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Integrated Molecular Identification and Quantitative Analysis of Diet-Dependent Venom Variation in *Leiurus quinquestriatus* Scorpion Species

Mennat-Allah G. Elbeheri

Egypt-Japan University of Science and Technology

Ehab El-Dabaa

Theodor Bilharz Research Institute

Sherif F. Hammad

Egypt-Japan University of Science and Technology
Helwan University

Ahmed Osman

Egypt-Japan University of Science and Technology
Ain Shams University

Abstract: Scorpions are ancient and successful arthropods that have lived on Earth for millions of years. A key feature for their evolutionary success is the presence of specialized venom glands, which produce the venom. Scorpion venom is a complex mixture of bioactive compounds, and its composition and yield are influenced by various ecological factors. This study investigated the effect of prey type on venom yield and protein profile in the medically significant scorpion *Leiurus quinquestriatus* (the "deathstalker"), a species renowned for venom containing therapeutic peptides such as chlorotoxin. Specimens were molecularly identified using 16S and 12S rDNA sequencing to confirm species identity. A four-month feeding experiment (n = 50) was conducted to evaluate two different dietary regimes. The results demonstrated that the mitochondrial 16S and 12S rDNA markers are reliable tools for scorpion identification. Dietary variation significantly influenced venom yield; scorpions fed a super mealworm diet exhibited a 64% increase in venom yield compared to the alternative regime. Notably, no cannibalistic behavior was observed among the specimens studied during the experiment. Furthermore, SDS-PAGE analysis revealed distinct protein banding patterns between the dietary groups, indicating diet-induced alterations in venom protein composition. These findings highlight the critical impact of diet on scorpion venom production. Understanding these factors is essential for optimizing venom yield for ecological studies and venom-based biodiscovery.

Keywords: Scorpion, 16S, 12S rDNA genes, Scorpion venom, Prey, Venom yield, SDS-PAGE.

Introduction

Scorpions (phylum Arthropoda, class Arachnida) are ancient species, existing on earth for 430 million years. Distributed all over the earth, they are adaptive, venomous animals. 19 of scorpions, which include more than 2200 species, have been identified so far. Out of them, the Buthidae family is well known for its hazardous envenomation, which also possesses medicinal importance (Hassan et al., 2024; Lafnoute et al., 2022). Naturally, scorpions use the stinger at the end of their tail to inject venom into predators as a defense mechanism or into prey to paralyze and capture them. Scorpion venom—that produced from venom glands—is composed of a heterogeneous mixture of compounds, including varying concentrations of proteins, enzymes, lipids, free amino acids, and others (Nasr et al., 2023).

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In the last decades, scorpion venoms have been identified as a potential natural resource for designing antitumor, antibacterial drugs. Several lines of evidence have revealed that scorpion venoms and venom-derived peptides suppressed tumor cell proliferation, metastasis, and induced tumor cell apoptosis (Sadeghi et al., 2024). Understanding the factors that influence venom composition and yield is therefore essential for both ecological studies and venom-based biodiscovery.

The scorpion genus *Leiurus* is one of the most medically important species in North Africa and the Middle East. The *Leiurus quinquestriatus* species, commonly known as “death stalker,” found in upper Egypt and Sudan in dry habitats and is one of the most dangerous species; it causes severe envenoming and high death rates because its venom is highly neurotoxic (Borges & Lomonte, 2024; Salama & Sharshar, 2013).

Accurate species identification represents a fundamental step in venom research, as venom composition can vary significantly among closely related scorpion species. Traditional morphological identification is often limited by phenotypic similarity and intraspecific variation. Consequently, molecular approaches, particularly the use of mitochondrial markers such as the 16S and 12S rDNA gene, have become powerful tools for reliable species identification and phylogenetic analysis in scorpions.

By integrating molecular identification, quantitative venom analysis, and protein profiling, this work provides a comprehensive evaluation of diet-associated venom variation. The results contribute to a better understanding of how nutritional factors influence venom production and demonstrate the importance of combining experimental biology with statistical and data-oriented analysis in venom research.

In this study, mitochondrial 16S, 12S rDNA sequencing was employed to confirm species identity, while controlled dietary experiments were conducted to investigate the effects of different prey types on venom yield. Demonstrated scorpion behavior. Venom samples obtained under different feeding regimes were analyzed using SDS-PAGE to compare protein profiles. By integrating molecular, biochemical, and ecological approaches, this research aims to enhance our understanding of scorpion venom dynamics and contribute to optimized scorpion rearing practices and venom-based biomedical research.

Methods

Scorpion Collection

Adult scorpions, *L. quinquestriatus* (n=50), were collected at night by professional hunters in the Sinai region of Egypt using UV light and extra-long forceps. They were observed walking on sand or burying themselves beneath rocks and wood materials. After observation, the scorpions were placed in separate containers filled with sand and equipped with holes for adequate ventilation before being transported to the laboratory.

Molecular Identification

DNA Extraction

DNA was extracted from the muscle of the scorpion tail by using Nucleic Acids extraction RealBest-Vet Gamma, (Novosibirsk, Russia). According to the manufacturer's protocol, briefly, muscles were isolated and chopped, 400 µl of lysis buffer were added, and they were placed in a heat block at 65°C for 10 minutes. After adding 400 µl of precipitating solution, the mixture was pipetted and then transferred to the spin column and centrifuged at 10,000 xg for one minute. Then wash twice with buffer 1 and 2. Finally, 200 µl of elution buffer was used. Concentration and quality of the DNA was determined by nanodrop (Thermo Scientific NanoDrop 2000C Spectrophotometer).

Real-Time Polymerase Chain Reaction

Amplification of 16S rDNA gene by using primer pairs, Sc- 16F 5'-CGATTTGAACTCAGATCA-3' and Sc-16R 5'- GTGCAAAGGTAGCATAAT-3, For amplification of 12S rDNA, the primers Sc-12F 5'-AGAGTGACGGGCAATATGTG-3' and Sc-12R 5'- CAGCGGCTGCGGTTATAC-3' were used. (Macrogen, Seoul, South Korea). (Soltan-Alinejad et al., 2021). The qPCR amplification reaction was performed with a final

mixture of 20 µl, 5 µl of 4X CAPITAL qPCR Green Master Mix (Berlin, Germany), 5 µl of purified DNA, 1.5 µl of each forward and reverse primers, and completed to 20 µl with PCR-grade water.

The amplification conditions for all fragments were as follows: Hold the temperature at 95°C for 2 minutes, then 45 cycles of 95°C for 10 seconds and 46°C for 30 seconds. Then the melt setup conditions were a ramp from 50°C to 90°C raised by 1 degree at each step, wait for 90 seconds of pre-melt conditioning on the first step and 5 seconds for each step afterwards to measure the fluorescence.

Gradient PCR

For the PCR amplification reaction performed in 50 µl of the final mixture, consisting of 25 µl COSMO PCR red master mix (Birmingham, UK), 2 µl of each primer, 5 µl of the purified DNA, and completed to 50 µl with 16 µl of PCR-grade water. Confirmation for amplifications and purity of the genes were electrophoresed on 1.5 agarose gel and then properly sized PCR product were sent for sequencing.

Sequence Analysis

The sequence obtained from 16S rRNA was trimmed using Bioedit Software and then compared to sequences from the GenBank, Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), to find the similarity. All the sequences received from the NCBI database together with the sequences from the present study, were aligned by ClustalW implanted in MEGA12 software. Phylogenetic trees were inferred by using the Maximum Likelihood (ML) tree, and the best-fit nucleotide substitution model of each was selected by using the Bayesian information criterion (BIC).

For 16S and 12S, genes from *Leiurus. quinquestriatus*, the phylogeny was inferred using the Maximum Likelihood method and Tamura (1992) model, with the evolutionary rate differences among sites modeled using a discrete Gamma distribution across 4 categories. The initial tree for the heuristic search was selected by choosing the tree with the superior log-likelihood between a Neighbor-Joining (NJ) tree and a maximum parsimony tree (MP). The NJ tree was generated using a matrix of pairwise distances computed using the Tamura model. The partial deletion was applied to eliminate all positions with less than 90% site coverage. The robustness of the inferred tree topology was assessed by bootstrap analysis with 1000 replicates, for the evolutionary history of 12S gene from *L. quinquestriatus* was inferred using the Neighbor-Joining method (NJ), the evolutionary distances were computed using the Tamura 3-parameter method.

Scorpion Rearing

In the laboratory, scorpions were transferred to wide, clean boxes, filled with 2 inches of sand. Properly sized stones and leaves were placed in each box. The boxes and their lids were designed to provide sufficient adequate ventilation for the scorpions and were positioned away from direct sunlight. conduct daily monitoring of environmental conditions such as temperature and 12:12 light cycle. Females bearing small juveniles were each placed in separated clean boxes. After 2-3-week period, once the young scorpions were off their mother's back, the young were separated into another boxes, fed with mealworms.

Behavioral Observation

During the first two weeks following the arrival of the scorpion species, a group of 20 scorpions were assembled to observe their interactions with one another. There were three boxes designated for *L. quinquestriatus*, accommodating a total of 50 scorpions. During this observation period, minimal interference was implemented to avoid any external influences on their natural behavior while keeping records of cannibalism incidents and their movements.

Impact of Dietary Intake on Venom Yield

Scorpion species had received two types of prey: live super mealworms and live red runner roaches once a week. Fifty scorpions were divided into two independent dietary groups (n = 25 each). One group was fed on

super mealworms, while the other was fed on roaches for four months. Venom volume data were expressed as mean $\pm$ and standard deviation (SD). Differences between dietary groups were analyzed using independent two-sample t-test assuming equal variances to compare venom yield between dietary groups, with statistical significance set at $p < 0.05$. Water sources were ad libitum. Sand must be changed monthly; any remnants of food must be removed; and the boxes should be in a hygienic condition.

Extraction and Preparing the Venom

Venom was collected by applying electrical stimulation (10– 12 V) to the articulation of the telson while carefully holding the scorpion with the left hand and gripping the stinger at the end of its tail between the index finger and thumb. Venom was extracted twice per month. The extracted venom collected in a clean Eppendorf tube, solubilized in distilled water in 1:4 ratio, centrifuged at 10,000 xg, 4°C, for 15 min to remove debris, and then the supernatant. The supernatants were pooled, lyophilized, and stored at -80 °C until use (Yoshimoto et al., 2019).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE):

SDS-PAGE was performed following the method from Laemmli 1970 (Laemmli, 1970), Venom samples were collected from scorpion species with different diets; the first venom sample was obtained from scorpions that consumed super mealworms, while the second sample was from scorpions that were fed with red runner roaches. the two venom samples were analyzed using a Tris-glycine running buffer system under reducing conditions. Samples were mixed with 3x loading dye and Bio- HELIX IRIS11 prestained as the protein ladder (3-260 KDa). 15%, resolving gel (29% acrylamide:1% bis acrylamide mix) and 5% stacking gel was carried out for protein bands' molecular weight analysis. The gels were stained overnight with Coomassie Brilliant blue R250, and the molecular weight of the separating bands was determined by GelAnalyzer 23.1.1 software.

Results and Discussion

Molecular Identification

Real-Time PCR Amplification

The DNA extracted from the tail muscle of scorpion species was analyzed first on Nanodrop, the concentration of the DNA was 200 ng/μl. Real-time PCR amplification was performed by using SYBR green. The melting curve, as in (Figure 1), showed a single distinct peak indicating amplification specificity for the gene without primer dimers or non-specific products.

Gradient PCR

The PCR product of the gene was electrophoresed on a 1.5% agarose gel, as shown in Figure 2; single, clear, distinct bands resulted with sizes of about 452 kDa and 563 kDa for 16S and 12S rDNA, respectively, which indicate the purity of the amplified gene.

Sequence Analysis

Molecular identification for *Leiurus quinquestriatus* was performed using 16S and 12S rRNA. The obtained sequences, which were made with triplicates, from the scorpion species were aligned with the sequences obtained from the GenBank. Blast analyses revealed 100% sequence identity, as in (Figure 3A). Regarding the 16S phylogenetic tree, the resulting ML tree consisted of thirty-three sequences formed from multiple *Leiurus quinquestriatus* together with outgroup taxa such as *Androctonus*, *Buthus*, and *Odontobuthus*. The scorpion specimen formed a basal lineage relative to *L. quinquestriatus* sequences; it was clearly separated from *Buthus* and *Androctonus* species. however, the deeper node of the specimen connected with the *L. quinquestriatus* lineage with a 100% bootstrap value (Figure 3B). For the 12S gene phylogenetic analysis, it was found to be directly clustered within the *L. quinquestriatus* clade with 78% bootstrap support, clearly separated from *Buthus* and *Androctonus* species (Figure 3 C).

Behavior of the Scorpions

In the study of the scorpion *L. quinquestriatus*, no aggression was observed among individuals, and cannibalism did not occur. They tend to hide under rocks and leaves during the day and do not exhibit vigorous movement at night. Consequently, we house every 10 scorpions together in the same box.

Dietary Intake Effect on Venom Yield

After offering the two different types of prey for scorpion species, venom yield after consuming super mealworms was higher than after roaches. Scorpions fed on super mealworms produced significantly higher venom volumes compared to those fed on roaches. The mean venom volume for the Super mealworms group was 137.5 μ L, while the roaches group produced a mean of 83.75 μ L. Statistical analysis revealed a highly significant difference between the two dietary treatments (independent t-test, $t = 11.93$, $df = 14$, $p < 0.0001$), table 1, (Figure 4).

SDS-PAGE

Lane L1, which was venom from the *L. quinquestriatus* scorpion, fed with super mealworms; lane L2, which was from the same scorpion species but fed with red runner roaches. Two protein bands appear in both venom samples, common between L1, and L2 samples, of sizes 213, 50 kDa. A bulk of Unseparated small peptides appear in both samples (Figure 5).

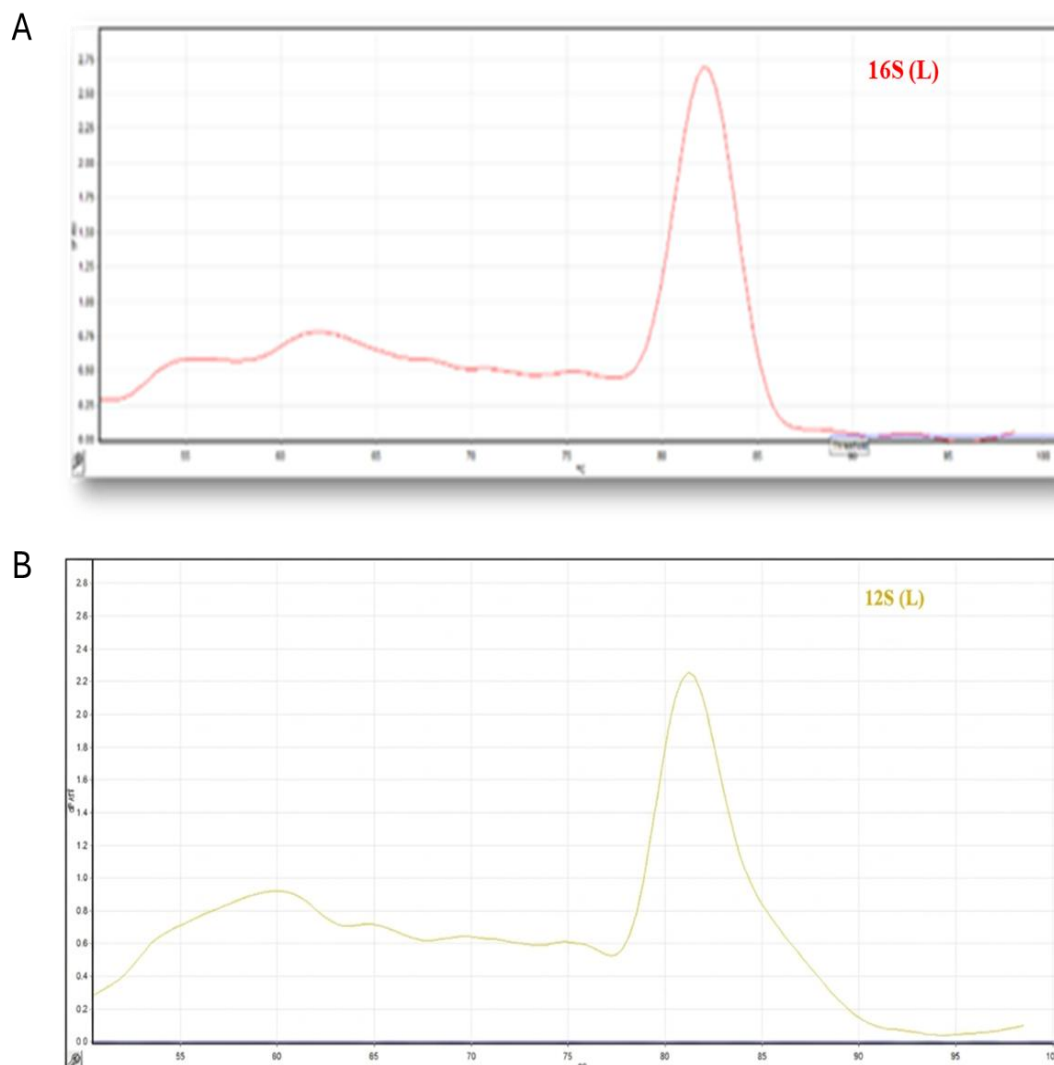


Figure 1. A: Melting curve of 16S rDNA. B: 12S rDNA for *L. quinquestriatus*

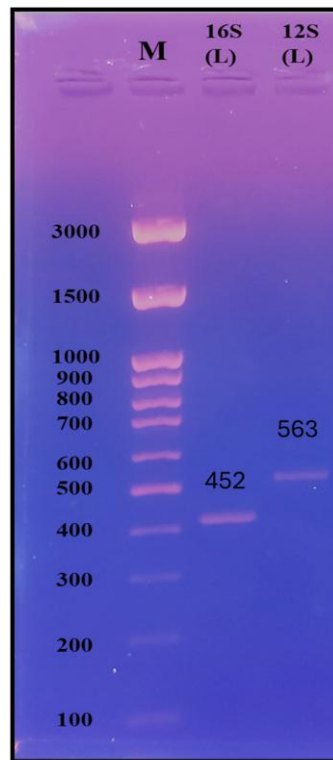


Figure 2. 1.5 agarose gel of the PCR product, M: marker protein. 16S (L): 16S and 12S rRNA gene of *L. quinquestriatus* scorpion.



Figure 3. A: Studied scorpions (*L. quinquestriatus* highlighted in yellow color) occur within leirus lineage: 16S rRNA gene sequences analysis, the yellow arrow is the tested scorpion (labelled Scorpion 1 16S), clustered with 100% bootstrap support with leirus lineage. C: 12S rRNA gene sequences analysis of the tested scorpion (yellow arrow) clustered within *L. quinquestriatus* clade.

Table 1. Comparison of mean venom yield between *L. quinquestriatus* scorpions fed on super worms androaches over a four-month period.

Dietary group	Super mealworms	Roaches
Standard Deviation (SD)	10.35	7.44
Variance	107.14	55.36
Mean yield (μL)	137.5	83.75
N (Sessions)	8	8
P-value	< 0.001	

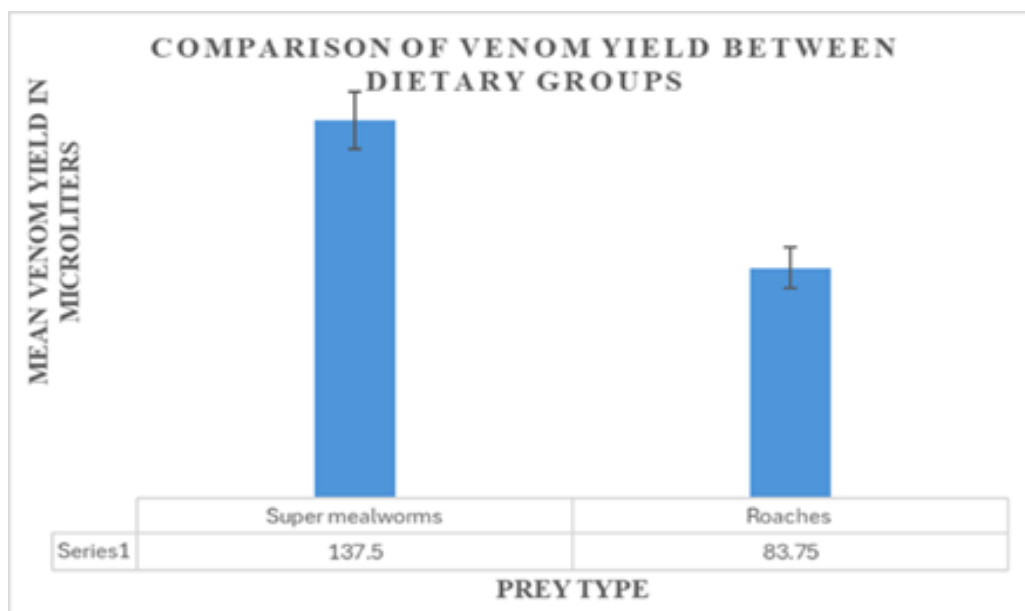
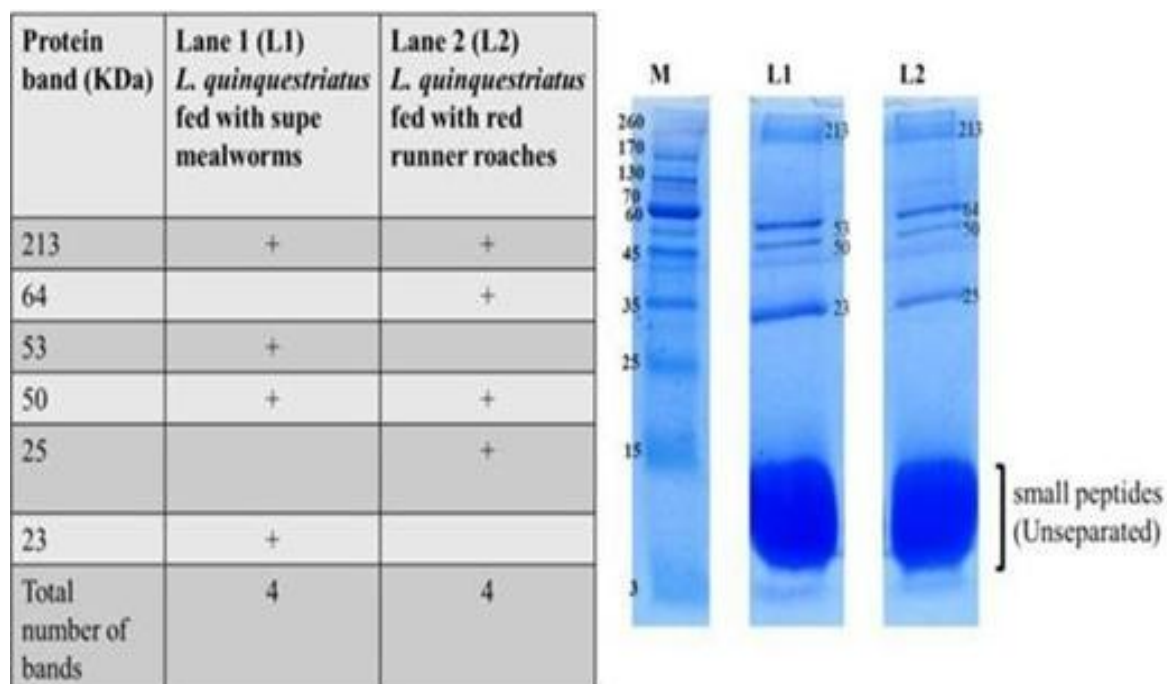


Figure 4. Comparison of mean venom yield between scorpions fed on superworms and those fed on cockroaches over a 4-month period. Data is presented as mean $\pm$ standard deviation (SD) of 8 milking sessions per group.

Statistical significance was determined using an independent two-sample t-test assuming equal variances



This study successfully combined molecular, behavioral, and physiological approaches to provide an integrated understanding of scorpion biology and venom production. Molecular identification using the mitochondrial 16S rRNA marker proved to be a reliable and effective tool for accurate species. Identification, particularly in cases where morphological differentiation may be challenging. Accurate molecular identification is essential for venom studies, as even closely related species may exhibit substantial variation in venom composition and potency. Confirming its suitability for taxonomic and evolutionary studies of scorpions, the scorpion specimens studied were confirmed to belong to a single species, *Leiurus quinquestriatus*, ensuring that observed differences in venom profiles were not due to taxonomic misidentification.

Dietary variation was found to have a significant influence on venom yield, highlighting the role of nutritional factors in venom production. Scorpions fed on different prey types exhibited measurable differences in venom quantity; scorpions that were fed on super mealworms produced significantly higher venom volumes compared to those fed on roaches. This difference was supported by statistical analysis. This finding suggests that nutritional composition plays a crucial role in venom synthesis and replenishment, likely due to differences in protein and energy availability required for venom production. Such results are consistent with previous studies indicating that venom is metabolically expensive and closely linked to feeding efficiency and prey quality, as in (Tobassum et al., 2018), where it was found that grasshopper nymphs and adults were the best diet for the scorpions to get a higher yield of venom as compared to house crickets, moths, and house flies.

In addition, behavioral observations revealed distinct feeding, defensive, and activity patterns associated with each diet, indicating a close relationship between nutrition, behavior, and venom utilization. Scorpions subjected to different feeding regimes exhibited variations in feeding behavior, activity levels, and defensive responses. These behavioral changes may reflect adaptive strategies aimed at optimizing venom use in relation to prey types and availability. The association between diet, behavior, and venom characteristics underscores the importance of considering ecological factors when studying venom dynamics. Biochemical analysis using SDS-PAGE and the comparative analysis revealed distinct banding patterns and variations in protein abundance between individuals fed super mealworms and those fed red runner roaches, indicating that prey type can modulate venom expression.

Overall, the integration of molecular, biochemical, and behavioral data provides a comprehensive understanding of how dietary variation influences scorpion venom expression. These findings have important implications for scorpion rearing protocols, venom extraction efficiency, and venom-based biodiscovery. Understanding venom variability under controlled conditions may enhance the reproducibility and biomedical relevance of future venom research.

Conclusion

In conclusion, this research bridges molecular taxonomy, nutritional ecology, and toxicology, highlighting the need to consider dietary history in both basic venom studies and applied bioprospecting efforts. *L. quinquestriatus* fed on super mealworms produced significantly higher venom volumes compared to those fed on roaches, indicating a strong diet-dependent effect on venom yield. SDS-PAGE analysis revealed observable differences in band intensity between the two dietary groups, suggesting potential variation in venom protein profile.

Furthermore, the use of 16S and 12S rRNA molecular barcoding proved to be an efficient and reliable tool for species identification of venomous scorpions. This technique enabled accurate taxonomic classification of collected specimens, which is critical for ensuring reproducibility in venom research and avoiding misidentification-related errors in downstream pharmacological applications.

Recommendations

Future studies are recommended to apply Tris-Tricine SDS-PAGE to achieve better resolution of low-molecular weight peptides present in scorpion venom. This technique would allow more accurate separation and characterization of venom peptides, which are often biologically active and of high pharmacological interest. Incorporating Tris-Tricine analysis alongside conventional SDS-PAGE may provide deeper insights into venom peptide diversity and enhance the potential for identifying novel bioactive compounds relevant to therapeutic applications.

And future work should expand to functional assays (e.g., antimicrobial or cytotoxic activity) to determine whether dietary-induced changes in venom also translate into altered biological activity.

Scientific Ethics Declaration

* The authors declare that the scientific ethical and legal responsibility of this article published in EPSTEM journal belongs to the authors.

Conflict of Interest

* The authors declare that they have no conflicts of interest.

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Author(s) Information

Mennat-Allah Gamal Elbeheri

Egypt-Japan University of Science and Technology (E-JUST), Faculty of Basic and Applied Science, Biotechnology program, New Borg El-Arab City, Alexandria, Egypt.

Zoology department, Faculty of Science, Alexandria, Egypt.

ORCID iD: <https://orcid.org/0009-0008-2495-020X>

* Contact e-mail: Mennaallah.gamal@ejust.edu.eg

Ehab El-Dabaa

Theodor Bilharz Research Institute (TBRI), Biochemistry and Molecular Biology Department, 30 Imbaba, P.O box 12411, Giza, Egypt

ORCID iD: <https://orcid.org/0000-0002-6080-5631>

Sherif Hammad

Egypt-Japan University of Science and Technology (E-JUST), Medicinal Chemistry Department, PharmD Program, New Borg El-Arab City, Alexandria, Egypt.

Pharmaceutical Chemistry Department, Faculty of Pharmacy, Helwan University, Ain Helwan, Egypt

ORCID iD: <https://orcid.org/0000-0001-8869-5967>

Ahmed Osman

Egypt-Japan University of Science and Technology (E-JUST), Faculty of Basic and Applied Science Biotechnology program, New Borg El-Arab City, Alexandria, Egypt.

Biochemistry department, Faculty of Science, Ain Shams University, Cairo, Egypt

ORCID iD: <https://orcid.org/0000-0002-3506-8254>

*Corresponding author(s) contact email

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