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"Effects of Chronic Clothianidin Exposure on Various Aspects of Oncorhynchus mykiss (rainbow trout) Development"

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Effects of Chronic Clothianidin Exposure on Various Aspects of *Oncorhynchus mykiss* (rainbow trout) Development

By

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Effects of Chronic Clothianidin Exposure on Juvenile *Oncorhynchus mykiss* (rainbow trout

Jacob Schmid

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
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ABSTRACT

Agricultural run-off is one of the leading sources of environmental pollution in the United States. One major pollutant within this is insecticides, and among these, neonicotinoids which have become the most used insecticide class across the world. Neonicotinoids act as an agonist on the nicotinic acetylcholine receptor (nAChR) in the central nervous system of insects. Neonicotinoids are perceived to have low toxicity on non-target organisms however recently extensive literature has been published showing various effects on non-target organisms. Clothianidin is one of the most applied compounds belonging to the neonicotinoid class, yet few studies have investigated its chronic effects on fishes. In the Midwest, one species that is at particular risk of exposure is rainbow trout *(Oncorhynchus mykiss)* as their habitats are often intertwined with agricultural practices. This study aimed to expose developing rainbow trout (15-100 days post fertilization) to chronic environmentally relevant concentrations of Clothianidin (0, 0.3, 3, 30 µg/L) and analyze the effects on gross spinal abnormality development, swimming performance, behavior, and kidney and muscle histology. Gross spinal abnormality development was analyzed upon mortality of individuals before 72 dpf. At 72 dpf, rainbow trout were randomly selected and euthanized for histological analysis, Swimming performance was conducted on individuals aged 72-93 days post fertilization (dpf), followed by behavioral analysis (97-100 dpf). The current study found that in the highest concentration group, swimming performance significantly decreased, and development of gross spinal abnormalities significantly increased compared to the control. Clothianidin exposure also caused a significant decrease in myofiber size in the lowest and highest concentration group and a significant increase in intermyofiber distance in the two highest concentration groups compared to the control. Chronic Clothianidin exposure did not alter behavior or kidney development. These data were mixed with similar studies which show the varying effects that Clothianidin has on non-target organisms based on procedural methods, species, life stage, and concentration and duration of exposure.

Introduction and Literature Review:

Agricultural run-off is the leading cause of non-point source and surface water pollution across the United States (EPA 1996, 1998). Agricultural activity comes with the usage of a vast array of pesticides and fertilizers which is associated with greater crop yield necessary for modern farming demands (Reganold et al. 1990). Agricultural run-off including pesticides, fertilizers, and livestock waste influences aquatic systems within range of agricultural operations (Carpenter et al. 1998; Reid et al. 2019; Allen et al. 2021) which can vary greatly based on topography, substrate, and floral densities. These toxins, nutrients, and waste are shown to cause decreases in invertebrate community biodiversity (Schäfer et al. 2012; Beketov et al. 2013), increases in eutrophication rates in water bodies (Bennett et al. 2001; Schindler 2006), increases in reproductive abnormalities in vertebrates (Folmar et al. 1996; McCoy et al. 2008), and many other ecological and physiological disturbances.

Pesticides (herbicides, fungicides, insecticides, etc.) have toxic effects on target pests, and often also have adverse effects on non-target organisms such as fish, crustaceans, insects, and mollusks (Deneer 2000). Herbicides are shown to cause teratogenicity in fish (Mhadhbi and Beiras 2012; Yusof et al. 2014), amphibians (Paganelli et al. 2010), and mice (Dallegrave et al. 2003). Fungicides are shown to alter hormonal concentrations in fish (Afonso et al. 1999; Teng et al. 2018), and in some classes are highly toxic to amphibians (Belden et al. 2010) and invertebrates (Ochoa-Acuna et al. 2009) causing mass mortality at environmentally relevant conditions. Insecticides of varying classes have demonstrated endocrine disruption through elevated concentrations of corticosterone/cortisol in reptiles (Mestre et al. 2019), fish

(O ̈zgu ̈r et al. 2011; Dogan and Can 2011; Fırat and Tutus 2020) as well as altering thyroid function through significantly effecting plasma concentrations of T3 (Triiodothyronine) and T4 (Thyroxine) in reptiles (Wang et al. 2020), rats (Akhtar et al. 1996), amphibians (Lajmanovich et al. 2019), birds (Pandey and Mohanty 2015), and fish (Dey and Saha 2014; Ghayyur et al. 2021).

One class of pesticides is neonicotinoids which were first synthesized in the 1970's (Jeschke and Nauen 2008) and are six-membered saturated-nitromethylene heterocycle molecules that can act on the nicotinic acetylcholine receptor (Jeschke and Nauen 2008). The use of this class of pesticides has grown considerably. For example, in 1990 neonicotinoids made up less than 2% of the world's insecticide economy. In 2008 neonicotinoids made up over 23% of the world's insecticide economy and by 2015 neonicotinoids had become the most widely used class of insecticides on earth (Jeschke et al. 2011; Simon-Delso et al. 2015). One main reason neonicotinoids have gained such popularity in such a short time span is their perceived low toxicity on non-target organisms (Jeschke and Nauen 2008). Although this may be the case when compared to traditional pesticides such as pyrethroids, organophosphates, and carbamates, there is still sufficient evidence that neonicotinoids are toxic to many non-target organisms.

Neonicotinoids act as agonists to the nicotinic acetylcholine receptor (nAChR) which is found in the central nervous system of invertebrates and within the central nervous system and peripheral nervous system of vertebrates (Yamamoto et al. 1995, 1998). Although neonicotinoids are capable of acting on nAChRs of vertebrates and invertebrates, they are considered to be fairly non-toxic within vertebrates due to their

low binding affinity for vertebrate nAChR (Yamamoto et al. 1995, 1998). The receptors vary greatly among and within vertebrates and invertebrates, but the presence of a partial positive charge within vertebrates seems to allow the neonicotinoid to distinguish and selectively bind to the invertebrate nAChR at a much greater frequency than vertebrate nAChR (Tomizawa et al. 1995; Yamamoto et al. 1998).

Neonicotinoids are designed to control populations of insects which have a particular affinity for consumption of crops such as corn, vegetables, fruit, cotton, potatoes, tobacco, and more (Jeschke and Nauen 2008). The primary insect targets include members of the order Hemiptera such as aphids, whiteflies, and plant hoppers, as well as members of the order Coleoptera which are beetles (Nauen and Denholm 2005; Jeschke and Nauen 2008). With such a wide range of species affected, there are concerns about the effects on non-target insects. For example, it has been shown that acute doses of Clothianidin, Thiamethoxam, and Imidacloprid (neonicotinoids) that can occur in nature can have lethal effects on honeybees (Decourtye et al. 2003; Girolami et al. 2009) and lower chronic doses can have impacts on bee learning, motor function and behavior (Lambin et al. 2001; Decourtye et al. 2003; Williamson et al. 2014).

Not only are single individuals or populations affected by neonicotinoid presence, but neonicotinoids also affect whole invertebrate communities. Outdoor and indoor mesocosm studies exposing Imidacloprid to invertebrates show a decrease in abundance and diversity, as well as effects on reproduction and movement behavior among invertebrates in neonicotinoid exposed streams (Pestana et al. 2009; Berghahn et al. 2012; Böttger et al. 2013). Neonicotinoid (Clothianidin, Imidacloprid, and Thiamethoxam) application to row crops also highly correlates with reduced native bee

richness (Main et al. 2020), and reduced aquatic invertebrate biomass (Schepker et al. 2020).

Although unfortunate, these effects may not be surprising as these groups of insects have similar nervous systems to target species. However, there is evidence that the direct effects of neonicotinoids do impact vertebrate species. Amphibians such as salamanders and frogs show a decrease in thyroid receptor β (TRβ) mRNA expression levels and swimming performance when exposed to acute and chronic neonicotinoid concentrations (Holtswarth et al. 2019; Sweeney et al. 2021). Mammals such as mice have also demonstrated negative effects from exposure to neonicotinoids including sperm production and development in vitro, to amino acid metabolism disorders, lipid accumulation, and oxidative stress (Gu et al. 2013; Yan et al. 2020). All these findings demonstrate the vast array of impacts that neonicotinoid exposure can have on a great variety of non-target organisms.

There are currently seven commercially available and widely used neonicotinoids including Imidacloprid, Clothianidin, Thiamethoxam, Thiacloprid, Dinotefuran, Acetamiprid, and Nitenpyram (Jeschke and Nauen 2008; Uneme 2011). The current study only examines the effects of Clothianidin which falls into the open-chained molecular category, as opposed to the 5 to 6-membered ring system category (Jeschke and Nauen 2008; Uneme 2011). One reason Clothianidin is selected for this study is it is considered a super agonist due to its open-chained structure (Tan et al. 2007). Clothianidin induces a greater response in the nAChR than other neonicotinoids and acetylcholine itself (Ihara et al. 2004).

Additionally, clothianidin was selected for this study because it is one of the most prevalent neonicotinoids found in surface and drinking water in the United States and Canada (Bradley et al. 2017; Montiel-León et al. 2018; Sultana et al. 2018; Thompson et al. 2021). Through uranalysis, Clothianidin has been shown to appear in over 7% of individuals and about 50% of individuals were shown to have recent contact with neonicotinoids across the United States (Ospina et al. 2019). Clothianidin has been measured in surface water near agricultural practices in concentrations as high as 43.6 µg/L (Schaafsma et al. 2015). A study done in Wisconsin revealed that concentrations as high as 3.43 µg/L of Clothianidin were found in groundwater monitoring wells which was higher than any of the other neonicotinoids examined (Huseth and Groves 2014). In Iowa, Clothianidin had the highest presence and peak concentration at 75% of all samples and a 0.257 µg/L respectively, when compared to other neonicotinoids (Hladik et al. 2014). In 2013, Clothianidin was the most used insecticide compound across the Midwest with an estimated almost 10^5 kg used with the next most used neonicotinoid (Thiamethoxam) estimated at just under 10^4 kg (Baker and Stone 2014). Even with Clothianidin use as high as it is, the estimations of prevalence within the environment may still be underestimated as Clothianidin is the main metabolite of Thiamethoxam when it breaks down within organisms or the environment (Nauen et al. 2003; Jeschke 2016; Fan and Shi 2017; Liu et al. 2018b).

Clothianidin, like most other neonicotinoids, has various effects on a variety of organisms. In some reptiles such as the Mongolia racerunner (*Eremias argus*), Clothianidin has been shown to up-regulate expression of the genes cyp17 and cyp19 when exposed to 20 mg of Clothianidin per kg of body weight. The cyp17 and cyp19

genes are responsible for estrogen synthesis and more specifically, androgenesis and androgen conversion to aromatic estrogen respectively (Chace et al. 2012; Munawar Lone et al. 2021). The upregulation of these genes ultimately led to a significant decrease in testosterone and an increase in estrogen in males, while having the opposite effect in females (Wang et al. 2019). Similarly, juvenile amphibians, such as tadpoles of various species, exposed to Clothianidin exhibited lower corticosterone levels, anemia (at 2.5 and 250 µg/L of clothianidin), and decreased movement in terms of mean velocity and total displacement (0.375, 0.75, 1.5, 3.0, 6.0 µg/L of Clothianidin) (Gavel et al. 2019; Holtswarth et al. 2019).

Within mammals, Clothianidin has also been extensively researched in mice and rats (EPA and Pesticide Programs 2003). In juvenile mice, cognitive and learning functions decreased when exposed to the EPA-deemed “no observed adverse effect level” (NOAEL, 24mg/kg) of clothianidin (EPA and Pesticide Programs, 2003; Özdemir et al. 2014). In another study, male mice exhibited increased anxiety through emittance of audible sounds and increased c-fos-positive nuclei in granule cells of the hippocampus at a NOAEL of 50 mg of clothianidin per kg of body mass (Hirano et al. 2018). C-fos is a proto-oncogene that when expressed within areas of the brain often indicates stress (Martinez et al. 1998; Azevedo et al. 2020). These results show that NOAELs may need further inspection and that even at low doses behavior is subject to impact from Clothianidin. These as well as other previously mentioned studies demonstrate the exaggerated effect that Clothianidin can have on developing vertebrates.

Comparatively few studies have examined the effects of Clothianidin in fish. In sockeye salmon exposure to Clothianidin at environmentally relevant concentrations (0.15, 1.5, 15, and 150 µg/L) during early stages of life caused an increase in whole body 17β-estradiol (Marlatt et al. 2019). At exposure of 150 mg/L of Clothianidin, glucocorticoid gene expression was reduced, yet survival, hatching, growth, edema, fin, skeletal, and craniofacial deformities were unaffected compared to controls (Marlatt et al. 2019). In rainbow trout exposure to environmentally relevant concentrations resulted in increased lipid peroxidation in the muscles, brain, gills, and kidneys (Dogan et al. 2021). Histological evaluation also revealed Clothianidin-induced necrosis, atrophy, and edema in muscles, as well as hydropic degeneration in the brain, development of melanomacrophage centers in kidneys. Feeding also decreased in Clothianidin exposed groups and observed qualitative swimming performance decreased in environmentally relevant concentrations (3, 15, and 30 µg/L) of Clothianidin (Dogan et al. 2021). These findings highlight the variety of effects that environmentally relevant concentrations of Clothianidin have on salmonids, and the need for more investigation into morphology, quantitative swimming performance, and behavior.

The focus of this study was on rainbow trout as it is one of the top sport fish in North America (Hardy 2002) and an important commercial food resource. Rainbow trout have anadromous migratory life history strategies meaning they spend much of their lives in the open ocean but return to freshwater streams to spawn (Hardy 2002; Arostegui et al. 2019). Hatching time can vary among fertilized eggs, but on average hatching can take approximately a month at 10 degrees Celsius (Hardy 2002). These fish emerge as sac-fry, where they will consume their yolk-sac until they become swim-

up fry and start to feed (Hardy 2002). Growth of rainbow trout can vary throughout the world based on climate, habitat, competition, and food availability but most fish reach sexual maturity at 3-4 years of age (Hardy 2002).

The current study analyzed group, individual, and cellular metrics to determine the comprehensive effects that Clothianidin has on rainbow trout at the eyed egg, alevin, and swim-up fry life stages. Survival rates, condition factors, and percent occurrence of spinal abnormalities were analyzed for trends among the Clothianidin and control treatments. Swimming performance was analyzed because swimming is the basic form of locomotion for all fish and is directly related to health (Jain et al. 1998). Rainbow trout are a migratory species (Hardy 2002; Arostegui et al. 2019), implying the necessity of prolonged swimming in their life history. Critical swimming speed (U_{crit}), which is the most direct measure of prolonged swimming performance, was used for this metric (Cano-Barbacil et al. 2020). Histological analysis was performed on skeletal muscle in the caudal peduncle region and kidney to determine cellular, tissue, and organ level changes across treatment groups. Skeletal muscle atrophy was assessed as it has impacts on organismal health and fitness as myofiber size is directly related to force output (Weber 1846, Krivickas et al. 2011). The kidneys were histologically assessed through measurement of tubule epithelial cell size and accumulation of melanomacrophage centers as swelling of tubule these effects are common signs of toxicity within the kidney (Roberts 1975, Agius and Roberts 2003; Steinel and Bolnick 2017; Liu et al. 2018a; Dogan et al. 2021) Behavioral analysis was also conducted on individuals by recording undisturbed behavior and then analyzing recordings with EthoVision XT 16 software (Noldus IT, Wageningen, Netherlands). We hypothesized

that velocity, acceleration, and distance moved will decrease as concentrations increase as qualitatively observed by Dogan et al. 2021.

Materials and Methods:

Chemicals:

≥ 98% pure Clothianidin ((*E*)-1-(2-Chloro-5-thiazolylmethyl)-3-methyl-2-nitroguanidine) was purchased from Millipore Sigma (PESTANAL® CAS Number 210880–92-5.).

Study Organisms:

Rainbow trout were purchased as eyed eggs from Spring Lake Trout Farm (3409 W 12300 S, Payson, UT 84651). The photoperiod was maintained at 24 hours of darkness until ≥ 95% hatch, after which a 12h/12h light/dark regime was implemented, consistent with Capkin et al. (2009) and Dogan et al. (2021). At first signs of fish entering the swim-up fry stage, approximately 3% of their body weight was fed daily, broken up into 2-3 feeding sessions with commercial trout food similar to Capkin et al. (2009).

Pesticide Exposure:

Fish (eggs) were exposed to their respective treatment immediately upon arrival (15 days post fertilization, dpf) and remained in exposure throughout the duration of the experiment (Figure 1). Treatment groups consisted of a control group (0 µg/L), and 0.3, 3, and 30 µg/L of Clothianidin groups similar to Marlatt et al. (2019) and Dogan et al.

(2021) and all treatment concentrations represent environmentally relevant concentrations (Schaafsma et al. 2015; Dogan et al. 2021). As Clothianidin is soluble in water up to 0.327 g/L at 20 °C (Epa and of Pesticide Programs, 2003), a 0.1g/L stock solution was prepared, and experimental concentrations were prepared by serial dilution. Concentrations were stored as aliquots at -80 °C and were administered through water changes.

Aquatic Exposure Systems:

Rainbow trout eggs were reared in 11.4L tanks inside Penn-Plax net breeders to allow for maximum egg surface-water exposure area. Water was oxygenated using air stones and sponge filters without activated carbon which additionally provide particulate filtering to improve water quality without affecting Clothianidin concentrations. Fifty eggs were reared in each 11.4 L tank, and each treatment was replicated across three tanks. Dead eggs and egg debris were removed daily and recorded. Upon ≥ 95% hatching, yolk fry were moved to a 28.39 L glass tanks with corresponding Clothianidin concentration treatments. Distilled water was mixed with 1.25 g/L of Instant Ocean salt to achieve proper freshwater salinity levels which was maintained at 10-12°C throughout the entire experiment. Water changes were performed once every two days or as needed with ~20% water volume replacement per water change. Water quality analysis on general hardness (GH), carbonate hardness (KH), acidity (pH), nitrite (NO_2^-), and nitrate (NO_3^-) levels was conducted weekly using API® 5 in 1 Aquarium Test Strips to ensure general water quality is within normal levels.

Survival, Condition Factor, and External Morphology

Inspection of tanks for dead eggs/fish occurred daily and was analyzed for survival rates (16-72 dpf). Ten individuals were randomly selected from each treatment and euthanized for histological and condition factor analysis (72 dpf). After euthanasian and prior to histological analysis, standard lengths and mass of all surviving individuals were measured and recorded. Condition factor (*k*) is an index derived from the weight and length of a given fish that indicates relative overall health of a fish, which assumes that the heavier the fish, the healthier the fish (Robinson et al. 2008,Fulton, 1904). Fulton's index (Fulton, 1904) equation where $k = (W/L^3) \times 100$, (*W* = weight in g, *L*= length in mm; Miller et al. 2007; Ridanovic et al. 2015; Dogan et al. 2021) was selected for simplicity as all fish are the same age and species. Gross spinal abnormalities including lordosis, kyphosis, scoliosis, and other undeveloped abnormalities were quantified by presence or absence among individuals upon mortality (Vignet et al. 2019; Von Hellfeld et al. 2022). Due to the low frequency of occurrence of specific abnormality types, all previously stated spinal abnormality conditions were grouped together as gross spinal abnormalities. All individuals displaying spinal abnormalities died prior to condition factor analysis.

Swimming Performance

Swimming performance was analyzed with the U_{crit} as described in (Brett 1964). The test measures the maximum prolonged swimming speed and occurs in a current and velocity-controlled flume. All swim tests were conducted in a modified Brett-type flume described in (Brett 1964) and tests were conducted on individuals aged 72-93 dpf.

This consisted of subjecting the individual to 20 minutes of acclimation time at no flow, followed by 20 minutes of acclimation time with 3 cm/s^{-1} to acclimate the fish to flow and environment. Immediately after acclimation flow was increased by 1 cm/s^{-1} every 5 minutes until the fish failed to continue swimming, completing the trial. The trial ended when the fish touched the back barrier five times in a single increment or if the fish was stuck on the back barrier for 5 or more seconds. The U_{crit} values were standardized among individuals by the standard length of each fish. Individuals who participated in swim trials were also analyzed for condition factor.

Histology

Prior to histology, 10 fish from each treatment were randomly selected and bisected from the anus to the posterior end of the dorsal fin, removing the caudal peduncle for muscle sectioning (72 dpf). Fish bodies were serially sectioned sagitally for analysis of the kidney (Figure 2A). The caudal peduncle was sectioned anterior to posterior for analysis of musculature (Figure 2B). Myofiber size and intermyofiber connective tissue size were analyzed within muscles to assess atrophy as found by Dogan et al. (2021). Myofiber size was quantified using minimum Feret's diameter which is the minimum distance of parallel tangents on the border of the cell (Briguet et al. 2004). Melanomacrophage centers and tubular epithelium cells on kidneys as found in Altinok and Capkin (2007) and Dogan et al. (2021) were analyzed. Melanomacrophage centers were quantified by calculating the total area within a given section of the kidney. Cell length was used to determine Clothianidin's effect on tubular epithelial cells as tubular epithelial cell swelling is indicative of a toxicity response

(Velmurugan et al. 2007; Esmaeillou et al. 2013; Dogan et al. 2021). Fish were chosen randomly for histology.

Behavior:

Fish (97-100 dpf) were placed in a 15x15 cm arena with a 5x5 cm box drawn in the center and allowed 5 minutes to acclimate with no people or disturbances present. Fish were recorded from a superior view for 20 minutes undisturbed. After fish were recorded, each fish was tracked using EthoVision XT 17 software (Noldus IT, Wageningen, Netherlands) with a sampling rate of 3 samples/sec. Track data were then smoothed using LOWESS (locally weighted scatterplot smoothing) method. The following variables were measured or calculated: total time at rest (below 0.25 cm/s), total distance traveled, mean acceleration while in motion (above 0.5 cm/s), mean velocity while in motion, maximum acceleration, maximum velocity, total time spent in the center zone, mean distance to the center point of the center zone, mean absolute meander (change of direction relative to distance moved) while in motion, and mean absolute turn angle.

Statistics:

Intraspecies treatment groups were compared quantitatively using a variety of statistical tests depending on the assumptions of the test and the spread of the data. Log-rank Mantel-Cox test was used for survivorship analysis. One-way analysis of variance (ANOVA) test with Dunnet's post hoc test was used for condition factor, swimming performance, and muscle and kidney histological analysis. One-tail binomial

distribution test of ratios was used for analysis of spinal abnormality development. Principal component analysis (PCA) was used for analysis of behavior. All analyses were conducted at an $\alpha \leq 0.05$ significance level.

Results:

Survival, Condition Factor, and External Morphology

Chronic Clothianidin exposure at environmentally relevant concentrations did not significantly affect survivorship (Mantel-Cox test, df = 3, n = 600, p = 0.4892, Figure 3) or condition factor (ANOVA, df = 3 and 95, n = 139, p = 0.8516, Table 1) in juvenile rainbow trout. Spinal abnormality development was affected by chronic Clothianidin exposure in a dose-dependent response (Figure 4A). The highest treatment had more than three times the spinal abnormalities (kyphosis, lordosis, scoliosis, or undeveloped) of the control group (One-tail binomial distribution test of ratios, df = 3 and 146, n = 600, $p < 0.001$, Figure 4A).

Histology

Muscle tissue structure was affected by chronic Clothianidin exposure (Figure 6). Mean minimum Feret's diameter of myofibers decreased in all treatments compared to the control. Mean minimum Feret's diameter significantly decreased by 19% in the lowest concentration and 21% in the highest concentration compared to the control (ANOVA, df = 3 and 36, n = 40, P = 0.0038, Figure 7A). A dose-dependent decrease in the minimum distance between myofibers was observed. A significant difference was

determined by comparing the two highest concentrations to the control with each treatment exhibiting over a 35% increase compared to the control (ANOVA, df = 3 and 36, n = 40, P = 0.0005, Figure 7B). Chronic Clothianidin exposure did not affect renal tissue structure (Figure 8). Renal tubular epithelial cell length was not significantly affected (ANOVA, df = 3 and 36, n = 40, P = 0.3821, Figure 9A). Total area of melanomacrophage centers within renal tissue was also not affected by chronic Clothianidin exposure (ANOVA, df = 3 and 36, n = 40, P = 0.5926, Figure 9B).

Performance and Behavior

Exposure to chronic Clothianidin affected the swimming performance of rainbow trout in a dose-dependent manner. An 18% decrease in U_{crit} occurred in the 30 µg/L group when compared to the control (ANOVA, df = 3 and 55, n = 59 p = 0.0385, Figure 5). In terms of behavior, mean absolute meander, mean absolute turn angle, time spent not moving, total distance moved, and mean distance to center zone variables all loaded into (with the total distance moved and mean distance to the center zone loading negatively) PC1 (Table 2). PC1 accounted for almost 45% of the total variance and described how fish swam, that is: how often and how far they moved, the location of the fish within the arena, and turning metrics. Maximum acceleration, maximum velocity, and mean velocity loaded into PC2 which accounted for almost 20% of the variance and described how fast the fish swam (Table 2). Mean acceleration and time spent in the center zone did not load into either PC. There were no significant changes in behavior comparing the treatment groups to the control group for principal component 1 (PC1)

(ANOVA df = 3 and 92, n = 96, P = 0.852) or for principal component 2 (PC2) (ANOVA df = 3 and 92, n = 96, P = 0.776. Figure 10).

Discussion:

Spinal analysis revealed that the occurrence of gross spinal abnormalities significantly increased in the highest concentration when compared to the control. It should be noted that the occurrence of gross spinal abnormalities was highly associated with mortality, similar to Oh et al. 2002, as all individuals (except for one) died prior to condition factor analysis at 72 dpf. The literature on the effect that exposure to Clothianidin has on spinal development is mixed. One study found zebra fish exposed to acute Clothianidin for 72 and 96 hours post fertilization (hpf) exhibited greater rates of developing lordosis and kyphosis (Von Hellfeld et al. 2022). However, a study exposing sock-eye salmon to similar concentrations as the current study but earlier in development and for a longer period of time saw no change in the rate of spinal abnormality (kyphosis, lordosis, scoliosis, and 2-headed fish) (Marlatt et al. 2019). Environmental toxicity-induced skeletal abnormities in fish have two broad mechanisms: 1) altered biochemistry within the bone tissue which affects bone integrity and position and 2) neuromuscular effects which cause positional alteration of vertebrae without affecting the biochemistry of the bone tissue (Jesu Arockia Raj et al. 2004). Nicotinic acetylcholine receptors are shown to play various roles within the bone tissue of vertebrates (Sato et al. 2010; Mandl et al. 2016), however, there is extreme limitation in the literature on nAChR's role within fish bones as even the presence or expression of the nAChR has yet to be determined. As for the 2nd broad mechanism of

environmentally induced spinal abnormalities, the current study showed the skeletal muscle of the treatment groups were significantly altered compared to the control. The effects that Clothianidin had on skeletal muscle degeneration likely played a role in the increased occurrence in development of spinal abnormalities (Granito et al. 2012; Hong et al. 2022).

Skeletal muscular analysis revealed myofiber size significantly decreased (atrophied) in the lowest and highest concentration and intermyofiber space significantly increased in the two highest concentrations compared to the control. These findings are consistent with previous literature exposing rainbow trout to chronic, environmentally relevant concentrations of Clothianidin (Dogan et al. 2021), and another study that investigated the histological effects that pesticides from industrial wastewater have on Asian stinging catfish (*Heteropneustes fossilis*). Conversely, other literature shows that chronic exposure to Dinotefuran increased the size of myofibers in zebrafish (Vignet et al. 2019). One study exposed a fish hybrid species to varying regiments of exercise and found that fish exposed to longer exercise regiments exhibited smaller and denser myofibers (reduced intermyofiber space) (Zhang et al. 2023). Because ACh is released at the neuromuscular junction during muscle activation, this may provide evidence that Clothianidin acts as an agonist to the nAChR and disturbs neuromuscular signaling leading to the effects on myofibers seen in this study (Timchenko 2013; Furukawa et al. 2013; Bauché et al. 2013).

Within the intermyofiber space in muscle tissue resides various interstitial cells, most of which have primary functions involved in myogenesis (Mauro 1961; Dellavalle et al. 2007; Joe et al. 2010; Bosurgi et al. 2012), with some cells causing fibrosis and

adipogenesis (Uezumi et al. 2011). Although increased intermyofiber space indicates atrophy of muscles (Cluff et al. 2013), more intermyofiber space could indicate a greater presence of these interstitial cells, providing further evidence of degeneration within the muscle tissue. It should be noted that electron microscopy and immunofluorescences microscopy are generally used for the identification of muscle interstitial cells (Mauro 1961; Dellavalle et al. 2007; Bosurgi et al. 2012) which can't be easily identified or distinguished using H&E staining, which is a limit to this study.

Swimming performance analysis revealed that Clothianidin exposure reduced U_{crit} scores in the highest concentration compared to the control. One study that exposed juvenile sockeye salmon (*Oncorhynchus nerka*) to similar concentrations of Clothianidin as the current study but only for 96 hours, found that burst swim velocity (U_{max}) was not affected (Engelking 2014). U_{max} is a comparable test to U_{crit} as it has the same methods regarding materials used, acclimation time, and stepwise progression of current velocity, except it consists of shorter time increments resulting in comparatively higher velocities reached at failure (Farrell 2008). Other studies found that exposure to Thiamethoxam resulted in increased average swim velocity in zebra fish (Yang et al. 2023), but decreased swim rate in catfish (*Clarias gariepinus*) (Erhunmwunse et al. 2023) compared to unexposed fish.

Although the current literature is inconsistent in its findings on whether neonicotinoids affect swimming performance, it should be mentioned that each of the studies investigated different chemicals, at different concentrations, at different time points, on different species, using different methods. The current study showed that Clothianidin had an atrophy effect on muscle in the treatment groups, most notably the

highest concentration which also exhibited a decrease in swimming performance. This provides reason to believe that the effect Clothianidin had on muscle tissue was caused or contributed to the decrease in swimming performance at the highest concentration. Further, it can be hypothesized that Clothianidin exposure affects the peripheral nervous system more than the central nervous system in rainbow trout as differences in muscle development were observed, which was not the case for behavior. Other studies exposing fish to acute (Benli and Çelik 2021) and chronic (Dogan et al. 2021) concentrations of Clothianidin found decreases in AChE activity in muscle and brain tissue alike.

Kidney analysis revealed that chronic exposure to Clothianidin had no effect on the kidney epithelial cell length or accumulation of melanomacrophage centers. This is inconsistent with current literature that found Clothianidin exposure at similar concentrations increased tubular hydropic degeneration (swelling of tubule cells) and melanomacrophage presence in the kidneys of rainbow trout (Dogan et al. 2021). Dogan et al. 2021 also qualitatively observed changes in behavior in the highest concentrations (15 and 30 μg/L) through reduced movement which is inconsistent with the current study which found no behavioral differences across the control and treatments. The current study differed as it exposed rainbow trout for a longer period than the aforementioned. A possible reason for the discrepancy in results is that kidneys may have adaptive features that allow organisms to adapt to chronic toxin exposure over time (Reeves 1995), especially in the early life stages of fishes (Bonatesta et al. 2022).

Rainbow trout exposed to chronic Clothianidin did not significantly differ in survival rate compared to the non-exposed rainbow trout. This aligns with a previous study that found sock-eye salmon exposed from fertilization through 119 dpf experienced no significant change in survival across similar concentrations of Clothianidin when compared to the control (Marlatt et al. 2019). Other literature indicates that exposure to environmentally relevant concentrations of Thiamethoxam did affect survival rates of embryonic fathead minnow (*Pimephales promelas*) and zebra fish however these studies exposed the fish earlier in development (Victoria et al. 2022b, 2022a). Condition factor of exposed rainbow trout was not significantly affected by chronic Clothianidin exposure. This is consistent with a similar study exposing developing sockeye salmon to similar chronic concentrations of Clothianidin (Marlatt et al. 2019). The conflicting and confirming results seen in this study to previous literature suggest a complicated relationship between the concentration, chemical, timing of exposure and species-specific effects.

The findings in this study may have broader implications on the ecology where trout reside. Rainbow trout provide benefits to the ecosystems they inhabit and many members that share its genus are considered keystone species (Willson et al. 1998; Gende et al. 2002; Helfield and Naiman 2006; Bergum 2016). For example, one study found that up to 24% of nitrogen present in a southwestern Alaskan stream (Lynx Creek) ecosystem was derived from Pacific salmon (*Oncorhynchus spp.*) and brown bear (*Ursus arctos*) interactions (Helfield and Naiman 2006). Every year Pacific salmon returning to spawn and die in tributaries consist heavily of marine-derived nutrients which are essential to many aspects of the ecosystem (Helfield and Naiman 2006).

Prolonged swimming is highly utilized in Pacific salmon migration (Hinch et al. 2002), and detriments to salmon prolonged swimming performance could affect the arrival of these fish to their inland destinations. The effects that Clothianidin had on prolonged swimming performance demonstrated in this study should raise concern for migratory salmon and the ecosystems they nourish.

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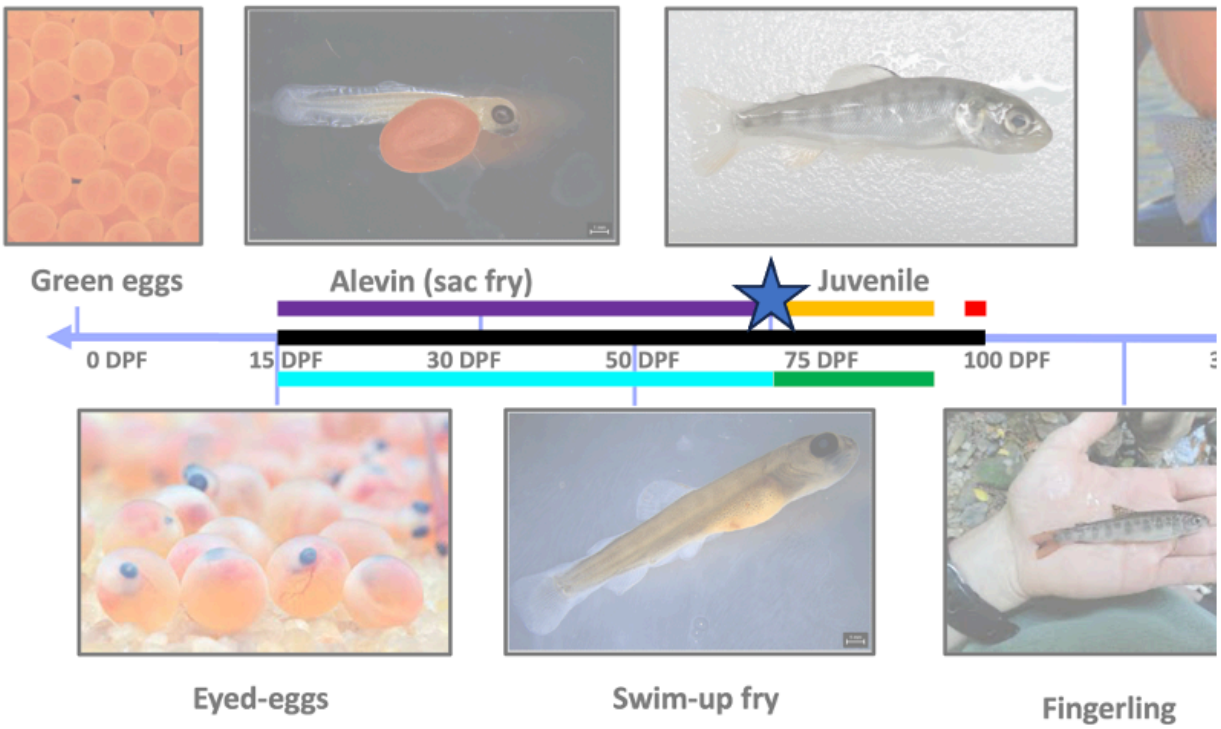

Figure 1: Early life stage timeline of rainbow trout with overlayed variable timeline showing data collection time points for each of the analyzed variables. Purple = survivorship (15 - 72 dpf), cyan = spinal abnormality (15 – 72 dpf), blue star = histology (72 dpf), yellow = swimming performance (72 – 93 dpf), green = condition factor (72 – 93 dpf), red = behavior (97 – 100 dpf), black = exposure period (15 – 100 dpf).

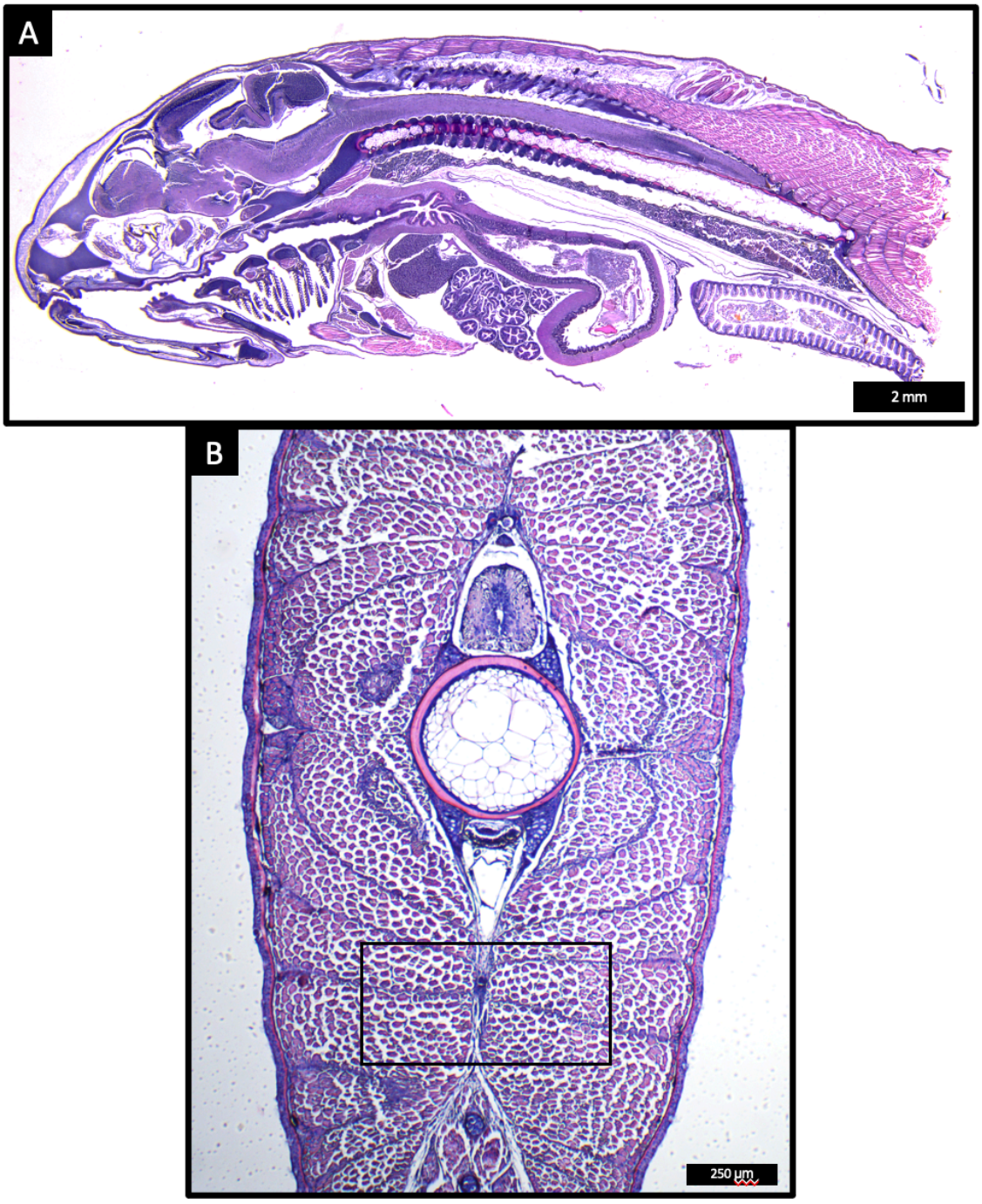

Figure 2: Histological images of sections of a rainbow trout stained with hematoxylin and eosin and sectioned at 10 µm thick. (A) sagittal view of the head and thoracic region of a rainbow trout (72 dpf, left = anterior, right = posterior, scale bar = 2 mm). (B) Cross-sectional view of caudal peduncle region of a rainbow trout. Black box denotes the region (medial-hypaxial) where myofibers were selected for analysis (72 dpf, top = dorsal, bottom = ventral, scale bar = 250 µm).

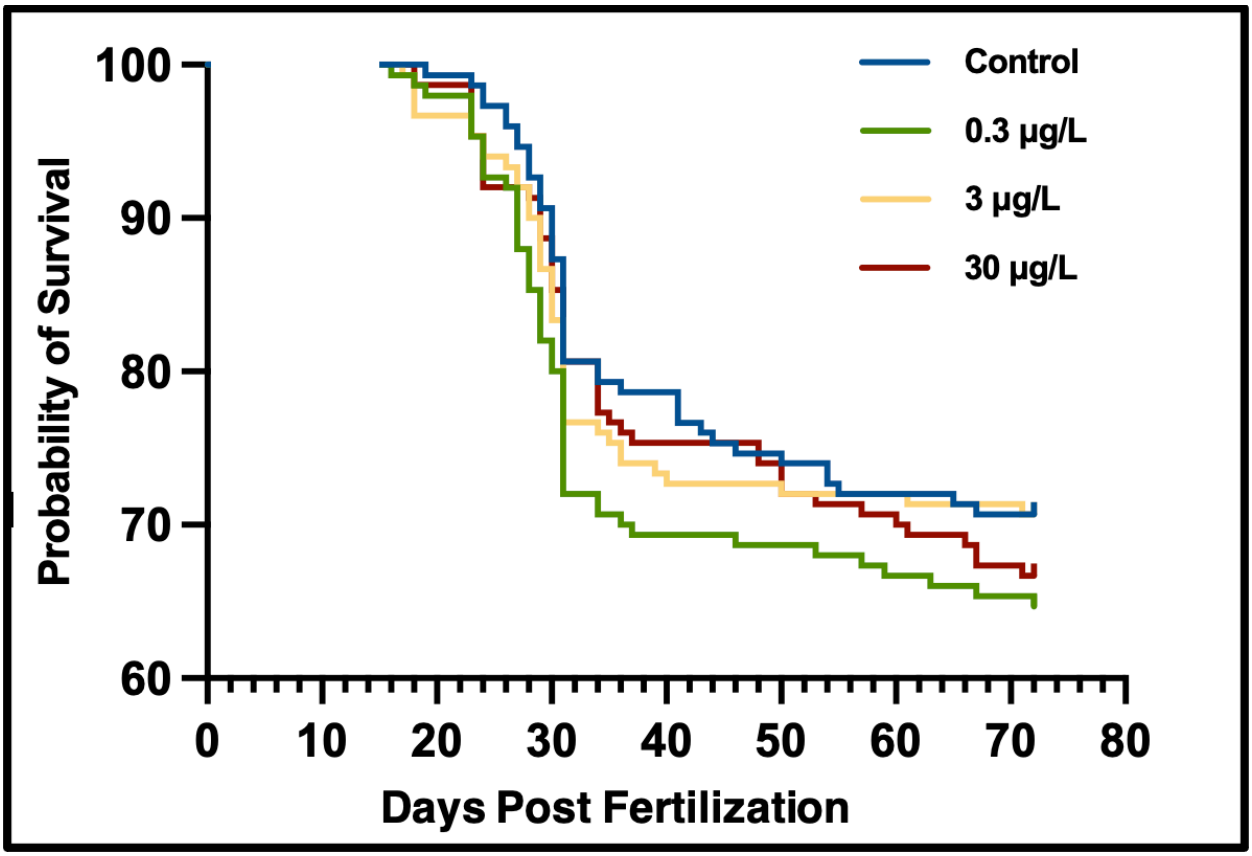

Figure 3: Probability of survival of rainbow trout was not affected by chronic Clothianidin exposure. Survival was examined from 15 – 72 days post fertilization. Mantel-Cox test was conducted df = 3, n = 600, p = 0.4892.

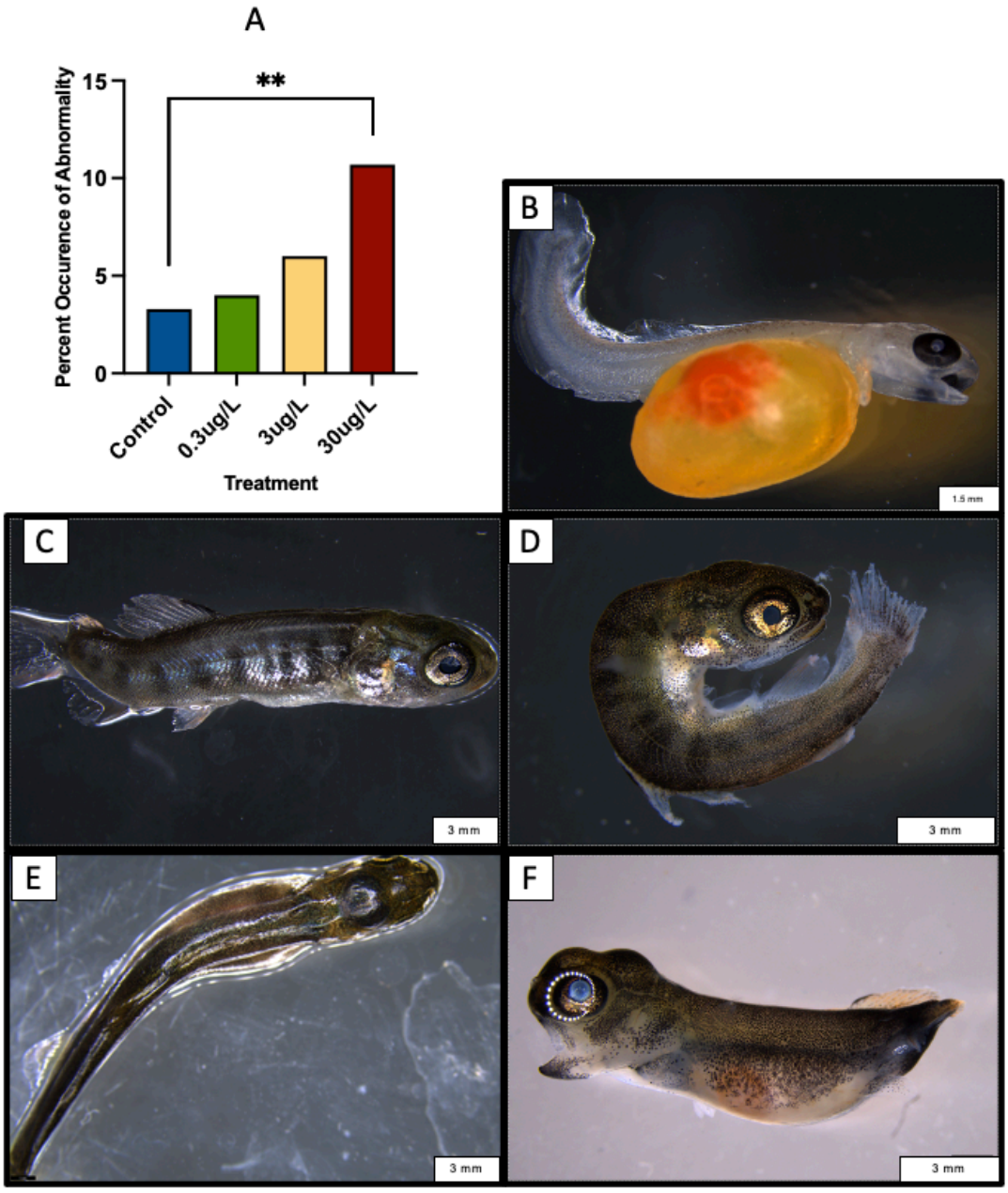

Figure 4: Chronic exposure to CLO increased the percent occurrence of spinal abnormalities in a dose-dependent manner. (A) Bar graph showing percent occurrence of spinal abnormalities in each treatment, classified as: lordosis, kyphosis, scoliosis, and Undeveloped. (**) indicate a significant difference between groups using a one-tail binomial distribution test of ratios (df = 3 and 146, n= 600, $p < 0.001$). (B and C) Profile view of individuals expressing lordosis (B: 30 µg/L at 32 dpf, scale bar = 1.5 mm. C: 3 µg/L at 85 dpf, scale bar = 3 mm). (D) Profile view of individual expressing kyphosis (30 µg/L at 61 dpf, scale bar = 3 mm). (E) Superior view of individual expressing scoliosis (30 µg/L at 60 dpf, scale bar = 3 mm). (F) Profile view of individual expressing undeveloped posterior spine (0.3 µg/L at 59 dpf, scale bar = 3 mm).

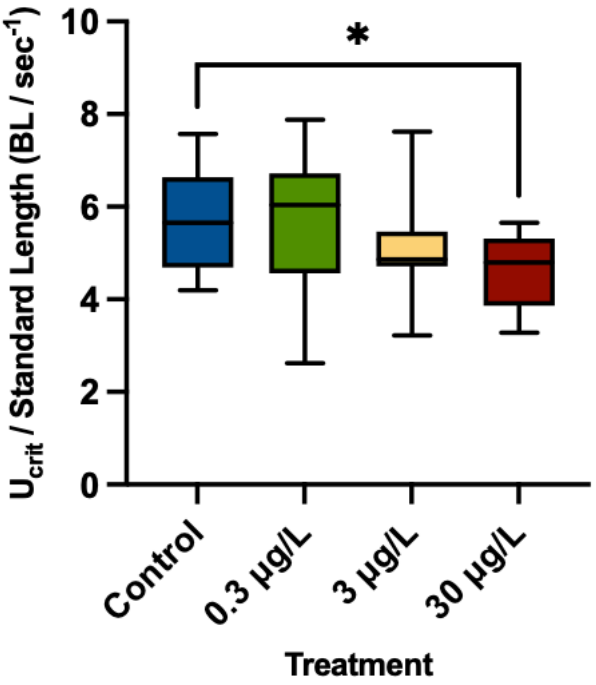

Figure 5: Exposure to chronic CLO affected U_{crit} in a dose-dependent manner**.** Box and whiskers plot showing U_{crit} normalized by standard length with the box indicating interquartile range and whiskers indicating range. ** indicated a significant difference between groups using a one-way ANOVA test with Dunnett's post hocs (df = 3 and 55, control, 3 µg/L and 30 µg/L n = 15, 0.3 µg/L n = 14, p = 0.0385).

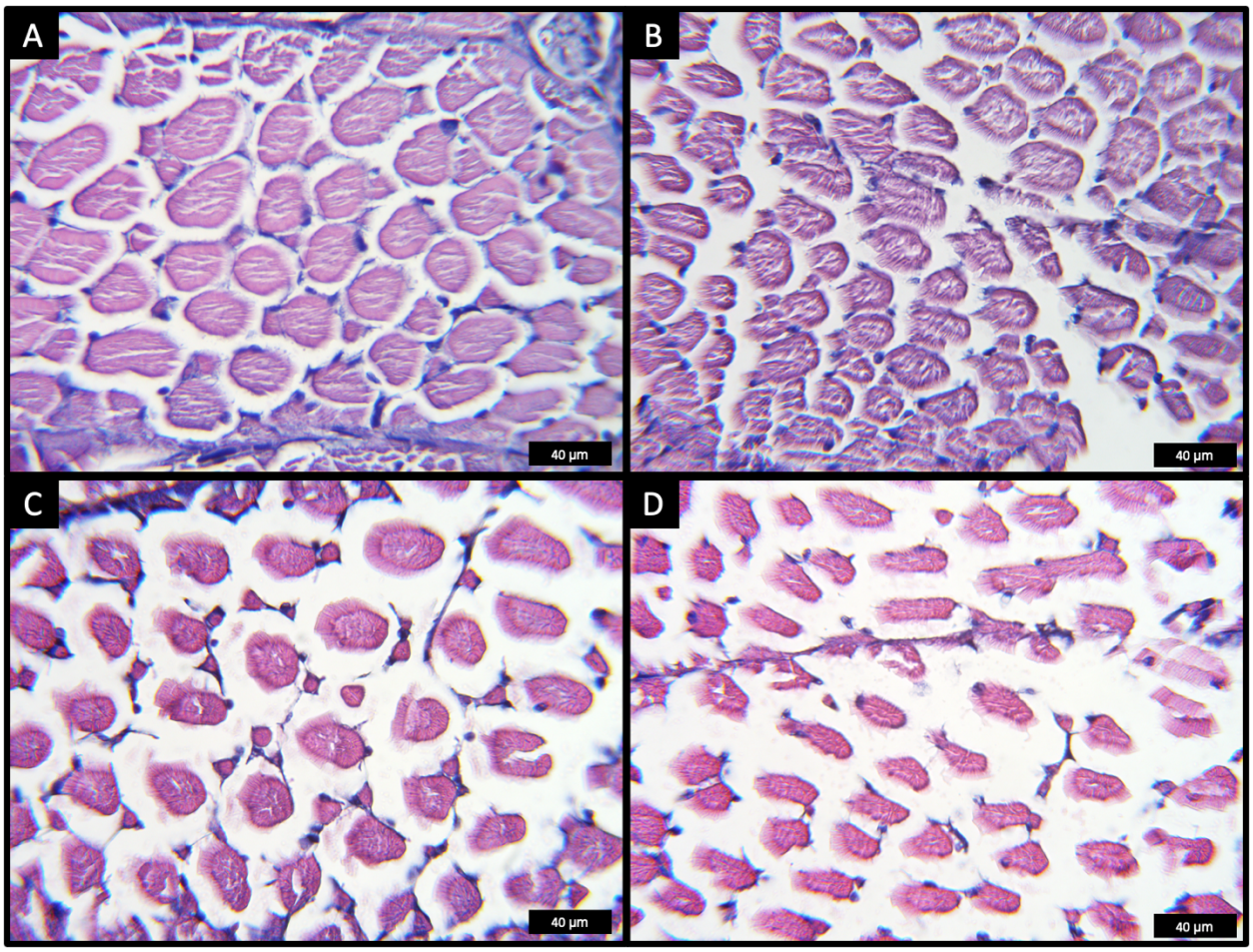

Figure 6: Cross-sectional view of rainbow trout medial-hypaxial caudal peduncle muscle tissue histology using hematoxylin and eosin staining techniques. (A) control, (B) 0.3 µg/L, (C) 3 µg/L, (D) 30 µg/L, scale bar = 40 µm

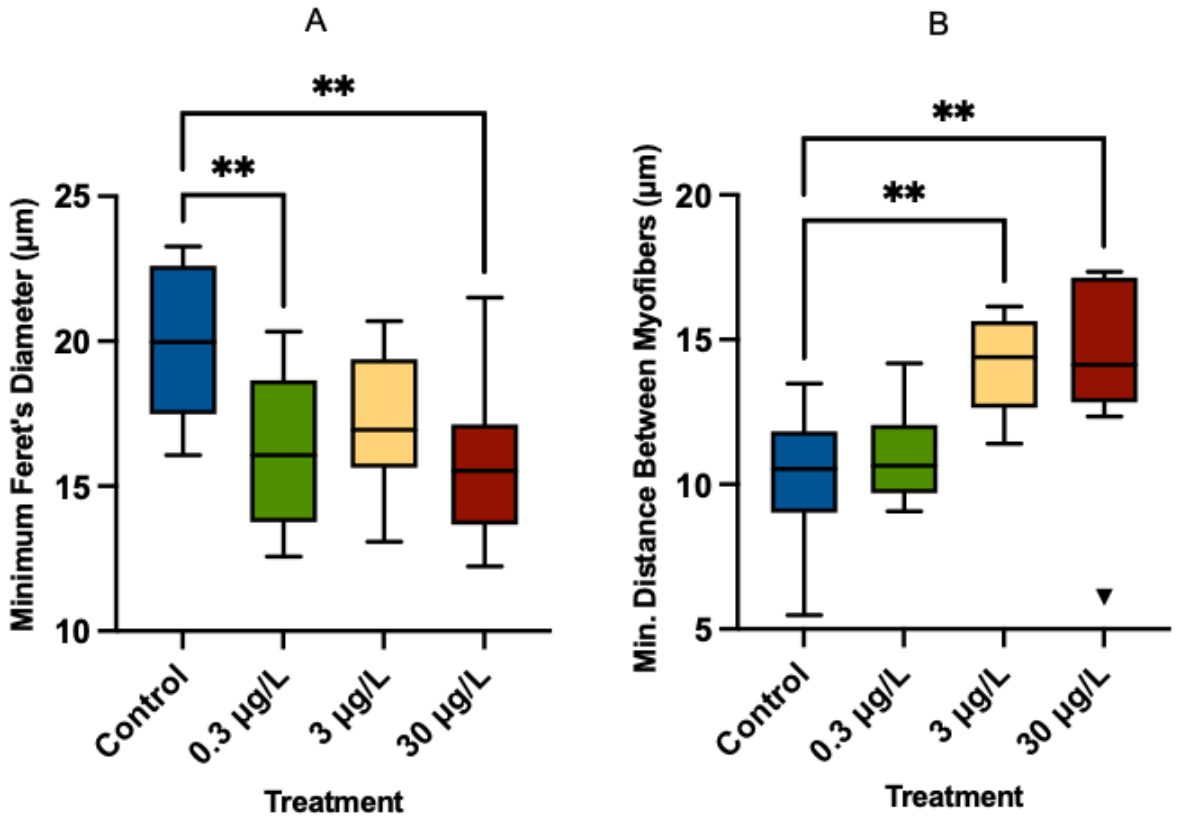

Figure 7: Chronic exposure to CLO affected minimum Feret's diameter (A) and minimum intermyofiber space (B) in muscle tissue. (**) indicate a significant difference between groups using a one-way ANOVA with Dunnett's post hocs (df = 3 and 36, n = 40, $p < 0.01$). Box indicates interquartile range, whiskers indicate range, and upside-down triangles indicate outliers (still used in analysis).

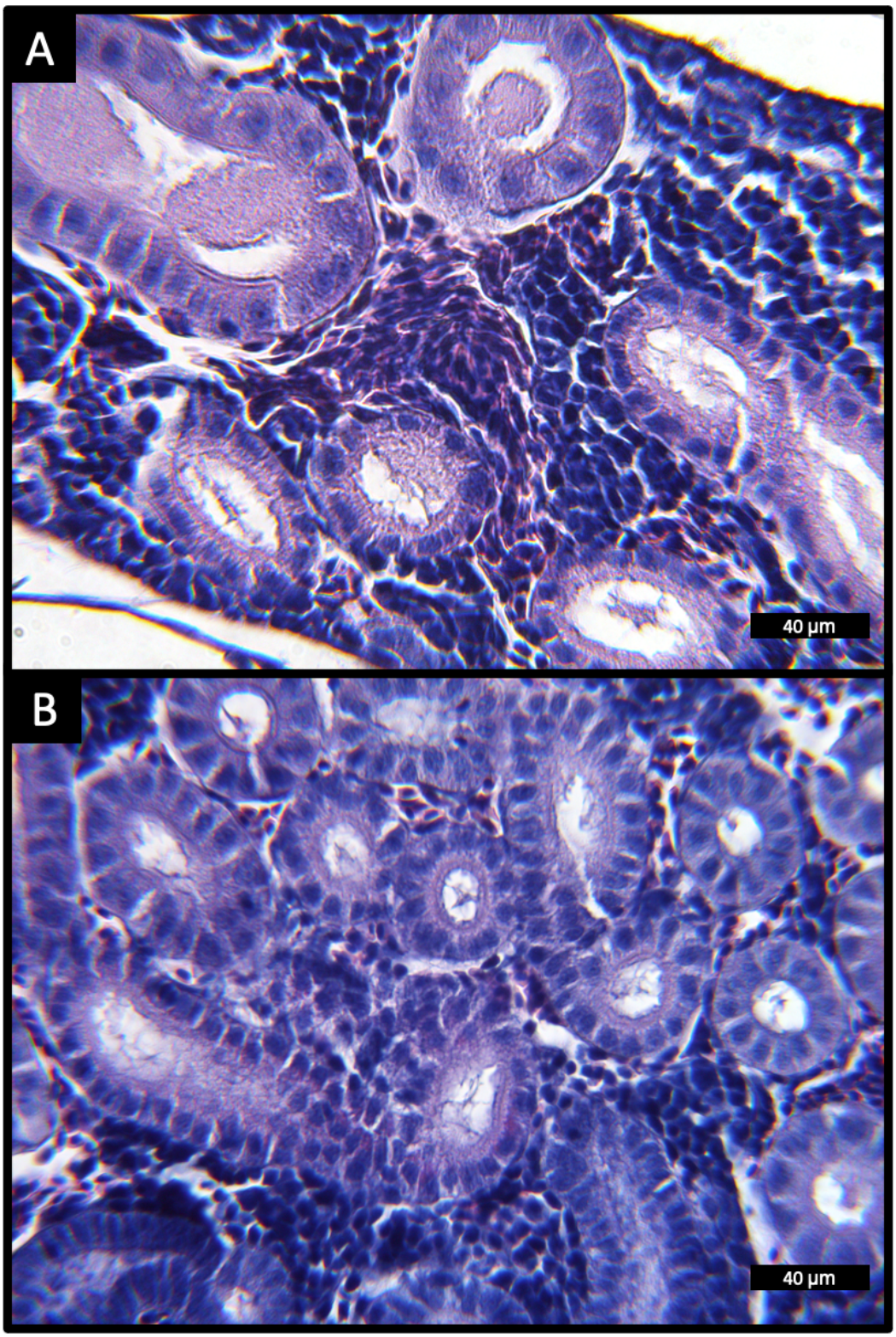

Figure 8: Lateral view of *O. mykiss* kidney tissue histology using hematoxylin and eosin techniques. (A) control, (B) 30 µg/L, scale bar = 40 µm.

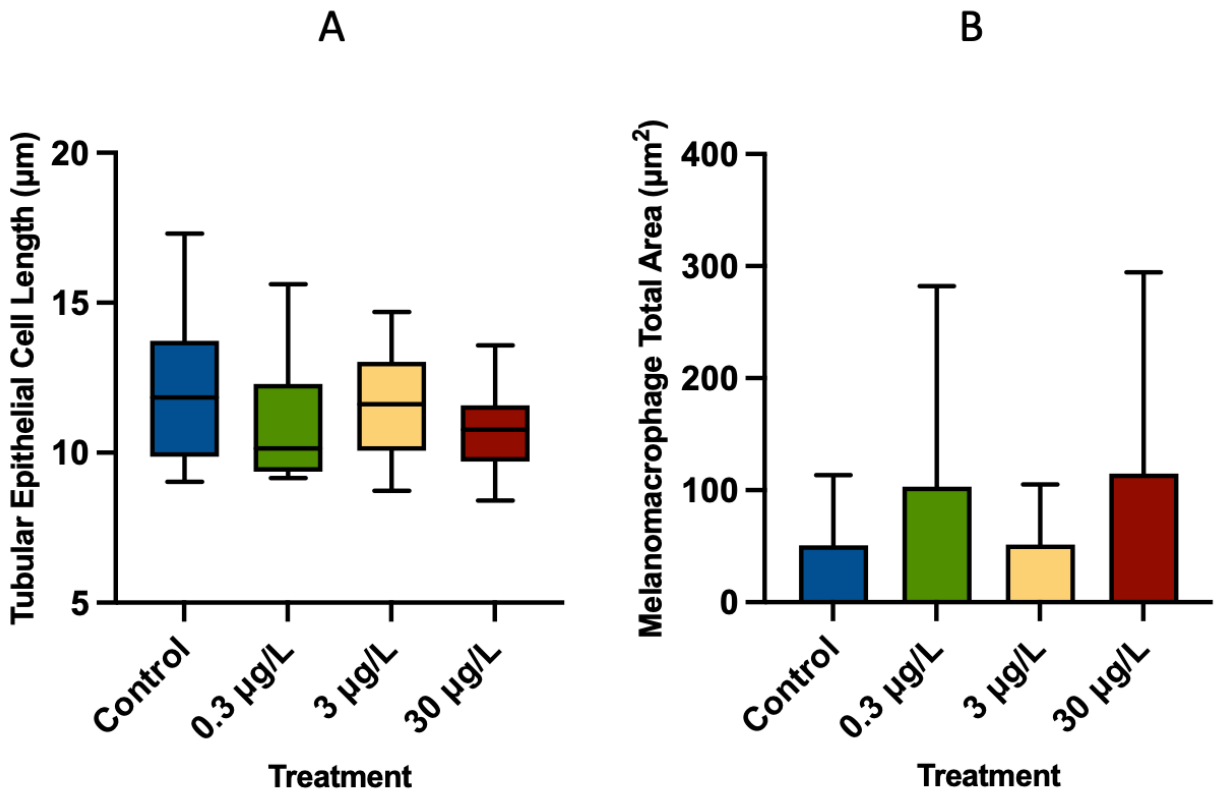

Figure 9: Chronic Clothianidin exposure did not affect tubular epithelial cell length (A) or melanomacrophage center area (B) in kidney tissue. One-way ANOVA test indicated no significant difference in tubular epithelial cell length or melanomacrophage center total area among treatments, A: (df = 3 and 36, n = 40, p = 0.3821), B: (df = 3 and 36, n = 40, p = 0.5926).

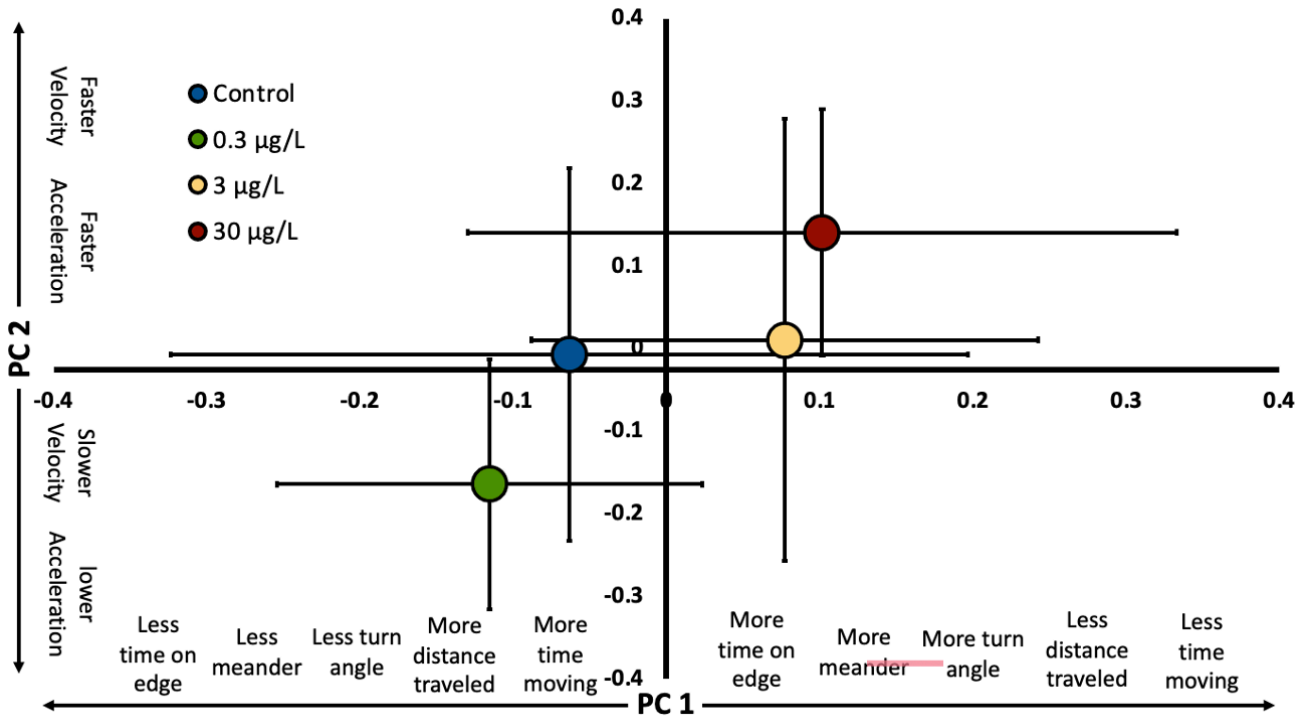

Figure 10: Chronic Clothianidin exposure did not affect behavior in rainbow trout. Cartesian plot displaying means (colored point) and standard error (error bars) of PC1 and PC2 data compared across treatments. One-way ANOVA test indicates no significant difference in PC1 or PC2 values comparing the treatments to the control (PC1 ANOVA: df = 3 and 92, n = 96, p = 0.85. PC2 ANOVA: df = 3 and 92, n = 96, p = 0.78).

Table 1: Condition factor (*k*) of rainbow trout was not affected by chronic Clothianidin exposure. *k* was examined at 72 – 92 days post fertilization. One-way ANOVA test was conducted (df = 3 and 95, $p > 0.05$).

	Control	0.3 ug/L	3 ug/L	30 ug/L
Mean	21.92	21.78	22.06	21.86
SE	2.34	2.20	1.88	1.78
n	35	34	35	35

Table 2: Analyzed behavioral variables and how they were loaded into the principal component analysis (PCA). PC1 accounted for 44.68% of total variance and PC2 accounted for 19.68% of total variance.

Variable	PC1	PC2
Mean absolute meander (°/cm)	0.901	
Mean absolute turn angle (°)	0.828	
Time spent not moving (sec)	0.801	
Total distance moved (cm)	-0.657	
Mean distance to center zone (cm)	-0.599	
Maximum Acceleration (cm/sec^{-2})		0.944
Maximum Velocity (cm/sec)		0.920
Mean Velocity (cm/sec)		0.668
Mean Acceleration (cm/sec^{-2})		
Time spent in center zone (sec)		

Table 3: Data summary table of tested variables except for survivorship, spinal abnormality development, and behavior. All variables were statistically analyzed using one-way ANOVA tests with Dunnett's post hocs comparing exposure treatments to the control. Bold font with * indicates a significant difference compared to the control ($p < 0.05$).

Variable	Treatment	N	Average +/- one SEM	Test Stat and P-value	Dunnett's Post Hoc P-value
Condition Factor	0 µg/L	35	21.92 ± 0.39		
	0.3 µg/L	34	21.78 ± 0.37	F = 0.2634	-
	3 µg/L	35	22.06 ± 0.31	P = 0.8516	-
	30 µg/L	35	21.87 ± 0.30		-
Swimming Performance (U_{crit}/Standard length)	0 µg/L	15	5.625 ± 0.27		
	0.3 µg/L	14	5.679 ± 0.38	F = 3.180	0.9983
	3 µg/L	15	5.039 ± 0.24	**P = 0.0310***	0.3227
	30 µg/L	15	4.629 ± 0.20		**0.0385***
Minimum Feret's Diameter (µm)	0 µg/L	10	19.90 ± 0.84		
	0.3 µg/L	10	16.16 ± 0.83	F = 5.328	**0.0076***
	3 µg/L	10	17.29 ± 0.76	**P = 0.0038***	0.0793
	30 µg/L	10	15.64 ± 0.84		**0.0022***
Minimum Distance Between Myofibers (µm)	0 µg/L	10	10.34 ± 0.76		
	0.3 µg/L	10	10.96 ± 0.49	F = 7.202	0.8862
	3 µg/L	10	14.15 ± 0.52	**P = 0.0007***	**0.0026***
	30 µg/L	10	14.01 ± 1.06		**0.0037***
Tubular Epithelial Cell Length (µm)	0 µg/L	10	12.17 ± 0.82		
	0.3 µg/L	10	10.88 ± 0.64	F = 1.050	-
	3 µg/L	10	11.51 ± 0.59	P = 0.3821	-
	30 µg/L	10	10.75 ± 0.46		-
Melanomacrophage Total Area ($µm^2$)	0 µg/L	10	50.86 ± 19.78		
	0.3 µg/L	10	103.3 ± 56.59	F = 0.6426	-
	3 µg/L	10	51.46 ± 17.00	P = 0.5926	-
	30 µg/L	10	114.9 ± 56.78		-