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Original Article

Biochemical alteration of golden apple snails, *Pomacea canaliculata* (Lamarck, 1822), and giant African snails, *Achatina fulica* (Bowdich, 1822) post-infection by indigenous Thai entomopathogenic nematodes

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Abstract

Golden apple snails (*Pomacea canaliculata* (Lamarck, 1822)) and giant African snails (*Achatina fulica* (Bowdich, 1822)) are among the most harmful invasive species that have spread across Thailand and numerous other countries. This study investigated the biochemical changes in these snails after infection by indigenous Thai entomopathogenic nematodes (EPNs). Five indigenous EPN isolates were used: *Heterorhabditis indica* (eAUT13.2_TH), *H. bacteriophora* (eALN18.2_TH), *Steinernema lamjungense* (eALN11.5_TH), *S. siamkayai* (eAPL10.3_TH), and *S. surkhetense* (eALN6.3_TH). Two-, three-, and four-month-old golden apple snails and three- and five-month-old giant African snails were infected with the EPNs at a density of 300 IJs/1.0 ml per snail. Biochemical analyses was conducted at 12 hours, 24 hours, and ten days after infection to assess the impact of EPNs on the snails' biochemistry. The results revealed significant changes in biochemical parameters after infection. After 12 and 24 hours, the average concentrations of total protein and uric acid decreased, while AST (Aspartate Transaminase) and ALT (Alanine Transaminase) levels increased. Furthermore, after ten days, AST and ALT concentrations continued to increase, while total protein and uric acid levels further declined. All tests indicated statistically significant differences between the control and infected groups. These findings demonstrate that EPN infections can significantly affect biochemical parameters in non-primary host snails, ultimately leading to the death of the infected snails.

Keywords: *Pomacea canaliculata, Achatina fulica,*Biochemical alteration, Mollusk pests

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Introduction

Among the most destructive invasive mollusk pests that have spread to many countries are the golden apple snail (*Pomacea canaliculata* (Lamarck, 1822)) and the giant African snail (*Achatina fulica* (Bowdich, 1822)). Initially introduced to Thailand for specific purposes, these snails were subsequently abandoned and have since become invasive species. Their ability to feed on various plant species, including economically important crops, makes them significant agricultural pests in Thailand (Printrakoon and Bullangpoti, 2021). Furthermore, both species serve as intermediate hosts for *Angiostrongylus cantonensis*, a parasite causing human angiostrongyliasis (Eamsobhana, 2014; Dumidae et al., 2021). Chemical molluscicides are the primary and most effective intervention to control mollusk pests in Thai agriculture. Molluscicides are an effective rapid and convenient solution to reduce the spread and damage caused by snails (Zheng et al., 2021). However, their use risks the environment and threatens non-target organisms, including plants, animals, and even humans, such as farmers and consumers (El-Gendy et al., 2021; Sudmoon et al., 2023).

Biological control agents have been used worldwide to protect crops from insect pests, providing an alternative to toxic chemical pesticides (Dongjing et al., 2024). Among these agents, entomopathogenic nematodes (EPNs) from the Heterorhabditidae and Steinernematidae families are particularly effective. These EPNs carry symbiotic bacteria that are released into the host's hemolymph once they enter. Once inside the host, usually an insect, the symbiotic bacteria rapidly induce host death (Grewal et al., 2011; Loulou et al., 2023). They do this by releasing endotoxins and producing secondary metabolites, such as enzymes, which disrupt the host's physiological functions. Additionally, the bacteria utilize various defensive mechanisms to evade and suppress the host's immune system (De Mandal et al., 2021; Li et al., 2007). EPN infections not only kill insect hosts but also influence their enzymatic activities and alter biochemical parameters, such as total protein, cholesterol, and glucose levels (El-Sadawy et al., 2009; Shaurub et al., 2015). Given their effectiveness, EPNs and their symbiotic bacteria are widely employed to manage a variety of insect pests (Askary and Abd-Elgawad, 2021). Moreover, EPNs have shown potential to control mollusks, such as slugs, land snails, and freshwater snails, including pest species and intermediate mollusk hosts like *Bradybaena similaris* (Férussac, 1822), *Pseudosuccinea columella* (Say, 1817), and *Lymnaea columella* (Say, 1817) (Saeedizadeh and Niasti, 2020; Schurkman and Dillman, 2021).

Symbiotic bacteria associated with EPNs have been shown to interfere with the biochemical and physiological processes of various hosts. The ability of EPNs to disrupt host survival depends on factors such as nematode-host interactions, host specificity, parasitism physiology, and resistance mechanisms (Poinar and Grewal, 2012). Previous studies have investigated physiological changes in hosts following EPN infection, which can lead to behavioral changes in the snails (Tunholi et al., 2017a). Although snails are not definitive (primary) hosts for EPNs, the nematodes can still cause significant mortality in these organisms (unpublished data). Consequently, understanding how EPNs affect the metabolic pathways of snails is an important research area. Both snail species are harmful pests to many crops in Thailand, where management strategies, including the use of chemical molluscicides, have been employed. However, chemical molluscicides pose environmental risks, particularly to waterways and aquatic organisms. Therefore, biological control agents, such as EPNs, are considered safer alternatives to chemical molluscicides. The ability of EPNs to kill indefinitive (non-primary) hosts, such as snails, remains unclear. This study aimed to investigate the impact of indigenous Thai EPNS on the biochemical parameters of golden apple snails and giant African snails which may contribute to their death. These findings may help clarify the potential use of EPNs as biological control agents against indefinite hosts which are agricultural pests like snails.

Material and Methods

Collection and rearing of golden apple snails and giant African snails

Snail samples were collected from Uttaradit and Phitsanulok provinces in northern Thailand. Golden apple snails were gathered from various habitats, including paddy fields, canals, and rivers, and were primarily identified based on external shell morphology, as described in previous studies by Dumidae et al. (2021). Giant African snails were collected from gardens, undergrowth, rubble, and crevices, where they typically seek shelter, between

May and September, the rainy season in Thailand. Identification of giant African snails was based on shell morphology according to Jena et al. (2017).

Four-five clusters of golden apple snail eggs were reared in glass aquariums (25 x 50 x 30 cm) filled halfway with dechlorinated water, under laboratory conditions at 30 ± 2°C, 60-75% humidity with a 12:12 light-to-dark cycle until they hatched. After hatching, the snails were kept in similar conditions. All snails were raised from hatching in the laboratory, under similar conditions to verify their ages before being transferred to testing containers at the appropriate developmental stage. Adult golden apple snails fed duckweed (*Lemna minor* L.) and water lettuce (*Pistia stratiotes* L.), while giant African snails were provided with fresh lettuce (*Lactuca sativa* L.) and other soft-stemmed or leafy vegetables. The aquariums were maintained by regularly changing the water, cleaning the glass, and replacing the food every two-three days. Dead snails were promptly removed when found. All snails were reared for 2-3 generations before being used in the experiments.

Multiplication of the entomopathogenic nematodes

This study used five isolates of indigenous Thai EPNs i.e., *Heterorhabditis indica* (eAUT13.2_TH), *H. bacteriophora* (eALN18.2_TH), *Steinernema lamjungense* (eALN11.5_TH), *S. siamkayai* (eAPL10.3_TH), and *S. surkhetense* (eALN6.3_TH) (Ardpairin et al., 2023). The nematodes were obtained from a culture collection and propagated using the final instar larvae of the greater wax moth (*Galleria mellonella* L.) as hosts. Following the White trap method as quoted by Manzoor et al. (2017), the caterpillar cadavers were placed in sterilized Petri dishes. After seven-ten days, the EPNs emerged into the surrounding water in their infective juvenile (IJ) stage. The IJs were then transferred to sterile flasks containing sterilized distilled water and stored at 12-15°C for approximately one week before being used.

Infection of snails under laboratory conditions

Infection experiments were conducted on golden apple snails aged two, three, and four months and giant African snails aged three and five months, as age influences susceptibility and the severity of EPN infection. Each snail was placed in an individual cylindrical plastic container (10 cm diameter × 5 cm height). The experiment followed a completely randomized design (CRD) with two treatments: 1) EPN infection, in which each golden apple snail was inoculated with ten ml of distilled water containing 300 IJs/ml, and each giant African snail was inoculated with five ml of distilled water containing 600 IJs/ml, in which EPN density was determined from preliminary experiments, and 2) a control treatment using the same volume of sterilized distilled water.

Collection of the hemolymph

Hemolymph was collected from both control and infected snails after being anesthetized on ice for 15-20 minutes. Sampling occurred at three time points: 12 hours, 24 hours, and upon death (up to ten days after infection). For golden apple snails, hemolymph was collected via cardiac puncture following the method of Accorsi et al. (2013), while for giant African snails, the procedure described by Ademolu et al. (2004) was used. A maximum of one ml of hemolymph was collected from each snail, transferred to an Eppendorf tube, and stored at -10°C around 1 week until biochemical analyses could be performed.

Determination of total proteins

The Biuret method was used to determine the total protein content in the snails, following the procedure described by Fenk et al. (2007). Protein concentration was measured by recording the absorbance at 550 nm using a spectrophotometer, and the results were expressed in mg/dl.

Determination of uric acid

Following Bishop et al. (1996), the determination of uric acid levels was carried out as follows; 50 µl of hemolymph was mixed with 2 ml of dye reagent. The mixture was homogenized and incubated at 37ºC for 5 minutes. The absorbance was measured at 505 nm using a spectrophotometer, and the results were expressed in mg/dl.

Determination of aspartate aminotransferase (AST) and Alanine transaminase (ALT)

AST and ALT concentrations were determined following the protocol of Tunholi et al. (2014). Absorbance was measured at 505 nm using a spectrophotometer, and the results were expressed as U/ml.

Statistical analysis

Each treatment was replicated three times, with five snails per replication. Statistical analyses were performed using SPSS Statistics (version 17.0). The concentrations of total protein, uric acid, AST, and ALT were expressed as mean values. One-way ANOVA followed by Tukey's HSD test was applied to assess significant differences between treatment means ($p < 0.05$).

Results

Total protein concentration

This study is the first to evaluate the biochemical changes in golden apple snails (*Pomacea canaliculata* (Lamarck, 1822)) and giant African snails (*Achatina fulica* (Bowdich, 1822)) after infection by five isolates of Thai indigenous EPNs. The results revealed that, although snails are not definitive hosts for these EPNs, all tested snails were killed within ten days. Both snail species showed a decrease in total protein concentration after infection, with a slight decrease observed during the first 12 hours and a significant decline from 24 hours until death. When comparing golden apple snails across different age groups, older snails (three and four months old) exhibited a more rapid decrease in protein levels than younger snails (two months old) (Figure 1A-C). In two-month-old snails, total protein levels began to decline at 12 hours post-infection but did not significantly differ from the control group (2.57±0.24 mg/dl). However, at 24 hours, the decline accelerated significantly, with levels remaining lower than the control group until death, within ten days of infection ($p < 0.05$) (Figure 1A). In contrast, three-month-old (Figure 1B) and four-month-old snails (Figure 1C) showed significant reductions in total protein at 12 hours after infection, persisting through the experiment. These reductions differed significantly from the control group (3.61±0.33 mg/dl) and between isolates ($p < 0.05$). All isolates followed a similar trend in reducing protein content across different age groups of golden apple snails. Notably, for two-month-old golden apple snails at 12 hours after infection, *S. siamkayai* (eAPL10.3_TH) (0.12±0.95 mg/dl) was more effective in reducing protein levels than other isolates. However, no significant diffferences in protein reduction were observed among the EPN isolates for three- and four-month-old snails.

Similar to the golden apple snails, the total protein content in giant African snails decreased after infection, with both age groups showing only a slight downward trend at 12 and 24 hours after infection (Figure 1D-E). However, the age-related trend was opposite of that observed in golden apple snails: in giant African snails, the younger group (three months old) had a greater decrease in total protein levels compared to the older group (five months old) by the end of the ten-day post-infection period. At this point, protein levels had decreased to approximately half of their initial values. (Figure 1D-E). In both age groups, all EPN isolates showed a comparable ability to significantly decrease total protein content compared to controls ($p < 0.05$).. In five-month-old snails, *S. lamjungense* (eALN11.5_TH) was particularly effective in reducing protein levels (4.32±0.65 mg/dl) at 12 hours and 24 hours after infection, showing a significant difference from the control group (6.54±0.19 mg/dl). However, no significant differences were observed between isolates at the end of the experiment ($p < 0.05$).

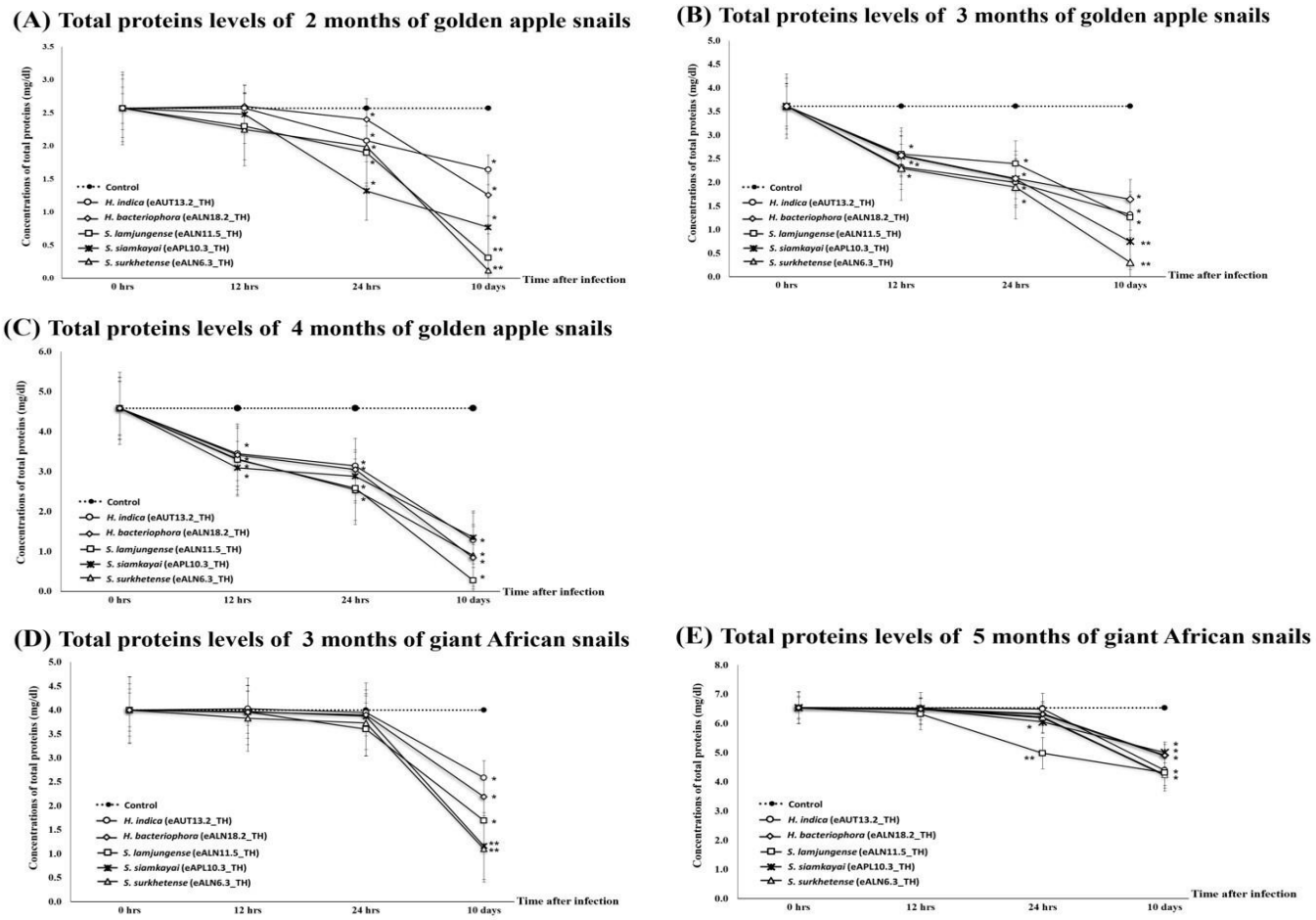

Figure 1. Mean total protein levels (mg/dl) in the hemolymph of snails after infection with EPNs: (A) two-, (B) three-, and (C) four-month-old golden apple snails, and (D) three-, and (E) five-month-old giant African snails. Each data point represents the mean from 15 samples (N=15).

Uric acid concentration

Uric acid levels were found to differ between infected and control groups of the golden apple snails, indicating changes in nitrogen excretion metabolism. In all infection stages of the golden apple snails, uric acid levels only slightly changed 12 hours after infection, but a notable decrease was observed at 24 hours, continuing through the end of the experiment at ten days (Figure 2). These reductions were statistically significant compared to the control group (p<0.05). Interestingly, in three-month-old snails infected with *S. surkhetense* (eALN6.3_TH), uric acid levels initially increased 12 hours after infection, then decreased at 24 hours, and continued to decline until the snail's death (Figure 2B). All isolates reduced uric acid concentrations, with *S. surkhetense* (eALN6.3_TH) causing a significantly greater reduction compared to other isolates and the control group (p < 0.05). In giant African snails, EPN infections resulted in similar trends in uric acid concentration across both age groups (Figure 2D-E). *S. surkhetense* (eALN6.3_TH) showed a greater capacity to lower uric acid levels in both age groups of snails, with results significantly different from both the control and other isolates.

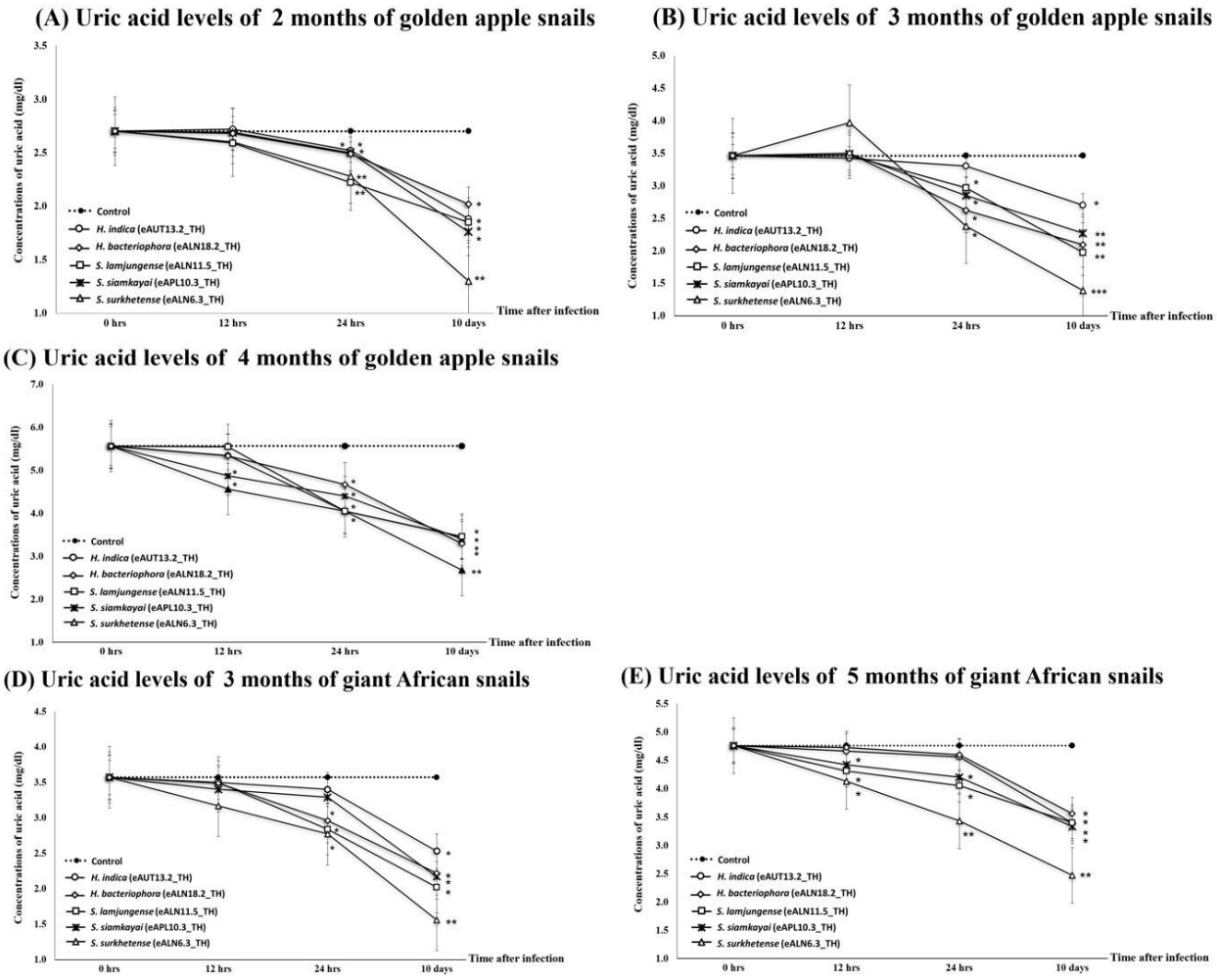

Figure 2. Mean uric acid levels (mg/dl) in the hemolymph of snails after EPN infection: (A) two-, (B) three-, (C) four-month-old golden apple snails, (D) three- and (E) five-month-old giant African snails (N=15).

AST concentration

The concentration of AST in the hemolymph of the golden apple snails slightly increased 12 hours after infection and significantly increased 24 hours after infection, up until the time of death. In all age groups of the golden apple snails (Figure 3A-C), the AST levels in the infected groups were slightly higher 12 hours after infection but did not significantly differ from the control group. However, at 24 hours, AST levels continuously increased, with *S. surkhetense* (eALN6.3_TH) having the greatest increase, while *H. bacteriophora* (eALN18.2_TH) exhibited the smallest rise from baseline. AST levels for *S. surkhetense* (eALN6.3_TH) were significantly different compared to other isolates and the control group ($p < 0.05$).

In three-month-old giant African snails (Figure 3D), AST levels showed a slight increase 12 hours after infection, although the change was not statistically significant compared to the control. By 24 hours, AST levels had gradually risen and continued to rise until the ten-day mark. Similar results were observed in the five-month-old snails (Figure 3E), and the results were significantly different from the control group. All EPN isolates showed similar effects on AST levels with *S. surkhetense* (eALN6.3_TH) causing the most pronounced increase and were significantly different from other isolates and the control group ($p < 0.05$).

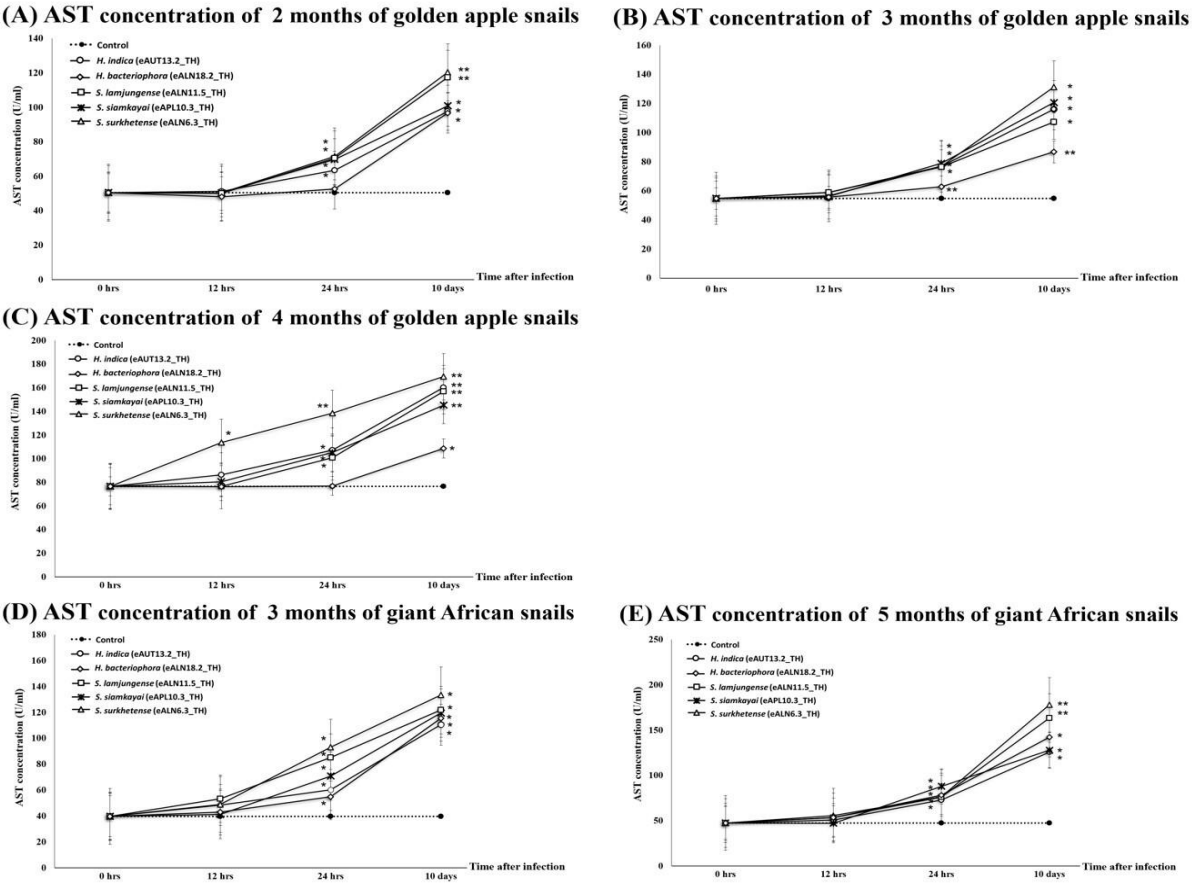

Figure 3. Mean concentrations of AST (U/ml) in the hemolymph of snails following EPN infection: (A) two-, (B) three-, (C) four-month-old golden apple snails, (D) three-, and (E) five-month-old giant African snails (N=15).

ALT concentration

Like AST levels, the ALT levels in two-month-old golden apple snails (Figure 4A) gradually increased over time to a level approximately 50 percent higher than baseline upon death within ten days of infection. Similar alterations were found in the three-month-old (Figure 4B) and four-month-old snails (Figure 4C), with ALT gradually increasing after infection. The increased levels were statistically significant ($p < 0.05$) for all age groups and for all isolates compared to the control group. Comparing the different EPN isolates' effects on ALT levels, *S. surkhetense* (eALN6.3_TH) resulted in the highest increase across all snail ages, while *H. bacteriophora* (eALN18.2_TH) had the smallest effect. There were no significant differences in ALT levels between different EPN isolates. However, there was a significant difference between the infected groups and the control group ($p < 0.05$).

Similar to golden apple snails, ALT levels in three- and five-month-old giant African snails were much higher after 24 hours until the end of the examination (Figure 4D-E). The ALT levels in the infected snails were significantly higher than the control group at the end of the experiment. Among EPN isolates, *S. surkhetense* (eALN6.3_TH) caused the greatest increase in ALT levels in both age groups of giant African snails. The lowest increase in ALT levels was observed with *H. bacteriophora* (eALN18.2_TH) in the three-months-old snails (Figure 4D) and with *S. siamkayai* (eAPL10.3_TH) in the five-month-old snails (Figure 4E)

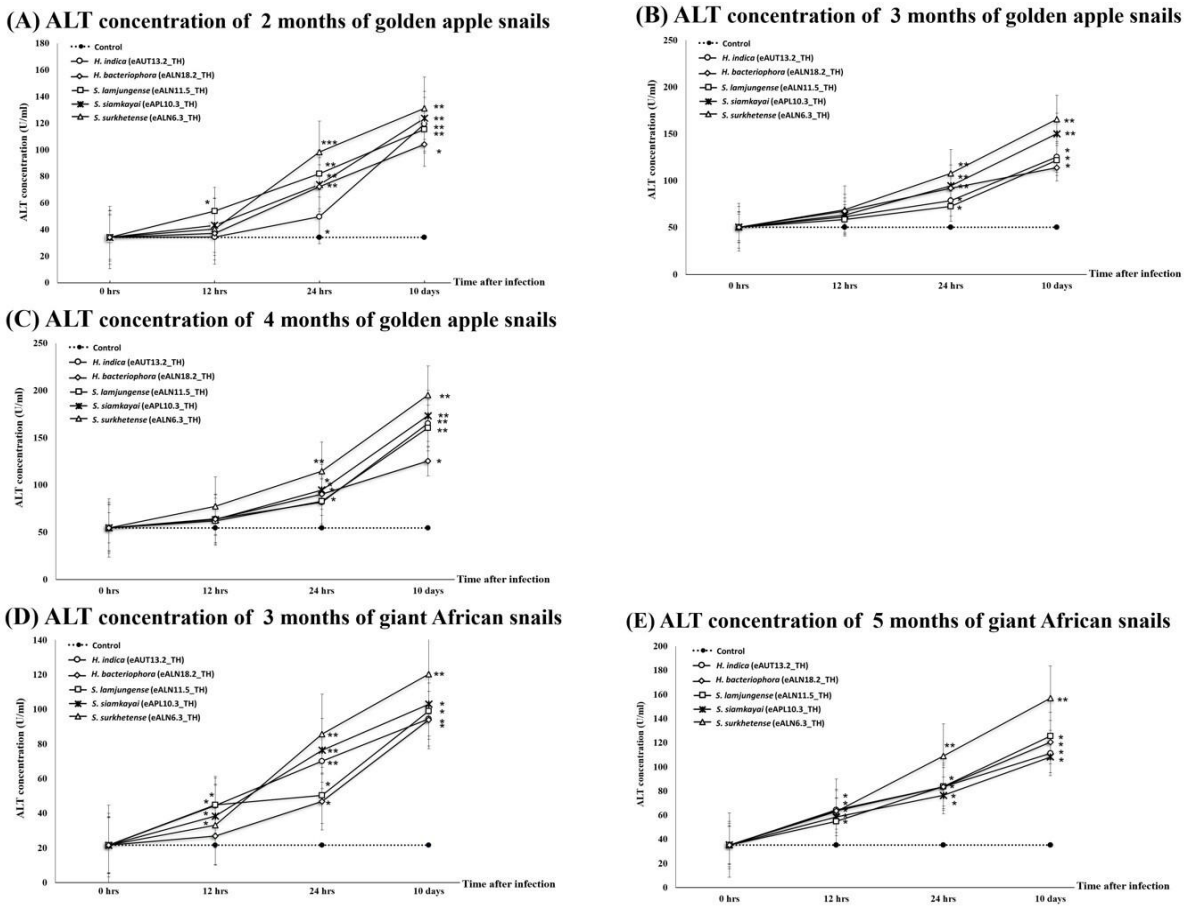

Figure 4. Mean concentrations of ALT (U/ml) in the hemolymph of snails following EPN infection: (A) two-, (B) three-, (C) four-month-old golden apple snails, (D) three-, and (E) five-month-old giant African snails (N=15).

Discussion

The results indicate that all five isolates of entomopathogenic nematodes (EPNs) affected the biochemical parameters for all life stages of infected golden apple snails and giant African snails. During EPN infection in insect hosts, their symbiotic bacteria produce endotoxins. that contribute to the mortality and biochemical changes observed in both snail species. Upon EPN infection, the the nematodes release symbiotic bacteria into the snails' hemolymph. These bacteria produce toxins, enzymes, antibiotics, and bacteriocins, such as toxin complex a (Tca), Txp40, depsipeptides, xenocoumacins, proteases, and lipases. These substances can impair the host's immune response by preventing hemocyte adhesion and inhibiting various immune related enzymes, leading to severe immune suppression and septicemia in the infected hosts (Abd-Elgawad, 2021; Dreyer et al., 2018).

Invertebrate immune responses against pathogens exhibit immunological memory and specificity that are comparable to vertebrate adaptive immunity, with mechanisms such as phagocytosis, cytotoxicity, aggregation, pathogen encapsulation, and the release of cytokines and other inflammatory proteins to eliminate foreign agents (Wang et al., 2023). Following septicemia, various systems are disrupted, leading to tissue degradation, cavity formation, and cellular disintegration that impairs normal bodily function (Hallem et al., 2007).

Although EPNs' symbiotic bacteria can alter the biochemistry of snails and eventually kill them, their secondary metabolites are tailored for the insect immune system, rendering them ineffective at suppressing the snail immune system. Histological analysis revealed no evidence of EPN proliferation within snail cadavers, despite biochemical alterations during infection (unpublished data). While damaged tissues were evident, there were no signs of

encapsulation or EPN remnants in the infected snails' tissue.s The symbiotic bacteria may induce septicemia in the hemolymph, but unlike insect hosts, they may not supply the necessary factors for EPNs to grow and reproduce in snails, which are not natural hosts. This mechanism differs from that of *Phasmarhabditis*, a parasitic nematode capable of killing slugs and certain snails, and can proliferate in snail cadavers (MacMillan et al., 2009).

The different isolates of EPNs tested in this study exhibited different levels of severity on both snail species. Although all stages of both snails underwent similar changes, two EPN isolates -*H. bacteriophora* (eALN18.2_TH) and *S. surkhetense* (eALN6.3_TH)- caused the most significant alterations in the biochemical parameters. *H. bacteriophora* is an important species used globally for the biological control of various insect pests, and previous research has shown its ability to infect snails such as *Pseudosuccinea columella* and *Biomphalaria glabrata*, inducing metabolic and reproductive changes (Sperandio et al., 2023). Younger snails of both species showed a greater response in all parameters than older snails. This is due to younger snails being more susceptible to parasitic infections, indicating that the age and size of the snails also affect their susceptibility (Williams and Rae, 2015).

The depletion of total protein in the hemolymph of both snail species occurred throughout the observation period (12, 24 hours, and up to ten days after infection), indicating that the symbiotic bacteria of EPNs caused physiological stress, leading to protein degradation through deamination. The resulting carbon structures were either used for gluconeogenesis or utilized in the Kerbs cycle,providing additional energy for the infected snails. The reduction in protein levels could also be related to cell death or destruction, which impairs protein synthesis. Similar results were observed in the Mediterranean fruit fly (*Ceratitis capitata)* by Shaurub et al. (2015) and are consistent with findings reported by Tunholi et al. (2014), which showed that increased catabolismin infected snails resulted from a shift in nitrogen extretion. The snails changed from uricotelic to ureotelic excretion, likely as a detoxification strategy to eliminate excess nitrogenous waste. Similar findings were observed in another snail species (*Biomphalaria glabrata*) by Tunholi et al. (2011; 2012) after infection. The results indicated an inversion in the hemolymph's excretion pattern, marked by an increase in urea levels and a decrease in uric acid.

AST and ALT are important enzymes in the metabolic pathways of amino acids and play an important role in energy production. The gluconeogenesis pathway depends on aminotransferases, an essential group of enzymes (Ngo et al., 2022). Both AST and ALT can be considered biomarkers of inflammation, as their levels rises in response to cell or organ damage. The secretion of these enzymes increases during inflammation, tissue injury, or cellular metabolic failure (Abobakr et al., 2021; Huang et al., 2006). The observed increase in aminotransferases levels (AST and ALT) from the initial time of infection to the end of the experiment, across all ages groups of infected snails, indicated metabolic disturbances, tissue damage, and likely inflammation after infection. Within ten days of infection, at the time of death, AST and ALT concentrations had increased by approximately 50 percent from the initial levels, showing the harmful effects of the infection caused by the symbiotic bacteria of EPNs.

There have been recorded uses of EPNs and their symbiotic bacteria as effective biocontrol agents against a wide range of pests, including insect and mollusks. However, the use of EPNs for controlling golden apple snails and giant African snails has not been previously documented. The results of this study demonstrated that EPN infection causes considerable biochemical alterations in both golden apple snails and giant African snails, ultimately leading to their death. The severity of these alterations in each biochemical marker varied depending on the EPN isolate and/or the developmental stage of the snails.

The results of this investigation revealed that, although snails are not definitive hosts for EPNs, infection can still cause severe alterations in biochemical markers in these non-target hosts, ultimately leading to their death. Additionally, the inability of EPNs to reproduce within snails may be advantageous for their use as biopesticides to control snail pests in agricultural settings, as this reduces the risk of impacting other non-target snail species.

Conclusion

This study investigated the effects of exposing golden apple snails and giant African snails to infective juveniles of Thai indigenous EPNs. The results indicate that EPN infections and their symbiotic bacteria significantly disturb the snails' biochemical parameters. Although snails are not the primary hosts for EPNs, these infections can still lead to their death. Further research is needed to fully understand the

mechanisms by which substances released by the symbiotic bacteria contribute to these biochemical disturbances.

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Disclaimer: None

Conflict of Interest: None

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Contribution of Authors

Wanitsumran P: Collected samples, carried out the experiments, performed data collection and analysis, and prepared the manuscript.

Wattanachaiyingcharoen D, Vitta A, Kenthao A & Lopin P: Contributed to data analysis, result interpretation, discussion and manuscript preparation.

Wattanachaiyingcharoen W: Initiation of the research, carried out the experiments, performed data analysis and interpretation and prepared the manuscript.

All authors discussed the results and contributed to the final version of the manuscript.

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