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Hormetic response of cholinesterase from *Daphnia magna* in chronic exposure to triazophos and chlorpyrifos

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Abstract

In vivo activity of cholinesterase (ChE) in *Daphnia magna* was measured at different time points during 21-day exposure to triazophos and chlorpyrifos ranging from 0.05 to 2.50 μg/L and 0.01 to 2.00 μg/L, respectively. For exposure to triazophos, ChE was induced up to 176.5% at 1.5 μg/L and day 10 when measured by acetylthiocholine (ATCh), whereas it was induced up to 174.2% at 0.5 μg/L and day 10 when measured by butyrylthiocholine (BTCh). For exposure to chlorpyrifos, ChE was induced up to 134.0% and 160.5% when measured by ATCh and BTCh, respectively, with both maximal inductions detected at 0.1 μg/L and day 8. Obvious induction in terms of ChE activity was also detected in daphnia removed from exposures 24 hr after their birth and kept in a recovery culture for 21 days. Results indicated that the enzyme displayed symptoms of hormesis, a characteristic featured by conversion from low-dose stimulation to high-dose inhibition. In spite of that, no promotion in terms of reproduction rate and body size was detected at any tested concentrations regardless of whether the daphnia were collected at end of the 21-day exposure or at end of a 21-day recovery culture. This suggested that induction of ChE caused by anticholinesterases had nothing to do with the prosperity of the daphnia population.

Key words: *Daphnia magna*; anticholinesterase; ChE; enzyme promotion; exposure; recovery culture; population prosperity

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Introduction

Anticholinesterases such as organophosphorus and carbamate insecticides (OPs and CBs) are known as potential inhibitors of cholinesterase (ChE) enzymes. Inhibition of these enzymes results in accumulation of neurotransmitters in synaptic regions and thus blocks neural transmission. This blockage leads to respiratory restraint and may result in the death of the organism through asphyxia. Because anticholinesterases can inhibit ChE indicates that these enzymes can serve as biomarkers for the environmental presence of anticholinesterases. As one of the earliest employed biomarkers, ChE has gained a reputation for efficiency during field applications (Sanchez-Hernandez et al., 1998; Stien et al., 1998; Cajaraville et al., 2000; den Besten et al., 2001; Abdel-Halim et al., 2006). Despite this, care must be taken in utilizing these biomarkers to explain adverse effects following sublethal exposure to anticholinesterases (Roex et al., 2003; Hackenberger et al., 2008).

Daphnia are a group of zooplankton that belongs to phylum Arthropoda, order Crustacea, and family Daphniidae. In aquatic areas, they feed on phytoplankton and serve as prey for aquatic vertebrates and macro-arthropods. Due to their significance in both economy and ecology, daphnia's *in vivo* response of ChE during exposure to anticholinesterases has been a source of research (Gälli et al., 1994; Bond and Bradley, 1995; Guilhermino et al., 1996; Sturm and Hansen, 1999; Barata et al., 2001, 2004; Carvalho et al., 2003; Duquesne, 2006; Rosa et al., 2006; Damásio et al., 2007; Printes et al., 2008). Such studies have confirmed that, to behave in the same way as fish and other aquatic animals, daphnia obviously inhibited their ChE in exposures adjacent to lethal concentrations of anticholinesterases. What has remained unclear, however, is the *in vivo* fluctuation of the enzymes in low-dose or chronic exposures and the biological consequences of such exposures.

Triazophos and chlorpyrifos are two of the most commonly used OPs in rice fields in China and Southeast Asia. Therefore, it is possible for them to enter aquatic ecosystems and impose hazards on aquatic organisms. In regards to this, triazophos and chlorpyrifos were taken as surrogates. We studied two daphnia populations: one

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underwent 21-day exposure to either triazophos or chlorpyrifos, the other was removed from OP exposures 24 hr after their birth and kept in recovery culture for 21 days thereafter. The main purpose of this study was: (1) to clarify *in vivo* responses of ChE along with time of exposure, (2) to clarify if the active level of ChE related to the prosperity of daphnia populations, and (3) to explore the relationship between *in vivo* ChE activity and ambient concentration of anticholinesterases. Results from this study benefit understanding the potential of ChEs in predicting the hazards of OPs or CBs from long-term exposures.

There may be more than one type of ChE in animal bodies, with some responsible for maintaining neurotransmission, and others serving as detoxification factors to scavenge OPs or CBs and prohibit anticholinesterases from reaching the targets (Villatte and Bachmann, 2002; Wang et al., 2004; Lenz et al., 2005). According to Diamantino et al. (2003), ChE from *Daphnia magna* shows characteristics of both acetylcholinesterases (AchE, EC 3.1.1.7) and pseudocholinesterases (PchE, EC 3.1.1.8). Since no ChE has been previously purified from daphnia, it remains uncertain whether daphnia contain one type of atypical ChE or whether they contain both AchE and PchE. Consequently, two commonly used aliphatic cholines, that is ATCh and BTCh, were taken as substrates to scale the activity of ChE (s) in the hope of finding clues about the type constitution of the enzyme in daphnia.

1 Material and methods

1.1 Daphnia

The daphnia used in this study were a pure breed of *Daphnia magna* (i.e., 62 D.M.) obtained from the Institute of Environmental Health and Related Product Safety, Chinese Center for Disease Control and Prevention. The breed was cultured in M_4 medium with pH of 7.8 ± 0.2 and was fed with unicellular algae *Scemedesmus subspicatus*. Water temperature, light intension, and light cycle were set as (21 ± 1)°C, 1500–2500 Lux, and 16 hr light/8 hr dark, respectively.

1.2 Chemicals

Standard triazophos (O,O-Diethyl O-(1-phenyl-1H-1,2,4-triazol-3-yl) phosphorothioate) and chlorpyrifos (O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) were bought from Dr. Ehrenstorfer GmbH; Amplex® Red Reagent (10-acetyl-3,7-dihydroxyphenoxazine) was a product of invitrogen™ (USA); horseradish peroxidase (HRP, EC 1.11.1.7), *Alcaligenes* sp.-originated choline oxidase (ChO, EC 1.1.3.17), acetylthiocholine-iodide (ATCh), butyrylthiocholine-iodide (BTCh), resorufin (7-hydroxyphenoxazone), and bovine serum albumin (BSA) were products of Sigma-Aldrich® (USA). All other reagents were domestic products of analytical degree unless mentioned specifically.

1.3 Experiments

1.3.1 Chronic exposure

Exposure lasted for 21 days, during which the daphnia were raised individually according to the Organisation for Economic Cooperation and Development (OECD, 1998) guideline No. 211. Concentrations adopted were 0.05, 0.10, 0.50, 1.00, and 1.50 μg/L for triazophos and 0.01, 0.05, 0.10, 0.50, and 1.00 μg/L for chlorpyrifos. Dilutions were prepared by spiking acetone solutions of different concentrations of either chlorpyrifos or triazophos into the M_4 medium. Measures were taken to ensure that each dilution (including the control) contained acetone of 2.0 μL/L. To start the exposure, 6–24 hr old daphnia were held in a 100 mL beaker with dilution of 30 mL. Each beaker contained 2–3 daphnia. Reallocation was made the moment that sex of the daphnia could be identified. Twenty repetitions were set for each treatment including the control. Mortality and number of produced offspring were recorded daily. Newly-born offspring were removed immediately after recording. Body length of adults was measured at the end of the 21-day exposure. The dilutions were renewed three times a week during the exposure. Ambient conditions were the same as that described in Section 1.1.

For investigating *in vivo* responses of ChE in daphnia, specimens were exposed to 0.05, 0.10, 0.50, 1.00, 1.50, 2.00, and 2.5 μg/L of triazophos or 0.01, 0.05, 0.10, 0.50, 1.00, 1.50, and 2.00 μg/L of chlorpyrifos. Dilutions were prepared by spiking acetone solutions of either chlorpyrifos or triazophos into the M_4. Measures were taken to ensure that each dilution (including control) contained 2.0 μL/L of acetone. To start the exposure, the 6–24 hr old daphnia were introduced into a 500 mL beaker with dilution of 400 mL. Each daphnia shared dilution of at least 4 mL. Subsamples were taken at day 1, 4, 8, 10, 12, 15, 18, and 21 of the exposures. The dilutions were renewed three times a week during the whole period of exposure. Ambient conditions were the same as that described in Section 1.1.

1.3.2 Recovery culture

The 6–24 hr old daphnia were bred in concentration series. Ambient conditions were the same as that described in Section 1.3.1. Offspring that belonged to the first and the third batch, i.e., F_1(1st) and F_1(3rd), were collected 24 hr after birth. The collected offspring were separated into two groups: one for measuring *in vivo* ChE activity and the other was kept in insecticide-free medium (i.e., M_4) to go through a recovery culture of 21 days.

To start the culture, 6–24 hr old daphnia were held in a 100 mL beaker at a dilution of 30 mL. Each beaker contained 2–3 daphnia. Reallocation was made the moment that sex of the daphnia could be identified. Twenty repetitions were set for each treatment, including the control. Mortality and number of produced offspring were recorded daily. Newly-born offspring were removed immediately after recording. At end of the recovery culture, surviving adults were removed for body length measurement and

enzymatic analysis. The dilutions were renewed three times a week during the culture. Ambient conditions were the same as that described in Section 1.1.

1.4 Enzyme preparation

Daphnia collected from Section 1.3.1 or 1.3.2 were homogenized in 0.025 mol/L tris-HCl buffer at pH 8.0. The homogenate was centrifuged for 10 min at 10,000 $\times g$. The supernatant was collected to serve as source of the enzyme. All above performances were conducted at 4°C. About 50 daphnia were used for preparation conducted at the beginning of exposure, while the number of daphnia used decreased with increasing body size (though never numbered less than three) for preparation conducted at other time points of exposure.

1.5 Enzyme activity determination

Since ChE activity is much lower in daphnia than in many species of aquatic vertebrates, a more sensitive method was applied in this study. This method involved the fluorometric detection of H_2O_2, an oxidative product of choline derived from the enzymatic hydrolyzing of aliphatic choline and which reacts with 10-acetyl-3,7-dihydroxyphenoxazine to create highly fluorescent resorufin (Zhou et al., 2000). Compositions of 150 μL of 0.025 mol/L tris-HCl buffer (pH 8.0) were added in the following sequence: (1) 50 μL of enzyme solution prepared as described in Section 1.4; (2) 25 μL of 0.05 mol/L ATCh or BTCh; (3) 25 μL of 0.1 U/mL ChO dissolved in 0.05 mol/L of phosphate buffer (pH 7.4); (4) 25 μL of 1 U/mL HRP dissolved in 0.05 mol/L of phosphate buffer (pH 7.4); and (5) 25 μL of 0.05 mol/L Amplex® Red Reagent dissolved in dimethysulfoxide. After incubation for 30 min at 25°C, the mixture was taken for measuring fluorescence at excitation wavelength of 530 nm and emission wavelength of 600 nm. The amount of resorufin produced was determined based on a linear regression equation established by plotting OD values against resorufin concentrations.

Protein content of the enzyme solution was determined by the Folin-phenol method as described by Lowry et al. (1951).

1.6 Data analysis

We preformed ANOVA and LSD tests using DPS© 16.0 to evaluate the effect of either triazophos or chlorpyrifos on ChE activity and on the biological parameters of daphnia.

2 Results

2.1 Activity of ChE in OP exposure

Activities of ChE related to groups exposed to different concentrations of triazophos and chlorpyrifos are shown in Fig. 1.

2.1.1 Induction

For triazophos exposure, induction up to 176.5% was detected when ChE was measured by ATCh (Fig. 1a), and induction up to 174.2% was detected when ChE activity was measured by BTCh (Fig. 1b). As shown in Fig. 1a, maximal induction was detected at 1.50 μg/L and day 10 when activity was measured by ATCh, whereas in Fig. 1b, maximal induction was detected at 0.50 μg/L and day 10 when activity was measured by BTCh. Obvious induction in terms of ChE activity was detected in groups exposed to triazophos concentrations ranging from 0.05 to 1.50 μg/L.

For chlorpyrifos exposure, induction up to 134.0% was detected when ChE activity was measured by ATCh (Fig. 1c), and induction up to 160.5% was detected when activity was measured by BTCh (Fig. 1d). As shown in Fig. 1c and d, maximal inductions were detected at 0.10 μg/L and day 8 regardless of whether ATCh and BTCh were employed. Obvious promotion ChE activity was detected in groups exposed to chlorpyrifos concentrations ranging from 0.05 to 0.50 μg/L.

2.1.2 Inhibition

Inhibition of ChE was found in groups exposed to relatively high concentrations of either triazophos or chlorpyrifos. Regardless of whether enzyme activity was measured by ATCh (Fig. 1c) or BTCh, initial inhibition was detected at 2.00 μg/L during exposure to triazophos (Fig. 1d). During exposure to chlorpyrifos, the initial inhibition was detected at 1.00 and 1.50 μg/L when activity was measured by ATCh (Fig. 1c) and BTCh (Fig. 1d), respectively.

At the end of triazophos exposure, no obvious inhibition of ChE activity was detected at any tested concentration when activity was measured by ATCh (Fig. 1a). When activity was measured by BTCh, however, initial inhibition was detected at 2.50 μg/L (Fig. 1b). At the end of chlorpyrifos exposure, initial inhibition was detected at 1.50 μg/L regardless of whether activity was measured by ATCh (Fig. 1c) or BTCh (Fig. 1d).

2.2 Activity of ChE at the beginning and end of the recovery culture

In vivo ChE activity in groups born in triazophos exposures are listed in Table 1, and *in vivo* ChE activity in those born in chlorpyrifos exposures are shown in Table 2.

2.2.1 Induction

For triazophos exposure, induction of ChE was detected in F1 (3rd) but not in F1 (1st) (Table 1). As indicated in Table 1, in samples collected before recovery culture, inductions up to 147.19% and 176.86% were detected when ATCh and BTCh were employed as substrates, and in samples collected at the end of the recovery culture inductions up to 126.93% were detected when BTCh was employed as the substrate.

For chlorpyrifos exposure, induction of ChE was detected in both F1(1st) and F1(3rd), although it was obvious in F1 (3rd) than F1(1st) (Table 2). As indicated in Table 2, inductions up to 121.30% were detected in F1(1st) collected at the beginning of the recovery culture when ATCh was employed as the substrate, while no obvious induction was detected in those collected at the end of the recovery

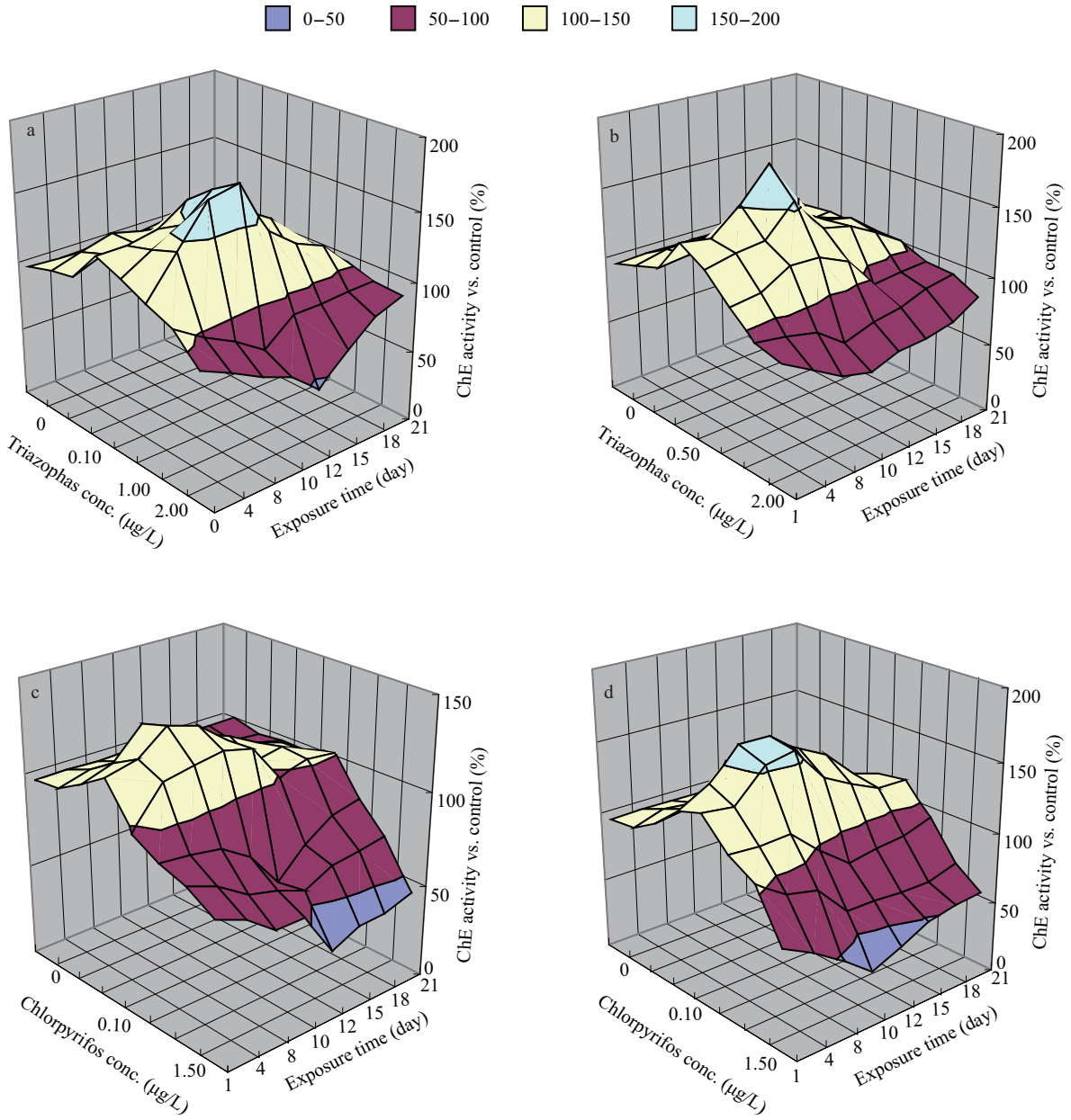

Fig. 1 *In vivo* activity of cholinesterase in *Daphnia magna* along with concentrations of anticholinesterases and time of exposures. Values are the average of three measurements. (a) Percentage activity vs. control in triazophos exposure using acetylthiocholine as substrate, (b) percentage activity vs. control in triazophos exposure using butyrylthiocholine as substrate, (c) percentage activity vs. control in chlorpyrifos exposure using acetylthiocholine as substrate, and (d) percentage activity vs. control in chlorpyrifos exposure using butyrylthiocholine as substrate.

culture. As shown in Table 2, inductions up to 188.15% were detected in F1 (3rd) collected before recovery culture when ATCh was employed as the substrate, while maximal induction was 148.42% when BTCh was employed as the substrate. In those collected at the end of the culture, inductions up to 136.25% were detected when ATCh was employed as the substrate.

2.2.2 Inhibition

Inhibition of ChE activity was detected in both the F1 (1st) and F1 (3rd) groups born in relatively high concentrations of either triazophos or chlorpyrifos, and the inhibition was detectable even though the groups went through a 21 day recovery culture (Tables 1 and 2).

Initial inhibition of ChE was detected at the end of the recovery culture in the F1 (1st) groups born in triazophos exposure of 2.50 μg/L when enzyme activity was measured by ATCh. Despite this, no obvious inhibition was detected in F1 (1st) at any tested concentrations when activity was measured by BTCh (Table 1). As for F1 (3rd), initial inhibition was detected in groups born in 2.50 μg/L exposure regardless of whether enzyme activity was measured by ATCh or BTCh (Table 1).

Initial inhibition of ChE was detected at the end of the recovery culture in the F1 (1st) groups born in chlorpyrifos exposure of 1.50 μg/L when enzyme activity was measured by ATCh. Despite this, the initial inhibition was detected in the F1 (1st) groups born in 2.00 μg/L exposure when enzyme activity was measured by BTCh (Table 2). As for F1 (3rd), no obvious inhibition was detected at any tested concentrations regardless of whether enzyme activity measured by ATCh or BTCh (Table 2).

Table 1 Activity of cholinesterase in *Daphnia magna* of F_1 (1st and 3rd) pretreated with triazophos

Exposure concentration (μg/L)	Cholinesterase activity (nmol/(mg·min))			
	Using ATCh as substrate		Using BTCh as substrate	
	Before recovery culture	21 days after recovery culture	Before recovery culture	21 days after recovery culture
The first batch				
0	42.26 ± 3.69 ab (100)	39.00 ± 4.05 a (100)	40.90 ± 3.73 a (100)	37.92 ± 4.05 a (100)
0.05	41.87 ± 3.34 ab (99.07)	38.58 ± 4.23 a (98.92)	39.84 ± 4.85 ab (97.40)	36.55 ± 4.90 a (96.38)
0.10	49.07 ± 3.16 a (116.12)	37.54 ± 3.94 a (96.26)	35.83 ± 4.92 ab (87.60)	35.44 ± 4.91 a (93.45)
0.50	40.91 ± 4.22 b (96.81)	36.39 ± 4.92 a (93.30)	32.84 ± 4.67 ab (80.30)	35.22 ± 5.87 a (92.89)
1.00	38.19 ± 5.23 b (90.37)	34.19 ± 4.09 ab (87.66)	30.88 ± 5.04 b (75.50)	34.51 ± 5.24 a (91.80)
1.50	37.92 ± 3.88 b (89.73)	34.26 ± 5.56 ab (87.84)	31.83 ± 5.87 b (77.60)	36.09 ± 3.63 a (95.18)
2.00	36.88 ± 3.20 bc (87.28)	32.18 ± 4.58 ab (82.52)	32.16 ± 4.06 ab (78.64)	32.27 ± 5.90 a (85.10)
2.50	30.11 ± 5.20 c (71.24)	27.47 ± 4.42 b (70.43)	30.90 ± 3.20 b (75.84)	28.54 ± 4.90 a (75.26)
The third batch				
0	41.36 ± 3.46 b (100)	39.86 ± 5.46 a (100)	39.96 ± 4.62 d (100)	38.86 ± 4.46 bc (100)
0.05	44.66 ± 3.36 b (107.97)	41.30 ± 5.63 a (103.61)	41.41 ± 4.45 cd (103.63)	41.05 ± 3.26 bc (105.63)
0.10	54.31 ± 4.60 a (131.32)	40.31 ± 3.86 a (101.13)	43.76 ± 3.36 cd (109.51)	42.44 ± 4.01 abc (109.22)
0.50	60.88 ± 5.56 a (147.19)	42.59 ± 5.23 a (106.85)	49.89 ± 4.36 bc (124.84)	46.12 ± 3.98 ab (118.69)
1.00	57.06 ± 4.64 a (137.96)	40.70 ± 3.69 a (102.11)	70.67 ± 6.23 a (176.86)	49.32 ± 4.08 a (126.93)
1.50	45.18 ± 5.26 b (109.23)	38.59 ± 4.01 a (96.81)	63.69 ± 4.62 a (159.38)	43.95 ± 3.76 ab (113.10)
2.00	43.66 ± 5.86 b (105.56)	36.73 ± 2.96 a (92.15)	54.05 ± 5.65 b (135.26)	35.06 ± 4.89 cd (90.23)
2.50	20.58 ± 5.23 c (49.75)	26.93 ± 3.23 b (67.56)	49.37 ± 5.65 bc (123.55)	31.05 ± 5.06 d (79.98)

Values enclosed in brackets are percentages of activity vs. control.
Data are presented as mean ± SD (n = 3). Values in each column marked with the same letter(s) are not significant ($p > 0.05$); whereas those marked with different letter(s) are significant ($p < 0.05$).

Table 2 Activity of cholinesterase in *Daphnia magna* of F1 (1st and 3rd) pretreated with chlorpyrifos[a]

Exposure concentration (μg/L)	Cholinesterase activity (nmol/(mg·min))			
	Using ATCh as substrate		Using BTCh as substrate	
	Before recovery culture	21 days after recovery culture	Before recovery culture	21 days after recovery culture
The first batch				
0	39.46 ± 2.99 bc (100)	38.91 ± 3.05 ab (100)	39.04 ± 5.73 a (100)	39.04 ± 3.05 a (100)
0.01	40.62 ± 2.34 ab (103.00)	42.93 ± 5.23 a (108.18)	39.38 ± 4.85 a (100.87)	38.98 ± 5.90 a (99.84)
0.05	38. 55 ± 4.16 bc (97.69)	37.44 ± 4.94 abc (96.22)	29.76 ± 4.92 b (76.31)	34.50 ± 3.91 ab (88.37)
0.10	39. 20 ± 5.22 bc (99.34)	32.15 ± 4.95 bcd (82.63)	27.90 ± 4.67 b (71.46)	31.69 ± 6.87 ab (81.17)
0.50	47.85 ± 5.23 a (121.30)	38.37 ± 5.09 ab (98.61)	27.75 ± 5.04 b (71.07)	34. 26 ± 4. 45 ab (109.00)
1.00	32.79 ± 4.88 bcd (83.10)	32.42 ± 4.56 bcd (83.32)	26.10 ± 5.87 bc (66.86)	31.42 ± 6.63 ab (80.48)
1.50	31.63 ± 4.20 cd (80.16)	28.07 ± 5.58 d (72.15)	20.93 ± 5.06 bc (53.60)	28.60 ± 6.90 ab (73.25)
2.00	26.50 ± 3.46 d (67.16)	28.88 ± 4.58 cd (74.22)	17.80 ± 5.90 c (45.60)	25.40 ± 4.90 b (65.06)
The third batch				
0	40.86 ± 5.46 c (100)	39.96 ± 5.62 c (100)	38.96 ± 5.62 cd (100)	37.96 ± 3.62 a (100)
0.01	41.26 ± 4.36 c (100.97)	42.21 ± 3.62 bc (102.3)	38.50 ± 4.45 d (98.81)	38.21 ± 4.72 a (100.65)
0.05	44.29 ± 4.60 c (112.33)	44.66 ± 4.92 bc (108.23)	38.86 ± 4.36 cd (99.75)	41.36 ± 6.01 a (108.96)
0.10	57.45 ± 5.56 b (145.60)	46.40 ± 6.01 bc (112.46)	52.97 ± 4.36 ab (135.96)	42.65 ± 3.80 a (112.36)
0.50	74.24 ± 6.64 a (188.15)	56.21 ± 3.62 a (136.25)	57.82 ± 5.23 a (148.42)	45.26 ± 4.63 a (119.23)
1.00	49.75 ± 5.26 bc (126.07)	49.45 ± 5.60 abc (119.86)	47.29 ± 4.62 bcd (121.38)	41.34 ± 2.65 a (108.90)
1.50	49.34 ± 5.86 bc (125.04)	50.35 ± 5.68 ab (122.03)	48.02 ± 5.65 bc (123.26)	41.49 ± 3.94 a (109.30)
2.00	49.94 ± 3.65 bc (122.23)	49.48 ± 4.63 abc (120.23)	42.70 ± 4.62 cd (109.59)	41.48 ± 5.73 a (109.26)

Values enclosed in brackets are percentages of activity vs. control.
Data are presented as mean ± SD (n = 3). Values in each column marked with the same letter(s) are not significant ($p > 0.05$); whereas those marked with different letter(s) are significant ($p < 0.05$).

2.3 Biological responses in exposure and in recovery culture

As seen from Table 3, declination of the reproduction rate was detected in the F0 groups born and developed in triazophos concentrations of ⩾ 0.10 μg/L, while declination of the body length of adults was detected in the F1 (1st) and F1 (3rd) groups born in triazophos concentrations of ⩾1.50 μg/L and ⩾2.00 μg/L, respectively. No declination and promotion in the body length of adults was detected in F0, F1 (1st), and F1 (3rd) at any tested concentrations.

As seen from Table 4, declination of the reproduction rate was detected in the F0 groups born and developed in chlorpyrifos concentrations of ⩾0.10 μg/L, while declination of the reproduction rate was detected in the F1 (1st) and F1 (3rd) groups born in chlorpyrifos concentrations of ⩾0.50 μg/L. No declination and promotion in the body length of adults was detected in F0, F1 (1st), and F1 (3rd) at any tested concentrations.

3 Discussion

3.1 Change of *in vivo* ChE activity and biological parameters

3.1.1 Induction

As indicted in this study, *in vivo* ChE activity was induced significantly at relatively low concentrations during

Table 3 Reproduction rate and body length of *Daphnia magna* treated with triazophos

Exposure concentration (μg/L)	Total number of offspring reproduced by per adult			Body length of adults (mm)		
	F0	F1 (1st)	F1 (3rd)	F0	F1 (1st)	F1 (3rd)
0	119.8 ± 4.3 a	118.0 ± 5.7 a	115.2 ± 8.0 a	2.82 ± 0.08 a	3.00 ± 0.08 a	2.90 ± 0.07 a
0.05	117.4 ± 7.2 a	117.0 ± 5.8 a	114.4 ± 7.8 a	2.78 ± 0.09 a	2.96 ± 0.07 a	2.92 ± 0.03 a
0.10	105.1 ± 5.3 b	113.0 ± 3.4 a	114.2 ± 7.0 a	2.80 ± 0.07 a	2.94 ± 0.09 a	2.90 ± 0.07 a
0.50	96.8 ± 5.6 bc	110.8 ± 5.7 ab	113.4 ± 6.3 a	2.72 ± 0.08 a	2.90 ± 0.07 a	2.88 ± 0.08 a
1.00	91.2 ± 6.0 c	108.0 ± 6.8 ab	112.8 ± 4.2 a	2.80 ± 0.16 a	2.94 ± 0.11 a	2.87 ± 0.16 a
1.50	74.7 ± 9.0 d	102.0 ± 2.9 b	106.4 ± 3.8 a	2.82 ± 0.08 a	2.90 ± 0.04 a	2.88 ± 0.08 a
2.00	–	91.6 ± 3.6 c	94.6 ± 4.2 b	–	2.95 ± 0.08 a	2.85 ± 0.11 a
2.50	–	74.2 ± 7.1 d	90.0 ± 9.5 b	–	2.90 ± 0.10 a	2.88 ± 0.10 a

Data are presented as mean ± SD (n = 20).
Values in each column with the same letter(s) are not significant ($p > 0.05$), whereas those with different letter(s) are significant ($p < 0.05$).

Table 4 Reproduction rate and body length of *Daphnia magna* treated with chlorpyrifos

Exposure concentration (μg/L)	Total number of offspring reproduced by per adult			Body length of adults (mm)		
	F0	F1 (1st)	F1 (3rd)	F0	F1 (1st)	F1 (3rd)
0	112.6 ± 4.3 a	116.2 ± 5.9 a	118.5 ± 6.5 a	2.76 ± 0.05 a	2.78 ± 0.08 a	2.82 ± 0.02 a
0.01	107.1 ± 7.2 ab	115.9 ± 5.6 a	113.2 ± 7.5 a	2.76 ± 0.05 a	2.78 ± 0.78 a	2.81 ± 0.01 a
0.05	102.6 ± 5.3 ab	116.8 ± 7.0 a	110.4 ± 6.7 ab	2.76 ± 0.15 a	2.78 ± 0.08 a	2.82 ± 0.01 a
0.10	98.3 ± 5.6 bc	113.8 ± 4.1 a	107.6 ± 5.3 abc	2.80 ± 0.14 a	2.74 ± 0.05 a	2.79 ± 0.11 a
0.50	90.6 ± 6.0 cd	100.6 ± 8.9 b	99.2 ± 6.4 bcd	2.66 ± 0.13 a	2.74 ± 0.21 a	2.80 ± 0.03 a
1.00	84.8 ± 9.0 d	96.8 ± 7.7 bc	94.6 ± 5.0 cd	2.70 ± 0.10 a	2.72 ± 0.22 a	2.79 ± 0.12 a
1.50	–	87.6 ± 3.9 c	90.4 ± 10.9 d	–	2.74 ± 0.21 a	2.78 ± 0.13 a
2.00	–	70.2 ± 11.1 d	85.8 ± 9.4 d	–	2.72 ± 0.16 a	2.78 ± 0.21 a

Data are presented as mean ± SD (n = 20).
Values in each column with the same letter(s) are not significant ($p > 0.05$), whereas those with different letter(s) are significant ($p < 0.05$).

21-day exposure before it became inhibited at relatively high concentrations. This changing pattern displayed characteristics of hormesis, a dose-response relationship featured by conversion from low-dose stimulation to high-dose inhibition (Calabrese, 2002; Chapman, 2002). Hormesis in terms of ChE activity was detected not only in groups that developed in anticholinesterase exposures but also in groups removed from the exposures within 24 hr of their birth and maintained in recovery culture for 21 days thereafter. Despite the frequent induction of ChE, corresponding induction in terms of biological parameters such as reproduction rate and body size were not found in daphnia collected at the end of the exposure or collected at the end of the recovery culture. This suggested that induction in terms of ChE activity did not influence population prosperity of daphnia, or to say, induction in terms of ChE activity was unrelated to prosperity of daphnia population.

Anticholinesterases have promoted population development of some terrestrial arthropod pests under field conditions. These anticholinesterases include four organophosphorus compounds and three methyl carbamates, and the pests involved include the two-spotted spider mite, the brown plant-hopper, and the green peach aphid (Cohen, 2006). Despite this, no induction of the reproduction rate or body size were detected in daphnia from this study. The promotion mentioned by Cohen (2006) might result from improvement of life conditions for these pests due to the removal of natural enemies by the application of anticholinesterases, or due to providing better growing host plants stimulated by the non-toxic metabolites of anticholinesterases. Nonetheless, in this study promotion did not result from induction of ChE by anticholinesterases.

3.1.2 Inhibition

Results from this study indicated that triazophos and chlorpyrifos concentration levels at which initial declination of reproduction rate was detected were obviously lower than those at which initial inhibition of ChE activity was detected, regardless of whether the samplings were taken at the end of the 21-day exposure or at end of the 21-day recovery culture. This suggested that induction made ChE less sensitive in predicting declination of reproduction rate in long-term exposure.

3.2 Possible reason for induction of ChE by anticholinesterases

There are contradictory findings on the response of *in vivo* ChE in aquatic organisms exposed to sub-lethal doses of anticholinesterases. A study conducted with mosquito fish (*Gambusia affinis*) revealed enhanced inhibition in terms of activity of brain ChE along with time of exposure when fish were exposed to relatively high concentrations of fenitrothion (i.e., 0.6 and 1.3 mg/L), whereas when fish were exposed to relatively low concentrations of fenitrothion (i.e., 0.3 mg/L), the extent of inhibition reduced dramatically with exposure time (Sorsa et al., 1999). Concentrations of fenitrothion adopted by Sorsa et al. (1999) were in the range of 1/8–1/3 of the 96 hr LC_{50}, and concentrations of triazophos and chlorpyrifos adopted in this study were in the range of 1/276–1/6 and 1/777–1/4 of the 48 hr EC_{50} (Tan et al., 2004). It is reasonable to infer, therefore, that the difference in margin between the adopted concentrations and the EC_{50} (or LC_{50}) resulted in marked induction in terms of ChE activity was detected in this study but not by Sorsa et al. (1999).

Induction of ChE might be explained by so-called "compensating metabolism", a process that rectifies functional disability on condition that part of the activity was inhibited by anticholinesterases (Lapadula et al., 1990). In a study of Japanese quail (*Coturnix coturnix*), a 40% increase in immunoreactive ChE (ChE-IR) quantity was detected in serum of birds orally administered with 20 mg/kg of monocrotophos (Khattab et al., 1994). A study conducted with red-legged partridges (*Alectoris rufa cross*) revealed a 26.5% increase in ChE-IR quantity in serum for birds orally administered with 167 mg/kg of malathion (Khattab and Ali, 2007). A 50% increase in ChE-IR quantity was detected in the brain of freshwater fish (*Psudorasbora parva*) for specimens exposed to 0.1 to 48 μg/L of triazophos (Li et al., 2005). All the above studies suggested accelerating synthesis of ChE when animals were exposed to anticholinesterases. Evidence for the existence of this acceleration was seen in the significant increase in ChE-related mRNA in mice (*Mus pahari*) administered with sub-lethal dosages of anticholinesterases (Kaufer et al., 1999).

The inducible nature of ChE seems to benefit daphnia in their attempt to mitigate the impact of anticholinesterases, but the nature is disadvantageous for ChE to act as a predictive biomarker in sub-lethal or long-term stresses. Firstly, it promotes total activity of ChE so conceals the actual inhibition of the enzyme resulting from anticholinesterase exposure. Secondly, due to the induction, enzyme activities change with time, as it was shown in this study, which means it is difficult to determine the actual relationship between the degrees of ChE inhibition and ambient concentration of anticholinesterases – which is critical for diagnosing field presence of anticholinesterases.

Results from this study suggested that in determining the field presence of anticholinesterases, it is better to scale *in vivo* ChE activity with content of the enzyme itself, as per Khattab and Ali (2007) and Li et al. (2005), rather than with that of total protein, as is done prevalently nowadays.

3.3 Types of ChE in daphnia

The ChE involved in this study could hyrolyze ATCh and BTCh at comparable rates, whereas ChE hydrolyzed ATCh at a much quicker rate than it hydrolyzed BTCh (Diamantino et al., 2003). This difference might result from the fact that daphnia contain both AchE and PchE, and the populations involved in this study possessed a higher portion of PchE than in the work by Diamantino et al. (2003). This also suggestted that daphnia had multi types of ChE in their bodies.

The presumption that daphnia contained multiforms of ChE was further evidenced by the fact that changes in patterns of the enzyme with concentrations and time of exposures were inconsistent when ATCh and BTCh were applied as substrates.

Considering the possible diversity of different types of ChE (Villatte and Bachmann, 2002; Wang et al., 2004; Lenz et al., 2005) in their responses to anticholinesterase exposure, it is necessary to separate ChEs from daphnia and to explore their dynamic properties and their sensitivity towards different types of anticholinesterases to better understand the efficiency of this biomarker in predicting the hazards of anticholinesterases in long-term exposure.

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