

RESEARCH ARTICLE

Effects of insecticides, fipronil and imidacloprid, on the growth, survival, and behavior of brown shrimp *Farfantepenaeus aztecus*

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Abstract

Increased use of pesticide is causing detrimental effects on non-target species worldwide. In this study, we examined the lethal and sub-lethal effects of fipronil and imidacloprid, two commonly used insecticides, on juvenile brown shrimp (*Farfantepenaeus aztecus*), one of the most commercially and ecologically important species in the United States. The effects of six concentrations of fipronil (0.0, 0.005, 0.01, 0.1, 1.0, and 3.0 µg/L) and six concentrations of imidacloprid (0.0, 0.5, 1.0, 15.0, 34.5, 320.0 µg/L) were tested in a laboratory. We examined five different endpoints: growth, moulting interval, survivorship, behavioral change, and body color change. Growth of shrimp was reduced significantly under higher concentrations of both insecticides. Under fipronil exposure, shrimp in control showed the shortest inter-moult interval (7.57 ± 2.17 day) compared with other treatments; similarly, in the imidacloprid experiment, moulting increased from 8.43 ± 2.52 day in control to 11.95 ± 4.9 day in 0.5 µg/L treatment. Higher concentrations of fipronil (1.0 and 3.0 µg/L) showed a 0.0% survival rate compared with 100% survival in the control and 0.005 µg/L treatment. Under imidacloprid, survivorship decreased from 100% in the control to 33.33% in the 320.0 µg/L treatment. The 96-h LC₅₀ of fipronil was 0.12 µg/L, which makes brown shrimp one of the most sensitive invertebrates to the pesticide. Changes in behavior and body color were observed under both insecticides after different durations of exposures depending on concentrations. We conclude that, at the corresponding EPA benchmark concentrations, fipronil had more lethal effects than imidacloprid, and imidacloprid had more sub-lethal effects than fipronil. Both effects are of serious concern, and we suggest monitoring is necessary in estuaries.

Introduction

The use of chemical pesticides has become critically important to assure both quality and productivity of agricultural products [1, 2] and to control household pests such as termites, fire

ants, and mosquitoes [3–5]. However, pesticides also have negative effects on non-target organisms [6–9], which may be considered beneficial organisms. Because chemical pesticides eventually flow into the surface water, it is expected these toxicants to affect aquatic environments more than terrestrial environments [10].

Although these negative effects could be acute (lethal) or chronic (sub-lethal) and vary depending on species [11, 12], the majority of ecotoxicological studies neglect their sub-lethal effects [13, 14] and focus on selected model species, such as daphnia *Daphnia magna* and zebrafish *Danio rerio* [2, 15]. Here, we present the results of experimental studies on the effects of fipronil (5-amino-1-[2, 6-dichloro-4-(trifluoromethyl) phenyl]-4[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile) and imidacloprid (1-[(6-Chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine), two of the most commonly used chemical pesticides worldwide on brown shrimp *F. aztecus*.

Brown shrimp is considered one of the most important commercial species for fisheries along the Atlantic Coast of the southeastern United States and in the Gulf of Mexico [16, 17]. Its commercial landing value was estimated at \$166,542 million in 2016 [18]. In addition to its economic importance, brown shrimp also has an essential ecological role as prey for many important fish species in the region [17, 19, 20]. In particular, these shrimp are abundant along the coasts of Texas and Louisiana, U.S.A. and inhabit the estuaries during their juvenile stage [16, 17]. However, because of the increased use of fipronil and imidacloprid in coastal communities, as well as the detection of pesticides residues in estuaries [21], the effects of these insecticides on penaeid shrimp specifically brown shrimp are particularly a serious concern.

Phenylpyrazoles (including fipronil) and neonicotinoids (including imidacloprid) are applied in a large scale, such as protecting plants from agricultural pests, controlling household pests, and controlling parasites on domesticated animals [22]. Presently, they account for approximately one third of the world insecticide market [23]. Although both fipronil and imidacloprid operate by disrupting neural transmission in the central nervous system of invertebrates [23], each product has a different mode of action. Fipronil interferes with the passage of chloride ions by binding to a specific site within the gamma-aminobutyric acid (GABA) receptor, while imidacloprid binds to postsynaptic nicotinic acetylcholine receptors (nAChR) [24, 25]. Compared to other types of insecticides, fipronil and imidacloprid are considered safer because of their low toxicity on fish and mammals. However, fipronil and imidacloprid are very effective on arthropods in small concentrations [26]. Their increased use in recent years [22, 23, 26], moderate to high solubility [27, 28], and persistence in water [29–31] pose a serious concern regarding the potential adverse impacts on non-target aquatic invertebrates.

Fipronil in the environment has been reported in the U.S. and other parts of the world, and its environmental concentrations can be as high as 10.004 µg/L [12, 32–35]. Among the sites where imidacloprid are actively used, its concentration has ranged between 0.016 µg/L and 320.0 µg/L [12, 30, 36–38]. Many of these studies reported the detection of fipronil and imidacloprid, or one or more of their degradation products, in aquatic environments exceeding their chronic levels of the U.S. EPA aquatic life benchmark for invertebrates (0.01 µg/L for both insecticides) [39].

In recent years, many studies have investigated the potential adverse effects of these insecticides on non-target organisms; however, the majority of these studies have focused on a limited number of commercially beneficial terrestrial invertebrates or on selected model organisms in aquatic ecosystems. For example, Pisa, *et al.* [22] found that, of 376 papers reviewed, the majority focused on the effects of fipronil and neonicotinoids on honeybees, and very few studied aquatic invertebrates, particularly marine species.

Both of fipronil and imidacloprid have been detected in aquatic environments in Texas, U.S. in recent years due to their increased use in coastal communities [40]. No previous study published in the peer-reviewed literature has reported the potential effects of these widely-used insecticides on the estuarine-dependent penaeid shrimp such as the brown shrimp *F. aztecus*, which are commercially and ecologically important. Therefore, determining the adverse effects of these insecticides on this particular species was critically important. The objective of this study was to evaluate both lethal and sub-lethal effects of the phenylpyrazole fipronil and neonicotinoid imidacloprid on the juvenile brown shrimp under the concentrations observed in the aquatic environment using multiple endpoints: growth (weight and length), moulting, survivorship, and behavioral change. This research complemented many recent studies that highlighted the risks of commonly used pesticides on different species of aquatic organisms, especially not-targeted marine species that have high ecological or commercial impact and limited ecotoxicology data. In addition, this study is the first to investigate the effects of imidacloprid on brown shrimp, and the previous experiments investigating the effects of fipronil on brown shrimp [41] was done only at higher concentrations, where all shrimp individuals died during the first weeks of the experiment and no information about the effects of lower concentrations of fipronil are available.

Materials and methods

Collection of brown shrimp

Juvenile brown shrimp were collected on June 12, 2017 (Scientific research permit, SPR-0396-777, Texas Parks and Wildlife Department) from Gangs Bayou in Galveston Bay (on Sportsman Road, N 29.25549; W 94.91575), Texas, using hand nets and a 3-m bag seine of 0.6 cm mesh size. Shrimp were transported to a laboratory in Texas A&M University, College Station, Texas in 45-liter coolers supplied with portable air pumps. After 4–5 hours of water temperature equilibration (between transportation coolers and room temperature), active shrimp were selected for the fipronil experiment (weight 0.57 ± 0.008 g and total length 4.41 ± 0.03 cm) and imidacloprid experiment (weight 0.81 ± 0.01 g and total length 5.31 ± 0.03 cm). After equilibrating temperature, the shrimp were moved to larger plastic tanks of 53-liter filled with artificial brackish water made using Instant Ocean[®] Sea Salt and dechlorinated tap water. In this study, no vertebrate species, endangered or protected species was involved; no ethical approval was required under Texas A&M University Animal Use Protocol.

Acclimation period

Shrimp were acclimated for 9 days to laboratory conditions. During the acclimation period, API[®] Bottom Feeder Shrimp Pellets were used to feed shrimp twice a day to maintain the nutritional requirements of shrimp [42] and to ensure the palatability and acceptability of the pellets by the shrimp. In order to remove excrement and remaining food, the acclimation tanks were cleaned and approximately 50% of their water was replaced on the daily basis. Water quality parameters of acclimation tanks were as follows: dissolved oxygen, 5.6 ± 0.42 mg/L; salinity, 15.50 ± 0.06 ‰; temperature, 24.53 ± 0.04 °C; and pH, 8.03 ± 0.06 for the fipronil experiment, and dissolved oxygen, 5.5 ± 0.66 mg/L; salinity, 15.73 ± 0.04 ‰; temperature, 24.43 ± 0.04 °C; and pH, 8.19 ± 0.01 for the imidacloprid experiment. We maintained controlled photoperiod of 12-h: 12-h light: dark cycle. After the acclimation period, shrimp were moved to aquariums used for experiment (S1A Fig).

Fipronil and imidacloprid experimental solutions

Both fipronil, purity limit $\geq 97\%$ (HPLC), and imidacloprid, purity limit 99.5% (HPLC), were purchased from Fisher Scientific Co. L.L.C., PA, U.S. Based on previously reported concentrations in the environment in other studies (Table 1), six concentrations of fipronil (0.0, 0.005, 0.01, 0.1, 1.0, and 3.0 $\mu\text{g/L}$) and six concentrations of imidacloprid (0.0, 0.5, 1.0, 15.0, 34.5, 320.0 $\mu\text{g/L}$) were selected. Three replicates were used for each concentration (treatment).

For each experiment, test solutions of the six nominal concentrations were prepared by a series of dilutions, beginning with mixing a specific amount of the insecticide powder (0.1 g fipronil, and 0.01 g imidacloprid) in 1 liter of artificial brackish water to create a highly homogenized 100 mg/L fipronil suspension and 10 mg/L imidacloprid solution using a magnetic stirrer. For both experiments, the specific dilutions of the nominal concentrations are provided in S1 and S2 Tables. We prepared 21 liters of test solutions for each concentration used in both experiments to fill three aquariums (replicates) of seven liters each. Although the hydrolysis half-life of these compounds at 25°C is much greater than 48 hours, >100 days for fipronil [24] and >30 days for imidacloprid [43], 100% of test solutions were changed every other day to maintain the concentrations relatively constant during the experiment.

Aquariums and experimental system

Experimental systems were designed in the same way for both fipronil and imidacloprid experiments using new aquariums and other supplies in both experiments. Each system contains 18 glass aquariums of 9.5 liter (30.7 X 15.4 X 20.5 cm), and each aquarium was considered as one replicate (S1A Fig). In order to keep track of shrimp moults individually and to prevent deaths from cannibalism among shrimp, each aquarium was divided into six cells of the same size, and one individual was assigned to each cell (S1B Fig). A screen was placed on each divider to allow water to flow and dissolved oxygen to be distributed evenly among the cells (S1C Fig). The dividers were made of fiberglass screen and polypropylene plates, materials that are commonly used in aquaculture studies.

Each aquarium was filled with 7 liters of test solution, and an air pump was used to provide air. In addition, all aquariums were covered on the top with glass lids to prevent shrimp from escaping. All sides of the aquariums were also covered with aluminum foils to reduce the degradation of the insecticides from exposure to light (S1A Fig). The aquariums were organized in three parallel rows. In each experiment, one of the six treatments was placed randomly in one of the two-column blocks of aquariums.

Table 1. Fipronil and imidacloprid concentrations observed in the aquatic environment and reported in previous studies.

Fipronil			Imidacloprid		
Detected concentrations ($\mu\text{g/L}$)	Location	Reference	Detected concentrations ($\mu\text{g/L}$)	Location	Reference
0.3–0.8	USA	[21]	0.016	Canada	[37]
1.0	Japan	[12]	0–0.22	Vietnam	[44]
0.28–2.11	USA	[34]	0.2–0.42	Australia	[38]
0.01–4.2	USA	[45]	0–3.3	USA	[46]
3.00–4.54	USA	[47]	1.0–14.0	Slovenia	[36]
0.15–5.0	USA	[48]	17.0–36.0	USA	[49]
0.007–6.0	USA	[50]	49.0	Japan	[12]
0.004–6.41	USA	[32]	320.0	Netherlands	[30]
0.0018–10.004	USA	[33]			
0.09–10.004	USA	[35]			

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The fipronil experiment lasted 34 days from June 20, 2017 to July 24, 2017, and the imidacloprid experiment lasted 36 days from June 20, 2017 to July 26, 2017. During both experiments, shrimp were fed on API[®] Bottom Feeder Shrimp Pellets twice daily. The amounts of food were adjusted weekly (according to the body weight of shrimp) and daily (according to the weight of dead shrimp) using shrimp feeding tables [42]. The temperature (°C), dissolved oxygen (mg/L), salinity (‰), and pH (water quality parameters) were measured with the YSI[®] Professional plus Multi-parameter Meter every other day.

Measurements

We conducted all experimental measurements according to the U.S. EPA guidelines [51] and using static-renewal method, with the test solutions being replaced periodically during the experiments. The sample size was determined based on previous studies [52, 53].

1. Weight gain and total length. We measured the weight of shrimp in each treatment every week by gently weighing each shrimp individually. First, shrimp was moved from its cell in the aquarium and placed on paper towel to remove remaining water on the body, and then the weight of shrimp was measured by placing the shrimp in a beaker containing a known amount of water. By weighing shrimp, we were able to monitor the effect of insecticide on growth of shrimp and to gain information for adjusting the amount of food.

Weight gain of shrimp was calculated using the following equation:

$$\% \text{Weight gain of shrimp} = \left\{ \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \right\} \times 100 \quad (1)$$

We also used a caliper (± 0.1 mm) to measure the total length of each shrimp by straighten the body of shrimp carefully on the table and measure the total length from the tip of the head to the end of the tail as shown in S1D Fig.

2. Moulting. Dates of moults of each individual shrimp in both experiments were recorded and used to calculate inter-moult intervals of shrimp in each treatment. Because each shrimp was placed in its own cell, we were able to count the number of days between the two consecutive moults of a particular individual.

3. Shrimp survival and LC₅₀. Shrimp movements were monitored multiple times every day, and the numbers of live shrimp were recorded to measure shrimp survivorship during both experiments. Shrimp were considered dead if they did not react or show any response during feeding time, if they did not swim, jump, escape from a net, or move their swimming legs when trying to pick them up for weighing, or if they were in abnormal position such as laying down on the bottom of the aquarium on their back or side without any motion. All dead shrimp were removed, counted, and weighed. Data gained by measuring the survivorship of shrimp were used to estimate the survival rate under both insecticides and the 96-h acute toxicity levels (LC₅₀) of fipronil.

4. Behavioral and other physical changes. In both experiments, shrimp were monitored multiple times every day in order to record any abnormal activity. The physical appearance of shrimp in each aquarium was monitored, and changes such as malformations and changes in body color were recorded in comparison with shrimp in the control treatments.

5. Statistical analysis. Non-parametric statistics were used for some measurements when data were not normally distributed. We used Kaplan–Meier estimator to measure the survivorship function of shrimp and non-parametric Log-Rank test to compare the survivorship distribution among treatments. We also used the non-parametric Kruskal-Wallis test followed by the pairwise Wilcoxon rank sum test to compare moulting intervals. LC₅₀ value of fipronil was estimated by fitting a generalized linear model to the proportion of individuals died against

the pesticide concentration with a logit link and binomial distribution. For all other measurements, we used linear regression analysis and One-way Analysis of Variance (ANOVA) to determine the significance of differences among treatments compared to the control. JMP[®] Pro 2016 [54] was used to calculate the LC₅₀ toxicity test and its 95% confidence intervals, Kruskal-Wallis, ANOVA, and Kaplan–Meier tests, and Microsoft Excel 2016 was used for linear regression analysis and to draw all figures. All of these statistical analyses were conducted at $\alpha = 0.05$ significance level.

Results

Weight gain and total length

The initial weight (at the first day of the experiment) of shrimp exposed to fipronil was not significantly different among all treatments, and the treatment means ranged between 0.56 ± 0.04 g in the 0.01 $\mu\text{g/L}$ treatment and 0.59 ± 0.03 g in the 0.005 $\mu\text{g/L}$ treatment (ANOVA, $P = 0.973$). Fipronil had a significant effect on the growth of shrimp during the experiment. Final weight of shrimp ranged between 1.02 ± 0.12 g in the 0.1 $\mu\text{g/L}$ treatment and 1.31 ± 0.07 g in the control (0.0 $\mu\text{g/L}$), which was significantly different from other treatments except the 0.005 $\mu\text{g/L}$ treatment under which the final weight was 1.30 ± 0.03 g (ANOVA, $P < 0.0001$) (Fig 1A and S3 Table). Fipronil also affected the percent weight gain of shrimp, and significant differences were observed between the control ($125.92 \pm 28.42\%$) and the 0.1 $\mu\text{g/L}$ treatment ($77.007 \pm 21.83\%$ weight gain) (ANOVA, $P < 0.0001$) whereas there was no significant difference between lower fipronil concentrations (0.005 $\mu\text{g/L}$ and 0.01 $\mu\text{g/L}$) and the control. The percent weight gain under the latter two concentrations was $120.17 \pm 15.16\%$ and $104.18 \pm 28.62\%$, respectively. We could not calculate the final weight and the percent weight gain under the 1.0 $\mu\text{g/L}$ and 3.0 $\mu\text{g/L}$ treatments because all shrimp died during the first days in these treatments (Fig 1A and S3 Table).

Shrimp exposed to imidacloprid also exhibited a reduction in growth during the experiment. The initial mean weight of shrimp ranged between 0.80 ± 0.06 g in the 15.0 $\mu\text{g/L}$ treatment and 0.84 ± 0.06 g in the 34.5 $\mu\text{g/L}$ treatment, and there was no significant difference among all treatments (ANOVA, $P = 0.971$) (Fig 1B and S4 Table). Final weight ranged between 1.04 ± 0.13 g in the 320.0 $\mu\text{g/L}$ treatment (calculated based on 5 survived shrimp) to 1.95 ± 0.12 g in the control (calculated based on 15 survived shrimp). The final weight under the control was significantly different from all treatments except the 0.5 $\mu\text{g/L}$ treatment (ANOVA, $P < 0.0001$) (S4 Table). There were also significant differences between the percent weight gain of the control ($140.3 \pm 16.15\%$) and three higher imidacloprid concentrations (15.0 $\mu\text{g/L}$, 34.5 $\mu\text{g/L}$, and 320.0 $\mu\text{g/L}$), which showed a reduction in their percent weight gains ($64.40 \pm 17.14\%$, $44.01 \pm 12.09\%$, and $29.48 \pm 16.43\%$, respectively) (ANOVA, $P = 0.0008$), but both 0.5 $\mu\text{g/L}$ and 1.0 $\mu\text{g/L}$ treatments had no significant effect on the percent weight gain compared with the control (S4 Table).

There was no significant difference in the initial body length of shrimp exposed to fipronil, and it ranged between 4.37 ± 0.15 cm in the 1.0 $\mu\text{g/L}$ treatment and 4.46 ± 0.09 cm in the control (0.0 $\mu\text{g/L}$) treatment (ANOVA, $P = 0.794$) (S5 Table). Starting from week 1 to week 4, significant differences were observed between the control and all other fipronil concentrations except the 0.005 $\mu\text{g/L}$ treatment (ANOVA, $P = 0.004$ – 0.04). At the end of the experiment (fifth length measurement), body length of the control was 6.32 ± 0.10 cm (S5 Table). Body length was not measured in shrimp under both 1.0 $\mu\text{g/L}$ and 3.0 $\mu\text{g/L}$ treatments because the shrimp died during the first days of fipronil exposure.

In the imidacloprid experiment, the initial body length of shrimp was not significantly different among all treatments and ranged between 5.28 ± 0.1 cm in the control and 5.38 ± 0.11

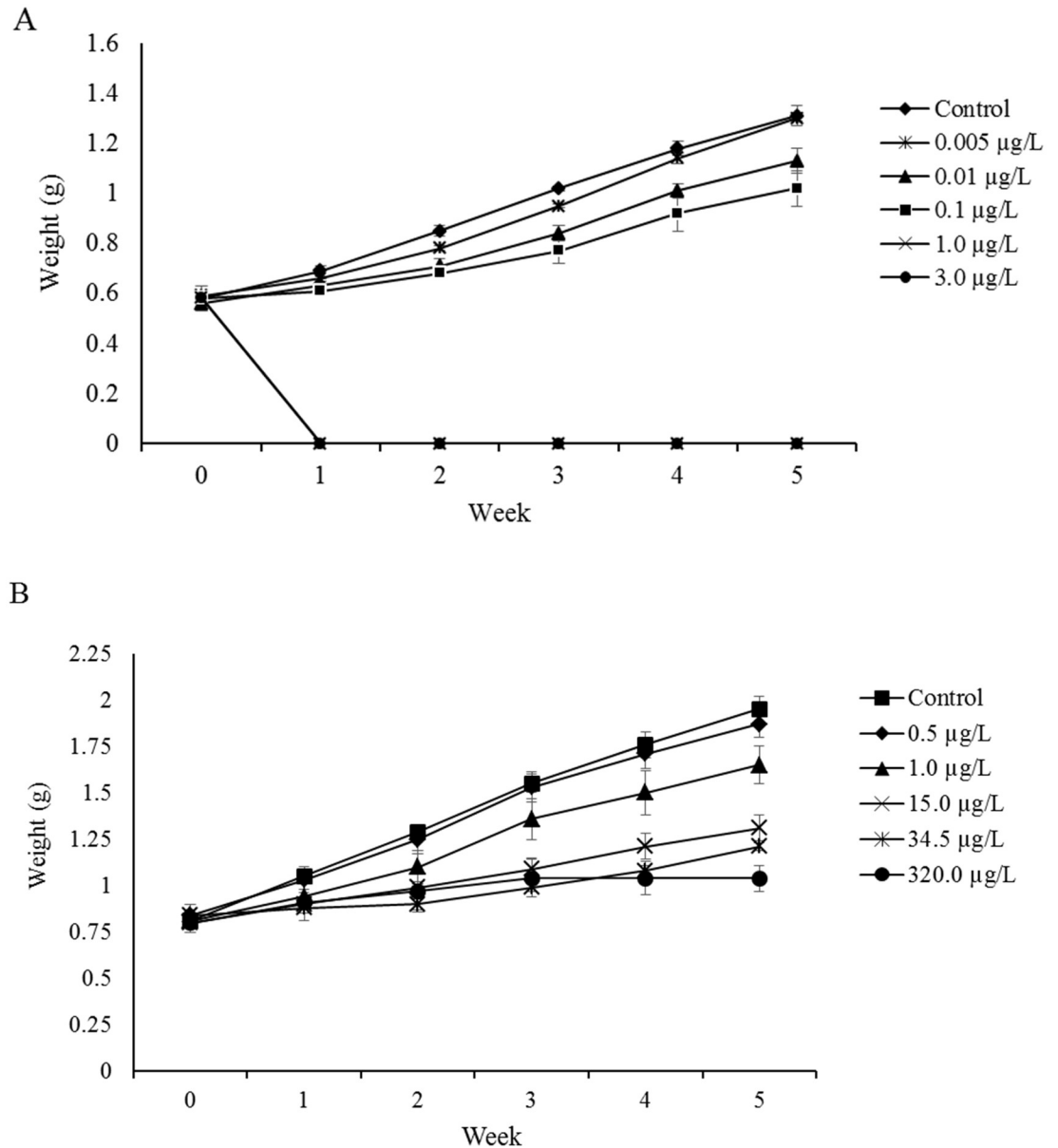


Fig 1. Wet weight of individual juvenile shrimp (g) during 5 weeks of the experiments. The vertical axis is the wet weight (g) of individual shrimp in each treatment, and the horizontal axis is the week from the beginning of experiment. Error bars indicate the standard errors (n = 18 in fipronil exp. and n = 15 in imidacloprid exp.). (A) Fipronil experiment, (B) Imidacloprid experiment.

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cm in the 34.5 µg/L treatment (ANOVA, P = 0.96) (S6 Table). After the second length measurement (week 2) and until the final measurement (week 5), there were significant differences between the control and other treatments except for the 0.5 µg/L and 1.0 µg/L treatments (ANOVA, P < 0.0001–0.019). At the end of the experiment, body length ranged between 5.91 ± 0.23 cm in the 320.0 µg/L treatment and 7.07 ± 0.12 cm in the control (S6 Table).

Moulting

In the fipronil experiment, shrimp under the control (0.0 µg/L) showed the shortest intermolt interval (7.57 ± 2.17 day) compared with other treatments, 0.005 µg/L, 0.01 µg/L, and

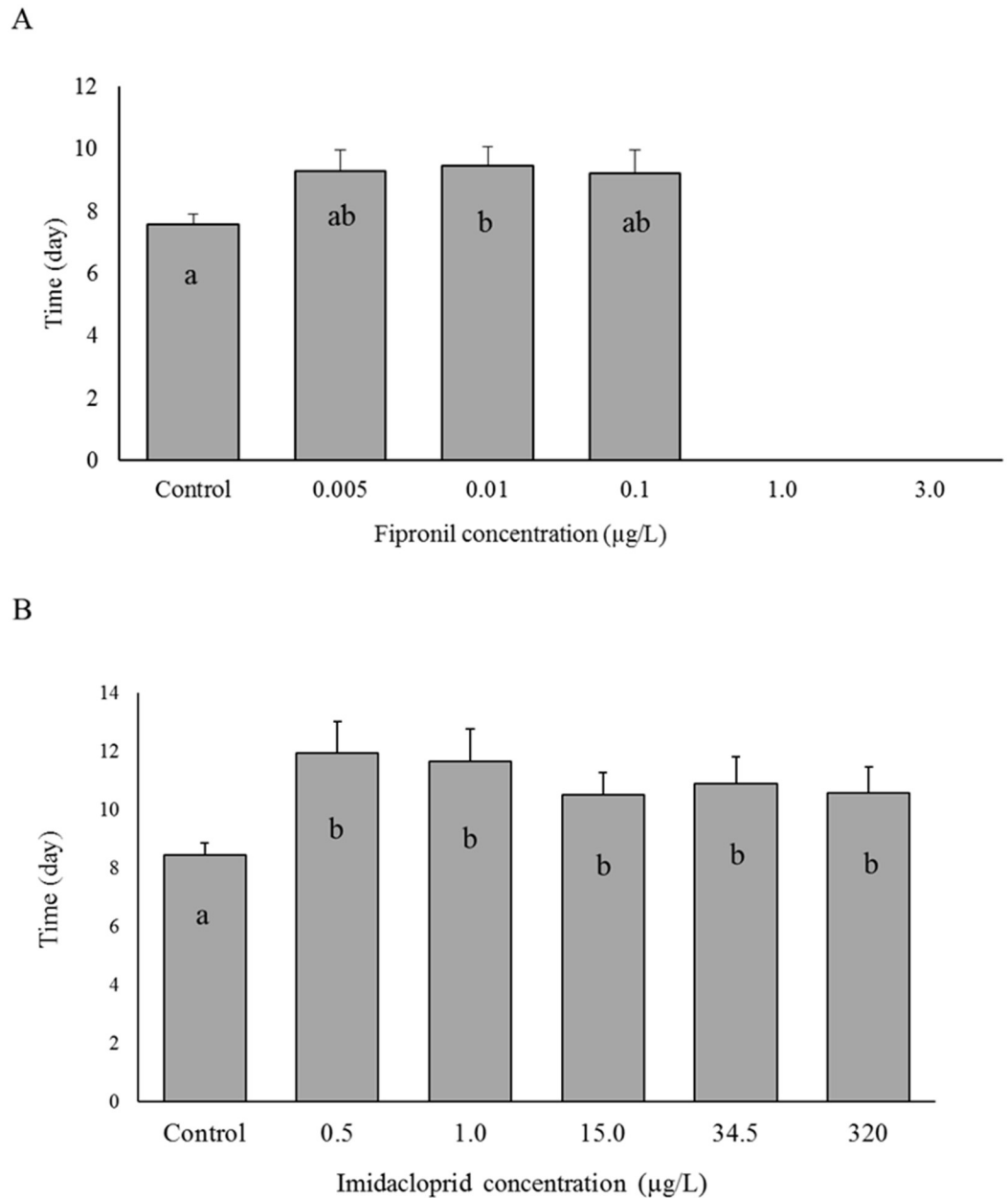


Fig 2. Inter-moult interval of juvenile shrimp under different concentrations of fipronil and imidacloprid. The vertical axis is the time (days) between consecutive moults of the same individual, and the horizontal axis is the six insecticide concentrations (µg/L) including the control. Error bars indicate the standard errors (n = 18 in fipronil exp. and n = 15 in imidacloprid exp.). (A) Fipronil experiment, (B) Imidacloprid experiment.

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0.1 µg/L, which had inter-moult intervals of 9.29 ± 4.22 day, 9.47 ± 2.73 day, and 9.20 ± 2.93 day, respectively (Fig 2A). The inter-moult interval of the control group differed significantly from treatment 0.01 µg/L ($P = 0.0117$); whereas, there was no difference with treatments 0.005 µg/L and 0.1 µg/L. Shrimp under the 1.0 µg/L and 3.0 µg/L treatments died during the first days of the experiment; thus we could not observe consecutive moults to calculate the inter-moult intervals.

Table 2. Percentage of individuals alive (mean ± standard deviation) of juvenile shrimp in fipronil experiment starting from day1 to the end of the experiment.

Fipronil concentrations (µg/L)	n	Survival %					
		Day 1	Week 1	Week 2	Week 3	Week 4	Week 5
Control	18	100	100	100	100	100	100
0.005	18	100	100	100	100	100	100
0.01	18	100	77.77 ± 9.62	77.77* ± 9.62	72.21* ± 9.62	72.21* ± 9.62	72.21* ± 9.62
0.1	18	100	44.44* ± 34.69	33.33* ± 16.67	33.33* ± 16.67	33.33* ± 16.67	33.33* ± 16.67
1.0	18	100	0	0	0	0	0
3.0	18	100	0	0	0	0	0

n = number of shrimp individuals in each treatment (6 shrimp per replicate aquarium, 3 aquariums per treatment). Values with star (*) indicate treatment is significantly different from the control (P < 0.0001–0.004).

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Shrimp under imidacloprid exposure showed a significant difference in inter-moult interval between the control (8.43 ± 2.52 day) and all other treatments (P = 0.0020–0.045). Inter-moult intervals under imidacloprid exposure ranged between 10.50 ± 4.04 day in the 15.0 µg/L treatment and 11.95 ± 4.9 day in the 0.5 µg/L treatment (Fig 2B).

Shrimp survival and LC₅₀

In the fipronil experiment, survivorship of shrimp under higher fipronil concentrations (1.0 µg/L and 3.0 µg/L) decreased rapidly during the first week of the exposure, and all shrimp died by day 6 and day 1, respectively. During the first week, survival rate in the control (100%) was significantly different from that in the 0.1 µg/L treatment (44.44%, P = 0.0006) (Table 2). The survivorship after week 2 differed significantly between the two lowest concentrations (control and 0.005 µg/L) and two higher concentrations (0.01 µg/L and 0.1 µg/L) (P < 0.0001) (Table 2). Lethal concentration of fipronil to reach 50% mortality of shrimp within 96 hours (96-h LC₅₀) of the juvenile brown shrimp was 0.12 µg/L with 95% confidence intervals 0.06–0.24 (S2 Fig).

In the imidacloprid experiment, shrimp showed higher survivorship percentage during the first two weeks compared with those in the fipronil experiment, and there was no significant difference among treatments including the control (Table 3). Starting from week 3, however, significant differences were observed in the survivorship of shrimp in treatments of higher concentrations. At the end of the experiment (36 days), the control treatment showed significant differences in the survival rate (100%) compared with the 15.0 µg/L, 34.5 µg/L, and

Table 3. Percentage of individuals alive (mean ± standard deviation) of juvenile shrimp in imidacloprid experiment starting from day1 to the end of the experiment.

Imidacloprid concentrations (µg/L)	n	Survival %					
		Day 1	Week 1	Week 2	Week 3	Week 4	Week 5
Control	15	100	100	100	100	100	100
0.5	15	100	100	93.33 ± 11.54	93.33 ± 11.54	93.33 ± 11.54	93.33 ± 11.54
1.0	15	100	93.33 ± 11.54	93.33 ± 11.54	93.33 ± 11.54	93.33 ± 11.54	86.66 ± 11.54
15.0	15	100	100	100	100	73.33 ± 23.09	66.66* ± 30.55
34.5	15	100	100	100	73.33* ± 23.09	60.0* ± 20.0	40.0*
320.0	15	100	100	100	80.0 ± 20.0	60.0* ± 34.64	33.33* ± 11.54

n = number of shrimp individuals in each treatment (5 shrimp per replicate aquarium, 3 aquariums per treatment). Values with star (*) indicate treatment is significantly different from the control (P < 0.0001–0.039).

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320.0 µg/L treatments, which had the survival rates of 66.6%, 40%, and 33.3%, respectively (Kaplan-Meier survival analysis followed by the non-parametric Log-Rank test, $P < 0.0001$). However, there were no significant differences among the control (100% survival), 0.5 µg/L (93.3% survival) and 1.0 µg/L treatment (86.6% survival) according to the Kaplan-Meier survival analysis (Table 3).

Behavioral and other physical changes

Swimming and feeding behaviors of brown shrimp under the exposure of fipronil and imidacloprid changed noticeably in comparison with those in the control treatments. These changes were consistent between fipronil and imidacloprid exposures, and a sequence of changes in behaviors was observed from the first day of the exposure (in some treatments) until the death of the affected shrimp. First, affected shrimp became unable to swim normally, and they started exhibiting circle-like movements. After that, shrimp stopped moving and sprawled on the bottom of an aquarium at the same time their swimming legs kept moving involuntarily. Then, their swimming legs stopped moving, and they died. Shrimp in the fipronil experiment showed these behavioral changes after only one day of the exposure and even in the lowest concentration (0.005 µg/L). Similarly, shrimp under imidacloprid exposure showed same behavioral changes by day one in all treatments except in the 0.5 µg/L treatment (lowest imidacloprid concentration). Under the lowest imidacloprid concentration, these changes started in day 5. During all of these abnormal swimming behaviors, shrimp exhibited difficulty in feeding, and food remained in aquariums were cleaned routinely.

At the end of the experiments, visible changes in color were noticed in shrimp bodies under the exposure to either pesticide. Fig 3 shows the changes in color of shrimp from the normal bright color under the control to gray and dark body color of shrimp in treatments of high fipronil and imidacloprid concentrations. In the fipronil experiment, shrimp exposed to higher concentrations (1.0 µg/L and 3.0 µg/L) died during the first days and did not show color changes.

Water quality

In both fipronil and imidacloprid experiments, all water quality parameters were within suitable ranges that fit the environmental requirements of brown shrimp [55]. Statistical analysis of these parameters showed no significant differences among treatments during the experiments. In the fipronil experiment, the mean values of water quality parameters were the following: temperature, 24.12 ± 0.06 °C; dissolved oxygen (DO), 5.82 ± 0.41 mg/L, salinity, 15.02 ± 0.14 ‰; and pH, 8.01 ± 0.06 (S7 Table). In the imidacloprid experiment, mean values were the following: temperature, 24.34 ± 0.03 °C; dissolved oxygen (DO), 5.87 ± 0.06 mg/L, salinity, 15.48 ± 0.08 ‰; and pH, 8.15 ± 0.05 (S8 Table).

Discussion

In the present study, all nominal concentrations of fipronil and imidacloprid were within the range of the concentrations reported in recent studies [12, 30, 35, 37] for both insecticides (Table 1). Our results showed that, for both insecticides, significant differences were observed in the final weight and final length (length at week 5) of shrimp under many of these concentrations compared with the control (S3–S6 Tables) suggesting the insecticides have effects to reduce growth of shrimp. Reduction in growth of aquatic arthropods resulting from contaminants has been demonstrated in several other studies: e.g. the Glyphosate-based herbicide (Roundup®) on freshwater shrimp *Caridina nilotica* [56], petroleum hydrocarbons from oil spill on juvenile brown shrimp *F. aztecus* and white shrimp *L. setiferus* [57], and imidacloprid

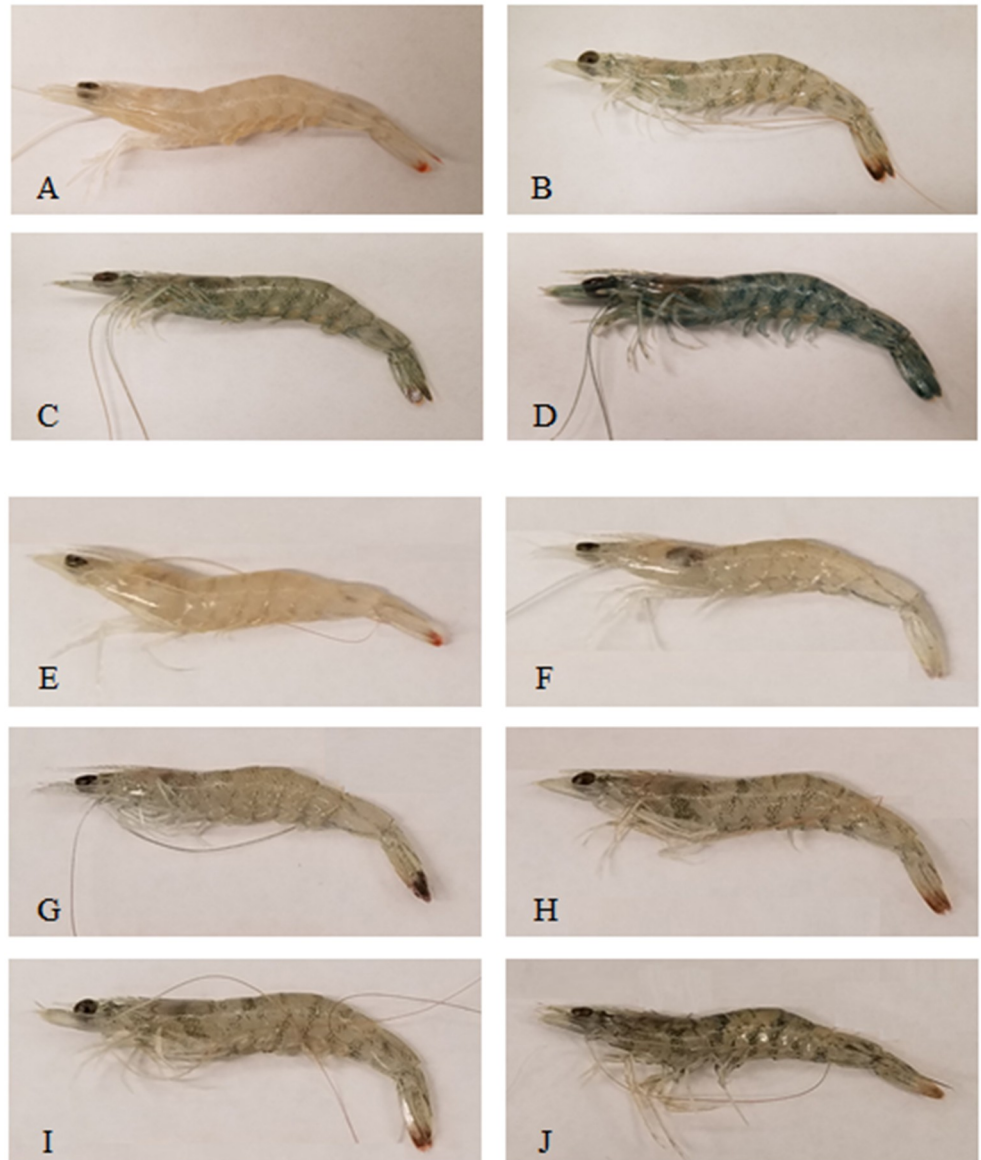


Fig 3. Color change of shrimp in different concentrations ($\mu\text{g/L}$) of the insecticides at the end of the experiments. (A) Control 0.0 $\mu\text{g/L}$ fipronil, (B) 0.005 $\mu\text{g/L}$ fipronil, (C) 0.01 $\mu\text{g/L}$ fipronil, (D) 0.1 $\mu\text{g/L}$ fipronil, (E) Control 0.0 $\mu\text{g/L}$ imidacloprid, (F) 0.5 $\mu\text{g/L}$ imidacloprid, (G) 1.0 $\mu\text{g/L}$ imidacloprid, (H) 15.0 $\mu\text{g/L}$ imidacloprid, (I) 34.5 $\mu\text{g/L}$ imidacloprid, (J) 320.0 $\mu\text{g/L}$ imidacloprid.

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on midge *Chironomus tentans*, amphipod *Hyaella azteca* [58], and harlequin fly *Chironomus riparius* [59]. However, we note that growth of juvenile blue crabs *Callinectes sapidus* showed a significant increase under all treatments of fipronil compared to the control in a short-term (96-h) experiment [60], suggesting the effects of insecticides on growth may not be always negative.

Many potential reasons exist for the reduced growth of juvenile brown shrimp under fipronil and imidacloprid exposures in our study. For example, organisms in polluted environments use the metabolic energy to detoxify the contaminant. Therefore, this will affect their growth performance by affecting the metabolism of protein and carbohydrate [61]. Shrimp is

known to derive energy more efficiently from protein compared with carbohydrates and lipids [62]; consequently, fipronil and imidacloprid in our study may have affected brown shrimp growth by reducing protein levels in bodies of those under exposure compared with shrimp in the control, which in turn affected their growth at the end of the experiment (S3–S6 Tables). Alternatively, the reduction in growth may also be caused by reduced feeding. Both insecticides are neurotoxins, which act by disrupting the central nervous system activity of exposed arthropods either by blocking the chloride channels at the gamma-aminobutyric acid (GABA) by fipronil [63] or by binding strongly to the nicotinic acetylcholine receptor (nAChR) by imidacloprid [64]. These effects on the nervous system activities may suppress feeding of invertebrates [65]. Regardless of the underlying mechanisms, the reduced growth from the insecticide is a great concern because the survival of juvenile brown shrimp is thought to be size dependent [66] and the abundance of adult white shrimp, which has very similar life history as brown shrimp has been demonstrated to be very sensitive to survival during a juvenile stage [67].

Moulting in arthropods is useful endpoint to test sub-lethal exposure of chemicals, and considered one of the most important physiological processes for these animals because in order to grow normally they have to cast their exoskeleton periodically [68, 69]. Moulting process is regulated by hormones and nervous system secretions; therefore, it is susceptible to the negative effects of endocrine disrupting chemicals EDCs including many pesticides [69], such as fipronil [70] or those who act like EDCs such as imidacloprid [71]. In our study, both fipronil and imidacloprid affected moulting of brown shrimp with significant differences between the control and other treatments. Inter-moult interval of shrimp under fipronil exposure was significantly delayed under the 0.01 µg/L treatment compared with the control (Fig 2A). This result is consistent with our previous study, which showed the prolonged inter-moult intervals under the 0.1 µg/L and 1.0 µg/L treatments compared with the control [41]. In our previous study, we evaluated the adverse effects of higher concentrations of fipronil (0.0, 0.1, 1.0, 3.0, 6.4, 10.0 µg/L) on brown shrimp juveniles, and because of the higher concentrations used, the exposure to all of the fipronil treatments resulted in all individuals dying before the end of the experiment. Under imidacloprid exposure in the current study, shrimp under all treatments showed a significant delay ($P = 0.0020$ – 0.045) in their inter-moult intervals (10.89 ± 3.97 day in 34.5 µg/L to 11.95 ± 4.90 day in 0.5 µg/L) compared with shrimp in control (8.43 ± 2.52 day) (Fig 2B). Many studies have reported similar delay in moulting of marine and freshwater arthropods after exposing them to different pesticides [41]. Such delay in moulting of shrimp may be linked to the reduction in growth and suggests that normal development of shrimp was affected even under concentrations below chronic level (0.01 µg/L) of fipronil.

Our study showed that survival of juvenile brown shrimp was concentration-dependent under both insecticides (Tables 2 and 3). Fipronil and imidacloprid caused significant lethal and sub-lethal effects on shrimp especially in higher concentrations. Under fipronil exposure, all shrimp died during first few days in the 1.0 µg/L and 3.0 µg/L treatments and survivorship declined significantly ($P < 0.0001$) under the 0.1 µg/L (33.33%) and 0.01 µg/L (72.21%) treatments compared with the control (100% survival) (Table 2). Under imidacloprid exposure, survivorship declined significantly ($P < 0.0001$) in the 320.0 µg/L (33.33%), 34.5 µg/L (40.0%), and 15.0 µg/L (66.66%) treatments compared with control (100%) (Table 3).

The nominal 96-h LC_{50} of fipronil for brown shrimp was 0.12 µg/L (0.06–0.24). This suggests brown shrimp is one of the most sensitive crustaceans to fipronil among all aquatic invertebrates studied so far. This 96-h LC_{50} for brown shrimp is less than that for estuarine mysid shrimp *Neomysis americana* $LC_{50} = 0.14$ µg/L reported in Gan, *et al.* [33]. Other sensitive marine invertebrates include estuarine grass shrimp *Palaemonetes pugio* with 96-h LC_{50} of 0.32 µg/L [72, 73], estuarine copepod *Amphiascus tenuiremis* with 96-h LC_{50} of 6.8 µg/L [21],

and estuarine Chinese mitten crab *Eriocheir sinensis* with 96-h LC_{50} of 8.56 $\mu\text{g/L}$ [52]. Many previous studies reported the greater sensitivity of marine invertebrates to fipronil compared with freshwater invertebrates, such as the copepod *Acanthocyclops robustus* with 48-h LC_{50} of 194.2 $\mu\text{g/L}$ [74], the water flea *D. magna* with 48-h LC_{50} of 190.0 $\mu\text{g/L}$ [27], and the red swamp crayfish *Procambarus clarkia* with 96-h LC_{50} of 163.5 $\mu\text{g/L}$ [73]. As for imidacloprid, we could not measure the LC_{50} for brown shrimp because there was not enough mortality in shrimp during the first 96 hours of the exposure under the concentrations used in this study.

We also note that the LC_{50} of fipronil measured in this study (0.12 $\mu\text{g/L}$) was below the LC_{50} measured in the previous study in 2016, 1.3 $\mu\text{g/L}$ [41]. This may be because of the difference in the temperature between the two studies: 20.84°C \pm 0.24 (in 2016 study) and 24.12°C \pm 0.06 (in present study). Although temperature was increased, these water temperatures are considered within the optimum temperature range for the brown shrimp development 18°C and 25°C [55]. We increased water temperature from the previous study because we expected shrimp to experience higher temperature during summer months in estuaries and previous shrimp experiments by others were often conducted at higher temperature e.g. [75–77]. Effect of temperature on toxicity of pesticides was observed in other studies, for example, Russo, *et al.* [78] noted that, of many environmental parameters investigated, temperature was the only parameter that magnified the effect of pesticide exposure on the crustacean *Gammarus pulex* in streams. Willming *et al.* [79] also reported that the 10 days LC_{50} for the crustacean *H. azteca* exposed to the fungicide chlorothalonil was lower under the fluctuating temperature regime compared with that under the constant temperature regime. We suggest further studies to investigate the effects of temperature on the effects of insecticides in the future study as the temperature in the subtropical estuaries (habitat for juvenile brown shrimp) can change greatly among seasons.

Behavioral changes are useful biomarkers to evaluate sub-lethal exposure effects of contamination [80]. Long-term behavioral changes can be detected even at low doses of pesticides, and the behavior can reveal a great deal about the systems and processes influenced by pesticides [81]. In the current study, shrimp in both experiments exhibited behavioral changes such as restricted swimming and mobility, paralysis, and feeding delay, starting from the first day of exposure even in treatments of low fipronil concentrations (0.005 and 0.01 $\mu\text{g/L}$) whereas those in treatment of low imidacloprid concentration (0.5 $\mu\text{g/L}$) did not show any behavioral changes until day 5 of the exposure. Similar behavioral changes were reported in studies that tested the effect of fipronil, imidacloprid, and other pesticides on aquatic invertebrates. For example, Overmyer *et al.* [26] observed abnormal behavior and muscle control in the aquatic insect *Simulium vittatum* under all tested fipronil and imidacloprid concentrations. Similarly, Stratman *et al.* [82] showed that the chironomid midge *Cricotopus betis* exposed to different concentrations of fipronil exhibited abnormal behaviors such as movement restriction and feeding reduction at all tested concentrations. Behavioral changes could be direct consequences of pesticides on the central nervous system of organisms [83]. These changes may have substantial ecological effects to the organisms by shifting them to unfavorable habitats or even by making them more sensitive to predators, and eventually leading to indirect lethal responses of pollutants at sub-lethal levels [69]. In particular, the major mortality of juvenile brown shrimp is considered to be predations [66], and small effects on their behavior may cause substantial reduction in their in situ mortality rate.

In both experiments, shrimp showed darker body color in treatments of higher concentrations of insecticides compared with those in control, which had normal bright color as they are in nature. The color also appears to be a concentration-dependent, and shrimp under fipronil exposure were much darker than those under imidacloprid exposure. In other study, Martinez *et al.* [84] reported that Pacific white shrimp *Litopenaeus vannamei* exposed to low

concentrations of heavy metals such as copper were significantly redder than those in controls. In shrimp and other crustaceans, many environmental factors are known to affect body color by affecting pigment dispersion within the chromatophores [85], which are regulated by neurosecreted hormones [86]. However, in our study, factors such as light intensity, background color, and temperature which are known to have an effect on body color of shrimp were carefully controlled. Color changes under fipronil and imidacloprid insecticides were observed in the exoskeleton and abdominal muscle of juvenile brown shrimp as shown in Fig 3. Body color may be used as an indicator of the health of shrimp [84] and environment [87]; consequently, we suggest that color change of penaeid shrimp needs more investigation because it could be used as an indicator of long-term effects of sub-lethal exposure to environmental neurotoxins such as fipronil, imidacloprid or other commonly used pesticides.

Conclusions

In summary, fipronil and imidacloprid had both lethal and sub-lethal effects on brown shrimp at concentrations that have been reported in the natural environment. According to the 96-h LC_{50} of fipronil, brown shrimp is one of the most sensitive invertebrate species to fipronil exposure among all marine and freshwater species studied to date. Although brown shrimp exhibited less lethal effects on imidacloprid than fipronil under the U.S. EPA benchmark concentrations, sub-lethal effects such as delayed moulting and reduced growth were significant. Both types of effects are of serious concern because of the commercial and ecological importance of brown shrimp, their dependency on estuaries, and increased use of these pesticides in coastal areas. We recommend continuous monitoring of both chemicals, and it may also be necessary to reduce the use of these chemicals when brown shrimp are abundant in estuaries.

Supporting information

S1 Fig. Experimental systems and the aquariums used in fipronil and imidacloprid experiments. (A) System of 18 glass aquariums covered with aluminum foil sheets and glass lids; (B) aquarium was divided into six cells of same size; (C) dividers made of polypropylene plates and fiberglass screen to distribute the dissolved oxygen among the cells; (D) total length of shrimp measured every week during the experiments.

(DOCX)

S2 Fig. Lethal concentration of fipronil to reach 50% mortality of shrimp within 96 hours (96-h LC_{50}) of the juvenile brown shrimp.

(DOCX)

S1 Table. Dilution procedures for all nominal fipronil concentrations used in the experiment. For steps 1 and 2, magnetic stirrer was used to homogenize the mixture.

(DOCX)

S2 Table. Dilution procedures for all nominal imidacloprid concentrations used in the experiment. For steps 1 and 2, magnetic stirrer was used to homogenize the mixture.

(DOCX)

S3 Table. Initial weight (g), final weight (g), and percent weight gain (mean \pm standard deviation) of juvenile shrimp under different concentrations of fipronil. n = number of shrimp in each treatment at the measurement time. Values were calculated based on the wet weight per individual shrimp. Means in columns not sharing the same letter are significantly different (ANOVA, $P < 0.05$).

(DOCX)

S4 Table. Initial weight (g), final weight (g), and percent weight gain (mean \pm standard deviation) of juvenile shrimp under different concentrations of imidacloprid. n = number of shrimp in each treatment at the measurement time. Values were calculated based on the wet weight per individual shrimp. Means in columns not sharing the same letter are significantly different (ANOVA, $P < 0.05$).

(DOCX)

S5 Table. Length (cm) of juvenile shrimp (mean \pm standard deviation) exposed to fipronil over five weeks. n = number of shrimp in each treatment. Means in columns not sharing the same letter are significantly different (ANOVA, $P < 0.05$).

(DOCX)

S6 Table. Length (cm) of juvenile shrimp (mean \pm standard deviation) exposed to imidacloprid over five weeks. n = number of shrimp in each treatment. Means in columns not sharing the same letter are significantly different (ANOVA, $P < 0.05$).

(DOCX)

S7 Table. Water quality parameters of shrimp aquariums during 34 days of fipronil experiment. Values are Mean \pm standard deviation for each parameter of all fipronil concentrations. Treatment 1.0 $\mu\text{g/L}$ has no standard deviation because the number of aquariums was reduced to 1 due to deaths of shrimp during first days of the experiment. Treatment 3.0 $\mu\text{g/L}$ has no water quality parameters because all shrimp died during the first day of the exposure.

(DOCX)

S8 Table. Water quality parameters of shrimp aquariums during 36 days of imidacloprid experiment. Values are Mean \pm standard deviation for each parameter of all imidacloprid concentrations.

(DOCX)

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