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DARLAN GUSSO

EFEITO DO PIRIPROXIFENO SOBRE O DESENVOLVIMENTO, PARÂMETROS COMPORTAMENTAIS E ENDÓCRINOS EM LARVAS E ADULTOS DE PEIXE-ZEBRA (DANIO RERIO)

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Efeito do Piriproxifeno sobre o desenvolvimento, parâmetros comportamentais e endócrinos em larvas e adultos de Peixe-zebra (*Danio rerio*)

Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular como requisito para obtenção do título de Mestre.

DARLAN GUSSO

Orientadora: Profa. Dra. Carla Denise Bonan

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Joseph Addison

RESUMO

Mosquitos são responsáveis pela transmissão de vários agentes patogênicos. Nos últimos três anos, o mundo testemunhou epidemias que afetam todas as faixas etárias, tais como dengue, febre amarela, chikungunya e zika vírus. Para controlar e eliminar vetores do gênero *Aedes*, responsável pela transmissão destas doenças, é necessário usar larvicidas e inseticidas como o piriproxifeno. O piriproxifeno é um larvicida usado para eliminar mosquitos, principalmente do gênero *Aedes*. Este estudo avaliou os efeitos do piriproxifeno sobre a sobrevivência, morfologia, comportamento e parâmetros endócrinos em diferentes estágios de desenvolvimento de peixe-zebra. Nós demonstramos que o piriproxifeno pode causar alterações na taxa de sobrevivência, comportamento e morfologia de larvas expostas, adultos expostos no estágio larval e descendentes adultos de pais expostos. As larvas expostas ao piriproxifeno durante 1-96 hpf (horas pós-fertilização) mostraram uma diminuição na taxa de sobrevivência, frequência cardíaca 3 dpf e comprimento corporal nos 5 e 8 dpf (dias pós-fertilização). A distância percorrida e a velocidade média das larvas expostas foram reduzidas em relação ao grupo veículo (1% de DMSO). Portanto, o piriproxifeno altera a morfologia e o comportamento do peixe-zebra em estágios iniciais de desenvolvimento e pode afetar as próximas gerações. No entanto, quando as larvas expostas foram mantidas até o estágio adulto (6 meses), não observamos diferenças nos parâmetros locomotores. A exposição parental ao piriproxifeno induziu uma diminuição na locomoção da prole adulta, bem como um comportamento do tipo ansiolítico. Nós também avaliamos o efeito da exposição ao piriproxifeno em peixe-zebra na fase adulta durante 96 horas sobre a locomoção, ansiedade, memória e parâmetros endócrinos, como expressão gênica do receptor de glicocorticoide (GR) e fator de liberação de corticotrofina (CRF). Os resultados demonstraram que não houve diferença significativa na locomoção, ansiedade e parâmetros endócrinos. Além disso, houve um prejuízo da memória de habituação do peixe-zebra adulto exposto à piriproxifeno. Portanto, é importante controlar o uso de larvicidas devido aos seus efeitos tóxicos em espécies não-alvo. Nossos achados demonstram a importância de estudos relacionados com a utilização de larvicidas, uma vez que são potenciais causadores de alterações morfológicas e comportamentais em espécies aquáticas, como o peixe-zebra.

Palavras-chave: comportamento; memória; larvicida; Piriproxifeno; peixe-zebra

ABSTRACT

Mosquitoes are responsible for the transmission of various pathogens. Over the past three years, the world has witnessed epidemics affecting all age groups, such as dengue fever, yellow fever, chikungunya and zika virus. To control and eliminate *Aedes* vectors responsible for the transmission of these diseases, it is necessary to use larvicides and insecticides such as pyriproxyfen. Pyriproxyfen is a larvicide used to control mosquitoes, mainly of the genus *Aedes*. This study evaluated the effects of pyriproxyfen on survival, morphology, behavior, and endocrine parameters at different stages of zebrafish development. We have demonstrated that pyriproxyfen can cause changes in the survival rate, behavior and morphology of exposed larvae, adults exposed at the larval stage, and adult offspring of exposed parents. Larvae exposed to pyriproxyfen for 1-96 hpf (hours post-fertilization) showed a decrease in survival rate, heart rate 3 dpf (days post-fertilization), and body length at 5 and 8 dpf. The distance traveled and the mean velocities of the exposed larvae were reduced in comparison to the vehicle group (1% DMSO). Therefore, pyriproxyfen changes the morphology and behavior of zebrafish in early stages of development and may affect the next generations. However, when the exposed larvae were maintained until the adult stage (6 months), we did not observe differences in locomotor parameters. Parental exposure to pyriproxyfen induced decrease in locomotion of zebrafish adult offspring as well as an anxiolytic-like behavior. We also tested pyriproxyfen exposure for 96 hours in adult zebrafish and the locomotion, anxiety, memory and endocrine parameters were analyzed as well as the gene expression of the glucocorticoid receptor (GR) and corticotropin-releasing factor (CRF). These results showed that there was no significant difference in locomotion, anxiety and endocrine parameters. In addition, there was an impairment of habituation memory in adult zebrafish exposed to pyriproxyfen. Therefore, it is important to control the use of larvicides due to their toxic effects on non-target species. Our findings demonstrated the importance of studies related to the use of larvicides, since they are potential causes of morphological and behavioral alterations in aquatic species, such as zebrafish.

Keywords: behavior; development; memory; larvicide; Pyriproxyfen; zebrafish

LISTA DE ABREVIATURAS

BHE - Barreira hematoencefálica

CHKU - Chikungunya

CL50 – Concentração letal

DENV - Vírus da dengue

DMSO - Dimetilsulfóxido

dpf - dias pós-fertilização

GFP - Proteína fluorescente verde

hpf - hora pós-fertilização

JH - Hormônio juvenil

P34 - Piriproxifeno

ppb - parte por bilhão

ppt – parte por trilhão

SNC – Sistema nervoso central

ZIKV - Zika vírus

LISTA DE FIGURAS

SUMÁRIO

CAPÍTULO 1

INTRODUÇÃO, JUSTIFICATIVA E OBJETIVOS

1. INTRODUÇÃO

1.1. Transmissão do Zika vírus

Mosquitos são responsáveis pela transmissão de uma variedade de agentes infecciosos potenciais causadores de doenças. Esses vetores estão bem adaptados em ambientes domésticos, principalmente nas regiões tropicais. Os vetores do gênero *Aedes* estão disseminados em todos os estados do Brasil (KRAEMER et al., 2015). Desde o ano de 2015, o Brasil foi acometido por três principais epidemias, vírus da dengue (DENV), chikungunya (CHKV), e zika vírus (ZIKV), sendo que o chikungunya e zika vírus podem atingir os níveis de incidência do vírus da dengue, o qual está endêmico em quase todos os estados do Brasil desde a década de 1980 (MACHADO et al., 2017). Além dos vírus da dengue (DENV), chikungunya (CHKV) e zika (ZIKV), apresentam-se também o vírus da febre amarela e o parasita causador da malária, tendo como principal forma de transmissão os mosquitos do gênero *Aedes,* principalmente das espécies *A. aegypti* e *A. albopictus* (TILAK et al., 2016). O zika vírus demandou acompanhamento pelos serviços de saúde pública devido à epidemia generalizada ocorrida no Brasil entre 2015-2016 (IMPERATO, 2016), descrita como a maior nas Américas (ARAUJO et al., 2016). A epidemia pelo zika vírus teve um maior foco de disseminação, principalmente nas áreas mais quentes com temperaturas que favorecem a criação exacerbada do mosquito e que ultrapassam as formas de controle. Outras vias foram também descritas como possíveis formas de disseminação do vírus, através do contato durante relações sexuais e pela via intrauterina (Igbinosa et al., 2017).

Os principais obstáculos a serem contornados são os casos assintomáticos ou que desenvolvem sinais brandos, os quais consistem em febre em menos de 20% dos casos (CHAN et al., 2016), dor de cabeça, mal-estar, artralgia, conjuntivite e erupções maculopapulares (IMPERATO, 2016). A transmissão vertical foi observada onde bebês estavam nascendo em áreas afetadas pelo zika vírus e o RNA do zika foi identificado no líquido amniótico de duas mulheres cujos fetos foram diagnosticados com microcefalia por ultrassonografia pré-natal (DE ARAÚJO et al., 2016; SCHULER-FACCINI et al., 2016). Além disso, casos de microcefalia aumentaram consideravelmente em estados onde o zika vírus havia sido reportado como endêmico (Figura 1) (MAESTRE et al, 2016; MARGOTTO, 2016).

O primeiro caso de zika vírus nas Américas foi confirmado em janeiro de 2014 no Chile. No Brasil, foi identificado em maio de 2015 (PAHO, 2015) e, a partir da confirmação desse caso, o Ministério da Saúde desenvolveu estratégias de controle, tais como a intensificação no uso de larvicidas, inclusive em água potável (REIS, 2016), a qual é utilizada diariamente nas residências.

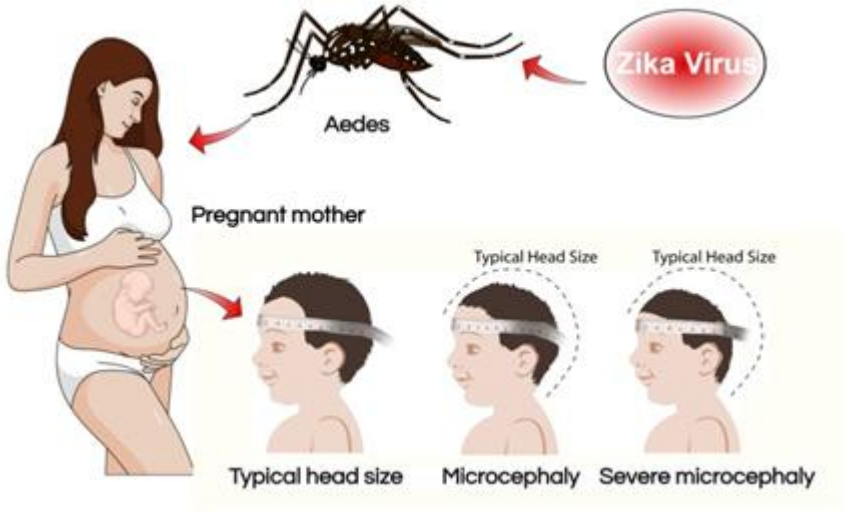

Figura 1- Zika vírus associado com microcefalia nos fetos em mulheres grávidas infectadas. Fonte: Adaptado de RATHER, 2017.

1.2. Controle populacional de vetores

O controle populacional de vetores é a forma mais eficaz para prevenção de epidemias. Uma diversidade de métodos que vão desde repelentes até larvicidas e inseticidas são recomendados pelo Ministério da Saúde (BRASIL. MINISTÉRIO DA SAÚDE, 2014; FAWELL et al., 2008). A limitação do uso de repelentes se deve ao fato de que estas substâncias apenas afastam temporariamente os mosquitos, deixando ainda assim a população exposta, agindo apenas como um método complementar.

A melhor opção para o controle de insetos é a destruição de criadouros e interrupção do ciclo de vida dos mosquitos. Para isso, utiliza-se larvicidas e inseticidas rotineiramente em muitos países (WANG et al., 2013). Testes laboratoriais demonstraram que o uso de larvicidas e inseticidas expressaram eficácia no controle de populações de mosquito (DEVILLERS., et al. 2015). Quando aplicados de forma combinada, larvicidas e inseticidas com diferentes mecanismos de ação aumentam a eficácia, atuando em todos os estágios de desenvolvimento dos insetos (WANG et al., 2013).

Durante muitos anos, o larvicida mais utilizado para controle de mosquitos tendo como principal foco o gênero *Aedes* foi o Temefós, um organofosforado que atua como inibidor irreversível da acetilcolinesterase (CAVALIERI et al., 1996). Além do potencial efeito inseticida, a aplicação do Temefós indiretamente proporciona neurotoxicidade em vertebrados (JUREWICZ., et al. 2013), e alterações genéticas nos camundongos onde o temefós induz a formação de micronúcleos em eritrócitos policromáticos (PCEMN) nos animais testados (MELO et al., 2008). A prática de utilização de larvicidas e inseticidas permite que, cada vez mais, se expandam as plantações e também o controle de insetos que são potenciais vetores para doenças. No entanto, estes compostos podem prejudicar a população com efeitos negativos à saúde e ao meio ambiente (BURATTI et al., 2003).

O controle de populações específicas de animais exige cuidados generalizados para que não haja interferência negativa em espécies não-alvo. Além disso, o fato de mosquitos do gênero *Aedes* terem formado seleções de gerações resistentes ao Temefós (ABE et al., 2014; LIMA et al., 2006) contribui para a substituição deste larvicida. Em função disso, como protocolo de prevenção e eliminação de focos do mosquito foi implementada a utilização de Diflubenzuron, uma benzoilfeniluréia que tem como mecanismo de ação a inibição da síntese de quitina (BELINATO., et al. 2015). Nos últimos anos, o Ministério da Saúde intensificou a prática de aplicação do larvicida fisiológico Piriproxifeno, principalmente em locais de maior incidência de zika vírus (BRASIL. MINISTÉRIO DA SAÚDE, 2014; FAWELL et al., 2008).

1.3. Piriproxifeno

O Piriproxifeno (P34) pertence ao grupo químico éter piridiloxipropílico e é classificado como um inseticida, sendo considerado um análogo do hormônio juvenil de insetos e um potente inibidor da embriogênese e metamorfose (FAWELL et al., 2008; ISHAAYAa; HOROWITZ, 1992). O hormônio juvenil (JH) regula muitos processos biológicos e metabólicos, e tem ação na regulação de *kr-h1* fator de transcrição regulado pelos (JH) (XU; ROY; PALLI, 2018). Este processo de regulação não está bem elucidado, mas sabe-se que há correlação entre a reprodução e o fator de transcrição *kr-h1* e esse pode afetar diretamente a função reprodutiva de insetos, reduzindo em até 46% a quantidade de ovos (OJANI et al., 2018). O inseticida piriproxifeno impede que os insetos desenvolvam características da fase adulta, alterando processos fisiológicos essenciais (SIDALL, 1976). Atuando no sistema

endócrino de insetos como análogo do hormônio juvenil, o piriproxifeno dificulta a ecdise e, subsequentemente inibe a reprodução de insetos (MAOZ et al., 2017). Para os JH, sua alta estabilidade lhes permite competir pelos receptores do site de ligação do JH (RIDDIFORD, 1994). Em exposição tópica de abelhas ao piriproxifeno, observou-se que ocorrem anormalidades morfológicas, incluindo a deformação das asas (FOURRIER et al., 2015). Sua estrutura química é composta por 4-fenoxifenil(RS)-2-(2-piridiloxi)propil-éter, e sua fórmula molecular é $C_{20}H_{19}NO_3$. O piriproxifeno na formulação granulada para utilização em água potável é composto de areias vulcânicas e pode bioacumular por um período que ultrapassa oito semanas e se manter biodisponível no ambiente (BRASIL. MINISTÉRIO DA SAÚDE, 2014; FAWELL et al., 2008). A classificação toxicológica de risco está registrada em classe IV, sendo autorizado para uso (FAWELL et al., 2008).

Dentre seus benefícios, essa molécula possui uma maior persistência no ambiente e na sua composição encontram-se surfactantes, os quais mantém a liberação lenta na água, atuando por um período que pode ultrapassar oito semanas (FAWELL et al., 2008).

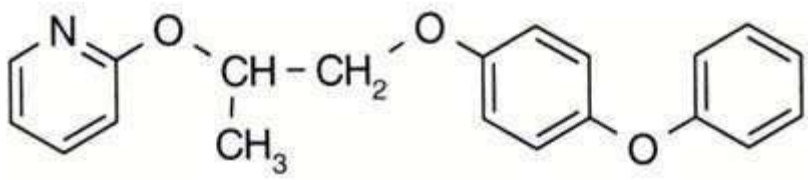

Figura 2 – Estrutura química do larvicida Piriproxifeno, Fonte: ANVISA, 2008.

Sua utilização tem por objetivo prevenir a disseminação de infestações danosas causadas por insetos em diversas plantações (MELLO, 2008), e também promover o controle de mosquitos do gênero *Aedes* (BRASIL. MINISTÉRIO DA SAÚDE, 2014; FAWELL et al., 2008; PALMA; MEOLA; MEOLA, 1993) com ampla distribuição geográfica. Atualmente, sua utilização está direcionada para prevenção da proliferação de *Aedes aegypti* e *Aedes albopictus* (TILAK et al., 2016). Muitos países utilizam esse composto em agricultura diversificada, como é o caso da Argentina, onde comumente é usado em culturas hortifrutícolas para o controle de pragas e considerado como inseticida seletivo por ter um menor impacto ambiental do que os inseticidas convencionais (FOGEL et al., 2016).

Em *Aedes aegypti*, estudos demonstraram que o Piriproxifeno na concentração de 0,012 partes por bilhão (ppb) promoveu um efeito considerado suficiente para definir a CL50 (ISHAAYA; HOROWITZ, 1992). Quando sua eficácia foi testada após a exposição em pulgas adultas *Ctenocephalides felis* durante 70 horas, observou-se que os ovos estavam desprovidos de gema e frequentemente colapsavam (PALMA; MEOLA; MEOLA, 1993). Em ratos, foi observado que a utilização do piriproxifeno provocou alterações tóxicas no fígado, quando administrado durante seis meses através da dieta, causando hipertrofia do hepatócito e aumento de tamanho dos rins (KOYAMA et al., 1989), sugerindo a ocorrência de efeitos tóxicos em mamíferos.

O piriproxifeno é utilizado para combater a proliferação de muitas espécies de insetos, sendo que seu uso aumentou consideravelmente após sua introdução no mercado dos agroquímicos (SIHUINCHA et al., 2005). É indicado principalmente em locais identificados como focos para proliferação de mosquitos, como também na concentração de 0,01 mg/L, sendo permitido o uso na água potável (BRASIL. MINISTÉRIO DA SAÚDE, 2014; FAWELL et al., 2008). Com objetivo de quantificar os níveis de inseticidas em amostras de água coletadas do rio Júcar, situado na Espanha, análises determinaram concentrações aproximadas de 90 ng/L de piriproxifeno (BELENGUER et al., 2014). Estes achados devem-se, principalmente, a aplicações contínuas deste larvicida, mas também pela persistência do produto no meio aquático, o qual pode permanecer por várias semanas.

1.4. Peixe-zebra (*Danio rerio*)

Dentre os modelos *in vivo* com aplicabilidade para estudos científicos, encontra-se o Peixe-zebra (*Danio rerio*). É um teleósteo de água doce, da família Cyprinidae e proveniente do continente asiático que, nas últimas décadas, tem sido utilizado para o desenvolvimento de estudos em diversas áreas, tais como farmacologia (ABREU et al., 2014; IDALENCIO et al., 2015; SIEBEL et al., 2015), endocrinologia (BARCELLOS et al., 2014; GIACOMINI et al., 2015), biologia do desenvolvimento (VILLAMIZAR et al., 2014) e hematologia (THISSE, 2002). O peixe-zebra também tem sido utilizado na forma adulta ou larval para estudos em neurociências, visando uma maior compreensão da função cerebral e também na modulação farmacológica (KALUEFF; STEWART; GERLAI, 2014; MIKLÓSI; ANDREW, 2006).

Diversas tarefas comportamentais já foram caracterizadas nesta espécie, permitindo avaliar a atividade locomotora, memória e aprendizado (AL-IMARI; GERLAI, 2008; BILOTTA et al., 2005; ZIMMERMANN et al., 2015), agressividade (OLIVEIRA et al., 2013) e comportamento social (MILLER; GERLAI, 2008).

O peixe-zebra mostra-se um excelente modelo com bastante confiabilidade em estudos toxicológicos (BARROS et al., 2008a). Essa espécie tem sido utilizada para analisar a toxicidade de substâncias químicas (HILL, 2005) e de fármacos em estágios iniciais de desenvolvimento (BARROS et al., 2008b). Um dos importantes aspectos na neurotoxicologia em larvas é o conhecimento sobre o desenvolvimento e transporte de compostos pela barreira hematoencefálica (BHE) (UMANS; TAYLOR, 2012). Em larvas de peixe-zebra com 3 dias pós-fertilização (dpf), já ocorre uma passagem restritiva de compostos (JEONG et al., 2008), sendo que a barreira hematoencefálica é funcionalmente similar à de mamíferos, reforçando que o peixe-zebra é um modelo confiável para estudos sobre efeitos de compostos químicos (ELICEIRI; GONZALEZ; BAIRD, 2011; UMANS; TAYLOR, 2012).

Múltiplos fatores direcionam a escolha desta espécie como um modelo biológico experimental, tais como a alta fertilidade e prolificidade, onde uma única fêmea pode produzir um total de 200 ovos por semana quando mantida sob condições favoráveis (DAHM; GEISLER, 2006). Em condições de laboratório, o peixe-zebra desova em todos os períodos do ano (BRAND; GRANATO; NÜSSLEIN-VOLHARD, 2002), com maturidade sexual a partir dos 3 - 4 meses e expectativa de vida de 2 - 4 anos, sendo que uma fêmea pode atingir o total de 15.000 a 35.000 ovos (BRAND; GRANATO; NÜSSLEIN-VOLHARD, 2002; DAHM; GEISLER, 2006). Outra vantagem desta espécie é o tamanho relativamente pequeno, em torno de 3-4 cm (KALUEFF; STEWART; GERLAI, 2014). Além disso, larvas podem viver em placas de 96 poços (GOLDSMITH, 2004), o que facilita a manipulação e distribuição de compostos químicos em menor quantidade. A fecundação e o desenvolvimento externos, além da transparência dos embriões (ELICEIRI; GONZALEZ; BAIRD, 2011; LIESCHKE; CURRIE, 2007) permitem imagens em tempo real do desenvolvimento desta espécie bem como a ocorrência de patologias em fases iniciais do desenvolvimento (LIESCHKE; CURRIE, 2007).

A utilização do peixe-zebra como modelo de estudo nas mais diversas áreas biomédicas se deve também à alta homogeneidade genética, apresentando cerca de 70% dos genes homólogos aos genes humanos (HOWE et al., 2013).

1.5. Peixe-zebra e Piriproxifeno

Por ser um modelo bem elucidado em estudos relacionados a toxicologia e contaminação ambiental, o peixe-zebra é apropriado para estudos de moléculas onde a exposição é através da diluição em água, como é o caso do piriproxifeno. Os peixes estão constantemente expostos a diversas moléculas que podem ser nocivas ou induzir a efeitos que podem ser prejudiciais a proliferação da espécie. Nesse sentido, considerando a necessidade de estudar os efeitos do piriproxifeno na exposição ao peixe-zebra, foram reportados alguns trabalhos relacionados a esse tema. Em estudos com o objetivo de testar a toxicidade do piriproxifeno foi demonstrado que, na concentração de 0,01 mg/L (0.1 µg/mL) indicada para utilização em água potável (FAWELL et al., 2008), houve diminuição da taxa de sobrevivência sem afetar a morfologia e desenvolvimento. Além disso, o piriproxifeno não é capaz de causar microcefalia em peixe-zebra (DZIECIOLOWSKA et al., 2017). Nesse mesmo estudo, foram analisadas linhagens de peixes transgênicos GFP para possíveis alterações na morfologia do encéfalo, mas não foram encontradas diferenças significativas (DZIECIOLOWSKA et al., 2017). Para entender melhor o potencial de toxicidade no desenvolvimento do peixe-zebra exposto ao piriproxifeno, os animais foram expostos em fase inicial de desenvolvimento na concentração de 0,64 µM, a qual demonstrou ser neurotóxica para larvas com 120 hpf e capaz de alterar parâmetros comportamentais (TRUONG et al., 2016).

2. JUSTIFICATIVA

O desenvolvimento de estratégias de combate aos mosquitos do gênero *Aedes* envolve a utilização de larvicidas e inseticidas, tais como o Piriproxifeno. Entretanto, existem poucos estudos avaliando o impacto do uso deste larvicida em ambientes aquáticos, os quais podem promover um efeito tóxico no desenvolvimento e ecologia de peixes. Além disso, pouco se sabe sobre os efeitos tóxicos da exposição a esta substância, especialmente no Sistema Nervoso Central (SNC). Neste sentido, torna-se relevante a realização de estudos que contribuam para um maior conhecimento sobre a toxicidade deste composto.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Avaliar os efeitos das exposições aguda e crônica ao piriproxifeno em parâmetros reprodutivos, comportamentais, endócrinos e morfológicos em peixe-zebra no estágio larval e adulto.

3.2. OBJETIVOS ESPECÍFICOS

Avaliar os efeitos do piriproxifeno em peixe-zebra sobre:

A) taxa e tempo para eclosão de ovos.

B) taxa e tempo de eclosão de ovos provenientes de peixe-zebra adultos expostos.

C) taxa de sobrevivência/mortalidade de larvas e adultos expostos.

D) morfologia de larvas expostas.

E) atividade locomotora em larvas e adultos expostos, bem como da prole adulta oriunda da exposição parental.

F) memória de habituação de peixes adultos expostos.

G) expressão gênica do receptor de glicocorticoide *crf* e do fator de liberação de corticotrofina *gr* em peixes adultos expostos.

CAPÍTULO 2

ARTIGO CIENTÍFICO

GUSSO D., BONAN C.D.

Parental and larval exposure to pyriproxyfen causes morphological and behavioral changes in Zebrafish

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Parental and larval exposure to pyriproxyfen causes morphological and behavioral changes in Zebrafish

Darlan Gusso and Carla Denise Bonan[1]

[1] Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[*] Corresponding author: Carla Denise Bonan

Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil

Phone: +55 51 3353 4158

E-mail address: cbonan@pucrs.br

Abstract

Pyriproxyfen is a larvicide used to eliminate mosquitoes, mainly of the genus *Aedes*. Here we show that pyriproxyfen can cause changes in the survival rate, behavior and morphology of exposed larvae, adults exposed in the larval stage and adult offspring of exposed parents. Exposed larvae to pyriproxyfen during 1-96 hpf (hours post fertilization) showed a decrease in survival rate, heart rate 3 dpf (days post fertilization) and body length at 5 and 8 dpf. The distance traveled and mean speed of exposed larvae were reduced when compared to vehicle (1% DMSO). However, when exposed larvae were maintained until the adult stage (6 months), we did not observe differences in locomotor parameters. Parental exposure to pyriproxyfen induced decrease in locomotion of zebrafish adult offspring as well as an anxiolytic-like behavior. Therefore, pyriproxyfen alters morphology and behavior of zebrafish in early stages of development and may affect the next generations.

Keywords: behavior; development; larvicide; Pyriproxyfen; zebrafish

Introduction

Insects are vectors responsible for the transmission of various infectious diseases, such as dengue fever, yellow fever, malaria, chikungunya and zika virus. Among the insects, the *Aedes aegypti* mosquito is a vector well recognized for being responsible for most infections. The reproduction of this mosquito occurs mainly in containers with standing water and is in higher proportions near the residences (Agha et al., 2017; Hiscox et al., 2013). In Brazil, *Aedes* is dissipated in all states (Kraemer et al., 2015), causing risks to public health (Garcia Serpa Osorio-de-Castro et al., 2017). Strategic measures for the control and elimination of mosquito outbreaks are constantly implemented using larvicides and insecticides (Wang et al., 2013). The use of conventional chemical insecticides and larvicides forms selections of resistant mosquitoes (Abe et al., 2014; Lima et al., 2006). For this, the need arises to implement new control protocols (Braga e Valle, 2007).

After the epidemic occurred in Brazil in 2015, where the babies were being diagnosed with microcephaly in areas affected by the Zika virus (Schuler-Faccini et al., 2016), the Ministry of Health elaborated a protocol of control and prevention using as effective larvicide the pyriproxyfen (Fawell et al., 2008). Pyriproxyfen is a physiological insecticide that acts as an analogue of juvenile insect hormone preventing embryogenesis and adult characteristics (Dhadialla et al., 1998; Fawell et al., 2008). Currently, its use is directed for preventing the proliferation of mosquitoes of the genus *Aedes* (Tilak et al., 2016). However, many countries use it in diversified agriculture by restricting their use in horticultural crops (Fogel et al., 2016), mainly because it is safe for humans with toxicological risk in class IV (Fawell et al., 2008). However, studies have shown toxicity in mice receiving pyriproxyfen in the diet, characterized by hepatocyte hypertrophy and kidney size increase (Koyama et al., 1989).

The development of strategies to combat vector mosquitoes involves the use of larvicides and insecticides, such as pyriproxyfen. However, there are few studies evaluating the impact of the use of this larvicide on aquatic environments, which may promote a toxic effect on the development and ecology of fish. In this sense, our objective was to investigate toxic effects of exposure to this substance on morphological, behavioral and physiological parameters in larvae, adults exposed when larvae and offspring of exposed adults. For this, we used as model the zebrafish (*Danio rerio*), a teleost implemented in several lines of study mainly for endocrinology (Barcellos et al., 2007; Giacomini et al., 2015), toxicology (Barros et al., 2008) and developmental biology studies (Villamizar et al., 2014).

Methodology

Fish

Zebrafish wild type; male/female were distributed in tanks containing 2 liters of water (10 animals per tank) where they remained for 96 hours exposed to pyriproxyfen. The water temperature was maintained at 28 ± 2 ° C; pH 7.0 ± 0.2; dissolved oxygen at 6.2 ± 0.3 mg/L; total ammonia <0.01 mg/L, nitrite, nitrate absent as an indication for this species (Westerfield, 2000). The water used in the experiments was obtained from a reverse osmosis apparatus (18 MΩ/cm) and was reconstituted with sea salt (Crystal Sea ™, Marinemix, Baltimore, MD, USA) at 0.4 ppt. Adult fish were fed three times daily with TetraMin (TetraMin Tropical Flake Fish) and supplemented with saline artemia. Larvae were fed with paramecium and TetraMin (TetraMin Tropical Flake Fish) powdered feed three times a day. During the trial, we did not change the water or

remove the exposed adult residues. For larvae, water was partially exchanged daily (20%).

This experimental protocol was approved by the Ethics Committee for Animal Use of the Pontifical Catholic University of Rio Grande do Sul, Brazil (Protocol No. 7546/2016 - CEUA - PUCRS) and followed the guidelines of the National Animal Experimentation Control Council (CONCEA).

Larvicide exposure

We performed the larvicide exposure in three conditions:

a) Zebrafish were exposed during 1 - 96 hpf subchronic (long-time exposure) and larvae were analyzed:

Zebrafish embryos immediately post fertilization (≤ 1 hour post fertilization, hpf) were exposed to 0.01 mg/L, 0.5 mg/L, 1 mg/L and 2 mg/L pyriproxyfen (Pestanal®) for 96 hours and maintained for analysis of survival rate (until 15 dpf, morphology (5 and 8 dpf), heart rate 3 and 5 dpf, and locomotion at 5 and 8 dpf.

b) Zebrafish exposed during 1 - 96 hpf and analyzed in the adult stage.

Zebrafish embryos were exposed immediately after fertilization (≤ 1 hour post fertilization, hpf) to 0.01 mg/L, 0.5 mg/L and 2 mg/L pyriproxyfen for 96 hours and maintained until adulthood (6 months) to be analyzed for behavioral parameters.

c) Zebrafish adult exposed for 96 h and the offspring was analyzed in the adult stage.

Zebrafish adults were exposed for 96 h at concentrations of 0.01 mg/L, 0.5 mg /L, 2 mg/L and 3.5 mg/L pyriproxyfen. After 96 h of exposure to pyriproxyfen, fish were mated to obtain offspring adults of exposed parents.

All concentrations of pyriproxyfen were diluted in dimethyl sulfoxide (DMSO) at a final concentration of 1%. The groups were also divided into control only with water and vehicle 1% DMSO. The concentrations were obtained considering indications

of use (Brasil. Ministério da Saúde, 2014) of the larvicide and previous studies (Dzieciolowska et al., 2017; Koyama et al., 1989; Lim and Lee, 2005; Truong et al., 2016).

During the experimental procedure, the larvae were left in Petri dishes and the water changed daily. The adults were exposed in aquarium without the use of filter, for that, we use aerator. The water was not changed during the 96 hours. After 96 h exposed, fish were mated to mate and obtain offspring.

Larvae analysis

Heart rate

Heart rate was assessed at 3 and 5 dpf in all groups during the morning, under the stereomicroscope. Treated larvae and controls were placed in Petri dishes with bottled spring water, and their heart rates were monitored for 60 s (n = 65). For all procedures, water temperature was kept stable at 28 °C by a thermo-plate coupled to the stereomicroscope.

Morphology

Morphological evaluation of pyriproxyfen exposure was evaluated in chronically treated larvae (n = 196). Larvae were evaluated at 5 and 8 dpf under a stereomicroscope (3×). The body length (μm) and ocular distance (μm) were measured after photographic registration with the software NIS-Elements D 3.2 for Windows, supplied by Nikon Instruments Inc. (Melville, USA). The body length was defined as the distance from the center of the eyes to the tip of the tail bud. The ocular distance was defined as the distance between the inner edges of the two eyes (Capiotti et al., 2011).

Exploratory behavior

Zebrafish larvae were analyzed at 5 dpf (n =154). Animals were individually placed in a 24-well plates, each with 3 mL of bottled spring water, for a 5-min session of exploratory behavior analysis following 1 minute acclimation (Colwill e Creton, 2011). The performance was video recorded for automated analysis by Ethovision XT (version 11.5, Noldus Information Technology., Wageningen, Holanda), which is able to track the swimming activity of the animals at a rate of 30 positions per second. Total distance traveled (m), mean speed (m/s, ratio between distance traveled and time mobile) were considered the main parameters of exploration of a new environment.

Adult analysis

Novel tank test

Animals were placed individually in experimental tanks (30 cm long × 15 cm high × 10 cm wide) with water. After 60 s of acclimatization period, the locomotion and exploratory patterns of the fish were recorded for 5 min for subsequent analysis with the software EthoVision XT (version 11.5, Noldus) at rate of 30 positions per second. The following behavioral parameters were analyzed: distance traveled (m), velocity (m/s), time spent in each tank zone (bottom vs. upper levels) (s) and the number of entries in the upper zone.

Statistical analysis

Survival percent rates throughout the 15 days for larvae and 4 days for adults were analyzed by a Kaplan-Meier test. Data from heart rate and morphological evaluation were evaluated by one-way ANOVA followed by Bonferroni post hoc test.

Locomotor behavior was evaluated using one-way ANOVA followed by Tukey's post-hoc.

Results

The pyriproxyfen exposure decreased the survival rate of treated larvae compared to control group and vehicle in treatment regimens ($p < 0.0001$) (Fig. 1A). The concentrations of 1 and 2 mg/L pyriproxyfen were excluded because they were highly toxic to larvae, showing an increase in the immobility time, and caused elevated mortality at 2 mg/L after 15 dpf. However, it does not change the survival rate of exposed adults in lower concentrations (Fig. 1B). Larvae exposed to pyriproxyfen showed a decreased heart rate at 3 dpf. We observed a significant decrease in the group exposed to 0.5 mg/L in relation to control and vehicle ($F_{(3,31)} = 21.11$, $p < 0.0001$) in 3 dpf (Fig. 2A) but does not observe difference on 5 dpf ($F_{(2.26)} = 28.90$, $p > 0.05$) (Fig. 2B)..

The potential teratogenic effects of exposure to pyriproxyfen on larval morphology were evaluated at 5 and 8 dpf. There were visible morphological alterations in chronically treated animals at 5 and 8 dpf. The most prominent effects of exposure to pyriproxyfen were reduced body length at 0.01 mg/L compared with vehicle ($F_{(3,94)} = 2.909$ $p < 0.05$) (Fig. 3A). For 8 dpf larvae, we observed changes at the concentrations of 0.5 mg/L in relation to vehicle and 0.01 mg/L in relation to 0.5 mg/L ($F_{(3,94)} = 3.593$, $p = 0.01$) (Fig. 3B). The ocular distance has no difference at 5 and 8 dpf ($F_{(3,94)} = 2.607$; 8 dpf ($F_{(3,94)} = 0.1856$) (Fig. 3C and 3D).

The exploratory behavior of the larvae was examined at 5 dpf to determine if exposure to pyriproxyfen could alter the locomotion and orientation of the larvae. The group 0.5 mg/L achieved a slower mean velocity in relation to the vehicle ($F_{(3,150)} = 3.221$ $p < 0.05$) (Fig. 4A). The distance parameter (m) covered decreases in all

concentrations of pyriproxyfen compared to control and vehicle ($F_{(3,150)}$ = 6.783, p < 0.001) (Fig. 4B).

Adults exposed on stage larval

Adult fish exposed to pyriproxyfen in the larval stage showed no changes in distance covered ($F_{(3,34)}$ = 0.1716 p > 0.05) (Fig. 5A), time spent in the upper region ($F_{(3,34)}$ = 0.8734 p > 0.05) (Fig. 5B) and mean speed ($F_{(3,34)}$ = 1.199 p > 0.05) (Fig. 5C).

Adult offspring of exposed parents

Adult zebrafish, which are offspring of exposed parents, have altered locomotor parameters. At concentrations of 0.5 and 2 mg/L pyriproxyfen, the animas displayed reduced locomotor behavior when compared to the control group ($F_{(4,52)}$ = 7.748; p < 0.0001) (Fig. 6A). Moreover, there was also change in number of entries in upper zone, suggesting that pyriproxyfen is able to alter the anxiolytic-like behavior at concentrations of 2 mg/L compared to the control ($F_{(4,52)}$ = 3.627, p = 0.01) (Fig. 6B). Mean speed parameters showed a decrease at the concentrations of 0.5 and 2 mg/L in relation to the control and the vehicle 1% DMSO ($F_{(4,52)}$ = 10.07 p <0.0001) (Fig. 6C). There were no changes in time spent in upper zone ($F_{(4,52)}$ = 1.244 p > 0.05) (Fig. 6D).

Discussion

Here we demonstrated that pyriproxyfen in water can negatively affect the survival rates of exposed larvae and impair behavioral habits not only in the exposed generations but also in the offspring of exposed parents. However, when we exposed

larvae and maintained until adulthood to perform behavior tests, we did not obtain a difference in the novel tank tests.

These findings demonstrated that larvae exposed to the insect growth regulator pyriproxyfen for 96 hours are able to cause mortality at almost all concentrations (0.01, 0.5, 1 and 2 mg/L). In addition to high lethality, it causes severe abnormalities in surviving fish and this condition has also been found in previous work, where it was observed pericardial edema, yolk sac edema, hyperemia and spinal deformity (Maharajan et al., 2018). At the concentration of 0.01 mg/L, which is the concentration of pyriproxyfen added to the drinking water and recommended by the Ministry of Health (Brasil. Ministério da Saúde, 2014), there was a decrease in the survival rate and it slows down the growth of exposed larvae. Previous studies did not observe difference in the lethality of pyriproxyfen at a concentration of 0.01 mg/L (0.01 μg/ml), but observed difference in survival at high concentrations (Dzieciolowska et al., 2017). The differences in the survival rates may occur due to different sensitivities of zebrafish strains to pyriproxyfen, since our study was conducted testing wild-type zebrafish, unlike other studies using transgenic fish.

In addition, larvae showed a decrease in heart rate at 3 dpf at concentrations of 0.5 mg/L. However, this condition was not changed at 0.01 mg/L, as shown in previous studies (Maharajan et al., 2018). At 5 dpf, the concentration of 0.5 mg/L had no significant difference in relation to control and vehicle. These findings indicate that zebrafish larvae can restore normal cardiac condition, since animals were removed from pyriproxyfen treatment after exposure during 96 hours.

Our study also demonstrated a decrease in body length to 5 dpf at a concentration of 0.01 mg/L when compared to vehicle. In 8 dpf, our results showed that larvae decreased body length at the concentration of 0.5 mg/L in comparison to 0.01 mg/L. In contrast,

this study did not coincide with recently presented results where there was no difference in body length. In relation to the ocular distance, we did not observe significant difference, which is in agreement with previous studies (Dzieciolowska et al., 2017), where they did not observe changes in eye diameter in 7 dpf larvae. Since we observed teratogenic effects, such as a reduction in body length and heart rate, it is important to monitor the use of pyriproxyfen to insect control and its impact in the environment and health.

In this study, we also observed that exposure to pyriproxyfen for 96 h was able to reduce the exploratory capacity of zebrafish larvae when analyzed at 5 dpf. Exposure to pyriproxyfen at 0.5 mg/L caused alterations in mean speed and distance; however, at the concentration of 0.01 mg/L, only distance was affected. Other studies demonstrated the occurrence of abnormal behavior in embryos after pyriproxyfen, such as swimming in circular movements (Maharajan et al., 2018). However, this is the first study reporting the locomotor parameters, such as distance and mean speed of zebrafish larvae exposed to pyriproxyfen. Adults exposed on larval stage did not change locomotor parameters when tested in adulthood. We suggested that zebrafish can reestablish its natural behavior by high regenerative capacity (González-Rosa et al., 2018). This is a finding that does not occur in adult zebrafish, which are offspring of exposed parents, since there is a change in locomotor parameters. The groups 0.5 and 2 mg/L pyriproxyfen showed a decrease in the distance traveled in adult offspring after parental exposure in relation to control and a reduction in the mean speed in relation to the control and vehicle. In previous studies, zebrafish adults were exposed to difenoconazole to analyze the effect on the next generation (F1). This fungicide is capable of altering the hypothalamic-pituitary-gonadal-liver axis and induces changes in the genes related to the ovary and testicles (Teng et al., 2018). These changes still directly affect the rate of

hatching and offspring survival (Teng et al., 2018). The relationship of agrochemicals, larvicides and insecticides that is capable of affecting the next generation (F1) has already been studied. Our hypothesis is that exposure to pyriproxyfen may be related to glucocorticoid cortisol levels directly in the hypothalamic-pituitary-interrenal axis or in the *gr* and *crf* genes. Cortisol is important in development, however, when deregulated can cause deformity and change in heart rate (Nesan e Vijayan, 2012). Our findings coincide with previous studies showing a change in heart morphology and frequency induced by pyriproxyfen (Maharajan et al., 2018).

In summary, our findings suggest that a decrease in exploratory behavior in zebrafish exposed to pyriproxyfen could become the animals more susceptible to predation, which could impact the survival of the animal in the environment. In addition, it can also affect subsequent generations and impact the ecology of this species.

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Figure Legends

Fig 1. Zebrafish larvae and adults exposed to pyriproxyfen. (**A**) Survival rates of larvae throughout the 15 experimental days after treatment during 96 h were analyzed by a Kaplan Meier test. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (n = 118). (**B**) Survival rates of adults throughout 96-h treatment were analyzed by a Kaplan Meier test.

Fig 2. Heartbeat rate of treated larvae measured at 3 dpf (A) and 5 dpf (B). Data are expressed as mean ± S.E.M. (n = 65). One-way ANOVA was used, followed by Bonferroni post hoc test. Significant differences are indicated by $^{****}p = 0.0001$ in relation to control and vehicle.

Fig 3. The effects of exposure to pyriproxyfen on morphological parameters of zebrafish larvae. Mean body length (μm) at 5 (**A, C**) and 8 dpf (**B, D**); and mean ocular distance (μm) at 5 (**A, C**) and 8 dpf (**B, D**). Data are showed as mean ± S.E.M (n = 98) and analyzed by One-way ANOVA, followed by Bonferroni posthoc test. Significant differences are indicated by $^{*}\ p \leq 0.05$ in relation to control and vehicle at the same developmental phase.

Fig 4. Effects of exploratory behavior of 5 dpf larvae. Mean speed is expressed in meters per seconds (**A**) and distance in meters (**B**). Data are showed as mean ± S.E.M (n = 154) and analyzed by one-way ANOVA, followed by Bonferroni posthoc test. Significant differences are indicated by $^{*}\ p < 0.05$ and $^{**}\ p < 0.01$ in relation to control and vehicle.

Fig 5. Locomotor activity in adults exposed during the larval phase. Distance in meters (A), time in upper zone (B), mean speed (C). Data are presented as mean ± S.E.M (n = 34); Locomotor activity was analyzed by One-way ANOVA, followed by Tukey's post hoc test.

Fig 6. Locomotor parameters on adult offspring of exposed parents. Distance in meters (**A**), number of entries in the upper zone (**B**), mean speed (**C**), time in upper zone (**D**). Data are presented as mean ± S.E.M (n = 57); Locomotor activity was analyzed by One-way ANOVA, followed by Bonferroni post hoc test. Significant difference are represented by * $^{\#}$ $p < 0.05$; ** $p < 0.01$; *** $^{\#\#\#}$ $p < 0.001$; **** $p < 0.0001$.

Figure 1

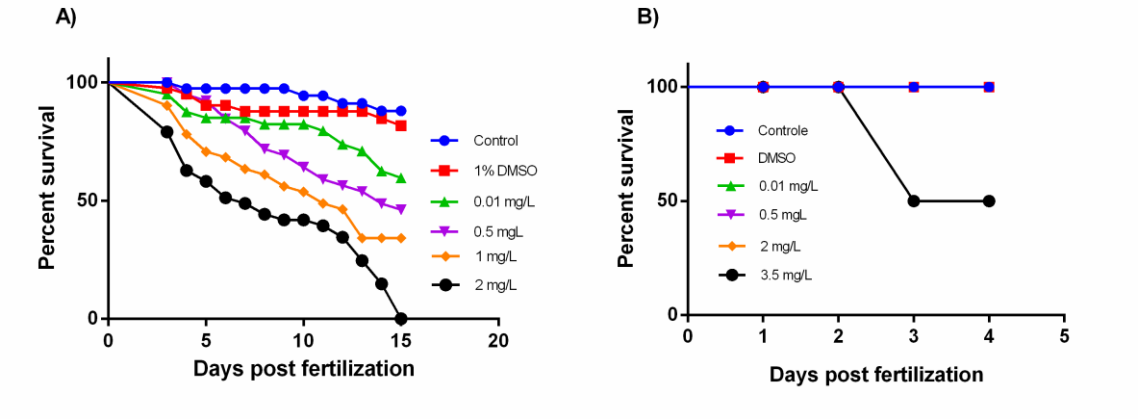
A)
Percent survival
100
50
0
0
5
10
15
20
Days post fertilization
Control
1% DMSO
0.01 mg/L
0.5 mgL
1 mg/L
2 mg/L
B)
Percent survival
100
50
0
0
1
2
3
4
5
Days post fertilization
Controle
DMSO
0.01 mg/L
0.5 mgL
2 mg/L
3.5 mg/L

Figure 2

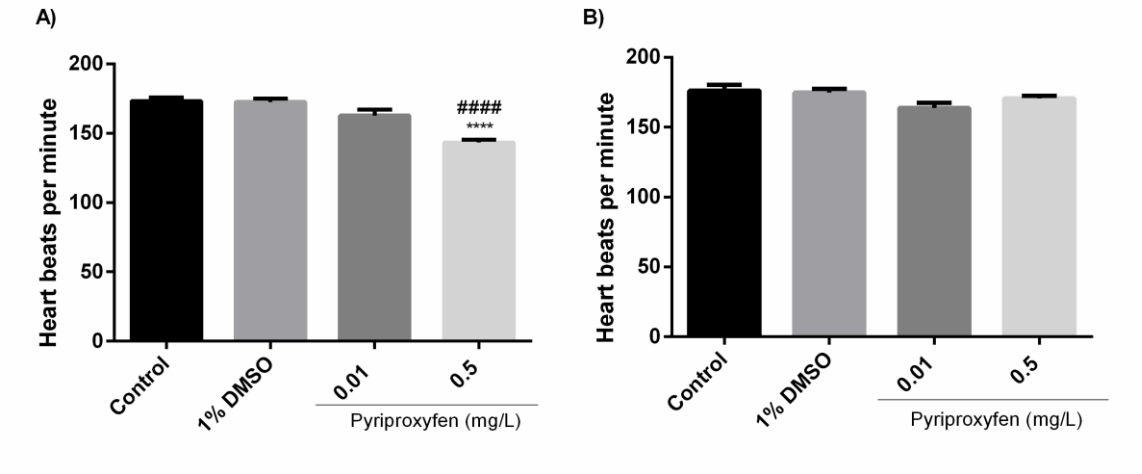
A)
Heart beats per minute
200
150
100
50
0
####
****
Control
1% DMSO
0.01
0.5
Pyriproxyfen (mg/L)
B)
Heart beats per minute
200
150
100
50
0
Control
1% DMSO
0.01
0.5
Pyriproxyfen (mg/L)

Figure 3

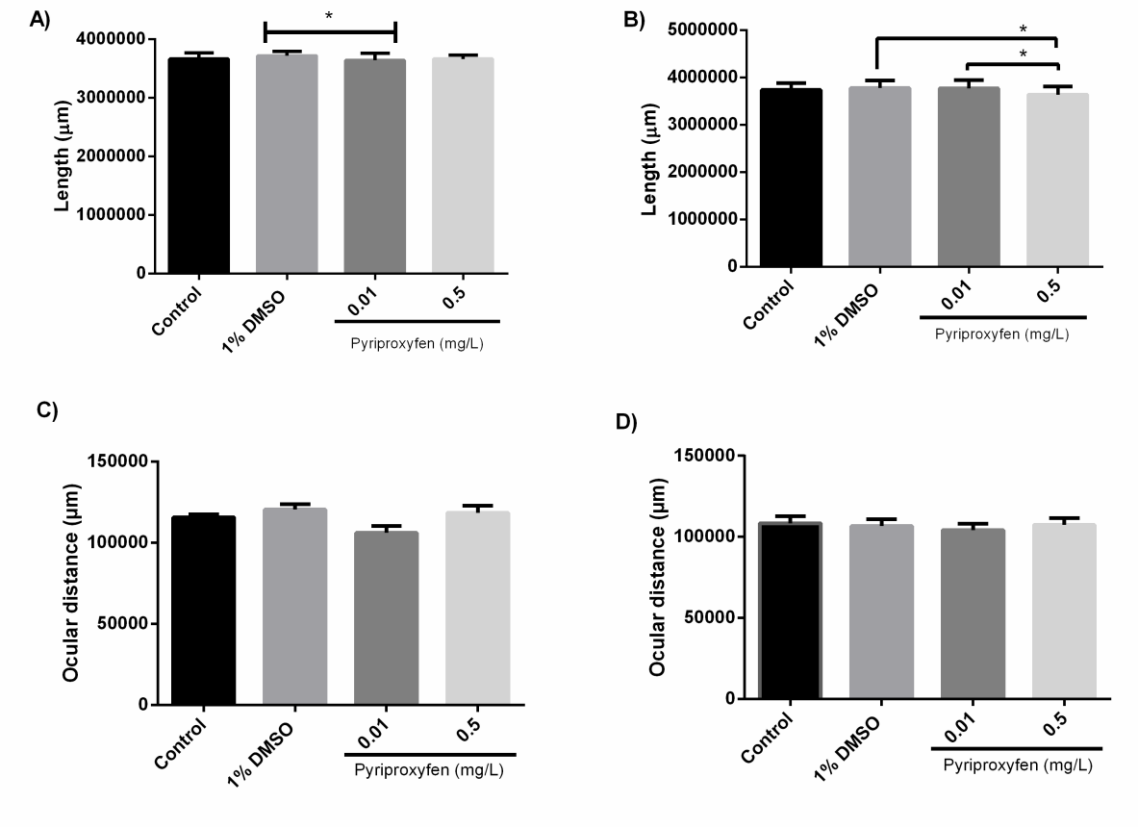
A)
Length (µm)
4000000
3000000
2000000
1000000
0
*
Control
1% DMSO
0.01
0.5
Pyriproxyfen (mg/L)
B)
Length (µm)
5000000
4000000
3000000
2000000
1000000
0
*
*
Control
1% DMSO
0.01
0.5
Pyriproxyfen (mg/L)
C)
Ocular distance (µm)
150000
100000
50000
0
Control
1% DMSO
0.01
0.5
Pyriproxyfen (mg/L)
D)
Ocular distance (µm)
150000
100000
50000
0
Control
1% DMSO
0.01
0.5
Pyriproxyfen (mg/L)

Figure 4

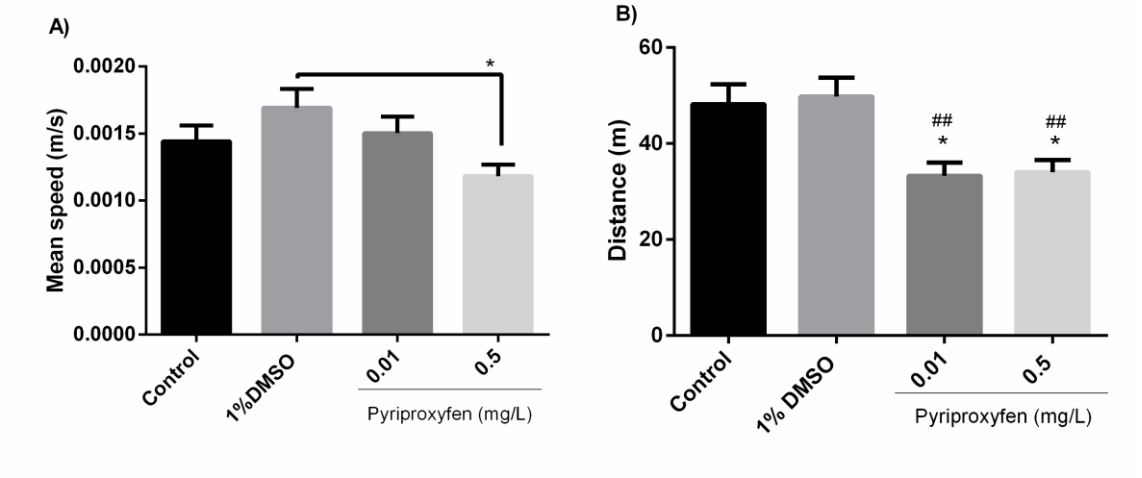
A)
Mean speed (m/s)
0.0020
0.0015
0.0010
0.0005
0.0000
*
Control
1%DMSO
0.01
0.5
Pyriproxyfen (mg/L)
B)
Distance (m)
60
40
20
0
##
*
##
*
Control
1% DMSO
0.01
0.5
Pyriproxyfen (mg/L)

Figure 5

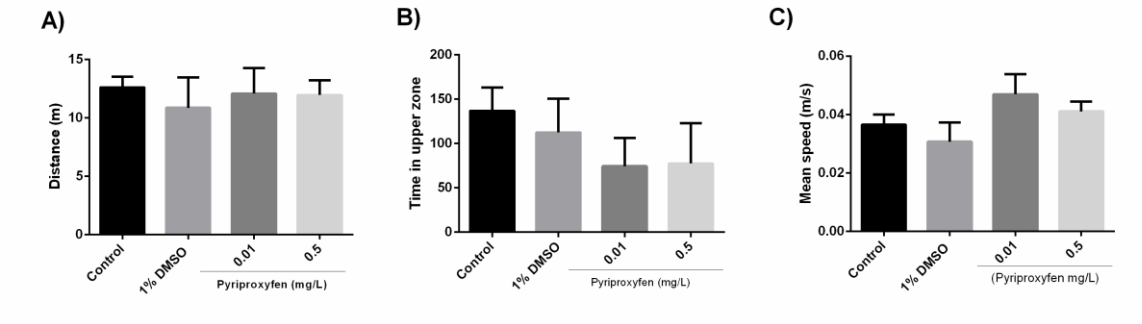
A)
Distance (m)
15
10
5
0
Control
1% DMSO
0.01
0.5
Pyriproxyfen (mg/L)
B)
Time in upper zone
200
150
100
50
0
Control
1% DMSO
0.01
0.5
Pyriproxyfen (mg/L)
C)
Mean speed (m/s)
0.06
0.04
0.02
0.00
Control
1% DMSO
0.01
0.5
(Pyriproxyfen mg/L)

Figure 6

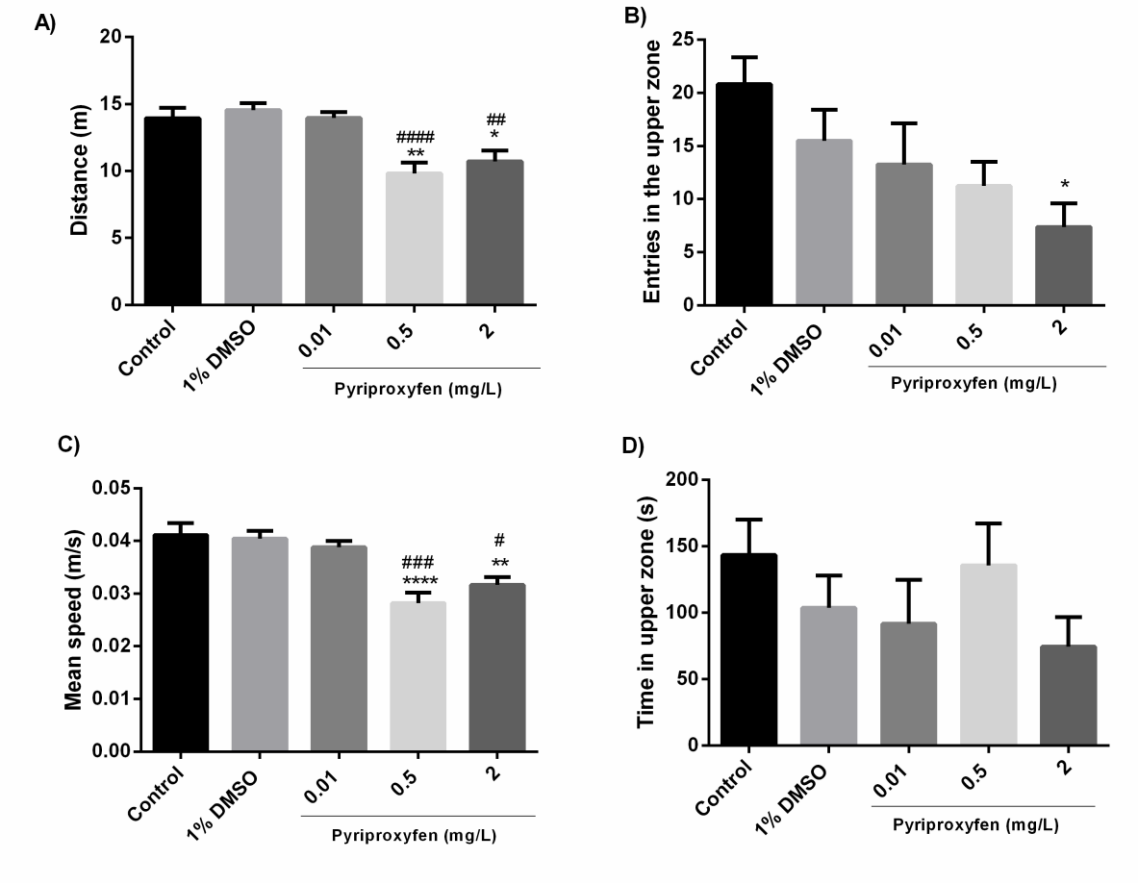
A)
Distance (m)
20
15
10
5
0
####
**
##
*
Control
1% DMSO
0.01
0.5
2
Pyriproxyfen (mg/L)
B)
Entries in the upper zone
25
20
15
10
5
0
*
Control
1% DMSO
0.01
0.5
2
Pyriproxyfen (mg/L)
C)
Mean speed (m/s)
0.05
0.04
0.03
0.02
0.01
0.00
###
****
#
**
Control
1% DMSO
0.01
0.5
2
Pyriproxyfen (mg/L)
D)
Time in upper zone (s)
200
150
100
50
0
Control
1% DMSO
0.01
0.5
2
Pyriproxyfen (mg/L)

Figure legends

Fig 1. Zebrafish larvae and adults exposed to pyriproxyfen. (**A**) Survival rates of larvae throughout the 15 experimental days after treatment during 96 h were analyzed by a Kaplan Meier test. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (n = 118). (**B**) Survival rates of adults throughout 96-h treatment were analyzed by a Kaplan Meier test.

Fig 2. Heartbeat rate of treated larvae measured at 3 dpf (A) and 5 dpf (B). Data are expressed as mean ± S.E.M. (n = 65). One-way ANOVA was used, followed by Bonferroni post hoc test. Significant differences are indicated by $^{****}p = 0.0001$ in relation to control and vehicle.

Fig 3. The effects of exposure to pyriproxyfen on morphological parameters of zebrafish larvae. Mean body length (μm) at 5 (**A, C**) and 8 dpf (**B, D**); and mean ocular distance (μm) at 5 (**A, C**) and 8 dpf (**B, D**). Data are showed as mean ± S.E.M (n = 98) and analyzed by One-way ANOVA, followed by Bonferroni post hoc test. Significant differences are indicated by $^{*}\ p \leq 0.05$ in relation to control and vehicle at the same developmental phase.

Fig 4. Effects of exploratory behavior of 5 dpf larvae. Mean speed is expressed in meters per seconds (**A**) and distance in meters (**B**). Data are showed as mean ± S.E.M (n = 154) and analyzed by one-way ANOVA, followed by Bonferroni posthoc test. Significant differences are indicated by $^{*}\ p < 0.05$ and $^{**}\ p < 0.01$ in relation to control and vehicle.

Fig 5. Locomotor activity in adults exposed during the larval phase. Distance in meters (A), time in upper zone (B), mean speed (C). Data are presented as mean ± S.E.M (n = 38); Locomotor activity was analyzed by One-way ANOVA, followed by Tukey's post hoc test.

Fig 6. Locomotor parameters on adult offspring of exposed parents. Distance in meters (**A**), number of entries in the upper zone (**B**), mean speed (**C**), time in upper zone (**D**). Data are presented as mean ± S.E.M (n = 57); Locomotor activity was analyzed by One-way ANOVA, followed by Bonferroni post hoc test. Significant difference are represented by * $^{\#}$ $p < 0.05$; ** $p < 0.01$; *** $^{\#\#\#}$ $p < 0.001$; **** $p < 0.0001$.

PÍTULO 3

ARTIGO CIENTÍFICO

Effect of pyriproxyfen on habituation to novelty and on gene expression of stress biomarkers in adult zebrafish

Manuscrito em preparação

Effect of pyriproxyfen on habituation to novelty and on gene expression of stress biomarkers in adult zebrafish

Darlan Gusso[1], Gustavo Kellermann Reolon, Jonas Brum Gonzalez, Luiza Wilges Kist[2], Mauricio Reis Bogo[2] and Carla Denise Bonan [1]

[1] Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[2]Laboratório de Biologia Genômica e Molecular, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul - PUCRS, Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil.

[*] Corresponding author: Carla Denise Bonan

Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil

Phone: +55 51 3353 4158

E-mail address: cbonan@pucrs.br

Abstract

Pyriproxyfen is one of the most commonly used larvicides and insecticides to insect control. Pyriproxyfen is mainly used to eliminate outbreaks of mosquitoes of the genus *Aedes*. In this study, we evaluated the effect of pyriproxyfen (0.01 mg/L, 0.5 mg/L, 2 mg/L and 3.5 mg/L) on locomotion, habituation memory, and gene expression of GR (Glucocorticoid receptor) and CRF (corticotrophin-released factor). Our results demonstrated that pyriproxyfen causes no changes in locomotor and anxiety parameters. However, pyriproxyfen impaired the memory habituation of fish exposed for 96 h at a concentration of 3.5 mg/L. We also analyzed the gene expression of GR and CRF to investigate whether pyriproxyfen alter the HPI-axis in zebrafish. Together, these findings demonstrated that pyriproxyfen may impair memory responses, but not interfere in molecular parameters related the HPI-axis responses in zebrafish.

Keywords: locomotion; memory; pyriproxyfen; zebrafish

Introduction

Pyriproxifen is a larvicide used mainly for the control of mosquitoes of the genus *Aedes*. Mosquitoes are responsible for the transmission of various infectious diseases, such as dengue fever, yellow fever, chikungunya, and zika virus (Tilak et al., 2016). All vector-borne diseases cause alertness to initiate control and prevention procedures. Some epidemics are prevalent, such as the zika virus, which was considered the largest epidemic in the Americas in 2015 (Araujo et al., 2016). In addition, other countries such as France, Polynesia, Yap Islands, Cook Island, Easter Island and New Caledonia have also identified an outbreak of zika virus (Duffy et al., 2009). This virus has also been isolated in other species of the genus *Aedes*, such as *A. albopictus*, *A. africanus* and *A. lutepcephalus* (Cao-Lormeau et al., 2014). In 2015, the increase in microcephaly cases was observed in Brazil due to zika virus infection (Schuler-Faccini et al., 2016). The Ministry of Health, following the guidelines of the World Health Organization, promoted campaigns to control mosquitoes populations and the main urban larvicide used was pyriproxifen (Brasil. Ministério da Saúde, 2014).

Pyriproxyfen is an analogue of juvenile insect hormone and is considered a growth regulator. This larvicide does not allow insects to form adult characteristics and interfere with the reproduction and proliferation of the species (Fawell et al., 2008; Ishaaya and Horowitz, 1992). In addition, pyriproxyfen is approved by the World Health Organization (WHO) as a safe physiological larvicide for use even in potable water (Fawell et al., 2008). In Brazil, many problems related to the drainage of rainwater and sewage networks make it a starting point for the unsuccessful control of mosquitoes populations and demand intensive control. However, the use of larvicides and insecticides can also cause damage to non-target species. The organophosphorus Temephos was used for many years (EPA, 2015), and only in 2015 was prohibited. Studies reported that Temephos, at sublethal concentrations, causes enzyme changes and

genotoxicity in fish of the species *Poecilia reticulate* (Pereira and de Campos Júnior, 2015). It has been also shown that marine fish were contaminated with agrochemical wastes, containing Metribuzine, a triazine that was discovered in the 1970s and has since been widely used in many cultures. Metribuzine was found in higher concentrations in muscle of several fish species (Polat et al., 2017). Despite the increase in the number of toxicological studies about larvicides and insecticides with indication of continuous use, there are few studies about the effects of pyriproxyfen in fish species.

For this reason, we performed experiments with zebrafish (*Danio rerio*), a teleost that shares a high degree of sequence and functional homology with mammals, including humans, has a complex behavioral repertoire and many zootechnical advantages, such as easy maintenance and high fertitlity (Howe et al., 2013; Orger and de Polavieja, 2017). This species is largely studied in several areas related to behavior (Bortolotto et al., 2014; Giacomini et al., 2016, 2015), developmental biology (Nery et al., 2014), cognition (Grossman et al., 2011; Zimmermann et al., 2015), endocrinology and toxicology (Abreu et al., 2015; Idalencio et al., 2015).

The development of studies about effects of the use of larvicides and insecticides is important to understand the impact of these compounds on non-target organisms. Therefore, this study evaluated the effects of pyriproxyfen on behavioral, cognitive and molecular parameters in zebrafish. Zebrafish is a species with extensive knowledge in stress neurobiology and serves as a model for endocrinology-related analyzes (Abreu et al., 2015; Giacomini et al., 2016; Mocelin et al., 2015). This supports the usefulness of zebrafish paradigms for toxicological testing.

Materials and methods

Animals

Adult (6–7 months) wild-type zebrafish (*Danio rerio*) were used. Animals were obtained from our breeding colony and kept in automated recirculating systems (Zebtec, Tecniplast, Italy) with reverse osmosis-filtered water equilibrated to reach the recommended temperature (28 °C ± 2 °C), pH (7.0–7.5), conductivity (300–700 μS), ammonia (< 0.02 mg/L), hardness (80–300 mg/L), nitrite (< 1 mg/L), nitrate (< 50 mg/L) and chloride (0 mg/L) levels for the species. The animals were maintained in a light/dark cycle of 14/10 h. Animals were fed with commercial flakes (TetraMin Tropical Flake Fish®) three times a day supplemented with brine shrimp (Westerfield, 2000) after 14 dpf. All protocols were approved by the Institutional Animal Care Committee from Pontifícia Universidade Católica do Rio Grande do Sul (CEUA-PUCRS, protocol number 7546/2016).

Pyriproxyfen treatment

The animals were exposed to pyriproxyfen at 0,01 mg/L, 0,5 mg/L, 2 mg/L and 3,5 mg/L during 96 hours. The control group and vehicle were exposed to water and 1% DMSO, respectively, during 96 hours. We used concentrations of pyriproxyfen based on indications from the World Health organization (Fawell et al., 2008), Brazilian Ministry of health (Brasil. Ministério da Saúde, 2014) and previous analysis of water collection in rivers containing pyriproxyfen (Belenguer et al., 2014).

Novel tank tests

Adult exploration was evaluated at the end of treatment. Animals were placed individually in experimental tanks (30 cm long × 15 cm high × 10 cm wide) with water. After 60 s of habituation, the locomotion and exploratory patterns of the fish were

recorded for 5 min for subsequent analysis with the software EthoVision XT (version 11.5, Noldus) at rate of 15 positions per second (Altenhofen et al., 2017). The following behavioral parameters were analyzed: distance travelled (m), mean speed (cm/s), time spent in upper zone (bottom vs. upper levels) (s), crossings, and latency to entry in the upper zone. The time spent in upper zone was considered an indicator of anxiety-like behavior, since when introduced into a new environment zebrafish tend to spend more time at the bottom of the tank, until gradually moving to the upper zone after a few minutes (Levin et al., 2007).

Novel tank habituation

For the habituation memory analysis, the total response was analyzed during 6-minute videos. Zebrafish adults were analyzed for 6 minutes and compared the habituation memory between the first vs. sixth minute (Grossman et al., 2011). The new tank test was validated for application on sensitive models, such as zebrafish, for intra/inter-session habituation reflecting its short- or long-term spatial memory phenotypes (Grossman et al., 2011). Animal behaviors were recorded as described above and analyzed for their distribution per minute using EthoVision XT software (version 11.5, Noldus). The habituation memory is measured as the time spent in the bottom and upper zone during the first and last minute of the test.

RNA isolation and Real-time RT-qPCR

The gene expression of corticotropin releasing factor (*crf*) and glucocorticoid receptor (*gr*) were determined by RT-qPCR. The total RNA was isolated from zebrafish brain with TRIzol® Reagent (Life Technologies) in accordance with the manufacturer's instructions. RNA purity (Abs 260/280nm ~2.0) and concentration were determined by Nanodrop® and after treated with Deoxyribonuclease I (Sigma-Aldrich) to eliminate

genomic DNA contamination in accordance with the manufacturer's instructions. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of the total RNA, following the manufacturer´s instruction. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis on the 7500 Real-time PCR System (Applied Biosystems). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C to confirm the specificity of primers and absence of primer-dimers and showed in all cases one single peak. All real time assays were carried out in quadruplicate and, in all cases, a reverse transcriptase negative control was included by substituting the templates for DNAse/RNAse-free distilled water in each PCR reaction. β-*actin, EF1α* and *Rpl13α* were used as reference genes for normalization. The sequences of reverse and forward primers are (Table 1). The efficiency per sample was calculated using LinRegPCR 2017.0 Software (http://LinRegPCR.nl) and the stability of the references genes, and the optimal number of reference genes according to the pairwise variation (*V*) was analyzed by GeNorm 3,5 Software (http://medgen.ugent.be/genorm/). Relative mRNA expression levels were determined using the 2-ΔΔCq method (Bustin et al., 2013).

Statistical analysis

Behavioral and molecular data were expressed as means ± S.E.M. Molecular data was analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, considering ($p < 0.05$) as statistical significance. Locomotor parameters were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Habituation memory was evaluated by the Wilcoxon matched pairs test. For all

comparisons, the level of significance was defined as $p \leq 0.05$. For analyzes of *crf* and *gr*, we used one-way ANOVA and the Dunn multiple-comparison test significance level was defined as $p \leq 0.05$. The GraphPad (GraphPad, La Jolla, CA, USA) software was used.

Results

Exploratory behavior

Pyriproxyfen at the concentration of 0.01, .,5, 2 and 3.5mg/L did not cause changes in locomotor and anxiety parameters after 96h exposure when compared to control, 1%DMSO groups. There were no significant changes in line crossing ($F_{(5.100)}$ = 1.197, $p > 0.05$) (Fig 1A), distance($F_{(5.100)}$ = 1.869, $p > 0.05$) (Fig 1B), mean speed ($F_{(5.100)}$ = 0.8892, $p > 0.05$) (Fig 1C), latency to entry in upper zone ($F_{(5.100)}$ = 2.331,($p > 0.05$) (Fig 1D), and ($F_{(5.100)}$ = 1.613, $p > 0.05$) (Fig 1E time in the upper zone).

Novel tank habituation analysis

We show that Pyriproxyfen at 0.01, 0.5 and 2mg/L did not alter the spatial memory of habituation, when compared the first and sixth minute of habituation. The 3.5 mg/L concentration was able to change the habituation memory, since there is no difference between the first and sixth minute of habituation ($F_{(11.219)}$ = 3.634, $p > 0.05$) (Fig 1F). The Figure 2 demonstrates representative track plot of the experimental groups obtained by video-tracking software EthoVision XT (version 11.5, Noldus), displaying the specific patterns of their exploratory behavior during the first and sixth minutes.

Glucocorticoid receptor GR and corticotrophin releasing hormone CRF

To investigate whether pyriproxyfen was able to alter cortisol stress responses through the quantification of the *CRF* and *GR* genes, the effects of 0.01 mg/L, 0.5 mg/L,

and 3,5mg/L pyriproxyfen during 96 h on the *CRF* ($F_{(5,66)}$ = 1.330, p >0.05) (Fig 4A) and *GR* ($F_{(5,66)}$ = 1.393, p > 0.05) (Fig 4B) gene expression pattern were analyzed. We demonstrated that there was no significant difference in CRF and GR mRNA transcript levels.

Discussion

In the present study, we show that pyriproxyfen does not alter locomotor and anxiety parameters in zebrafish exposed for 96 h. Previous studies showed that larvae exposed to pyriproxyfen presented a decreasing in the survival rate and severe morphological changes in development, which may directly impact the locomotor parameters in adulthood (Maharajan et al., 2018). Furthermore, parental exposure to pyriproxyfen induced changes in locomotor parameters of adult offspring, with a decrease in distance and mean speed (Gusso and Bonan, unpublished data).

Anxiety parameters did not alter in adults exposed to pyriproxyfen for 96h. However, the animals exposed to pyriproxyfen at 3.5mg/L did not demonstrate differences in the time spent in the bottom and upper zone in the first and sixth minute of analysis, indicating that this larvicide may be causing cognitive deficits. The habituation memory is a well consolidated test (Grossman et al., 2011; Wong et al., 2010) and provides reliable results, since the changes observed in fish performance are related to memory, which is confirmed by the absence of alterations in anxiety and locomotion.

To investigate whether pyriproxyfen may modulalte HPI-axis responses, we evaluate the gene expression of glucocorticoid receptor (GR) and corticotrophin-releasing factor (CRF). However, our results demonstrated that exposure to pyriproxyfen does not cause changes in CRF and GR. In contrast, studies demonstrated possible interactions between fungicides and insecticides with the interrenal system of exposed fish (Aquilino et al., 2018). Rare minnow (*Gobiocypris rarus*), exposed to vinclozolin, a

commonly used fungicide, caused a decline in CRF and GR in male and females (Yang et al., 2011).

In summary, our study demonstrated that pyriproxyfen exposure promoted alterations in habituation memory of zebrafish, which may be due to neurotoxic effects induced by this larvicide. In addition, these changes may significantly affect the survival of zebrafish in natural habitats and pose risks to other aquatic organisms, such as algae and microcrustaceans that are natural foods of various fish species.

Acknowledgments

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Figure legends

Fig 1. Zebrafish adults exposed to pyriproxyfen. (**A**) Line crossing, $p > 0.05$. (n = 106). (**B**) Distance in meters, $p > 0.05$. (n = 106). (**C**) Mean speed $p > 0.05$. (n = 18). (**D**) Latency to enter the upper zone $p > 0.05$. (n = 106). (**E**) Time in upper zone, $p > 0.05$. (n = 106). All locomotor parameters were measured after 96h-pyriproxyfen exposure and the data was analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. The habituation memory (**F**) was tested in adult zebrafish at first and sixth min of exposure to a novel tank test (n = 18 per group) and the results were analysed by Wilcoxon matched pairs test. Significant difference are represented by * $p < 0.05$; ** $p <$ 0,01; *** $p < 0.001$ between first and sixth min for each group tested.

Fig 2. Schematic representation of the novel test tank. The figure demonstrates representation of zebrafish movements during the first and sixth minute of the session.

Fig 3. Effects 96 h exposure to pyriproxyfen on CRF (fig. 3A) and GR (fig. 3B) gene expression. Data are expressed as mean ± S.E.M (n = 72) and analyzed by one way ANOVA, followed by Dunn's post hoc test.

Table 1. Primer sequences for RT-qPCR experiments included in the study

Gene	Forward primer	Reverse primer	Reference
Crf	5'-CAATTACGCACAGATTCTCCTCG-3'	5'-GAAGTACTCCTCCCCCAAGC-5'	(Khezri et al., 2017)
Gr	5'-ACTCCATGCACGACTTGGTG-3'	5'-GCATTTCGGGAAACTCCACG-3'	(Manuel et al., 2014)
β-actin	5'-CGAGCTGTCTTCCCATCCA-3'	5'-TCACCAACGTAGCTGTCTTTCTG-3'	(Tang et al., 2007)
EF1α	5'-CTGGAGGCCAGCTCAAACAT-3'	5'-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3'	(Tang et al., 2007)
Rpl13α	5'-TCTGGAGGACTGTAAGAGGTATGC-3'	5'-AGACGCACAATCTTGAGAGCAG-3'	(Tang et al., 2007)

Figure 1

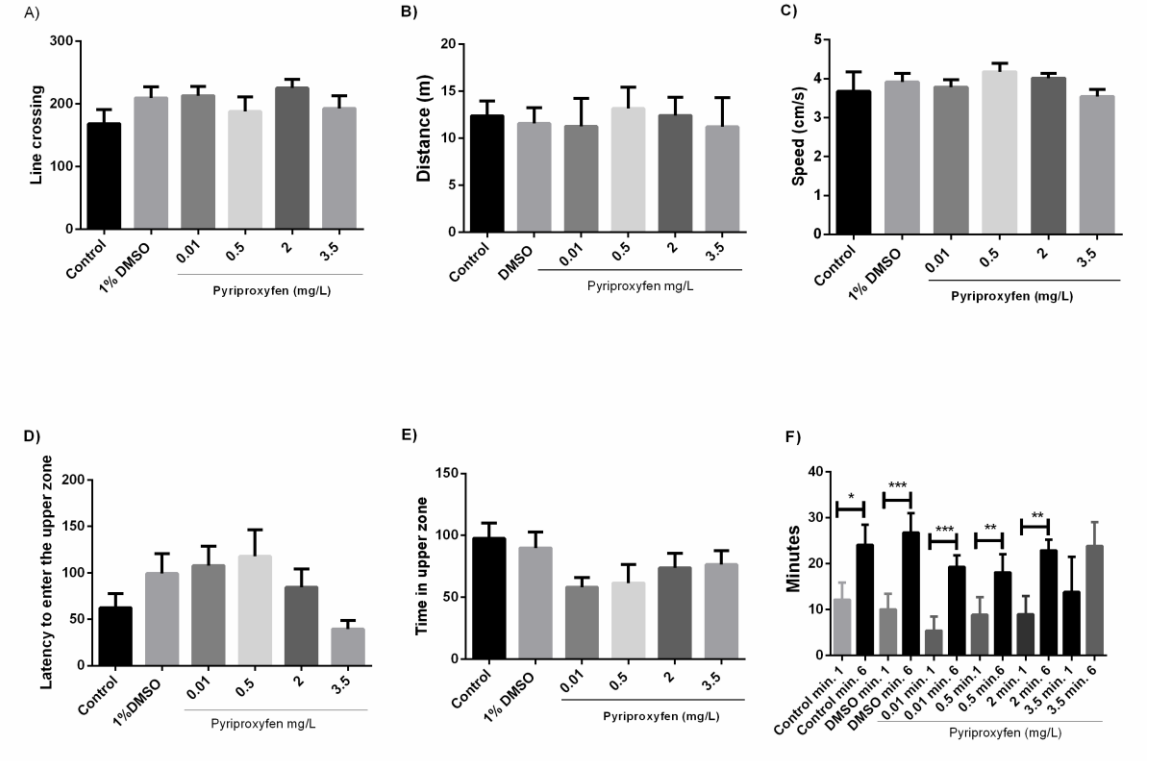

A)
Line crossing
300
200
100
0
Control
1% DMSO
0.01
0.5
2
3.5
Pyriproxyfen (mg/L)
B)
Distance (m)
20
15
10
5
0
Control
DMSO
0.01
0.5
2
3.5
Pyriproxyfen mg/L
C)
Speed (cm/s)
5
4
3
2
1
0
Control
1% DMSO
0.01
0.5
2
3.5
Pyriproxyfen (mg/L)
D)
Latency to enter the upper zone
200
150
100
50
0
Control
1%DMSO
0.01
0.5
2
3.5
Pyriproxyfen mg/L
E)
Time in upper zone
150
100
50
0
Control
1% DMSO
0.01
0.5
2
3.5
Pyriproxyfen (mg/L)
F)
Minutes
40
30
20
10
0
*
***
***
**
**
Control min. 1
Control min. 6
DMSO min. 1
DMSO min. 6
0.01 min. 1
0.01 min. 6
0.5 min.1
0.5 min.6
2 min. 1
2 min. 6
3.5 min. 1
3.5 min. 6
Pyriproxyfen (mg/L)

Figure 2

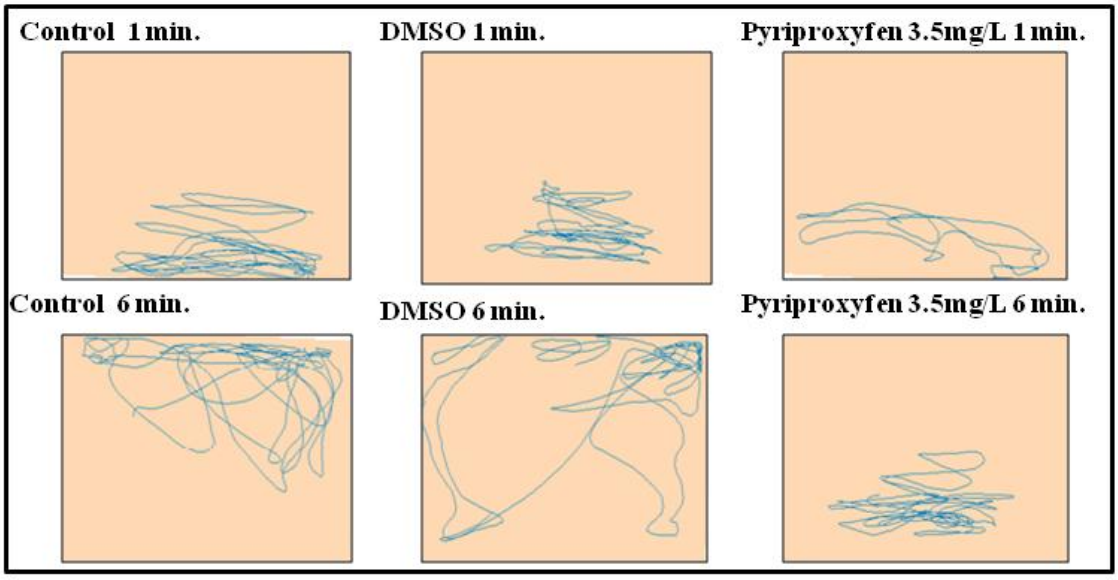
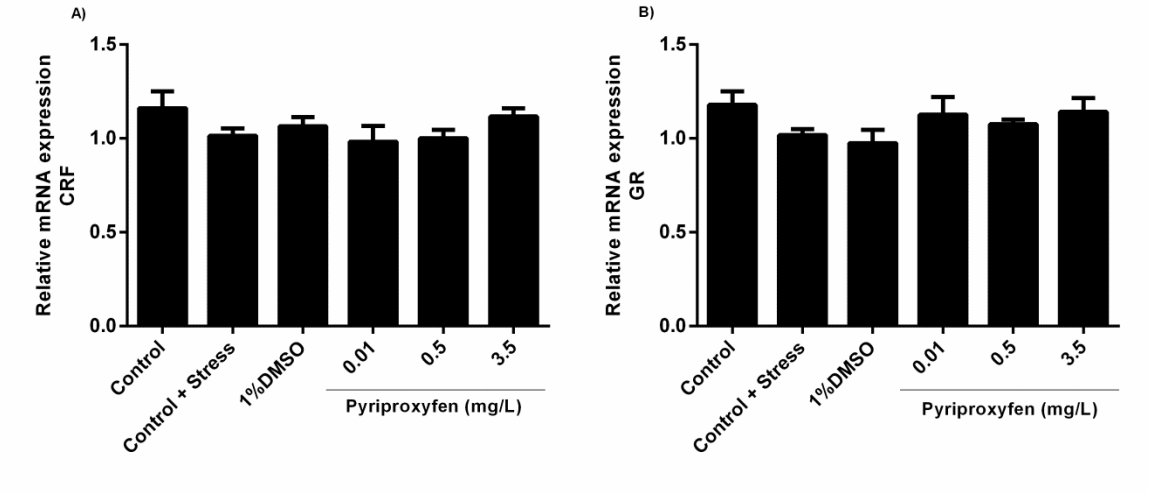

Control 1 min.
DMSO 1 min.
Pyriproxyfen 3.5mg/L 1 min.
Control 6 min.
DMSO 6 min.
Pyriproxyfen 3.5mg/L 6 min.

Figure 3


A)
Relative mRNA expression
CRF
1.5
1.0
0.5
0.0
Control
Control + Stress
1%DMSO
0.01
0.5
3.5
Pyriproxyfen (mg/L)
B)
Relative mRNA expression
GR
1.5
1.0
0.5
0.0
Control
Control + Stress
1%DMSO
0.01
0.5
3.5
Pyriproxyfen (mg/L)

n

CAPÍTULO 4

CONSIDERAÇÕES FINAIS

4. CONSIDERAÇÕES FINAIS

Inseticidas e larvicidas são compostos comumente utilizados para controle de vetores e pragas que transmitem doenças e causam danos em plantações. O piriproxifeno é um larvicida de uso contínuo para proteção de culturas e principalmente para controle de insetos que são potenciais transmissores de doenças, como é o caso dos mosquitos do gênero *Aedes.* No entanto, a utilização descontrolada em combinação com o período em que o piriproxifeno pode se manter no ambiente, que pode atingir um tempo aproximado de oito semanas, pode de forma indireta, causar danos ao meio ambiente (SUMAN et al., 2018).

No capítulo 2 do presente estudo, foram avaliados os efeitos da exposição de peixe-zebra ao piriproxifeno durante 96 h em três diferentes condições: a) larvas foram expostas ao piriproxifeno durante 96h, com o objetivo avaliar a taxa de sobrevivência, locomoção, morfologia e batimentos cardíacos; b) Adultos que foram expostos ao piriproxifeno durante a fase larval, foram testados após 6 meses em parâmetros como distância percorrida, tempo na zona superior e velocidade média; c) Adultos filhos de pais expostos, em que foram analisados os parâmetros de distância percorrida, velocidade média, tempo na zona superior e número de entradas na zona superior.

Nossos resultados demonstraram que o piriproxifeno afeta diretamente a taxa de sobrevivência de larvas expostas, inclusive na concentração de 0,01 mg/L, que é indicada para utilização em água potável (FAWELL et al., 2008), mas não causam diferença significativa na taxa de sobrevivência de adultos expostos. Também observamos que os batimentos cardíacos são afetados no terceiro dia nas concentrações de 0,5 mg/L, 1 mg/L e 2mg/L em relação ao grupos controle e veículo (DMSO 1%). No quinto dia, somente as concentrações de 1 mg/L e 2 mg/L causam diminuição dos batimentos cardíacos em relação aos grupos controle e veículo.

Os aspectos morfológicos mensurados no quinto dia resultaram em diminuição do comprimento de larvas expostas a 0,01 mg/L em relação ao grupo veículo (DMSO 1%). No entanto, no oitavo dia pós-fertilização, nós observamos diminuição do comprimento corporal na concentração de 0,5 mg/L em relação a 0,01 mg/L e grupo veículo, porém, não houve alteração na concentração de 0,01 mg/L em relação ao grupo veículo. Em outros estudos, foi observado que o piriproxifeno causa anormalidades severas nos peixes

sobreviventes e também apresenta alterações, tais como edema pericárdico, edema de saco vitelino, hiperemia e deformidade espinhal nas larvas (MAHARAJAN et al., 2018).

Após análise de parâmetros locomotores, demonstramos que larvas expostas ao piriproxifeno apresentam redução na distância percorrida nas concentrações de 0,01 mg/L e 0,5 mg/L em relação aos grupos controle e veículo. Nesse mesmo contexto, foi observado que a velocidade média também pode estar comprometida pelo efeito do piriproxifeno, uma vez que larvas expostas a 0,5 mg/L apresentaram diminuição na velocidade média em relação ao veículo (DMSO 1%). Em ambientes aquáticos, inevitavelmente ocorre a relação presa-predador. Mudanças em parâmetros, tais como comprometimento da locomoção, alteração na morfologia e déficit de memória podem tornar a presa um alvo mais fácil e levar a um desequilíbrio da espécie (OLIVEIRA et al., 2013).

Peixes que foram expostos ao piriproxifeno na fase larval e testados durante a fase adulta não demonstraram alterações comportamentais em parâmetros locomotores, como distância percorrida e velocidade média, e também não apresentaram mudanças relacionadas à ansiedade. Como o peixe-zebra possui uma alta capacidade regenerativa, a exposição na fase larval pode fazer com que larvas expostas nas primeiras 96 hpf consigam reestabelecer seu comportamento normal até a fase adulta. No entanto, isso não ocorre com filhos de pais expostos, uma vez que a prole adulta proveniente de pais expostos, nas concentrações de 0,5 mg/L e 2 mg/L diminuíram a velocidade média e a distância percorrida quando comparados com os grupos controle e veículo. Estudos prévios demonstraram que o fungicida difenoconazol tem a capacidade de afetar a geração de filhos de pais expostos e alterar o eixo hipotálamo-hipófise-gonadal-fígado, além de induzir mudanças nos genes relacionados ao ovário e testículos (TENG et al., 2018).

No capítulo 3 do presente estudo foram avaliados os efeitos da exposição de peixe-zebra adulto ao piriproxifeno durante 96 h e analisados os parâmetros locomotores, memória e expressão gênica do receptor de glicocorticóide (GR) e hormônio liberador de corticotrofina (CRF). Nossos resultados demonstraram que a exposição ao piriproxifeno durante 96h não causou alteração nos parâmetros comportamentais como ansiedade, e locomoção. No entanto, peixes analisados no teste de memória de habituação, utilizando o primeiro e o sexto minuto para avaliar a memória espacial apresentaram déficits de memória na concentração de 3,5 mg/L. Esse resultado, demonstra que não houve

diferença estatisticamente significativa no tempo de permanência na zona superior, sugerind que não há alteração na ansiedade. Os peixes normalmente passam o primeiro minuto no fundo do aquário e então começam a explorar a zona medial e superior. Peixe-zebra quando introduzido em um ambiente novo permanece por mais tempo na parte inferior e, posteriormente inicia a exploração de todo o ambiente. Na formação de memória de habituação, o peixe-zebra reconhece o ambiente e sabe que não tem um efeito nocivo e então inicia a exploração. O teste de memória de habituação está bem consolidado e representa alta confiabilidade (GROSSMAN et al., 2011; WONG et al., 2010).

As análises da expressão gênica de CRF e GR não apresentaram diferenças significativas entre os grupos testados. Entretanto, estudos demonstram possíveis interações entre fungicidas e inseticidas no sistema inter-renal de peixes expostos (AQUILINO et al, 2018). Estudos futuros serão realizados para quantificar os níveis de cortisol de peixes expostos ao piriproxifeno e se poderá haver interação no eixo hipotálamo-hipófise-interrenal.

Nossos resultados demonstram que peixes expostos ao piriproxifeno apresentam mudanças severas na taxa de sobrevivência e comportamento, o que pode impactar diretamente na sobrevivência dos animais em ambientes aquáticos. Além disso, nós observamos também que o piriproxifeno, além de afetar os parâmetros de peixes diretamente expostos, pode causar alterações nas gerações futuras, o que pode diretamente impactar os ecossistemas aquáticos.

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6. ANEXOS

S I P E S Q

Sistema de Pesquisas da PUCRS

Código SIPESQ: 7546 Porto Alegre, 6 de outubro de 2016.

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou o Projeto de Pesquisa "Efeito do Piriproxifeno sobre o desenvolvimento, parâmetros comportamentais e celulares em Peixe-zebra (Danio rerio)" coordenado por CARLA DENISE BONAN.

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está autorizada a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Nº de Animais	Espécie	Duração do Projeto
3462	Peixe-Zebra (Danio rerio)	06/10/2016 - 06/02/2018