Effects of diazinon based-diazan 60EC on cholinesterase in early stages of snakehead fish (*Channa striata*)

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Abstract. Pesticides containing diazinon are popular in rice cultivation. Diazinon was detected in different water bodies, concentrations varying between 8–711 µg L^{-1} in rice field water. Snakehead fish (*Channa striata*) is a local species in the Vietnamese Mekong delta (VMD). The species reproduces many times a year, but mainly in the rainy season. Approximately 50% of reproduction occurs in paddy fields. After spawning, embryos or larvae float in surface water and ordinarily spend 4 days feeding from the yolk. With these ecological features, snakeheads have a high risk to insecticide exposure. Effects of diazinon based-Diazan 60EC on cholinesterase (ChE) of snakehead fish (*C. striata*) at early stages (larval, finished yolk, and started air-breath) were studied under laboratory conditions. Four concentrations of diazinon (2.3, 4.6, 23, and 57.5 µg L^{-1}) and control were completely randomized, designed using 15 L glassware tank containers with 5 replications. Each replication contained 2 L of Diazinon solution and 50 fishes. Fish were sampled at 12, 24, 48, 72, and 96 hours after exposure for ChE assay. Results showed that ChE activity of snakehead at the started air breath stage was more sensitive to diazinon than at younger stages (larval, finished yolk stages). ChE was adversely inhibited at environmentally realistic concentrations at all early stages. Diazinon at a concentration of 100 times lower than the 96h-LC50 is not safe for snakehead life cycle. Further studies of the effect of enzyme P450 in the early development stages of snakehead is needed for understanding the sensitivity of early stages to diazinon.
Key Words: organophosphate pesticide, sensitivity, striped snakehead, toxicology.

Introduction. Pesticides containing diazinon are often used to protect agricultural crops (Stadlinger et al 2018; Khatun et al 2023; Ore et al 2023). Half-life of diazinon in neutral conditions is 185 days (Tomlin 1994). Therefore, it is considered a persistent compound. Diazinon has been detected in air ($4x10^{-7}$ – $2.7x10^{-6}$ µg m^{-3}), soil (8.8–175.5 µg kg^{-1}) and water (0.000013-768.9 µg L^{-1}) (Ore et al 2023). In the Vietnamese Mekong Delta (VMD), diazinon is widely used in rice cultivation (Berg 2001; Stadlinger et al 2018). After applying the commercial Diazan 60EC (containing 60% diazinon) for rice in the VMD, concentrations of diazinon in rice field water fluctuated between 8–711 µg L^{-1} at one hour post application and decreased to 1.5–14.3 µg L^{-1} at day 1, and below detection limit (0.3 µg L^{-1}) to 2.27 µg L^{-1} at day 3 after application (Cong & Linh 2010). In the VMD, diazinon was detected in 6 out of 8 water samples collected randomly, with concentrations ranging from 3.5 to 42.8 ng L^{-1} (Carvalho et al 2008).

Diazinon is an organophosphate inhibiting the enzyme cholinesterase (ChE) (Stenersen 2004). When the enzyme has been inhibited in a percentage of 70% or more, almost all aquatic organisms die, while abnormal activities are typically found most times when ChE inhibition is over 30% (Fulton & Key 2001; Aprea et al 2002). At sub-lethal concentrations, diazinon has been found to cause prolonged brain ChE inhibition in adult snakehead (*Channa striata*) (Cong et al 2006) and in giant shrimp (*Macrobrachium rosenbergii*) (Cong et al 2022) and growth inhibition in snakehead (Cong et al 2009).

Toxicity of chemicals for organisms is age dependent. Hamm et al (2001) reported that the 96h-LC50 value of diazinon was 32 µM for the medaka (*Oryzias latipes*) larvae (day 11), while it was 102 µM for the embryo stage 11 (day 1) and 111 µM for the embryo stage 34 (day 8), respectively. This suggests that the larvae are more sensitive to diazinon than the embryo stages. Dutta et al (1995) reported that brain ChE activity of the fingerling stage of *Heteropneustes fossilis* was more sensitive to organophosphate malathion than the adult stage. Lund et al (2000) found that ChE of the embryo of *Palaemonetes pugio* stage VII was more sensitive to malathion than stage VI. Hamm et al (2001) found that ChE inhibition by diazinon in the medaka 7-day old larvae was higher than in the medaka 24-hours old larvae. Therefore, different development stages of organisms would have different risk levels in exposure to chemicals such as pesticides.

Snakehead is a local inhabitant in the VMD. It can be found in ponds, canals, and paddy fields (Khoa & Huong 1993). The species reproduces numerous times a year, but the main season is the rainy season (Ali 1999). In the rainy season, approximately 50% of the reproduction of snakehead fish was found to occur in paddy fields (Amilhat & Lorenzen 2005). After spawning, embryos or larvae float in surface water and ordinarily it takes 4 days for the yolk sack to disappear (Singh et al 1988). With the natural behavior of spawning in shallow water and rice fields as preferable habitats, early stages of this species would be at a high risk of exposure to pesticides such as diazinon for rice crops in the VMD. The median lethal concentration for 96 h of diazinon was 0.23 mg L^{-1} for snakehead at the beginning of the air–breathing stage (Cong et al 2008). This study aims to assess the sublethal effects of diazinon on ChE activity of snakehead at early stages (larval, finished yolk, and started air–breath). The results contribute to assessing the risk of snakehead in the VMD.

Material and Method

Tested animals. Embryos of snakehead were obtained from the hatchery at the college of Aquaculture and Fisheries, Can Tho University, Vietnam. All embryos originated from a single parent snakehead fish. Embryos were incubated in composite tanks with de-chlorinated tap water. The water from the tank was continuously aerated and changed daily until hatching. When the yolk disappeared, fish were fed with crustaceans (mainly *Cladocera*) until larvae used air breathing. Afterward, fish was fed red worms (*Tubifex* sp.). Feeding stopped one day before exposure to avoid water quality degradation due to exerted products.

Chemicals. Commercial trade name, Diazan 60EC containing 60% diazinon (common name), was purchased from An Giang Plant Protection and Services Company. Di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) and sodium hydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) were purchased from Merck for use to prepare buffer solutions (pH 7.4 and pH 8). Acetylthiocholine iodide (Sigma Aldrich, Germany) and 5,5′-dithiobis (2 nitrobenzoic acid) ($C_{14}H_8N_2O_8S_2$) (Sigma Aldrich, Germany) were used for ChE assay.

Experimental designs

Effect of diazinon on ChE activity of snakehead at larval stage. Four diazinon treatments (2.3, 4.6, 23, 57.5 μg L^{-1}) corresponding to 1, 2, 10, and 25% of the 96h-LC50 of snakehead at the onset of the air-breathing stage (Cong et al 2008) and a control (de-chlorinated tap-water) were prepared. The experiment was conducted for four days using glass aquaria (0.3x0.25x0.5 m^3) containing 2 L of treatment solution. Each treatment was replicated five times. 20 snakehead larvae (2 d post-hatch) were carefully placed into each aquarium. The larvae were sampled at 12, 24, 48, 72, and 96 h post exposure for ChE assay. At each sampling time, two larvae were collected from each replication.

Effect of diazinon on ChE activity of snakehead at the finished yolk stage. With a similar design to the first experiment, 20 finished yolk snakeheads (the yolk under the abdomen disappeared and the fish began swimming normally) from the same parent as the in first experiment were carefully placed into each glass aquarium. Fish were sampled at 12, 24, 48, 72, and 96 h after exposure to analyze the ChE activity.

Effect of diazinon on ChE activity of snakehead at the air breathing stage. 4 treatments with 4 similar concentrations of diazinon as in the first 2 experiments and control treatments were randomly conducted in 15 L glass aquariums. Each treatment was replicated 5 times. Twenty air breathing snakehead fish from the same broodstock as in the first and second experiments were exposed into each replication. Fish were sampled at 12, 24, 48, 72, and 96 h to analyze ChE activity.

Cholinesterase assay techniques. The entire body of each fish from the first and second experiments was weighed and then separately homogenized in 0.1 M phosphate buffer (pH 7.4) for ChE assay, while the brain of each fish from the third experiment was used for detecting ChE activity. After homogenizing, the homogenate of each sample was transferred into an Eppendorf tube and centrifuged at 2000 rpm for 20 min at 4°C in a centrifuge (Sigma, Germany). A part of supernatant from the homogenate solution was used for ChE assay using a spectrophotometer (U2900, Japan) at 412 nm for 200 s following the method described by Ellman et al (1961) and modified by Cong et al (2022). ChE enzyme activity was calculated by the following equation (Cong et al 2020).

$$At=(AxVcxVb)/(FxLxVsxWs)$$

Where: At - activity of the enzyme (μM/g/min); A - absorption of the sample per 1 min (Abs/min) or blank sample absorption (Abs/min); Vc - volume of the cuvette or total volume of measured solution (mL); Vb - volume of the buffer solution used for the homogenized sample (mL); F - a factor of 13.6; L - length of cuvette (cm); Vs - volume of sample used for measurement after centrifugation (mL); Ws - fresh weight of a homogenized sample (g).

Data analysis. In order to compare the sensitivity of ChE at the different development stages of snakehead, inhibition rate was calculated with the following equation (Cong et al 2020).

$$I=100-(At/A0)x100$$

Where: I - inhibition rate (%); At - ChE activity of exposed fish (μM/g/min); A0 - average ChE activity of fish in control treatment (μM/g/min).

Differences in ChE inhibition among treatments and among stages were analyzed using a one-way analysis of variance and the Dunnett C test for comparing with control. Duncan's test was used for comparison among treatments when differences were observed. Significant differences were considered at $p<0.05$.

Results

Effect of diazinon on ChE activity of snakehead at larval stage. The results from the first experiment showed that the ChE inhibition rate is dose dependent; a higher diazinon concentration produces more ChE inhibition (Figure 1). No sign of recovery was seen during the 96 h of exposure. In the two highest concentrations, ChE inhibition rates were significantly different to the control ($p<0.05$) from 12 h of the exposure and prolonged to the end of experimentation. At 24 h of exposure, there was a significant difference ($p<0.05$) in ChE inhibition rate between the diazinon treatment of 4.6 μg L^{-1} and the control. However, at 48 h of exposure, significant ChE inhibition was observed in the diazinon treatment of 2.3 μg L^{-1}. In general, a higher diazinon concentration produces earlier significant effects in snakeheads. The peak of ChE inhibition reached 65.6 and 76.6% in the diazinon 23 μg L^{-1} and 57.5 μg L^{-1}, respectively, while the highest ChE inhibition in the diazinon 2.3 μg L^{-1} and 4.6 μg L^{-1} was 38 and 46.6%, respectively.

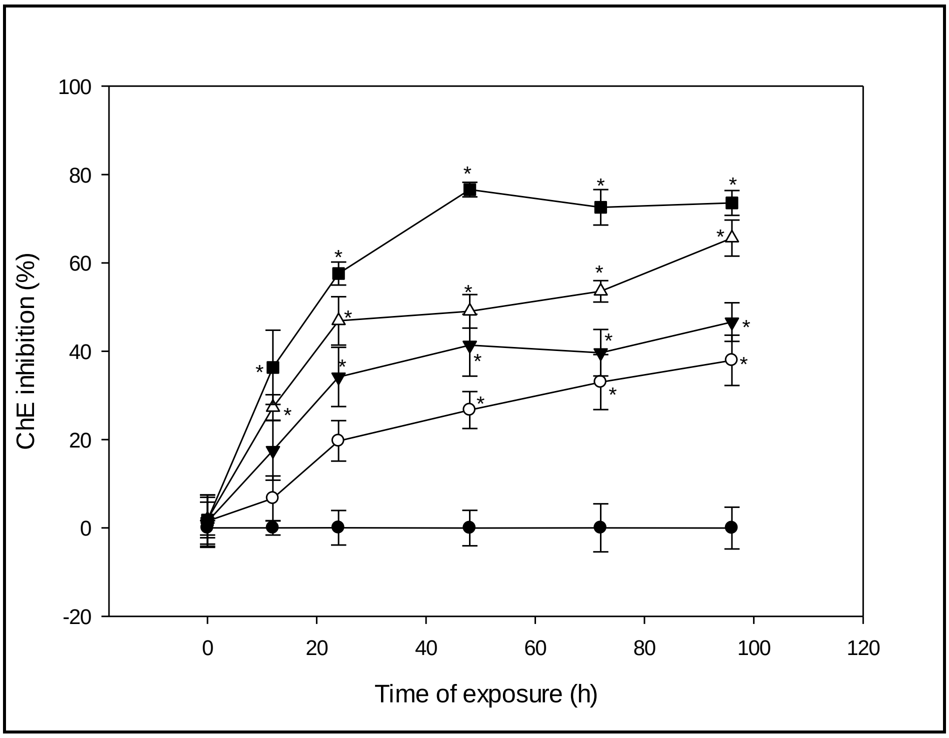

Figure 1. Cholinesterase inhibition rate (%) of the larval stage (1 d-old stage) of snakehead (*Channa striata*) during the exposure period; Control (●), 2.3 μg L^{-1} (o), 4.6 μg L^{-1} (▼), 23 μg L^{-1} (Δ), 57.5 μg L^{-1} (■). Data are presented as mean±SE (n=5), and an asterisk (*) indicates significant difference from the control ($p<0.05$, Dunnett C test) at the same sampling time.

Effect of diazinon on ChE activity of snakehead at the finished yolk stage. ChE activity of snakehead at the finished yolk stage from the nursery aquaria varied between 6.96 and 7.14 μM/g/min. The results from the second experiment showed that following 12 h of exposure, the ChE inhibition rates differed to the control ($p<0.05$) in all diazinon treatments except the diazinon treatment of 2.3 μg L^{-1} ($p>0.05$). In the lowest diazinon treatment, the ChE inhibition rate was significantly different from the control value at 48 h of exposure. Trends of ChE inhibition increased in the longer exposure time with no signal of recovery during 96 h of exposure (Figure 2). The peak of ChE inhibition reached 75.5 and 85.7% in the diazinon 23 μg L^{-1} and 57.5 μg L^{-1}, respectively. The highest ChE inhibition in the diazinon 2.3 μg L^{-1} and 4.6 μg L^{-1} was 40.5 and 57.7%, respectively.

Effect of diazinon on ChE activity of snakehead at the air breathing stage. Before exposure to diazinon, the average ChE activity of the snakehead at the beginning of the air-breathing stage in the nursery aquaria ranged from 10.3 to 12.6 μM/g/min ($p>0.05$). After 12 h of exposure, ChE activity was greatly inhibited and reached 24.1 and 45% in the diazinon treatment 2.3 and 4.6 μg L^{-1}, respectively. At the higher diazinon dose (23 μg L^{-1}), ChE inhibition was 68.3% and in the highest concentration (57.5 μg L^{-1}) ChE inhibition rate was 89.4% (Figure 3). All cases were significantly different ($p<0.05$) from the control and prolonged to the end of 96 h of exposure. The peak of ChE inhibition reached 54.2 and 61.2% in the diazinon 2.3 μg L^{-1} and 4.8 μg L^{-1}, respectively, whereas the highest ChE inhibition in the diazinon 23 μg L^{-1} and 57.5 μg L^{-1} was 82.7 and 89.8%, respectively.

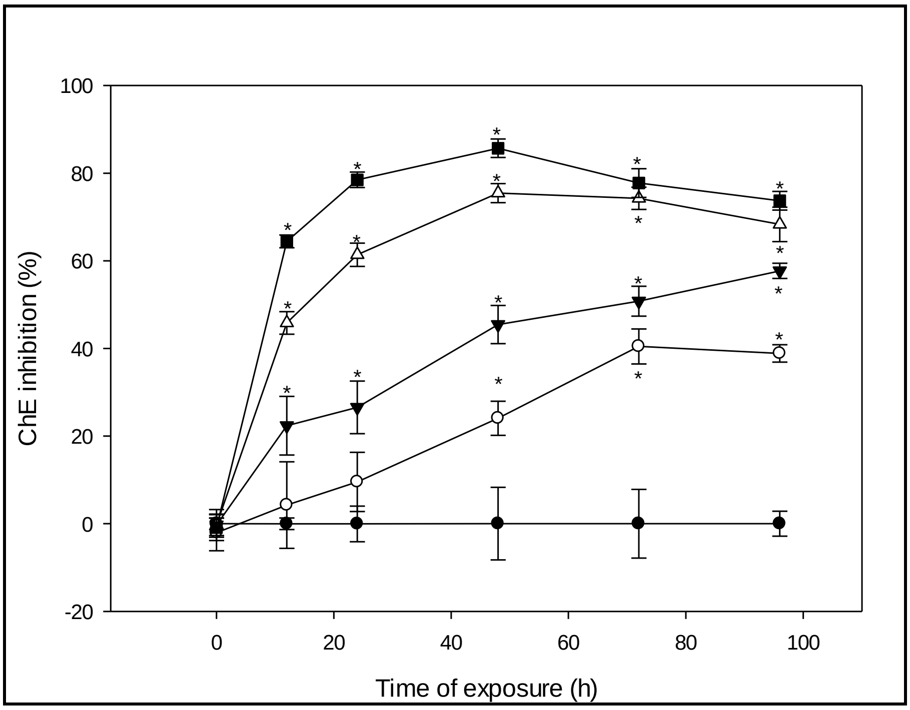

Figure 2. Cholinesterase activity of snakehead (*Channa striata*) at the finished yolk stage (4 d-old stage) in the exposure diazinon time; Control (•), 2.3 μg L^{-1} (o), 4.6 μg L^{-1} (▼), 23 μg L^{-1} (Δ), 57.5 μg L^{-1} (▪). Data are presented as mean±SE (n=5), and an asterisk (*) indicates significant difference from the control (p<0.05, Dunnett C test) at the same sampling time.

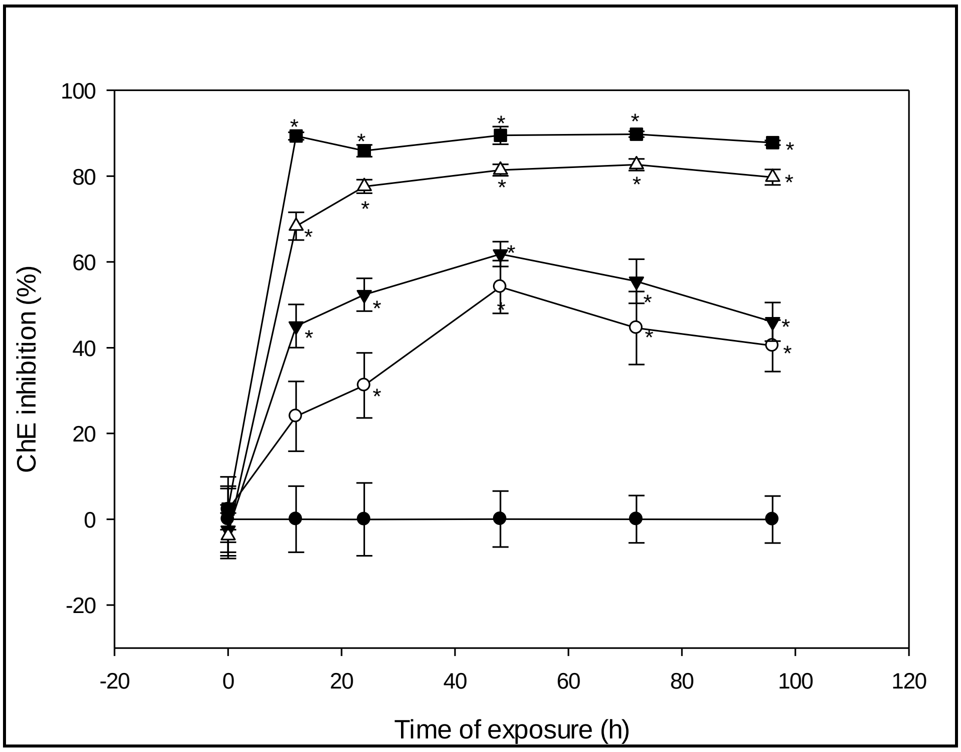

Figure 3. Cholinesterase activity of snakehead (*Channa striata*) at the air breathing stage (20 d-old) in the exposure diazinon time. Control (•), 2.3 μg L^{-1} (o), 4.6 μg L^{-1} (▼), 23 μg L^{-1} (Δ), 57.5 μg L^{-1} (▪). Data are presented as mean±SE (n=5), and an asterisk (*) indicates significant difference from the control (p<0.05, Dunnett C test) at the same sampling time.

Discussion. Findings from this study show that the early stages of snakehead development have a high risk because of pesticide exposure in the rice fields of the VMD. The results from three experiments showed that, although ChE of snakehead was significantly inhibited in all stages, the ChE inhibition rate of fish at the onset of the air breathing stage was higher than in the younger stages. After 12 h of the exposure, in the lowest diazinon treatment (2.3 μg L^{-1}), the ChE inhibition rate was 24% in the oldest stage, while it was 6.7% and 4.3% for the 1-day old stage and 4-day old stage, respectively. In the highest diazinon treatment (57.5 μg L^{-1}), ChE of the oldest stage was inhibited 89.3%, while the ChE inhibition rate in the 1-day old stage was 36.4% and in the onset of the air breathing stage it was 64.4%. This is clearer when comparing data from the diazinon treatment of 23 μg L^{-1} (Figure 4). Trends of ChE inhibition were completely separated among three stages, with ChE inhibition always highest for the onset of the air breathing stage and lowest for the 1-day old stage.

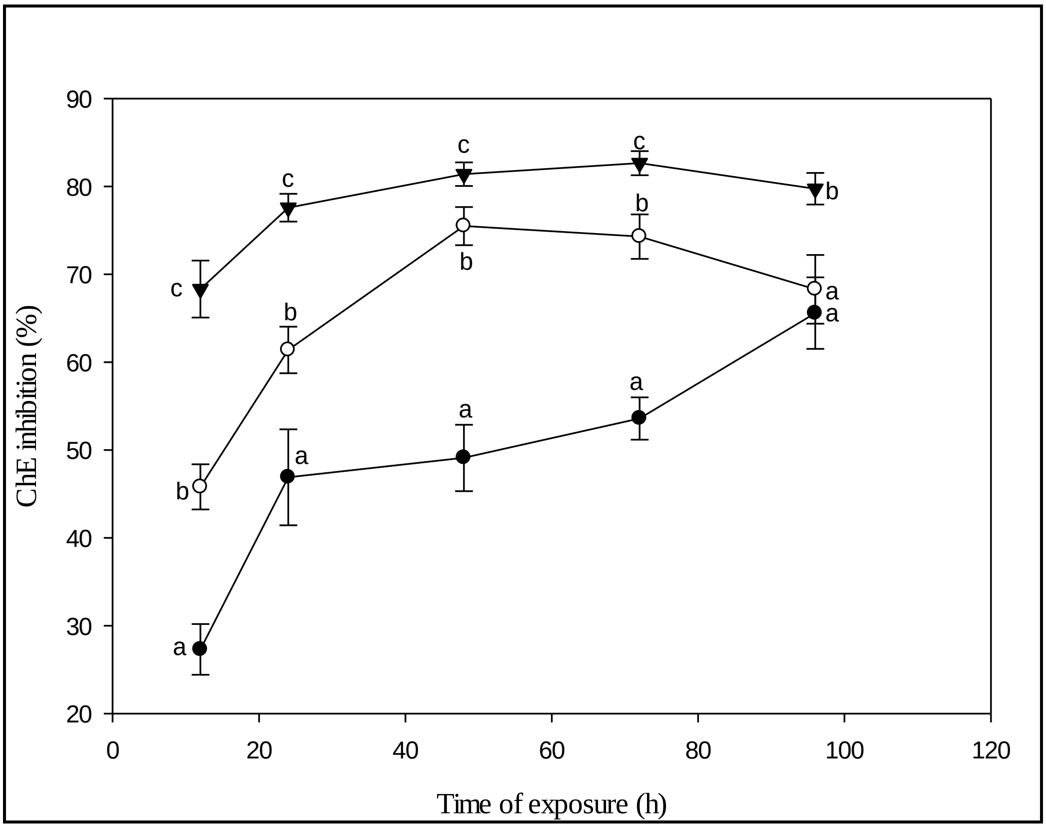

Figure 4. Cholinesterase activity inhibition at different stages of snakehead (*Channa striata*) when exposed to diazinon at a concentration of 23 μg L^{-1}. The 1-day old stage (●), the 4-day old stage (o), the onset of air breathing stage (▼). Data is presented as mean±SE (n=5); the letters a, b, c show significant differences ($p<0.05$, Duncan test) at the same time of sampling.

The mode of action of organophosphorus pesticides is ChE inhibition (Stenersen 2004). However, an organophosphorus pesticide with a P=S structure like diazinon produces less ChE inhibition, unless it is oxidized to the oxon form (diazinoxon, P=O structure) (Keizer et al 1995). After uptake, the oxidation or bio-transformation process is performed by enzyme P450 in phase I (Manikandan & Nagini 2018). Therefore, high activity P450 would lead to oxidation of an organophosphorus compound with the P=S form to the P=O form quicker than the low case and cause more ChE inhibition. Activity of the P450 is age dependent (Zane et al 2018) and also affected by other factors such as ambient temperature and sex (Andersson & Forlin 1992). In the shrimp *Palaemonetes pugio*, Lund et al (2000) found that ChE of the stage VII of the embryo was more sensitive to malathion (P=S structure) than stage VI. In medaka, Hamm et al (2001) reported that

ChE of the medaka larvae was more sensitive to diazinon (P=S structure) than the embryo stage. Viant et al (2006) reported that juvenile salmon (*Oncorhynchus tshawytscha*) was more susceptible to diazinon than the embryo stage. Lee et al (2002) showed that after oxidizing diazinon (P=S structure) to P=O structure, the inhibitory rate of ChE activity increased from 13 to 73%. The rate of oxidation depends on the organism and the activity of the enzyme P450 (Keizer et al 1991; Keizer et al 1995). The activity of the enzyme P450 increases gradually from the embryonic stage to the mature stage (Andersson & Forlin 1992). In this study, we did not measure P450 enzyme activity at the different development stages of snakehead fish. It is recommended that future investigation should be undertaken to confirm our implications.

In the present study, at the lowest diazinon concentration (2.3 μg L^{-1}), ChE was significantly inhibited and the maximum ChE inhibition was 38% for the 1-day old stage, 40.6% for the 4-day old and 54.7% at the onset of the air breathing stage. Fulton & Key (2001) reported that over 30% ChE inhibition often led to abnormal activity in organisms. Brewer et al (2001) found that the swimming speed of trout was positively correlated with ChE activity after exposing the trout to diazinon or malathion for 24 or 96 h. Snakehead was found to have muscle convulsions after exposure to diazinon (Cong et al 2006). This suggests that a higher ChE inhibition will produce a lower swimming speed. Activity of ChE was significantly inhibited by over 30% even at very low diazinon concentration (2.3 μg L^{-1}) with no signals of recovery at the end 96 h of exposure in the present study. It means that the diazinon concentration of 2.3 μg L^{-1} is not safe for snakehead. Inhibition of swimming speed or muscle convulsion may lead to an inability to escape predators or feed. Diazinon was detected at 8-711 μg L^{-1} in rice fields after 1 h of spraying in the VMD (Cong & Linh 2010). It implies that snakehead fish would be at a very high risk for their entire life cycle from water residues of pesticide in general and diazinon in particular. Many other factors affect snakehead production, such as loss of natural habitats due to intensification in rice cultivation, overfishing, and environmental pollution (Edwards et al 1997). Although there was no data found for wild fish production, farmer interviews in the VMD revealed that the majority feel that fish production from rice fields decreased, mainly due to pesticides (Berg & Tam 2018). Findings from the present study provide evidence to confirm the perception of famers on the reduction of fish production in the rice fields of the VMD.

Conclusions. Cholinesterase activity of snakehead at the onset of the air breathing stage was found to be more sensitive to insecticide diazinon than that of the younger stages (1-day old and 4-day old stages). ChE was adversely inhibited at environmentally realistic concentrations in all early stages. Diazinon at a concentration 100 folds lower than the 96h-LC50 was found to be not safe for snakeheads over their entire life cycle. Further study of the enzyme P450 on the early stages of snakehead fish is needed for the improved understanding of the sensitivity of early stages of this species to diazinon.

Acknowledgements. The authors would like to thank the laboratory of Environmental Toxicology at the College of Environment and Natural Resources, Can Tho University, Vietnam for the supply of equipment. The authors would like to thank Nigel K. Downes—Researcher for Integrated Water Resource Management (IWRM) for proofreading the article.

Conflict of Interest. The authors declare that there is no conflict of interest.

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Received: 26 February 2024. Accepted: 09 April 2024. Published online: 10 May 2024.
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How to cite this article:
Cong N. V., Diem H. T., Hang B. T. B., 2024 Effects of diazinon based-diazan 60EC on cholinesterase in early stages of snakehead fish (*Channa striata*). AACL Bioflux 17(3):876-884.