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Original Article

Adverse effects of cypermethrin on golden apple snails *(Pomacea canaliculata)* and their eggs, and application of Acetylcholinesterase (AChE) as biomarker

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Abstract

This study aimed to evaluate the effects of cypermethrin on hatching rate of golden apple snail eggs, mortality rate of the golden apple snails, and acetylcholinesterase (AChE) expression applied as a bio-indicator. The results showed that cypermethrin concentration did not affect hatching rate or development of the eggs and larvae in comparison with the control. The mortality rate depended on the exposure concentration. Median lethal concentration (LC_{50}) (95% confidence) at 96 h was approximately 8.99 (8.93-9.06). The concentration of cypermethrin had an effect on AChE expression in both the snails and their eggs. The molecular weight of AChE found was 71 kDa, as studied by SDS-PAGE and Western blot techniques. The ELISA technique revealed that AChE contents in both the snails and their eggs were significantly different from the control ($p < 0.05$). Based on our results, AChE could be applied to assess cypermethrin exposure in the snails and their eggs, in order to plan contamination management of such pesticides in the snails and reduce the risks to consumers.

Keywords: ELISA technique, mortality rate, toxicity testing, western blot

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1. Introduction

The application of agro-chemicals, especially pesticides, causes toxic residues in the environment and affects organisms in the ecosystem. It results in the accumulation and biomagnification of various toxins through the food chain, and they are ultimately harmful to humans as the top consumers. Freshwater mussels, such as pond snail and golden apple snail, are considered primary consumers in the food chain (Thanomsit *et al*., 2017). They have a risk of exposure to pesticides through feeding like when filtering planktons or consuming various aquatic plants, including rice in paddy fields that are generally sprayed with pesticides. They can be exposed to pesticides in water from accumulation in the bottom sediment. An extensive survey of cypermethrin in water samples of Welsh streams found that cypermethrin was present at concentrations ranging within 0.4-9.5 µg/l. Cypermethrin is very toxic to aquatic invertebrate and fish at nanogram per liter concentrations (Environment Agency Wales, 2010). Therefore, these animals have the potential to receive and accumulate these toxicants in their bodies. Also, they can be used as biological indicators of exposure to various chemicals such as heavy metals, Tributyltin, and insecticides (Piyatiratitivorakul & Boonchamoi, 2008; Putkome, Cheevaporna, & Helandera, 2008).

Cypermethrin is a synthetic pesticide based on pyrethroids. It is among the most effective pyrethroid preparations (Velisek, Stara, & Svobodova, 2011). It has been widely applied in agricultural areas. Hartnik and Styrishave (2008) revealed that its concentration in water is rather low because of poor water solubility. It tends to adsorb onto suspended particles and thus it remains unavailable to many aquatic organisms. In Thailand, it was reported that cypermethrin was the 4th top import of insecticides in 2010. Although it is considered a substance that has a low tendency to accumulate in living organisms, it is likely to spread and toxic to aquatic organisms. Cypermethrin is rapidly degraded and found in small amounts in water sources, but it is highly toxic to aquatic organisms (Liadprathom, Tiamtud, Thumvicharn, & Hirunsadjalerd, 2016). The fundamental data in terms of toxicity or residues of cypermethrin in the environment and its effects on hatchery of the eggs as well as on embryo development remain unavailable, so further studies of these details are needed. Cypermethrin contamination may affect also consumers. Moreover, Putkome *et al*. (2008) indicated that the golden apple snail was appropriate for monitoring insecticide contamination because its AChE expression is inhibited by insecticide exposure. Thus, the AChE expression can be used as a bio-indicator of environmental contamination (Nitu, 2014).

Golden apple snail (*Pomacea canaliculata*) is a freshwater snail, having its origin in Africa and then having spread into Asia (Dai, Wang, Dong, Hu & Nan, 2011). In Thailand, it is an invasive alien species, widely found in paddy fields. It causes severe damage to farming by feeding on the rice plants. Farmers need to apply pesticides in their fields for eliminating it, which results in toxicant accumulation in the surrounding environment (Putkome *et al*., 2008). There are many reports indicating that most pesticides, applied in order to increase crop production, tend to inhibit AChE, which is a neurotransmitter, in the case of long-term exposure. However, the exposed organisms are stimulated to synthesize more AChE in a short-term exposure. Thus, they can be applied in a short-term to detecting AChE as bio-indicators (Thanomsit *et al*., 2018). In this study, we investigated the hatching rates of golden apple snail eggs after exposure to cypermethrin, and the toxicity levels of cypermethrin causing 50% mortality (LC_{50}) in the snails after 96 h of exposure. And, we applied AChE as a bio-indicator to detect exposure using antibody techniques: dot blot, Western blot, and ELISA. A suitable candidate species is the golden apple snails**,** because they are tolerant and have been widely consumed (Thanomsit *et al*., 2019).

2. Materials and Methods

2.1 Chemicals and animal husbandry

The chemical applied to test golden apple snails was cypermethrin [(RS) -α-cyano-3-phenoxybenzyl (1RS) -cis, trans-3- (2, 2-dichlorovinyl) -2, 2-dimethyl cyclopropane carboxylate in a commercial solution form. Generally, all chemicals used were analytical grade. Chemicals used in protein pattern and AChE studies were the products of Bio-Rad Company.

In this study, golden apple snails and their eggs were collected from Surin Fishery Office, Thailand. The snails used were of adult size having an average length of 4.4 ± 1.2 cm, an average width of 2.8 ± 0.8 cm, and an average weight of 23.98 ± 3.1 g. They were acclimatized in a 200 L concrete pond for 7 days.

2.2 Hatching rates of golden apple snail eggs and physiological appearance

The study of hatching rate percentage and physiological appearance of snail eggs exposed to cypermethrin at concentrations of 0, 0.5, 1, 1.5, 2, and 2.5 ppm, compared to the control, was performed following Thanomsit *et al*. (2019). Briefly, they were placed on a Petri dish with 40 ml of water containing cypermethrin then poured in. Each test run was performed in 6 replicates. The plate was examined for hatching every 24 h and then compared for differences in the average percentage of hatching rate by treatment. The morphology of the snail eggs was examined under microscope. Then, 100 eggs were randomly collected in triplicate for assessing characteristics of eggs and abnormal embryos after exposure to cypermethrin.

2.3 Mortality rates of golden apple snails after exposure to cypermethrin

The snails (n=16) were put in a glass jar having cypermethrin in concentrations of 0, 5, 6.25, 7.5, 8.75 and 10 ppm. After that, mortality rates were recorded at 24, 48, 72, and 96 h and then taken to calculate cumulative mortality percentages and determine the toxicity level causing LC_{50} at 96 h, using probit analysis with Minitab software.

2.4 Extraction of AChE from eggs and golden apple snails

The extraction protocol of AChE from the eggs and the snails was modified from the study of Thanomsit *et al*. (2018). Briefly, the eggs and the snails collected from Huai-Saneng Reservoir, Surin, Thailand, were rapidly transferred to the laboratory and washed. Then, they were exposed to cypermethrin, and the control group were cut and mashed with 0.02 M of Tris-HCl (pH 7.2) and 0.01 M of phenyl methylsulfonyl fluoride (PMSF) in the egg:buffer ratio of 1 g:3 ml. Then, the sample was centrifuged at 350 rpm for 65 min and the supernatant was kept at 4°C until AChE expression measurement was performed.

2.5 Protein determination

Protein determination was performed following Thanomsit *et al*. (2017). The BSA standard protein was diluted to 1, 0.5, 0.25, 0.0625, and 0.03125 mg of protein per ml. After that, the protein extracted from the snails and their eggs was diluted with distilled water at the ratio of 1:10. A 10 µl aliquot of BSA solution and protein samples from the snails and their eggs were each added with 200 µl of diluted dye reagent in the wells. Next, the absorbances were determined at 595 nm wavelength. The absorbances at various BSA concentrations were plotted into a standard curve of the BSA concentration vs. absorbance.

2.6 Protein pattern study using SDS-PAGE, and AChE specificity study using Western blot

AChE from the snails and their eggs were detected using 10 % SDS-PAGE and transferred to a nitrocellulose membrane. Samples were electrophoresed at 110 V for 1.45 h followed by staining with Coomassie brilliant blue R-250. The Western blot assay was based on the protocol of Thanomsit *et al*. (2018).

2.7 AChE expression study using dot blot technique

Dot blot technique was developed from the study of Prasatkaew and Nanthanawat (2018). The samples were prepared for protein concentrations of 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 µg/µl. A drop of sample (1 µl) was imbibed into nitrocellulose membrane. Then, it was immersed in 5% skimmed milk in PBS for 1 h. Next, it was incubated in antibody specific to AChE (1:200) for 12 h and then the nitrocellulose membrane was soaked in secondary antibody (GAR-HRP) at the dilution level of 1:2,000 and left in 4ºC overnight. The nitrocellulose membrane was developed for reaction color in the substrate solution (0.03% 3, 3'-diaminobenzidine tetrahydrochloride (DAB), 0.06% H_2O_2, and 0.05% $CoCl_2$ in 0.15 M PBS, at pH = 7.4).

2.8 Evaluation of AChE using ELISA

ELISA technique was used to evaluate AChE in the snails. The protocol was modified based on the study of Prasatkaew, Nanthanawat, Khongchareonporn and Kingtong (2019). The purified AChE from hybrid catfish was applied as a positive control. Briefly, AChE from the snails was diluted in blocking solution (5% skim milk in 0.15 M PBS, pH = 7.2) and then applied to the inner surface of each well (100 µl/well). Next, the plate was incubated at 4°C overnight. The pre-incubation step was performed by adding PAb-AChE (1:200) with the dilution of AChE (0.03–30,000 ng/mL) at 4°C overnight. Next, it was washed and GAR-HRP (1:5,000) was added and then incubated for 3 h. A 100 µL of chromogenic substrate solution containing 0.025% 3, 3′, 5, 5′-tetramethylbenzidine (TMB), 2.5% DMSO and 0.35% H_2O_2 in 0.1 M of citrate buffer, at pH 4.5, was added and incubated for 10 min. Next, the reaction was stopped by adding 100 µL of 1 mol/L H_2SO_4 to each well. The plate was measured for absorbances at 450 nm using a microtiterplate reader (Versamax, USA).

2.9 Statistical analysis

The hatching rates, morphological alterations of the eggs, protein contents and evaluation of the amount of AChE using ELISA technique were compared with the control by using SAS University edition (Order number 1095069) to run Duncan's Multiple Range Test ($p<0.05$).

3. Results

3.1 Hatching rates and morphological alterations of golden apple snail eggs

After the eggs had been exposed to cypermethrin at the concentrations of 0, 0.5, 1, 1.5, 2, and 2.5 ppm, the results showed that hatching rates were not different from the rate in the control (i.e., $p>0.05$). The first hatching occurred after 7 days of exposure (Figure 1A). We found that there was no difference in percentage of morphological alterations between the exposed group and the control ($p>0.05$) (Figure 1B). Figure 2 shows the effects on physiological appearances of larvae (Figure 2B) in the control and exposed group.

3.2 AChE expression in golden apple snail eggs after exposure to cypermethrin

Samples of the eggs were extracted, measured for protein amount and determined for protein form, and the results indicated that the protein concentration in the eggs in the exposed group was not different from the control ($p>0.05$) (Figure 3A). Molecular weight of AChE was 71 kDa. For the eggs exposed to cypermethrin at concentrations of 0.5, 1, 1.5, 2, 2.5 ppm for 96 h, we found that AChE could not be measured in the treatments with high concentrations of 1 and 2.5 ppm (Figure 3B-3C). Dot blot was applied to study AChE expression with detection limits of 0.156, 1.25, 2.5, 5, 5 and 10 µg/µl in the eggs of the control, and the groups exposed to cypermethrin at concentrations of 0.5, 1, 1.5, 2 and 2.5 ppm, respectively (Figure 3D). The amount of AChE in the snails of the control studied by ELISA technique was 1,130.84±15.00 ng/ml while the groups exposed to cypermethrin at concentrations of 0.5, 1, 1.5, 2 and 2.5 ppm had respectively 1,163.12±68.02, 1,044.67±80.30, 988.90±1.40, 884.56±8.17 and 780.48±30.40 ng/ml (Figure 3E).

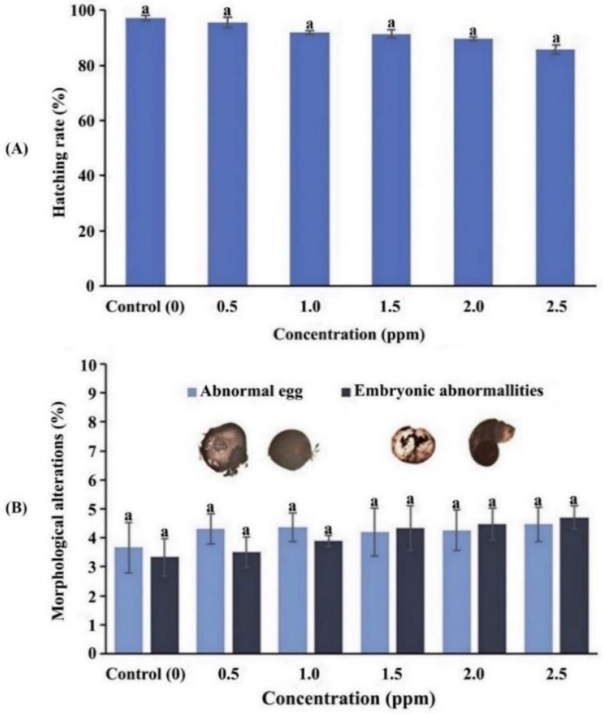

Figure 1. Hatching rates and morphological alterations of eggs in the group exposed to cypermethrin compared to the control. Different letters indicate a significant difference with the control ($p<0.05$).

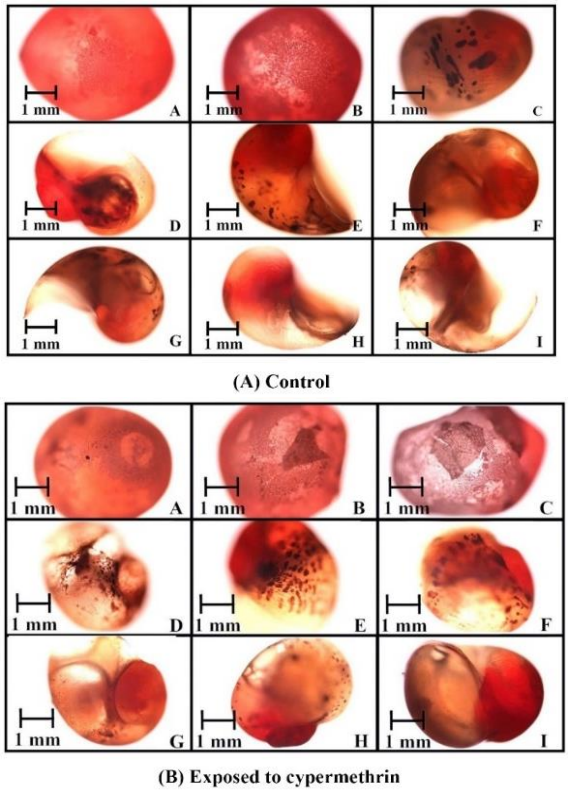

Figure 2. Physiological appearance of larvae in the control (A), the group exposed to cypermethrin (B), egg abnormality (A-C) and larvae (D-I) (10x).

3.3 Toxicity testing of cypermethrin on golden apple snail

Golden apple snails of large size were used to study cypermethrin toxicity by evaluating cumulative mortality rates after 96 h of exposure. The results indicate that at a concentration of 5 ppm, cumulative mortality rate was 20.83±7.22%, while at the concentrations of 6.25, 7.50, 8.75 and 10 ppm those rates were 25.00±0.00%, 33.33±7.22%, 45.83±7.22% and 62.50±0.00 %, respectively. Cumulative

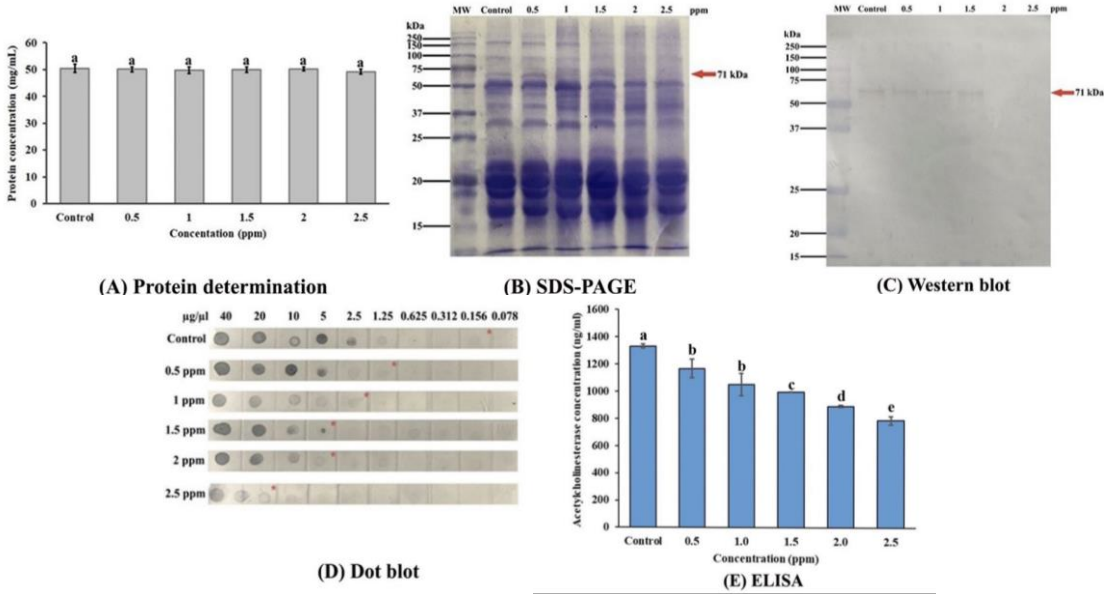

Figure 3. (A) Protein concentration of golden apple snail eggs exposed to cypermethrin, (B) protein patterns using SDS-PAGE, (C) specificity of AChE using Western blot technique, (D) sensitivity and specificity of AChE using dot blot technique, and (E) AChE concentrations using ELISA technique. Different letters indicate significant differences.

mortality rates were used to estimate LC_{50} at 96 h after exposure to cypermethrin by probit analysis run in Minitab software, and the results indicated that LC_{50} (95% confidence) at 96 h was 8.99 (8.93-9.06) and the coefficient of determination (R^2) was 0.8926 (Table 1 and Figure 4).

After studying the toxicity of cypermethrin causing mortality to the snails, they were divided into two groups to study the expression of total protein and AChE, and morphological changes were studied. The snails were exposed to cypermethrin in both sub-lethal and lethal concentrations. The results indicate that at the lethal concentrations, morphologies did not differ from the control and were similar as with cypermethrin effects on the snails at sub-lethal concentration (Figure 5A). On studying the total protein content, the snails exposed to cypermethrin in both the lethal and sub-lethal concentrations did not differ from the control ($p > 0.05$) (Figure 5B). The protein contents measured were in the range 39.28±1.2-40.75±2.3 mg/ml.

Table 1. Mortality levels and toxicity parameters caused by exposure to cypermethrin of golden apple snails after 96 h.

Concentration (ppm)	Average percent mortality ±SD
0	-
5.00	20.83±7.22
6.25	25.00±0.00
7.50	33.33±7.22
8.75	45.83±7.22
10.00	62.50±0.00
Correlation coefficient (R^2)	0.8926
LC_{50}	8.99 (8.93-9.06) (95% confidence)
Regression equation	Y= 134.46x-78.458

Note: Average based on three replicates (Mean±SD)

3.4 Patterns of protein and specificity of AChE of golden apple snails exposed to cypermethrin by Western blot technique

In the golden apple snails exposed to cypermethrin at both lethal and sub-lethal concentrations, we found that AChE in both the control and the exposed groups had an isoform with a 71 kDa molecule size. When studied by Western blot technique, the protein patterns were similar in all treatments, but the detected AChE content decreased with increased exposure concentration (Figure 6).

The dot blot technique was used to assess the sensitivity of the specificity of AChE that was detected after the snails were exposed to cypermethrin. In non-lethal concentrations, the detection limits were consecutively 0.78, 0.078, 0.156, 0.625, 2.5 and 2.5 µg/µl; in contrast, in lethal concentrations, those were 0.625, 0.625, 1.25, 2.5, 2.5 and 10 µg/µl, respectively (Figure 7).

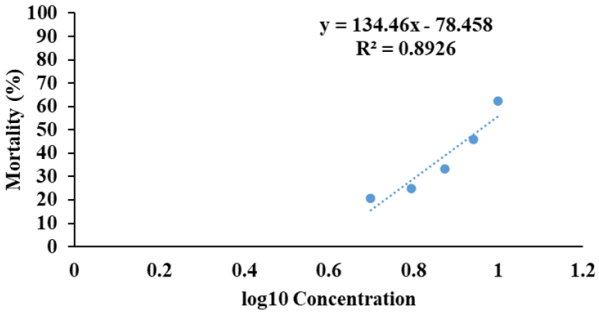

Figure 4. Model fit to the mortality rate of golden apple snails exposed to cypermethrin

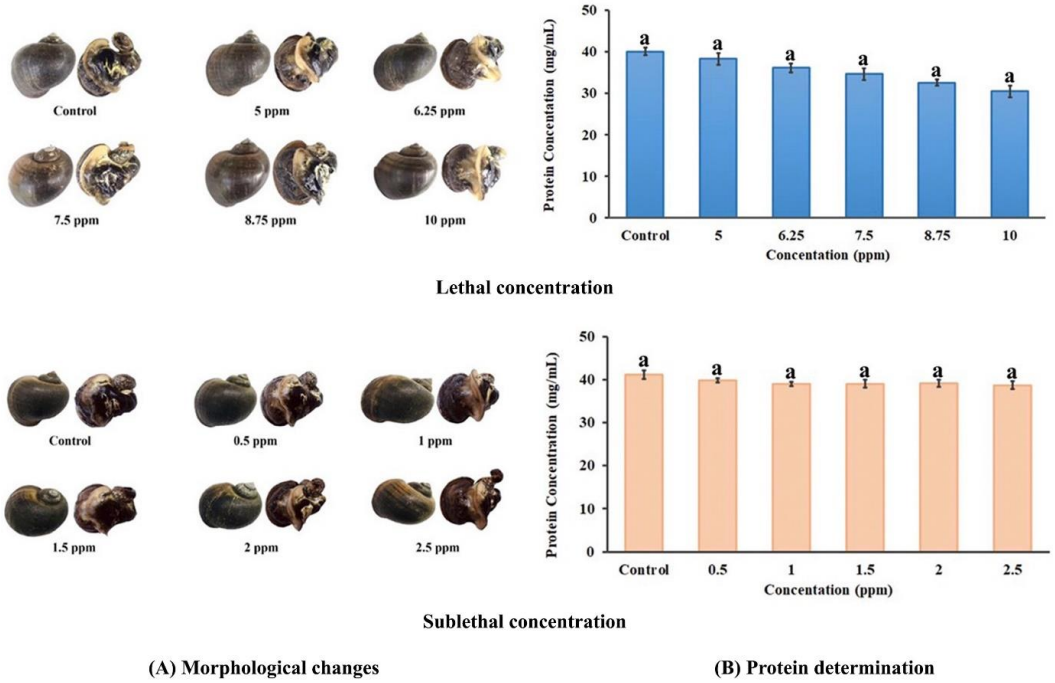

Figure 5. (A) Morphological changes of golden apple snails, and (B) protein concentrations of golden apple snails exposed to cypermenthrin. Different letters indicate significant differences.

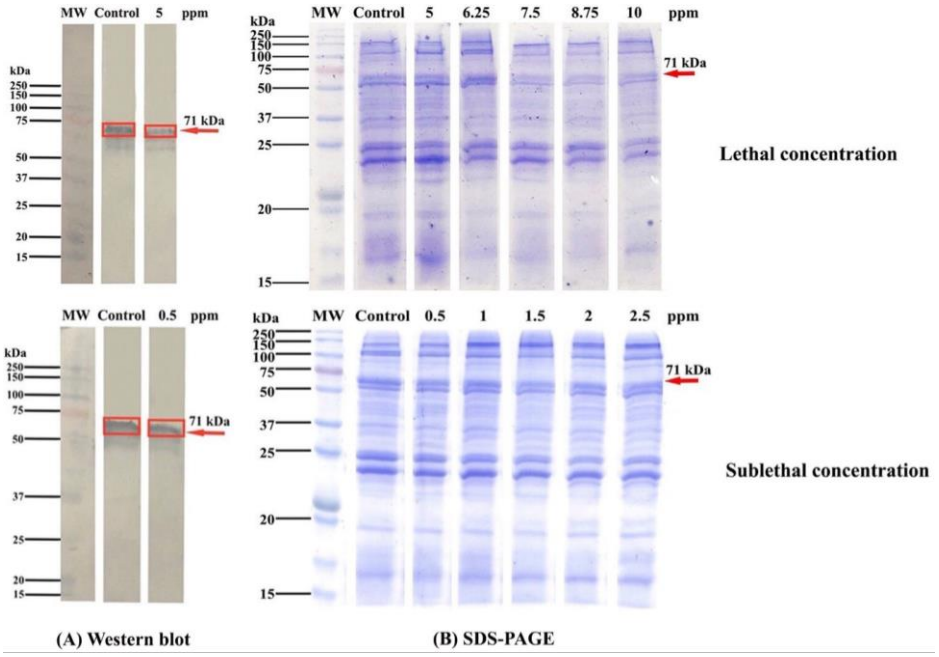

Figure 6. (A) Western blot analysis of AChE in golden apple snails, and (B) 10% SDS-PAGE shows the patterns of protein from golden apple snails exposed to cypermethrin.

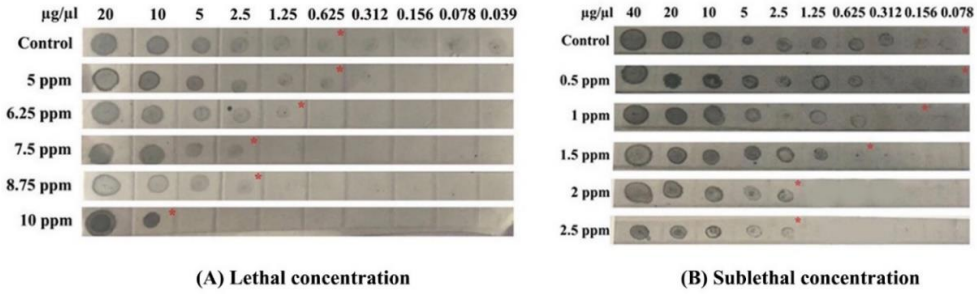

Figure 7. Sensitivity and specificity of AChE expression in golden apple snails exposed to cypermethrin at sub-lethal and lethal concentrations for 96 h, by dot blot technique.

The study of the AChE expression in the snails exposed to cypermethrin in comparison with the control was performed using ELISA technique. When exposure at lethal concentration was compared to the control, we found that AChE concentrations decreased with cypermethrin concentration (Figure 8A). The amount of measured AChE was approximately 1,506.59 ± 88.19 ng/ml in the control. After exposures at concentrations of 5, 6.25, 7.5, 8.75 and 10 ppm, the measured AChE was 1,286.95 ± 78.02, 1,195.45 ± 78.02, 1,003.93 ± 7.13, 837.175 ± 73.77 and 528.59 ± 42.97 ng/ml, respectively, and for all treatments significantly different from the control ($p < 0.05$). In contrast for snails exposed to cypermethrin at sub-lethal concentrations there were only three treatment groups, with the concentrations 1.5, 2 and 2.5 ppm, these being significantly different from the control ($p < 0.05$) (Figure 8).

4. Discussion

After the snails were exposed to cypermethrin in laboratory conditions, we found a decreasing in hatching rate indicating that cypermethrin affected egg hatchery. We also found egg and embryo abnormalities, but there was no difference from the control. This result is similar to the study of Thanomsit *et al*. (2019), which reported that the eggs that were exposed to pesticides including cypermethrin had a hatching rate of up to 90% and were not significantly different from the control ($p>0.05$). Moreover, there were alterations in the egg and embryo development. For hatchery process, the eggs in the group exposed to pesticides had a high hatching rate close to the control because the eggs have self-defense ability. The first step of defense is the egg coating which is a protein called perivitellin 2 (PV2). This substance affects the neurotransmitter proteins (Neurotoxin) consisting of lactin and pore-forming chains. However, perivitellin 2 is a slow-acting active ingredient, so the snails need more protection from surrounding environment and predators (Cadierno, Dreon, & Heras, 2017; Dreon *et al*., 2013). The mentioned protection is the red-clamshell eggs, which come from a protein called ovorubin. Garin, Heras and Pollern (1996) reported that ovorubin is a protein found in eggs with its fraction up to 65%, having an important function as a food source and in

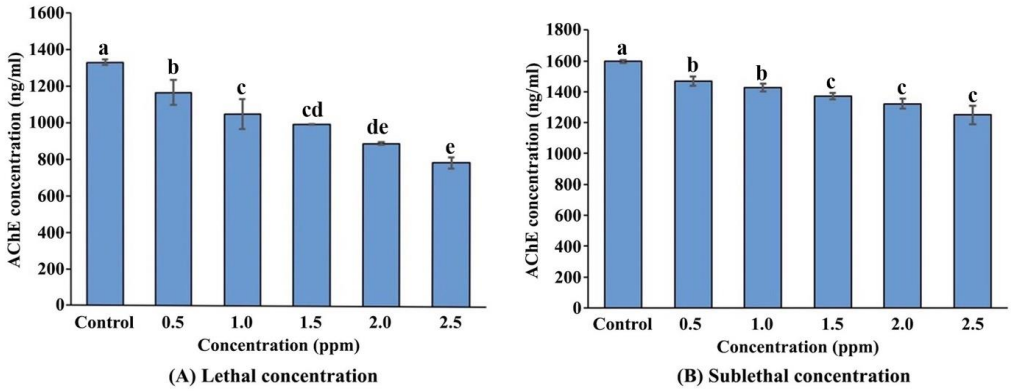

Figure 8. AChE concentrations detected by ELISA technique from golden apple snails exposed to cypermethrin at lethal concentration (A), and at sub-lethal concentration for 96 h (B). Different letters indicate significant differences.

embryo protection. Ovorubin is a protein of 28 to 35 kDa molecular size.

In the toxicity study causing mortality to the snails, we found that the LC_{50} at 96 h was approximately 8.99 (8.93-9.06) ppm (95% confidence), which was different from the study performed in freshwater snail (*Melanoides tuberculatus*) for which the LC_{50} value at 96 h of exposure to cypermethrin was 3.81 ppm (Fakeye & Olatunji, 2016). In addition, our result is different from the study in Estuarine Clam, Marcia Opima (Gmelin, 1791), showing the LC_{50} value was 2.75 ppm (Mukadam & Kulkarn, 2014). The difference might be due to the different types of molluscs and the different forms of the cypermethrin applied.

In the snails and their eggs, the AChE expression tended to decrease with exposure to cypermethrin at high concentrations. In this study, we applied antibody techniques to detect AChE expression in both the snails and their eggs because these techniques are highly sensitive and specific, easy, and inexpensive. They can be applied to a lot of samples and many times, and to study AChE expression both qualitatively and quantitatively (Thanomsit *et al*., 2020). Therefore, these are effective techniques. Besides, we found only one isoform of AChE (71 kDa) in the eggs exposed to cypermethrin. This is similar to a previous study that found the AChE isolated from snails being 71 kDa (Thanomsit *et al*., 2017; Thanomsit *et al*., 2018). In the eggs, we found only one isoform having molecular weight of 71 kDa different from the report of Thanomsit *et al*. (2018) which found two isoforms: 66 kDa and 71 kDa. In this study, Western blot, dot blot and ELISA techniques showed clear results that the concentration of cypermethrin exposure of the snails had an effect on the AChE expression, but did not affect the total protein content in the eggs. This is consistent with the study of Thanomsit *et al*. (2018) in that the exposure to pesticides reduced AChE in the golden apple snail eggs. Moreover, it is consistent with the previous studies reporting that pesticides inhibit AChE expression in snails, e.g. *Xreopicta aerbentina*. In addition, the concentration of substance had effects in inhibiting AChE expression in snails, e.g. in *Lymnae acuminate*, which when exposed to Trimyristin and Myristicin had the measured AChE activity reduced from 0.73 ± 0.00 to 0.66 ± 0.0 and 0.057 ± 0.00 after 24 h of exposure (Jaiwal, Kumar, Singh & Singh, 2010). Our result is also similar to that on *H. aspersa*, in which AChE was inhibited when exposing to thiamethoxam (Smina, Samira, Mohamed, & Houria, 2016). These data suggest that AChE is a specific bio-indicator of exposure to organophosphate pesticides in the snails and their eggs.

5. Conclusions

Cypermethrin is a pesticide widely applied in many countries including Thailand, causing contamination in the environment and accumulation to living organisms. Our study results demonstrated the effects of cypermethrin on both golden apple snails and their eggs. In the case of direct exposure, it resulted in mortality and changes in biochemical levels relating to AChE. However, cypermethrin concentration did not affect the hatching rate or the development of eggs. This exposure could be assessed via AChE as bio-indicator observed by antibody techniques, consisting of dot blot, Western blot, and ELISA. We found that all these techniques could be applied to detect AChE expression. The expression was related to the concentration of cypermethrin exposure. This information should be utilized to assess the cypermethrin exposure of the golden apple snails and the contamination by cypermethrin of water sources, to benefit golden apple snail consumption and related water quality management.

Acknowledgements

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References

Cadierno, M. P., Dreon, M. S., & Heras, H. (2017). Apple snail perivitellin precursor properties help explain predators' feeding behavior. *Physiological and Biochemical Zoology*, *90*(4), 461-470.

Dai, L., Wang, W., Dong, X., Hu, R., & Nan, X. (2011). Molluscicidal activity of cardiac glycosides from *Nerium indicum* against *Pomacea canaliculata* and its implications for the mechanisms of toxicity. *Environmental Toxicology and Pharmacology, 32*, 226−232.

Dreon, M. S., Frassa, M. V., Ceolin, M., Ituarte, S., Qiu, J., Sun, J., . . . Heras, H. (2013). Novel animal defenses against predation: A snail egg neurotoxin combining lectin and pore–forming chains that resembles plant defense and bacteria attract toxins. *PLoS One*, *8*(5), e63782.

Environment Agency Wales. (2010). An evaluation of the impact of cypermethrin use in forestry on Welsh streams.

Environment Agency Wales (2010). An evaluation of the impact of cypermethrin use in forestry on Welsh streams. Retrieved from http://aquaticcommons.org/10900/1/01-An_evaluation_of_the_impact_of_cypermethrin_use_in_forestry_on_Welsh_streams.pdf

Fakeye, O. D., & Olatunji, E. O. (2016). A study of the acute toxicity of some selected heavy metal and pesticides on a fresh water snail (*Melanoides tuberculatus*). *International Journal of ChemTech Research*, *11*, 28-43.

Garin, C. F., Heras, H., & Pollern, R. J. (1996). Lipoprotein of the egg perivitellin fluid of *Pomacea canaliculata* snails (Mollusca: Gastropoda). *Journal of Experimental Zoology*, *276*, 307-314.

Hartnik, T., & Styrishave, B. (2008). Impact of biotransformation and bioavailability on the toxicity of the insecticides α-Cypermethrin and Chlorfenvin phos in earthworm. *Journal of Agricultural and Food Chemistry*, *56*(22), 11057-11064.

Jaiwal, P., Kumar, P., Singh, V. K., & Singh, D. K. (2010). Enzyme inhibition by Molluscicidal components of *Myristica fragrans.* Houtt. in the nervous tissue of snail Lymnaea acuminate. *Enzyme Research*, *2010*, 1-6. doi:10. 4061/2010/478746

Liadprathom, N., Tiamtud, N., Thumvicharn, J., & Hirunsadjalerd, R. (2016). The effect of salinity on the acute toxicity of Cypermethrin and Paraquat in Asian Seabass. *Journal of Fisheries Technology Research*, *7*(2), 82-93.

Mukadam, M., & Kulkarni, A. (2014). Acute toxicity of synthetic Pyrethroid Cypermethrin on protein content in Estuarine Clam, Marcia Opima (Gmelin, 1791). *Journal of Environmental and Analytical Toxicology*, *4*(2), 1-4. doi:10.4172/2161-0525. 1000209

Nitu, S. (2014). Acetylcholinesterase activities in the nervous system of snail Lymnaea acuminate as biomarker of water pollutants in Ramgarh Lake, Gorakhpur, µP. India. *International Research Journal of Environmental Science*, *3*(2), 20-26.

Piyatiratitivorakul, P., & Boonchamoi, P. (2008). Comparative toxicity of mercury and cadmium to the juvenile freshwater snail, *Filopaludina martensi martensi*. *Science Asia*, *34*(4), 367-370.

Prasatkaew, W., & Nanthanawat, P. (2018). Development of dot blot technique for detection of vitellogenin as a biomarker of endrocrine disrupting chemicals. *Burapha Science Journal*, *23*(3), 1458-1457.

Prasatkaew, W., Nanthanawat, P., Khongchareonporn, N., & Kingtong, S. (2019). A monoclonal antibody against *Lates calcarifer* vitellogenin and a competitive ELISA to evaluate vitellogenin induction after exposure to xenoestrogen. *Journal of Environmental Science*, *75*, 325–333.

Putkome, S., Cheevaporna, V., & Helandera, H. F. (2008). Inhibition of Acetylcholinesterase Activity in the Golden Apple Snail (*Pomacea canaliculata Lamarck*) Exposed to Chlorpyrifos, Dichlorvos or Carbaryl Insecticides. *Environment Asia*, *2*, 15-20.

Smina, A. H., Samira, B., Mohamed, D., & Houria, B. (2016). Evaluation of acetylcholinesterase, Glutathione s-transferase and catalase activities in the land snail Helix aspersa exposed to thiamethoxam. *Journal of Entomology and zoology studies*, *4*(5), 369-374.

Thanomsit, C., Maprajuab, A., Prasatkaew, W., Ocharoen, Y., Wattanakornsiri, A., Nanuam, J., & Nanthanawat, P. (2017). Application of Acetylcholinesterase as biomarker for pesticide exposure to reduce health risk in consuming pon snail and golden apple snail. *Proceeding of The Innovation and Technology Conference. 2017* (pp. A221-A227). Rajamangala university of Technology Surin campus, Surin, Thailand.

Thanomsit, C., Maprajub, A., Saowakoon, S., Prasatkaew, W., Ocharore, Y., Wattanakornsiri, A., Nanuam, J., & Nanthanawat, P. (2018). Acetylcholinesterase (AChE): Potential Biomarker for Evaluating Pesticide Exposure on Egg and Tissue of Golden Apple Snail (*Pomacea Canaliculata*) from Huai−Saneng Reservoir, Surin Province, Thailand. *Thai Journal of Agricultural Science*, *51*(3), 104−117.

Thanomsit, C., Boonpok, L., Preabsenadee, T., Wattana kornsiri, A., Ocharoen, Y., Nanuam, J., Prasatkaew, W., & Nanthanawat, P. (2019). Hatching rate, morphological characteristic and alteration of golden apple snail's egg (*Pomacea canailculata*) after being exposed to agricultural chemicals. *Journal of Fisheries Technology Research*, *13*(1), 24-40.

Thanomsit, C., Saowakoon, K., Wattanakornsiri, A., Khongchareonporn, N., Nanuam, J., Prasatkaew, W., & Nanthanawat, P. (2020). Acetyl choline sterase (AChE) polyclonal antibody from hybrid catfish (*C. macrocephalus* × *C. gariepinus*): Specification, sensitivity and cross reactivity. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, *237*, 108837. doi:10.1016/ j.cbpc.2020.108837

Velisek, J., Stara, A., & Svobodova, Z. (2011). The Effects of Pyrethroid and Triazine pesticides on fish physiology. In M. Stoytcheva (Eds.), *Pesticides in the modern world pests control and pesticides exposure and toxicity assessment* (pp. 377-402). Rijeka, Croatia: InTech.