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PROGRAMA DE PÓS-GRADUAÇÃO EM BIOEXPERIMENTAÇÃO

EXPOSIÇÃO EMBRIONÁRIA A GENISTEÍNA INDUZ COMPORTAMENTO ANSIOLÍTICO E ANTISSOCIAL EM *ZEBRAFISH*: EFEITOS PERSISTENTES ATÉ A FASE ADULTA

DISSERTAÇÃO DE MESTRADO

Aloma Santin Menegasso

Passo Fundo, RS, Brasil
2020

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Bioexperimentação, Área de Concentração em Bioexperimentação, da Faculdade de Agronomia e Medicina Veterinária da Universidade de Passo Fundo (UPF), como requisito parcial para a obtenção do grau de **Mestre em Bioexperimentação**

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Dedico este trabalho a minha família e a todos os meus professores, pilares da minha formação como ser humano.

EPÍGRAFE

Prefiro sempre o temor do entendimento ao temor da ignorância.

Douglas N. Adams

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3. CAPÍTULO 1

LISTA DE SIGLAS E ABREVIATURAS

α	Alfa
β	Beta
BPM	Batimentos por minuto
CEUA	Comitê de Ética em Pesquisa
CONCEA	Conselho Nacional de Controle de Experimentação Animal
DMSO	Dimetilsufóxido
dpf	Dias pós-fecundação
ER	*Estrogenic receptor*
FAMV	Faculdade de Agronomia e Medicina Veterinária
hpf	Horas pós-fecundação
g/mol	Gramas por mol
LDT	Teste Claro/Escuro
min	Minuto
MSRE	Modulador Seletivo de Receptores de Estrogênio
PST	Teste Preferência Social
ng/L	Nanograma por litro
NTT	Teste Tanque Novo
μg/L	Microgrma por litro
UPF	Universidade de Passo Fundo

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo

EXPOSIÇÃO EMBRIONÁRIA A GENISTEÍNA INDUZ COMPORTAMENTO ANSIOLÍTICO E ANTISSOCIAL EM *ZEBRAFISH*: EFEITOS PERSISTENTES ATÉ A FASE ADULTA

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Orientador: Luciana Grazziotin Rossato-Grando
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Passo Fundo, 16 de dezembro de 2020.

A genisteína é um fitoestrógeno presente em diversos alimentos e em maior abundância na soja e em seus derivados. Possui estrutura química semelhante ao estrogênio 17β-estradiol e tem a capacidade de ligação nos mesmos receptores, desencadeando ação estrogênica ou antiestrogênica dependendo do tecido alvo, concentração de estrógenos endógenos e número de receptores livres. Por ser uma substância desreguladora endócrina, pode trazer prejuízos ao sistema reprodutor, tanto em humanos quanto em animais. O principal objetivo deste trabalho foi elucidar os efeitos que a genisteína, em concentrações similares às encontradas em efluentes, gera no comportamento e na diferenciação sexual de peixes expostos durante o período embrionário. Os efeitos da exposição embrionária foram avaliados na fase larval e na fase adulta. Ovos de *zebrafish* foram expostos durante as primeiras 72 horas pós-fecundação (hpf) a 3 diferentes concentrações de genisteína: 10μg/L, 40μg/L e 80μg/L. Às 48hpf foi realizada a verificação da frequência cardíaca (BPM) e durante as primeiras 72hpf foi realizada a taxa de mortalidade. Os testes comportamentais claro-escuro e campo novo foram aplicados nas larvas no 6dpf, e os testes tanque novo, preferência social e claro-escuro nos peixes adultos no 90dpf. A sexagem foi realizada após realização dos testes comportamentais (90dpf). A exposição embrionária à genisteína causou um comportamento tipo-ansiolítico tanto em larvas quanto na fase adulta. Na fase adulta observamos um aumento na atividade locomotora e um comportamento antissocial na concentração de 40μg/L. Houve um aumento na taxa de mortalidade, nas concentrações de 10, 40 e 80μg/L quando comparados com o controle e houve um aumento na frequência cardíaca na concentração de 80μg/L. A exposição a 10μg/L apresentou uma maior frequência de fêmeas quando comparado à frequência encontrada nos controles. Nossos resultados evidenciam que a exposição a genisteína durante a fase embrionária

traz prejuízos a curto e longo prazo uma vez que aumenta a taxa de mortalidade e leva a distúrbios de comportamento tanto na fase larval quanto na fase adulta. O efeito tipo-ansiolítico e a menor interação social são efeitos que trazem prejuízo à sobrevivência, devido à perda de noção de predador, afetando o desempenho social e consequentemente a busca por alimentos e a reprodução dos peixes.

Palavras-chave: Fitoestrógenos. Genisteína. Isoflavonas. Comportamento. *Zebrafish*.

ABSTRACT

Master's Dissertation
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo

EMBRYONIC EXPOSURE TO GENISTEIN INDUCES ANSIOLYTIC AND ANTISOCIAL BEHAVIOR IN ZEBRAFISH: PERSISTENT EFFECTS UNTIL ADULT STAGE

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Passo Fundo, 16 de dezembro de 2020.

Genistein is a phytoestrogen present in several food and in bigger abundance in soybean and its derivatives. It has a chemical structure similar to 17β-estradiol and has the ability to bind to the same receptors, triggering estrogenic or antiestrogenic action depending on the target tissue, concentration of endogenous estrogens and number of free receptors. Because it is an endocrine disrupting substance, it may cause damage to the reproductive system, both in humans and animals. The main objective of this work was to elucidate the effects that genistein, in similar concentrations found in effluents, causes in behavior and sexual differentiation of fish exposed during the embryonic period. The effects of embryonic exposure were evaluated in the larval stage and in adult stage. Zebrafish eggs were exposed during the first 72 hours post-fertilization (hpf) to 3 different concentrations of genistein: 10μg/L, 40μg/L e 80μg/L. At 48hpf, the heart rate was checked (BPM) and during the first 72hpf, the mortality rate was performed. The light/dark and open field behavioral test were applied to the larvae at 6 day post-fertilization (dpf), and the novel tank, social preference and light/dark tests to adult fish at 90dpf. Sexing was performed after conducting behavioral tests (90dpf). Embryonic exposure to genistein caused anxiolytic-like behavior both in larvae and in adult stages. In adult stages, we observed an increasing in locomotor activity and antisocial behavior in concentration of 40μg/L. There was an increase in mortality rate, in concentrations of 10, 40 and 80μg/L when compared to the control and there was an increase in heart rate at the dosage of 80μg/L. Exposure to 10μg/L presented a higher rate in females when compared to the rate found in controls. Our results show that exposure to genistein during embryonic phase brings short and long term damages as it increases mortality rate and leads to behavioral disorder both in the larval and adult stages. The anxiolytic-like effect and less social interaction are effects that harm survival, due to the loss of notion of predator, affecting social performance and, therefore, the search for food and the reproduction of fish.

Key-words: Phytoestrogens. Genistein. Isoflavones. Behavior. Zebrafish.

1. INTRODUÇÃO

A soja e os demais alimentos derivados possuem alto teor de proteína e gordura e baixo teor de carboidratos, o que a difere de outras leguminosas, sendo esse um dos motivos do seu consumo ser alto na dieta vegetariana e asíatica. Possui em sua composição as isoflavonas (1), que são biosintetizadas em vários tipos de plantas como, por exemplo, no trevo vermelho, feijão vermelho, broto de feijão mungo, feijão marinho, araruta japonesa (Kudzu), porém encontrando-se de forma mais abundante na soja (2).

Das isoflavonas, as mais utilizadas como suplementação alimentar são a genisteína e a daidzeína. Estruturalmente ambas são similares ao estrogênio 17β-estradiol e podem se ligar aos seus receptores, porém a genisteína possui uma afinidade e potencial de ligação superior (3).

Uma substância desreguladora endócrina pode ser um composto natural ou sintético com capacidade de alterar os sistemas homeostático e hormonal, os quais permitem que o organismo responda corretamente ao ambiente. A exposição a um desregulador endócrino em diferentes fases da vida podem ter consequências muito diferentes. A exposição durante a fase inicial da vida (embrionária) pode interferir nos genes do indivíduo e determinar a propensão de desenvolvimento de doenças ou disfunções na fase adulta (4).

Estudos mostram que tanto efluentes quanto reservatórios de água tratada usada para consumo humano apresentam substâncias desreguladoras endócrinas, entre elas o estrogênio e a genisteína (fitoestrógeno)(5–7). As isoflavonas são um dos principais compostos encontrados em efluentes, provenientes majoritariamente do escoamento agrícola (8). A exposição embrionária a fitoestrógenos afeta a diferenciação sexual e o comportamento de aves (9) e aumenta a apoptose por mecanismos independentes de receptores estrogênicos em embriões de peixes (8). Porém, os efeitos deste padrão de exposição sobre o comportamento de peixes quando adultos ainda não foram elucidados.

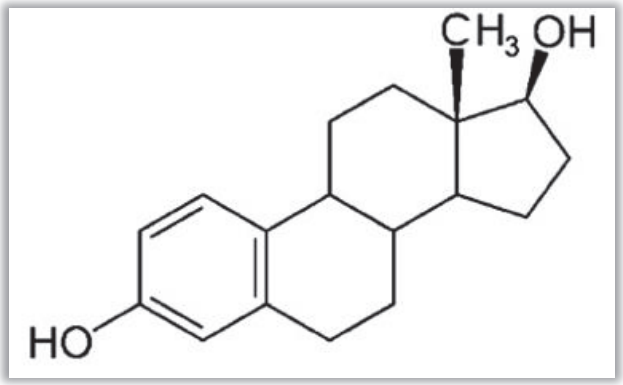
Considerando estes aspectos, esta dissertação teve como objetivo avaliar se a exposição embrionária à genisteína provoca alterações comportamentais na fase larval e na fase adulta, e/ou a feminilização em *zebrafish*. A presente dissertação encontra-se dividida em introdução, revisão de literatura abordando os principais aspectos do fitoestrógeno genisteína, riscos e benefícios, além de dados sobre sua presença em

efluentes. Os resultados obtidos estão apresentados no capítulo 1 em forma de artigo científico intitulado: Embryonic Exposure to Genistein Induces Ansiolytic and Antisocial Behavior in Zebrafish: Persistent Effects Until Adult Stage, o qual será submetido ao periódico *Archives of toxicology*. Por fim apresentamos as conclusões do estudo, as considerações finais e as referências bibliográficas.

2. REVISÃO DE LITERATURA

Os fitoestrogênios são polifenóis naturais não esteroidais estruturalmente similares ao estrogênio 17β-estradiol (Figura 1) (10). Possuem, portanto, a capacidade de desencadear atividade estrogênica e dependendo do tecido, dos receptores e da concentração de estrógenos endógenos circulantes, essa atividade será agonista ou antagonista (11).

Figura 1: Estrutura química 17β-estradiol.



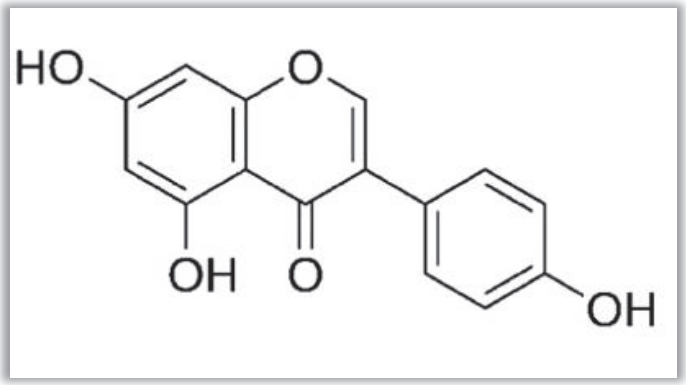
Fonte: (12).

Durante os últimos anos, os fitoestrógenos, em especial a genisteína, entraram em evidência na área de pesquisa e saúde devido aos seus potenciais efeitos benéficos, entre eles: o alívio dos sintomas da menopausa, prevenção da osteoporose, melhora na saúde cardiovascular, diminuição do risco de diabetes, redução dos distúrbios neurológicos, prevenção de cânceres incluindo câncer de mama e endometrial (13).

A soja e seus derivados possuem uma grande quantidade de isoflavonas e a mais abundante é a genisteína (Figura 2) (14). Seus efeitos estrogênicos são amplamente reconhecidos tanto em animais quanto em seres humanos e atingem o sistema reprodutivo, neurológico, bem como o desenvolvimento celular (15), sendo classificada como um fitoestrógeno.

A capacidade da genisteína, em alguns casos de simular a atividade do hormônio 17β-estradiol e em algumas células ter o efeito contrário, a classifica como Modulador Seletivo de Receptores de Estrogênio (MSRE) (10).

Figura 2: Estrutura química da genisteína.



Fonte: (12).

A genisteína (5,7-dihydroxy-3-(4-hydroxyphenyl)-4-benzopyrone) tem uma estrutura difenol, com distância entre os grupos OH nos lados opostos da molécula semelhantes ao17β-estradiol, o que a torna capaz de se ligar aos subtipos de receptores de estrogênio alfa e beta. Sua fórmula molecular é: C15H10O5, possui peso molecular de 270,24g/mol, ponto de fusão: 297–298ºC, possui solubilidade em: dimetilsulfóxido (DMSO), praticamente insolúvel em água, se apresenta como um pó esbranquiçado a levemente amarelado, sensível à luz (16,17).

A ligação do fitoestrógeno com o receptor celular irá depender da concentração de estrógenos endógenos, ou seja, quando a concentração de estrógeno endógeno estiver baixa, como por exemplo na menopausa ou na infância, haverá mais receptores livres para que ocorra a ligação com os fitoestrógenos consumidos, como por exemplo, a genisteína (18). Assim, as isoflavonas podem desregular o sistema endócrino, gerando uma ameaça ao sistema reprodutivo masculino (19). Recentemente nosso grupo de pesquisa demonstrou este efeito em ratos machos expostos a uma dieta rica em leite de soja durante todo o período pré-pubertal. Esta dieta causou uma diminuição da testosterona e na qualidade dos espermatozoides quando os animais atingiram a adolescência (20).

A exposição neonatal à genisteína afeta negativamente também o sistema reprodutor feminino de ratos, comprometendo a resposta uterina a estímulos hormonais, levando à falha na implantação fetal e infertilidade (21).

O estrogênio possui variadas funções fisiológicas no organismo, as mais comuns são: desenvolver características sexuais secundárias, regulação da secreção de gonadotrofina para ovulação, manutenção da massa óssea, regulação da síntese de

lipoproteínas, regulação da resposta à insulina, manutenção de funções cognitivas, entre outros. Sabe-se que diversos tecidos possuem a capacidade de sintetizar estrogênio a partir de hormônio androgênico, em ambos os sexos (22). A enzima P450 aromatase faz essa conversão (23). Os efeitos do estrogênio são modulados através de dois diferentes receptores (ER), ERα e ERβ, ambos possuem afinidade similar a estrogênios naturais e sintéticos, conforme descrito em estudos in vitro. Porém diferem na sua expressão conforme localização nos tecidos. O ERα é predominantemente expresso na mama, útero, colo do útero, vagina e vários outros órgãos alvo. Já o ERβ possui uma menor expressão, e se encontra majoritariamente no ovário, próstata, testículo, baço, pulmão, hipotálamo e timo. Quanto ao cérebro são expressos em maior ou menor quantidade conforme região cerebral (24).

Os mecanismos pelos quais o estrogênio age no cérebro ainda não estão totalmente elucidados, porém pode se afimar que este hormônio é capaz de modular neurotransmissores como a serotonina, noroepinefrina e dopamina (25).

A via de ligação do estrogênio pode ser intracelular ou extracelular. A via intracelular pode envolver a área genômica, de transcrição de genes alvo, ou a não genômica, que traduz rapidamente os sinais mediados por receptores ligados a membrana. Já a via extracelular envolve outros hormônios, citoquinas, fatores de crescimento e a sinalização autócrina ou parácrina (26).

Além da atividade estrogênica, a genisteína pode atuar inibindo a proteína tirosina quinase, modulando o sistema imunológico e através de atividade antioxidante. (27,28). A tirosina quinase possui atividade em diversas vias de sinalização celular, incluindo transmissões neuronais, modificando canais iônicos e transportadores através da fosforilação da tirosina (29).

Substâncias químicas desreguladoras do sistema endócrino presentes no meio ambiente são uma preocupação crescente. Essas substâncias são originadas de diferentes fontes, como por exemplo, produtos industriais, pesticidas, resíduo de medicamentos, resíduos de lavouras e promotores de crescimento (6,8). Em um estudo na Alemanha a genisteína foi detectada em 60% das amostras de água analisadas com uma concentração média de 2,7ng/L e máximo de 38ng/L (30), em Portugal os níveis encontrados dessa substância foi de em média 230ng/L (31), nos EUA os níveis detectados em um lago urbano foi de 1,4 +/- 0,5 ng/L e em efluentes de águas residuais tratadas de 1,6 +/- 0,4ng/L (32), em outro estudo também em Portugal foram encontrados concentrações que variam entre 96,3 a 135ng/L (33), no Canadá, em

Ontário, foram encontradas concentrações que variavam de 13,1μg/L a 10,5μg/L em efluentes não tratados e tratados respectivamente, ou seja, a substância permanecia em concentrações semelhantes mesmo após o tratamento (7). No Japão em Osaka foram detectados níveis médios de genisteína de 143.4μg/L (34). Em amostras de águas residuais provenientes da produção de leite de soja, foram detectados 20μg/L e <1μg/L em afluentes e efluentes respectivamente (35). Estudo realizado em reservatórios de água tratada usada para consumo humano na China demonstrou a presença de outros desreguladores endócrinos, como o 17β-estradiol, em todas as 23 fontes testadas (5). Essas substâncias possuem a capacidade de alterar as funções endócrinas normais levando, por exemplo, à feminilização dos peixes em locais onde há resíduos provenientes dos esgotos (36).

O *zebrafish* é um dos modelos mais úteis nas pesquisas genéticas, fenotípicas e da influência de substâncias químicas devido a alta homologia do seu genoma com o genoma humano (37). Esse peixe possui alta fecundidade, com capacidade de fertilizar 200 a 300 ovos, chegando a fase adulta em cerca de 90 dias. Permitem um estudo rápido e econômico quando comparado a outros modelos animais (38). As drogas ou compostos a serem estudados podem ser adicionados diretamente ao ambiente da água do *zebrafish*, facilitando a manipulação (37). Para estudos de exposição embrionária é considerado um dos melhores modelos devido à sua alta sensibilidade. Além disso, o embrião de *zebrafish* tem inigualável clareza óptica, permitindo o rastreamento visual de destinos de células individuais ao longo da organogênese (38).

O *zebrafish* é um organismo modelo cada vez mais utilizado para estudar mecanismos fisiológicos, genéticos e neurobiológicos, além de ser amplamente utilizado para rastreio de medicamentos, devido a sua fenotipagem neurocomportamental quanto a ansiedade e estresse (39). São altamente sensíveis a estressores ambientais, que variam desde a exposição a predadores, a feromônios de alarme, ambientes novos, exposição a drogas ou a sua retirada. Podem ser utilizados em estudos de exposição aguda ou crônica tanto na fase larval como no estágio adulto com a mesma validade (40,41).

3 CAPÍTULO 1

EMBRIONIC EXPOSURE TO GENISTEIN INDUCES ANXIOLITIC AND ANTISOCIAL BEHAVIOR IN ZEBRAFISH: PERSISTENT EFFECTS UNTIL THE ADULT STAGE

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Abstract

Genistein is a phytoestrogen, similar to 17β-estradiol. It is present in plants, food, and as a contaminant in effluents. In this article, we demonstrate the ecotoxicity of embryonic exposure to three different concentrations of genistein, similar to those found in effluents: 10µg/L, 40µg/L and 80µg/L. Zebrafish eggs were exposed during the first 72 hours post-fertilization (hpf). At 48hpf the heart rate was evaluated and during the first 72hpf the mortality rate was performed. The light/dark (LDT) and open field (OPT) behavioral tests were applied to the larvae (6dpf), and the novel tank (NTT), social preference (SPT), light-dark (LDT) and sexing tests were performed on adult fish (90dpf). Embryonic exposure to genistein caused anxiolytic-like behavior in both larvae and adult animals. In adult stage we observed an increase in locomotor activity and antisocial behavior in the concentration of 40µg/L. There was an increase in the mortality rate in all concentrations when compared to the control and also an increase in heart rate at the concentration of 80µg/L. Exposure to 10µg/L generated a higher frequency of females when compared to the control group. Our results show that exposure to genistein during the embryonic phase brings damage in short and long term as it increases the mortality rate and leads to behavioral disorders both in the larval stage, with perpetuation until adult stage. The anxiolytic-like effect and less social interaction are effects that harm fish survival.

Keywords: Phytoestrogens. Genistein. Isoflavones. Behavior. Zebrafish.

1. Introduction

Phytoestrogens are a group of substances naturally present in several types of plants. Genistein is an example of these phytoestrogens, abundantly present in soybeans and its derivatives (McClain et al. 2007). It has a chemical structure and molecular weight very similar to those of steroidal hormone 17β-estradiol and bind to the same receptors, mimicking its functions in the organism (Meza et al. 2015). They can also establish antiestrogenic action, depending on concentration of circulating endogenous estrogens, free receptors and target tissue (Gaffer et al. 2018).

Benefits of consuming genistein have already been described, such as the relief of menopausal symptoms, the prevention of osteoporosis, the improvement of cardiovascular health, the reduction of the risk of diabetes, the reduction of neurological disorders and the prevention of cancers (Van Duursen 2017; Mukund et al. 2017; Spagnuolo et al. 2015; Weng et al. 2019). However, genistein is also considered an endocrine disruptor (Lintelmann et al. 2003). Its presence as contaminant in effluents and water for human consumption has already been described (Sassi-Messai et al. 2009; Rearick et al. 2014). In Germany, genistein was detected in 60% of the analyzed water samples with an average concentration between 2,7ng/L e 38ng/L (Spengler et al. 2001). In Portugal, there are records of levels ranging from 96 to 230ng/L (Ribeiro et al.2016; Rocha et al. 2013). In wastewater samples from soy milk production or close to soy plantations the level of these contaminants are higher (20µg/L) (Ferrer et al. 2009). In Japan, in industrial and agricultural regions, average genistein levels of 143.4µg/L were detected (Kawanishi et al. 2004). Conventional treatment of effluents is not effective in removing these residues, as demonstrated in a study in Ontario, Canada, where concentrations ranging from 10,5µg/L to 13,1µg/L were found in untreated and treated effluents respectively (Kiparissis et al. 2001).

The impact of embryonic fish exposure to genistein residues remains unknown. The present work aims to assess the effects of embryonic exposure to genistein residue, in concentrations already described in the environment, both in larvae and in adult fish.

2. Materials and Methods

2.1 Study strategy

To assess the effects of genistein, zebrafish was exposed during the embryonic period, that is, in the first 72 hours post-fertilization (hpf) at the following concentrations: 10µg/L, 40µg/L and 80µg/L. The concentrations chosen were based on those already found in effluents (Ferrer et al. 2009; Kawanishi et al. 2004; Kiparissis et al. 2001; Sassi-Messai et al. 2009). After hatching the eggs, open field and light/dark behavioral tests were performed with the larvae at 6 dpf. Mortality rate and heart rate (beats per minute – BPM) were also assessed. After ninety days, in adult stage, behavioral tests novel tank, light/dark and social preference were performed, followed by euthanasia and sexing to determine the frequency of female and male fish. All the tests were equally performed in the control group (unexposed). The methodology is outlined in Figures 1 e 2.

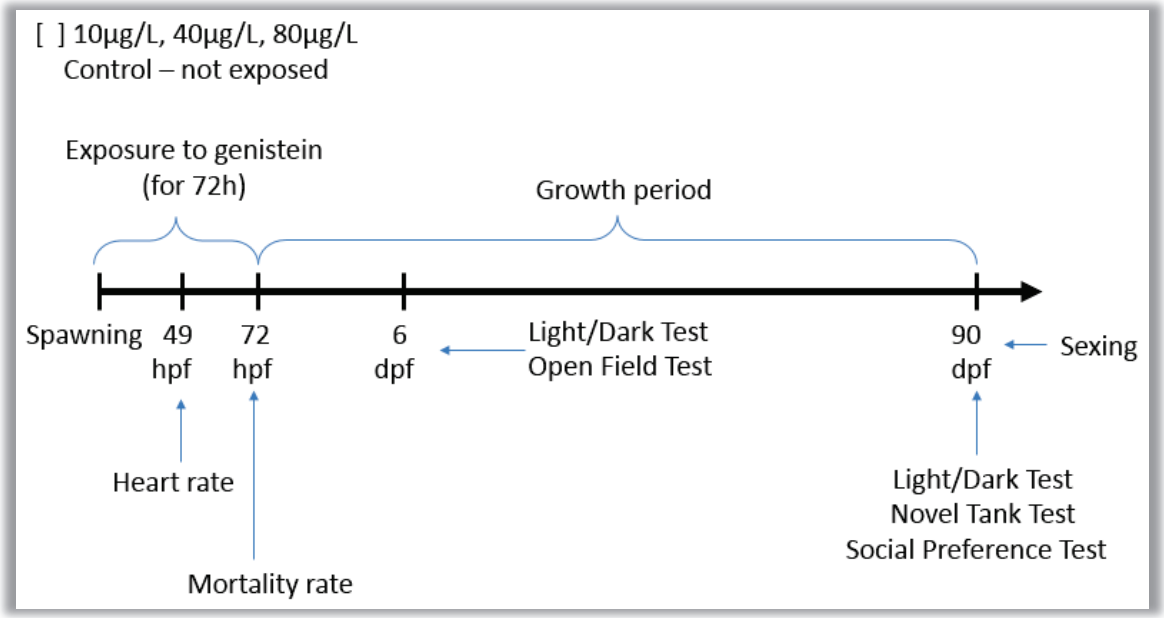

Figure 1: Experimental design.

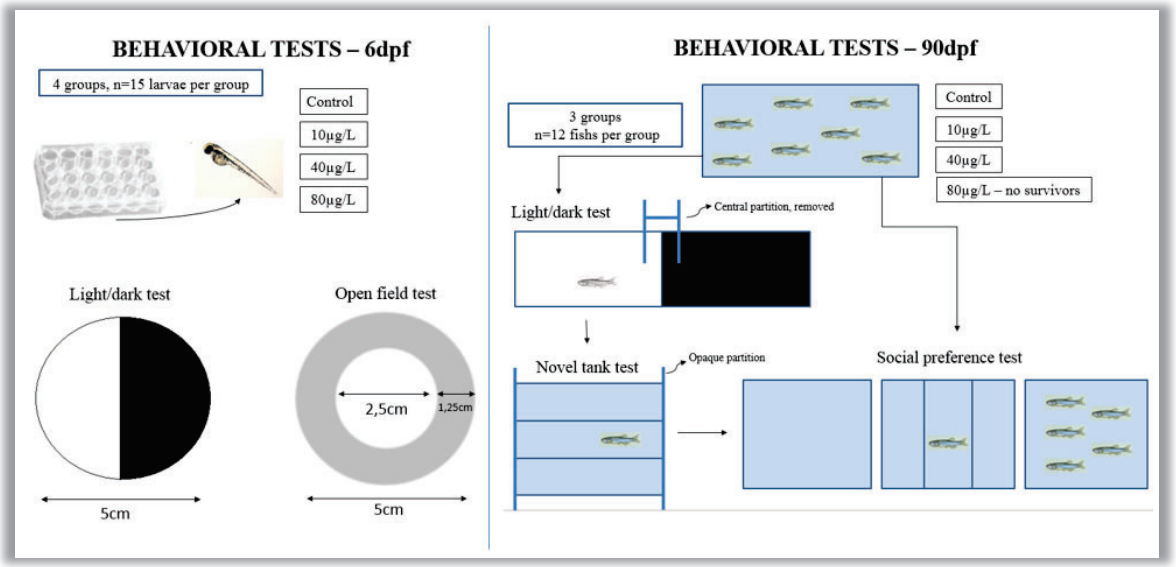

Figure 2: Methodological flowchart.

2.2 Animals and maintenance conditions

Adult male and female zebrafish (*Danio rerio*) were placed in breeding tanks specifically designed to prevent predation of eggs, separating adult animals from eggs with the aid of a grid with a 1 x 1 mesh inside each breeding tank. Adult animals were removed and the fertilized eggs were collected by siphoning (Spence et al. 2007; Buske and Gerlai 2012). Afterwards, fertilized eggs were transferred to cell culture plates. The plates were kept in an incubator at 28°C +/- 2ºC and monitored daily. Unfertilized and dead eggs were identified and removed. Were kept in the environment: reverse osmosis water, oxygen concentrations at 6.2 ± 0.4 mg/L, pH 7.0 ± 0.25, with natural photo period (14h light, 10h dark), they were fed twice a day (Avdesh et al. 2012).

2.3 Drug and concentrations

The embryos were divided into four (300 by group), one being a negative control group (unexposed to genistein), and the other three groups exposed to genistein, with the chosen concentrations based on those found in effluents: 10µg/L, 40µg/L, 80µg/L (Ferrer et al. 2009; Kawanishi et al. 2004; Kiparissis et al. 2001; Sassi-Messai et al. 2009). The drug (genistein) was added directly to the water, without adding solvents.

2.4 Embryonic evaluations

The classification of “hatched eggs” were to those that had exposure of all or most of the tail present (Kimmel et al. 1995). The mortality rate was calculated to reflect the ratio of dead to living embryos/larvae. Non-transparent, coagulated embryos, with no formation of somites and with no cardiac movement and blood circulation were considered dead embryos. Furthermore, the absence of reflexive response to stimuli or unborn eggs after 72 hours were also considered dead (Kimmel et al. 1995).

The heart rate was assessed in all groups at 49hpf. Four embryos, randomly selected, were assessed per group and they were tested twice. The counting of the heartbeat was performed in 1 minute (min) using a stereomicroscopic (Kimmel et al. 1995; Kalichak et al. 2016).

2.5 Evaluation of behavior parameters

Behavioral tests were performed on the animals in larval and adult stages. The tests performed in larval stage (at 6dpf) were: open field (OFT) and light-dark (LDT), to evaluate parameters of locomotion and anxiety. In survival animals in adult stage (90 dpf) the performed tests were: novel tank (NTT), social preference (SPT) and light-dark (LDT), to assess parameters of locomotion, anxiety and response to social stimulus. After each test, the water in the apparatus was completely changed, to receive the new individual to be tested (Colwill and Creton 2011; Dametto et al. 2018; Kalueff 2017; Kalichak et al. 2017; Kysil et al. 2017; Maximino et al. 2010; Magno et al. 2015; Steenbergen et al. 2011).

2.5.1 Open Field Test (larval phase)

To perform the open field test, 15 larvae were used per group. Individually placed in 10mL wells, the larvae were filmed for 6 minutes, with Canon EOS Rebel T5 Lens Macro EF 100mm cameras, according to Figure 2. The test was divided between initial phase (0 to 3min) and final phase (3 to 6min). In the videos, the well was virtually divided into a central and peripheral area (Colwill and Creton 2011). The distance travelled in the peripheral area, latency to enter the peripheral area, time in the

central area and entries in the central area were analyzed using the ANY-maze software (Kalichak et al. 2017; Kysil et al. 2017).

2.5.2 Light-Dark Test (larval phase)

It was used a 6-well cell culture plate, as shown in Figure 2. One well (5mL) was divided between a dark zone (black) and a light zone (white). 15 larvae (6dpf) per group were placed in the testing area and individually filmed for 12 minutes (Steenbergen et al. 2011).

The permanency time in the light zone, the number of entries, the distance traveled and the absolute turn angle were evaluated using ANY-maze software. The test was divided between initial phase (0 to 6min) and final phase (6 to 12 min) (Kysil et al. 2017; Steenbergen et al. 2011).

2.5.3 Novel Tank Test and Social Preference test (adult stage)

Novel tank and social preference were performed concomitantly, with twelve individuals per group. First, the novel tank test was applied. Each fish individually placed in a tank (24x8x20cm, length, width and height), the sides were covered with an opaque partition according to Figure 2, and each fish was filmed for 6 minutes. The parameters analyzed using ANY-maze software were: total distance, number of crossings, absolute turn angle, time spent in the upper zone, number of entries and latency for the first entry in the upper zone, time spent in the lower zone and time of immobility (Dametto et al. 2018; Kalueff 2017; Kirsten et al. 2018; Saverino and Gerlai 2008). After 6 minutes of test, the partition between the tanks were removed to being the social preference test. One of the sides of the apparatus was filled with water, with no fish, and on the other side of the apparatus 10 adult fish were placed without sex specification. The behavior of each fish was filmed for 1 minute, individually. The parameters analyzed using ANY-maze software were: total distance, number of crossings, absolute turn angle, time spent in isolation zone, latency for the first entry in the interaction zone, time and number of entries in the social interaction zone (Dametto et al. 2018; Kalueff 2017; Kirsten et al. 2018; Kysil et al. 2017).

2.5.4 Light/Dark Test (adult stage)

To perform the light-dark behavioral test, the apparatus described in Figure 2 was used, a glass tank (15 cm × 10 cm × 45 cm, height × width × length) divided equally into two parts, one-half white and one-half black, both the walls and the bottom. The tank contained two central separations linked together, each separation with the color of the tank where it sits, creating a central compartment of 15 cm × 10 cm × 10 cm. 12 adult fish per group were individually filmed for 7 minutes. In the first minute, individuals were in the central containment area for adaptation, after this area was opened and they remained in the test for another 6 minutes (Maximino et al. 2010; Magno et al. 2015).

The permanency time in the light zone, number of entries and latency for the entry in the light zone, total distance traveled, absolute turn angle and number of rotations were evaluated using ANY-maze software (Kysil et al. 2017).

2.6 Euthanasia

After behavioral tests, all fish in the study were euthanized. First, they were transferred to a container with water at a temperature close to 0ºC in order to anesthetize them. After, the spinal cord dislocation was performed (Matthews and Varga 2012).

2.7 Sexing

After euthanasia, an analysis of the sexual differentiation of the animals was performed, through dissection and morphological visualization of the reproductive organs, the presence of testicles (male) or ovaries (females) (Yossa et al. 2013).

2.8 Statistical analysis

The results were evaluated for data distribution using the Shapiro-Wilk test. Parametric data were compared using ANOVA followed by Dunnet's test. Nonparametric data were compared by Kruskal-Wallis followed by Dunn´s test. Sexing

was performed by analyzing the frequency and comparisons using Binomial test. The groups will be considered different when $p<0.05$.

3. Results

3.1 Behavior parameters

3.1.1 Embryonic exposure to genistein causes anxiolytic-like behavior in both the larval and adult stages

Larvae (6 dpf) exposed to 10μg/L and 80μg/L entered a bigger number of times in the light zone (Fig. 3B), where they spend less time than control animals (Fig. 3A). The 80μg/L group traveled a greater distance when compared to the 40μg/L group (Fig. 3C), and had a greater absolute turn angle when compared to the group exposed to a 10μg/L (Fig. 3D).

Adult animals exposed to 40μg/L had an increase in the number of entries in the light zone (Fig. 4B), traveled a greater distance (Fig. 4D) and showed a greater number of rotations (Fig. 4F) when compared to the controls. The group exposed to 10ug/L spent more time in the light zone (Fig. 4A), and both the 10μg/L and the 40μg/L had an increase in latency to enter the light zone (Fig. 4C) and an increase in absolute turn angle (Fig. 4E) when compared to the controls.

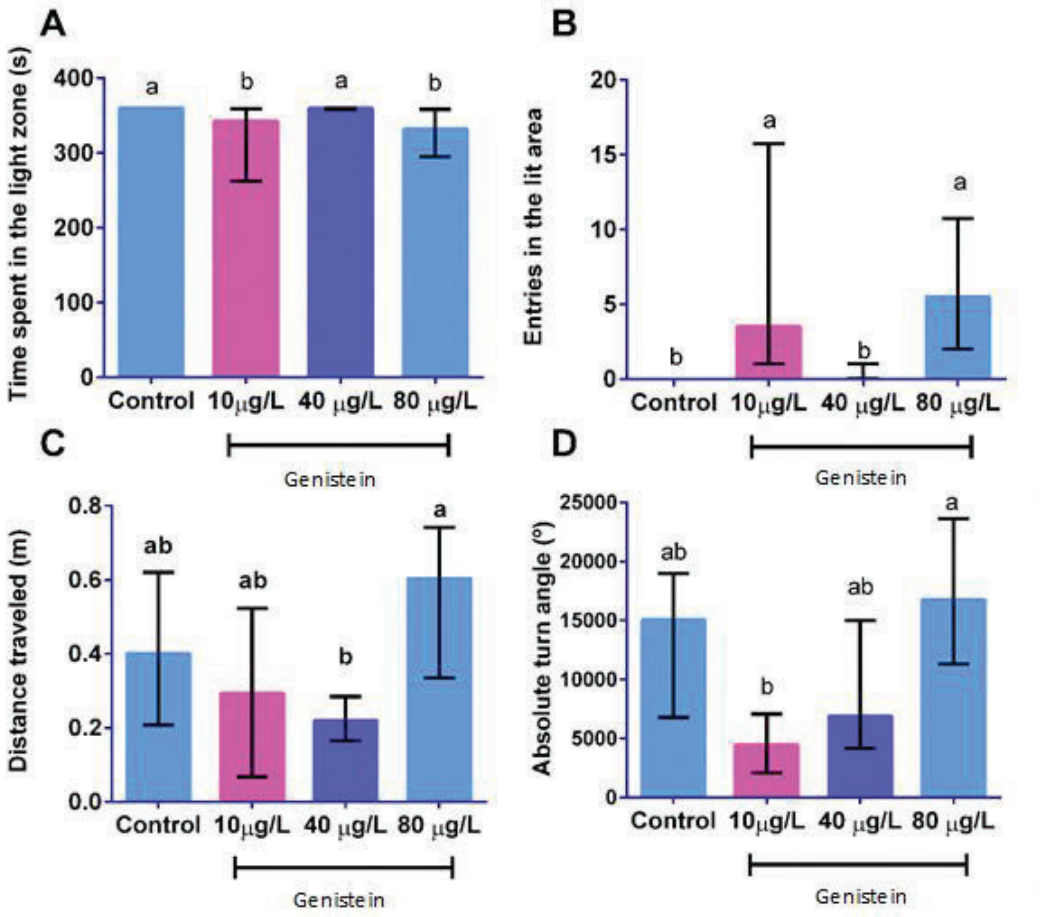

Figure 3: Behavioral parameters of the light/dark test (larvae) in the control groups, 10µg/L, 40µg/L and 80µg/L. Time spent in the light zone (A), Number of entries in the light zone (B), Distance traveled (C), Absolute turn angle (D). Data expressed as median ± interquartile range, analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test. Different letters indicate statistical difference at $p < 0.05$.

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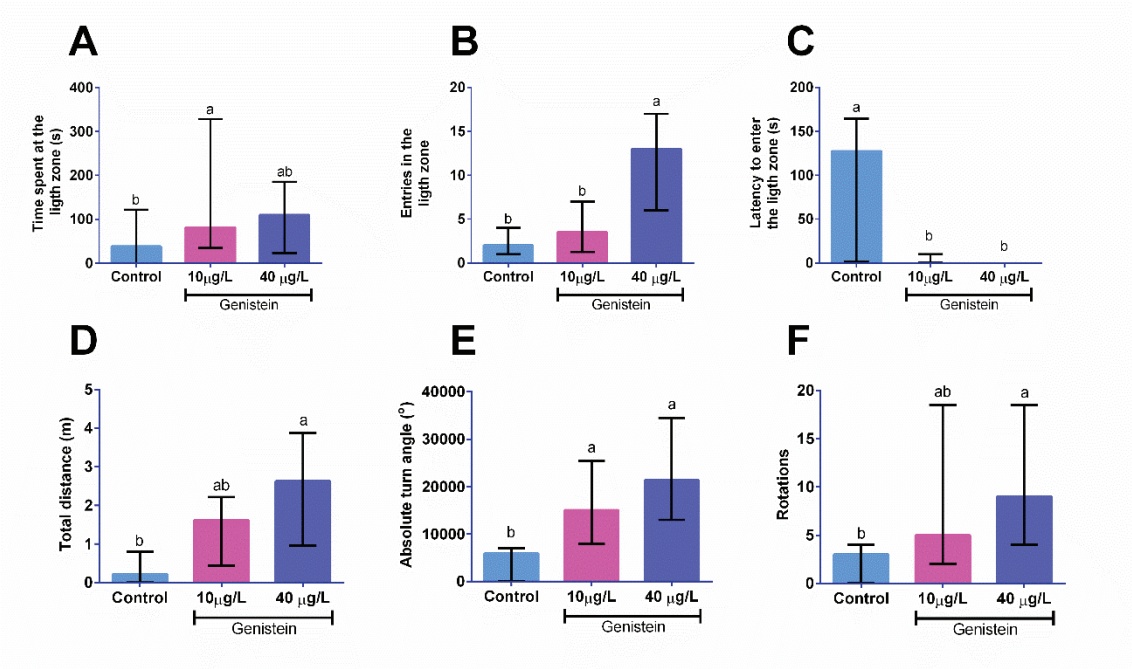

Figure 4: Behavioral parameters of the light-dark test (adults) in the control groups, 10µg/L and 40µg/L. Time spent in the light zone (A), Number of entries in the light zone (B), Latency to enter the light zone (C), Traveled distance (D), Absolute turn angle (E), Rotations (F). Data expressed as median ± interquartile range, analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test. Different letters indicate statistical difference at $p < 0.05$.

Larvae exposed to 40µg/L had a longer latency time for the first entry into the peripheral area, in the open field test, (Fig. 5D) and the larvae of the groups exposed to 40µg/L and 80µg/L remained longer in the central area compared to that exposed to 10µg/L (Fig. 5B). There was no significant difference between groups in the number of entries in the central area (Fig. 5A) and in the distance traveled in the peripheral area (Fig. 5C).

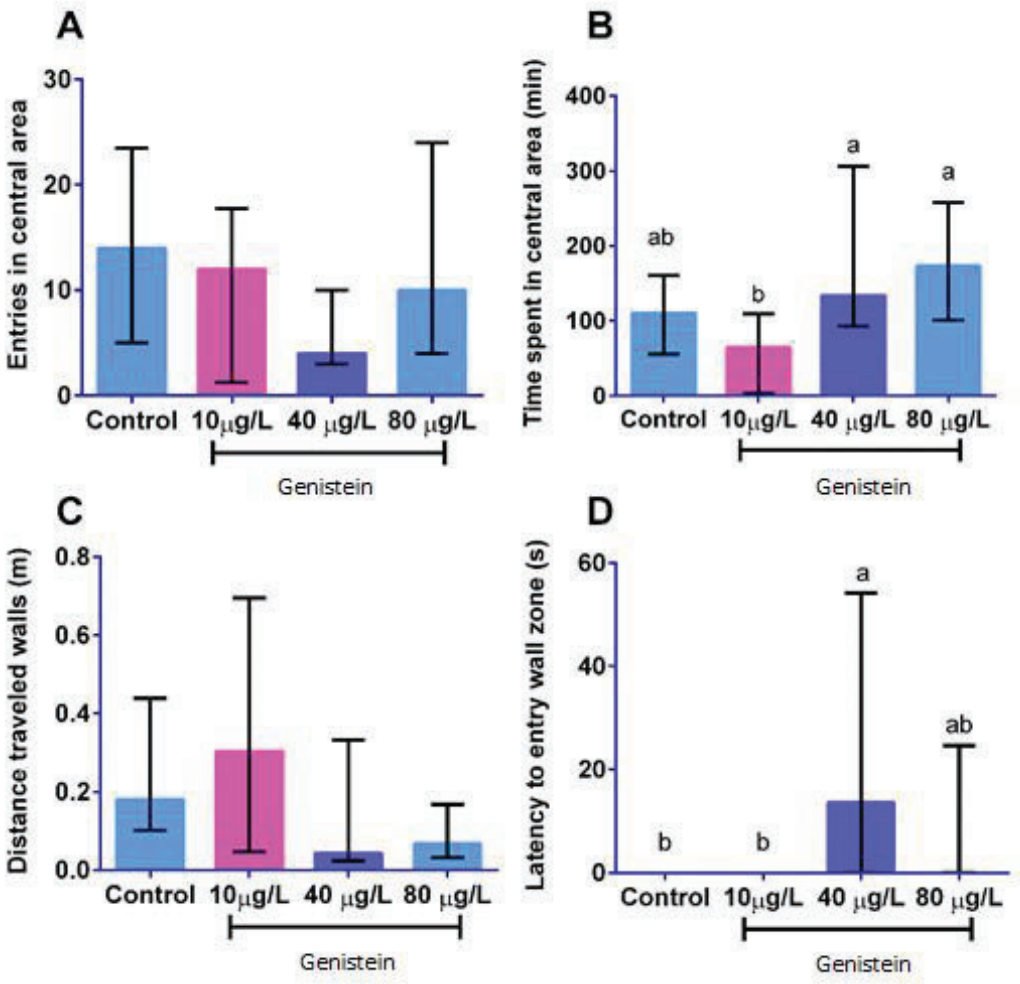

Figure 5: Behavioral parameters of the open field test of the larvae in the control groups, 10µg/L, 40µg/L and 80µg/L. Number of entries in the central area (A), Time spent in the central area (B), Distance traveled in the peripheral area (C), Latency to the first entry in the peripheral area (D). Data expressed as median ± interquartile range, analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test. Different letters indicate statistical difference at $p < 0.05$.

3.1.2 Embryonic exposure to genistein induces antissocial behavior and locomotor effect in adult stage

Adults exposed to 10µg/L had a higher number of crossings, in the novel tank test, when compared to the control group (Fig. 6B), and a greater turn angle when compared to the group exposed to 40µg/L (Fig. 6D). Other parameters showed no significant difference.

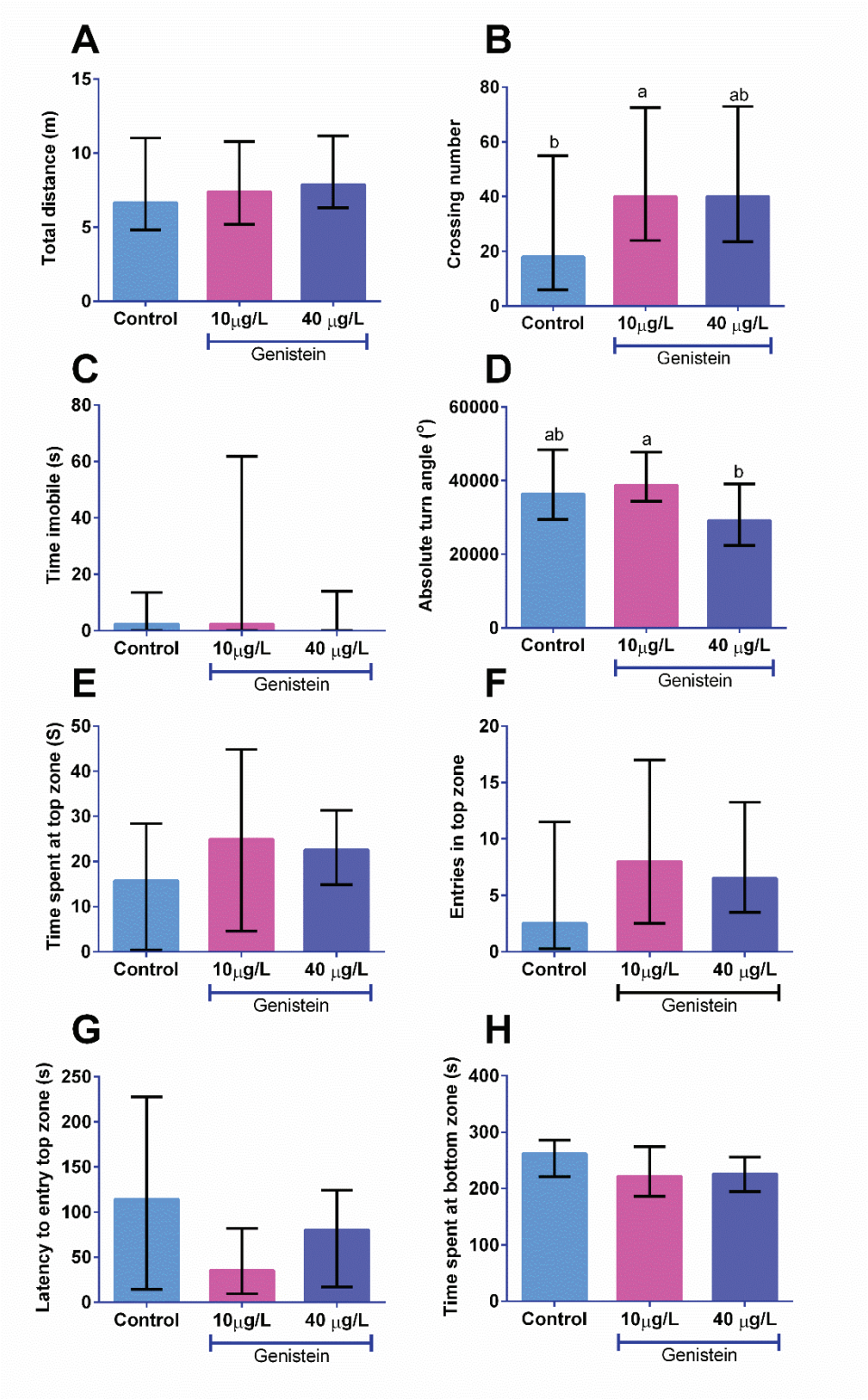

Figure 6: Behavioral parameters of the novel tank test in adults in control groups 10µg/L and 40µg/L. Total distance (A), Number of crossings (B), Time immobile (C), Absolute turn angle (D), Time in the upper zone (E), Number of entries in the upper zone (F), Latency for entry to the upper zone (G) and Time spent in the lower zone (H). Data expressed as median ± interquartile range, analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test. Different letters indicate statistical difference at $p < 0.05$.

The group exposed to 40μg/L showed a longer latency time, in the social preference test, to enter the interaction zone (Fig. 7A), spent less time in the interaction zone and more time in isolation (Fig. 7D) and had a greater number of crossings between zones (Fig. 7F) when compared to the other groups. When compared to the 10μg/L groups, animals exposed to 40μg/L entered the interaction zone more often (Fig. 7B), (Fig. 7C). As for the absolute turn angle, the group exposed to 10μg/L had a higher rate compared to the other groups. (Fig. 7G). There was no significant difference between groups in the total distance traveled (Fig. 7E).

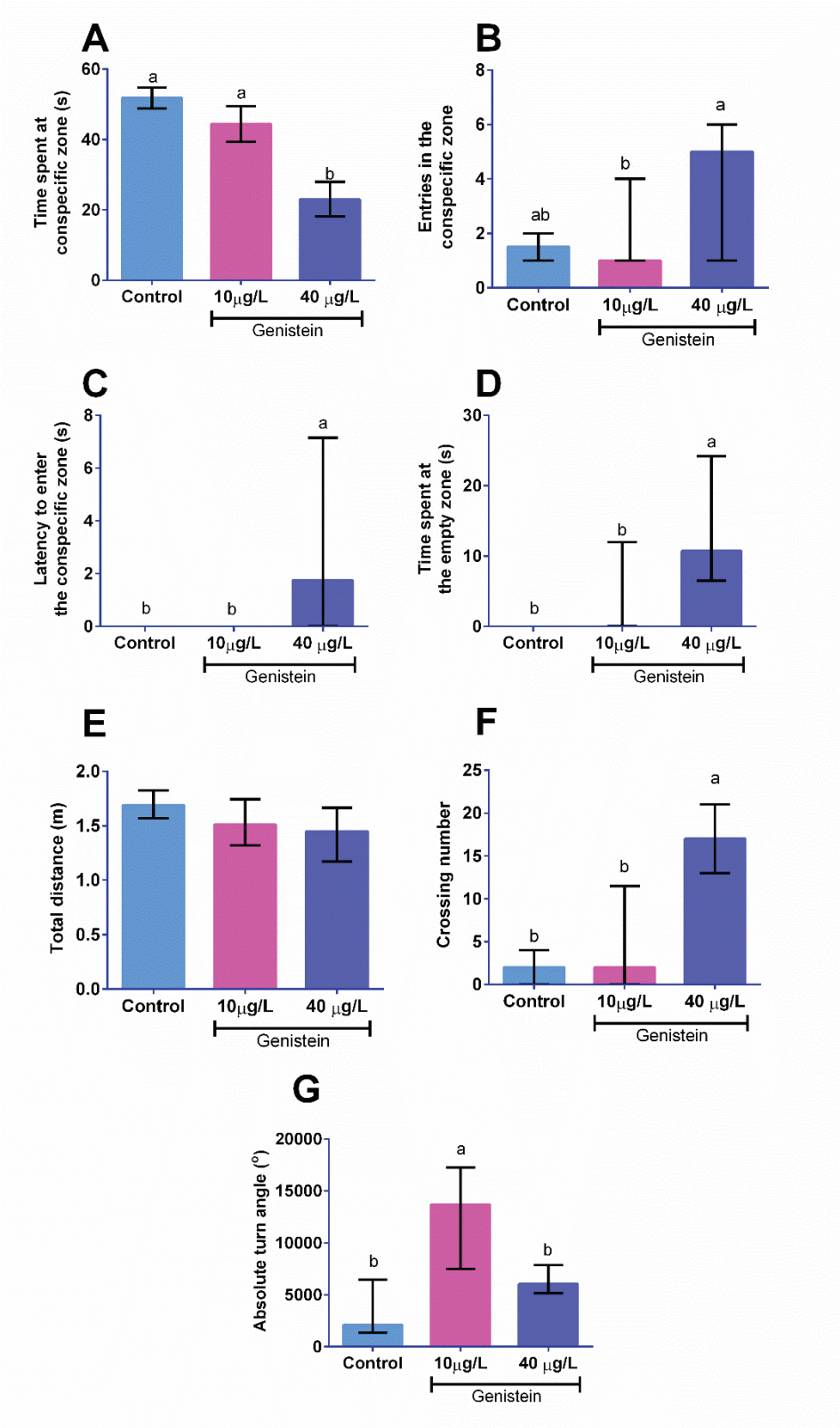

Figure 7: Behavioral parameters of the social preference test in the control groups, 10µg/L and 40µg/L. Time in the interaction zone (A), Number of entries in the interaction zone (B), Latency to enter the interaction zone (C), Time in isolation zone (D), Total distance (E), Number of crossings (F), Absolute turn angle (G). In panels A and E, data were expressed as mean ± SEM, and analyzed by One-way Anova followed by Dunnet's multiple comparison test; while on panels B, C, D, F and G as median ± interquartile range, analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test. Different letters indicate statistical difference at $p < 0.05$.

3.2 Embryonic exposure to genistein increases heart rate and mortality

The larvae in the group exposed to 80µg/L showed an increase in the number of heart beats per minute compared to the control, as shown in Figure 8.

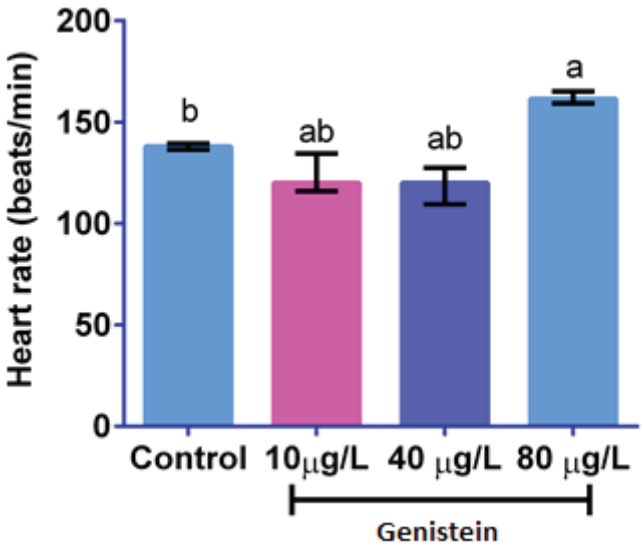

Figure 8: Heart rate in the larvae (49 hpf) exposed to 10µg/L, 40µg/L and 80µg/L. Data expressed as median ± interquartile range, analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test. Different letters indicate statistical difference at $p < 0.05$.

The groups exposed to genistein had a decrease in the percentage of survival (Fig.9), with the highest concentration tested (80µg/L) having the highest mortality rate and no survivors beyond 10 dpf.

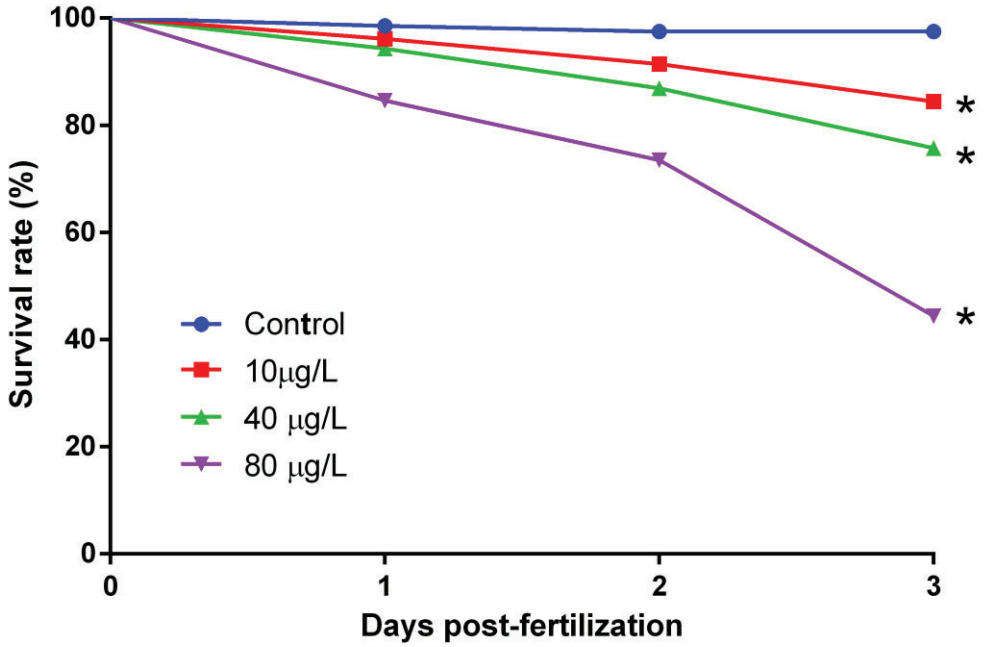

Figure 9: Percentage of survival in the control groups, 10µg/L, 40µg/L and 80µg/L. Data expressed as a percentage of living animals and analyzed by Mantel-Cox test. Asterisks indicate that the specific mortality curve of exposed embryos is different from the control embryo curve ($p < 0.05$).

3.3 Embryonic exposure to genistein and increased frequency of females

At the concentration of 10µg/L there was a significant increase in the frequency of females (52.63%) when compared to the frequency observed in the control group (16.67%). This difference was not significant in the group exposed to the concentration of 40 µg/L (8.33% of females).

Table 1: Analysis of sexing in control groups, 10µg/L and 40µg/L.

SEXING (after 90dpf)			
	Control n=12	**10µg/L** n=19	**40µg/L** n= 24
Number of males	10	9	22
Number of females	2	10	2
Percentage of males	**83,33%**	**47,36%**	**91,67%**
Percentage of females	**16,67%**	**52,63%**	**8,33%**

Table 1 shows a compilation of the results obtained in larval and adult stages, emphasizing that in the adult stages there is no group exposed to 80µg/L, as they did not survive beyond 10dpf.

Table 2: Summary of zebrafish responses to genistein.

COMPILATION OF RESULTS			
Genistein	**10 µg/L**	**40 µg/L**	**80 µg/L**
Embryonic Evaluations			
Heart Rate	*	*	↑
Percent survivors	↓	↓	↓
Behavioral Parameters (larval phase)			
Anxiety	↓	*	↓
Locomotors effect	*	*	*
Behavioral Parameters (adult stage)			
Anxiety	↓	↓	†
Social Behavior	*	↓	†
Locomotors Effect	↑	↑	†
Sexual Differentiation			
Number of females x males	↑	*	†
Legend:			
↑ – increase			
↓ – decrease			
* – not changed			
† – No survivors on adult stage			

4. Discussion

Here we show that embryonic exposure to genistein residues alters important aspects of behavior in the larval phase, effects that persist until the adult phase of fish. After embryonic exposure to concentrations of 10µg/L and 80µg/L, the larvae showed an anxiolytic pattern. When the animals reached adult stage, the anxiolytic-like behavior persisted and, in fish that had been exposed to 40µg/L, an effect was verified that can be classified as antisocial behavior (more time in the isolation zone). In addition,

embryonic exposure to genistein caused hypermotility in fish as adults. Embryonic exposure to genistein residues also caused an increase in the dose-dependent mortality rate, an increase in the frequency of females (exposure to 10 µg/L) and an increase in heart rate in individuals exposed to 80µg/L.

Anxiolytic-like effects were observed both in the larval and adult stages of embryos previously exposed to genistein in the light-dark test. In the larval phase, individuals remained and entered a greater number of times in the dark zone, which symbolizes the shadow of a predator. Adult individuals, on the other hand, entered and/or stayed longer in the light zone and the expected normal pattern is that adult animals prefer the bottom and dark zones (scototaxis) where they are less visible to possible predators (Kysil et al. 2017; Stewart et al. 2011; Maximino et al. 2010). This anxiolytic-like behavior was also observed in the open field test in the larval stage, where the larvae exposed to 40µg/L and 80µg/L remained most of the time in the central area, an unusual behavior, since the animals tend to remain in the periphery (thigmotaxis) (Collier et al. 2017; Stewart et al. 2012). The preference of these organisms for dark environments, instead of bright and light places, is due to a defense mechanism, where the zebrafish seeks to explore the substrate by camouflaging itself in the place and thus avoiding potential predators. Such behavior can represent anxiety or fear (Blaser and Gerlai 2006; Maximino et al. 2010). These innate mechanisms are important in maintaining the survival of these animals. In exposure to novelty, as in the novel tank and open field tests, the animals have an anxious behavior response, preferring to stay on the periphery and at the bottom, away from the surface where there is greater exposure to predators, an essential mechanism for the survival of the species (Colwill and Creton 2011; Egan et al. 2009; Maximino et al. 2010).

Anxiolytic effects like the one found in this study have also been reported in studies with rats, where genistein had an effect similar to 17β-estradiol, acting on ERβ receptors (Rodríguez-Landa et al. 2017). Genistein as well as estrogen can modulate serotonin receptors, generating an anxiolytic effect (Toyohira et al. 2009). There is an interaction between estrogen and serotonin in brain regions involved mainly in mood and cognition (Amin et al. 2005).

When fish from embryos exposed to genistein (40µg/L) reached adult phase, they showed impairment in social behavior, which can even be classified as antisocial behavior, since the fish remained significantly longer in the isolation zone. In fact, the zebrafish is an extremely sociable animal that lives in schools, as a protective strategy

against predators, better detection of food and of partners (Orger and Polavieja 2017; Saverino and Gerlai 2008). The isolation behavior observed in fish exposed to genistein is another damage to their survival and reproduction.

Adult animals also expressed hipermotility. This effect may be related to the interaction with noradrenaline receptors, causing an excitatory brain response (Toyohira et al. 2009).

The threats to the survival of aquatic organisms were most evident in mortality studies. Larvae exposed to higher concentration (80µg/L) showed an increase in heart rate and there was a decrease in the percentage of dose dependent survival, indicating toxicity. These findings are consistent with teratogenicity, increased mortality, pericardial edema, among other effects (Kim et al. 2009).). Similarly elevated levels of genistein in urine have also been linked to cardiovascular problems and increased mortality in humans (Marcelo et al. 2019). Besides that genistein has the capacity to increase dose-dependent cell apoptosis, through different mechanisms not yet fully elucidated (Sarasquete et al. 2018).

We evidenced an increase in the frequency of females in the group exposed to 10µg/L when compared to the control group, that is, there was a feminization of the fish, a result similar to other researches, as in the study with the fish *Oryzias latipes*, where an impact of exposure to genistein was identified on gonadal differentiation and on the development of secondary sexual characteristics (Kiparissis et al. 2003). And an increase in the expression of the vitellogenin gene in male zebrafish, a protein normally expressed by females, responsible for the formation / maturation of eggs (Kausch et al. 2008; Scholz et al. 2004; Zhang et al. 2002), as well as a decrease in circulating testosterone production and levels in males *Oryzias latipes* (Zhang et al. 2002). These results are worrisome since endocrine disrupting substances are found in most water and effluent treatment plants worldwide (Spengler et al. 2001), from different sources such as: industrial products, pesticides, medicine residues, crop residues and fattening agents (Sassi-Messai et al. 2009; Tashiro et al. 2003) and remain present even after treatment. The studies carried out in this work used environmentally relevant concentrations of genistein (Ferrer et al. 2009; Kiparissis et al. 2001; Kawanishi et al. 2004).

When analyzed together, our findings (Table 2) demonstrate that embryonic exposure to genistein causes behavioral changes in larvae and that these effects are persistent until adult phase. The set of these alterations can bring a clear loss to the survival of these organisms, since they can lead to the loss of the ability to detect the

presence of predators, as well as in the anti-predatory reactions such as immobile permanence in the background and social group. In addition to these persistent behavioral effects, exposure to genistein causes immediate damage to individuals by increasing the mortality rate and causing cardiac changes, evidencing its toxicity. Besides that, genistein still leads to an increase in the proportion of females and cause indirect damage to reproduction, due to behavioral changes in socialization.

5. Ethical Note

This study was approved by Animal Use Ethics Committee (Comissão de Ética em Uso Animal - CEUA) of the University of Passo Fundo, UPF, Passo Fundo, RS, Brazil (protocol nº: 041/2019) which fully follows the guidelines of the National Council for the Control of Animal Experimentation (CONCEA) and SisGen (National System for the Management of Genetic Heritage and Associated Traditional Knowledge).

6. Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. References

Amin Z, Canli T, Epperson CN (2005) Effect of Estrogen-Serotonin Interactions on Mood and Cognition. Behavioral and Cognitive Neuroscience Reviews. 4(1):43-58. doi: 10.1177/1534582305277152.

Avdesh A, Chen M, Martin-Iverson MT, Mondal A, Ong D, Rainey-Smith S, Taddei K, Lardelli M, Groth D M, Verdile G, Martins RN (2012). Regular care and maintenance of a zebrafish (Danio rerio) laboratory: an introduction. Journal of visualized experiments: JoVE, (69), e4196. https://doi.org/10.3791/4196

Buske C, Gerlai R (2012) Maturation of shoaling behavior is accompanied by changes in the dopaminergic and serotoninergic systems in zebrafish. Developmental psychobiology, 54(1), 28–35. https://doi.org/10.1002/dev.20571

Collier AD, Kalueff AV, Echevarria DJ (2017) Zebrafish Models of Anxiety-Like Behaviors. In: Kalueff A. (eds) The rights and wrongs of zebrafish: Behavioral phenotyping of zebrafish. Springer, Cham. https://doi.org/10.1007/978-3-319-33774-6_3

Colwill RM, Creton R (2011) Imaging escape and avoidance behavior in zebrafish larvae. Reviews in the Neuroscience. 1; 22(1): 63–73. DOI: 10.1515/RNS.2011.008

Dametto FS, Fior D, Idalencio R, Rosa J, Fagundes M, Marqueze A, Barreto R E, Piato A, Barcellos L (2018) Feeding regimen modulates zebrafish behavior. PeerJ, 6, e5343. https://doi.org/10.7717/peerj.5343

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV (2009) Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res. 14;205(1):38-44. doi: 10.1016/j.bbr.2009.06.022

Ferrer I, Barber LB, Thurman EM (2009) Gas chromatographic-mass spectrometric fragmentation study of phytoestrogens as their trimethylsilyl derivatives: identification in soy milk and wastewater samples. J Chromatogr A. 7;1216(32):6024-32. doi: 10.1016/j.chroma.2009.06.042

Gaffer G G, Elgawish R A, Abdelrazek H M A, Ebaid H M, Hend M (2018) Dietary soy isoflavones during pregnancy suppressed the immune function in male offspring albino rats. Toxicology Reports 296–301. https://doi.org/10.1016/j.toxrep.2018.02.002

Kalichak F, Idalencio R, Rosa JGS, Barcellos HHA, Fagundes M, Piato A, Barcellos LJG (2017) Psychotropic in the environment: risperidone residues afect the behavior of fish larvae. Scientific Reports, 7: 14121. https://doi.org/10.1038/s41598-017-14575-7

Kalueff AV, Stewart AM, Gerlai R (2014) Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol Sci. 35(2):63-75. doi: 10.1016/j.tips.2013.12.002

Kausch U, Alberti M, Haindl S, Budczies J, Hock B (2008) Biomarkers for exposure to estrogenic compounds: gene expression analysis in zebrafish (Danio rerio). Environ Toxicol. 23(1):15-24. doi: 10.1002/tox.20306

Kawanishi M, Takamura-Enya T, Ermawati R, Shimohara C, Sakamoto M, Matsukawa K, Matsuda T, Murahashi T, Matsui S, Wakabayashi K, Watanabe T, Tashiro HY, Yagi T (2004) Detection of genistein as an estrogenic contaminant of river water in Osaka. Environ Sci Technol. 1;38(23):6424-9. doi: 10.1021/es049764v.

Kim DJ, Seok SH, Baek MW, Lee HY, Na YR., Park SH, Lee HK, Dutta NK, Kawakami K, Park JH (2009) Developmental toxicity and brain aromatase induction by high genistein concentrations in zebrafish embryos. Toxicology mechanisms and methods, 19(3), 251–256. https://doi.org/10.1080/15376510802563330

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn. 203(3):253-310. doi: 10.1002/aja.1002030302.

Kiparissis Y, Hughes R, Metcalfe C (2001) Identification of the Isoflavonoid Genistein in Bleached Kraft Mill Effluent. Environmental Science & Technology. Vol. 35, nº. 12. doi: 10.1021/es001679

Kiparissis Y, Balch GC, Metcalfe TL, Metcalfe CD (2003) Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka Oryzias latipes. Environ Health Perspect. 111(9):1158-63. doi: 10.1289/ehp.5928

Kirsten K, Soares SM, Kaokiski G, Kreutz LC, Barcellos LJ (2018) Characterization of sickness behavior in zebrafish, Brain, Behavior, and Immunity. Volume 73, Pages 596-602, ISSN 0889-1591. https://doi.org/10.1016/j.bbi.2018.07.004

Kysil EV, Meshalkina DA, Frick EE, Echevarria DJ, Rosemberg DB, Maximino C, Lima MG, Abreu MS, Giacomini AC, Barcellos LJG, Song C, Kalueff AV (2017) Comparative Analyses of Zebrafish Anxiety-Like Behavior Using Conflict-Based Novelty Tests. Zebrafish. 14 (3):197-208. doi: 10.1089/zeb.2016.1415

Lintelmann J, Katayama A, Kurihara N, Shore L, Wenzel A (2003) Endocrine disruptors in the environment (IUPAC Technical Report), Pure and Applied Chemistry, 75(5), 631-681. doi: https://doi.org/10.1351/pac200375050631

McClain RM, Wolz E, Davidovich A, Edwards J, Bausch J (2007) Reproductive safety studies with genistein in rats. Food Chem Toxicol.; 45(8):1319-32. doi: 10.1016/j.fct.2007.01.009.

Magno LD, Fontes A, Gonçalves BM, Gouveia A Jr (2015) Pharmacological study of the light/dark preference test in zebrafish (Danio rerio): Waterborne administration. Pharmacol Biochem Behav.; 135:169-176. doi:10.1016/j.pbb.2015.05.014.

Marcelo C, Warwick M, Marcelo C, Qayyum R (2009) The association between urinary genistein levels and mortality among adults in the United States. PLoS One. 25; 14 (1): e0211368. doi: 10.1371/journal.pone.0211368

Mathews M, Varga ZM (2012) Anesthesia and Euthanasia in Zebrafish, ILAR Journal, Volume 53, Issue 2, Pages 192–204, https://doi.org/10.1093/ilar.53.2.192

Maximino C, Marques de Brito T, Dias CA, Gouveia A Jr, Morato S (2010) Scototaxis as anxiety-like behavior in fish. Nat Protoc. 5 (2):209-216. doi:10.1038/nprot.2009.225.

Maximino C, de Brito TM, Colmanetti R, Pontes AA, de Castro HM, de Lacerda RI, Morato S, Gouveia A Jr (2010) Parametric analyses of anxiety in zebrafish scototaxis. Behav Brain Res. 26;210(1):1-7. doi: 10.1016/j.bbr.2010.01.031

Meza DLM, Mercado CR, Barraza CA (2015) Efecto de las isoflavonas de la soja en la salud ósea de adultos y niños. Barranquilla Salud. vol. 31, n. 1, p. 138-152. http://www.scielo.org.co/scielo.php?script=sci_arttext&pid=S0120-55522015000100014&lng=en&nrm=iso&gt Accessed 26 August 2019

Mukund V, Mukund D, Sharma V, Mannarapu M, Alam A (2017) Genistein: Its role in metabolic diseases and cancer. Crit Rev Oncol Hematol.; 119:13-22. doi:10.1016/j.critrevonc.2017.09.004.

Orger MB, Polavieja GG (2017) Zebrafish Behavior: Opportunities and Challenges. Annu Rev Neurosci. 25; 40:125-147. doi: 10.1146/annurev-neuro-071714-033857

Rearick DC, Fleischhacker NT, Kelly MM, Arnold WA, Novak PJ, Schoenfuss HL (2014) Phytoestrogens in the environment, I: occurrence and exposure effects on fathead minnows. Environ Toxicol Chem. 33(3):553-9. doi: 10.1002/etc.2461.

Ribeiro AR, Maia A, Santos M, Tiritan ME, Ribeiro CM (2016) Occurrence of Natural Contaminants of Emerging Concern in the Douro River Estuary, Portugal. Arch Environ Contam Toxicol. 70(2):361-71. doi: 10.1007/s00244-015-0212-1.

Rocha MJ, Cruzeiro C, Rocha E (2013) Quantification of 17 Endocrine Disruptor Compounds and Their Spatial and Seasonal Distribution In the Iberian Ave River and Its Coastline. Toxicological and environmental chemistry, v. 95,.3 pp. 386-399. doi: 10.1080/02772248.2013.773002

Rodríguez-Landa JF, Cueto-Escobedo J, Puga-Olguín A, Rivadeneyra-Domínguez E, Bernal-Morales B, Herrera-Huerta EV, Santos-Torres A (2017) The Phytoestrogen Genistein Produces Similar Effects as 17β-Estradiol on Anxiety-Like Behavior in Rats at 12 Weeks after Ovariectomy. Biomed Res Int.2017:9073816. doi: 10.1155/2017/9073816. Epub 2017 Oct 31. PMID: 29226152; PMCID: PMC5684542.

Sarasquete C, Úbeda-Manzanaro M, Ortiz-Delgado JB (2018) Toxicity and non-harmful effects of the soya isoflavones, genistein and daidzein, in embryos of the zebrafish, Danio rerio. Comp Biochem Physiol C Toxicol Pharmacol. 211:57-67. doi: 10.1016/j.cbpc.2018.05.012. Epub 2018 Jun 2. PMID: 29870789.

Sassi-Messai S, Gilbert Y, Bernard L, Nishio S, Lagneau K F F, Molina J, Aandersson-Lendahl M, Benoit G, Balaquer P, Laudet V (2009) The Phytoestrogen Genistein Affects Zebrafish Development through Two Different Pathways. Plos One, Vol. 4. https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0004935&type=printable. Accessed 22 August 2019

Saverino C, Gerlai R (2008) The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. Behav Brain Res. 5;191(1):77-87. doi: 10.1016/j.bbr.2008.03.013

Scholz S, Kordes C, Hamann J, Gutzeit HO (2004) Induction of vitellogenin in vivo and in vitro in the model teleost medaka (Oryzias latipes): comparison of gene expression and protein levels. Mar Environ Res. 57(3):235-44. doi: 10.1016/S0141-1136(03)00082-5

Spagnuolo C, Russo G L, Orhan I E, Habtemariam S, Daglia M, Sureda A, Nabavi S F, Devi K P, Loizzo M R, Tundis R, Nabavi S M (2015) Genistein and cancer: current status, challenges, and future directions. Advances in nutrition (Bethesda, Md.), 6(4), 408–419. https://doi.org/10.3945/an.114.008052

Spence R, Gerlach G, Lawrence C, Smith C (2008) The behaviour and ecology of the zebrafish, Danio rerio. Biol Rev Camb Philos Soc. 83(1):13-34. doi: 10.1111/j.1469-185X.2007.00030.x.

Spengler P, Körner W, Metzger JW (2001) Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 1. Chemical analysis. Environ Toxicol Chem. 20(10):2133-41. https://doi.org/10.1002/etc.5620201001

Steenbergen PJ, Richardson MK, Champagne DL (2011) Patterns of avoidance behaviours in the light/dark preference test in young juvenile zebrafish: A pharmacological study. Behavioural Brain Research. Volume 222, Issue 1, 12, Pages 15-25. DOI: 10.1016/j.bbr.2011.03.025

Stewart A, Maximino C, Brito TM, Herculano A, Gouvêia A, Morato S, Cachat J, Gaikwad S, Elegante MF, Hart PC, Kalueff A (2011). Neurophenotyping of Adult Zebrafish Using the Light/Dark Box Paradigm. https://doi.org/10.1007/978-1-60761-953-6_13

Stewart AM, Gaikwad S, Kyzar E, Kalueff AV (2012) Understanding spatio-temporal strategies of adult zebrafish exploration in the open field test. Brain Res. 27;1451:44-52. doi: 10.1016/j.brainres.2012.02.064

Tashiro Y, Takemura A, Fujii H, Takahira K, Nakanishi Y (2003) Livestock wastes as a source of estrogens and their effects on wildlife of Manko tidal flat, Okinawa, Marine Pollution Bulletin, Volume 47, Issues 1–6, Pages 143-147, ISSN 0025-326X. https://doi.org/10.1016/S0025-326X(03)00053-5

Toyohira Y, Ueno S, Tsutsui M, Itoh H, Sakai N, Saito N, Takahashi K, Yanagihara N (2010) Stimulatory effects of the soy phytoestrogen genistein on noradrenaline

transporter and serotonin transporter activity. Mol Nutr Food Res. 54(4):516-24. doi: 10.1002/mnfr.200900167

Van Duursen M B M. (2017). Modulation of estrogen synthesis and metabolism by phytoestrogens in vitro and the implications for women's health. The Royal Society of Chemistry, Toxicol. Res., 6, 772–794. https://doi.org/10.1039/c7tx00184c

Zhang L, Khan IA, Foran CM (2002) Characterization of the estrogenic response to genistein in Japanese medaka (Oryzias latipes). Comparative Biochemistry and physiology. Toxicology & Pharmacology: CBP. 132(2):203-211. DOI: 10.1016/s1532-0456(02)00067-4.

Weng L, Zhang F, Wang R, Ma W, Song Y. (2019). A review on protective role of genistein against oxidative stress in diabetes and related complications. Chem Biol Interact.1; 310:108665. doi: 10.1016/j.cbi.2019.05.031.

Yossa R, Pallab K. Sarker EP, Vishal S, Marc E, Grant WV (2013) A Practical Approach for Sexing Zebrafish, Danio rerio, Journal of Applied Aquaculture, 25:2, 148-153, DOI: 10.1080/10454438.2013.792170

4. CONCLUSÕES

As principais conclusões deste trabalho são:

1. A genisteína aumenta a taxa de mortalidade em todas as concentrações de exposição quando comparado ao controle.
2. Na concentração de 80μg/L há aumento na frequência cardíaca e na toxicidade levando à morte a totalidade dos indivíduos após no máximo 10dpf.
3. Na fase larval nas concentrações de 10μg/L e de 80μg/L a genisteína induz comportamento ansiolítico.
4. Na fase adulta nas concentrações de 10μg/L e 40μg/L o efeito tipo-ansiolítico também é observado.
5. Na fase adulta na concentração de 40μg/L a genisteína gera alteração no comportamento social, diminuindo a interação do peixe exposto com o cardume.
6. A exposição embrionária à genisteína (10μg/L e 40μg/L) aumenta a atividade locomotora nos peixes adultos.
7. A genisteína provoca feminilização dos peixes na concentração de 10μg/L.

5. CONSIDERAÇÕES FINAIS

Nesse estudo demonstramos que a genisteína, um desregulador endócrino já identificado em efluentes de diversos países, é um risco ao ecossistema, pois provoca alterações comportamentais que persistem na fase adulta e que influenciam na sobrevivência dos animais testados, além disso, pode levar a feminilização dos peixes.

Embora existam diversos estudos sobre os benefícios desse fitoestrógeno, há ressalvas quanto à segurança do seu uso e também sobre os reflexos do seu descarte no meio ambiente e quais os danos oriundos a curto e longo prazo sobre o ecossistema aquático.

6. REFERÊNCIAS

1. Messina M, Messina V. The role of soy in vegetarian diets. Nutrients. 2010;2(8):855–88.
2. Zaheer K, Humayoun Akhtar M. An updated review of dietary isoflavones: Nutrition, processing, bioavailability and impacts on human health. Crit Rev Food Sci Nutr. 2017;57(6):1280–93.
3. Sosic-Jurjevic B, Filipović B, Ajdžanović V, Savin S, Nestorović N, Milošević V, et al. Suppressive effects of genistein and daidzein on pituitary-thyroid axis in orchidectomized middle-aged rats. Exp Biol Med. 2010;235(5):590–8.
4. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-disrupting chemicals: An Endocrine Society scientific statement. Endocr Rev. 2009;30(4):293–342.
5. Jiang W, Yan Y, Ma M, Wang D, Luo Q, Wang Z, et al. Assessment of source water contamination by estrogenic disrupting compounds in China. J Environ Sci [Internet]. 2012;24(2):320–8. Available from: http://dx.doi.org/10.1016/S1001-0742(11)60746-8
6. Tashiro Y, Takemura A, Fujii H, Takahira K, Nakanishi Y. Livestock wastes as a source of estrogens and their effects on wildlife of Manko tidal flat, Okinawa. Mar Pollut Bull. 2003;47(1–6):143–7.
7. Kiparissis Y, Metcalfe C, Ternes T. Identification of the Isoflavonoid Genistein in Bleached Kraft Mill Effluent. Vol. 35. 2001.
8. Sassi-messai S, Gibert Y, Bernard L, Nishio S, Lagneau KFF, Balaguer P, et al. The Phytoestrogen Genistein Affects Zebrafish Development through Two Different Pathways. PLoS One. 2009;4(3):e4935.
9. Viglietti-panzica C, Mura E, Panzica G. Effects of early embryonic exposure to genistein on male copulatory behavior and vasotocin system of Japanese quail. Horm Behav. 2007;51:355–63.
10. Meza DLM, Mercado CR, Barraza CA. The effects of soybean isoflavones over the bone health of adult and children. Salud Uninorte. 2015;31(1):138–52.
11. Gaffer GG, Elgawish RA, Abdelrazek HMA, Ebaid HM, Tag HM. Dietary soy isoflavones during pregnancy suppressed the immune function in male off spring albino rats. Toxicol Reports. 2018;5(April 2017):296–301.
12. Marini H, Polito F, Adamo EB, Bitto A, Squadrito F, Benvenga S. Update on genistein and thyroid: An overall message of safety. Front Endocrinol (Lausanne). 2012;3(JUL):94–7.
13. Van Duursen MBM. Modulation of estrogen synthesis and metabolism by phytoestrogens in vitro and the implications for women's health. R Soc Chem. 2017;6:772–94.
14. Zin SR, Omar SZ, Khan N laqat A, Musameh NI, Das S, Kassim NM. Effects of the phytoestrogen genistein on the development of the reproductive system of Sprague Dawley rats. Clinics. 2013;68(2):17–20.
15. Zhang L, Gao M, Zhang T, Chong T, Wang Z, Zhai X, et al. Protective Effects of

Genistein against Damage in Prepubertal Sertoli Cells. Biomed Res Int. 2017;2017:12.

16. ECHA ECA. Substance Information - Genistein. https://echa.europa.eu/pt/substance-information/-/substanceinfo/100.006.524. 2020. p. 2020.

17. Ganai AA, Farooqi H. Bioactivity of genistein: A review of in vitro and in vivo studies. Biomed Pharmacother [Internet]. 2015;76:30–8. Available from: http://dx.doi.org/10.1016/j.biopha.2015.10.026

18. Torrezan R, Gomes RM, Ferrarese ML, De Melo FBH, Ramos AMD, Mathias PCF, et al. O tratamento com isoflavonas mimetiza a ação do estradiol no acúmulo de gordura em ratas ovariectomizadas. Arq Bras Endocrinol Metabol. 2008;52(9):1489–96.

19. Whirledge SD, Kisanga EP, Oakley RH, Cidlowski JA. Neonatal Genistein Exposure and Glucocorticoid Signaling in the Adult Mouse Uterus. Environ Health Perspect. 2012;126:1–17.

20. Nardi J, Moras PB, Koeppe C, Dallegrave E, Leal MB, Rossato-Grando LG. Prepubertal subchronic exposure to soy milk and glyphosate leads to endocrine disruption. Food Chem Toxicol [Internet]. 2017;100:247–52. Available from: http://dx.doi.org/10.1016/j.fct.2016.12.030

21. Jefferson WN, Padilla-Banks E, Goulding EH, Lao SPC, Newbold RR, Williams CJ. Neonatal exposure to genistein disrupts ability of female mouse reproductive tract to support preimplantation embryo development and implantation. Biol Reprod. 2009;80(3):425–31.

22. Nelson LR, Bulun SE. Estrogen production and action. J Am Acad Dermatol. 2001;45(3):S116–24.

23. Damiani D, Damiani D. Pharmacological management of children with short stature: the role of aromatase inhibitors. J Pediatr (Rio J). 2007;83(5 Suppl):172–7.

24. Hall JM, Couse JF, Korach KS. The Multifaceted Mechanisms of Estradiol and Estrogen Receptor Signaling. J Biol Chem. 2001;276(40):36869–72.

25. Amin Z, Canli T, Epperson CN. Effect of estrogen-serotonin interactions on mood and cognition. Behav Cogn Neurosci Rev. 2005;4(1):43–58.

26. Kiyama R, Wada-Kiyama Y. Estrogenic endocrine disruptors: Molecular mechanisms of action. Environ Int [Internet]. 2015;83:11–40. Available from: http://dx.doi.org/10.1016/j.envint.2015.05.012

27. Duffy C, Perez K, Partridge A. Implications of Phytoestrogen Intake for Breast Cancer. CA Cancer J Clin. 2007;57(5):260–77.

28. Toyohira Y, Ueno S, Tsutsui M, Itoh H, Sakai N, Saito N, et al. Stimulatory effects of the soy phytoestrogen genistein on noradrenaline transporter and serotonin transporter activity. Mol Nutr Food Res. 2010;54(4):516–24.

29. O'Dell thomas J, Kandel ER, Grant SGN. Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. Nature. 1991;354:737–40.

30. Spengler P, Körner W, Metzger JW. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 1. Chemical

analysis. Environ Toxicol Chem. 2001;20(10):2133–41.

31. Rocha MJ, Cruzeiro C, Rocha E. Quantification of 17 endocrine disruptor compounds and their spatial and seasonal distribution in the Iberian Ave River and its coastline. Toxicol Environ Chem. 2013;95(3):386–99.

32. Rearick DC, Fleischhacker NT, Kelly MM, Arnold WA, Novak PJ, Schoenfuss HL. Phytoestrogens in the environment, I: Occurrence and exposure effects on fathead minnows. Environ Toxicol Chem. 2014;33(3):553–9.

33. Ribeiro AR, Maia A, Santos M, Tiritan ME, Ribeiro CMR. Occurrence of Natural Contaminants of Emerging Concern in the Douro River Estuary, Portugal. Arch Environ Contam Toxicol. 2016;70(2):361–71.

34. Kawanishi M, Takamura-Enya T, Ermawati R, Shimohara C, Sakamoto M, Matsukawa K, et al. Detection of genistein as an estrogenic contaminant of river water in Osaka. Environ Sci Technol. 2004;38(23):6424–9.

35. Ferrer I, Barber LB, Thurman EM. Gas chromatographic-mass spectrometric fragmentation study of phytoestrogens as their trimethylsilyl derivatives: Identification in soy milk and wastewater samples. J Chromatogr A [Internet]. 2009;1216(32):6024–32. Available from: http://dx.doi.org/10.1016/j.chroma.2009.06.042

36. Gross-Sorokin MY, Roast SD, Brighty GC. Assessment of feminization of male fish in english rivers by the environment agency of England and Wales. Environ Health Perspect. 2006;114(SUPPL.1):147–51.

37. Bootorabi F, Manouchehri H, Changizi R, Barker H, Palazzo E, Saltari A, et al. Zebrafish as a model organism for the development of drugs for skin cancer. Int J Mol Sci. 2017;18(7):1–15.

38. Spitsbergen JM, Kent ML. The State of the Art of the Zebrafish Model for Toxicology and Toxicologic Pathology Research—Advantages and Current Limitations. Toxicol Pathol. 2004;31(1_suppl):62–87.

39. Kalueff A V, Stewart AM, Gerlai R. Zebrafish as an emerging model for studyng complex brain disorders. Trends Pharmacol Sci. 2015;35(2):63–75.

40. Collier AD, KALUEFF A V, Echevarria DJ. Zebrafish models of anxiety-like behaviors. The Rights and Wrongs of Zebrafish: Behavioral Phenotyping of Zebrafish. 2017. 1–317 p.

41. Alsop D, Vijayan MM. Development of the corticosteroid stress axis and receptor expression in zebrafish. Am J Physiol - Regul Integr Comp Physiol. 2008;294(3):711–9.