INVESTIGATION OF LETHAL AND SUB-LETHAL EFFECTS OF COMMON INSECTICIDES, FIPRONIL AND IMIDACLOPRID, ON JUVENILE BROWN SHRIMP, *FARFANTEPENAEUS AZTECUS*, AND WHITE SHRIMP, *LITOPENAEUS SETIFERUS*

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2019

Major Subject: Wildlife and Fisheries Sciences

ABSTRACT

Chemical pesticides are widely used around the world, but at the same time, they may pose direct or indirect risks to many non-target organisms. Recent increased use of insecticides in coastal areas to control invasive species raises concern that insecticides may affect ecologically and/or commercially important species found in estuaries.

In this study, a series of laboratory experiments was conducted to evaluate short-term (lethal) and long-term (sub-lethal) effects of fipronil and imidacloprid on juveniles of brown shrimp and white shrimp. Various concentrations of fipronil and imidacloprid in each experiment were used. The concentrations were determined based on previously observed concentrations in the aquatic environment by other researchers. In the first experiment, five nominal concentrations of fipronil (0.1, 1.0, 3.0, 6.4, and 10.0 μg/L) were used; whereas, in the second and third experiments, lower concentrations of fipronil (0.005, 0.01, 0.1, 1.0, and 3.0 μg/L) and five nominal concentrations of imidacloprid (0.5, 1.0, 15.0, 34.5, 320.0 μg/L) were used.

The endpoints of the studies were survivorship, the nominal median lethal concentration (LC_{50}), the nominal median lethal time (LT_{50}), development (weight gain and inter-molt intervals), behavioral and physical changes, and whole-body chemical composition. The main results were as following:

(1) Both insecticides affected brown shrimp and white shrimp growth, survival, body composition, body color, and behavior in a concentration-dependent manner; (2) Brown shrimp juveniles were more sensitive to fipronil exposure than white shrimp,

with 96-hour LC_{50} = 0.12 μg/L, which makes brown shrimp one of the most sensitive invertebrates to fipronil studied so far; (3) Under their environmental concentrations, fipronil showed higher impact on juvenile brown shrimp compared with imidacloprid; (4) Fipronil and imidacloprid caused noticeable sub-lethal effects to brown shrimp and white shrimp at concentrations lower than their chronic levels in the aquatic life benchmark of the U.S. EPA.

Our results suggest that monitoring of fipronil and imidacloprid should be recommended in estuaries and other areas along the coast near the locations where either fipronil or imidacloprid is used. In addition, it is of importance to reduce the usage of these insecticides especially during the seasons of penaeid shrimp migration to inshore annual nursery areas. Revising the acute and chronic levels of the U.S. EPA aquatic life benchmarks for fipronil and imidacloprid is also recommended to improve the health of estuaries and increase the abundance of shrimp populations in the Gulf of Mexico region.

DEDICATION

To my father (in memoriam), who never stopped supporting me until his last day

My loving mother, my wonderful wife and children, and all my family

For their unlimited love, support, and encouragement that helped me get through this long journey.

ACKNOWLEDGEMENTS

Over the years of my PhD study in Texas A&M University, I have been fortunate to meet many special people who helped me substantially to reach this point of my life. First, I would like to express my deepest appreciation and gratitude to my advisor and committee chair, Dr. Masami Fujiwara for his trust, mentorship, time and immeasurable support. For me, he is the best advisor any graduate student could have, and I consider myself very lucky to have had the honor of being one of his PhD students. Every moment I spent with him, I have learned an important lesson that improved me personally and professionally, and without him, this endeavor would not have been possible.

I would like to extend my special thanks and appreciation to my committee members, Dr. Delbert Gatlin, Dr. Miguel Mora, and Dr. David Wells for their guidance, help and valuable input throughout the course of my study and research.

I would like to thank all the faculty and staff in the Department of Wildlife and Fisheries Sciences (WFSC) at Texas A&M University for their kindness, help and support. Special thanks to Dr. Michael Masser, Dr. Frances Gelwick, Dr. Thomas DeWitt, Cheree Steward, Shirley Konecny, Dawn Miles, Selina Garcia, Royce Robinson, and Lindsay Hutchins for their continued help during my study. I would also like to thank all the graduate students in the WFSC department, especially those who I have had the pleasure to meet and work with, in particular, I would like to extend my special thanks to Fiala Bumpers from Dr. Fujiwara Laboratory for her help in the GIS

work, and Clement de Cruz from Fish Nutrition Laboratory for his help in the chemical composition analyses.

I would also like to thank the faculty and staff of the English Language institute (ELI) at Texas A&M University, especially those outstanding instructors who I was fortunate enough to meet and learn from during the 2012-2013 academic year. Without their invaluable efforts, I would not be able to pursue my dream. I would like to express my gratitude to all my friends in College Station, Texas, for their friendship and support during my time in College Station, in particular, many thanks to those who helped me during my first weeks in the city. They were more than brothers to me, and I greatly appreciate that.

I am grateful to the U.S. National Marine Fisheries Service (NMFS), Galveston Laboratory, for their help in providing shrimp for the experiments. Special thanks to the Higher Committee for Education Development in Iraq (HCED) who chose me for this scholarship among many candidates and supported me during almost all the time of my study in the United States. I would also like to extend my thanks to the Office of Graduate and Professional Studies (OGAPS) of the Texas A&M University, who awarded me a Dissertation Fellowship which funded me during last year of my study while writing this dissertation. Many thanks also to the Texas Sea Grant at Texas A&M University, Grants-In-Aid of Graduate Research Program, for partial funding for the supplies used in my research.

Finally, my profound gratitude to my family: my beautiful wife, and wonderful children, Mom, Dad (in memoriam), brothers, sister, A., A. GH., M., for their love,

patience, support and encouragement throughout all these years, without them I would have not reached this far.

For all of you, thank you so much and god bless.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professor Masami Fujiwara (advisor) and Professors Delbert Gatlin and Miguel Mora of the Department of Wildlife and Fisheries Sciences and Professor David Wells of the Department of Marine Biology.

All work conducted for the dissertation was completed by the student, under the advisement of Professor Masami Fujiwara of the Department of Wildlife and Fisheries Sciences.

Funding Sources

Graduate study was supported by the Higher Committee for Education Development in Iraq (HCED) and partially by a dissertation fellowship from the Office of Graduate and Professional Studies (OGAPS) of the Texas A&M University, and a Grants-In-Aid of Graduate Research Program from the Texas Sea Grant at Texas A&M University.

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CHAPTER I

INTRODUCTION

This dissertation research complemented many recent studies that highlighted the risks of pesticides on aquatic organisms. The objective of the research was to determine the lethal and sub-lethal effects of the insecticides fipronil (5-amino-1-[2, 6-dichloro4-4(trifluoromethyl) phenyl]-4[(trifluoromethyl) sulfinyl]-1H- pyrazole-3-carbonitrile) and imidacloprid (1-[(6-Chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine) on brown shrimp *Farfantepenaeus aztecus* and white shrimp *Litopenaeus setiferus* under the concentrations previously found in the environment. This objective was addressed through a series of laboratory experiments to evaluate the effects of fipronil and imidacloprid on survivorship, growth, inter-molt intervals, body chemical composition, and behavioral changes of juvenile shrimp.

1.1. Background

Pesticides are poisons designed to control various insects, weeds, and other pests. Pesticides protect human health, property, crops, and domestic animals, and they can also protect our drinking water from contamination caused by algae and other hazardous organisms (Stevenson et al., 1997; Opsahl, 2012). However, these chemical compounds

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This dissertation follows the style of *Environmental Pollution*.

can also be an environmental hazard, adversely affecting non-target organisms (Boran et al., 2007). The use of pesticides in the United States has increased rapidly in recent years. For example, over 453,592 metric ton of pesticides are used annually in the U.S.; this is approximately one-fifth of global use (Donaldson et al., 2002; Alavanja, 2009). The U.S. Geological Survey (USGS) reported that 97% of U.S. urban streams had one or more pesticides detected sometime during the ten years of their study (Ensminger et al., 2013).

Pesticides are classified into different categories based on their attributes. For example, they are categorized according to their use (e.g., insecticides, herbicides, fungicides, molluscicides, nematicides, miticides, and rodenticides), toxicity (e.g., extremely dangerous, highly dangerous, moderately dangerous, and slightly dangerous), and median lifetime (e.g., permanent, persistent, moderately persistent, and not persistent). Another common way of classification is based on their chemical structure, for which there are four main groups: organochlorines, organophosphates, carbamates, and pyrethroids (Garcia et al., 2012), in addition to relatively new compounds such as the neonicotinoids and phenylpyrazoles.

In 1996, the United States Environmental Protection Agency (U.S. EPA) issued the Food Quality Protection Act (FQPA), which restricted and canceled many widely used organophosphate insecticides due to their adverse effects on humans and other animals. These insecticides have been replaced by synthetic pyrethroids and relatively new chemical compounds such as phenylpyrazoles and neonicotinoids for agricultural and household uses. Two of these new chemical insecticides that gained wide popularity

are the phenylpyrazole fipronil and the neonicotinoid imidacloprid (Overmyer et al., 2005; Goodman, 2011).

Fipronil and imidacloprid are neurotoxins that operate by disrupting neural transmissions in the central nervous system of exposed invertebrates; however, they have different modes of action. For example, fipronil interferes with the passage of chloride ions by binding to a specific site within the gamma-aminobutyric acid (GABA) receptor; whereas, imidacloprid binds to postsynaptic nicotinic acetylcholine receptors (nAChR) (Gunasekara et al., 2007; Mortensen et al., 2015; Simon-Delso et al., 2015). Currently, fipronil and imidacloprid account for approximately one-third of the world insecticide market with large-scale applications including protecting plants from agricultural pests, controlling household pests, and controlling parasites on domesticated animals (Pisa et al., 2015; Simon-Delso et al., 2015).

These insecticides are considered safer than other types of old generation pesticides because of their low toxicity on vertebrates. However, actual detrimental effects of fipronil and imidacloprid on non-target organisms have been reported in many studies conducted on various species such as mammals (Bhardwaj et al., 2010; Gu et al., 2013), birds (Avery et al., 1997; Berny et al., 1999), fish (Beggel et al., 2010; Clasen et al., 2012), amphibians (Feng et al., 2004; Gripp et al., 2017), reptiles (Peveling and Demba, 2003; Cardone, 2015), and invertebrates (Yang et al., 2008; Hladik et al., 2016; Al-Badran et al., 2018).

The use of fipronil and neonicotinoids (including imidacloprid) was banned recently in many countries such as Canada, the European Union countries, China, and

Taiwan (Wu et al., 2014; CCM, 2017; CBC, 2018; Stokstad, 2018). However, both insecticides are still used commonly in many states in the United States, including Texas, California, Ohio, Louisiana, and South Carolina (USEPA, 2016; Hladik et al., 2018; Troiano et al., 2018). With their increased use in recent years and highly persistent nature in soils, they are frequently detected in surface and groundwater (Overmyer et al., 2005; Bonmatin et al., 2015; Simon-Delso et al., 2015). For example, it has been reported that fipronil was detected in the aquatic environment in concentrations as high as 12.62 μg/L in the U.S. and other parts of the world (Chandler et al., 2004; Wirth et al., 2004; Mize et al., 2008; Gan et al., 2012; Ruby, 2013). Similarly, the highest reported environmental concentration of imidacloprid reached 320.0 μg/L (Hayasaka et al., 2012b; Van Dijk et al., 2013; Main et al., 2014; Hook et al., 2018). These concentrations exceed the acute toxicity levels of the U.S. EPA aquatic life benchmark for invertebrates for both insecticides, 0.11 μg/L for fipronil and 0.38 μg/L for imidacloprid (USEPA, 2019).

However, information is still very limited with regard to the risk assessment and environmental toxicology of these chemicals in marine ecosystems. For example, in a review conducted by Pisa et al. (2015) on the effects of fipronil and neonicotinoids on non-target invertebrates, the authors found that, of 376 papers being reviewed, there were a small number of studies on aquatic invertebrates and even less on marine species. The majority of the studies focused on terrestrial invertebrates such as honeybees.

1.2. Current study

In this dissertation, I present the results from the studies that investigated the toxicity of fipronil and imidacloprid on two penaeid shrimp species in the Gulf of Mexico. Brown shrimp and white shrimp are the most important penaeid shrimp species for commercial harvest along the Atlantic coast of the southeastern United States and in the Gulf of Mexico (DeLancey et al., 2005; Ditty, 2011; Montero et al., 2016). Between 2016 and 2018, the annual commercial landing of both species in the United States was 105,833 metric ton, worth about $456 million (NMFS, 2019). In addition to their economic importance, brown shrimp and white shrimp play a critical ecological role in energy transfer from benthic to pelagic food web systems, and also as prey for various fish species that support important fisheries in the Gulf of Mexico region (Sheridan and Ray, 1981; Patillo et al., 1997; Daewel et al., 2011; Fujiwara et al., 2016; Montero et al., 2016).

Fipronil and imidacloprid are increasingly used in coastal communities in Texas, both in agriculture and household use, and these insecticides and their degradation products in aquatic environments are detected in levels exceeding the acute levels of the U.S. EPA. Because brown shrimp and white shrimp are estuarine-dependent during their juvenile stage, they are vulnerable to pesticides that are used on land and near their nursery habitat. Therefore, the effects of these insecticides on brown shrimp and white shrimp are a serious concern, particularly after the recent decline of the fishery for both shrimp species below their average annual harvest. No study has been conducted to assess the impact of fipronil and imidacloprid on brown shrimp and white shrimp.

1.3. Study significance

This study addresses a critical issue in coastal management: effects of insecticides on the major commercial fishery species in Texas. Insecticides are often designed to kill insects, but not vertebrates or plants. This leads to the notion that they are safe. However, we often overlook the fact that insecticides can also kill other groups of beneficial invertebrates such as shrimp. The adult stock abundance of brown shrimp and white shrimp is known to be very sensitive to changes in survival rate during estuarine stages (Baker et al., 2014; Leo et al., 2016). Therefore, a small change in survival rates will have a large impact on the stock abundance and fishery yield. The shrimp fishery is the most valuable fishery in the Gulf of Mexico; it is worth $588 million USD and accounts for 65% (by weight) of the total U.S. shrimp landing (NMFS, 2016). Therefore, understanding the lethal and sub-lethal effects of widely used insecticides such as fipronil and imidacloprid on brown shrimp and white shrimp is critically important.

On the other hand, the use of insecticides is beneficial at the same time. Although the quantification of the benefit may not be straightforward, and beyond the scope of this study, more than $24.3 billion of pesticides were used between 2006 and 2007 in the U.S. (Grube et al., 2011), far exceeding the commercial value of shrimp stocks. Therefore, it is not reasonable to expect complete cessation of pesticide use in the near future. However, if we can assess the negative impacts of pesticides, it will allow an informed decision balancing the cost and benefit of the use of these pesticides.

This study is innovative because it focuses on economically important resources and long-term effects. Sub-lethal effects of toxicants are often discussed in the literature; however, they are rarely investigated with economically important species. This study quantifies these effects on growth, development, behavior, and body composition in addition to lethal effects. The current study is also timely because the increased use of insecticides is expected in Texas. This results from increased risks of vector-borne diseases such as Zika and West Nile viruses and increased invasive ants (tawny crazy ants) and termites in many parts of Texas, especially in coastal areas (USEPA, 2016). Also, this study investigates the effects on commercially important fishery species, which are unlikely to be investigated by the U.S. EPA. Because of these reasons, this study is expected to yield high impact outcomes.

In this dissertation, I present the results of multiple toxicity experiments to evaluate the adverse effects of commonly used insecticides on two penaeid shrimp species from a coastal area in Texas. The dissertation is divided into three main chapters (Chapters 2-4). Each of the three chapters was written as an independent paper and represented a different set of experiments with a specific research goal. Consequently, there are repetitions in some information among the three chapters.

CHAPTER II

LETHAL AND SUB-LETHAL EFFECTS OF THE INSECTICIDE FIPRONIL ON JUVENILE BROWN SHRIMP *FARFANTEPENAEUS AZTECUS**

2.1. Introduction

Chemical pesticides are commonly used world-wide for both agricultural and household purposes to control pests. However, they are known to have negative side effects on non-target organisms, including terrestrial organisms such as birds (Mineau and Palmer, 2013; Goulson, 2014; Hallmann et al., 2014) and insects (Krupke et al., 2012; Whitehorn et al., 2012; Krupke and Long, 2015) as well as aquatic organisms such as fish (Ghisi et al., 2011; Beggel et al., 2012; Clasen et al., 2012) and arthropods (Osterberg et al., 2012; Roessink et al., 2013). A rapid increase in pesticides use in recent years has resulted in enormous pressure on the ecosystems (Tano, 2011; Simon-Delso et al., 2015). Pesticides are expected to have a much greater effect on the aquatic environments compared with terrestrial environments, because water bodies are the eventual recipients of these chemicals (Pritchard, 1993). The adverse effects of chemical pesticides may be lethal (acute) or sub-lethal (chronic), and the effects can vary depend-

* Reprinted with permission from Al-Badran, A. A., Fujiwara, M., Gatlin, D. M., Mora, M. 2018. Lethal and sub-lethal effects of the insecticide fipronil on juvenile brown shrimp *Farfantepenaeus aztecus*. Scientific Reports 8, https://doi.org/10.1038/s41598-018-29104-3, Copyright 2018.

ing on species (Laboy-Nieves et al., 2009). However, the majority of ecotoxicological studies have focused on the investigation of their lethal effects, neglecting sub-lethal effects. These studies also focus on a few selected model organisms, neglecting effects on other non-target organisms, which may play an important role for ecosystem functions and/or are important for commercial purposes (Shaw et al., 2008; Abbott, 2013; Ottinger et al., 2013).

Currently, fipronil is considered one of the most effective phenylpyrazole insecticides, which are used widely, and it is considered to affect arthropods selectively (Hainzl et al., 1998; Chaton et al., 2002). It is used increasingly for the protection of crops such as rice, corn, cotton, potatoes, turnips, and rutabagas from herbivorous insects and for controlling ticks and fleas on animals (Wirth et al., 2004; Mize et al., 2008; Simon-Delso et al., 2015). In particular, the use of fipronil has increased in the U.S.A. in recent years in many different states such as California, Louisiana, South Carolina, and Texas. For example, the U.S. EPA issued a quarantine exemption to the Texas Department of Agriculture in 2016, allowing the expanded use of fipronil in southeastern counties of Texas to control tawny crazy ants *Nylanderia fulva* (Fig. 1) (USEPA, 2016).

Fipronil can flow into creeks, rivers, and estuaries because it is mobile in soils and soluble in water (USEPA, 1996b). Many recent studies have demonstrated the occurrence of fipronil and its degradation products, which have the same or greater toxic properties and are more stable than fipronil itself (Tingle et al., 2003; Wirth et al., 2004; Gunasekara et al., 2007; Stratman et al., 2013), in the aquatic environment at levels ranging between 0.001 - 12.62 µg/L, often exceeding the acute level (0.1 µg/L) of fipr-

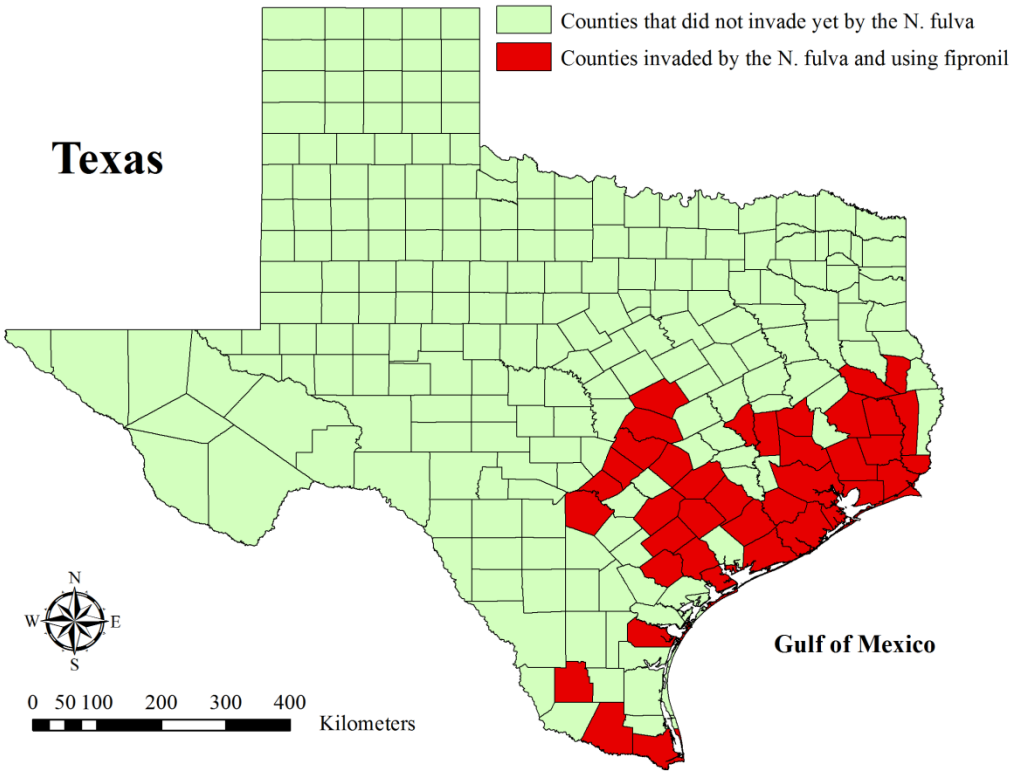


Figure 1. Texas counties map. Red color indicate the counties that have been invaded by the tawny crazy ants *N. fulva* and that are using fipronil based on the exemption issued by the U.S. EPA in 2016 to control the invasion.

onil in the aquatic life benchmark of the U.S. EPA (Mize et al., 2008; Gan et al., 2012; Ensminger et al., 2013; Ruby, 2013; Budd et al., 2015). A nationwide survey from 2002 to 2011 conducted by Stone et al. (2014) found that fipronil concentrations exceeded its chronic aquatic life benchmark concentration (0.01 µg/L) in about 70% of 125 monitored streams sometime during the survey.

Several studies tested the toxicity of fipronil on non-target aquatic crustaceans such as estuarine mysid shrimp *Americamysis bahia* (USEPA, 1996a), Chinese mitten crab *Eriocheir sinensis* and giant river prawn *Macrobranchium rosenbergii* (Shan et al.,

2003), red swamp crayfish *Procambarus clarkia* (Schlenk et al., 2001; Biever et al., 2003), white river crayfish *Procambarus zonangulus* (Schlenk et al., 2001), grass shrimp *Palaemonetes pugio* (Key et al., 2003; Volz et al., 2003), water flea *Daphnia pulex* (Stark and Vargas, 2005), and blue crab *Callinectes sapidus* (Goff et al., 2017). However, the number of species studied is still limited, and most focused on lethal effects.

The aim of this study was to investigate both lethal and sub-lethal effects of fipronil on the brown shrimp *F. aztecus*. Brown shrimp is one of the most important commercial fishery species in the U.S., found along the Atlantic coast of the southeastern United States and in the Gulf of Mexico (GOM) (Ditty, 2011; Montero et al., 2016) with a commercial landing value of $166,542 million in 2016 (NMFS, 2017). They are especially abundant along the coasts of Texas and Louisiana, U.S.A. In addition to their economic importance, brown shrimp play an important ecological role for supporting other species (Sheridan and Ray, 1981; Fujiwara et al., 2016; Montero et al., 2016). They are estuarine-dependent during a juvenile stage (Ditty, 2011; Montero et al., 2016); this potentially exposes them to pesticides that are used on land, because their residues end up in the runoff. The effects of fipronil on penaeid shrimp such as brown shrimp are particularly a concern because of its increased use in coastal communities. In this study, I estimated the effects of fipronil on survivorship, weight gain, inter-molt interval, behavioral changes, and body chemical composition under different nominal concentrations in controlled conditions. The concentrations were selected based on those previously reported for the aquatic environment. I also determined the nominal median

lethal concentration (LC_{50}) and the nominal median lethal time (LT_{50}) of fipronil. These results will fill our knowledge gap in potential effects of fipronil on estuarine crustacean.

2.2. Materials and Methods

2.2.1. Test organisms and acclimation to laboratory conditions

Juvenile brown shrimp *F. aztecus* (weight 0.80 ± 0.06 g, total length 5.0 ± 0.67 cm) were collected from Gangs Bayou, Sportsman Road (N 29.25549; W 94.91575) in Galveston Bay, Texas, using a 3-m bag seine (0.6 cm mesh size) on May 6, 2016. Shrimp were transported in 45-liter coolers equipped with air pumps to the laboratory in Texas A&M University, College Station, Texas. After equilibrating water temperature of the transportation coolers with laboratory temperature over approximately 5 hours, active shrimp were selected and moved to 53-liter plastic tanks filled with aerated artificial brackish water, which was prepared with dechlorinated tap water and Instant Ocean® Sea Salt (Supplementary Figs. B1a and B1b).

Shrimp were acclimated to laboratory conditions in the tanks for 10 days at temperature, 19.93 ± 0.15 °C; salinity, 15.75 ± 0.16‰; pH, 8.14 ± 0.18; and photoperiod, 12 hour: 12 hour light: dark cycle (Supplementary Fig. B1a). During the acclimation period, shrimp were fed on API® Bottom Feeder Shrimp Pellets, which fit the nutritional requirements of shrimp (Lovell, 1998), twice a day. The acclimation tanks were cleaned daily to remove feces and uneaten food and approximately 30-40% of

water was changed with newly prepared brackish water. At the end of the acclimation period, shrimp were moved to test aquariums to begin the experiment.

2.2.2. Experimental design and water quality parameters

The experiment lasted 29 days from May 17, 2016 to June 14, 2016. The system consisted of 18 glass aquariums (six treatments X three replicates) of 9.5 liter (30.7 X 15.4 X 20.5 cm) (Supplementary Fig. B1c), one aquarium was treated as one replicate. An aquarium was filled with 7 liters of test solution, equipped with air pumps (Topfin® AIR-8000), and covered with a glass lid to prevent shrimp from escaping. Each aquarium was divided equally into six cells (Supplementary Fig. B1d), and one individual was assigned to each cell to prevent cannibalism among shrimp and to follow molting of each shrimp individually (USEPA, 2007). The divider was made of a polypropylene plate and fiberglass screen (Supplementary Fig. B4b); both are commonly used for aquaculture purposes. The screen maintained the flow of water, which distributed dissolved oxygen among the cells. Additionally, aquariums were covered from all sides with aluminum foil sheets to minimize the degradation of fipronil due to light exposure during daytime (Supplementary Fig. B1c). The aquariums were placed randomly in three rows. During the experiment, shrimp were fed twice daily. Food amount was adjusted according to the body weight, which was measured weekly, based on the published feeding tables for shrimp (Lovell, 1998). Dissolved oxygen concentration (mg/L), salinity (‰), temperature (°C), and pH were measured every other day using YSI® Professional Plus Multi-parameter Meter.

2.2.3. Insecticide, concentrations and test solutions

Fipronil (5-amino-1-[2, 6-dichloro4-4(trifluoromethyl) phenyl]-4[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile), CAS number 120068-37-3 and purity limit ≥ 97% (HPLC), was purchased from Fisher Scientific Co. L.L.C., PA, US. Six nominal concentrations, including the control, were used for this experiment: 0.0, 0.1, 1.0, 3.0, 6.4, and 10.0 µg/L. These concentrations were selected based on those previously observed by other researchers in the environment (Mize et al., 2008; Gan et al., 2012; Hayasaka et al., 2012b; Ruby, 2013)(Supplementary Table A1). Each treatment (concentration) was conducted in triplicate.

The nominal experimental solutions were prepared by making a 1 liter of highly homogenized 100 mg/L fipronil suspension; this suspension was made by mixing 0.1 g of fipronil powder in 1 liter of artificial brackish water using a magnetic stirrer. Then, all of the nominal experimental concentrations (0.1, 1.0, 3.0, 6.4, and 10.0 µg/L) were prepared by diluting specific quantities of 100 mg/L fipronil suspension with artificial brackish water. For example, to prepare 0.1 µg/L fipronil solution, I took 10 ml of 100 mg/L fipronil suspension and mixed it with 990 ml of prepared water to create 1 mg/L fipronil solution, and then, I took 2.1 ml of 1 mg/L fipronil solution and mixed it with 21 liters of prepared water. Dilutions of all nominal experimental concentrations are shown in Supplementary Table A2. For each nominal concentration, 21 liters of fipronil solution was created for three aquariums (replicates). To maintain the fipronil concentrations under all treatments during the experiment, 100% of test solutions were replaced every two days.

2.2.4. Experimental measurements

All assays were conducted using the static-renewal method and according to the guidelines of the U.S. EPA (USEPA, 2002). The number of shrimp in each replicate and the number of replicates were determined referring to previous studies (Shan et al., 2003; USEPA, 2007).

2.2.4.1. Survival, median lethal time (LT_{50}), and acute toxicity test (LC_{50})

Survivorship of shrimp was measured by monitoring shrimp movements in the aquariums during feeding periods. Dead shrimp were removed, counted, and weighed. The weight of dead shrimp was used to adjust food amounts for remaining live shrimp. Shrimp were considered dead if they lay down on their side or back with no noticeable movement and they did not make any response (such as jumping, moving their legs, or flipping their tails) after taking them out of water. The dead individuals were placed in a freezer for later body chemical composition analysis. I used survivorship data to estimate the median lethal time (LT_{50}) and also the acute toxicity of fipronil (LC_{50}) on shrimp under 96-h of exposure.

2.2.4.2. Weight gain and growth rate

Shrimp were weighed every week to observe the effect of fipronil concentrations as well as to adjust the amount of food. Shrimp were weighed individually after gently removing water with paper towel and placed in a beaker with known amount of brackish water. The weekly weight gain of shrimp was calculated using following equation:

% Weight gain = {(Final weight – Initial weight) / Initial weight} X 100

2.2.4.3. Inter-molt interval

I calculated the inter-molt interval of shrimp under each concentration by counting the number of days between each two consecutive molts of the same individuals. This was possible because I isolated juvenile shrimp in cells (within the same aquarium) and covered the aquarium with a glass lid to prevent the movement of individuals among cells. Then, the date of molting of each individual was recorded.

2.2.4.4. Behavioral and physical changes

At each feeding time (morning and afternoon) and also at night, any abnormalities in shrimp activities as well as any changes in physical appearance compared with shrimp in the control were noted and recorded on video.

2.2.4.5. Analysis of whole-body composition

At the end of the experiments, live shrimp were collected, euthanized by freezing them, and kept in freezer (at -18 °C). Eighteen individuals under each treatment were combined to create two samples. For each sample, dry matter of whole body of shrimp was measured first by accurately weighing 2.0 g of shrimp in a pre-weighed porcelain crucible, placing the samples in an oven at 135 °C for 3 hours (AOAC, 1990), and weighing them again. Then, porcelain lab mortar and pestle were used to prepare a highly homogenized shrimp powder to be used in subsequent analyses. The crude

protein content of shrimp body was determined through Dumas protocol using a LECO protein analyzer to measure total nitrogen as described in (AOAC, 2005). Lipids were estimated using chloroform/methanol 2:1 extraction method (Folch et al., 1957). Ash was determined by placing dry matter samples in a muffle furnace at 550 °C for 3 hours (AOAC, 1990).

2.2.5. Statistical analysis

One-way Analysis of Variance (ANOVA) and linear regression were used to test for the significant differences among all treatments compared to the control. In some measurements such as the survivorship and inter-molt interval of shrimp, the data were not normally distributed, and non- parametric tests were used. Kaplan–Meier estimator was conducted to estimate shrimp survivorship followed by the non-parametric Log-Rank test to compare the survival distribution among treatments. Probit analysis described by Finney (Finney, 1952) was used to calculate the LC_{50}, using log concentration as dependent variable and probit as independent variable, then I used the parametric bootstrap method to calculate the 95% confidence intervals of the LC_{50} toxicity test (Finney, 1952) (Supplementary Fig. B2). Non- parametric Kruskal-Wallis test followed by the pairwise Wilcoxon rank sum test were used to test for differences among the means of treatments of the inter-molt intervals. All of these statistical analyses were conducted at significance level $\alpha = 0.05$ using JMP® Pro 2016 (JMP, 2016) (ANOVA, Kaplan–Meier, and Kruskal-Wallis tests), Matlab R2017a (MATLAB,

2017) (LC_{50} calculations), and Microsoft Excel 2016 (linear regression test, and to draw all figures).

2.3. Results

2.3.1. Water quality

Mean values of water quality parameters were the following: temperature, 20.84 ± 0.24 °C; salinity, 16.20 ± 0.10‰; pH, 8.69 ± 0.15; and dissolved oxygen (DO), 5.67 ± 0.24 mg/L (Table 1). There were no significant differences among treatments for all water quality parameters measured during the experiment, which lasted 29 days, and all of them were within appropriate ranges of the environmental requirements of shrimp (Lassuy, 1983).

Table 1. Water quality parameters of the shrimp aquariums during 29 days of laboratory experiments. * Values of Mean ± standard deviation for each parameter of all concentrations (treatments) of fipronil used during the trials. ** Values of fipronil concentration 10.0 µg/L have no standard deviation due to shrimp mortality during first few days of the trial.

Fipronil concentrations (µg/L)	**Water quality parameters**			
	Temp. °C	**Salinity ‰**	**pH**	**DO mg/L**
Control	21.11 ± 0.32*	16.11 ± 0.40	8.41 ± 0.16	5.43 ± 0.11
0.1	21.0 ± 0.33	16.16 ± 0.44	8.61 ± 0.12	5.43 ± 0.17
1.0	20.98 ± 0.25	16.23 ± 0.38	8.74 ± 0.03	5.60 ± 0.26
3.0	20.7 ± 0.10	16.30 ± 0.04	8.78 ± 0.02	5.72 ± 0.11
6.4	20.45 ± 0.49	16.34 ± 0.24	8.81 ± 0.02	5.85 ± 0.04
10.0**	20.8	16.09	8.81	6.04

2.3.2. Survival, median lethal time (LT_{50}), and acute toxicity test (LC_{50})

My results showed that survival of juvenile shrimp decreased significantly with fipronil concentration, from 0.1 µg/L to 10.0 µg/L (Kaplan-Meier survival curve analysis followed by the non-parametric Log-Rank test, $P < 0.0001$) as shown in Table 2 and Fig. 2. Starting from week 1, significant differences were detected between a control treatment (with survival rate of 100%) and all other fipronil treatments except 0.1 µg/L treatment, which showed a survival rate of 72.2% over the week. After week 1, all treatments were significantly different from the control (Kaplan-Meier survival curve analysis followed by the non-parametric Log-Rank test, $P < 0.0001$).

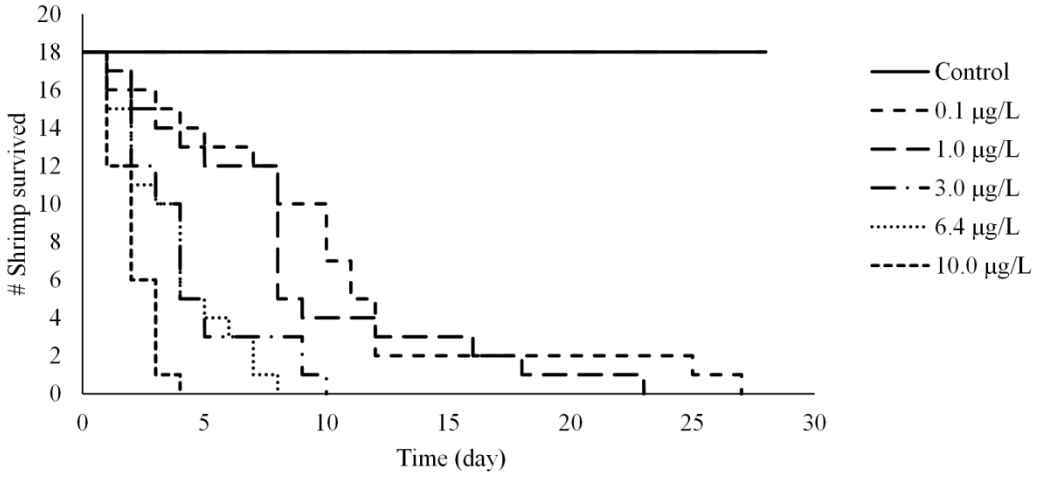


Figure 2. Kaplan-Meier survivorship curves of juvenile shrimp under different concentrations of fipronil during 29 days of exposure. Day 1 is 24-h after the beginning of the experiment. All treatments were significantly different from the control according to the non-parametric Log-Rank test ($P < 0.0001$).

Table 2. Weekly survival rate (mean ± standard deviation) of juvenile shrimp starting from day1 to the end of the experiment. n = number of shrimp individuals in each treatment (6 shrimp per replicate aquarium, 3 aquariums per treatment). LT_{50} is the time required for 50% of shrimp to die after the exposure to fipronil under each treatment, measured per day in this study. Values with star (*) indicate treatment is significantly different from the control ($P < 0.05$).

Fipronil concentrations (µg/L)	**n**	**LT_{50} (day)**	**Survival %**				
			Day 1	**Week 1**	**Week 2**	**Week 3**	**Week 4**
Control	18	/	100	100	100	100	100
0.1	18	6.66 * ± 3.51	100	72.20 ± 25.46	16.63 * ± 16.65	11.10 * ± 19.22	0
1.0	18	6.33 * ± 3.78	100	66.63 * ± 28.86	16.63 * ± 16.65	5.53 * ± 9.58	0
3.0	18	2.66 * ± 1.15	100	16.63 * ± 16.65	0	0	0
6.4	18	3.0 * ± 1.0	100	16.60 *	0	0	0
10.0	18	1.66 * ± 0.57	100	0	0	0	0

Under the higher concentrations of fipronil (6.4 µg/L and 10 µg/L), shrimp showed faster reduction in survivorship where all individuals died by day 8 and day 4, respectively. Under lower fipronil concentrations (0.1 µg/L and 1.0 µg/L), survivorship declined with time at a slower rate and all individuals died by day 28 and 23, respectively. Under all concentrations of fipronil, the survival rate of juvenile shrimp over the duration of the experiment was 0.0% (Kaplan-Meier survival curve analysis followed by the non-parametric Log-Rank test, $P < 0.0001$). In comparison, none of the shrimp died in any replicate under the control treatment (0.0 µg/L).

The median lethal time LT_{50} (the time required for 50% of the animals to die at a particular exposure concentration, and also called median time to death) of juvenile shrimp under fipronil treatments ranged between 1.66 ± 0.57 day in the 10.0 µg/L treatment to 6.66 ± 3.51 day in the 0.1 µg/L treatment. One-way Analysis of Variance ANOVA ($P < 0.05$) showed that all treatments were significantly different from the control which showed no mortality among the shrimp during the experiment (Table 2). Fipronil 96-h LC_{50} (lethal concentration to reach 50% mortality within 96 hours) of juvenile shrimp was 1.3 µg/L with the 95% confidence interval ranging from (1.0 to 1.5). Table 3 compares the results from this study with those from previous studies obtained by others.

Table 3. Median lethal concentration (LC_{50}) for *F. aztecus* and other estuarine and freshwater arthropods (Crustacean species) exposed to fipronil for 96-h in toxicity tests. Values with star (*) indicate LC_{50} for 48-h exposure. Table was modified from (Chandler et al., 2004).

Species	**Common name**	**Habitat**	**LC_{50} (µg/L)**	**Reference**
Americamysis bahia	Mysid shrimp	Estuarine	0.14	(USEPA, 1996a)
Palaemonetes pugio	Grass shrimp	Estuarine / Marine	0.32	(Overmyer et al., 2007)
Palaemonetes pugio	Grass shrimp	Estuarine / Marine	0.32 (adult) 0.68 (larvae)	(Key et al., 2003)
Macrobrachium rosenbergii	Giant river prawn	Brackish water / Freshwater	0.98	(Shan et al., 2003)
Farfantepenaeus aztecus	**Brown shrimp**	**Estuarine / Marine**	**1.3**	**Current study**
Macrobrachium nipponensis	Oriental river shrimp	Freshwater	4.32	(Shan et al., 2003)
Amphiascus tenuiremis	Copepod	Estuarine / Marine	6.8	(Chandler et al., 2004)
Diaptomus castor	Copepod	Freshwater	7.9 *	(Chaton et al., 2002)
Eriocheir sinensis	Chinese mitten crab	Estuarine / Freshwater	8.56	(Shan et al., 2003)
Procambarus clarkii	Red swamp crayfish	Freshwater	14.3	(Schlenk et al., 2001)
Ceriodaphnia dubia	Water flea	Freshwater	17.7 *	(Konwick et al., 2005)
Procambarus zonangulus	White river crayfish	Freshwater	19.5	(Schlenk et al., 2001)
Procambarus clarkii	Red swamp crayfish	Freshwater	163.5	(Overmyer et al., 2007)
Daphnia magna	Water flea	Freshwater	190.0*	(USEPA, 1996a)
Acanthocyclops robustus	Copepod	Freshwater	194.2*	(Chaton et al., 2002)

2.3.3. Weight gain and growth rate

At the beginning of the experiment, there was no significant difference in the initial weight among the treatments; initial weight of shrimp ranged between 0.78 ± 0.08 g in the 1.0 µg/L treatment and 0.82 ± 0.08 g in the 6.4 µg/L treatment (Table 4).

The final weight in Table 4 was calculated for each treatment by taking the final weight measured before the death of all shrimp. However, the week of death of the last shrimp was different among the treatments. For example, the final weights of the 0.1 µg/L and 1.0 µg/L treatments were measured at the end of week 3; whereas, final weights of 3.0 µg/L and 6.4 µg/L treatments were measured at the end of week 1 of fipronil exposure because all shrimp in these treatments died before reaching week 2. However, final weight and percent weight gain clearly showed the effect of fipronil. In all treatments, a significant reduction in growth was observed after the first week of fipronil exposure (ANOVA, $P < 0.05$) (Table 4 and Fig. 3).

The percent weight gain showed significant differences among all treatments (ranging from -21.42 g in the 1.0 µg/L treatment to 2.77 ± 19.64 g in the 3.0 µg/L treatment) and the control (60.19 ± 15.44 g) (ANOVA, $P < 0.05$). Weight loss occurred (- 8.97, -21.42, and -16.87 ± 29.57 g) in three concentrations (0.1, 1.0, and 6.4 µg/L, respectively), indicating the final weight was less than the initial weight under all of these treatments. Final weight and percent weight gain in the 10.0 µg/L treatment were not measured because all individuals died during the first days of exposure before measuring the weight in week 1 (Table 4).

Table 4. Initial weight (g), final weight (g), and % weight gain (mean ± standard deviation) of juvenile shrimp exposed to different concentrations of fipronil. n = number of replicates in each treatment. All values were calculated based on the wet weight per individual shrimp. Means in columns not sharing the same letter are significantly different ($P < 0.05$).

Fipronil concentrations (µg/L)	**Initial weight (g)**	**Final weight (g)**	**Week of final weight measurement**	**% Weight gain**
Control	0.80 ± 0.08 (n = 3) **a**	1.28 ± 0.08 (n = 3) **a**	4	60.19 ± 15.44 (n=3) **a**
0.1	0.79 ± 0.05 (n = 3) **a**	0.71 (n = 1) **b**	3	- 8.97 (n=1) **b**
1.0	0.78 ± 0.08 (n = 3) **a**	0.55 (n = 1) **b**	3	-21.42 (n=1) **b**
3.0	0.82 ± 0.09(n = 3) **a**	0.81 ± 0.24 (n = 2) **b**	1	2.77 ± 19.64 (n=2) **b**
6.4	0.82 ± 0.08 (n = 3) **a**	0.66 ± 0.17 (n = 3) **b**	1	-16.87 ± 29.57 (n=3) **b**
10.0	0.81 ± 0.06(n = 3) **a**			

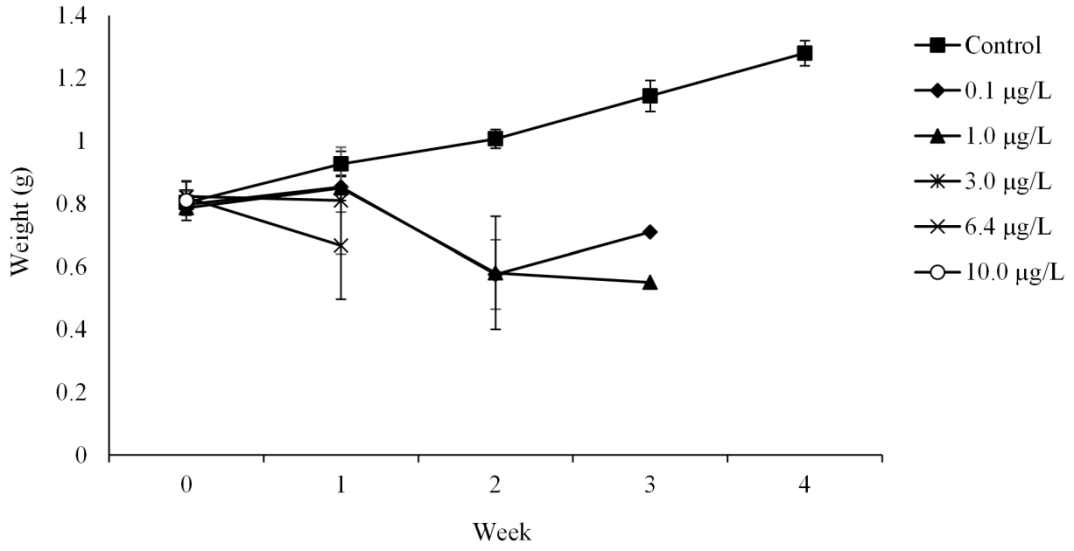


Figure 3. Average weight (g wet weight per individual) of juvenile shrimp as a function of the duration of the exposure to fipronil. The horizontal axis represents the experiment period per week (week 0 - week 4) while the vertical axis represents the average wet weight (g) per individual shrimp in each treatment. Error bars indicate the standard errors (n = 18). One-way Analysis of Variance ($P < 0.05$) showed that, after one week of fipronil exposure all treatments were significantly different from the control.

2.3.4. Inter-molt interval

Control shrimps showed the highest number of molts per individual (m = 2.6); whereas, the number of molts decreased progressively with fipronil concentration. Inter-molt interval of the control treatment (12.2 ± 1.64 day) was significantly shorter than 0.1 µg/L treatment (14.0 ± 0.85 day) and the 1.0 µg/L treatment (15.5 ± 0.53 day) according to the Kruskal-Wallis rank sum test ($P < 0.0001$) (Fig. 4).

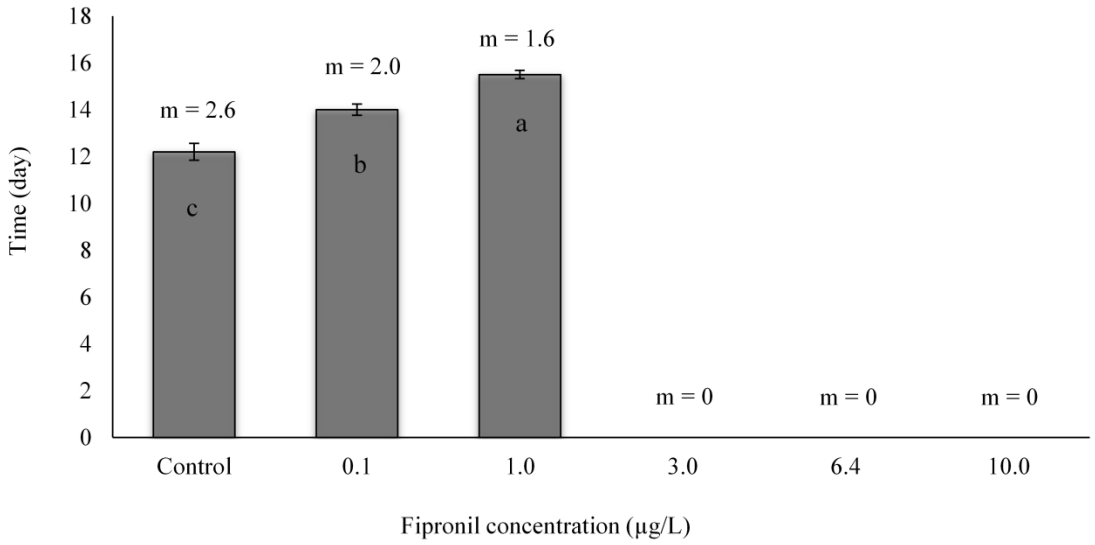


Figure 4. Inter-molt intervals of juvenile shrimp exposed to different concentrations of fipronil for 29 days. The horizontal axis represents the six fipronil concentrations (µg/L) including the control used during the experiment while the vertical axis represents time (per day) of the inter-molt intervals of juvenile shrimp. Error bars indicate the standard errors (n = 18). m is the average number of molts of individual shrimp in each treatment. Non- parametric Kruskal-Wallis test followed by the pairwise Wilcoxon rank sum test ($P < 0.0001$) showed that means in columns not sharing the same letter were significantly different.

I could not calculate the inter-molt intervals for shrimp in treatments of higher fipronil concentrations (3.0, 6.4, and 10.0 µg/L) because they died before they have two consecutive molts of any individual during the experiment.

2.3.5. Behavioral and physical changes

Shrimp under high fipronil concentrations (3.0, 6.4, and 10.0 µg/L) showed behavioral changes after only one day. These changes were observed in the following order: (1) shrimp in these treatments started moving in circles with no control on their

movements; (2) shrimp stopped moving in circles and sprawled on their sides or backs on the bottom of the aquarium with only their swimming legs moving in continuous involuntary movements; and (3) shrimp stopped moving their swimming legs and died. All of these abnormal swimming and feeding behaviors were recorded to compare them with shrimp in the control treatment. It is important to note that during all of these stages of abnormal behaviors, shrimp were not able to feed effectively. This was clearly observable under low fipronil concentrations (0.1 and 1.0 µg/L) in which shrimp survived for a longer period and exhibited the behavioral changes progressively and slowly.

The visual examinations of the physical changes in shrimp bodies at the end of the experiment indicated a clear difference in their body color. Fig. 5 shows shrimp body color gradient from bright color of shrimp in the control (0.0 µg/L) to gray and dark color of shrimp in the 0.1 µg/L and 1.0 µg/L treatments. This result indicates that fipronil affected shrimp body color in a concentration-dependent manner.

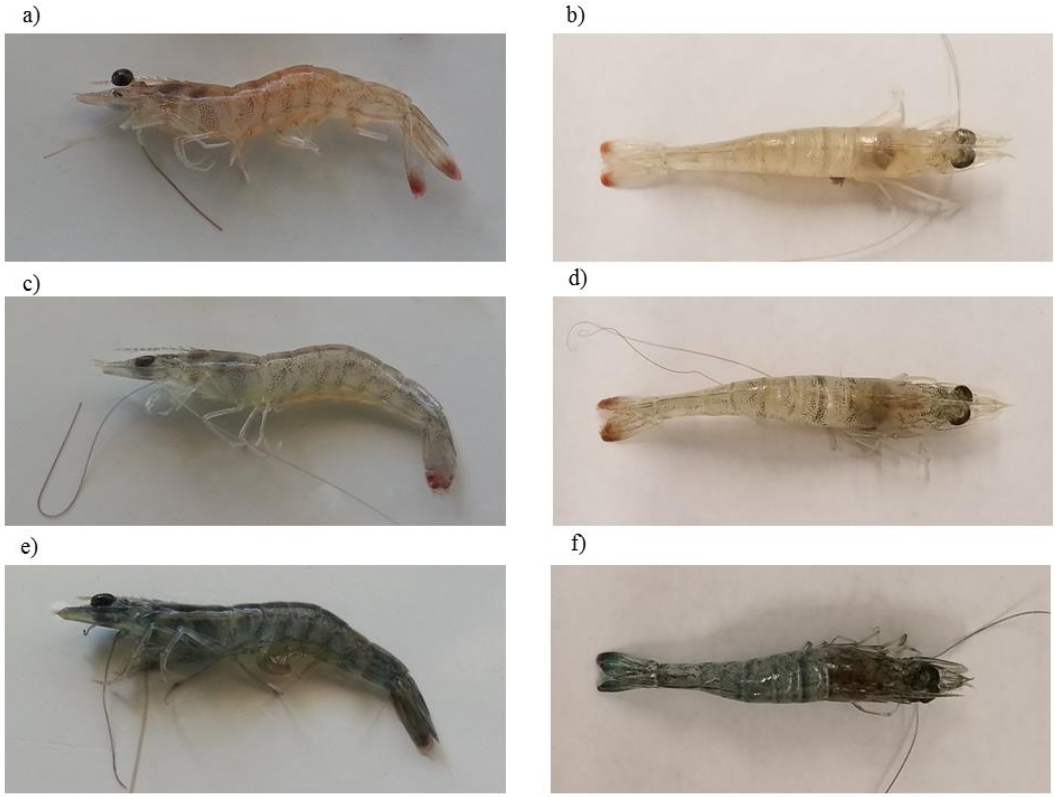


Figure 5. Body color of juvenile shrimp exposed to different concentrations of fipronil. Controls (a, b), 0.1 µg/L treatment (c, d), 1.0 µg/L treatment (e, f).

2.3.6. Analysis of whole-body composition

Fig. 6 shows the analysis of protein, lipid, and ash composition (dry basis) of juvenile shrimp in all treatments. My results revealed that there were some significant differences (ANOVA, $P < 0.05$) among treatments in all components analyzed. There was an overall significant decrease (ANOVA, $P < 0.05$) in the percentage of body protein for all treatments compared to the control (0.0 µg/L) which showed the highest level of protein 71.69 ± 0.23%, although there was not a clear trend (Fig. 6a).

The ANOVA ($P < 0.05$) of lipid percentage indicated that there was no difference among treatments with the five lower concentrations, including the control. Similarly, there was no difference among treatments (ANOVA, $P < 0.05$) with the four higher concentrations (Fig. 6b). However, a linear regression analysis ($P = 0.0017$) indicated that lipid percentage increased significantly with increasing concentration of fipronil (Supplementary Fig. B3).

For ash percentage, my analysis showed that the differences among groups in this case appear to be random and not associated with the insecticide exposure; although, control treatment had the lowest ash percentage (15.78 ± 0.53%) and differed significantly (ANOVA, $P < 0.05$) from most of the treatments (Fig. 6c).

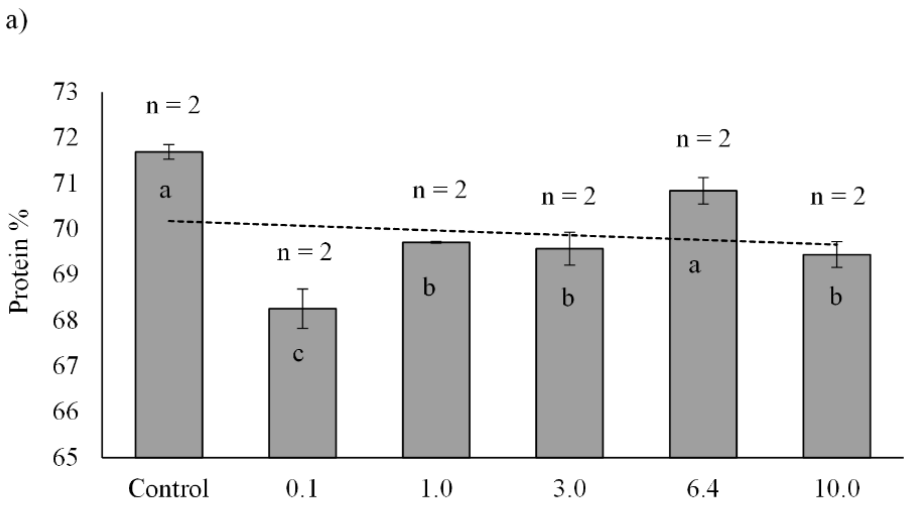
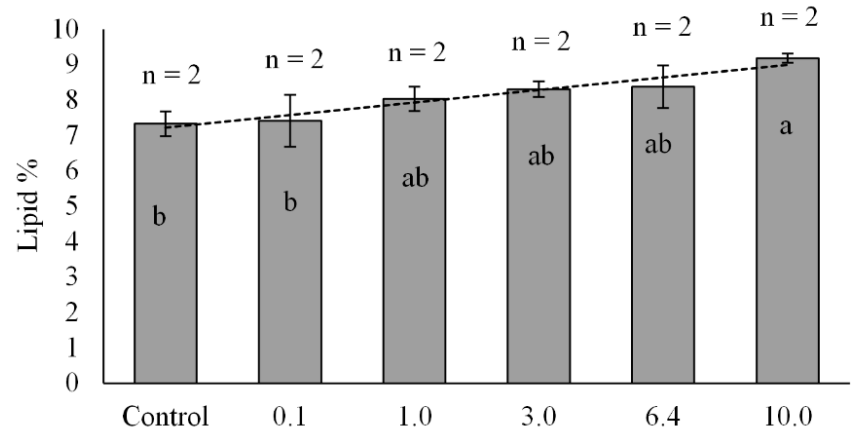


Figure 6. Analysis of body composition of juvenile shrimp under different fipronil concentrations. The horizontal axes in (6.a, 6.b, and 6.c) represent the six fipronil concentrations (µg/L) used during the experiment while the vertical axes represent the protein % (in 6.a), lipid % (in 6.b), and ash % (in 6.c) in bodies of juvenile shrimp measured at the end of the experiment. Dashed lines are fitted regression lines. Error bars indicate the standard errors (n = number of samples analyzed from each treatment). One-way Analysis of Variance ($P < 0.05$) showed that means in columns not sharing the same letter are significantly different.

b)



c)

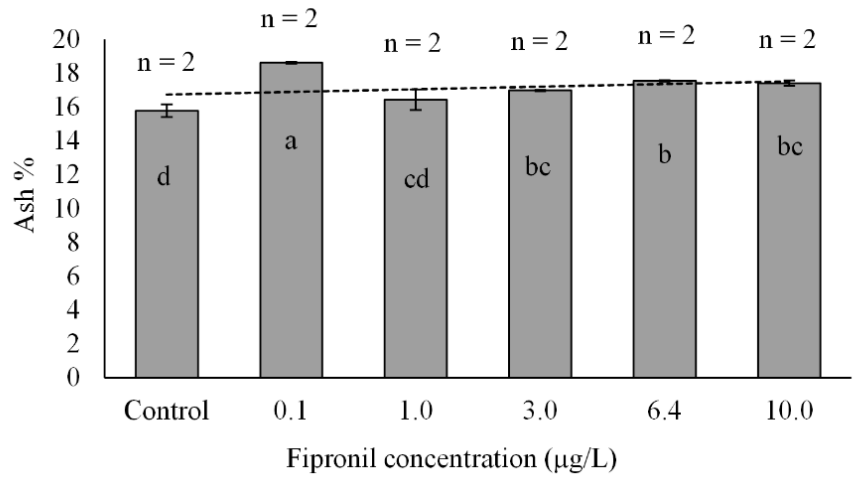


Figure 6 Continued.

2.4. Discussion

Fipronil is known to cause lethal and sub-lethal effects on non-target invertebrates in both aquatic and terrestrial ecosystems (Pisa et al., 2015). However, studies are often conducted with a limited number of model organisms. Consequently, there is a gap of knowledge in the effects on a large number of non-target invertebrates, especially from coastal and marine ecosystems (CCME, 2007). Fipronil desulfinyl (a photodegradation product of fipronil) was detected in the eggs of the Atlantic blue crab *Callinectes sapidus* off the coast of South Carolina (the Eastern coast of the United States), and it may be one of the causes of *C. sapidus* decline to the lowest historical levels over the past decade (Goff et al., 2017). In Texas, fipronil has been reported in several recent studies conducted by the U.S. Geological Survey (USGS) and U.S. EPA in different cities including Houston-Galveston (Sneck-Fahrer and East, 2007), Austin (Mahler et al., 2009), San Antonio (Opsahl, 2012), and College Station (Mosier, 2005), in concentrations ranging between 0.021 µg/L and 0.075 µg/L. All of these studies have reported the detection of fipronil and two or more of its degradation products (i.e., fipronil sulfide, fipronil sulfone, desulfinylfipronil, and desulfinylfipronil amide) in surface water and urban streams in levels exceeding the chronic level of the U.S. EPA aquatic life benchmark for invertebrates (0.01 µg/L). To the best of my knowledge, this study is the first to report the effects of fipronil on commercially and ecologically important penaeid shrimp *F. aztecus*.

All nominal fipronil concentrations tested in this study were within the range of concentrations found in the environment by other researchers in streams, rivers, and

estuaries in the U.S. and other countries (USGS, 2003; Mize et al., 2008; Hayasaka et al., 2012b; Ruby, 2013) (Supplementary Table A1). My results showed that fipronil caused significant lethal and sub-lethal effects on juvenile *F. aztecus*. Results also showed that survival of shrimp was concentration-dependent (Table 2 and Fig. 2). All individuals died during the 29 days of exposure under all the fipronil concentrations tested; whereas, no individual died in the control. The nominal 96-h LC_{50} of fipronil for juvenile *F. aztecus* was estimated at 1.3 μg/L. This result suggests *F. aztecus* have an intermediate sensitivity to fipronil among marine invertebrates, but they are far more sensitive than freshwater invertebrates studied so far (Table 3).

In my study, final weight and percent weight gain of shrimp showed significant differences ($P < 0.05$) between the control and all other concentrations (Table 4 and Fig. 3). Growth reduction of aquatic arthropods under the exposures to toxicants also has been reported in other studies with sand shrimp *Metapenaeus ensis* (Wong et al., 1995) and freshwater crayfish *Cherax quadricarinatus* (Frontera et al., 2011). A similar reduction in body growth of *F. aztecus* has been documented by Rozas et al. (2014), who found the reduction in the growth of juvenile *F. aztecus* and white shrimp *L. setiferus* held for 7 days in field mesocosms contaminated with the non-lethal concentrations of petroleum hydrocarbons from an oil spill. On the contrary, Goff et al. (2017) found that juvenile blue crabs *C. sapidus* exposed to different nominal concentrations of fipronil and fipronil desulfinyl resulted in significant increases in growth in all treatments compared to controls in a short-term (96-h) experiment.

There are several reasons that may explain the decrease in the growth of juvenile *F. aztecus* in my study. For instance, animals affected by environmental stressors, such as the chemical toxicants, utilize the energy in the detoxification processes, thus affecting the metabolism of protein and carbohydrate and eventually growth performance (Frontera et al., 2011). Shrimp derive energy more expeditiously from protein than from lipids and carbohydrate (Gauquelin et al., 2007); therefore, exposing *F. aztecus* to fipronil may have resulted in a reduced protein level in exposed shrimp compared with those in the control (Fig. 6a), which might have, in turn, reduced growth (Fig. 3). On the other hand, fipronil is a phenylpyrazole insecticide, which acts by blocking the chloride channels, disrupting the central nervous system activity (Simon-Delso et al., 2015), which may have inhibited feeding activity of juvenile *F. aztecus* under exposure.

Molting is one of the important physiological processes for arthropods allowing them to grow normally (Lachaise et al., 1993; Mensah et al., 2012a). Because molting in crustaceans is mainly controlled by the interaction of molt-stimulating hormones (ecdysteroids), molt-inhibiting hormones (produced in the eyestalks), and nervous system secretions, endocrine disrupting chemicals, including fipronil (Volz et al., 2003) in my study, are expected to have adverse effects on molting (OECD, 2005). In this study, fipronil affected *F. aztecus* molting process in a concentration-dependent manner. Inter-molt intervals of shrimp under the control (12.2 ± 1.64 day) were significantly shorter ($P < 0.0001$) than those in other fipronil treatments (Fig. 4). Increased inter-molt

intervals suggest the development of shrimp is delayed by exposure to sub-lethal levels of fipronil in water.

Similar delay in molting has been reported with other arthropods exposed to pesticides. Betancourt-Lozano et al. (2006) showed significant increase in inter-molt intervals of juvenile Pacific white shrimp *Litopenaeus vannamei* under the exposure to Tilt (a commercial formulation of the fungicide propiconazole). Snyder and Mulder (2001) showed delayed molting of American lobster *Homarus americanus* larvae exposed to cyclodiene pesticide heptachlor. Baldwin et al. (1995) reported that juveniles and adults of the freshwater crustacean *D. magna* exhibited reduced molting frequency after they were chronically exposed to diethylstilbestrol (DES). Moreover, there are also reports of reduced molting intervals, for example, with freshwater shrimp *Caridina nilotica* under exposure to the herbicide Roundup® (Mensah et al., 2012a) and grass shrimp *P. pugio* under exposure to sodium pentachlorophenate and Aroclor 1242 (Fingerman et al., 1998). These studies suggest potentially complex mechanisms of pesticides affecting the molting of arthropods.

Behavioral changes are often the first indication of the harmful impacts of pesticides on living organisms, and even at low doses of pesticides, long-term behavioral changes can be observed. This effect is magnified especially if the pesticide exposure occurred during the developmentally critical periods of the organism's life (Raley-Susman, 2014). In the present study, behavioral changes were observed under all fipronil concentrations compared with those under the control, starting from day one in the high concentration treatments and later in lower concentration treatments. Change in

swimming (mobility) and feeding activities were the main observed changes. Similar results have been reported by other researchers. For example, Stratman et al. (2013) showed that the chironomid midge *Cricotopusle betis* Sublette exposed to fipronil exhibited abnormal behaviors, movement restriction, and feeding reduction. Overmyer et al. (2005) observed abnormal behavior and muscle control in the aquatic insect *Simulium vittatum* under all fipronil concentrations tested in the study.

Color changes were clearly observed in both the exoskeleton and abdominal muscle (Fig. 5). Because the body color of shrimp under 1.0 µg/L fipronil (Fig. 5e, f) were darker than those under 0.1 µg/L fipronil (Fig. 5c, d), which were, in turn, darker than that in the control (Fig. 5a, b), I concluded that the effect of fipronil on the color of juvenile *F. aztecus* was concentration-dependent. In crustaceans, and especially shrimp, many environmental factors are known to affect body color by affecting pigment dispersion (movement) within the chromatophores (O'Halloran, 1990). However, I note that the factors that are known to have an effect on body color of shrimp, such as temperature, light intensity, and background color, were carefully controlled in my study (Table 1). Body color in shrimp is often considered a sign of shrimp health, and consequently, influencing its commercial value (Martinez et al., 2014); for the same reason, color change in crustaceans, which is a hormonally-regulated process, can be used as a biomarker of environmental health (Fingerman et al., 1998).

Some changes in body chemical compositions were observed under the exposure to fipronil in this study. A linear regression analysis showed a significant increase ($P = 0.0017$) in lipid percentage with fipronil concentration (Supplementary Fig. B3). Similar

results were found with juvenile mud crab *Rhithropanopeus harrisii* exposed to the insecticide fenoxycarb (Nates and McKenney Jr, 2000), Pacific white shrimp *L. vannamei* exposed to oxytetracycline (OTC) (Bray et al., 2006), and freshwater crayfish *C. quadricarinatus* exposed to glyphosate acid and polyoxyethylenamine (POEA) (Frontera et al., 2011), and freshwater amphipod *Gammarus pulex* exposed to the insecticide imidacloprid (Nyman et al., 2013). Protein percentage may also have been affected by fipronil (Fig. 6a). Although the protein percentage under the control (71.69 ± 0.23%) did not differ with those in the 6.4 µg/L treatment (70.84 ± 0.41%), it may be because of the fact that those in higher concentrations died early in the first days of the experiment, and they did not have enough time to exhibit a measurable reduction in protein percentage. Both protein and lipid metabolism are potentially affected by detoxification process (Frontera et al., 2011). If so, I would expect the effects to be concentration-dependent. However, they are also affected by the duration of exposure and feeding rate, which are also affected by toxicants. Further studies are needed for determining the existence of effects of fipronil on body chemical composition as well as potential mechanisms.

CHAPTER III

EFFECTS OF INSECTICIDES, FIPRONIL AND IMIDACLOPRID, ON THE GROWTH, SURVIVAL, AND BEHAVIOR OF BROWN SHRIMP *FARFANTEPENAEUS AZTECUS*

3.1. Introduction

Chemical pesticides have become critically important for the sustainability of commercial agricultural production, as well as improving the quality of our daily lives. The use of pesticides to protect crops against pests is indispensable to assure both quality and productivity of agriculture (Oerke and Dehne, 2004; Hayasaka et al., 2012b) and to control household pests such as termites, fire ants (such as *Solenopsis invicta*), and mosquitoes (Elliott and Barnes, 1963; Aktar et al., 2009; Drees, 2014). However, pesticides also have negative effects on non-target organisms, whether they are terrestrial or aquatic organisms (Clasen et al., 2012; Goulson, 2013; Roessink et al., 2013; Krupke and Long, 2015), that may be considered beneficial organisms. Because water bodies are the eventual recipients of chemical pesticides, it is expected that these toxicants pose a much greater adverse effects in aquatic environments compared with terrestrial environments (Pritchard, 1993).

Although these negative effects could be acute (lethal) or chronic (sub-lethal) and vary depending on the sensitivity of species (Laboy-Nieves et al., 2009; Hayasaka et al., 2012a), the majority of ecotoxicological studies neglect their sub-lethal effects (Shaw et al., 2008; Abbott, 2013) and instead focus on selected model species, such as water flea

D. magna and zebrafish *Danio rerio* (Hayasaka et al., 2012b; Dai et al., 2014). Here, I present the results of experimental studies on the effects of fipronil (5-amino-1-[2, 6-dichloro4-4(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*, one of the most commercially prominent marine species in the U.S.

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 (Ditty, 2011; Montero et al., 2016). Its commercial landing value was estimated at $166,542 million in 2016 (NMFS, 2017). In addition to its economic importance, brown shrimp also has an essential ecological role as prey for many important fishery species in the region (Sheridan and Ray, 1981; Fujiwara et al., 2016; Montero et al., 2016). In particular, these shrimp are abundant along the coasts of Texas and Louisiana, U.S.A. and inhabit the estuarine areas during their juvenile stage as part of their annual life cycle (Ditty, 2011; Montero et al., 2016). However, because of the increased use of fipronil and imidacloprid in coastal communities, as well as the detection of pesticides residues in estuaries water (Chandler et al., 2004; Hala, personal communication, May 2019), the effects of these insecticides on penaeid shrimp specifically brown shrimp are a particularly serious concern.

Phenylpyrazoles (including fipronil) and neonicotinoids (including imidacloprid) are widely used insecticides with large-scale applications, such as protecting plants from

agricultural pests, controlling household pests, and controlling parasites on domesticated animals (Pisa et al., 2015). Presently, they account for approximately one third of the world insecticide market (Simon-Delso et al., 2015). Although both fipronil and imidacloprid operate by disrupting neural transmission in the central nervous system of invertebrates (Simon-Delso et al., 2015), 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) (Gunasekara et al., 2007; Mortensen et al., 2015). Compared to other types of insecticides, fipronil and imidacloprid are considered safer because of their low toxicity on mammals. Fipronil and imidacloprid are very effective on insects (arthropods) in small concentrations (Overmyer et al., 2005). However, their increased use in recent years (Overmyer et al., 2005; Pisa et al., 2015; Simon-Delso et al., 2015), moderate to high solubility (USEPA, 1996b; Raby et al., 2018), and persistence in water (Tisler et al., 2009; Van Dijk et al., 2013; McMahen et al., 2016) pose a serious concern regarding the potential adverse impacts on non-target organisms, especially on aquatic invertebrates.

Fipronil has been reported in the U.S. and other parts of the world, and its environmental concentrations can be as high as 12.62 μg/L (Mize et al., 2008; Gan et al., 2012; Hayasaka et al., 2012a; Ensminger et al., 2013; Ruby, 2013). Among the sites where imidacloprid are actively used, the environmental concentrations of imidacloprid have ranged between 0.016 μg/L and 320.0 μg/L (Jemec et al., 2007; Hayasaka et al., 2012a; Van Dijk et al., 2013; Main et al., 2014; Hook et al., 2018). Many of these

studies reported the detection of fipronil and imidacloprid, or one or more of their degradation products, in aquatic environments in levels exceeding their chronic levels of the U.S. EPA aquatic life benchmark for invertebrates of 0.01 μg/L for both insecticides (USEPA, 2017). 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. In particular, Pisa et al. (2015) 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.

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 observed concentrations in the aquatic environment using multiple endpoints such as growth (weight and length), molting, survival, and behavioral change. This study was the first to investigate the effects of imidacloprid on brown shrimp. The previous experiments investigating the effects of fipronil on brown shrimp (Al-Badran et al., 2018) was done only at higher concentrations. The results of this study will fill the existing knowledge gap regarding the adverse effects of fipronil and imidacloprid insecticides on juvenile penaeid shrimp in estuaries.

3.2. Materials and Methods

3.2.1. Collection of brown shrimp

Juvenile brown shrimp were collected on June 12, 2017 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 equilibration, the shrimp were moved to larger plastic tanks of 53-liter filled with artificial brackish water made using Instant Ocean® Sea Salt and de-chlorinated tap water.

3.2.2. 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 meet the nutritional requirements of shrimp (Lovell, 1998) 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 a 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. I maintained a 12-h: 12-h light: dark cycle by controlling lights with a time. After the acclimation period, shrimp were moved to aquariums used for the experiments.

3.2.3. Fipronil and imidacloprid experimental solutions

Both of fipronil, purity limit ≥ 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 by other researchers (Table 5), five concentrations of fipronil (0.005, 0.01, 0.1, 1.0, and 3.0 µg/L) and five concentrations of imidacloprid (0.5, 1.0, 15.0, 34.5, 320.0 µg/L) were selected and three replicates were used for each concentration (treatment).

For each experiment, test solutions of the five nominal concentrations were prepared by a series of dilutions, beginning with mixing a specific amounts 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 Supplementary Tables A3 and A4. I prepared 21 liters of test solutions for each concentration used in both experiments to fill three aquariums (replicates) of 7 liters each. Although the hydrolysis half-life of these compounds at 25ºC is much greater than 48 h, fipronil > 100 days (Gunasekara et al., 2007) and imidacloprid > 30 days (Kollman and Randall, 1995), 100% of test

solutions were changed every 2 days to maintain the concentrations relatively constant during the experiment.

Table 5. Fipronil and imidacloprid concentrations observed in the aquatic environment and reported in previous studies.

Fipronil		Imidacloprid	
Detected concentrations (µg/L)	**Reference**	**Detected concentrations (µg/L)**	**Reference**
0.0006 - 0.0086	(Harman-Fetcho et al., 2005)	0.016	(Main et al., 2014)
0.0145 - 0.0274	(Weston et al., 2015)	0 - 0.22	(Lamers et al., 2011)
0.3 – 0.8	(Chandler et al., 2004)	0.2 – 0.42	(Hook et al., 2018)
1.0	(Hayasaka et al., 2012a)	0 - 3.3	(Starner and Goh, 2012)
0.28 - 2.11	(Ensminger et al., 2013)	1.0 - 14.0	(Jemec et al., 2007)
0.01 - 4.2	(Greenberg et al., 2010)	17.0 - 36.0	(Fossen, 2006)
3.00 - 4.54	(USGS, 2003)	49.0	(Hayasaka et al., 2012a)
0.15 - 5.0	(Wirth et al., 2004)	320.0	(Van Dijk et al., 2013)
0.007 - 6.0	(Gilliom et al., 2006)		
0.004 - 6.41	(Mize et al., 2008)		
0.09 - 10.004	(Ruby, 2013)		
0.0018 – 12.62	(Gan et al., 2012)		

3.2.4. Aquariums and experimental system

Experimental systems were designed in the same way for both fipronil and imidacloprid experiments but using new aquariums and other supplies in both experiments. Each system contained 18 glass aquariums of 9.5 liter (30.7 X 15.4 X 20.5

cm), and each aquarium was considered as one replicate (Supplementary Fig. B1c). In order to keep track of shrimp molts 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 (Supplementary Fig. B1d). A screen was placed on each divider to allow water to flow and dissolved oxygen to be distributed evenly among the cells (Supplementary Fig. B4b). 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 jumping out of the water. All sides of the aquariums were also covered with aluminum foil to reduce the degradation of the insecticides from exposure to light (Supplementary Fig. B1c). 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.

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, and 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 (Lovell, 1998). 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.

3.2.5. Measurements

I conducted all experimental measurements according to the U.S. EPA guidelines (USEPA, 2002) using the static-renewal method, with the test solutions being replaced periodically during the experiments. The sample size was determined based on previous studies (Shan et al., 2003; USEPA, 2007).

3.2.5.1 Weight gain and total length

I 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, I was able to monitor the effect of insecticide on growth and to gain information for adjusting the amount of food provided. Weight gain of shrimp was calculated using the following equation:

% Weight gain of shrimp = {(Final weight – Initial weight) / Initial weight} X 100

I 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 measuring the total length from the tip of the head to the end of the tail as shown in Supplementary Fig. B4c.

3.2.5.2. Molting

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

3.2.5.3. Shrimp survival and LC_{50}

Shrimp movements were monitored many 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 positions 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 96-h acute toxicity levels (LC_{50}) of fipronil.

3.2.5.4. 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.

3.2.6. Statistical analysis

Non-parametric statistics were used for some measurements when normal distribution of data was not achieved. For example, I used Kaplan–Meier estimator to measure the survivorship function of shrimp and non-parametric Log-Rank test to compare the survivorship distribution among treatments. I also used the non-parametric Kruskal-Wallis test followed by the pairwise Wilcoxon rank sum test to compare molting intervals. LC_{50} value of fipronil was estimated using logistic regression by fitting a generalized linear model to the proportion of dead individuals against the pesticide concentration with a logit link and binomial distribution using JMP. For all other measurements, I 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 (JMP, 2016) was used to calculate the LC_{50} 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.

3.3. Results

3.3.1. 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 µg/L treatment and 0.59 ± 0.03 g in the 0.005 µg/L treatment (ANOVA, P = 0.973). Fipronil had a significant effect on the growth of

shrimp during the experiment. Fipronil affected the percent weight gain of shrimp, and significant differences were observed between the control (125.9 ± 28.42%) and the 0.1 µg/L treatment (77.1 ± 21.83% weight gain) (ANOVA, P <0.0001); whereas, there was no significant difference between lower fipronil concentrations (0.005 µg/L and 0.01 µg/L) and the control. The percent weight gain under the latter two concentrations was 120.17 ± 15.16% and 104.18 ± 28.62%, respectively. I could not calculate the percent weight gain under the 1.0 µg/L and 3.0 µg/L treatments because all shrimp died during the first days in these treatments (Supplementary Table A5 and Fig. 7a).

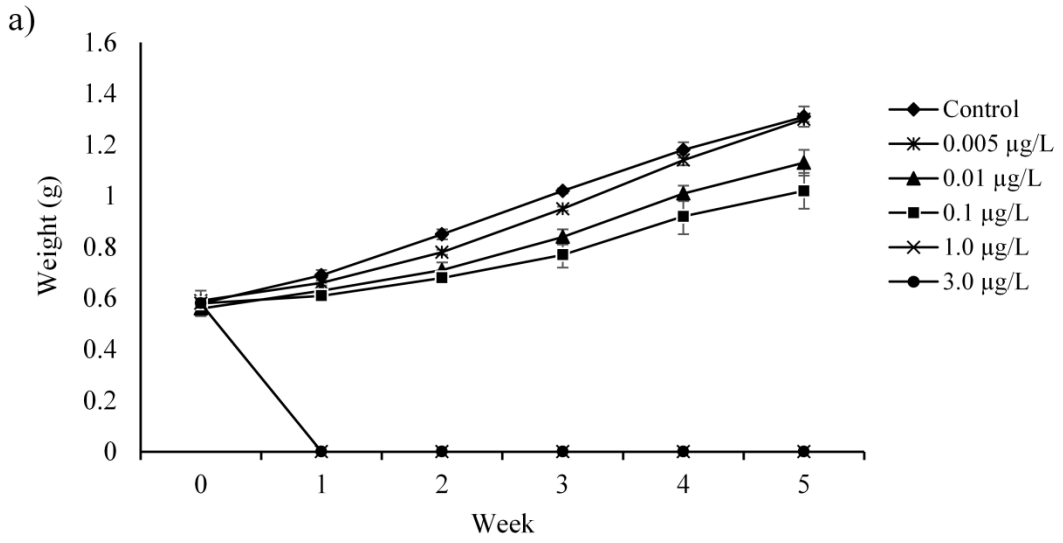


Figure 7. Wet weight of individual juvenile shrimp (g) during 5 weeks of the experiments. The vertical axis is the wet weight (g) of individual shrimp in each treatment, and the horizontal axis is the week from the beginning of experiment. Error bars indicate the standard errors (n = 18 in fipronil exp. and n = 15 in imidacloprid exp.). (a) Fipronil experiment, (b) Imidacloprid experiment.

b)

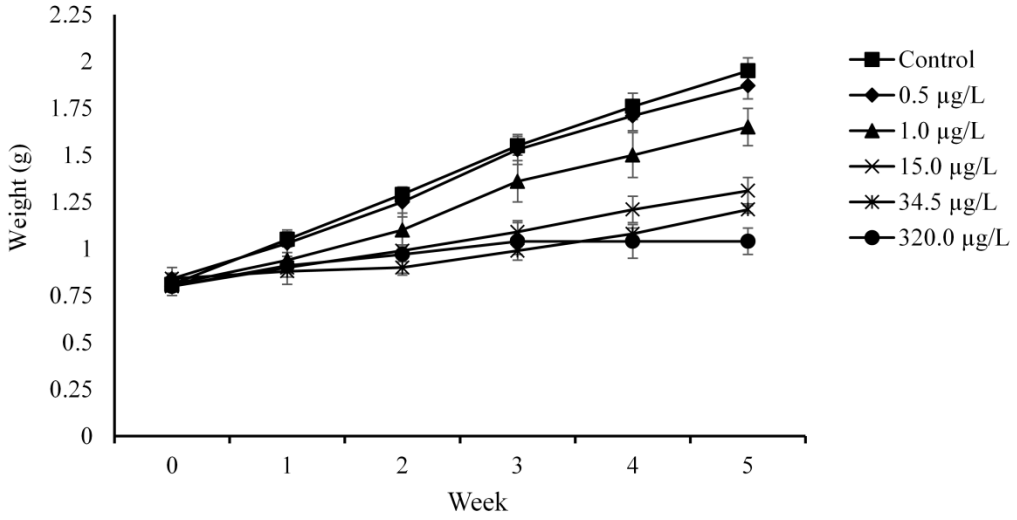


Figure 7 Continued.

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 µg/L treatment and 0.84 ± 0.06 g in the 34.5 µg/L treatment, and there was no significant difference among all treatments (ANOVA, P = 0.971) (Supplementary Table A6 and Fig. 7b). There were significant differences between the percent weight gains of the control (140.3 ± 16.15%) and three higher imidacloprid concentrations (15.0 µg/L, 34.5 µg/L, and 320.0 µg/L), which showed a reduction in their percent weight gains (64.4 ± 17.14 g%, 44.0 ± 12.09 g%, and 29.5 ± 16.43 g%, respectively) (ANOVA, P = 0.0008), but both 0.5 µg/L and 1.0 µg/L treatments had no significant effect on the percent weight gain compared with the control (Supplementary Table A6).

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 µg/L treatment and 4.46 ± 0.09 cm in the control (0.0 µg/L) treatment (ANOVA, P = 0.794) (Supplementary Table A7). Starting from week 1 to week 4, significant differences were observed between the control and all other fipronil concentrations except the 0.005 µg/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 (Supplementary Table A7). Body length was not measured in shrimp under both 1.0 µg/L and 3.0 µg/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 cm in the 34.5 µg/L treatment (ANOVA, P = 0.96) (Supplementary Table A8). 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 (Supplementary Table A8).

3.3.2. Molting

In the fipronil experiment, shrimp under the control (0.0 µg/L) showed the shortest inter-molt interval (7.57 ± 2.17 day) compared with other treatments, 0.005 µg/L, 0.01 µg/L, and 0.1 µg/L, which had inter-molt intervals of 9.29 ± 4.22 days, 9.47 ±

2.73 days, and 9.20 ± 2.93 days, respectively (Fig. 8a). The inter-molt 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 I could not observe consecutive molts to calculate the inter-molt intervals.

Shrimp under imidacloprid exposure showed a significant difference in inter-molt interval between the control (8.43 ± 2.52 day) and all other treatments (P = 0.0020 – 0.045). Inter-molt 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. 8b).

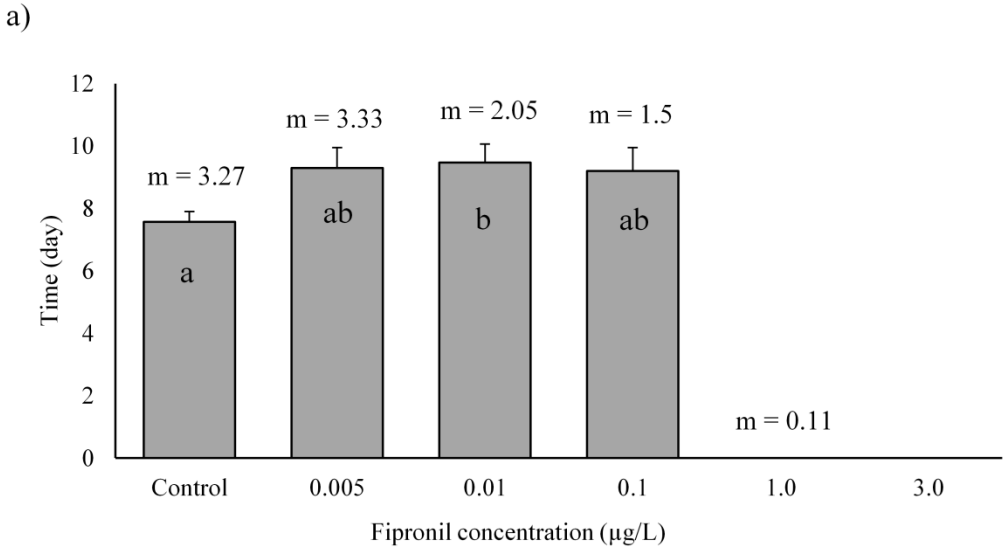


Figure 8. Inter-molt intervals of juvenile shrimp. The vertical axis is the time (days) between consecutive molts of the same individual, and the horizontal axis is the six insecticide concentrations (µg/L) including the control. Error bars indicate the standard errors (n = 18 in fipronil exp. and n = 15 in imidacloprid exp.). m is the average number of molts of individual shrimp in each treatment. (a) Fipronil experiment, (b) Imidacloprid experiment.

b)

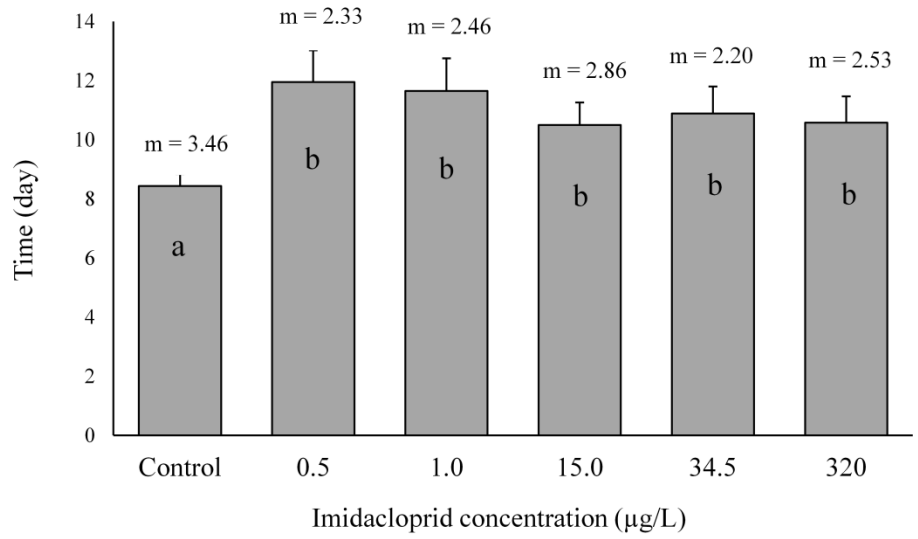


Figure 8 Continued.

3.3.3. Shrimp survival and LC_{50}

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) (Supplementary Table A9). 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) (Supplementary Table A9). Lethal concentration of fipronil to reach 50% mortality of shrimp within 96 hours (96-h LC_{50}) of the juvenile brown shrimp was 0.12 µg/L with 95% confidence intervals 0.06 - 0.24 (Supplementary Fig. B5).

In the imidacloprid experiment, shrimp showed higher survivorship percentage during the first 2 weeks compared with those in the fipronil experiment, and there was no significant difference among treatments including the control (Supplementary Table A10). 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 (34 days), the control treatment showed significant differences in the survival rate (100%) compared with the 15.0 μg/L, 34.5 μg/L, and 320.0 μg/L treatments, which had 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 (Supplementary Table A10).

3.3.4. Behavioral and other physical changes

Swimming and feeding behaviors of brown shrimp under exposure to 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 involuntary. Then, their swimming legs stopped

moving, and they died. Shrimp in the fipronil experiment showed these behavioral changes after only 1 day of exposure and even in the lowest concentration (0.005 µg/L). Similarly, shrimp under imidacloprid exposure showed the same behavioral changes by day 1 in all treatments except in the 0.5 µg/L treatment (lowest imidacloprid concentration). Under the lowest imidacloprid concentration, these changes started by day 5. During all of these abnormal swimming behaviors, shrimp exhibited difficulty in feeding, and food remained in aquariums that were cleaned routinely.

At the end of the experiments, visible changes in color were noticed in shrimp bodies under exposure to either pesticide. Fig. 9 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.

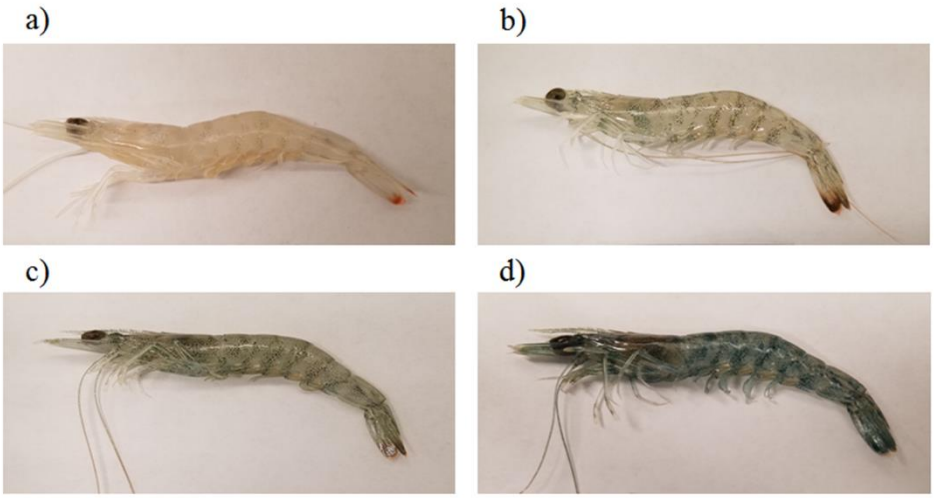


Figure 9. Shrimp in different concentrations (µg/L) of insecticide at the end of the experiments. (a) Control 0.0 µg/L fipronil, (b) 0.005 µg/L fipronil, (c) 0.01 µg/L fipronil, (d) 0.1 µg/L fipronil, (e) Control 0.0 µg/L imidacloprid, (f) 0.5 µg/L imidacloprid, (g) 1.0 µg/L imidacloprid, (h) 15.0 µg/L imidacloprid, (i) 34.5 µg/L imidacloprid, (j) 320.0 µg/L imidacloprid.

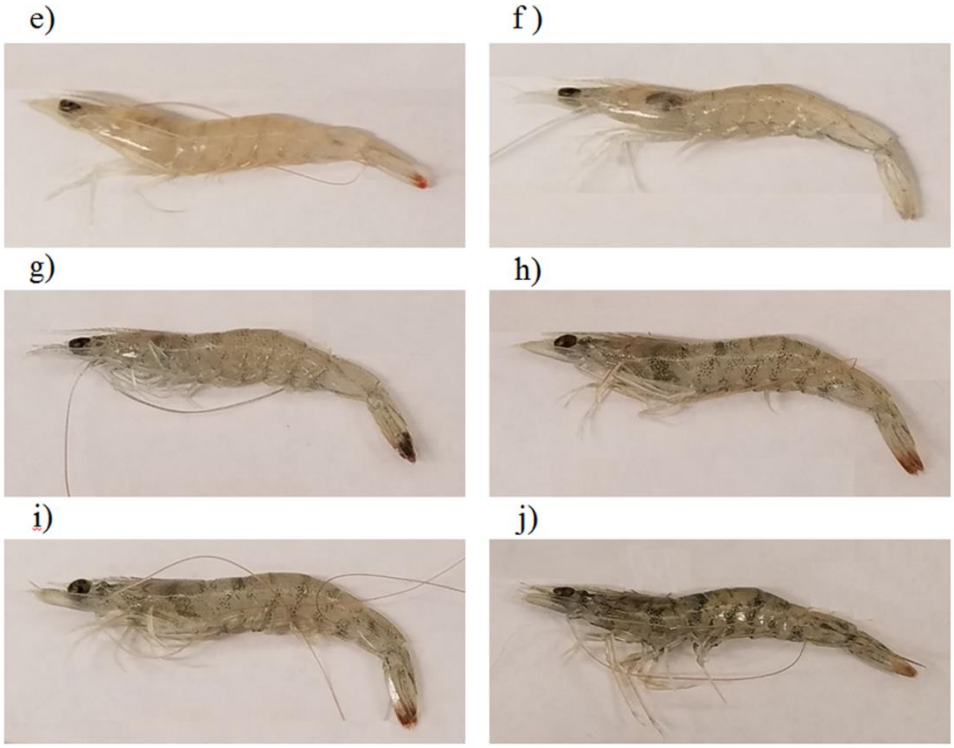


Figure 9 Continued.

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.

3.3.5. Water quality

In both fipronil and imidacloprid experiments, all water quality parameters were within suitable ranges that fit the environmental requirements of brown shrimp (Lassuy, 1983). 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 (Supplementary

Table A11). 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 (Supplementary Table A12).

3.4. Discussion

In the present study, all nominal concentrations of fipronil and imidacloprid were within the range of the concentrations reported in recent studies (Hayasaka et al., 2012a; Ruby, 2013; Van Dijk et al., 2013; Main et al., 2014) for both insecticides (Table 5). The 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 (Supplementary Tables A5, A6, A7, and A8) 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 *C. nilotica* (Mensah et al., 2012b), petroleum hydrocarbons from oil spill on juvenile brown shrimp *F. aztecus* and white shrimp *L. setiferus* (Rozas et al., 2014), and imidacloprid on midge *Chironomus tentans*, amphipod *Hyalella azteca* (Stoughton et al., 2008), and harlequin fly *Chironomus riparius* (Azevedo-Pereira et al., 2011). However, I note that growth of juvenile blue crabs *C. sapidus* showed a significant increase under all treatments of fipronil compared to the control in a short-term (96-h) experiment (Goff et al., 2017), suggesting the effects of insecticides on growth may not be always negative.

Many reasons could explain the reduced growth of juvenile brown shrimp under fipronil and imidacloprid exposures in the present 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 (Frontera et al., 2011). Shrimp is known to derive energy more efficiently from protein compared with carbohydrates and lipids (Gauquelin et al., 2007); consequently, fipronil and imidacloprid in this 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 (Supplementary Tables A5, A6, A7, and A8). 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 (Ecobichon, 1996) or by binding strongly to the nicotinic acetylcholine receptor (nAChR) by imidacloprid (Morrissey et al., 2015). These effects on the nervous system activities may suppress feeding of invertebrates (Hasenbein et al., 2015). 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 (Minello et al., 1989) 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 the juvenile stage (Baker et al., 2014).

Molting in arthropods is a 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 (Lachaise et al., 1993; OECD, 2005). The molting 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 (OECD, 2005), such as fipronil (Volz et al., 2003) or those that act like EDCs such as imidacloprid (Baines et al., 2017). In my study, both fipronil and imidacloprid affected molting of brown shrimp with significant differences between the control and other treatments. Inter-molt interval of shrimp under fipronil exposure was significantly delayed under the 0.01 μg/L treatment compared with the control (Fig. 8a). This result is consistent with my previous study, which showed the prolonged inter-molt intervals under the 0.1 μg/L and 1.0 μg/L treatments compared with the control (Al-Badran et al., 2018). Under imidacloprid exposure in the current study, shrimp exposed to all treatments showed a significant delay ($P = 0.0020 – 0.045$) in their inter-molt intervals (10.9 ± 3.97 day in 34.5 μg/L to 11.9 ± 4.90 day in 0.5 μg/L) compared with shrimp in the control (8.4 ± 2.52 day) (Fig. 8b). Many studies have reported similar delays in molting of marine and freshwater arthropods after exposing them to different pesticides (Al-Badran et al., 2018). Such delay in molting of shrimp may be linked to the reduction in growth and suggests that normal development of shrimp was affected even under concentrations below 0.01 μg/L of fipronil.

This study showed that survival of juvenile brown shrimp was concentration-dependent for both insecticides (Supplementary Tables A9 and A10). Fipronil and imidacloprid caused significant lethal and sub-lethal effects on shrimp especially in

higher concentrations. Under fipronil exposure, all shrimp died during the 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) (Supplementary Table A9). 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 the control (100%) (Supplementary Table A10).

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. (2012). Other sensitive marine invertebrates include estuarine grass shrimp *P. pugio* with a 96-h LC_{50} of 0.32 μg/L (Key et al., 2003; Overmyer et al., 2007), estuarine copepod *A. tenuiremis* with a 96-h LC_{50} of 6.8 μg/L (Chandler et al., 2004), and estuarine Chinese mitten crab *E. sinensis* with a 96-h LC_{50} of 8.56 μg/L (Shan et al., 2003). Many previous studies reported the greater sensitivity of marine invertebrates to fipronil compared with freshwater invertebrates, such as the copepod *A. robustus* with a 48-h LC_{50} of 194.2 μg/L (Chaton et al., 2002), the water flea *D. magna* with a 48-h LC_{50} of 190.0 μg/L (USEPA, 1996b), and the red swamp crayfish *P. clarkia* with a 96-h LC_{50} of 163.5 μg/L (Overmyer et al., 2007). As for imidacloprid, I 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.

I also note that the LC_{50} of fipronil measured in this study (0.12 µg/L) was below the LC_{50} measured in my previous study in 2016, 1.3 µg/L (Al-Badran et al., 2018). This may be because of the difference in the temperature between the two studies: 20.84°C ± 0.24 (in 2016 study) and 24.12°C ± 0.06 (in present study). Although temperature was increased, these water temperatures are considered within the optimum range for brown shrimp development (18°C to 25°C) (Lassuy, 1983). I increased water temperature from the previous study because I expected shrimp to experience higher temperatures during summer months in estuaries and previous shrimp experiments by others were often conducted at higher temperatures (e.g., Zein-Eldin and Aldrich, 1965; Adamack et al., 2012; Leo et al., 2018). Effect of temperature on toxicity of pesticides has been observed in other studies. For example, Russo et al. (2018) noted that, of many environmental parameters investigated, temperature was the only parameter that magnified the effect of pesticide exposure on the crustacean *G. pulex* in streams. Willming et al. (2013) also reported that the 10-day 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. I suggest further studies to investigate the effects of temperature on insecticides toxicity 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 (Tu et al., 2010). 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 (Raley-Susman, 2014). 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 µg/L); whereas, those exposed to a low imidacloprid concentration (0.5 µg/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. (2005) observed abnormal behavior and muscle control in the aquatic insect *S. vittatum* under all tested fipronil and imidacloprid concentrations. Similarly, Stratman et al. (2013) showed that the chironomid midge *C. lebetis* 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 (Roque et al., 2005). 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 (OECD, 2005). In particular, the major mortality of juvenile brown shrimp is considered to be predations (Minello et al., 1989), 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 do in nature. It also appears to be a concentration-dependent color change, and shrimp under fipronil exposure were much darker than those under imidacloprid

exposure. In another study, Martinez et al. (2014) reported that Pacific white shrimp *L. 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 (O'Halloran, 1990), which are regulated by neurosecreted hormones (Fernlund and Josefsson, 1972). However, in this 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. 9. Body color may be used as an indicator of the health of shrimp (Martinez et al., 2014) and environment (Fingerman et al., 1998); consequently, I 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.

CHAPTER IV

THE ADVERSE EFFECTS OF PHENYLPYRAZOLE FIPRONIL ON JUVENILE WHITE SHRIMP *LITOPENAEUS SETIFERUS*

4.1. Introduction

Pesticide use has increased in recent years especially in developed countries, and many studies have documented the adverse side effects of these chemical compounds on wildlife, especially on aquatic organisms (Pritchard, 1993; Clasen et al., 2012; Osterberg et al., 2012; Roessink et al., 2013; Mahmoud, 2017). Fipronil is a phenylpyrazole insecticide introduced to the market in 1993 (USEPA, 1996b; Gunasekara et al., 2007). Because of the widespread use, mobility in soil, solubility in water, and a moderate level of persistence in the environment, fipronil can flow into surface water, and many studies have reported its detection in aquatic environments reaching 12.62 µg/L, which exceeds the acute level of the aquatic life benchmark for invertebrates (0.1 µg/L) according to the U.S. EPA (USGS, 2003; Wirth et al., 2004; Harman-Fetcho et al., 2005; Gilliom et al., 2006; Mize et al., 2008; Greenberg et al., 2010; Gan et al., 2012; Hayasaka et al., 2012b; Ensminger et al., 2013; Ruby, 2013; Weston et al., 2015; USEPA, 2017). The pesticide runoff can eventually flow into estuaries.

In the last two decades, fipronil has been used commonly in many parts of the world and has played an essential role in pest control in both agricultural and non-agricultural uses because of its high effectiveness (especially on arthropods) at low field application rates (Chaton et al., 2002; Gunasekara et al., 2007; Kurz et al., 2013).

However, recent studies have reported the potential effects of fipronil and its degradation products on non-target terrestrial and aquatic organisms such as birds, honeybees, fish, and aquatic invertebrates. Consequently, fipronil use was banned in China in 2009 and the European Union countries in 2013 (Wu et al., 2014; Wu et al., 2015). Fipronil is still used in the United States, in many states such as Texas, Louisiana, California, and South Carolina for various purposes. Moreover, its use recently has been increased in some of these areas. In particular in Texas, the quarantine exemption was issued by the U.S. EPA to the Texas Department of Agriculture in 2016, permitting the expanded use of fipronil to control the infestation of tawny crazy ants *N. fulva* in southeastern counties of Texas, which are adjacent to estuaries in the Gulf of Mexico (Fig. 1) (USEPA, 2016).

White shrimp *L. setiferus* is an estuarine-dependent species distributed from the west coast of Florida to the Bay of Campeche, Mexico (Ball and Chapman, 2003; Wenner et al., 2005). It is considered one of the most important species for commercial and recreational harvest along the Atlantic coast of the southeastern United States and in the Gulf of Mexico (DeLancey et al., 2005). In 2017, the annual commercial landing of white shrimp in the United States was 58,144 metric ton worth about $263.5 million (NMFS, 2019). In addition to the economic importance of white shrimp, it has a significant ecological role as prey for many fish species such as the spotted seatrout *Cynoscion nebulosus*, red drum *Sciaenops ocellatus*, and southern flounder *Paralichthys lethostigma*, which support important fisheries throughout the Gulf of Mexico area (Fujiwara et al., 2016). Because white shrimp use estuaries as a nursery habitat during their juvenile stage, the increased use of fipronil in coastal communities as well as the

continuous detection of pesticides residues in estuarine waters are serious concerns (Chandler et al., 2004; Mace III and Rozas, 2015).

Consequently, the objective of this study was to investigate the impact of fipronil under concentrations that were previously observed in different aquatic environments on juvenile white shrimp. This type of study has not been done with this particular species despite its economic importance and its potential exposure to the pesticide. Therefore, I exposed juvenile white shrimp in a laboratory to different concentrations of fipronil and measured survival, development (percent weight gain and inter-molt intervals), body composition, and behavioral endpoints.

4.2. Material and Methods

4.2.1. Test organism and acclimation period

Juveniles white shrimp were sampled in the Gangs Bayou (on Sportsman Road N 29.25549; W 94.91575) in Galveston Bay, Texas (Fig. 10) on August 11, 2016. Bag seine (3-m length and 0.6 cm mesh size) and hand nets (0.1 cm mesh size) were used. Average initial weight and total length of collected shrimp were 0.80 ± 0.08 g and 5.23 ± 0.58 cm, respectively. The shrimp were transported to a laboratory at Texas A&M University, College Station, Texas, in aerated 45-liter coolers. In the laboratory, shrimp were kept in the coolers for approximately 5 hours for equilibrating to room temperature. Then, shrimp were counted and measured. Subsequently, shrimp were moved to 53-liter tanks with aerated synthetic brackish water prepared with Instant Ocean® Sea Salt and de-chlorinated tap water for further acclimation.

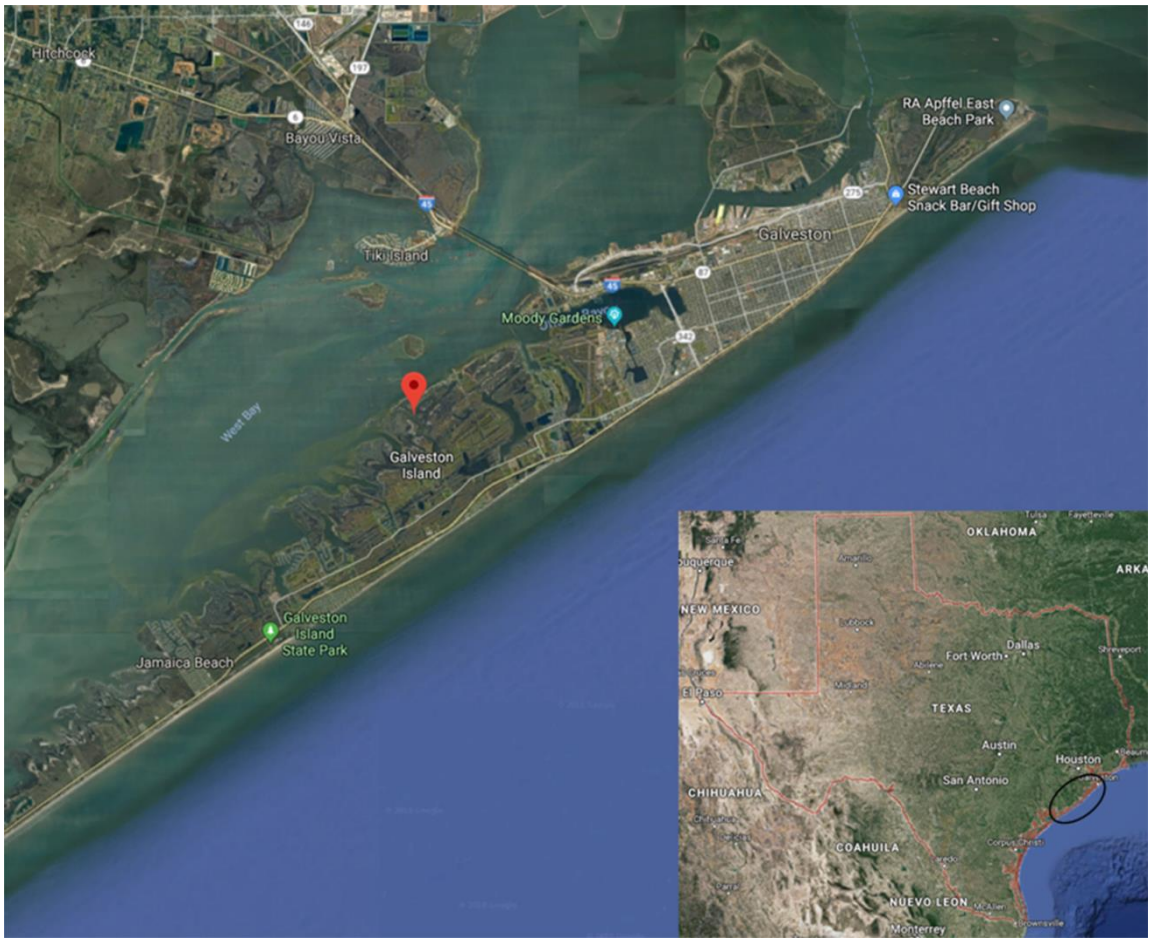


Figure 10. Location of the Gangs Bayou in Galveston Bay, Texas where juveniles white shrimp were sampled before the beginning of the experiments. Image is taken from Google Maps.

In the acclimation tanks, shrimp were kept under controlled laboratory conditions for 15 days. Water quality parameters were measured daily using the YSI® Professional plus Multi-parameter Meter. Photoperiod was controlled in a 12 hour light: 12 hour dark cycle. Furthermore, to be able to fulfill the daily nutritional requirements of shrimp as described in Lovell (1998), API® Bottom Feeder Shrimp Pellets were used to feed shrimp twice daily. This also ensured that shrimp were accustomed to the feed pellets before conducting the experiments. Using a siphon, uneaten feed and shrimp feces were

removed, and approximately 40-50% of the water in acclimation tanks was replaced on a daily basis.

4.2.2. Experimental solutions

Fipronil (purity limit ≥ 97%) was purchased from Fisher Scientific Co. L.L.C., PA, U.S. Depending on the environmental concentrations of fipronil reported previously in the published literature (Table 5), five nominal concentrations of fipronil (0.005, 0.01, 0.1, 1.0, and 3.0 μg/L) were chosen in the current study. Two replicate tanks of shrimp were used for each treatment.

All nominal experimental solutions were prepared by mixing specific quantities of the insecticide with water in multiple dilution steps starting with mixing 0.1 g fipronil in 1 liter of water to make 1 liter of highly homogenized 100 mg/L fipronil suspension. Then, for each nominal concentration (treatment), I created 14 liters of its test solution to be used in two aquariums (replicates) of 7 liters each. All dilution steps to prepare the nominal concentrations are provided in the Supplementary Table A13. Although the hydrolysis half-life of fipronil at 25ºC is > 100 days (Gunasekara et al., 2007), 100% of test solution of each concentration were replaced every 2 days in order to maintain the constant concentrations of fipronil in each treatment during the experiment.

4.2.3. Experimental system and exposure tests

The experimental system consists of 12 glass aquariums (9.5 liter, dimensions 30.7 X 15.4 X 20.5 cm), where each aquarium represented one replicate of the fipronil

treatments and the control (six treatments X two replicates) (Supplementary Fig. B4a). Each aquarium was filled with 7 liters of test solution, and a Topfin® AIR-8000 air pump was used to provide air to the aquaria via an airstone. To avoid cannibalism among shrimp and to monitor molting of each shrimp, barriers made of polypropylene plates and fiberglass screen (materials that are commonly used in aquaculture experiments) were used to divide each aquarium into six cells of equal size (Supplementary Fig. B4b and B1d), and one individual shrimp was placed in each cell. The screen in the middle of the barriers allowed flow of water and better distribution of dissolved oxygen among the cells.

Each aquarium in the experimental system was covered from the top with fiberglass screen and a glass lid to prevent shrimp from jumping into the adjacent cells or escaping outside the aquarium. Additionally, to avoid the photo-degradation of fipronil, all sides of the aquariums were fully covered using aluminum foil (Supplementary Fig. B4a). During the experiment, which lasted 45 days, shrimp were fed twice daily the same type of feed used during the acclimation period. Using shrimp feeding tables (Lovell, 1998), feed quantities were adjusted every day (if there was death of shrimp) and every 9 days (based on the changes in body weight). All water quality parameters, temperature (°C), dissolved oxygen (mg/L), salinity (‰), and pH, were measured every other day.

The number of shrimp in each aquarium and number of replicates used for each treatment were selected based on similar experiments done by other researchers (Shan et

al., 2003; USEPA, 2007). In addition, all exposure tests of this study were conducted using the static-renewal method according to the U.S. EPA guidelines (USEPA, 2002).

4.2.3.1. Survival

Shrimp mobility was monitored multiple times per day, i.e., at feeding times, measuring water quality parameters, and replacing the test solutions. Any dead shrimp found during the monitoring were removed from the aquarium, weighed (to adjust food amount for the remaining live shrimp), and kept in a freezer at −18 °C for later compositional analysis. Each dead shrimp was carefully checked for any sign of movement after being taken out of water to confirm death.

4.2.3.2. Growth

4.2.3.2.1. Weight gain

Percent weight gain of shrimp was calculated by measuring the weight of shrimp every 9 days. At the time of weight measurement, each individual shrimp was weighed separately by gently removing it from the water and using a paper towel to remove any excess water on its body. Then, the weight of shrimp was measured by placing it in a beaker containing water on a scale after zeroing it. The percent weight gain of each shrimp was calculated using this equation:

% Weight gain of shrimp = {(Final weight – Initial weight) / Initial weight} x 100

Based on the weight measurement, I adjusted food quantity in each aquarium to be consistent with increased or decreased weight of shrimp and based on the nutrition tables for shrimp (Lovell, 1998).

4.2.3.2.2. Inter-molt intervals

By assigning each individual shrimp in its own cell in an aquarium (Supplementary Fig. B4a), I was able to follow and record the dates of molting, which were used to calculate the inter-molt intervals of shrimp exposed to each concentration of fipronil.

4.2.3.3. Behavioral and body color changes

Any abnormal activities of shrimp were noted and recorded on video multiple times per day. In addition, any changes in the physical appearance of shrimp, such as change in body color, were monitored and compared later with shrimp in the control group.

4.2.3.4. Chemical analysis of body composition

Chemical composition of the whole body of shrimp was measured at the end of the experiment. On the last day, all remaining live shrimp in each treatment were collected and euthanized by freezing them at −18 °C. All shrimp used in each treatment, 12 shrimp per fipronil concentration, were used in the body chemical composition analysis. First, dry matter of each group of shrimp was measured by weighing a sample

of 2.0 g wet shrimp using a pre-weighed porcelain crucible and then weighing the crucible with the sample again after placing them in an oven at 135 °C for 3 hours (AOAC, 1990). Then dry matter was calculated using this equation:

% Dry matter = (Dry weight / Wet weight) x 100

Ash was measured using the muffle furnace by placing dry matter samples of shrimp at 550 °C for 3 hours (AOAC, 1990), then using the following equation to calculate % ash in shrimp:

% Ash = (Ash weight / Dry weight) x 100

Lipids were estimated by using the method of chloroform/methanol 2:1 extraction as described by Folch et al. (1957) and the following equation:

% Lipid = {(Weight of 10 ml aliquot/10 ml) x (15 ml/Dry weight)} x 100

Finally, a LECO protein analyzer was used to measure the total nitrogen in shrimp samples with the Dumas protocol (AOAC, 2005), and nitrogen was multiplied by 6.25 to estimate crude protein content.

4.2.4. Statistical analysis

Kaplan–Meier estimator followed by the non-parametric Log-Rank test was used to estimate the survivorship of shrimp exposed to each concentration of fipronil and to compare the survivorship distribution among treatments. I also used the non-parametric Kruskal-Wallis test followed by the pairwise Wilcoxon rank sum test to analyze shrimp molting data. For all remaining measurements, One-way Analysis of Variance (ANOVA) and linear regression analyses were conducted to estimate the significant

differences between the control group and all fipronil treatments. These statistical analyses were conducted at $\alpha = 0.05$ significance level using JMP® Pro 2016 (JMP, 2016) for the Kaplan-Meier, Kruskal-Wallis, and ANOVA tests. I used Microsoft Excel 2016 to test for the linear regression and to draw all related figures.

4.3. Results

4.3.1. Water quality parameters

During the experiment, water quality parameters were measured every other day, and their means were as following: temperature, 21.06 ± 0.14 °C; dissolved oxygen, 5.49 ± 0.19 mg/L; salinity, 15.15‰ ± 0.04; and pH, 8.21 ± 0.07 (Supplementary Table A14). In addition, all water quality parameters measured in this study were within the levels that satisfy the environmental needs of white shrimp according to Muncy (1984).

4.3.2. Survival

There was no significant difference in the survivorship of shrimp exposed to the control (survival rate 100%) and the three lowest fipronil concentrations (0.005 μg/L, 0.01 μg/L, and 0.1 μg/L), which showed final survival of 83.3% ± 23.57, 100%, and 83.3% ± 0.0, respectively. However, significant differences between the control and two higher concentrations (1.0 μg/L and 3.0 μg/L) were observed starting after 18 days (Kaplan-Meier survival analysis followed by the non-parametric Log-Rank test, $P < 0.0001$). In both 1.0 μg/L and 3.0 μg/L treatments, shrimp showed a gradual decrease in

survival from 100% to 25% in the 1.0 µg/L treatment, and to 0.0% in the 3.0 µg/L treatment (Table 6 and Fig. 11).

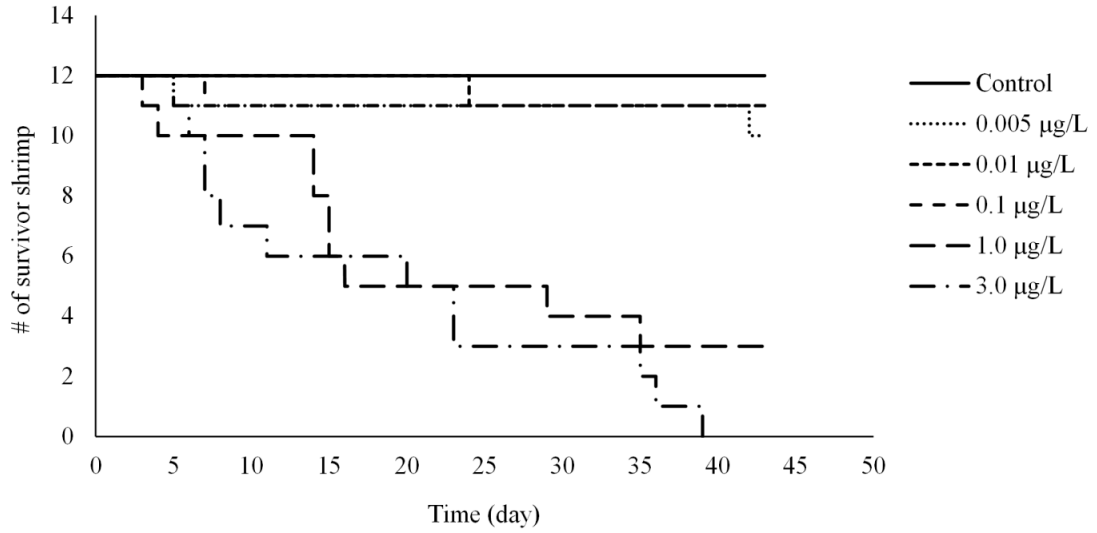


Figure 11. Kaplan-Meier survivorship curves of juvenile white shrimp under different concentrations of fipronil during 45 days of exposure. According to the non-parametric Log-Rank test, treatments of higher concentrations of fipronil were significantly different from the control ($P < 0.0001$). Day 1 is 24-h after the beginning of the experiment.

Table 6. Survival rate (mean ± standard deviation) of juvenile white shrimp starting from day 1 to the end of the experiment on day 45. n = number of shrimp in each treatment (6 shrimp per replicate aquarium, 2 aquariums per treatment). Values with star (*) indicate treatment is significantly different from the control ($p < 0.0001$). Time between each two consecutive measurements is 9 days.

Fipronil concentrations (µg/L)	n	Survival %					
		Day 1	Day 9	Day 18	Day 27	Day 36	Day 45
Control	12	100	100	100	100	100	100
0.005	12	100	91.66 ± 11.78	91.66 ± 11.78	91.66 ± 11.78	91.66 ± 11.78	83.33 ± 23.57
0.01	12	100	100	100	100	100	100
0.1	12	100	100	91.66 ± 11.78	83.33	83.33	83.33
1.0	12	100	83.33	83.33*	41.66 ± 58.92	33.33* ± 47.13	25.00* ± 35.35
3.0	12	100	83.33 ± 23.57	50.00*	24.99* ± 11.78	24.99* ± 11.78	

4.3.3. Growth

4.3.3.1. Weight gain

The wet weight of shrimp measured at the beginning of the experiment (initial weight) was 0.70 ± 0.02 g in the 0.005 µg/L treatment and 0.93 ± 0.04 g in the 3.0 µg/L treatment. The final weight of shrimp was 0.38 ± 0.53 g in the 1.0 µg/L treatment and 1.19 ± 0.01 g in the control, which was not significantly different from other treatments except the 1.0 µg/L treatment (ANOVA, $P < 0.05$). All the values of final weight and percent weight gain measured in this study were based on the wet weight of shrimp in the two replicates of each treatment, except treatment 1.0 µg/L where all shrimp died in one of its replicates before day 45, thus the final weight measured was for the remaining shrimp in one replicate (Supplementary Table A15).

Growth of shrimp was clearly affected by fipronil exposure under all concentrations used in this study, where the percent weight gain declined significantly with increasing fipronil concentration from 51.6% ± 2.29 in the control to -2.6% in the 1.0 µg/L treatment; whereas, treatments 0.01 µg/L and 0.1 µg/L showed percent weight gain of 33.8% ± 2.70 and 17.4% ± 0.11, respectively (Supplementary Table A15 and Fig. 12). Statistical analysis of the weight gain showed significant differences between the control and all treatments including the 0.005 µg/L treatment, which had percent weight gain of 41.2% ± 5.25 (ANOVA, $P < 0.05$).

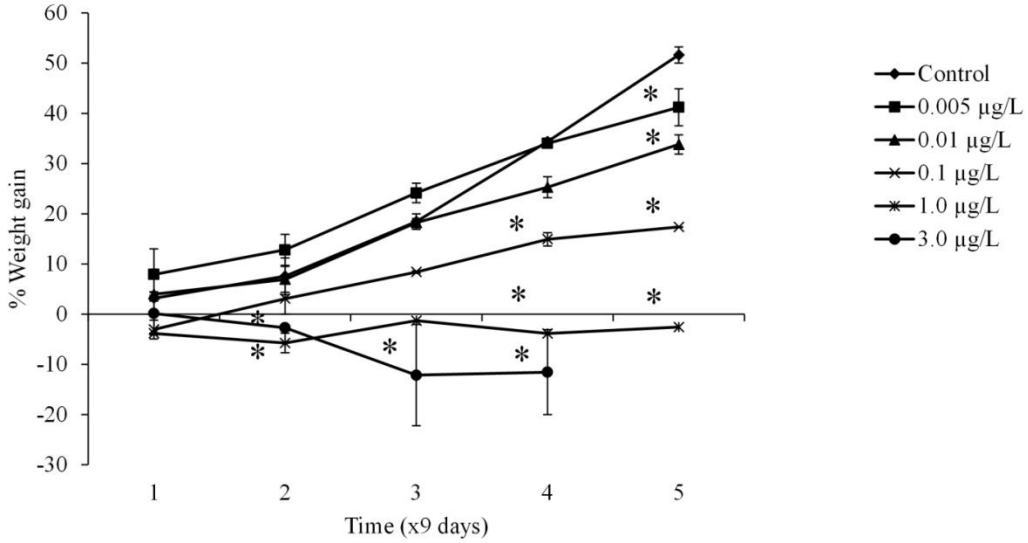


Figure 12. Percent weight gain of juvenile white shrimp at multiple time periods from the first day to the end of the experiments. The horizontal axis represents the experiment period where measurements were taken each 9 days while the vertical axis represents the % weight gain per individual shrimp in each treatment. Stars above the curve indicate that fipronil treatment was significantly different from the control at the time of measurement. One-way Analysis of Variance showed that all treatments were significantly different from the control by the end of the experiment ($P < 0.05$). Error bars indicate the standard errors ($n = 12$).

4.3.3.2. Inter-molt intervals

Shrimp in the control showed shorter inter-molt intervals (13.6 ± 1.98 day) compared with all other treatments, where the inter-molt intervals ranged between 14.8 ± 3.10 day in the 0.005 µg/L treatment and 20.0 ± 4.24 day in the 3.0 µg/L treatment. The regression analysis showed that inter-molt intervals of shrimp increased significantly with fipronil concentration ($P = 0.001$) (Fig. 13).

The number of molts per individual shrimp was affected by fipronil exposure. It was greatest in the control treatment ($m = 2.16$), and it showed a gradual decline as fipronil concentration increased (Fig. 13).

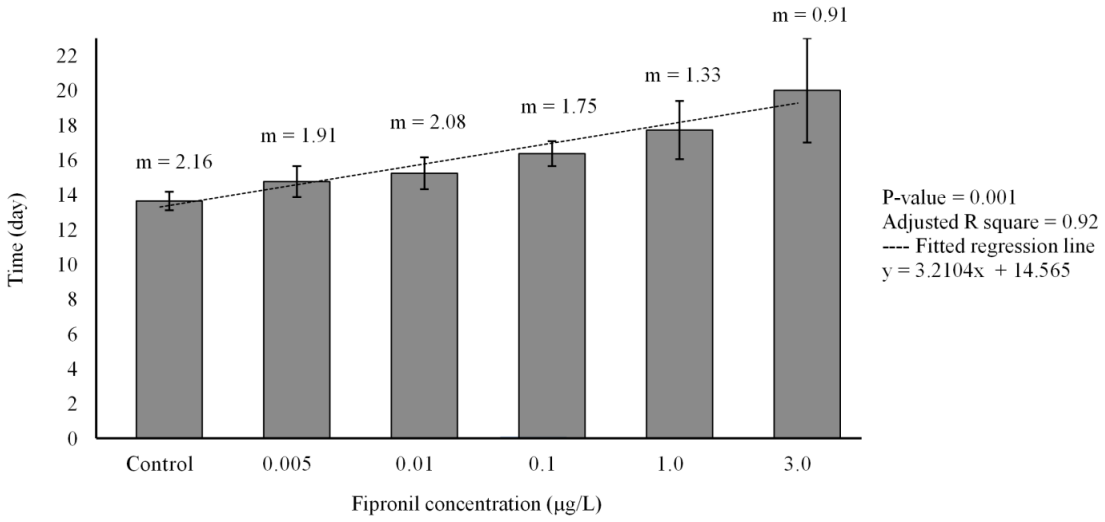


Figure 13. Inter-molt intervals of juvenile white shrimp exposed to multiple concentrations of fipronil for 45 days. The vertical axis represents time (per day) of the inter-molt intervals of shrimp while the horizontal axis represents the six fipronil concentrations (µg/L) used in the experiment. m is the average number of molts of individual shrimp in each treatment. Regression analysis showed that inter-molt intervals increased significantly with fipronil concentration (P = 0.001). Error bars indicate the standard errors (n = 12).

4.3.4. Behavioral and body color changes

Changes in swimming and feeding behavior of shrimp were observed during the 45 days of the experiment under all fipronil concentrations compared with those in the control. Shrimp started losing control of their swimming behavior and exhibited abnormal loop-like movements; then their swimming legs started moving fast involuntarily while shrimp were on their sides or backs on the bottom of the aquarium and unable to change their position. After that, they just stopped moving any parts of their bodies and died. During the exposure time and until their death, shrimp were unable to eat provided feed normally and remaining feed was noticed in the cells of all affected shrimp.

Timing of the onset of these abnormal behaviors differed among treatments, and it was concentration dependent. For example, shrimp in treatments of higher fipronil concentrations (1.0 µg/L and 3.0 µg/L) showed these abnormal behaviors by day 2; whereas, behavioral changes in lower fipronil concentrations (0.1 µg/L and 0.01 µg/L treatments) did not start until day 10. In the same way, these changes were only noticed among shrimp in the lowest fipronil concentration (0.005 µg/L) after 20 days from the beginning of the experiment.

In addition to the behavioral changes, changes in the body color of shrimp in some treatments were observed as well. At the end of the experiment, shrimp in treatments 1.0 µg/L and 3.0 µg/L showed a clear variation in their body color compared with shrimp in the control. Fig. 14 shows the difference between the gray color of shrimp in treatments 1.0 µg/L and 3.0 µg/L and bright color of shrimp in the control. Shrimp in all other treatments (0.005 µg/L, 0.01 µg/L, and 0.1 µg/L) had no noticeable changes in their body color by the end of the experiment.

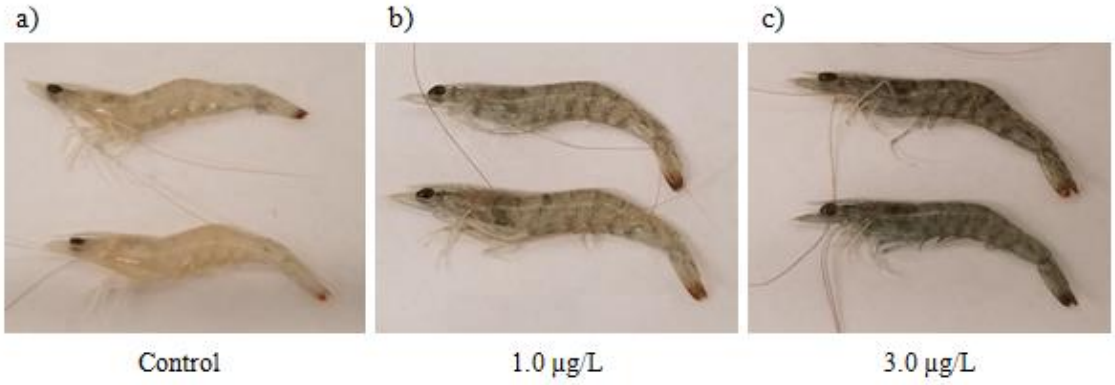


Figure 14. Changes of body color of juvenile white shrimp exposed to the highest concentrations of fipronil (µg/L) during the experiment compared to the control. (a) Control 0.0 µg/L fipronil, (b) 1.0 µg/L fipronil, (c) 3.0 µg/L fipronil.

4.3.5. Chemical analysis of body composition

Analysis of whole-body composition of shrimp is shown in Fig. 15. All of the lipids, ash, and protein contents were analyzed and expressed on a dry-matter basis. Lipid content in shrimp decreased significantly with increased concentration of fipronil (linear regression analysis, P = 0.027). Shrimp in the control showed the highest percentage of lipid 9.1% ± 2.45; whereas, the lowest percentages of lipid were in the treatments 3.0 µg/L and 1.0 µg/L (7.5% ± 1.15 and 8.0% ± 0.05, respectively; Fig. 15a).

a)

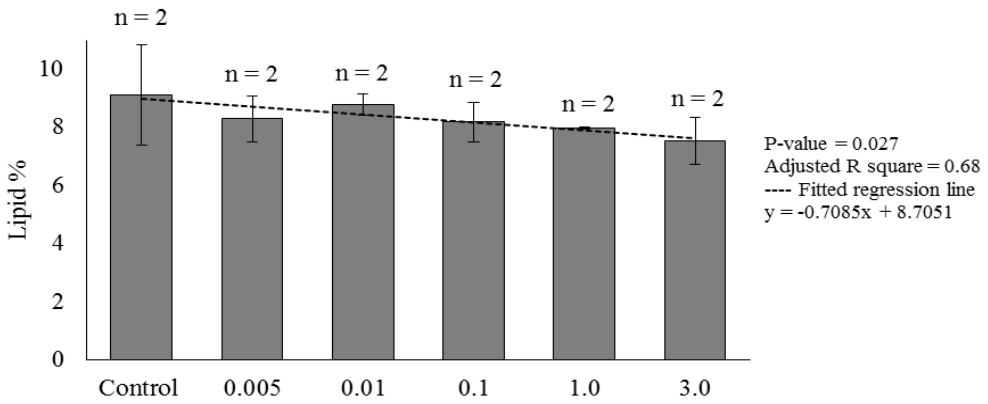
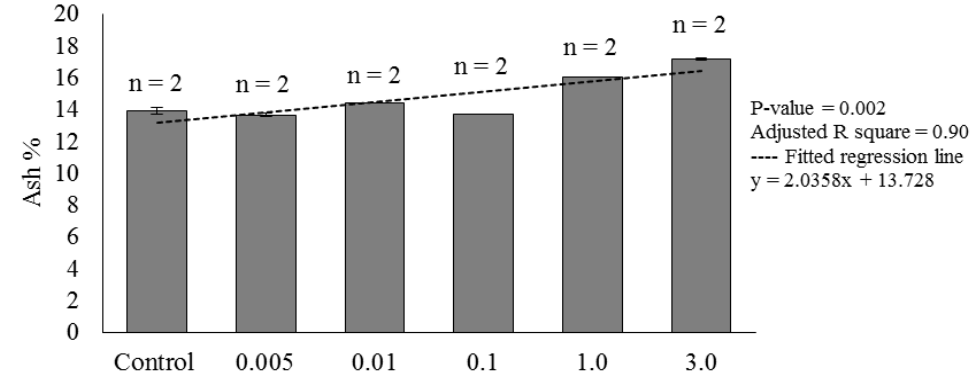


Figure 15. Body chemical composition analysis of juvenile white shrimp exposed to multiple concentrations of fipronil. The vertical axes represent the lipid % (in 15.a), ash % (in 15.b), and protein % (in 15.c) in bodies of shrimp which measured at the end of the experiment. The horizontal axes in (15.a, 15.b, and 15.c) represent the six different fipronil concentrations (µg/L) used in the experiment. Regression analysis showed that lipid content in shrimp decreased significantly with increased concentration of fipronil (P = 0.027), while ash content increased significantly (P = 0.002), with no significant difference among treatments regarding the protein content (P = 0.222). Error bars indicate the standard errors (n = number of samples analyzed for each treatment).

b)



c)

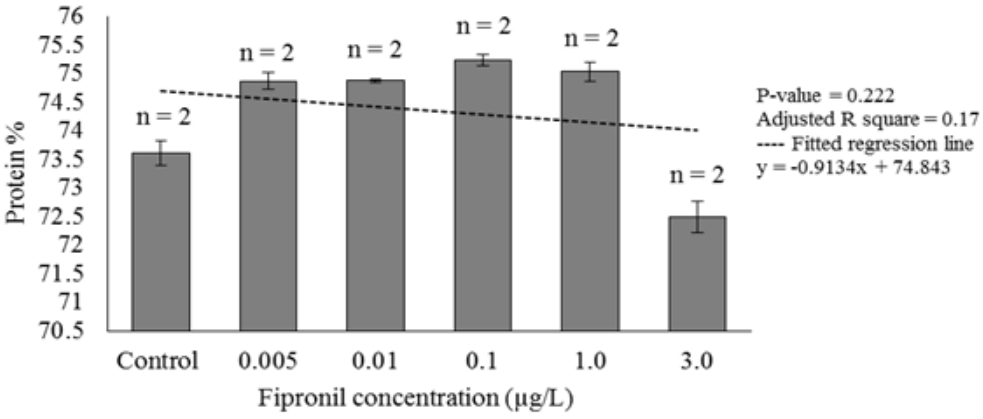


Figure 15 Continued.

On the contrary, ash content of shrimp increased with the increase of fipronil. Linear regression analysis showed that this increase was statistically significant (P = 0.002). Ash content of shrimp ranged between 13.7% ± 0.12 in the 0.005 µg/L treatment and 17.2% ± 0.12 in the 3.0 µg/L treatment (Fig. 15b). There was no clear pattern for the

effect of fipronil on the protein content of shrimp, and there was no significant difference among treatments according to linear regression analysis (Fig. 15c).

4.4. Discussion

The insecticide fipronil has been used as a successful alternative to the old generations of insecticides such as carbamates, organophosphates, and pyrethroids, and a part of its success is due to its effectiveness at low field application rates against insect pests that became resistant to other insecticides (Gunasekara et al., 2007; Simon-Delso et al., 2015). Fipronil acts by damaging the central nervous system of targeted pests through blocking the chloride channels controlled by gamma-aminobutyric acid (GABA) receptors, causing an excessive neuronal stimulation and finally death (Gunasekara et al., 2007). Due to the universality of the GABA receptors across arthropod species (Chandler et al., 2004), fipronil could result in unintentional and undesired adverse effects on non-target organisms including crustaceans. For example, many studies reported the toxicity of fipronil (or one or more of its degradation products, which have similar or more toxicity than fipronil itself) on crayfish production in rice-crayfish producing fields (e.g., Ngim and Crosby, 2001; Bedient et al., 2005). A recent study also has suggested that the decline of Atlantic blue crab *C. sapidus* may be due to the photo-degradation product of fipronil (fipronil desulfinyl), which was detected in their eggs off the coast of South Carolina (Goff et al., 2017).

In the last decade, there were detections of fipronil and its degradation products (e.g., desulfinyl fipronil, fipronil sulfide, fipronil sulfone) in the surface water in

different parts of the world at concentrations exceeding 0.01 μg/L, which is the chronic level according to the U.S. EPA aquatic life benchmark for invertebrates. Furthermore, fipronil use has increased over the world and in the United States, especially in Texas near estuaries, following the quarantine exemption by the U.S. EPA in 2016 (Sneck-Fahrer and East, 2007; Mahler et al., 2009; Opsahl, 2012).

The present study showed that fipronil caused significant lethal effects under higher concentrations (1.0 μg/L and 3.0 μg/L), in concentration- and time-dependent manners (Table 6 and Fig. 11). The survivorship of shrimp declined progressively from 100% in day 1 of the experiment to 25.0% and 0% on day 45 (last day) in the 1.0 μg/L and 3.0 μg/L treatments, respectively (Table 6). Brown shrimp *F. aztecus* tested in a previous study was more sensitive to fipronil in terms of their survivorship than white shrimp tested in the current study. Brown shrimp also was the most sensitive crustacean to fipronil exposure among all aquatic invertebrates studied to date with a nominal 96-h LC_{50} of 0.12 μg/L, where all shrimp in the 1.0 μg/L and 3.0 μg/L treatments died during the first few days of exposure, and survivorship of shrimp in low fipronil concentrations (0.1 μg/L and 0.01 μg/L) declined significantly compared with the control (Al-Badran et al., under review).

Currently, we do not understand the underlying mechanisms that cause the differences in sensitivity (responses) among species (Rubach et al., 2011). It may be due to the differences in their toxico-kinetic and/or toxico-dynamic processes (Mensah et al., 2014). For example, toxico-kinetic processes may enable some species to regulate uptake, to reduce, and/or to remove the stressor. In some cases, they detoxify a chemical

rapidly, greatly delaying the initial mortality of some species. On the other hand, different toxico-dynamic processes among species may cause variation in their ability to repair damage or in interaction between target enzymes and the chemical (stressor) (Rubach et al., 2011).

Reduced growth is the most common response to sub-lethal exposure to toxicants although it may not be a particularly sensitive endpoint (OECD, 2005). The decrease in growth as a result of the insecticide exposure is a great concern because the abundance of adult white shrimp has been demonstrated to be highly sensitive to survival during juvenile stage, and the survival of juveniles is thought to be size dependent (Baker et al., 2014). My results showed that fipronil adversely affected growth of white shrimp under all tested concentrations compared with the control group in a concentration-dependent manner (Figs. 12 and 13). The growth reduction also may be due to the influence of a toxicant on food metabolism. For example, animals affected by chemical toxicants use energy in detoxification processes, consequently affecting the carbohydrate and protein metabolism and finally growth performance (Frontera et al., 2011). The effect may be more pronounced with shrimp because they derive energy more efficiently from protein compared with lipids and carbohydrate (Gauquelin et al., 2007). My results are consistent with a study conducted by Rozas et al. (2014) who found similar reduction in growth of juvenile white shrimp and brown shrimp in field mesocosms contaminated with petroleum hydrocarbons from an oil spill. In a different study conducted by Mensah et al. (2012b), the Glyphosate-based herbicide (Roundup®) caused a significant growth reduction of the freshwater shrimp *C. nilotica*.

On the other hand, the reduction in growth also may be a consequence of reduced feeding. Fipronil is a neurotoxic insecticide disrupting the central nervous system activity (Ecobichon, 1996), which may have inhibited feeding activity of juvenile white shrimp. This type of effect was demonstrated by Hasenbein et al. (2015), who reported growth inhibition in *Chironomus dilutus* exposed to permethrin (18.21 ng/L), lambda-cyhalothrin (5.50 ng/L), or the mixture of three pesticides (15.47 ng/L chlorpyrifos, 1.04 ng/L lambda-cyhalothrin, and 3.15 ng/L permethrin) in 10-day exposures. The authors attributed part of the reduced growth to reduced feeding activities as all of the tested pesticides are neurotoxins.

In the present study, I observed reduced molting due to exposure to fipronil. Molting is a very crucial physiological process for the growth of arthropods, and because it is regulated by nervous system secretions and hormones in crustaceans, it is a potential negative sub-lethal effect of endocrine disrupting chemicals (EDCs) such as fipronil (Lachaise et al., 1993; Volz et al., 2003; OECD, 2005). My results showed that inter-molt intervals of white shrimp increased significantly from 13.6 ± 1.98 day in the control to 20 ± 4.24 day in the 3.0 µg/L treatment with increasing fipronil concentrations, and number of molts per individual shrimp decreased gradually from 2.16 molt/individual in the control to 0.91 molt/individual in the 3.0 µg/L treatment ($P = 0.001$) (Fig. 13).

The impacts of other pesticides on molting of aquatic arthropods were reported in previous studies. For example, both imidacloprid and fipronil caused a significant delay in the inter-molt intervals of brown shrimp (Al-Badran et al., under review). Molting of the American lobster *H. americanus* larvae also was delayed when they were exposed to

the cyclodiene pesticide heptachlor for 24 h (Snyder and Mulder, 2001). Similarly, the fungicide propiconazole (Tilt) increased inter-molt intervals of juvenile Pacific white shrimp *L. vannamei* (Betancourt-Lozano et al., 2006). Due to the fact that molting in crustaceans is mostly controlled by the neuroendocrine system, toxicants may affect the molting process either by inhibiting or stimulating this system (Weis et al., 1992). Thus, some crustaceans may exhibit an opposite reaction as a result of the exposure to toxicants. For example, Mensah et al. (2012a) reported that molting frequency of freshwater shrimp *C. nilotica* was higher in all groups exposed to sub-lethal concentrations of the herbicide Roundup®.

During the 45 days of the current experiment, various behavioral changes were noticed under all concentrations of fipronil, even those in the lowest concentration treatment (0.005 µg/L). Previously reported environmental concentrations of fipronil (tested in this study) mainly affected swimming (mobility) and feeding behaviors of white shrimp in concentration- and time-dependent manners. Many studies have reported similar changes exhibited by different species of non-target aquatic arthropods due to the contamination of fipronil and other neurotoxic pesticides in the environment (Overmyer et al., 2005; Al-Badran et al., 2018). For example, researchers found that post-larval Black Tiger shrimp *Penaeus monodon* exposed to fipronil, imidacloprid, and bifenthin for 20 days exhibited restricted movements and feeding inhibition, and they were more sensitive to fipronil than the other two chemicals (Hook et al., 2018). Similarly, exposing the chironomid midge *C. lebetis* to different concentrations of fipronil (0.5, 2, 5, 10, 15, and 20 µg/L) for 24 to 96 h led to various abnormal behaviors such as feeding depression

and movement limitation at all tested fipronil concentrations (Stratman et al., 2013). Under long-term exposure to low concentrations of DDT, white shrimp, brown shrimp, and pink shrimp showed lethargy, refused their feed, and died; additionally, those under acute concentrations showed the classic symptoms of DDT toxicity such as trembling, hyperkinetic, and paralysis (Couch, 1978).

Such behavioral changes might be a direct consequence of the action of pesticides on the central nervous system of affected organisms (Roque et al., 2005); pesticides cause a disruption of neuron signaling affecting the normal behavioral activities of crustaceans. Therefore, behavioral change is considered a useful biomarker of sub-lethal contamination. Behavioral endpoints, which combine endogenous and exogenous factors, can link physiological and biochemical processes of the animals, consequently providing insights into the effects of environmental contamination from individual to community levels (Tu et al., 2010).

Under the exposure of higher concentrations of fipronil (1.0 μg/L and 3.0 μg/L), juvenile white shrimp showed changes in body color from normal to a dark color distributed evenly on their exoskeleton and internal body parts (Fig. 14). Color change in crustaceans as a result of chemical exposure was reported previously in other studies. For example, Martinez et al. (2014) reported that Pacific white shrimp *L.vannamei* became significantly redder after exposing them to low concentrations (1 mg/L) of copper. In addition, in my previous study on brown shrimp (Al-Badran et al., under review), individuals under all concentrations of imidacloprid and fipronil including 0.005 μg/L exhibited color changes as a result of the exposure. In the current study, I did

not observe color change in white shrimp at the 0.005 μg/L concentration. Color change in crustaceans is a hormonally-regulated process and can be used as an indicator of environmental health (Fingerman et al., 1998; Martinez et al., 2014).

My analysis showed that body chemical composition of white shrimp also was affected by fipronil. Lipids decreased significantly with increasing fipronil ($P = 0.027$). In contrast, ash content showed a significant increase with fipronil concentrations ($P = 0.002$), Protein percentage showed no clear pattern, although higher fipronil concentration (3.0 μg/L) was associated with the lowest level of protein compared with all other treatments including the control (Fig. 15).

Similar findings about the negative effects of chemical pesticides on the body contents of non-target aquatic arthropods have been reported in several studies. In my previous study on brown shrimp juveniles, fipronil has significantly affected the protein and lipid contents of shrimp under exposure of all treatments. The control showed the highest level of protein 71.7 ± 0.23% compared with other treatments; whereas, lipid percentage increased significantly with increasing concentration of fipronil (Al-Badran et al., 2018). Frontera et al. (2011) found that juveniles of freshwater crayfish *C. quadricarinatus* exposed to sub-lethal levels of glyphosate acid and polyoxyethylen amine (POEA) for 50 days exhibited significant reduction in lipid reserves and muscle protein levels. In another study, juvenile mud crab *R. harrisii* exposed to different concentrations of fenoxycarb insecticide showed a significant reduction in total lipid content of their bodies (Nates and McKenney Jr, 2000). Nyman et al. (2013) also reported that lipid content of freshwater amphipod *G. pulex* was reduced significantly as

a result of exposing them to a constant imidacloprid concentration of 15 mg/L. The observed change in the body content and reduced growth of white shrimp in the current study confirm that these organisms use their food energy for detoxification processes rather than growth in polluted environments as reported in many studies (Mensah et al., 2012a; Rozas et al., 2014).

CHAPTER V

CONCLUSIONS

The phenylpyrazole fipronil and the neonicotinoid imidacloprid have been detected in aquatic environments in Texas in recent years due to their increased use in coastal communities (Sneck-Fahrer and East, 2007; Hala, personal communication, May 2019). 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* and white shrimp *L. setiferus*, which are commercially and ecologically important. Therefore, determining the lethal and sub-lethal effects of these insecticides on these particular species was critically important. To this extent, a series of long-term toxicity experiments were conducted to evaluate the adverse effects of fipronil and imidacloprid on brown shrimp and white shrimp, which inhabit the estuaries in the Gulf of Mexico. To better understand and compare these effects, various laboratory tests including estimates of survivorship (mortality), weight gain / growth rates, inter-molt intervals, body chemical composition, and measurement of behavioral and physical changes were conducted.

In Chapter II of this dissertation, I reported the lethal and sub-lethal effects of the previously observed concentrations of fipronil in the environment on juvenile brown shrimp under exposures to five nominal concentrations (0.1, 1.0, 3.0, 6.4, and 10.0 µg/L) over 29 days. Under all of the concentrations tested, fipronil caused both lethal (acute) and sub-lethal (chronic) effects on brown shrimp; exposure to fipronil resulted in all

individuals dying before the end of the experiment. In Chapter III, I investigated the effects of fipronil and imidacloprid, on the growth, survival, and behavior of juvenile brown shrimp over 36 days of exposure. In this experiment, I used the lower concentrations of fipronil than the first study reported in Chapter II. These concentrations included those below the chronic level of the U.S. EPA aquatic life benchmark for invertebrates. The findings from this study suggested that fipronil and imidacloprid had both lethal and sub-lethal effects on brown shrimp. Although brown shrimp were affected less by imidacloprid than fipronil in their survivorship, sub-lethal effects such as delayed molting and reduced growth were still significant under both insecticides. Also, brown shrimp was determined to be one of the most sensitive invertebrates to the exposure to fipronil according to the estimated nominal 96-h LC_{50} of fipronil (0.12 μg/L) in this study. In Chapter IV, I evaluated the adverse effects of fipronil on juvenile white shrimp using the same concentrations tested in Chapter III (0.005, 0.01, 0.1, 1.0, and 3.0 μg/L). The results of this study suggested that juvenile white shrimp were also affected by the exposure to fipronil in terms of all of the endpoints measured. By comparing the findings in Chapter III and IV, it is clear that brown shrimp are far more sensitive to fipronil than white shrimp under exposure to the same concentrations.

Any subtle impacts of fipronil or imidacloprid on the early life stages of brown shrimp and white shrimp could translate into a large impact on their abundance in a later stage, especially that the abundance of adults of some penaeid species has been demonstrated to be highly sensitive to survival during juvenile stage (Baker et al., 2014).

Consequently, this study recommends revising the acute and chronic levels of the U.S. EPA aquatic life benchmarks for fipronil and imidacloprid because of the significant impacts of these insecticides on brown shrimp and white shrimp discovered in this research. Furthermore, continuous monitoring of insecticides concentrations is recommended in estuaries and other areas along the coast of Texas. It will also be important to reduce their usage or their allowable quantities, especially during the seasons of penaeid shrimp migration to inshore annual nursery areas. Further studies of the effects of fipronil and imidacloprid and their major metabolites on other non-target organisms using concentrations below chronic levels established by the U.S. EPA for marine invertebrates are also recommended.

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APPENDIX A

SUPPLEMENTAL TABLES

Supplementary Table A1. Fipronil concentrations observed in the aquatic environment, year, and the place of the survey. * U.S. Geological Survey, Baton Rouge, LA, USA, unpublished data.

Detected concentrations of fipronil in environment (µg/L)	Year of the survey	State/Country	Reference
0.0006 - 0.0086	2002 - 2004	Florida, U.S.A	(Harman-Fetcho et al., 2005)
0.004 -6.41	2006	Louisiana, U.S.A	(Mize et al., 2008)
0.007 - 6.0	1992 - 2001	U.S.A	(Gilliom et al., 2006)
0.0145 - 0.0274	2014	California, U.S.A	(Weston et al., 2015)
0.03	2004 - 2005	Texas, U.S.A	(Sneck-Fahrer and East, 2007)
0.3 - 0.8 *****	1999 - 2001	Louisiana, U.S.A	(Chandler et al., 2004)
0.01 - 4.2	2007 - 2008	California, U.S.A	(Greenberg et al., 2010)
0.28 - 2.11	2008 - 2011	California, U.S.A	(Ensminger et al., 2013)
0.829 - 5.29	2000	Louisiana, U.S.A	(USGS, 2003)
1.0	2010 - 2011	Tsukuba, Japan	(Hayasaka et al., 2012b)
0.0018 - 10.004	2006 - 2008	California, U.S.A	(Gan et al., 2012)
0.09 - 10.004	2003-2012	California, U.S.A	(Ruby, 2013)

Supplementary Table A2. Dilutions of all nominal fipronil concentrations used in the experiment. For steps 1 and 2, I used 2 flasks of 1000 ml and magnetic stirrer to assure the fully homogenize of the experimental solutions.

Fipronil concentration µg/L	**Dilution steps**		
	Step 1	**Step 2**	**Step 3**
	100 mg/L Fipronil suspension	**1 mg/L Fipronil solution**	
0.1	Mix 0.1 g of fipronil powder in 1000 ml of brackish water	Mix 10 ml of 100 mg/L fipronil suspension in 990 ml of brackish water	Mix 2.1 ml of 1 mg/L fipronil solution in (21,000 ml – 2.1 ml) of water
1.0			Mix 21 ml of 1 mg/L fipronil solution in (21,000 ml – 21 ml) of water
3.0			Mix 63 ml of 1 mg/L fipronil solution in (21,000 ml – 63 ml) of water
6.4			Mix 134.4 ml of 1 mg/L fipronil solution in (21,000 ml – 134.4 ml) of water
10.0			Mix 210 ml of 1 mg/L fipronil solution in (21,000 ml – 210 ml) of water

Supplementary Table A3. Dilution procedures for all nominal fipronil concentrations used in the experiment. For steps 1 and 2, magnetic stirrer was used to homogenize the mixture.

Fipronil concentration µg/L	**Dilution steps**		
	Step 1	**Step 2**	**Step 3**
	100 mg/L Fipronil suspension	**0.1 mg/L Fipronil solution**	
0.005	Mix 0.1 g of fipronil powder in 1000 ml of brackish water	Mix 1.0 ml of 100 mg/L fipronil suspension in 999 ml of brackish water	Mix 10 ml of 0.1 mg/L fipronil solution in 990 ml of water to make 1.0 µg/L fipronil solution, then mix 105 ml of 1.0 µg/L fipronil in (21,000 ml – 105 ml) of water
0.01			Mix 10 ml of 0.1 mg/L fipronil solution in 990 ml of water to make 1.0 µg/L fipronil solution, then mix 210 ml of 1.0 µg/L fipronil in (21,000 ml – 210 ml) of water
0.1			Mix 21 ml of 0.1 mg/L fipronil solution in (21,000 ml – 21 ml) of water
1.0			Mix 210 ml of 0.1 mg/L fipronil solution in (21,000 ml – 210 ml) of water
3.0			Mix 630 ml of 0.1 mg/L fipronil solution in (21,000 ml – 630 ml) of water

Supplementary Table A4. Dilution procedures for all nominal imidacloprid concentrations used in the experiment. For steps 1 and 2, magnetic stirrer was used to homogenize the mixture.

Imidacloprid concentration µg/L	**Dilution steps**		
	Step 1	**Step 2**	**Step 3**
	10 mg/L Imidacloprid solution	**1000µg/L Imidacloprid solution**	
0.5	Mix 0.01 g of Imidacloprid powder in 1000 ml of brackish water	Mix 100 ml of 10 mg/L Imidacloprid solution in 900 ml of brackish water	Mix 10.5 ml of 1000 µg/L Imidacloprid solution in (21,000 ml – 10.5 ml) of water
1.0			Mix 21 ml of 1000 µg/L Imidacloprid solution in (21,000 ml – 21 ml) of water
15.0			Mix 315 ml of 1000 µg/L Imidacloprid solution in (21,000 ml – 315 ml) of water
34.5			Mix 724.5 ml of 1000 µg/L Imidacloprid solution in (21,000 ml – 724.5 ml) of water
320.0			Mix 67.2 ml of 10mg/L Imidacloprid solution in (21,000 ml – 67.2 ml) of water

Supplementary Table A5. Initial weight (g), final weight (g), and percent weight gain (mean ± 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, $P < 0.05$).

Fipronil concentration (µg/L)	Initial weight (g)	n	Final weight (g)	n	% Weight gain
Control	0.58 ± 0.04 a	18	1.31 ± 0.07 a	18	125.92 ± 28.42 a
0.005	0.59 ± 0.03 a	18	1.30 ± 0.03 a	18	120.17 ± 15.16 a
0.01	0.56 ± 0.04 a	18	1.13 ± 0.1 b	13	104.18 ± 28.62 ab
0.1	0.58 a	18	1.02 ± 0.12 b	6	77.007 ± 21.83 b
1.0	0.58 ± 0.08 a	18	0	0	0
3.0	0.58 ± 0.04 a	18	0	0	0

Supplementary Table A6. Initial weight (g), final weight (g), and percent weight gain (mean ± 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, $P < 0.05$).

Imidacloprid concentrations (µg/L)	Initial weight (g)	n	Final weight (g)	n	% Weight gain
Control	0.81 ± 0.03 a	15	1.95 ± 0.12 a	15	140.3 ± 16.15 a
0.5	0.84 ± 0.11 a	15	1.87 ± 0.13 ab	14	126.89 ± 46.88 a
1.0	0.81 ± 0.03 a	15	1.65 ± 0.18 b	13	103.39 ± 27.92 ab
15.0	0.80 ± 0.06 a	15	1.31 ± 0.12 c	10	64.40 ± 17.14 bc
34.5	0.84 ± 0.06 a	15	1.21 ± 0.05 cd	6	44.01 ± 12.09 c
320.0	0.80 ± 0.09 a	15	1.04 ± 0.13 d	5	29.48 ± 16.43 c

Supplementary Table A7. Length (cm) of juvenile shrimp (mean ± 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, $P < 0.05$).

Fipronil concentrations (µg/L)	Initial length (cm)	Length week 1 (cm)	Length week 2 (cm)	Length week 3 (cm)	Length week 4 (cm)	Length week 5 (cm)
Control	4.46 ± 0.09 (n = 18) a	4.84 ± 0.09 (n = 18) a	5.25 ± 0.05 (n = 18) a	5.62 ± 0.05 (n = 18) a	5.98 ± 0.12 (n = 18) a	6.32 ± 0.10 (n = 18) a
0.005	4.44 ± 0.01 (n = 18) a	4.79 ± 0.02 (n = 18) ab	5.07 ± 0.07 (n = 18) a	5.50 ± 0.04 (n = 18) ab	5.91 ± 0.05 (n = 18) ab	6.32 ± 0.03 (n = 18) a
0.01	4.38 ± 0.11 (n = 18) a	4.64 ± 0.15 (n = 14) bc	5.02 ± 0.18 (n = 14) a	5.27 ± 0.08 (n = 13) bc	5.69 ± 0.13 (n = 13) bc	5.99 ± 0.16 (n = 13) ab
0.1	4.43 ± 0.03 (n = 18) a	4.58 ± 0.10 (n = 8) c	4.63 ± 0.24 (n = 6) b	5.05 ± 0.25 (n = 6) c	5.49 ± 0.18 (n = 6) c	5.87 ± 0.33 (n = 6) b
1.0	4.37 ± 0.15 (n = 18) a	/	/	/	/	/
3.0	4.39 ± 0.07 (n = 18) a	/	/	/	/	/

Supplementary Table A8. Length (cm) of juvenile shrimp (mean ± 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, $P < 0.05$).

Imidacloprid concentrations (µg/L)	Initial length (cm)	Length week 1 (cm)	Length week 2 (cm)	Length week 3 (cm)	Length week 4 (cm)	Length week 5 (cm)
Control	5.28 ± 0.1 (n = 15) a	5.83 ± 0.09 (n = 15) a	6.22 ± 0.09 (n = 15) a	6.57 ± 0.09 (n = 15) a	6.92 ± 0.17 (n = 15) a	7.07 ± 0.12(n = 15) a
0.5	5.32 ± 0.09(n = 15) a	5.8 ± 0.21(n = 15) a	6.17 ± 0.2(n = 14) ab	6.55 ± 0.22(n = 14) a	6.77 ± 0.2(n = 14) ab	6.96 ± 0.18(n = 14) a
1.0	5.33 ± 0.15(n = 15) a	5.67 ± 0.13(n = 14) a	5.92 ± 0.26(n = 14) abc	6.28 ± 0.27(n = 14) a	6.48 ± 0.31(n = 14) bc	6.72 ± 0.24(n = 13) a
15.0	5.28 ± 0.21(n = 15) a	5.59 ± 0.15(n = 15) a	5.79 ± 0.18(n = 15) bc	5.86 ± 0.24(n = 15) b	6.12 ± 0.2(n = 11) cd	6.25 ± 0.24(n = 10) b
34.5	5.38 ± 0.11(n = 15) a	5.52 ± 0.09(n = 15) a	5.58 ± 0.13(n = 15) c	5.7 ± 0.08(n = 11) b	5.79 ± 0.11(n = 9) d	5.98 ± 0.05(n = 6) b
320.0	5.29 ± 0.2(n = 15) a	5.63 ± 0.29(n = 15) a	5.66 ± 0.35(n = 15) c	5.82 ± 0.32(n = 12) b	5.97 ± 0.31(n = 9) d	5.91 ± 0.23(n = 5) b

Supplementary Table A9. Percentage of live individuals (mean ± standard deviation) of juvenile shrimp in fipronil experiment starting from day1 to the end of the experiment. 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).

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

Supplementary Table A10. Percentage of live individuals (mean ± standard deviation) of juvenile shrimp in imidacloprid experiment starting from day1 to the end of the experiment. 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).

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

Supplementary Table A11. Water quality parameters of shrimp aquariums during 34 days of fipronil experiment. Values are Mean ± standard deviation for each parameter of all fipronil concentrations. Treatment 1.0 µg/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 µg/L has no water quality parameters because all shrimp died during the first day of the exposure.

Fipronil concentrations (µg/L)	**Water quality parameters**			
	Temp. °C	**DO mg/L**	**Salinity ‰**	**pH**
Control	24.24 ± 0.18	5.32 ± 0.59	15.16 ± 0.37	7.90 ± 0.17
0.005	24.08 ± 0.16	5.67 ± 0.27	14.95 ± 0.78	8.02 ± 0.13
0.01	24.07 ± 0.19	5.56 ± 0.49	15.12 ± 0.42	8.02 ± 0.12
0.1	24.14 ± 0.15	6.08 ± 0.25	15.14 ± 0.39	8.07 ± 0.12
1.0	24.10	6.5	14.77	8.07
3.0	/	/	/	/

Supplementary Table A12. Water quality parameters of shrimp aquariums during 36 days of imidacloprid experiment. Values are Mean ± standard deviation for each parameter of all imidacloprid concentrations.

Imidacloprid concentrations (µg/L)	Water quality parameters			
	Temp. °C	DO mg/L	Salinity ‰	pH
Control	24.34 ± 0.19 a	5.86 ± 0.52	15.51 ± 0.34	8.08 ± 0.09
0.5	24.4 ± 0.15	5.9 ± 0.29	15.36 ± 0.12	8.10 ± 0.05
1.0	24.32 ± 0.16	5.8 ± 0.26	15.39 ± 0.15	8.12 ± 0.04
15.0	24.34 ± 0.11	5.94 ± 0.75	15.60 ± 0.12	8.20 ± 0.04
34.5	24.34 ± 0.16	5.8 ± 0.26	15.53 ± 0.14	8.19 ± 0.06
320.0	24.3 ± 0.12	5.96 ± 0.48	15.49 ± 0.11	8.22 ± 0.07

Supplementary Table A13. Dilution procedures for the nominal concentrations of fipronil used in the experiment. Magnetic stirrer was used to homogenize the mixture in steps 1 and 2.

Fipronil concentration µg/L	Dilution steps		
	Step 1	Step 2	Step 3
	100 mg/L Fipronil suspension	0.1 mg/L Fipronil solution	
0.005	Mix 0.1 g of fipronil powder in 1000 ml of brackish water	Mix 1.0 ml of 100 mg/L fipronil suspension in 999 ml of brackish water	Mix 10 ml of 0.1 mg/L fipronil solution in 990 ml of water to make 1.0 µg/L fipronil solution, then mix 70 ml of 1.0 µg/L fipronil in (14,000 ml – 70 ml) of water
0.01			Mix 10 ml of 0.1 mg/L fipronil solution in 990 ml of water to make 1.0 µg/L fipronil solution, then mix 140 ml of 1.0 µg/L fipronil in (14,000 ml – 140 ml) of water
0.1			Mix 14 ml of 0.1 mg/L fipronil solution in (14,000 ml –14 ml) of water
1.0			Mix 140 ml of 0.1 mg/L fipronil solution in (14,000 ml – 140 ml) of water
3.0			Mix 420 ml of 0.1 mg/L fipronil solution in (14,000 ml – 420 ml) of water

Supplementary Table A14. Water quality parameters of shrimp aquariums during 45 days of the experiment. Values are Mean ± standard deviation.

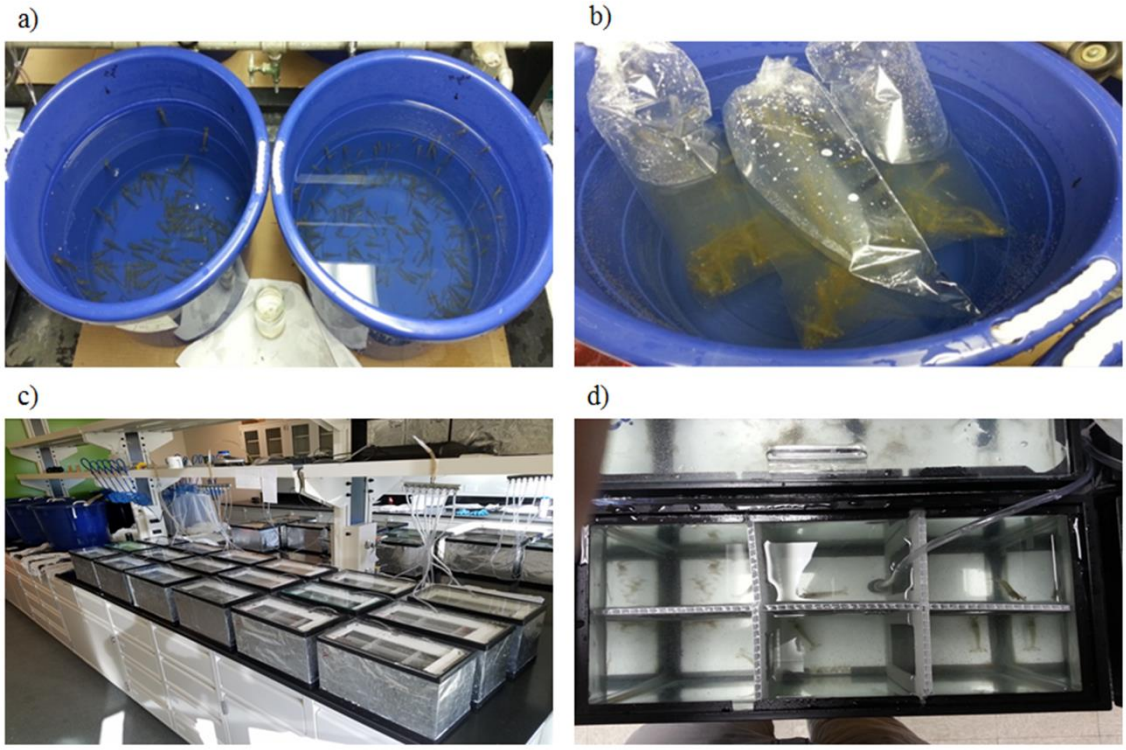
Fipronil concentrations (µg/L)	Water quality parameters			
	Temp. °C	DO mg/L	Salinity ‰	pH
Control	21.22 ± 0.44	5.39 ± 0.29	15.08 ± 0.23	8.09 ± 0.25
0.005	21.25 ± 0.17	5.36 ± 0.26	15.17 ± 0.39	8.18 ± 0.33
0.01	21.08 ± 0.22	5.49 ± 0.25	15.21 ± 0.18	8.23 ± 0.29
0.1	20.96 ± 0.25	5.28 ± 0.18	15.19 ± 0.30	8.25 ± 0.30
1.0	20.92 ± 0.33	5.63 ± 0.52	15.18 ± 0.22	8.27 ± 0.34
3.0	20.95 ± 0.29	5.81 ± 0.35	15.12 ± 0.23	8.28 ± 0.37

Supplementary Table A15. Initial weight (g), final weight (g), and % weight gain (mean ± standard deviation) of juvenile white shrimp exposed to different concentrations of fipronil. n = number of replicates in each treatment. All values were calculated based on the wet weight per individual shrimp. Values with star (*) indicate treatment is significantly different from the control (ANOVA, $P < 0.05$). All shrimp in treatment 3.0 µg/L died before reaching the final day of the experiment (day 45), thus no final weight or % weight gain calculated for this treatment.

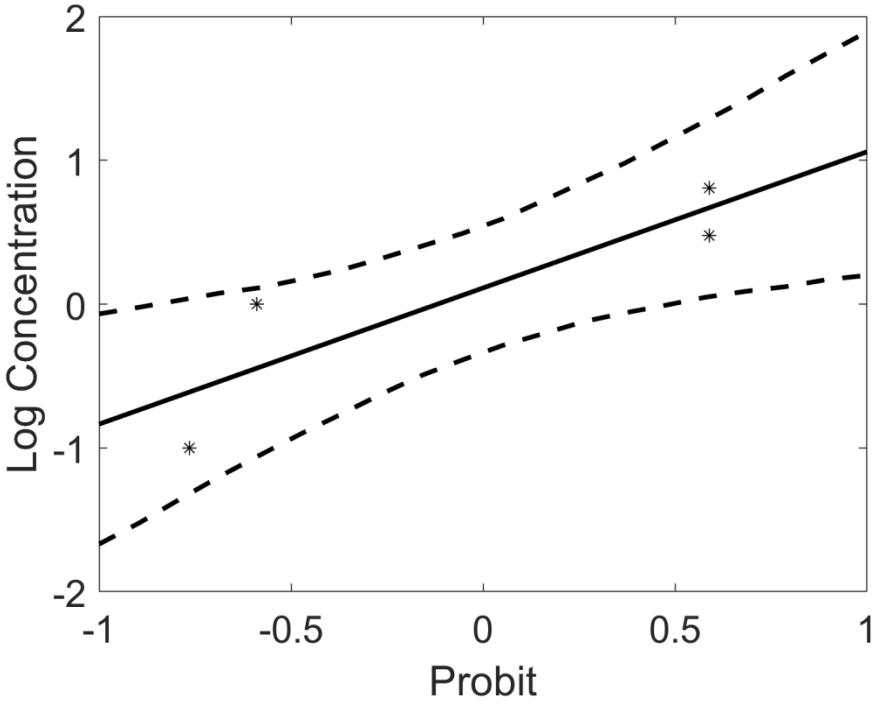
Fipronil concentrations (µg/L)	Initial weight (g)	Final weight (g)	% Weight gain
Control	0.78 ± 0.02 (n = 2)	1.19 ± 0.01 (n = 2)	51.62 ± 2.29 (n = 2)
0.005	0.70 ± 0.02 (n = 2)	0.99 ± 0.007 (n = 2)	41.21* ± 5.25 (n = 2)
0.01	0.76 ± 0.10 (n = 2)	1.02 ± 0.16 (n = 2)	33.79* ± 2.70 (n = 2)
0.1	0.83 ± 0.03 (n = 2)	0.98 ± 0.04 (n = 2)	17.36* ± 0.11 (n = 2)
1.0	0.78 ± 0.007 (n = 2)	0.38* ± 0.53 (n = 1)	-2.56* (n = 1)
3.0	0.93 ± 0.04 (n = 2)		

APPENDIX B

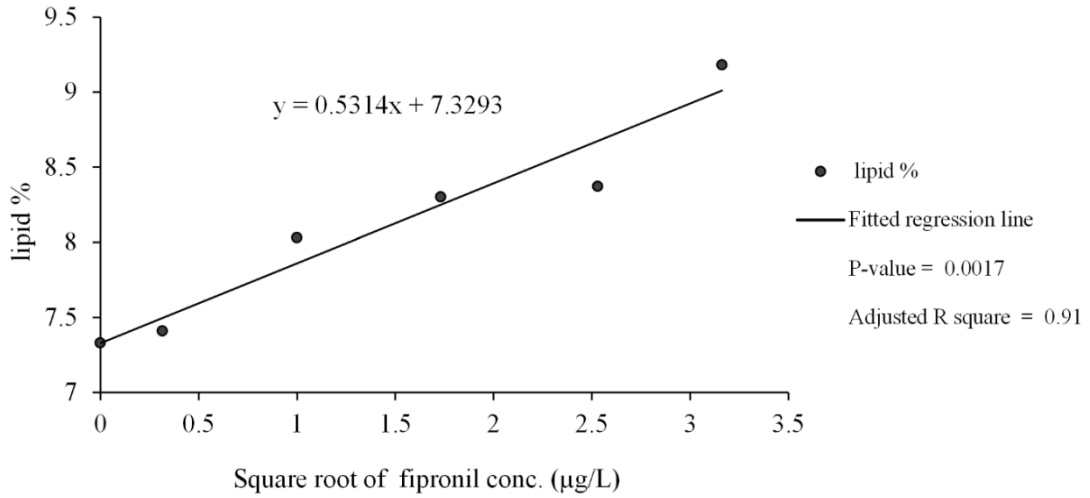
SUPPLEMENTAL FIGURES



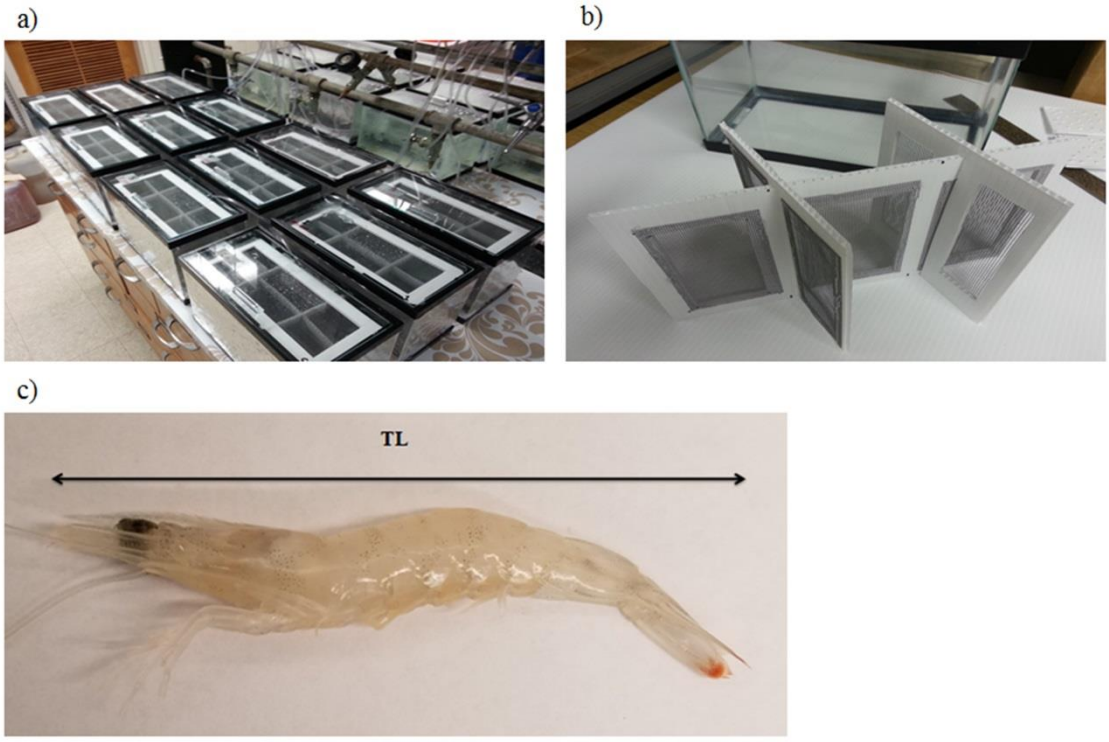
Supplementary Figure B1. Acclimation tanks and experimental system used for juvenile *F. aztecus* laboratory experiments: (a) large tanks used for water temperature equilibration and shrimp acclimation before starting the trials; (b) procedure used for moving shrimp to tanks of prepared brackish water for acclimation to laboratory conditions; (c) experimental system, glass aquariums covered with aluminum foil sheets and glass lids; (d) aquariums divided into six separate cells.



Supplementary Figure B2. Probit analysis used to calculate the 96-h LC_{50} of fipronil. The horizontal axis represents the probit as independent variable while the vertical axis represents the log concentration as dependent variables. Dashed lines show the 95% confidence intervals of the LC_{50} toxicity test calculated using the parametric bootstrap method.

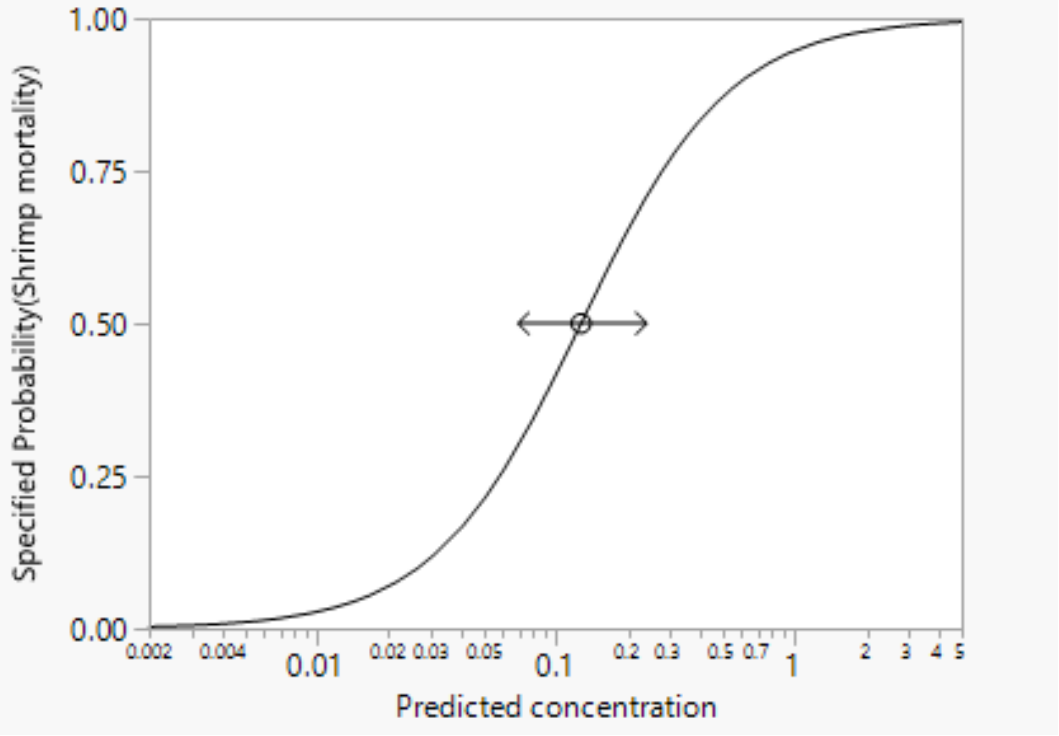


Supplementary Figure B3. Linear regression of lipids % of shrimp bodies measured under different concentrations of fipronil. The horizontal axis represents the square root of fipronil concentrations (µg/L), and the vertical axis represents the lipid % in bodies of juvenile shrimp measured at the end of the experiment (n = 2 samples analyzed from each treatment). Linear regression analysis (P = 0.0017) indicated that lipid % increased significantly with increasing concentration of fipronil.



Supplementary Figure B4. Experimental system and the aquariums used in the experiments: (a) experimental system consists of 12 glass aquarium covered from the top with fiberglass screen and glass lid; (b) barriers of polypropylene plates and fiberglass screen used to divide the aquarium; (c) total length of shrimp measured every week during the experiments.

Specified Probability(Shrimp mortality)	Predicted concentration	Lower 95%	Upper 95%
0.5000000	0.1275412	0.0694389	0.2404084



Supplementary Figure B5. Lethal concentration of fipronil to reach 50% mortality of shrimp within 96 hours (96-h LC_{50}) of the juvenile brown shrimp.