T. Bergauer, B. Kaufmann, et al., "Insights Into Effective RNAi Gained from Large-Scale siRNA Validation Screening," *Oligonucleotides* 17 (2007): 237–250.

42. D. Li, Y. Tang, J. Lin, and W. Cai, "Methods for Genetic Transformation of Filamentous Fungi," *Microbial Cell Factories* 16 (2017): 168.

43. E. T. Jordan, M. Collins, J. Terefe, L. Ugozzoli, and T. Rubio, "Optimizing Electroporation Conditions in Primary and Other

Difficult-to-Transfect Cells," *Journal of Biomolecular Techniques* 19 (2008): 328–334.

44. J. J. Sherba, S. Hogquist, H. Lin, J. W. Shan, D. I. Shreiber, and J. D. Zahn, "The Effects of Electroporation Buffer Composition on Cell Viability and Electro-Transfection Efficiency," *Scientific Reports* 10 (2020): 3053.

45. S. M. Elbashir, W. Lendeckel, and T. Tuschl, "RNA Interference is Mediated by 21- and 22-Nucleotide RNAs," *Genes & Development* 15 (2001): 188–200.

46. P. Y. Chum, G. Schmidt, M. Saloheimo, and C. P. Landowski, "Transient Silencing of DNA Repair Genes Improves Targeted Gene Integration in the Filamentous Fungus *Trichoderma reesei*," *Applied and Environmental Microbiology* 83 (2017): e00535–17.

47. S. S. Calkins, N. C. Elledge, K. E. Mueller, et al., "Development of an RNA Interference (RNAi) Gene Knockdown Protocol in the Anaerobic Gut Fungus *Pecoramyces ruminantium* Strain C1A," *PeerJ* 6 (2018): e4276.

48. N. Kalleda, A. Naorem, and R. V. Manchikatla, "Targeting Fungal Genes by Diced siRNAs: A Rapid Tool to Decipher Gene Function in *Aspergillus nidulans*," *PLoS One* 8 (2013): e75443.

49. J. Elmén, H. Thonberg, K. Ljungberg, et al., "Locked Nucleic Acid (LNA) Mediated Improvements in siRNA Stability and Functionality," *Nucleic Acids Research* 33 (2005): 439–447.

50. A. Koch, N. Kumar, L. Weber, H. Keller, J. Imani, and K. H. Kogel, "Host-Induced Gene Silencing of Cytochrome P450 Lanosterol C14α-Demethylase-Encoding Genes Confers Strong Resistance to *Fusarium* species," *Proceedings of the National Academy of Sciences of the United States of America* 110 (2013): 19324–19329.

51. X. Hu, S. Hipolito, R. Lynn, V. Abraham, S. Ramos, F. Wong-Staal, "Relative Gene-Silencing Efficiencies of Small Interfering RNAs Targeting Sense and Antisense Transcripts from the Same Genetic Locus," *Nucleic Acids Research* 32 (2004): 4609–4617.

52. C. Jöchl, E. Loh, A. Ploner, H. Haas, and A. Hüttenhofer, "Development-Dependent Scavenging of Nucleic Acids in the Filamentous Fungus *Aspergillus fumigatus*," *RNA Biology* 6 (2009): 179–186.

53. O. Abdulsalam, N. Ueberschaar, K. Krause, and E. Kothe, "Geosmin Synthase ges1 Knock-Down by siRNA in the Dikaryotic Fungus *Tricholoma vaccinum*," *Journal of Basic Microbiology* 62 (2022): 109–115.

54. M. Bulgarella, J. W. Baty, R. McGruddy, and P. J. Lester, "Gene Silencing for Invasive Paper wasp Management: Synthesized dsRNA Can Modify Gene Expression But Did Not Affect Mortality," *PLoS One* 18 (2023): e0279983.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.