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Mechanistic effects of microwave radiation on pupal emergence in the leafminer fly, *Liriomyza trifolii*

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Research Paper

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Abstract

Liriomyza trifolii is a significant pest of vegetable and ornamental crops across the globe. Microwave radiation has been used for controlling pests in stored products; however, there are few reports on the use of microwaves for eradicating agricultural pests such as *L. trifolii*, and its effects on pests at the molecular level is unclear. In this study, we show that microwave radiation inhibited the emergence of *L. trifolii* pupae. Transcriptomic studies of *L. trifolii* indicated significant enrichment of differentially expressed genes (DEGs) in 'post-translational modification, protein turnover, chaperones', 'sensory perception of pain/transcription repressor complex/zinc ion binding' and 'insulin signaling pathway' when analyzed with the Clusters of Orthologous Groups, Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes databases, respectively. The top DEGs were related to reproduction, immunity and development and were significantly expressed after microwave radiation. Interestingly, there was no significant difference in the expression of genes encoding heat shock proteins or antioxidant enzymes in *L. trifolii* treated with microwave radiation as compared to the untreated control. The expression of DEGs encoding cuticular protein and protein takeout were silenced by RNA interference, and the results showed that knockdown of these two DEGs reduced the survival of *L. trifolii* exposed to microwave radiation. The results of this study help elucidate the molecular response of *L. trifolii* exposed to microwave radiation and provide novel ideas for control.

Introduction

The leafminer fly, *Liriomyza trifolii* (Burgess), is an invasive, polyphagous insect pest that inflicts losses in both horticultural and agricultural crops worldwide (Spencer, 1973). The larvae of *L. trifolii* injure plants by forming tunnels in foliage, whereas the adults injure leaves during feeding and oviposition (Johnson *et al.*, 1983; Parrella *et al.*, 1985; Reitz *et al.*, 1999). The host range of *L. trifolii* is wide and includes crops in the Leguminosae, Solanaceae, Cucurbitaceae, and Cruciferae families (Spencer, 1973). The damage caused by *Liriomyza* spp. has escalated due to the increased use of indoor facilities for agricultural purposes. At present, chemicals are the most common method used to control *L. trifolii*; unfortunately, the frequent and often unreasonable use of chemical pesticides has led to increased resistance in *L. trifolii* (Gao *et al.*, 2012, 2017; Reitz *et al.*, 2013), and this has also led to changes in interspecies competition among *Liriomyza* spp (Gao *et al.*, 2012; Reitz *et al.*, 2013; Chang *et al.*, 2020*a*). Effective control of *Liriomyza* spp. has become increasingly difficult, thus warranting new strategies and approaches.

Physical control strategies, including microwave radiation, are an important part of integrated pest management and are safe, convenient and do not pollute the environment (Kang *et al.*, 2009; Sang *et al.*, 2022). Microwave radiation is generated by the violent vibration of molecules in a high-frequency electromagnetic field. With respect to killing insects, the biological effects of microwave radiation can be divided into thermal and nonthermal (Hoz *et al.*, 2005). Microwave technology has been used in studies aimed at preventing and controlling pests of stored plant products (Bedi and Singh, 1992; Zhang *et al.*, 2007; Lu *et al.*, 2010). For example, 100% mortality was observed for all developmental stages of the cowpea weevil when exposed to 400 W of power for 28 s (Purohit *et al.*, 2013). In another study, cowpea grains infested with larvae of the cowpea weevil were exposed to 240 W, which reduced the number of emerging insects and increased the egg-to-adult developmental period (Barbosa *et al.*, 2017). In contrast, there are relatively few studies using microwave radiation to prevent and control agricultural pests in the field (Chen *et al.*, 2018; Zhang *et al.*, 2020).

Microwave radiation was shown to increase the expression of genes encoding heat shock protein (Hsps) in the maize weevil, *Sitophilus zeamais* (Tungjitwitayakul *et al.*, 2016). Although we are unware of other studies on the mechanism of microwave radiation in insects, the effects of other nonionizing forms of radiation on insects indicated that genes encoding Hsps and antioxidant enzymes are involved (Tungjitwitayakul *et al.*, 2016; Su *et al.*, 2021; Yang *et al.*, 2021). For instance, the response of the tephritid fruit fly, *Bactrocera dorsalis,* to UV radiation indicated that antioxidant enzyme activity was irreversibly reduced (Cui *et al.*, 2021). In *Tribolium castaneum*, the expression of *Hsp27*, *Hsp68*, and *Hsp83* and the cytochrome P450 genes, *CYP6BQ4* and *CYP6BQ8*, were significantly increased during short-term exposure to UV-A (Sang *et al.*, 2012).

The effects of microwave radiation on growth, development and gene expression in *L. trifolii* have not been previously reported. In this study, the effects of microwave radiation on *L. trifolii* pupae were evaluated, and gene expression was investigated by comparative transcriptomics and RNA interference. The results of this study provide a reference for further study on the insecticidal mechanism of microwave radiation and its use to control pests.

Materials and methods

Insects and microwave treatments

Populations of *L. trifolii* were originally collected from Yangzhou (32.39°N, 119.42°E), China, and reared in the laboratory at 26°C with a 16:8 h (L: D) photoperiod as described previously (Chen and Kang, 2002). Larvae and adults were reared on kidney beans, and leaves with larval tunnels were collected for pupation. Pupae were collected in test tubes for microwave radiation, which was generated with a household microwave oven (P70D20TL-D4, Galanz, Foshan, China).

Two-day-old *L. trifolii* pupae were exposed to microwave radiation (700 W) at 0, 30, 60, 90, and 120 s. The microwave frequency is 2450 MHz and the wavelength is 122 mm. Pupae (n = 30) were collected and transferred to 1.5 ml tubes and placed in the middle of the microwave oven tray for microwave radiation and emergence rates were measured. Five biological replicates were evaluated for each treatment.

Transcriptome sequencing, annotation and verification of DEGs

Total RNA was extracted from microwave-irradiated *L. trifolii* pupae using the SV Total RNA Isolation System (Promega, Fitchburg, WI, USA). RNA integrity and purity were assessed as described previously (Chang *et al.*, 2020*b*), and three biological replicates were included for microwave treatments and the non-irradiated control. Libraries were generated and sequenced by Biomarker Technologies (Biomarker, Beijing, China) as described previously (Chang *et al.*, 2020*b*). RNA-seq data were deposited at the National Center for Biotechnology Information (NCBI) in the Sequence Read Archives, accession no. PRJNA823487.

Raw sequence data were filtered to remove adapter sequences, and clean data were analyzed for GC content, Q20, Q30, and sequence duplication. The clean data were assembled with Trinity v. 2.1.1 to obtain a high-quality unigene library (Grabherr *et al.*, 2011; Cui *et al.*, 2018). BLAST searches (e-value < 10^{-5}) of unigenes were queried against the following protein databases: COG (Clusters of Orthologous Groups), GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), KOG (euKaryotic Orthologous Groups), Pfam (Protein family), Swiss-Prot, TrEMBL, eggNOG and NR (NCBI nonredundant database).

The fragments per kilobase of transcript sequence per million (FPKM) nucleotides were calculated, read counts were mapped, and the assembled transcriptomes of microwave-treated samples were compared with controls. The DESeq2 R package was used to determine differential expression as described (Anders and Huber, 2010; Varet *et al.*, 2016). The Benjamini–Hochberg protocol for controlling false discovery rates (FDR) was used to adjust P values. FDR < 0.05 and fold-change $|FC| \geq 1.5$ were used to determine differential expression of unigenes. In addition, genes commonly associated with stress tolerance (e.g., genes encoding Hsps and antioxidant enzymes) were screened and compared, and heat maps were constructed using GraphPad Prism v. 8.0. Ten unigenes were chosen from sequencing results, and their expression was validated by quantitative real-time PCR (qPCR). Primers (table 1) were designed with Primer Premier v. 5.0. Total RNA (0.5 µg) was reverse-transcribed, and qPCR was performed in 20 µl reaction volumes as described (Chang *et al.*, 2017). Samples were assessed in triplicate.

Functional verification of key genes based on RNAi

Two highly expressed unigenes encoding cuticular protein and protein takeout (GenBank accession nos. ON716450 and ON716451, respectively) were selected for further study. These genes were analyzed with siDirect v. 2.0 (http://sidirect2.rnai.jp/) to select potential small interfering RNA (siRNA) sequences that could be used to design dsRNA primers. A T7 promoter sequence (TAATACGACTCACTATAGGGAGA) was incorporated into the 5′ end of sense and antisense primers to facilitate transcription from both cDNA strands. The control consisted of a dsRNA specific to green florescence protein (GFP) (table 1). Purified DNA templates (1.5 µg) were used to synthesize dsRNA, and products were purified with the MEGAscript™ RNAi Kit (Thermo, Waltham, MA, USA). The quality and quantity of dsRNA were evaluated by gel electrophoresis and spectrophotometry, respectively.

Prepupae that were newly emerged from leaf tissue were used in RNAi experiments; these were transferred to Petri dishes containing 600 ng µl^{-1} dsRNA and 1% RNATransMate (Sangon Biotech, Shanghai, China). Prepupae were immersed in this solution, after 10 s immersion, removed the excess droplets of dsRNA using a soft brush, to prevent it from blocking the stomata and affecting pupation, and the dsRNA-treated prepupae were used in subsequent experiments. Each treatment contained 10 prepupae and was repeated three times; dsGFP was used as the control. Pupae were collected for RNA extraction, and silencing efficiency was analyzed by qPCR. Emergence rates for pupae were calculated for each dsRNA group (n = 10 represented one repetition) and radiated at 90 s to recorded the numbers of eclosion. To evaluate the efficiency of delivering dsRNA, Ultra GelRed (Vazyme, Nanjing, China) was used to dye dsRNA, and the fluorescence of pupae was observed using a gel imaging system (fig. S1).

Statistical analysis

Expression levels of unigenes were evaluated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and *Actin* served as a

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Table 1. Primers used in RT-qPCR for validation of differentially expressed genes (DEGs) and functional verification of selected genes

	Gene	F/R	Primer sequences (5′ → 3′)
Transcriptome validation	*Unigene_23009*	F	GACTGGTGGGATGGTATGGACTT
		R	CGTCTGTAGCCGTAATGGTATCT
	Unigene_35101	F	CAAACAGCCAAGCAAAGAAAAT
		R	TTTCACAAGCAGCGAATACATC
	Unigene_31229	F	GGCTGAAAACAAACCAGTGACA
		R	AAATGGCGGTTTTCATCAGCAG
	Unigene_22373	F	AAGACCCTAACGATTGACGCTG
		R	ACATTTCATAGTTGCCTGCCAT
	Unigene_43624	F	ACCGATAGTTTGGGTTTTGGGA
		R	CGTAAAAGGTCTCCAGTGCTCA
	Unigene_08138	F	ATGGCATTTATGGTGAAGAGCA
		R	TGAAACCTCCATCGTCACTCTT
	Unigene_44261	F	GATGTTAGGATTACAGGACCCAA
		R	GCAGCAGACGACACCAGTTATTT
	Unigene_09422	F	AGCAAACGGGAGGTAGTCAGGT
		R	ACCACACTCTGAGCAGCGTTTT
	Unigene_24549	F	AAGTGCCAACAATGTATGCCG
		R	TAAACCAGTGCGATTGTAGGG
	Unigene_47390	F	AGATGGATTTTCTTGTGAGGGC
		R	ATGTTCCTTGCCATCACCGTAT
	Actin	F	TACCAACTGGGATGATATGGAA
		R	TCGACCAGCCAAGTCCAAACGC
Functional verification of selected genes	ds*CP*	F	TAATACGACTCACTATAGGGCATCAAGAAGAATACCGCACC
		R	TAATACGACTCACTATAGGGTCATAGCCATTACGGATTTTG
	ds*TO*	F	TAATACGACTCACTATAGGGGGCAACTATGAAATGTCTGG
		R	TAATACGACTCACTATAGGGTTGAGTCAAAGGGAATGTTT
	dsGFP	F	TAATACGACTCACTATAGGGCCTCGTGACCACCCTGACCTAC
		R	TAATACGACTCACTATAGGGCACCTTGATGCCGTTCTTCTGC
	qPCR-*CP*	F	CCACGAAGAAGCCCACAACA
		R	TTGGGAGGTGGTGGTGTAGTG
	qPCR-*TO*	F	GGCATACCACCATTGGAACCT
		R	TTTTTCAAATCAGTTCTGTAGCGT

Note: F, forward; R, reverse; underscored nucleotides indicate the T7 polymerase promoter sequence.

reference gene (Chang *et al.*, 2017). GraphPad Prism was used to analyze the correlation between qPCR and RNA-seq data. Using linear fitting, $P < 0.05$ was considered to represent significant correlation. For transcriptome validation and silencing efficiency, the relative abundance of target genes and survival rates were compared to the dsGFP control. The Student's *t*-test was used to compare differences in gene expression, and one-way ANOVA followed by Tukey's multiple comparison was used to compare differences in survival/mortality data with SPSS v. 16.0. For ANOVA, survival/mortality data were transformed by using arcsine square root, and differences were considered significant at $P < 0.05$.

Results

Microwave radiation bioassay

To examine microwave tolerance in *L. trifolii*, survival was evaluated in response to different time intervals of microwave radiation. Although survival rates at 30, 60 and 90 s were not significantly different from the control, survival of pupae dramatically decreased with increasing time and only 3.33% pupae emerged after a 120 s exposure ($F_{4,10} = 17.004$; $P < 0.05$). The 90 s treatment resulted in 60–70% survival rates and was used for transcriptome analysis (fig. 1).

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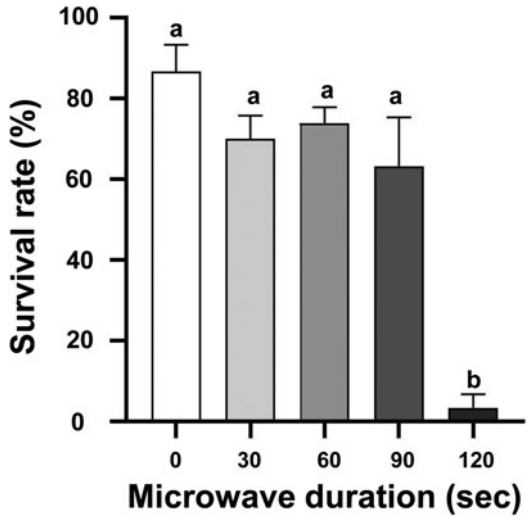

Figure 1. Impact of microwave radiation on *L. trifolii* survival. Different lowercase letters indicate significant differences among treatments. Tukey's multiple range test was used for pairwise comparison of means ($P < 0.05$).

Transcriptome sequencing and functional annotation of DEGs

RNA-seq was used to quantify *L. trifolii* gene expression in response to the presence and absence of microwave radiation, and 40.44 Gb of clean sequence was obtained. The GC content was 39.73–40.44%, and the Q30 values were ≥93.71% (table 2). Trinity software was used to assemble high-quality reads into transcripts. In total, 219,440 transcripts representing 33,247 unigenes were obtained. The average length of transcripts and unigenes was 1444.45 and 1380.34 bp and N50 lengths were 2628 and 2574 bp, respectively. Functional annotation revealed that 3846, 10,854, 11,173, 9525, 11,128, 10,383, 14,661, 12,286 and 14,687 unigenes mapped to COG, GO, KEGG, KOG, Pfam, Swiss-Prot, TrEMBL, eggNOG, and NR, respectively.

Table 2. Summary of statistics and annotation of *L. trifolii* transcriptomes

Sequencing/annotation	Data summary
Total number of transcripts	219,440
Total number of unigenes	33,247
Mean length of transcripts (bp)	1444.45
Mean length of unigenes (bp)	1380.34
N50 length of transcripts (bp)	2628
N50 length of unigenes (bp)	2574
COG annotated	3846
GO annotated	10,854
KEGG annotated	11,173
KOG annotated	9525
Pfam annotated	11,128
SwissProt annotated	10,383
TrEMBL annotated	14,661
eggNOG annotated	12,286
NR annotated	14,687
All annotated	14,951

Pairwise comparison of transcriptomes in irradiated and control treatments indicated that 62 unigenes were differentially expressed during microwave exposure; 48 unigenes were induced and the remaining 14 were repressed (fig. 2a). Fourteen, 45, 42, 36, 45, 39, 56, 49, and 56 DEGs were obtained and functionally annotated using the COG, GO, KEGG, KOG, Pfam, Swiss-Prot, TrEMBL, eggNOG, and NR databases, respectively. The COG classification results are shown in fig. 2b. DEGs were primarily assigned to 'post-translational modification, protein turnover, chaperones' (15.79%), 'secondary metabolite biosynthesis, transport and catabolism' (15.79%), 'lipid transport and metabolism' (10.53%), 'general function prediction only' (10.53%), 'defense mechanisms' (10.53%) and 'cell wall/membrane/envelope biogenesis' (10.53%). GO enrichment analysis results were categorized into three groups: e.g. biological processes, cellular components, and molecular functions. With respect to genes in biological processes, the results showed that 'sensory perception of pain', 'heterochromatin assembly', and 'somatic muscle development' were enriched and expressed. The cellular component categories were enriched for 'transcription repressor complex', 'extracellular region' and 'obsolete extracellular region part'. In the molecular function grouping, 'zinc ion binding', 'sequence-specific DNA binding' and 'chromatin binding' were the most frequent categories represented in response to microwave radiation (fig. 2c). DEGs were compared to KEGG database entries to further elucidate gene functions. A total of 31 pathways were identified for 37 DEGs; the 20 most-enriched KEGG pathways are shown in fig. 3. In response to microwave radiation, enriched pathways were assigned to the 'insulin signaling pathway', 'focal adhesion' and 'ABC transporters' (fig. 3).

Annotation and validation of DEGs

The top ten upregulated DEGs in *L. trifolii* exposed to microwave radiation are shown in table 3. DEGs related to reproduction, insect immunity, and growth and development pathways were significantly expressed in response to microwave radiation. Induced DEGs included 'ejaculatory bulb-specific protein 3', 'transmembrane protease serine 11D', and 'cuticle protein 2', whereas repressed DEGs encoded '20-hydroxyecdysone protein', 'serine protease inhibitor 42Dd' and 'chitin-binding type-2 domain-containing protein' (table 3). Interestingly, we compared two types of genes related to stress tolerance and found no significant difference between genes encoding Hsps and antioxidant-related enzymes after microwave radiation (fig. 4). Ten DEGs with distinct expression patterns were selected to validate RNA-seq data by qPCR. The expression of the ten genes by qPCR correlated with RNA-seq data ($R^2 = 0.9593$; $P < 0.05$) (fig. 5), indicating that the latter data are reliable.

Functional verification of selected DEGs by RNAi

DEGs encoding cuticular protein (*CP*, unigene_08138) and protein takeout (*TO*, unigene_22373) were chosen for RNAi experiments based on fold-change values (fig. 5). RNA interference studies were conducted by immersing *L. trifolii* prepupae in solutions containing dsRNA specific for *CP* and *TO*. There was a significant reduction in *CP* expression when prepupae were immersed with dsCP (24.76%) in comparison to the dsGFP control ($t = 3.030$; $P < 0.05$) (fig. 6a). Similar expression patterns were

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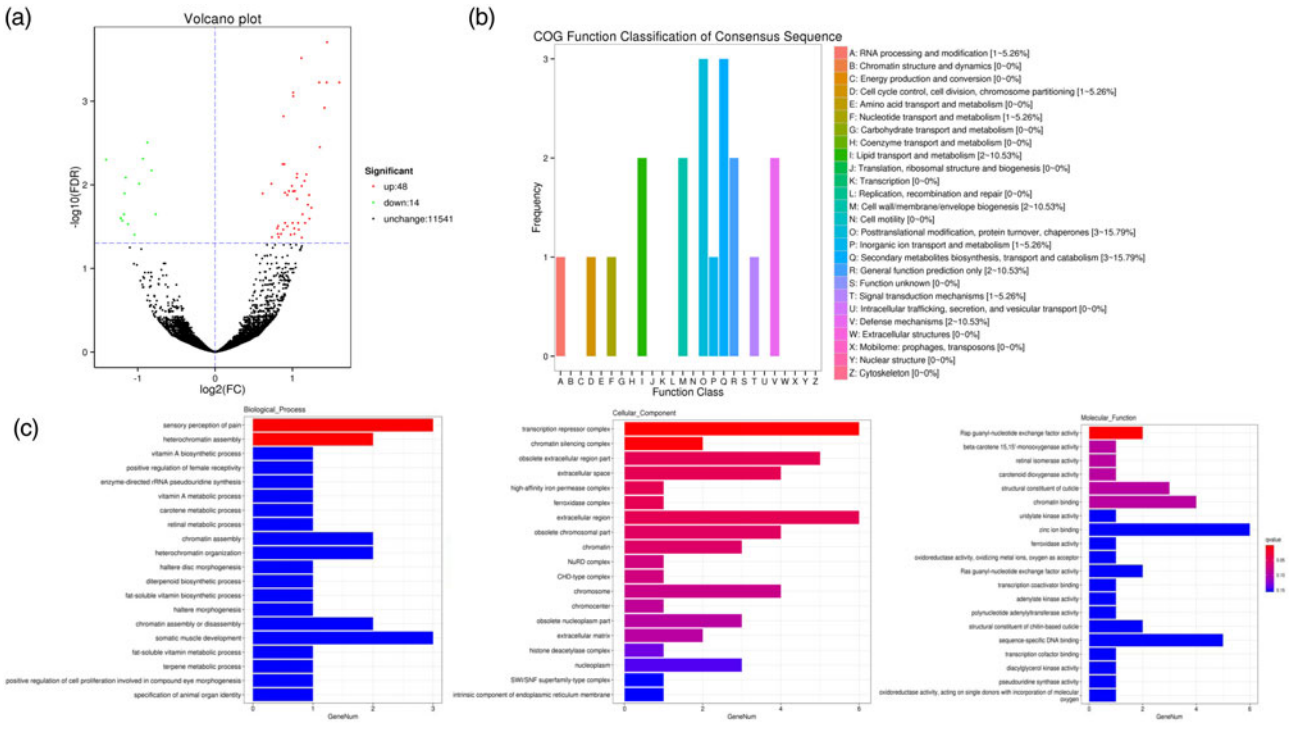

Figure 2. Differentially expressed genes (DEGs) and its functional annotation in *L. trifolii* under microwave radiation. (a) Volcano plot of microwave treatment vs. control. The *y*-axis represents $-\log_{10}$ significance, whereas the *x*-axis represents log2-fold change. Red dots indicate induced unigenes, whereas green dots indicate repressed unigenes; (b) Functional classification of DEGs into clusters of orthologous groups (COG); (c) Gene ontology (GO) enrichment assignments of annotated DEGs: biological processes, cellular components, molecular function.

observed for *TO* expression, which was significantly reduced (38.53%) when prepupae were immersed in dsTO ($t = 9.717$; $P < 0.05$) (fig. 6b). When *L. trifolii* was exposed to 90 s of microwave radiation after dsRNA treatment, mortality increased by 33.99 and 42.78% for dsCP and dsTO, respectively, as compared to dsGFP (26.48%) (fig. 6b). Mortality was further increased to 54.85% when prepupae were treated with both dsCP and dsTO and exposed to 90 s of microwave radiation, and this difference was significantly higher than the dsGFP control ($F_{3,8} = 5.569$; $P < 0.05$) (fig. 6b).

Discussion

It is well-established that microwave radiation negatively impacts insect colonization of stored products (Bedi and Singh, 1992; Zhang *et al.*, 2007; Lu *et al.*, 2010; Purohit *et al.*, 2013; Barbosa *et al.*, 2017); however, little research has been conducted to address the underlying mechanisms of microwave radiation on agricultural and invasive pests (Chen *et al.*, 2018; Zhang *et al.*, 2020). For example, the application microwave radiation at 600 W for 13 min was consisted to be a possible control method for *Empoasca onukii*, the tea green leafhopper (Chen *et al.*, 2018). In the current study, microwave radiation was shown to significantly inhibit the emergence of *L. trifolii* pupae. RNA-seq was used to study the effects of microwave radiation on gene expression, and the role of two DEGs in conferring tolerance to microwaves was evaluated using RNAi.

The biological effects of microwave radiation can be divided into thermal and non-thermal effects (Hoz *et al.*, 2005). The mechanisms involved in the lethal action of microwave radiation could be due to the high oscillation frequency of water molecules in the bodies of the insects. Microwave heating is based on the transformation of electromagnetic field energy into thermal energy and can kill insects (Lu *et al.*, 2010); however, there is no research on genome-wide changes in gene expression during microwave irradiation. The reproductive process of insects has been extensively studied because of its importance in species propagation and its potential as a target for control methods (Roy *et al.*, 2018). In this study, 'ejaculatory bulb-specific protein' was highly expressed, which suggests that microwave radiation impacts reproduction in *L. trifolii*. In another study, the expression of genes encoding vitellogenin and its receptor were upregulated in *Ostrinia furnacalis* during exposure to UV-A (Liu *et al.*, 2020). In *M. persicae,* exposure to microwave radiation at different frequencies and durations had variable effects on the mortality and reproduction of apterous adults. Short-term microwave radiation promoted the reproduction of one-day-old apterous adults of *M. persicae*; apterous aphids had the greater fertility when subjected to microwave radiation for 30 s but their reproduction was inhibited when subjected to radiation for 15 and 120 s (Zhang *et al.*, 2020). In addition, the use of microwave radiation is different from sterile insect technique (SIT), where ionizing radiation is used to produce sterile males of target insects, resulting in declining pest populations in defined regions (Knipling, 1959; Robinson, 2005). However, for microwave radiation, the detection of specific spawning at different radiation times needs to be supplemented to prove this point.

In response to UV-A radiation, comparative transcriptome analysis between UV-treated and control groups in *O. furnacalis* indicated the involvement of pathways associated with signal transduction, detoxification, the stress response, immune defense, and antioxidative systems (Su *et al.*, 2021). Meanwhile, antiviral and FcγR-mediated phagocytosis of immune-related genes were

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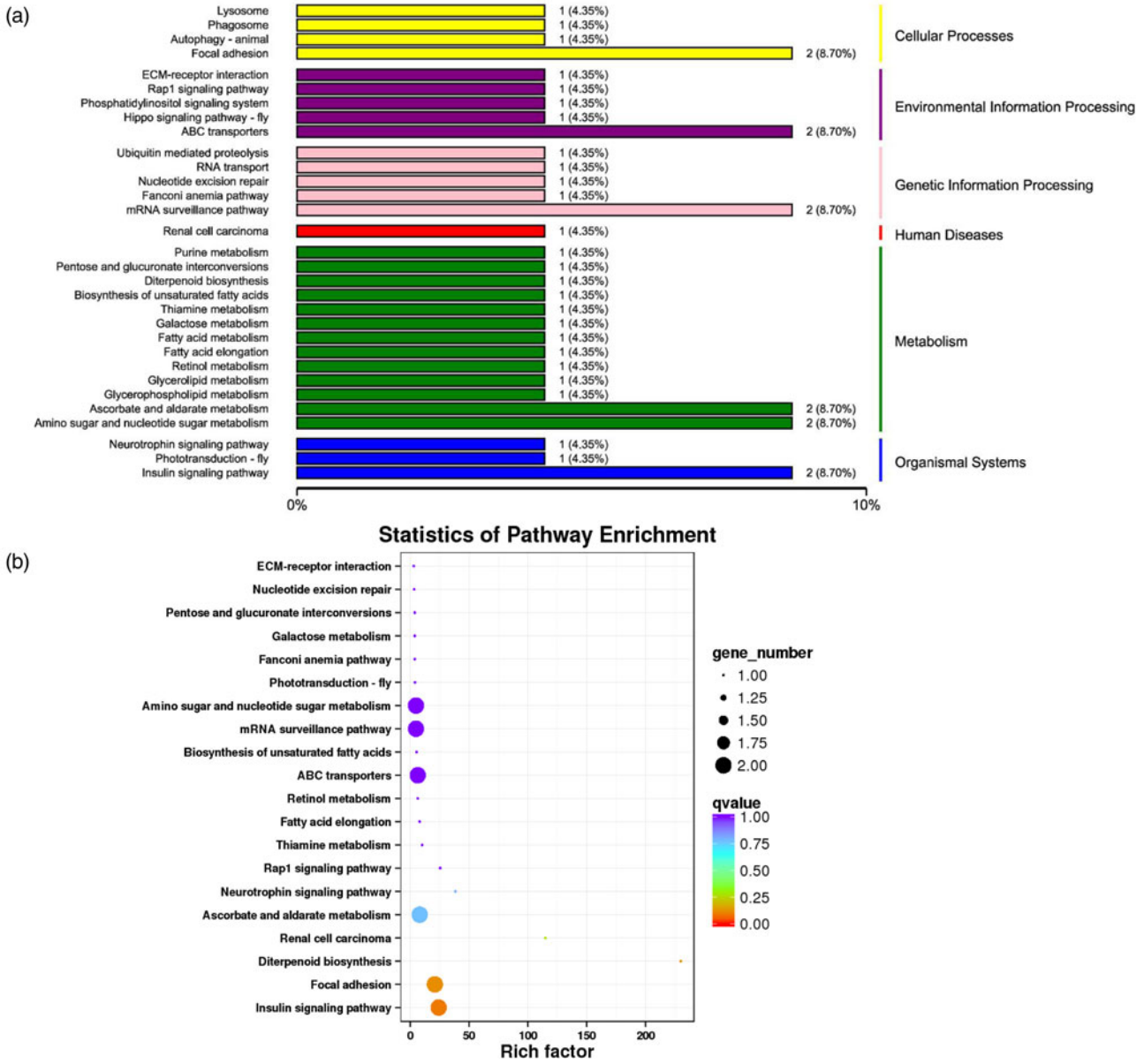

Figure 3. (A) DEGs based on pathways in the KEGG database. The *x*-axis indicates the percentages of genes that are annotated to a specific pathway; y-axes indicate the categories in the KEGG repository; (B) The twenty most enriched KEGG pathways among DEGs under microwave radiation. The Rich factors represent the ratio of DEG numbers vs. the number of genes annotated in the pathway. Larger Rich factors indicate a greater level of enrichment. The *q* values are corrected *P* values ranging from 0 to 1, with lower values indicating greater enrichment.

induced during exposure to UV-B radiation, which indicates that insects have strong immune-adaptive functions (Adamo, 2012, 2017; Yang *et al.*, 2021). In the current study, microwave radiation significantly reduced the expression of several immune-related genes, including serine protease inhibitor and serine/threonine-protein phosphatase. These results indicate that the underlying mechanism of microwave radiation is different from other nonionizing forms of radiation. The thermal effects of microwave radiation can accelerate cell division and reproductive rates in organisms, which are conducive to the cells in the division stage (Pang and Zhang, 2001). However, in our study, genes involved in growth and 20-hydroxyecdysone (20E) synthesis were down-regulated; the latter is especially significant since 20E controls and coordinates development in insects during metamorphosis (Riddiford *et al.*, 2000; Dubrovsky, 2005).

In *Myzus persicae* exposed to UV-A radiation, DEGs were associated with antioxidants and detoxification, metabolism and protein turnover, the immune response, and stress-related signal transduction; furthermore, the *shd* gene, which encodes 20-hydroxylase and converts ecdysone into 20E, was significantly down-regulated during UV-B exposure. The synthesis of 20E in *M. persicae* may be inhibited by UV-B, which needs to be confirmed by further experiments (Yang *et al.*, 2021). In the present study, genes encoding cuticular protein in *L. trifolii* were significantly up-regulated in response to microwave radiation. This is notable because the insect exoskeleton is an assembly of chitin and cuticular proteins (Charles, 2010). Interestingly, multiple genes encoding cuticular proteins genes were significantly down-regulated in *M. persicae* during UV-B stress (Shang *et al.*, 2020), suggesting that radiation stress impacts insects differentially.

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Table 3. Annotated DEGs of *L. trifolii* exposed to microwave treatment

#ID	FDR	Log_2FC	NR annotation	TrEMBL/SwissProt annotated
Up regulation				
Unigene_32292	0.000197	1.446399	PREDICTED: ejaculatory bulb-specific protein 3-like	Ejaculatory bulb-specific protein 3
Unigene_02934	0.000597	1.441445	CLIP domain-containing serine protease 14D	Transmembrane protease serine 11D
Unigene_22373	0.001199	1.414249	PREDICTED: protein takeout	Protein takeout
Unigene_43624	0.000597	1.346608	PREDICTED: adenylate kinase isoenzyme 1 isoform X1	Adenylate kinase isoenzyme 1
Unigene_23273	0.018817	1.245355	hypothetical protein RP20_CCG013604	Retrovirus-related Pol polyprotein from transposon TNT 1–94
Unigene_08138	0.013241	1.216481	PREDICTED: larval cuticle protein LCP-30 isoform X1	Cuticle protein 2
Unigene_05490	0.017336	1.199834	PREDICTED: uncharacterized protein LOC101893009	E3 ubiquitin-protein ligase SINAT2
Unigene_48759	0.007506	1.18015	Carotenoid isomerooxygenase	carotenoid isomerooxygenase
Unigene_48165	0.008974	1.156678	calmodulin-binding transcription activator 1, partial	Calmodulin-binding transcription activator 1
Unigene_44261	0.022566	1.130186	epidermal retinol dehydrogenase 2 isoform X1	Epidermal retinol dehydrogenase 2
Down regulation				
Unigene_09422	0.022566	−0.768	20-hydroxyecdysone protein	20-hydroxyecdysone protein
Unigene_12162	0.00313	−0.87628	cell wall protein DAN4	-
Unigene_24549	0.004866	−0.93302	PREDICTED: leukocyte elastase inhibitor	Serine protease inhibitor 42Dd
Unigene_09037	0.039456	−1.0434	uncharacterized protein LOC101452084	Serine/threonine-protein phosphatase beta isoform
Unigene_47607	0.029466	−1.12664	hypothetical protein FF38_10102	Laccase-14 OS = Arabidopsis thaliana
Unigene_01123	0.008206	−1.15591	PREDICTED: uncharacterized protein LOC106614389	Cuticlin-1
Unigene_47390	0.012647	−1.17125	fibrous sheath CABYR-binding protein	Chitin-binding type-2 domain-containing protein
Unigene_32593	0.025175	−1.21741	zinc carboxypeptidase A 1	Zinc carboxypeptidase A 1
Unigene_24207	0.024976	−1.22256	PREDICTED: early nodulin-75	Uncharacterized protein
Unigene_29933	0.005013	−1.40947	homeobox protein homothorax isoform X3	Homeobox protein homothorax

Microwave radiation induces thermal activity (Lu *et al.*, 2010), and it likely that the increased temperature due to microwave irradiation was responsible for the induction of *Szhsp70* and *Szhsp90* in the maize weevil, *S. zeamais* (Tungjitwitayakul *et al.*, 2016). Similarly, microwave radiation induced *Hsp70* expression in chick embryos (Shallom *et al.*, 2002) and human neuroblastoma cells (Calabrò *et al.*, 2012). The effects of other nonionizing radiation treatments have been reported in insect pests, and genes encoding Hsps and antioxidant enzymes have been studied as mechanisms that provide some tolerance to radiation (Tungjitwitayakul *et al.*, 2016; Su *et al.*, 2021; Yang *et al.*, 2021). In *S. zeamais*, *Szhsp70* and *Szhsp90* were induced after UV-C radiation, and *Szhsp70* was expressed at a much higher level than *Szhsp90* (Tungjitwitayakul *et al.*, 2016). The response of *Hsp70* to irradiation has also been reported for *T. castaneum* (Sang *et al.*, 2012), where *Hsp70* was the most highly up-regulated *Hsp* in response to microwave treatment. In another study, exposure to UV radiation significantly increased mortality of the tephritid fruit fly, *B. dorsalis*, during the pre-oviposition period, significantly reduced the number of eggs deposited, and lowered the activities of several antioxidant enzymes (Cui *et al.*, 2021). In contrast, our results showed no significant difference in the

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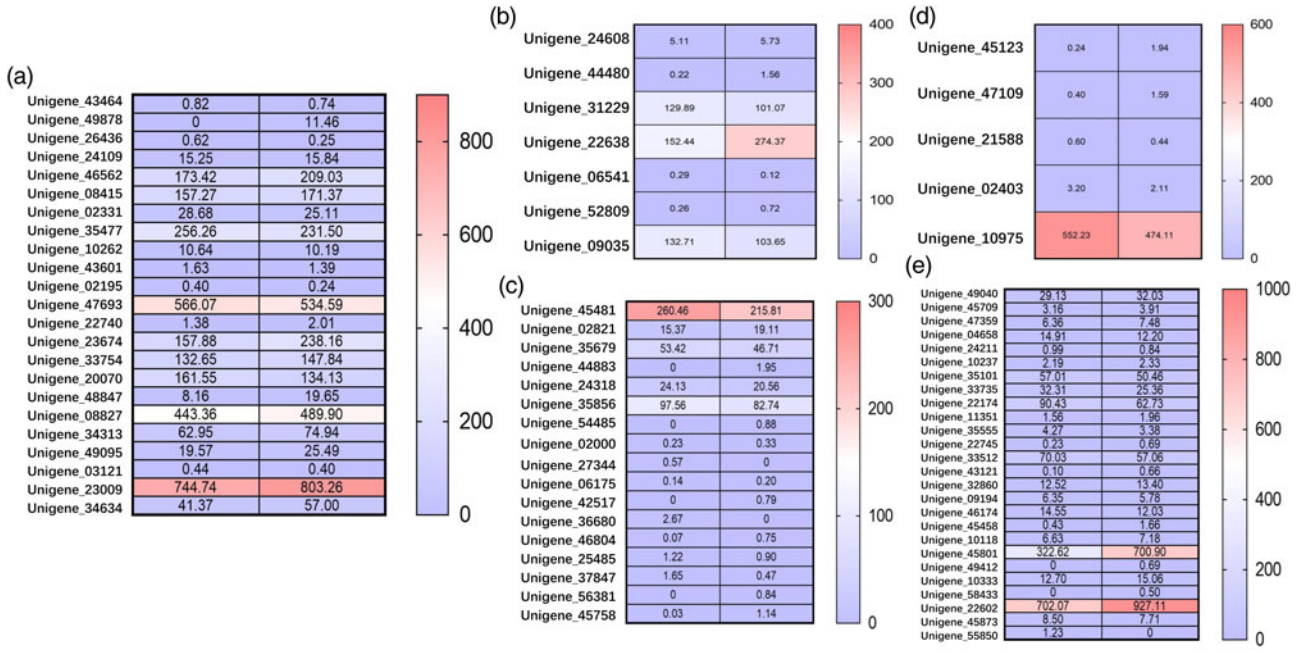

Figure 4. Heatmap of genes encoding *Hsps* (a) and antioxidant enzymes related genes (b: *CAT*; c: *POD*; d: *SOD*; E: *GST*) under microwave radiation. Color scale from red to blue indicates log2 transcription ratios.

expression of genes encoding antioxidants or Hsps when *L. trifolii* was exposed to microwave radiation.

Physical control techniques are important components of pest management and provide alternatives to chemical control. Physical methods are safe, convenient and do not pollute the environment (Sang *et al.*, 2022). Recently, the use of microwaves to sterilize insects has attracted attention (Bedi and Singh, 1992; Lu *et al.*, 2010; Purohit *et al.*, 2013; Barbosa *et al.*, 2017). In our study, two DEGs, namely *CP* and *TO*, were examined by RNAi to evaluate their role in response to microwave-mediated radiation stress. Our results showed that the knockdown of *CP* and *TO* significantly reduced the survival of *L. trifolii* exposed

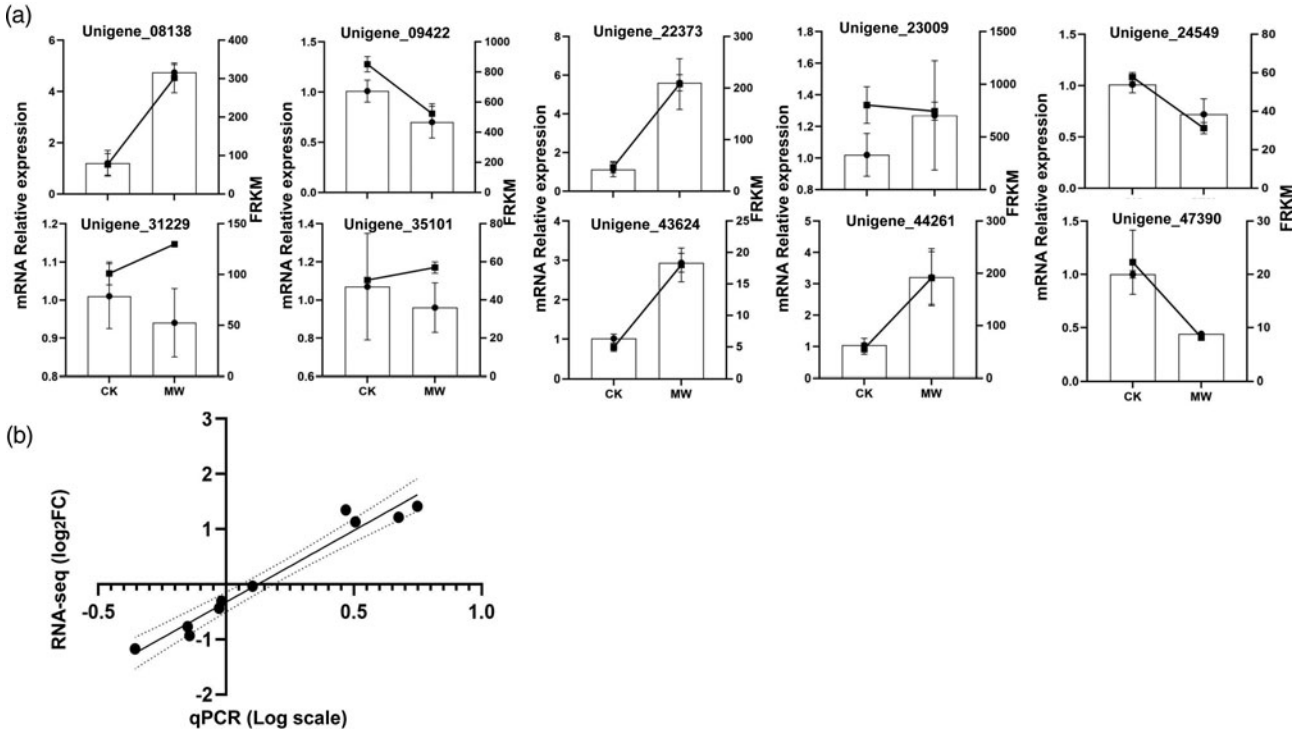

Figure 5. Validation of gene expression patterns by RT-qPCR. (a) Means (±SE) were used to determine transcript levels with the $2^{-\Delta\Delta}$Ct method. The y-axes indicate log-transformed FPKM values (right) and log-transformed relative expression (left). (b) Linear correlation analysis of RNA-seq and RT-qPCR data.

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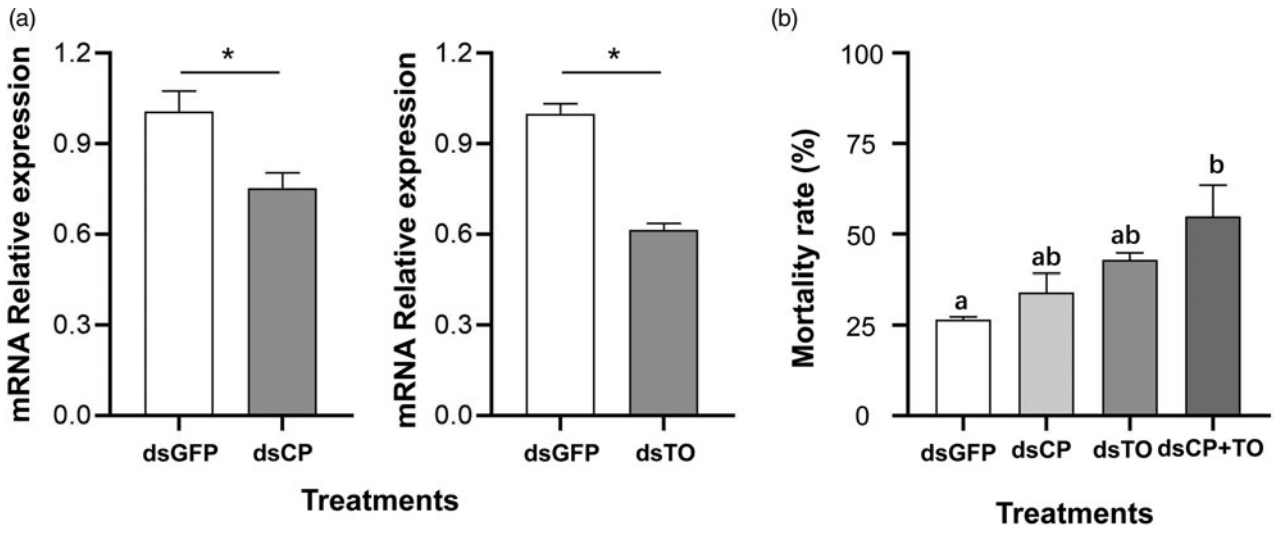

Figure 6. RNAi-mediated knockdown of *CP* and *TO* genes expressions in *L. trifolii* immersed with dsCP and dsTO (a) and mortality rate of *L. trifolii* after 90 s microwave radiation (b).

to microwave radiation. The combination of microwave radiation and RNAi has potential application in the control of insect pests. In RNAi, which is widely used to study gene function (Joga *et al.*, 2016); dsRNA is used to trigger the degradation of homologous mRNA. The dsRNA can be delivered in dietary form or by immersion or microinjection; however, the latter is limited due to the difficulty in using it for controlling pests in the field. Delivery of dsRNA to pests as a dietary supplement requires that insects feed and absorb dsRNA via the midgut; however, barriers to the midgut may reduce RNAi efficiency in some insects (Burand and Hunter, 2013; Baum and Roberts, 2014; Joga *et al.*, 2016). The immersion method is another potential method of delivering to RNAi to pests (Tabara *et al.*, 1998; Zhang *et al.*, 2015), In this study, dsRNA was delivered to prepupae by the direct immersion method. The prepupal stage can morph into pupae within a few hours (Parrella, 1987), and dsRNA may be encapsulated in the insect body to facilitate delivery. The development of portable devices to deliver microwave radiation and dsRNA would significantly improve the use of RNAi technology in the field. In terms of pest control, the potential impact of dsRNA on the quality of agricultural products should be evaluated, and recommended dosages would need to be determined for large-scale application trials.

In general, our study showed that microwave radiation can inhibit the emergence of *L. trifolii* pupae. Transcriptome analysis of *L. trifolii* exposed to microwave radiation revealed significant expression of genes involved in reproduction, immunity, and growth and development; these were generally more highly expressed than stress-related genes encoding Hsps or antioxidant enzymes. Microwave irradiation combined with RNAi would be facilitated by better comprehension of fundamental physiological mechanisms and represents a new direction for pest management, especially for invasive insects.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485322000578.

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Conflict of interest. The authors declare no conflict of interest.

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