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Research Article

Stability of double-stranded RNA in haemolymph and whole-body extracts of adult fall armyworm, *Spodoptera frugiperda* (J.E. Smith): Insights for RNAi-mediated fall armyworm management

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ABSTRACT: RNA interference (RNAi) is a promising tool for functional genomics and pest management; however, its efficiency largely depends on the persistence of double-stranded RNA (dsRNA) within insect tissues. The present study assessed the ex vivo stability of dsRNA in the haemolymph and whole-body extracts of adult *S. frugiperda*. Double-stranded RNA designed to target the α-tubulin gene was treated with haemolymph and whole-body extracts, and degradation was monitored over time. Double-stranded RNA persisted for longer durations in haemolymph, whereas rapid degradation was observed in whole-body extracts within 30-60 min. As whole-body extracts represent a composite of multiple tissues, including gut and other somatic tissues, the observed degradation suggests the collective action of tissue-associated dsRNases that may limit dsRNA persistence. In contrast, haemolymph provided a comparatively permissive environment for dsRNA stability. These findings highlight important tissue-level barriers to dsRNA persistence in adult *S. frugiperda* and provide a laboratory-based framework for functional validation of genes associated with adult physiology and reproduction. These findings provide useful insights for improving RNAi-mediated management strategies against fall armyworm and support the development of sustainable RNAi-based pest control approaches.

KEYWORDS: dsRNA degradation, fall armyworm management, haemolymph, RNA interference, *S. frugiperda*

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INTRODUCTION

RNA interference (RNAi) functions as a sequence-specific gene-silencing mechanism and is increasingly recognised as a valuable tool in functional genomics and insect pest management (Fire *et al.*, 1998; Scott *et al.*, 2013; Zotti *et al.*, 2018, He *et al.*, 2022). The success of RNAi largely depends on the stability of double-stranded RNA (dsRNA) within the insect internal environment. Following delivery, dsRNA is exposed to endogenous ribonucleases (RNases) present within insect tissues, which can influence dsRNA stability and RNAi efficiency (Singh *et al.*, 2017).

Spodoptera frugiperda (J.E. Smith) is a highly destructive and invasive lepidopteran pest of major agricultural importance worldwide (Goergen *et al.*, 2016; Early *et al.*, 2018). The pest causes a substantial economic loss to maize and other cereal crops, with yield losses up to 20-50% under severe infestations (Day *et al.*, 2017). While RNAi has been widely reported in larval stages of *S. frugiperda*, information on dsRNA stability and RNAi efficiency in adult insects remains limited (Terenius *et al.*, 2011; Cooper *et al.*, 2019). This knowledge gap is important because adults play a crucial role in reproduction, dispersal

and population buildup. Understanding dsRNA behaviour in the adult internal environment is therefore essential before designing RNAi-based functional studies targeting adults or reproductive genes.

Haemolymph and whole-body contents represent biologically relevant internal components that contain endogenous enzymes, including RNases, which influence dsRNA persistence (Christiaens *et al.*, 2014; Koo and Palli, 2025). Although examined under *ex vivo* conditions, these components are isolated from live, physiologically active insects. Enzymatic activity may remain functionally active for defined periods after extraction when samples are maintained under controlled conditions. Observed degradation of dsRNA in such assays reflects endogenous nuclease activity. Differences in degradation patterns between haemolymph and whole-body contents provide insights into tissue-specific nuclease activity and dsRNA susceptibility (Singh *et al.*, 2017).

Therefore, the objective of this study is to evaluate the stability of dsRNA in the haemolymph and whole-body contents of adult *S. frugiperda* under *ex vivo* incubation conditions. Double-stranded RNA persistence was characterised at defined time intervals up to 60 min. The study provides baseline information which is essential for RNAi-based functional studies and gene silencing approaches in adult *S. frugiperda* with potential relevance for RNAi-mediated fall armyworm management and sustainable pest control strategies.

MATERIALS AND METHODS

Insect culture and dsRNA synthesis

Adult *S. frugiperda* were obtained from a continuous laboratory culture maintained at ICAR-National Bureau of Agricultural Insect Resources (ICAR-NBAIR), Bengaluru, India, under controlled conditions (24 ± 1°C, 65 ± 5% relative humidity and a 16:8 h light: dark photoperiod). Freshly emerged adults (0-24 h old) were used as experimental material for all assays, which were conducted during November 2025.

Double-stranded RNA (dsRNA) specific to the α-tubulin gene was synthesised using the MEGAscript® RNAi Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. PCR-amplified templates containing T7 promoter sequences were included to facilitate *in vitro* transcription. Gene-specific primers appended with T7 promoter sequences were utilised for template amplification (Forward:5′-TAATACGACTCACTATAGGGAGTCGGCAAGGAAATCGTAG-3′; Reverse:5′-TAATACGACTCACTATAGGGAGGTATGTGACCAGAGGGAAG-3′). The amplified dsRNA fragment was approximately 366 bp in length. The synthesised dsRNA was purified and preserved at −80°C until use. The concentration and purity of dsRNA were determined using a NanoDrop spectrophotometer, and only preparations with A260/280 ratios between 1.9 and 2.1 were used. The final working concentration of dsRNA used for stability assays was 500 ng μL^{-1} (Cooper *et al.*, 2019).

Haemolymph and whole-body extract preparation

Haemolymph was sampled from adult *S. frugiperda* by gently puncturing the intersegmental membrane of the abdominal region using a sterile fine needle. Haemolymph droplets were immediately transferred into pre-chilled microcentrifuge tubes containing ice-cold phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.01% PTU to prevent melanisation and preserve sample integrity during downstream molecular analyses (Cooper *et al.*, 2019). For each biological replicate, haemolymph from ten adults was pooled and centrifuged at 12,000 × g for 10 min at 4°C to remove cellular debris, and the clear supernatant was used for further assays.

Whole-body extracts were prepared by removing wings and legs, and the remaining tissues were homogenised in pre-chilled PBS (pH 7.4) containing 0.01% PTU under ice-cold conditions using chilled microcentrifuge tubes to minimise melanisation, preserve sample integrity and reduce unwanted degradation during downstream molecular analyses. The homogenate was centrifuged at 12,000 × g for 10 min at 4°C, and the clear supernatant was collected for analysis (Singh *et al.*, 2017). All haemolymph and whole-body extract preparations were utilised for *ex vivo* assays within one hour of collection to ensure enzymatic activity.

Ex vivo dsRNA stability assay and analysis

Ex vivo dsRNA degradation assays were performed by incubating haemolymph or whole-body extracts (4 µL) with dsRNA (2 µL; 500 ng μL^{-1}), and the reaction volume was adjusted to 10 µL with phosphate-buffered saline supplemented with phenylthiourea (PBS-PTU). Reactions were incubated at 37°C and samples were collected at 0, 15, 30 and 60 min. At each time point, enzymatic activity was terminated by the addition of EDTA (final concentration 10 mM), followed by heat inactivation at 95°C for 5 min to inhibit divalent cation-dependent nuclease activity (Cooper

et al., 2019). Each experimental treatment was conducted using three independent biological replicates. Double-stranded RNA integrity was analysed by electrophoresis on 1.5% agarose gels prepared in 1× TAE buffer. Control reactions included dsRNA incubated with PBS-PTU alone and dsRNA incubated with heat-inactivated haemolymph or whole-body extracts to confirm enzyme-mediated degradation (Cooper *et al.*, 2019).

RESULTS

Haemolymph collected from adult *S. frugiperda* in PBS-PTU remained clear throughout the experimental period, enabling subsequent *ex vivo* assessment of dsRNA stability. *Ex vivo* dsRNA stability assays revealed distinct differences in dsRNA persistence between haemolymph and whole-body extracts. In buffer-only controls (dsRNA incubated with PBS-PTU in the absence of haemolymph or whole-body extracts), dsRNA bands remained intact throughout the incubation period, confirming intrinsic dsRNA stability under the assay conditions. Similarly, dsRNA incubated with heat-inactivated haemolymph or whole-body extracts (extracts subjected to heat treatment prior to dsRNA incubation to abolish enzymatic activity) showed minimal degradation, indicating that degradation observed in active samples was enzyme-mediated.

At the initial time point (0 min), intact dsRNA bands were detected in both haemolymph and whole-body extract samples. After 15 min of incubation at 37°C, dsRNA integrity

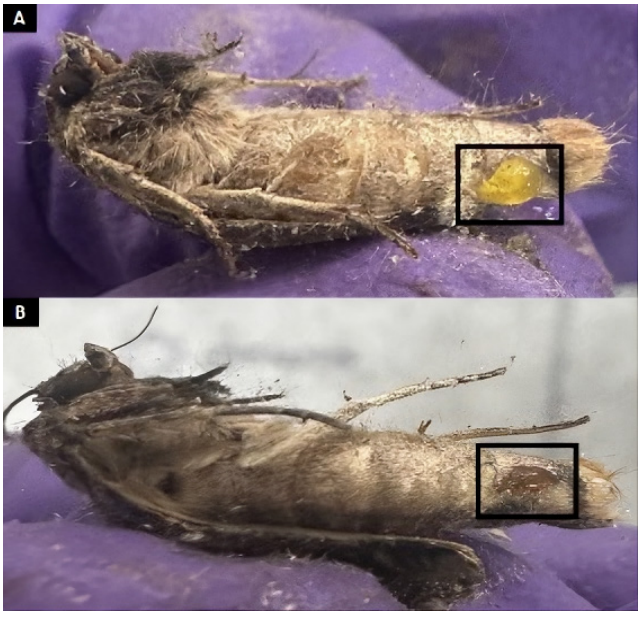


Figure 1. (A). Fresh haemolymph, **(B).** Melanized haemolymph exudates from adult *Spodoptera frugiperda*.

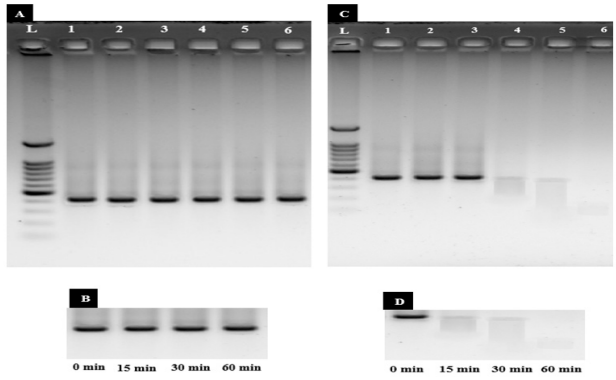


Figure 2. Agarose gel electrophoresis showing **(A, B).** preserved dsRNA band integrity in haemolymph and **(C, D).** reduced intensity with smearing in whole-body extracts.

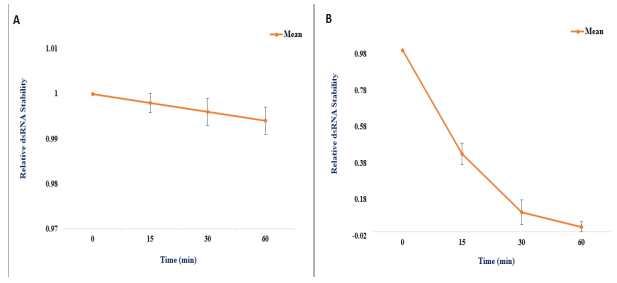


Figure 3. Densitometric analysis showing relative dsRNA band intensity in **(A).** haemolymph, **(B).** whole-body extracts.

was largely retained in haemolymph, whereas whole-body extracts exhibited a noticeable reduction in band intensity. With increasing incubation time, dsRNA degradation in whole-body extracts progressed rapidly, with faint to nearly undetectable dsRNA bands observed at 30 min and near-complete loss of intact dsRNA by 60 min. In contrast, dsRNA incubated with haemolymph remained detectable across all time points examined.

Densitometric analysis performed using Fiji software further supported these qualitative observations (Schindelin *et al.*, 2012). Relative dsRNA levels remained high in haemolymph samples throughout the incubation period, whereas whole-body extracts showed a sharp decline in dsRNA levels within 30 min, followed by near-complete degradation at 60 min.

DISCUSSION

The present study demonstrated marked differences in dsRNA stability between haemolymph and whole-body

extracts of adult *S. frugiperda*. Double-stranded RNA remained detectable for longer durations in haemolymph, whereas rapid degradation occurred in whole-body extracts within 30-60 min, indicating differential nuclease activity between internal tissue environments. These findings suggest that tissue-specific factors may influence dsRNA persistence and could affect the efficiency of RNA interference (RNAi)-based approaches for functional studies and pest management applications.

The prevention of haemolymph melanisation using Phenylthiourea (PTU) was essential for maintaining sample integrity during molecular assays. Melanisation is a well-recognised immune response mediated by phenoloxidase activity following tissue injury and exposure to oxygen. Suppression of haemolymph melanisation during collection is routinely employed in insect physiological studies to preserve sample quality for downstream molecular analyses (Admella and Torrents, 2022). The use of PTU in the present study ensured reliable assessment of dsRNA stability in haemolymph samples.

In contrast to whole-body extracts, dsRNA persisted for longer durations in haemolymph under identical conditions. Haemolymph does not directly contain gut-derived digestive nucleases, which likely contributes to comparatively reduced dsRNA degradation. The observed difference in dsRNA stability between haemolymph and whole-body extracts indicates that gut-associated tissue environments may play a dominant role in dsRNA degradation in adult insects. These observations suggest that delivery strategies targeting the haemocoel may improve dsRNA persistence and enhance RNAi efficiency in *S. frugiperda*.

The densitometric analysis further reaffirmed these findings, demonstrating rapid dsRNA degradation in whole-body extracts and sustained dsRNA detectability in haemolymph. Overall, these findings suggest that gut-associated and tissue-associated nucleases present within composite tissue extracts are key determinants of dsRNA instability, whereas haemolymph represents a comparatively less degradative internal environment for dsRNA. This stability may support the development of RNAi-based sustainable pest management approaches (Fletcher *et al*., 2020).

CONCLUSION

The present study demonstrates that dsRNA stability differs markedly between haemolymph and whole-body extracts of adult *S. frugiperda*. When haemolymph melanisation was effectively prevented, dsRNA remained detectable for longer durations in haemolymph, whereas rapid degradation occurred in whole-body extracts within 30 to 60 min.

These findings indicate that gut-associated nucleases present within composite tissue extracts represent a major constraint on dsRNA stability. In contrast, dsRNA exposure within the haemocoelic environment, which is not directly influenced by gut-derived degradative enzymes, supports greater dsRNA persistence. This work presents foundational information for future laboratory-based functional validation studies targeting adult-expressed genes in *S. frugiperda*. The findings further indicate that haemolymph provides a comparatively more permissive internal environment for dsRNA stability than gut-containing tissues, which may support the development of RNAi-based sustainable pest management approaches.

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AUTHOR CONTRIBUTION

Sabitha Chellem, D. V. Sai Ram Kumar, R. Gandhi Gracy, V. Prasanna Kumari and B. S. Manjunatha contributed to the design of the study, execution of experiments, data analysis, interpretation of results and preparation of the manuscript. All authors have read and agreed to the published version of the manuscript.