Possible Enzymatic Mechanism Underlying Chemical Tolerance and Characteristics of Tolerant Population in Scapholeberis Kingi

Makoto Ishimota (mota-4024will@hotmail.co.jp)
The Institute of Environmental Toxicology https://orcid.org/0000-0003-4686-0244
Mebuki Kodama
The Institute of Environmental Toxicology
Naruto Tomiyama
The Institute of Environmental Toxicology

Research Article

Keywords: Cladocera, Insecticide, Tolerance, Acetylcholinesterase, Peroxidase, Superoxide dismutase, Multi-generational study, Field population.

Posted Date: May 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-492302/v1

Version of Record: A version of this preprint was published at Environmental Science and Pollution Research on October 27th, 2021. See the published version at https://doi.org/10.1007/s11356-021-17071-8.

Abstract

To determine the potential effects of pesticides on aquatic organisms inhabiting a realistic environment, we explored the characteristics and mechanisms of chemical tolerance in *Scapholeberis kingi*. We established a chemical-tolerant population via continuous exposure to pirimicarb, an acetylcholinesterase (AChE) inhibitor, and examined the effects of pirimicarb concentration on the intrinsic growth rates (*r*) of tolerant cladocerans. We also explored the association between *r* and feeding rate and tested the involvement of antioxidant enzymes [peroxidase (PO) and superoxide dismutase] and AChE in pirimicarb sensitivity. *S. kingi* was continuously exposed to sublethal pirimicarb concentrations (0, 2.5, 5, and 10 μg/L) for 15 generations and changes (half maximal effective concentration at 48 h, 48 h-EC_{50}) in chemical sensitivity were investigated. In the F14 generation, the sensitivity of the 10 μg/L group was three times lower than that of the control group, suggesting the acquisition of chemical tolerance. Moreover, *r* was significantly and negatively correlated with 48 h-EC_{50}, suggesting a fitness cost for tolerance. Surprisingly, there was no significant correlation between *r* and feeding rate. Our generalized linear model indicated that elevated PO activity may be related to chemical tolerance. Therefore, oxidative stress regulation may be involved in the acquisition of chemical tolerance in cladocerans. These findings will help elucidate the characteristics and mechanisms of chemical tolerance in aquatic organisms inhabiting a realistic environment.

Introduction

The risk assessment of chemicals to aquatic organisms is primarily based on recommended tests on model species (Brock and Van Wijngaarden 2012; Taenzler et al. 2007). However, the chemical sensitivity of test species differs from that of their actual field clones. Because field clones may inhabit real environments with various stressors or chemicals, they often acquire chemical tolerance (Haap and Köhler 2009; Jansen et al. 2011; Muyssen et al. 2002). Despite their low sensitivity to chemicals, tolerant individuals encounter fitness costs for this adaptation, such as reduced population growth rate or shortened life span, compared with sensitive individuals (Heine-Fuster et al. 2017; Homem et al. 2020). Furthermore, insecticide-tolerant individuals require more energy and have greater fat body mass than sensitive individuals (Kliot and Ghanim 2012). However, several characteristics of chemical-tolerant populations remain unknown. Thus, further research is warranted to elucidate the characteristics of chemical-tolerant populations, which will help understand the true effects of chemicals on field clones.

To investigate the characteristics of chemical tolerance, organisms with different degrees of chemical sensitivity must be produced, and multigenerational exposure to chemicals is a strategy to obtain such organisms. In a previous study, we investigated changes in the sensitivity of the cladoceran species *Scapholeberis kingi* to the carbamate insecticide pirimicarb, an acetylcholinesterase (AChE) inhibitor, which is one of the most common insecticides detected in surface water (Struger et al. 2016; Ishimota and Tomiyama 2020); we performed intergenerational experiments (two generations) by exposing neonates (<24-hour old) to pirimicarb for 48 h and observed that pirimicarb sensitivity differed between the pirimicarb-treated and non-treated individuals. Although we measured AChE activity in clones, we

could not explain the intergenerational changes in pirimicarb sensitivity based on the activity of this enzyme. Pirimicarb has been reported to induce oxidative stress or produce genotoxic and cytotoxic effects in aquatic organisms, including fish, tadpoles, and snails (Natale et al. 2018; Raisi et al. 2018; Vera-Candioti et al. 2015), indicating the potential involvement of antioxidant enzymes in the chemical sensitivity of cladocerans.

Antioxidant enzymes serve as useful biomarkers for various chemicals, such as pyrethroid, organochlorine, pharmaceutical chemicals, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and pirimicarb (Connon et al. 2003; Oliveira et al. 2015; Raisi et al. 2018; Valavanidis et al. 2006; Van der Oost et al. 2003). Peroxidase (PO) catalyzes the peroxidation of halogen compounds (Cl^-, Br^-, and I^-) to reduce various peroxides (Hajishengallis and Russell 2015; Van der Oost et al. 2003). Superoxide dismutase (SOD) catalyzes the production of reactive superoxide radicals (O_2^-) to yield hydrogen peroxide (H_2O_2) and oxygen (Park et al. 2012). To understand mechanisms underlying chemical tolerance (i.e., enzymes involved in the alteration of sensitivity to AChE inhibitors), we continuously exposed *S. kingi* to various concentrations of pirimicarb and established populations with varying degrees of sensitivity to this chemical. Subsequently, we explored the involvement of antioxidant enzymes (PO and SOD) in pirimicarb tolerance of these population. We also measured AChE activity considering that it is the target enzyme for pirimicarb (Jeon et al. 2013).

S. kingi is a cladoceran commonly observed in paddy fields and ditches (Mano et al. 2010). It is considered a suitable model species for evaluating chemical toxicity owing to its higher sensitivity to AChE inhibitors than that of other test species, such as *Daphnia magna* (Ishimota et al. 2020b).

To this end, the objectives of the present study were (1) to assess changes in the intrinsic growth rate (*r*) of field cladocerans (*S. kingi*) depending on their degree of pirimicarb tolerance; (2) to determine the association between *r* and feeding rate; and (3) to explore the involvement of antioxidant enzymes (PO and SOD) and AChE in pirimicarb sensitivity.

We collected several *S. kingi* clones and selected them via continuous exposure to sublethal pirimicarb concentrations for 15 generations (F0–F14). To investigate changes in the sensitivity of *S. kingi*, the half maximal effective concentration at 48 h (48 h-EC_{50}) for neonates (<24-hour old) from the F0, F4, and F14 generations was calculated. Additionally, the *r* and feeding rate of the chemical-tolerant population were determined. Finally, the association of the 48 h-EC_{50} value with the activity of PO, SOD, and AChE was determined using a generalized linear model (GLM).

Methods

Chemical analysis

We obtained pirimicarb (99.9% purity) from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and used it as the model AChE inhibitor. To determine its pirimicarb concentration, we diluted the test

solution with acetonitrile in pure water (60:40 v/v). The pirimicarb concentration of each test solution was measured using liquid chromatography–mass spectrometry (LC-MS/MS) at a flow rate of 0.3 mL/min and column temperature of 40°C (LC: 1290 HPLC; MS/MS: 6460 Triple Quad LC-MS/MS, Agilent Technologies, CA, USA; column: Acquity UPLC HSS T3, 1.8 μm × 2.1 mm × 100 mm, Waters, MA, USA). The analytical conditions were set as described previously (Ishimota et al. 2020a). The limit of quantification was set as 0.5 μg/L.

In a previous study, we confirmed that recoveries and their relative standard deviations (RSDs %, SD divided by mean) for pirimicarb were within the acceptable range using this method (mean recovery rate, 90%–108%, RSD, 1%–2%) (Ishimota and Tomiyama 2020). Furthermore, the target chemical was very stable during the exposure period (48 h). Thus, we measured pirimicarb concentration only at the beginning of exposure in the multigenerational experiment. We set the acceptable recovery for pirimicarb concentrations as 70%–120% (US EPA 2012; Stamatis et al. 2013).

Test organisms and culture methods

In a previous study, we confirmed that *S. kingi* clones exhibited different degrees of sensitivity to pirimicarb (Ishimota and Tomiyama 2020). Thus, we used clones collected from four littoral sites, namely Lake Kasumigaura (36°04'59"N, 140°13'06"E), Lake Kitaura (36°04'16"N, 140°31'44"E), Tega pond (35°51'40"N, 140°02'16"E); and Moriya pond (35°57'04"N, 140°00'19"E), during the summer of 2015. A single *S. kingi* clone from each site was individually maintained in the ISO medium (ISO 1996) with slight modification (36.8 mg/L $CaCl_2 \cdot 2H_2O$, 6.1 mg/L $MgSO_4 \cdot 7H_2O$; 64.8 mg/L $NaHCO_3$, 8.60 mg/L KCl, 75.0 μg/L thiamine hydrochloride, 1.00 μg/L cyano-cobalamin, and 0.75 μg/L biotin) (Ishimota and Tomiyama 2020). For culture, including the acclimation period, 10 adults were incubated in 100 mL glass beakers containing 100 mL of the medium and fed at least five times per week with *Chlorella vulgaris* (Recenttec K. K, Japan, 5×10^5 cells/mL). The cultures were maintained under a 16:8 h light:dark photoperiod at 800 lx and 22 ± 1°C. Neonates from the third or fourth brood were used for stock culture. The culture medium was changed three times per week.

Multigenerational experiment

In a previous study, we noted changes in the chemical sensitivity of *S. kingi* following exposure to sublethal pirimicarb concentrations for several clonal lineages (Ishimota and Tomiyama 2020). Thus, we expected clonal differences in the acquisition of chemical tolerance in the test species. To normalize the ability to acquire pirimicarb tolerance, 10 individuals from each sampling site were pooled and cultured together (total individuals, 40) in 500 mL beakers containing 500 mL of the medium. These individuals were cultured as the test population for 1 month according to the standard culture conditions before starting the experiment.

We prepared a stock solution of 1.6 g/L pirimicarb with a methanol: pure water solution (50:50, v/v). Next, we diluted this stock solution with the culture medium to prepare six concentrations of the test solution (0, 2.5, 5.0, 10.0, 20.0, and 40.0 μg/L) in methanol (12.5 μL/L). We then conducted acute toxicity

experiments based on the OECD guideline no. 202 (OECD 2004). Five female neonates (<24-hour old) collected from the third or fourth brood were exposed to 50 mL of test solution in a 50 mL glass beaker without food under standard culture conditions (as previously described) for 48 h (n = 4; total individuals, 20). Following exposure, we counted the neonates that could not swim during 15 s of gentle agitation of the beaker as immobilized individuals at each concentration. We determined the 48 h-EC_{50} values and 95% confidence intervals for the first generation (F0) based on the immobilization data.

In the preliminary experiment, the 48 h-EC_{50} values for pirimicarb for each clone ranged from 7 to 16 µg/L. Considering the sublethal concentration for each clone, neonates (<24-hour old) in the third or fourth brood were continuously exposed to various pirimicarb concentrations (0, 2.5, 5.0, and 10.0 µg/L) until they reproduced third- or fourth-brood neonates. We exposed the test cladocerans (<24-hour old) to 100 mL of each test solution in a glass beaker containing 100 mL of the medium (n = 10) and cultured them according to the standard method (previously described). We collected the third- or fourth-brood neonates to maintain the next generation and to determine the 48 h-EC_{50} values and enzyme activity. Third- or fourth-brood neonates (<24-hour old) from the F0, F4, and F14 generations in each test group were exposed to various pirimicarb concentrations (0, 2.5, 5.0, 10.0, 20.0, and 40.0 µg/L) for 48 h to calculate the 48 h-EC_{50} values and 95% confidence intervals.

To estimate the variation in 48 h-EC_{50} values, we performed all experiments in duplicate.

Measurement of r

The *r* value is a representative life cycle parameter providing information at the population level (growth and reproduction) (Buhl et al. 1993; Silva et al. 2017). According to the OECD test guideline, TG 211 (OECD 1997) reproduced 12 neonates (<24-hour old) from the third brood in each test group (0, 2.5, 5, and 10 µg/L); neonates in each test group were exposed to the same pirimicarb concentrations until 21 days after birth. From each test group, one neonate was placed in a 10 mL glass beaker containing 10 mL of each test solution; for each test concentration, four replicates were set, and the experiment was repeated in triplicate (total neonates, 12 per test group). The survival and reproductive rates were counted daily for 21 days in each generation (Ishimota and Tomiyama 2020). To avoid density effects, we removed the neonates immediately after counting. The neonates were cultured under standard culture conditions and fed with *C. vulgaris* (5×10^5 cells/mL) every day. The *r* values were calculated using the daily age-specific survival and reproduction data. Means and standard deviations of the *r* values were calculated based on the rates in each replicate (n = 3). The *r* values in the F0, F4, and F14 generations were estimated using the dominant eigenvalue (λ) of the Leslie matrix (daily time step, 21 age classes) for each treatment, using the following equation (Case 2000):

$$r = loge(\lambda)$$

where *r* is the intrinsic population growth rate and λ is the dominant eigenvalue of the Leslie matrix.

Feeding experiment

To determine mechanisms underlying the generational changes in *r*, the feeding rate of mature *S. kingi* in each test group was calculated by comparing the food concentration at the beginning of the experiment with that at the end. This experiment was conducted using test individuals in the F0, F4, and F14 generations from each concentration group. Pirimicarb test solutions (0, 2.5, 5.0, and 10 µg/L) in methanol (12.5 µL/L), and *C. vulgaris* density was set at 5×10^5 cells/mL. One adult individual (7-day old) from each concentration group was placed in a 10 mL glass beaker containing 10 mL of each test solution, and five replicates were set for each test group. To confirm the decrease in algal cell density, a blank group (a 10 mL glass beaker containing 10 mL of the medium with the same density of algal cells but without *S. kingi*) was prepared. All experiments were performed in the dark at a controlled temperature (22 ± 1°C) to minimize algal growth (Agra et al. 2010). Algal cell density was measured using flow cytometry (Guava EasyCyte Mini, Millipore, USA) at the beginning and after 48 h of exposure. The feeding rate (*Fr*) was calculated as follows:

$$Fr\ \ (\%) = \left(\frac{(Ct0 - Ct48) \times 100}{Ct0}\right) - \left(\frac{(Cb0 - Cb48) \times 100}{Cb0}\right)$$

where *Fr* is feeding rate, *Ct* and *Cb* are the algal cell densities in the pirimicarb test (0, 2.5, 5.0, and 10 µg/L) and blank groups, respectively; and "0" indicates time at the beginning of pirimicarb exposure; "48" indicates time at 48 h after exposure (the end of the exposure).

Additionally, at the beginning of the test, the body sizes of *S. kingi* in each test group were measured (n = 5) and compared using ANOVA ($p = 0.05$). This experiment was conducted using test neonates in the F0, F4, and F14 generations from each concentration group.

Enzyme activity

Initially, pirimicarb test solutions (0, 2.5, 5.0, and 10 µg/L) in methanol (12.5 µL/L) were prepared. Since the body size of *S. kingi* is very small (approximately 0.3 mm), 100 neonates (<24-hour old) were required to measure the activity in a single sample. Thus, three sets of 100 neonates (<24-hour old) in the third brood were exposed to 1 L of each test solution in a 1 L glass beaker for 48 h. Then, the F0 neonates in each beaker were homogenized according to the analytical method described previously (n = 3; total individuals, 300) (Ishimota and Tomiyama 2020). In addition, 100 F4 and F14 neonates (<24-hour old) in the third brood were exposed to the same pirimicarb concentrations and homogenized; three replicates were set for each test group. All samples were filtered using a cell strainer (70 µm, FALCON®) in 100 µL of 1% phosphate-buffered saline containing 0.25 mg/mL Pefabloc® SC (Sigma analytical standard, Sigma Aldrich, UK). The samples were sonicated for 15 min and centrifuged for 10 min at 4°C and 15,000 × *g*. The supernatant of each sample was isolated, and the protein concentration was measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Next, enzyme (PO, SOD, and AChE) activity was determined using colorimetric assay kits [PO: Amplite™ Colorimetric Peroxidase (HRP) Assay Kit, AAT Bioques; SOD:

Superoxide Dismutase Assay Kit, Cayman Chemical, Michigan, USA; and AChE: Amplite™, AAT Bioquest, Sunnyvale, CA, USA] and measured using a spectrophotometer (SpectraMax® 190 Microplate Reader, Molecular Devices, San Jose, CA, USA) according to the manufacturer's protocol. Absorbances of PO, SOD, and AChE were measured at 410, 664, and 470 nm, respectively. The activity of each enzyme in the samples was expressed as a ratio of each protein [μmol/(min mg) of protein, n = 3]. Following calculation, activity in each test sample was divided by that in each control sample (0 μg/L) to determine the rate relative to the controls.

Statistical analysis

All data were analyzed using R 3.6.1 (R Development Core Team 2019). Using the medrc package, the 48 h-EC_{50} values with 95% confidence intervals were estimated by fitting the acute toxicity data to a two-parameter log-logistic model, considering duplicate testing of each test group as the random effect (Gerhard and Ritz 2017; Ishimota et al. 2020b). Significant differences in 48 h-EC_{50} values among the test groups were analyzed with the ratio test using the EDcomp function in the drc package (with a significance level: $p = 0.05$) (Ritz et al. 2006; Wheeler et al). The significance level for each pair was adjusted using Holm's method (Holm 1979). Activity of each enzyme in all generations was compared using a pairwise t-test function in R, and the significance level in each pair was adjusted using Holm's method (Holm 1979).

If we observed the negative effects of pirimicarb on *r* in the F0 group, we investigated the correlation between 48 h-EC_{50} and mean *r* for all generations using Pearson's correlation analysis with the Rcmdr package (Fox 2005). Pearson's coefficients (cor) were calculated with a significance level of 0.05, with a coefficient of 1 ($p < 0.05$) indicating a perfect positive correlation and a coefficient of -1 ($p < 0.05$) indicating a perfect negative correlation.

To determine the correlation between enzyme activity and 48 h-EC_{50} in multiple generations (F0, F4, and F14), a GLM was built. We selected the best model based on the lowest Akaike information criterion (AIC) (Akaike 1974). The full model included all variables (generational alteration; pirimicarb concentration; and PO, SOD, and AChE activity). In this GLM, gamma distribution (log-link) was assumed. Since there were no interactions, the variables were assumed to be independent of one another. The significance of variables was determined using the χ^2 test ($p < 0.05$) based on the Wald statistic (Hosmer and Lemeshow 2000; Ishimota et al. 2020b; Marques et al. 2008).

Results

Insecticide concentration and water quality in test solutions

In the intergenerational experiment, the ratio of the measured concentration to the nominal concentration in the water sample at the start of pirimicarb exposure ranged from 80% to 104%, which was within the acceptable range (70%–120%).

Temperature, pH, and dissolved oxygen concentration in the water ranged from 22.1 to 22.6°C, 8.11 to 8.44, and 8.10 to 9.36 mg/L, respectively, and variations in these parameters remained within the OECD test guideline no. 202 throughout the experiment (OECD 2004).

Multigenerational experiments

The 48 h-EC_{50} values in the control group (0 µg/L) were comparable during the test generations (Fig. 1), and no immobilized neonates were not found in any generations of controls (0 µg/L).

In the F4 and F14 generations, the 48 h-EC_{50} values in the 2.5 µg/L concentration group were comparable to those in the control group. In contrast, in the F4 generation, the 48 h-EC_{50} value in the 5.0 concentration group were significantly decreased compared with those in the control group. Likewise, in the F4 generation, the 48 h-EC_{50} value in the 10 µg/L concentration group were decreased compared with those in the control group, albeit non-significantly. In the F14 generation, the 48 h-EC_{50} values in the 5.0 and 10 µg/L concentration groups (12.2–25.8 µg/L) were increased compared with those in the control group (7.7-9.7 µg/L). In particular, the 48 h-EC_{50} values in the 10 µg/L concentration group were approximately three times higher than those in the control group (0 µg/L). These results confirmed that *S. kingi* acquired pirimicarb tolerance in the F14 generation.

Additionally, our GLM showed that the 48 h-EC_{50} values significantly increased as the generations proceeded (Table 1), but the exposure concentration was not related to the tolerance degree.

r and feeding rate

The *r* values in the control group (0 µg/L) were comparable throughout the experimental period (Fig. 2). The *r* values in the 10 µg/L concentration group were significantly lower than those in the control group in the F0 and F14 generations but comparable to those in the control group in the F4 generation. Additionally, the 48 h-EC_{50} value was significantly and negatively correlated with the *r* values (cor = -0.82, $p < 0.01$).

The feeding rates in the control group (0 µg/L) were comparable throughout the experimental period. In the F0 generation, the feeding rates in all test groups were decreased compared with those in the control group, albeit non-significantly (Fig. 3). In contrast, in the F4 generation, the feeding rates were comparable among the test and control groups. However, in the F14 generation, the feeding rates in the test groups were significantly higher than those in the control group. Contrary to our expectations, the feeding rate was not related to the *r value* (cor = -0.39, $p \geq 0.05$).

Furthermore, the body size of the test species (0.60-0.65 mm) did not significantly differ among the test groups, suggesting that body size hardly affected the feeding rate.

Enzyme activity

The PO activity of the F0 generation in the 2.5 and 5.0 µg/L concentration groups and of the F4 generation in the 5.0 and 10.0 µg/L concentration groups was decreased compared with that in the control group (0 µg/L) (Fig. 4). Although the PO activity of the F0 generation in the 10.0 µg/L concentration group did not decrease, this may be attributed to the large variation in the activity of this enzyme. The PO activity of the F14 generations in all test groups was comparable to that in the control group (0 µg/L).

The SOD activity of the F0 generation in all test groups was decreased compared with that in the control group (0 µg/L) (Fig. 5). In the F4 generation, although the SOD activity in the 5.0 and 10.0 µg/L concentration groups was decreased compared with that in the control group, the activity in the 2.5 µg/L concentration group was comparable to the control level. Finally, in the F14 generation, the SOD activity in all test groups was comparable to that in the control group.

The AChE activity in the test groups was significantly decreased compared with that in the control group in the F0 and F4 generations but remained comparable to the control level in the F14 generation (Fig. 6).

Overall, a significant decrease in the activity of all enzymes was confirmed in the F0 and F4 generations, but the activity recovered to the control level in the F14 generation. In the best GLM, elevated PO activity was significantly correlated to increased 48 h-EC_{50} (cor = 0.82, $p < 0.01$); however, the remaining two enzymes were not related to chemical tolerance (Table 1).

Table 1

Generalized linear model of the half maximal effective concentration at 48 h for estimating generational alternation, pirimicarb concentration, enzyme activity

Variable	Parameter coefficients in best model		AIC	
	Slope	*P* (>|t|)	Best model	Full model
Intercept	1.281	5.35×10^{-6}*	206	209
Generational alternation	0.048	6.09×10^{-5}*		
Pirimicarb concentration	0.038	0.05		
Peroxidase	0.834	6.23×10^{-4}*		
Superoxide dismutase	-	-		
Acetylcholinesterase	-	-		

Asterisks indicate the explanatory variables (* $p < 0.001$, Wald statistics).

-: Variables excluded from the best model.

Discussion

In F4 generation, individuals exposed to higher pirimicarb concentrations (5.0 and 10 µg/L) were more sensitive than the controls (Fig. 1), suggesting that they could not recover from the sublethal effects of the chemical even after a few generations. However, at the end of the experimental period (in the F14 generation), individuals exposed to the highest pirimicarb concentration (10 µg/L) were the most tolerant. These findings indicate that the chemical sensitivity of the test populations was strongly affected at the initiation of pirimicarb exposure, but they could adapt to the effects of this pollutant over generations.

In previous studies, when cladocerans were continuously exposed to various chemicals (nano-scaled titanium dioxide, fungicides, and organophosphate) over several generations, they produced more sensitive clones than controls (Jacobasch et al. 2014; Silva et al. 2017; Zalizniak and Nugegoda 2006). For instance, following continuous exposure to chlorpyrifos (organophosphate) over three successive generations, *D. carinata* produced almost two times more sensitive neonates than the original clones (Zalizniak and Nugegoda 2006), which supports our findings. In contrast, several reports have suggested that cladocerans could rapidly acquire chemical tolerance following exposure to synthetic chemicals (mercury and carbamates (pirimicarb and carbaryl)) (Tsui and Wang 2005, Ishimota et al. 2020b; Jansen et al. 2010). Given these contradictory findings regarding the changes in chemical sensitivity, we cannot definitively establish the number of generations required to obtain a chemical-tolerant population via exposure to chemicals. In a study by Wuerthner et al. (2019), *D. pulex* acquired higher pesticide tolerance when exposed to sublethal carbaryl concentrations at early life stages (carbaryl) than when exposed at later life stages. Thus, exposure at early life stages might be an important factor in the acquisition of chemical tolerance. In this study, we continuously exposed *S. kingi* to various chemical concentrations at early life stages, which possibly induced chemical tolerance of varying degrees in the test cladocerans.

The *r* value is a sensitive life cycle parameter that combines the number of broods, survival rate, and time to the first brood release (Silva et al. 2017; Zalizniak et al. 2006). Although the *r* value of the F0 generation in the 10 µg/L concentration group was decreased compared with that in the control group, it recovered to the control level in the F4 generation (Fig. 2). In several studies, the decreased *r* values of *D. magna* exposed to various chemicals (nickel and molinate) were increased following exposure over several subsequent generations (three to six generations) (Münzinger 1990; Sánchez et al. 2004), which supports our findings in the F4 generation. However, the recovered *r* values in the F4 generation decreased again in the F14 generation, suggesting that the potential effects of chemicals are underestimated by short-term (up to only five generations) multigenerational studies. Thus, longer exposure to chemicals may, in fact, negatively affect the growth rate of cladocerans. Additionally, the 48 h-EC_{50} value was significantly correlated with the *r* value, indicating a potential fitness cost for tolerance. In several studies, no fitness costs of chemical tolerance were detected (Santos-Amaya et al 2017; Saro et al. 2012);

therefore, our findings provide valuable information for understanding the characteristics of a chemical-tolerant population.

We hypothesized that the *r* value would be positively correlated to the feeding rate in *S. kingi*. In other words, decreased food intake may decrease the growth rate. Feeding behavior is an important index to explore the effects of pollutants on ecosystems (Liu et al. 2019). Several studies have evaluated chemical toxicity using feeding rate as the endpoint and reported decreased rates following exposure to chemicals (Liu et al. 2019; Ogonowski et al. 2016; Villarroel et al. 1999). In *D. longispina*, the feeding rate of metal-tolerant individuals was lower than that of sensitive controls (Agra et al. 2010). In this study, contrary to our expectations, there was no significant correlation between these two parameters.

In the F0 generation, the feeding rates in all test groups were significantly lower than those in the control group; however, in the F14 generation (chemical tolerant generation), the feeding rates in all test groups were higher than those in the control group (Fig. 3). Similarly, in previous studies, higher algal consumption in daphnids continuously exposed to lead or fungicides (carbendazim) over several generations has been observed (Araujo et al. 2019; Silva et al. 2017; Skjolding et al. 2014), suggesting that increased food consumption accelerates gut clearance and helps detoxification. Thus, our findings provide evidence that chemical-tolerant clones consume more energy to eliminate pollutants from their body. Energy and resource allocation for adaptation and survival is essential when organisms cope with the toxicity of insecticides (Kliot and Ghanim 2012). In fact, the total carbohydrate, protein, and lipid content in *D. magna* exposed to several chemicals, such as tributyltin chloride and linear alkylbenzene sulfonic acid, was related to survival, growth, and reproduction (De Coen and Janssen 2003). Therefore, these parameters should be investigated to determine the characteristics of chemical-tolerant clones in detail.

Finally, we discuss the enzymatic mechanism underlying the differential chemical sensitivity of various clones. Decreased AChE activity of the F0 and F4 generations in the 5.0 and 10.0 μg/L concentration groups suggests that pirimicarb inhibited AChE (Fig. 6). These observations are consistent with our previous findings of decreased AChE activity in *S. kingi* exposed to pirimicarb (Ishimota and Tomiyama 2020). In addition, pirimicarb acts via other toxic mechanisms, such as oxidative stress, genotoxicity, and cytotoxicity (Natale et al. 2018; Raisi et al. 2018; Vera-Candioti et al. 2015). Raisi et al. (2018) reported that the oxidative biomarker levels (catalase activity) in snails were altered following pirimicarb exposure. Similarly, the glutathione PO (a type of PO) and SOD activity was decreased in *D. magna* exposed to various insecticides (chlorantraniliprole, cyantraniliprole, and flubendiamide) (Cui et al. 2017). In the present study, in the F0 and F4 generations, the PO and SOD activity in the test groups was significantly lower than that in the control groups (Figs. 4 and 5), suggesting that pirimicarb induced oxidative stress in *S. kingi*.

In all test groups, the decreased activity of all enzymes in the F0 generation was recovered to the control level in the F14 generation (when the individuals had acquired pirimicarb tolerance) (Figs. 4–6). In the

best GLM, the elevation of the PO activity, but not the SOD and AChE activity, was significantly correlated with the changes in the pirimicarb sensitivity of *S. kingi* (Table 1).

The PO activity is related to chemical tolerance in many plants (Kim et al. 2008; Sreenivasulu et al. 1999; Wu et al. 2017); however, only a few studies have reported the association between the PO activity and chemical tolerance in animals, including insects (e.g., bed bags) (Mamidala et al. 2012). Moreover, we did not find any report on this association in cladocerans. Thus, our novel findings will contribute to elucidating the enzymatic mechanism underlying chemical tolerance in animals. The measurement of peroxidation levels in *S. kingi* (based on the levels of H_2O_2 and lipid peroxidation byproduct 4-hydroxynonenal) (Terhzaz et al. 2015) will provide useful insights to determine the oxidative mechanism of action of pirimicarb.

Although we could not ascertain the effects of SOD and AChE activity on changes in the chemical sensitivity of *S. kingi* based on our GLM, the activity of these enzymes was enhanced in the pirimicarb-tolerant population (F14). In aquatic invertebrates, SOD plays important roles in protection against oxidative damage, and its activity in invertebrates is equal to or higher than that in vertebrates (Valavanidis et al. 2006). Furthermore, increased AChE activity in other cladocerans (*Ceriodaphnia cornuta*) may be related to changes in pirimicarb sensitivity (Ishimota et al. 2020b). Therefore, although these enzymes were not directly related to changes in pirimicarb sensitivity in our test species, the recovery of the activity of various enzymes in the tolerant population suggests the regulation of chemical stress.

Overall, our findings shed light on the mechanism of insecticide tolerance in cladocerans (*S. kingi*) and the characteristics of chemical-tolerant population, including decreased *r* and increased feeding rate. In particular, the elevated PO activity may be related to chemical tolerance, suggesting that the regulation of oxidative stress is involved in the acquisition of chemical tolerance.

Conclusion

Following continuous exposure to sublethal concentrations of pirimicarb for 15 generations, the test *S. kingi* individuals became three times more tolerant than the controls at the highest exposure concentration (10 µg/L). The *r* value was significantly but negatively correlated with the 48 h-EC_{50} value, indicating the fitness cost for pirimicarb tolerance; however, we could not explain changes in the *r* value based on the feeding rates, and additional experiments are warranted to clarify these effects. Moreover, we explored the involvement of antioxidant enzymes (PO and SOD) and AChE in changes in the sensitivity of cladocerans to pirimicarb. Our GLM indicated that elevated PO activity may be related to chemical tolerance, although there was no association between chemical tolerance and the activity of other enzymes (SOD and AChE). In summary, the regulation of oxidative stress may be related to the acquisition of chemical tolerance in cladocerans. These findings will contribute to elucidating the characteristics and mechanisms of chemical tolerance in aquatic organisms.

Declarations

Ethics approval and consent to participate

All procedures involving animals performed in this study were in accordance with the ethical standards of the institution. This study did not involve any studies with human participants or animals performed by any of the authors.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

The authors did not receive support from any organization for the submitted work.

Authors' contributions

All authors contributed to the conception and design of the study. Data collection, material preparation, and data analysis were performed by Makoto Ishimota. The first draft of the manuscript was written by Makoto Ishimota. Mebuki Kodama and Naruto Tomiyama commented on the revised versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We would like to thank Aya Kitahara for technical support. We also thank Dr. Kazutoshi Ohyama and Dr. Hiroaki Aoyama for allowing us to conduct this study and providing helpful comments. We thank Editage (https://www.editage.com/) for editing and reviewing this manuscript for English language.

References

1. Agra AR, Guilhermino L, Soares AMVM, Barata C (2010) Genetic costs of tolerance to metals in *Daphnia longispina* populations historically exposed to a copper mine drainage. Environ Toxicol Chem 29:939–946. https://doi.org/10.1002/etc.97
2. Akaike H (1974) A new look at the statistical model identification. IEEE Trans Autom Control 19:716–723

3. Araujo GS, Abessa DMS, Soares AMVM, Loureiro S (2019) Multi-generational exposure to Pb in two monophyletic *Daphnia* species: Individual, functional and population related endpoints. Ecotoxicol Environ Safe 173:77–85. https://doi.org/10.1016/j.ecoenv.2019.02.001
4. Brock TCM, Van Wijngaarden RPA (2012) Acute toxicity tests with *Daphnia magna*, *Americamysis bahia*, *Chironomus riparius* and *Gammarus pulex* and implications of new EU requirements for the aquatic effect assessment of insecticides. Environ Sci Pollut Res 19:3610–3618. https://doi.org/10.1007/s11356-012-0930-0
5. Buhl KJ, Hamilton SJ, Schmulbach JC (1993) Chronic toxicity of the bromoxynil formulation Buctril® to *Daphnia magna* exposed continuously and intermittently. Arch Environ Contam Toxicol 25:152–159. https://doi.org/10.1007/BF00212126
6. Case TJ (2000) An Illustrated Guide to Theoretical Ecology. Oxford University, New York
7. Connon R, Dewhurst RE, Crane M, Callaghan A (2003) Haem peroxidase activity in *Daphnia magna*: a biomarker for sub-lethal toxicity assessments of kerosene-contaminated groundwater. Ecotoxicology 12:387–395. https://doi.org/10.1023/A:1026195621777
8. Cui F, Chai T, Qian L, Wang C (2017) Effects of three diamides (chlorantraniliprole, cyantraniliprole and flubendiamide) on life history, embryonic development and oxidative stress biomarkers of *Daphnia magna*. Chemosphere 169:107–116. https://doi.org/10.1016/j.chemosphere.2016.11.073
9. De Coen WM, Janssen CR (2003) The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ Toxicol Chem 22:1632–1641. https://doi.org/10.1002/etc.5620220727
10. Fox J (2005) The R Commander: A Basic Statistics Graphical User Interface to R. J Stat Softw 14:1–42. http://www.jstatsoft.org/v14/i09/
11. Gerhard D, Ritz C (2017) Marginalization in nonlinear mixed-effects models with an application to dose-response analysis. https://arxiv.org/abs/1707.02502
12. Haap T, Köhler HR (2009) Cadmium tolerance in seven *Daphnia magna* clones is associated with reduced hsp70 baseline levels and induction. Aquat Toxicol 94:131–137. https://doi.org/10.1016/j.aquatox.2009.06.006
13. Hajishengallis G, Russell MW (2015) Mucosal Immunology. Chapter 15: Innate Humoral Defense Factors. pp. 251–270
14. Heine-Fuster I, Aránguiz-Acuña A, Ramos-Jiliberto R (2017) Pesticide increases transgenerational cost of inducible defenses in a freshwater rotifer. Environ Sci Pollut Res 799:249–260. https://doi.org/10.1007/s10750-017-3221-4
15. Holm S (1979) A simple sequentially rejective multiple test procedure. Scand J Stat 6:65–70
16. Homem RA, Buttery B, Richardson EE, Tan Y, Field LM, Williamson MS, Davies TGE (2020) Evolutionary trade-offs of insecticide resistance - the fitness costs associated with target-site mutations in the nAChR of *Drosophila melanogaster*. Molecular Ecol 29:2661–2675. https://doi.org/10.1111/mec.15503

17. Hosmer D, Lemeshow S (2000) Applied logistic regression. Wiley, New York
18. Ishimota M, Tajiki-Nishino R, Fukuyama T, Tomiyama N (2020a) Rapid adaptation of *Chironomus yoshimatsui* to acetylcholinesterase inhibitors (pyraclofos and pirimicarb) in a multi-generation study. J Environ Sci Health B 55:429–437. https://doi.org/10.1080/03601234.2019.1708165
19. Ishimota M, Tajiki-Nishino R, Fukuyama T, Tomiyama N, Sakamoto M, Ohyama K (2020b) Long-term tolerance acquisition and changes in acetylcholinesterase activity in three cladoceran species after a 48-h pulsed exposure to pirimicarb. Wat Air Soi-Poll 231:287. https://doi.org/10.1007/s11270-020-04670-3
20. Ishimota M, Tomiyama N (2020) Increased acetylcholinesterase inhibitor sensitivity as an intergenerational response to short–term acetylcholinesterase inhibitor exposure in *Scapholeberis kingi*. Limnology 21:187–196. https://doi.org/10.1007/s10201-019-00598-8
21. ISO (International Organization for Standardization) (1996) Water quality- Determination of the Inhibition of the Mobility of *Daphnia magna* Straus (Cladocera, Crustacea)- Acute Toxicity test. ISO 6341
22. Jacobasch C, Völker C, Giebner S, Völker J, Alsenz H, Potouridis T, Heidenreich H, Kayser G, Oehlmann J, Oetken M (2014) Long-term effects of nanoscaled titanium dioxide on the cladoceran *Daphnia magna* over six generations. Environ Pollut 186:180–186. https://doi.org/10.1016/j.envpol.2013.12.008
23. Jansen M, Stoks R, Coors A, van Doorslaer W, de Meester L (2010) Collateral damage: rapid exposure-induced evolution of pesticide resistance leads to increased susceptibility to parasites. Evolution 65:2681–2691. https://doi.org/10.1111/j.1558-5646.2011.01331.x
24. Jansen M, Coors A, Stoks R, De Meester L (2011) Evolutionary ecotoxicology of pesticide resistance: a case study in *Daphnia*. Ecotoxicology 20:543–551. https://doi.org/10.1007/s10646-011-0627-z
25. Jeon J, Kretschmann A, Escher B, Hollender J (2013) Characterization of acetylcholinesterase inhibition and energy allocation in *Daphnia magna* exposed to carbaryl. Ecotoxicol Environ Safe 98:28–35. https://doi.org/10.1016/j.ecoenv.2013.09.033
26. Kim Y-H, Kim CY, Song W-K, Park D-S, Kwon S-Y, Lee H-S, Bang J-W, Kwak S-S (2008) Overexpression of sweetpotato *swpa4* peroxidase results in increased hydrogen peroxide production and enhances stress tolerance in tobacco. Planta 227:867–881. https://doi.org/10.1007/s00425-007-0663-3
27. Kliot A, Ghanim M (2012) Fitness costs associated with insecticide resistance. Pest Manag Sci 68:1431–1437. https://doi.org/10.1002/ps.3395
28. Li S, Xu J, Sheng L (2014) The trans-generation effect during pulsed cadmium exposure: Tolerance and induction of hsp70. Ecotoxicol Environ Safe 107:300–305. https://doi.org/10.1016/j.ecoenv.2014.06.003
29. Liu Y, Yan Z, Zhang L, Deng Z, Yuan J, Zhang S, Chen J, Guo R (2019) Food up-take and reproduction performance of *Daphnia magna* under the exposure of Bisphenols. Ecotoxicol Environ Safe 170:47–54. https://doi.org/10.1016/j.ecoenv.2018.11.106

30. Mano H, Sakamoto M, Tanaka Y (2010) A comparative study of insecticide toxicity among seven cladoceran species. Ecotoxicology 19:1620–1625. https://doi.org/10.1007/s10646-010-0547-3
31. Mamidala P, Wijeratne AJ, Wijeratne S, Kornacker K, Sudhamalla B, Rivera-Vega LJ, Hoelmer A, Meulia T, Jones SC, Mittapalli O (2012) RNA-Seq and molecular docking reveal multi-level pesticide resistance in the bed bug. BMC Genom 13:6. https://doi.org/10.1186/1471-2164-13-6
32. Marques SC, Azeiteiro UM, Leandro SM, Queiroga H, Primo AL, Martinho F, Viegas I, Pardal MA (2008) Predicting zooplankton response to environmental changes in a temperate estuarine ecosystem. Mar Biol 155:531–541. https://doi.org/10.1007/s00227-008-1052-6
33. Münzinger A (1990) Effects of nickel on *Daphnia magna* during chronic exposure and alterations in the toxicity to generations pre-exposed to nickel. Wat Res 24:845–852. https://doi.org/10.1016/0043-1354(90)90134-R
34. Muyssen BTA, Janssen CR, Bossuyt BTA (2002) Tolerance and acclimation to zinc of field-collected *Daphnia magna* populations. Aquat Toxicol 56:69–79. https://doi.org/10.1016/S0166-445X(01)00206-5
35. Natale GS, Vera-Candioti J, de Arcaute CR, Soloneski S, Larramendy ML (2018) Lethal and sublethal effects of the pirimicarb-based formulation Aficida® on *Boana pulchella* (Duméril and Bibron, 1841) tadpoles (Anura, Hylidae). Ecotoxicol Environl Safe: 147:471–479. https://doi.org/10.1016/j.ecoenv.2017.09.007
36. OECD (Organization for Economic Co-operation and Development) (1997) OECD guidelines for testing of chemicals, Sect. 2, test No.211: *Daphnia magna* reproduction test. https://doi.org/10.1787/20745761. Accessed 16 April 2021
37. OECD (Organization for Economic Co-operation and Development) (2004) OECD guideline for the testing of chemicals, Sect. 2, test No.202: *Daphnia* sp., acute immobilization test. https://doi.org/10.1787/20745761. Accessed 16 April 2021
38. Ogonowski M, Schür C, Jarsén Å, Gorokhova E (2016) The Effects of Natural and Anthropogenic Microparticles on Individual Fitness in *Daphnia magna*. PLoS ONE 11:e0155063. https://doi.org/10.1371/journal.pone.0155063
39. Oliveira LLD, Antunes SC, Gonçalves F, Rocha O, Nunes B (2015) Evaluation of ecotoxicological effects of drugs on *Daphnia magna* using different enzymatic biomarkers. Ecotoxicol Environ Safe 119:123–131. https://doi.org/10.1016/j.ecoenv.2015.04.028
40. Park SY, Nair PMG, Choi J (2012) Characterization and expression of superoxide dismutase genes in *Chironomus riparius* (Diptera, Chironomidae) larvae as a potential biomarker of ecotoxicity. Compara Biochem Physiol Part C 156:187–194. https://doi.org/10.1016/j.cbpc.2012.06.003
41. R Development Core Team (2019) R: A Language and Environment for Statistical Computing. Version 3.6.1. R Foundation for Statistical Computing, Vienna
42. Raisi M, Pourkhabbaz HR, Banaee M, Pourkhabbaz A, Javanmardi S (2018) Effects of Pirimicarb carbamate insecticide alone and in combination with lead (Pb) on biochemical parameters of soft tissues in freshwater snail, *Galba truncatula*. Int J Aquat Biol 6:126–137

43. Ritz C, Cedergreen N, Jensen JE, Streibig JC (2006) Relative potency in nonsimilar dose-response curves. Weed Sci 54:407–412. https://doi.org/10.1614/WS-05-185R.1
44. Sánchez M, Andreu-Moliner E, Ferrando MD (2004) Laboratory investigation into the development of resistance of *Daphnia magna* to the herbicide molinate. Ecotoxicol Environ Safe 59:316–323. https://doi.org/10.1016/j.ecoenv.2003.09.003
45. Santos-Amaya OF, Tavares CS, Rodrigues JVC, Campos SO, Guedes RNC, Alves AP, Alves AP, Pereira EJG (2017) Fitness costs and stability of Cry1Fa resistance in Brazilian populations of *Spodoptera frugiperda*. Pest Manag Sci 73:35–43. https://doi.org/10.1002/ps.4312
46. Saro L, Lopes I, Martins N, Ribeiro R (2012) Testing hypotheses on the resistance to metals by *Daphnia longispina*: differential acclimation, endpoints association, and fitness costs. Environ Toxicol Chem 31:909–915. https://doi.org/10.1002/etc.1762
47. Silva ARR, Cardoso DN, Cruz A, Pestana JL, Mendo S, Soares AMVM, Loureiro S (2017) Multigenerational effects of carbendazim in *Daphnia magna*. Environ Toxicol Chem 36:383–394. https://doi.org/10.1002/etc.3541
48. Silva ARR, Santos CSA, Ferreira NGC, Morgado R, Cardoso DN, Cruz A, Mendo S, Soares AMVM, Loureiro S (2018) Multigenerational effects of carbendazim in *Daphnia magna*: From a subcellular to a population level. Environ Toxicol Chem 38:412–422. https://doi.org/10.1002/etc.4307
49. Skjolding LM, Kern K, Hjorth R, Hartmann N, Overgaard S, Ma G, Veinot JGC
50. Baun A (2014) Uptake and depuration of gold nanoparticles in *Daphnia magna*
51. Ecotoxicology 23: 1172–1183. https://doi.org/10.1007/s10646-014-1259-x
52. Sreenivasulu N, Ramanjulu S, Ramachandra-Kini K, Prakash HS, Shekar-Shetty H, Savithri HS, Sudhakar C (1999) Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of fox-tail millet with differential salt tolerance. Plant Sci 141:1–9. https://doi.org/10.1016/S0168-9452(98)00204-0
53. Stamatis N, Hela D, Triantafyllidis V, Konstantinou I (2013) Spatiotemporal Variation and Risk Assessment of Pesticides in Water of the Lower Catchment Basin of Acheloos River, Western Greece. Sci World J 231610. https://doi.org/10.1155/2013/231610
54. Struger J, Grabuski J, Cagampan S, Sverko E, Marvin C (2016) Occurrence and Distribution of Carbamate Pesticides and Metalaxyl in Southern Ontario Surface Waters 2007–2010. Bull Environ Contam Toxicol 96:423–431. https://doi.org/10.1007/s00128-015-1719-x
55. Taenzler V, Bruns E, Dorgerloh M, Pfeifle V, Weltje L (2007) Chironomids: Suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. Ecotoxicology 16:221–230. https://doi.org/10.1007/s10646-006-0117-x
56. Terhzaz S, Cabrero P, Brinzer RA, Halberg KA, Dow JA, Davies SA (2015) A novel role of *Drosophila* cytochrome *P450-4e3* in permethrin insecticide tolerance. Insect Biochem Mol Biol 67:38–46. https://doi.org/10.1016/j.ibmb.2015.06.002
57. Tsui MTK, Wang W-X (2005) Multigenerational acclimation of *Daphnia magna* to mercury: Relationships between biokinetics and toxicity. Environmen Toxicol 24:2927–2933.

https://doi.org/10.1897/05-085R.1

58. US EPA (United States Environmental Protection Agency Office of Chemical Safety and Pollution Prevention) (2012) Ecological Effects Guideline, OCSPP 850.6100. Environmental Chemistry Methods and Associated Independent Laboratory Validation. U.S. Environmental Protection Agency, Washington, D.C
59. Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M (2006) Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol Environ Safe 64:178–189. https://doi.org/10.1016/j.ecoenv.2005.03.013
60. Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environ Toxicol Pharmacol 13:57–149. https://doi.org/10.1016/S1382-6689(02)00126-6
61. Vera-Candioti J, Soloneski S, Larramendy ML (2015) Pirimicarb-based formulation-induced genotoxicity and cytotoxicity in the freshwater fish *Cnesterodon decemmaculatus* (Jenyns, 1842) (*Pisces, Poeciliidae*). Toxicol Indust Health 31:1051–1060. https://doi.org/10.1177/0748233713486958
62. Villarroel MJ, Ferrando MD, Sancho E, Andreu E (1999) *Daphnia magna* feeding behavior after exposure to tetradifon and recovery from intoxication. Ecotoxicol Environ Safe 44:40–46. https://doi.org/10.1006/eesa.1999.1817
63. Wheeler MW, Park RM, Bailer AJ (2006) Comparing median lethal concentration values using confidence interval overlap or ratio tests. Environ Toxicol Chem 25:1441–1444. https://doi.org/10.1897/05-320R.1
64. Wu Y, Yang Z, How J, Xu H, Chen L, Li K (2017) Overexpression of a peroxidase gene (*AtPrx64*) of *Arabidopsis thaliana* in tobacco improves plant's tolerance to aluminum stress. Plant Mol Biol 95:157–168. https://doi.org/10.1007/s11103-017-0644-2
65. Wuerthner VP, Jaeger J, Garramone PS, Loomis CO, Pecheny Y, Reynolds R, Deluna L, Klein S, Lam M, Hua J, Meindl GA (2019) Inducible pesticide tolerance in *Daphnia pulex* influenced by resource availability. Ecol Evol 9:1182–1190
66. Zalizniak L, Nugegoda D (2006) Effect of sublethal concentrations of chlorpyrifos on three successive generations of *Daphnia carinata*. Ecotoxicol Environ Safe 64:207–214. https://doi.org/10.1016/j.ecoenv.2005.03.015

Figures

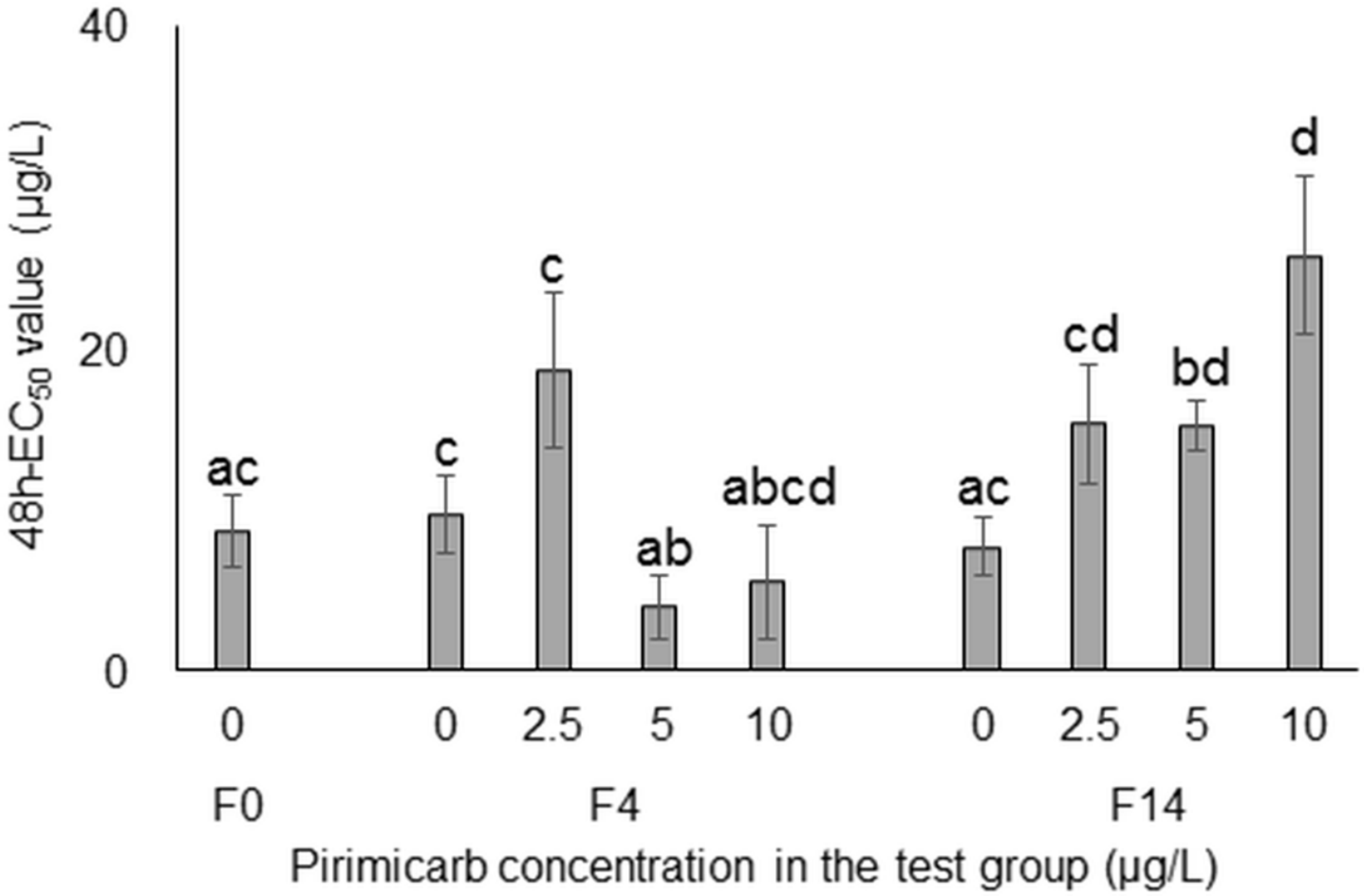

Figure 1

Half maximal effective concentration at 48 h (48 h-EC50) and 95% confidence intervals (error bars) for Scapholeberis kingi exposed to pirimicarb (0, 2.5, 5.0, and 10 µg/L) for 48 h in F0, F4, and F14 generations. Different letters indicate significant differences ($p < 0.05$, corrected by Holm's method).

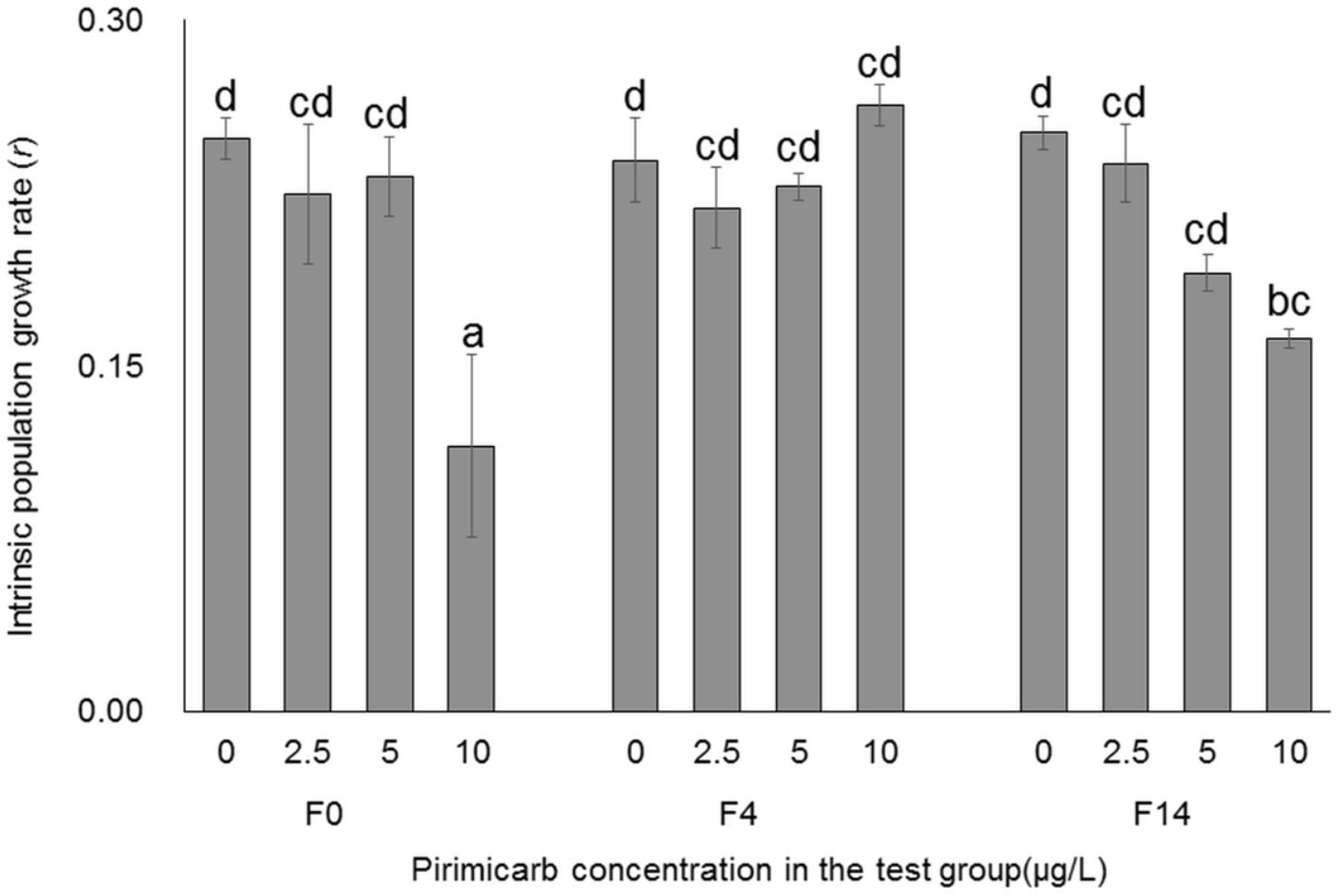

Figure 2

Mean intrinsic population growth rate (r) of Scapholeberis kingi continuously exposed to pirimicarb (0, 2.5, 5.0, and 10 μg/L) in F0, F4, and F14 generations. Error bars indicate standard deviation (n = 3). Different letters indicate significant differences ($p < 0.05$, corrected by Holm's method).

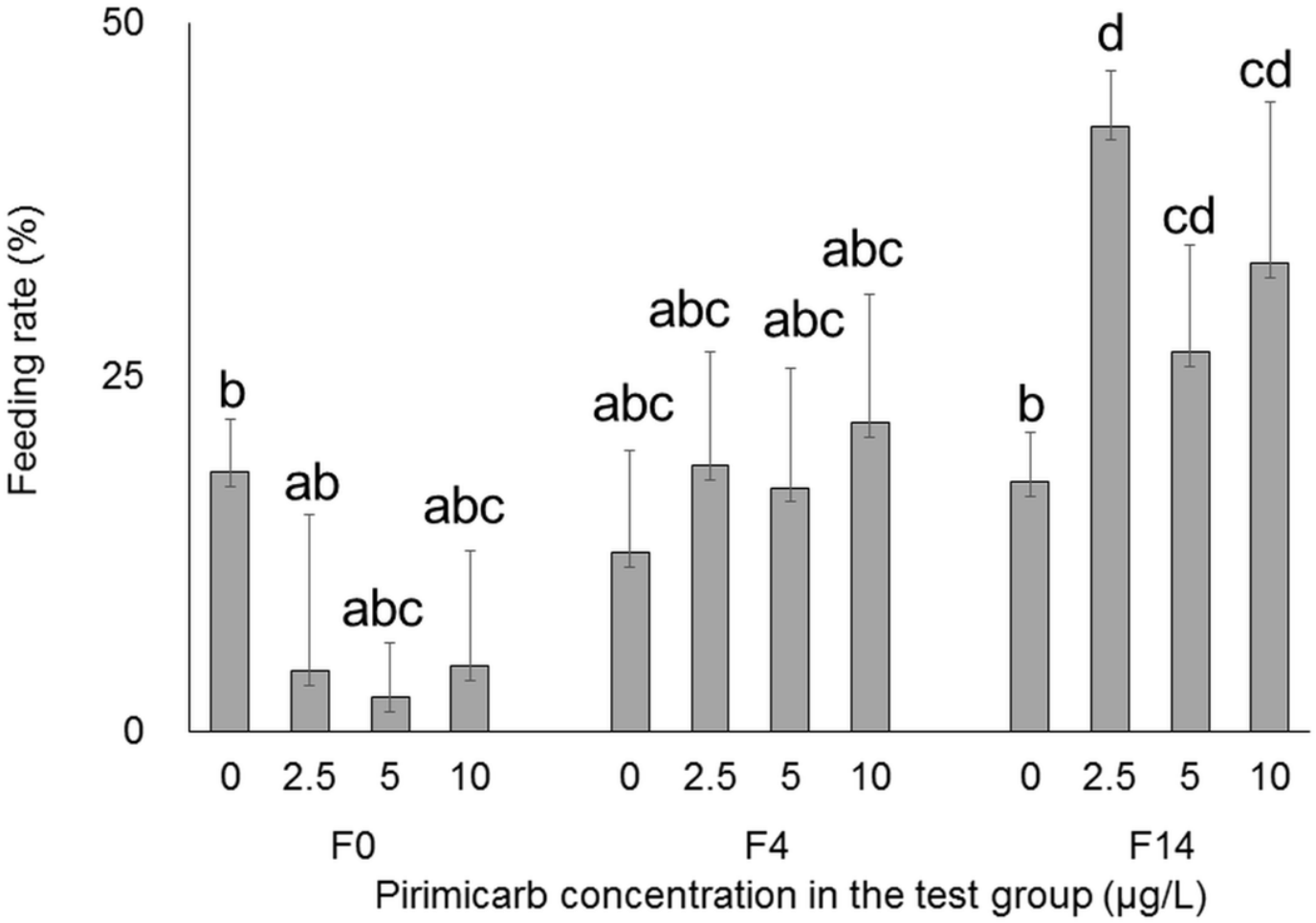

Figure 3

Mean feeding rate of adult Scapholeberis kingi (7-day old) continuously exposed to pirimicarb (0, 2.5, 5.0, and 10 μg/L) in F0, F4, and F14 generations. S. kingi individuals in each group (0, 2.5, 5.0, and 10 μg/L) were exposed to the same concentration of pirimicarb for 48 h in the presence of food (Chlorella vulgaris, 5 × 105 cells/mL), and the feeding rate was calculated based on the loss of food. Error bars indicate standard deviation (n = 5). Different letters indicate significant differences ($p < 0.05$, corrected by Holm's method).

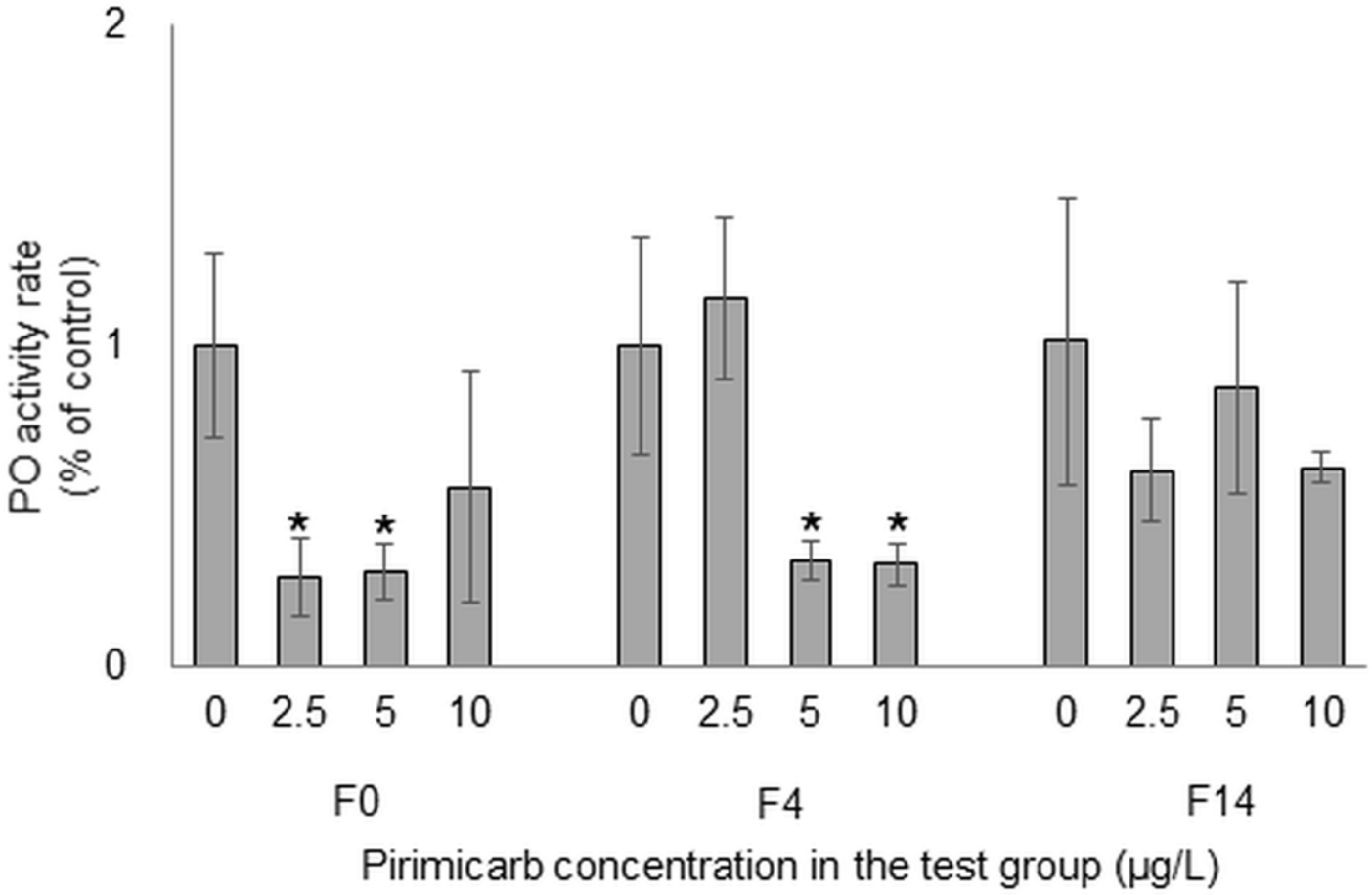

Figure 4

Peroxidase (PO) activity in third-brood neonates (<24-hour old) exposed to pirimicarb (0, 2.5, 5.0, and 10 µg/L) for 48 h in F0, F4, and F14 generations. Neonates were continuously exposed pirimicarb (0, 2.5, 5.0, and 10 µg/L) until 21 days after birth. The reproduced neonates (<24-hour old) from each test group were exposed to the same concentration of pirimicarb (0, 2.5, 5.0, and 10 µg/L) for 48 h and used for the estimation of PO activity. Data represent the mean ± standard deviation (% of control, 0 µg/L, n = 3). Asterisks indicate significant differences compared with the control in each generation ($p < 0.05$).

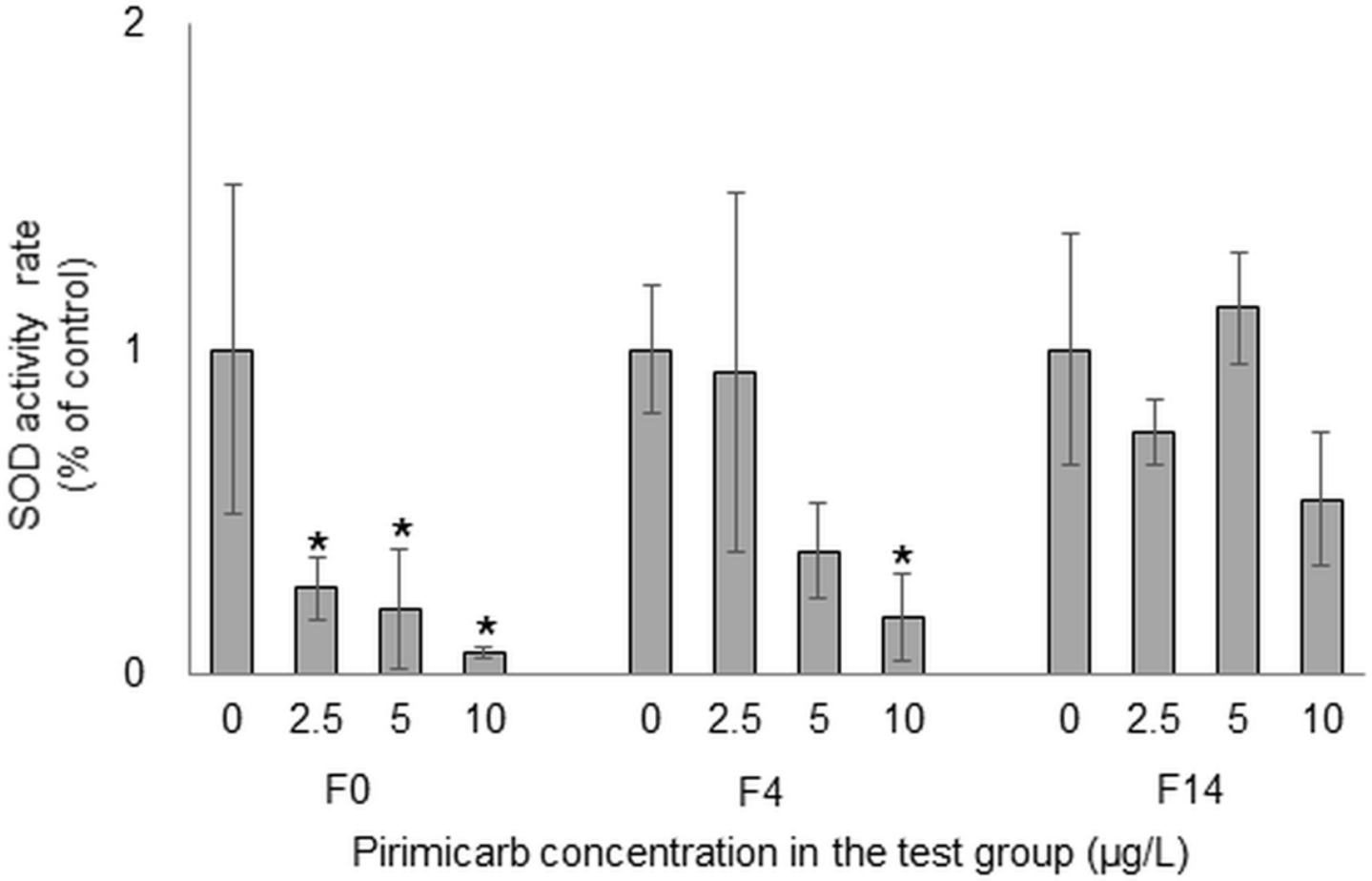

Figure 5

Superoxide dismutase (SOD) activity in third-brood neonates (<24-hour old) exposed to pirimicarb (0, 2.5, 5.0, and 10 µg/L) for 48 h in F0, F4, and F14 generations. Neonates were continuously exposed pirimicarb (0, 2.5, 5.0, and 10 µg/L) until 21 days after birth. The reproduced neonates (<24-hour old) from each test group were exposed to the same concentration of pirimicarb (0, 2.5, 5.0, and 10 µg/L) for 48 h and used for the estimation of SOD activity. Data represent the mean ± standard deviation (% of control, 0 µg/L, n = 3). Asterisks indicate significant differences compared with the control in each generation ($p < 0.05$).

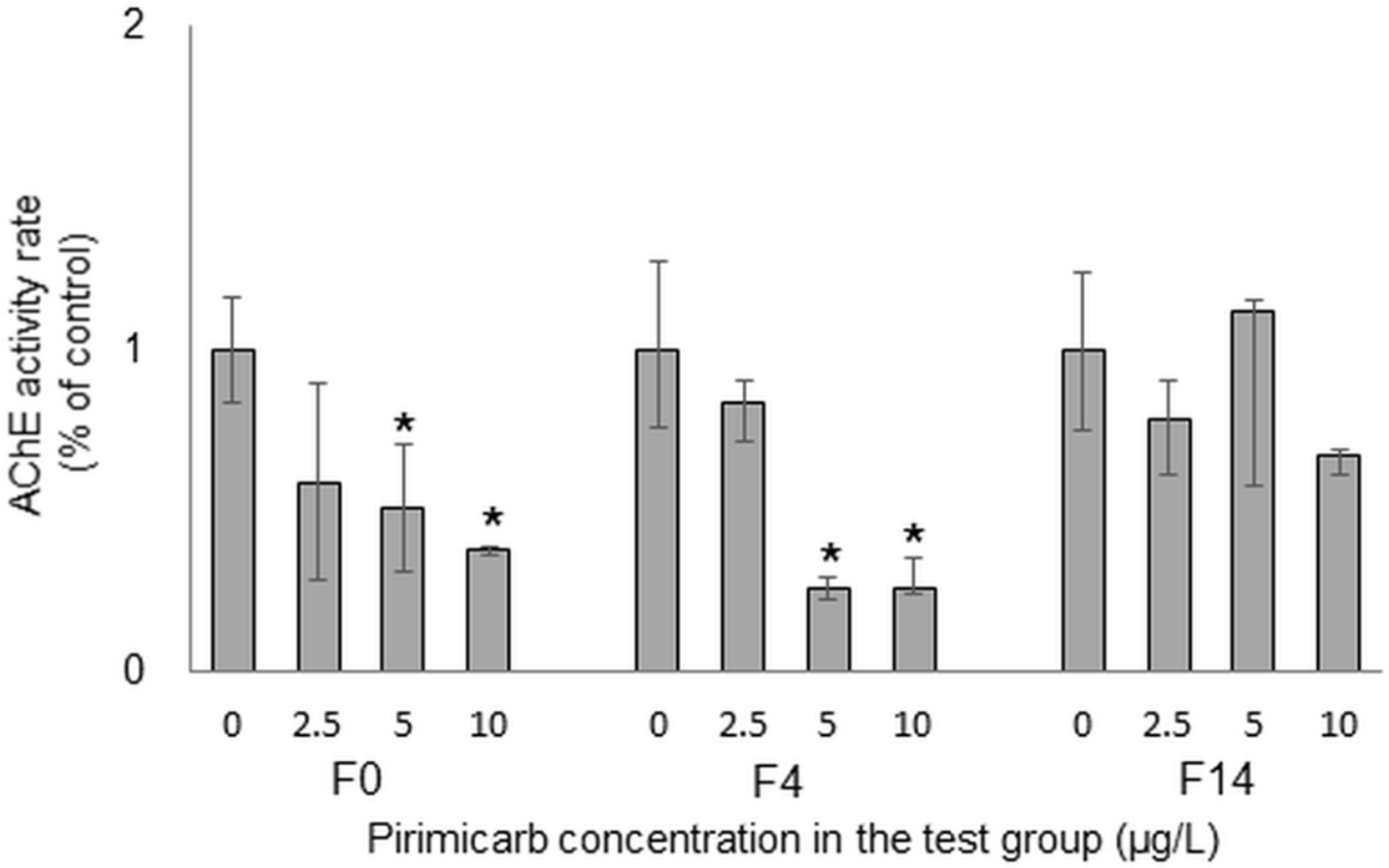

Figure 6

Acetylcholinesterase (AChE) activity in third-brood neonates (<24-hour old) exposed to pirimicarb (0, 2.5, 5.0, and 10 µg/L) for 48 h in F0, F4, and F14 generations. Neonates were continuously exposed pirimicarb (0, 2.5, 5.0, and 10 µg/L) until 21 days after birth. The reproduced neonates (<24-hour old) from each test group were exposed to the same concentration of pirimicarb (0, 2.5, 5.0, and 10 µg/L) for 48 h and used for the estimation of AChE activity. Data represent the mean ± standard deviation (% of control, 0 µg/L, n = 3). Asterisks indicate significant differences compared with the control in each generation ($p < 0.05$).