# The Effects of the $H_1$ Antagonist Chlorpheniramine on Anxiety in Zebrafish

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Abstract

Zebrafish (*Danio rerio*) have recently emerged as an excellent model organism to study the neurological basis of anxiety disorders. They display robust behavioural responses to external stimuli and possess all of the main vertebrate neurotransmitters. Research in rats has demonstrated that the histaminergic system plays a role in anxiety, possibly by interacting with other monoamines such as serotonin and dopamine. In zebrafish, however, the histaminergic system is not well characterized, so it is of interest to assess the role of histamine on anxiety in zebrafish. Chlorpheniramine, a histamine antagonist that has been tested multiple times in rodents and shown to decrease anxiety, was administered to fish with the expectation that we would observe similar anxiolytic effects in zebrafish. Chlorpheniramine was administered through immersion for ten minutes at two doses (20mg/L and 25mg/L), and zebrafish were tested using the shoaling test, which is a measure of anxiety based on the tendency of fish to form more cohesive shoals when anxious. We found that chlorpheniramine did not produce a significant anxiolytic effect at either dose; however previous research in our lab suggests that the 20mg/L dose reduces anxiety in the novel tank diving test. Further research using different doses and tests or other histamine antagonists should be conducted for a more thorough understanding of the histaminergic system in zebrafish.

*Keywords*: Chlorpheniramine, *Danio rerio*, shoaling, anxiety

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Anxiety is one of the most common psychological disorders, with a global prevalence of approximately 7.3% across all anxiety disorders in the DSM-IV and ICD-10 (Baxter, Scott, Vos, & Whiteford, 2012). Anxiety is an umbrella term that refers to any diagnosis in the DSM that is characterized by excessive fear and worry. This includes generalized anxiety disorder, social anxiety disorder, obsessive-compulsive disorder, post-traumatic stress disorder, panic disorder, and specific phobia (Baxter, Vos, Scott, Ferrari, & Whiteford, 2014). Anxiety has evolved as a mechanism that enables organisms to recognize and respond appropriately to potential threats; however, it is maladaptive if an individual responds to non-threatening stimuli (Bishop, 2007). Given the burden anxiety disorders pose on healthcare systems, it is important to identify effective therapies to help patients function on a day-to-day basis.

The etiology of anxiety continues to be characterized, but the major factors that appear to play a role in the development of anxiety disorders are learned behavioural responses, environmental stressors, psychosocial factors, and biological vulnerability (i.e. genetic and neurochemical factors) (Newman, Llera, Erickson, Przeworski, & Castonguay, 2016). The neurobiology of anxiety is not well understood, and there are several models to explain the neural factors that contribute to anxiety disorders (Bishop, 2007). Much of the current understanding of the biological bases of anxiety disorders comes from animal models, typically rodents, which are used to screen for genetic variants, abnormalities in neurotransmitters, and disrupted brain circuitry (Stewart et al., 2012). For example, abnormalities in the amygdala and the prefrontal cortex are linked to an increase in anxiety and altered selective attention (Bishop, 2007). Several neurotransmitters are linked to anxiety, including dopamine, serotonin, and GABA (Guo, 2004).

Recently, zebrafish (*Danio rerio)* have emerged as an excellent model organism for studying anxiety disorders as they possess all of the main vertebrate neurotransmitter systems and share physiological homology with humans (Egan et al., 2009). Furthermore, because zebrafish are a prey species, they display robust responses to threatening stimuli that are easily quantified in a lab setting, both through physiological endpoints of stress and behavioural measures (Egan et al., 2009; Stewart et al., 2012).The genetics of zebrafish are well characterized, and zebrafish can be studied both at the larval and adult stages (Guo, 2004). Additionally, because of their size and fecundity, they are an ideal lab animal to house and breed (Egan et al., 2009).

Based on rodent paradigms, several tests have been developed to assess zebrafish behaviour. Many paradigms used in zebrafish are based on an instinctual fear of novel situations (Egan et al., 2009). One of the most widely used tests is the novel tank diving test, which is based on the tendency of an anxious fish to swim at the bottom of a tank, whereas less anxious individuals engage in more exploratory behaviour and approach the surface of the water more frequently (Egan et al., 2009). This test is similar to the open field test in rodents, in that animals are subjected to an unfamiliar environment and anxiety is assessed based on the animal's willingness to explore the environment. Another test that has been used in both rodents and zebrafish is the light-dark test, which is based on the tendency of anxious animals to seek shelter in a dark environment (Blaser, Chadwick, & McGinnis, 2010). This test measures the amount of time an animal spends in the light zone in an arena vs. the darker zone, and it is expected that less anxious animals will spend more time exploring the light zone.

However, there are also behavioural tests that are unique to fish behaviour, such as the shoaling test. Shoaling is a social behaviour in fish that has been evolutionarily conserved, in

which fish will form tight groups known as shoals (Green et al., 2012). Shoaling may offer protection from predators, and thus when fish perceive a threat, they are more likely to form a more cohesive shoal (Miller & Gerlai, 2007). The shoaling test measures the distance between fish to quantify anxiety, and has been validated using pharmacologics (Green et al., 2012).

Through the administration of pharmacologics, anxiety can be readily manipulated in zebrafish (Maximino & Herculano, 2010). Zebrafish possess several receptors that have homology to human receptors, both physiologically and genetically (Maximino & Herculano, 2010), and the administration of drugs is typically non-invasive, as they can be dissolved in water and absorbed via the gills rather than injected (Stewart et al., 2011).

Several neurotransmitters and their receptors have been identified in zebrafish, including serotonin, dopamine, histamine, norepinephrine, and GABA, all of which contribute to the neurobiology of anxiety (Stewart et al., 2011). A number of these are implicated in other neurological disorders such as Alzheimer's disease and Parkinson's disease (Best & Alderton, 2008).

While it has been established that histamine has many roles in the brain, as of yet its role in anxiety is still not well characterized, especially compared with other monoamines. Further research should be conducted to better understand anxiety, as well as to further our understanding of histamine functions in the nervous system. In rats, chronic and acute stress caused increases in brain histamine, and blocking histamine transmission through histamine antagonists produced anxiolytic behavioural responses (Ito, 2000). Changes in histamine have been linked to neurodegenerative disorders; data suggest that increases of histamine may be a factor in the pathology of both Alzheimer's disease and Parkinson's disease (Shan, Bao, & Swaab, 2015). An emerging line of research is investigating histamine's role in

neurodevelopment, with much of the evidence being derived from studies in rats and zebrafish. Histamine has been shown to affect proliferation and differentiation of neural stem cells, and in zebrafish the histaminergic system is hypothesized to be plastic over an animal's lifetime (Panula, Sundvik, & Karlstedt, 2014).

In mammals, four histamine receptors have been identified, with $H_1$, $H_2$, and $H_3$ having been functionally defined in the human brain (Shan et al., 2015). In zebrafish, the $H_1$, $H_2$, and $H_3$ receptors have all been identified in the brain, and are highly conserved between humans and zebrafish (Peitsaro, Sundvik, Anichtchik, Kaslin, & Panula, 2007). Antagonists of each receptor in zebrafish produced behavioural changes, providing evidence that the receptors are functional in the CNS (Peitsaro et al., 2007). However, further studies comparing the functions of the zebrafish histaminergic system to human and rodent systems are required for better understanding. In humans, the $H_1$ receptor is involved in allergies and inflammatory processes, circadian rhythms, learning and memory (Shan et al., 2015). The $H_2$ receptor has functions in cognition (Shan et al., 2015), and may play a role in anxiety (Chee & Menard, 2013). The $H_3$ receptor is an autoreceptor, which is located postsynaptically on GABAergic, noradrenergic, glutaminergic, and cholinergic neurons (Shan et al., 2015). The $H_3$ receptor is involved in regulating release of histamine and other monoamines and is therefore involved in several cognitive processes (Shan et al., 2015).

The histaminergic system is highly conserved between mammals and zebrafish with regards to morphology, distribution, and projections in the CNS (Kaslin & Panula, 2001). Histaminergic neurons in zebrafish are primarily located posterior to the hypothalamus and project to the telencephalon, optic tectum, and brainstem (Panula et al., 2010). It is hypothesized that high densities of histaminergic and serotonergic neurons in the telencephalon are involved in

regulating arousal and activity and that these neurons are involved in activating other forebrain structures (Kaslin & Panula, 2001). Histamine has regulatory effects on other monoaminergic neurons; in mammals histamine regulates dopamine and tyrosine hydroxylase release. Given the significant overlap between histaminergic and other monoaminergic neurons in zebrafish, it is hypothesized that monoamines may regulate histamine release (Kaslin & Panula, 2001). Additionally, it has been found that histamine may interact with GABA, thyrotropin-releasing hormone (TRH) and neuropeptides including galanin and methionine-enkephalin (mENK) depending on the species (Sundvik & Panula, 2012). In zebrafish and rat, histamine colocalizes with GABA, galanin, and TRH. These transmitters may act to regulate histamine release, as well as having modulatory effects on the hypocretin/orexin system, which is responsible for arousal (Sundvik & Panula, 2012). Additionally, there is evidence that suggests that galanin and TRH may have roles in anxiety (Karlsson & Holmes, 2006; Zeng et al., 2007).

Currently, antihistamines are not used as a treatment for anxiety disorders, however evidence from rodents suggests that histamine antagonists may act as anxiolytics (Privou, Knoche, Hasenöhrl, & Huston, 1998; Zarrindast, Taheri, & Rezayof, 2005) . In particular, $H_1$ antagonists have produced anxiolytic effects. In conditioned place preference tasks (Privou et al., 1998) and the elevated plus maze (Zarrindast et al., 2005) rats showed decreased anxiety after treatment with $H_1$ antagonists. The data currently suggest that histamine may be involved in the formation of emotional memory and reinforcement through acting on receptors in the limbic system (Serafim, Russo, Fernandes, Gianlorenco, & Mattioli, 2016; Zarrindast, Valizadegan, Rostami, & Rezayof, 2008), possibly through the actions on other neurotransmitter systems. Evidence suggests that the $H_1$ antagonist chlorpheniramine may have effects on serotonin (Miyata, Hirano, Ohsawa, & Kamei, 2011), dopamine, and noradrenaline levels in the brain

(Hirano, Miyata, Onodera, & Kamei, 2007). There is also a case report of chlorpheniramine causing serotonin syndrome in humans, suggesting it may function as an SSRI (Ayoglu et al., 2009).

At this point in time, histamine antagonists have not been widely tested in zebrafish, with no reports on their function with regard to anxiety. Because of the advantages of the zebrafish model, it is of interest to further characterize the histaminergic system of this species, as well as to investigate the functionality of histamine antagonists for anxiety disorders and to better establish the role of histamine in anxiety disorders. This study is the first to administer chlorpheniramine to fish through immersion. We used the shoaling test and predicted that zebrafish will have a decreased shoal cohesion, indicative of lower anxiety, after treatment with chlorpheniramine. Based on the similarities between the rat histaminergic system and the zebrafish histaminergic system, we hypothesize that chlorpheniramine will act as an anxiolytic in zebrafish.

## Methods

### Animals and Housing

Wild-type zebrafish of mixed sex were obtained from a commercial distributor (Aquatic Imports, Calgary, Canada). Animals were kept in quarantine for six weeks and acclimated to the laboratory habitat for a minimum of 72 hours before testing. Fish were housed in an Aquatic Habitats three-tier benchtop system (AHAB, Aquatic Ecosystems, Inc., Apopka, FL., USA) with water being continuously circulated and filtered. Fish were kept in 10L tanks in groups of up to 50 and 3L tanks in groups of up to 20. Water was passed through a UV filter and an activated carbon filter, as well as being mechanically filtered. Water was prepared from reverse osmosis (RO) treated tap water, which was then buffered with non-iodized salt, sodium bicarbonate,

acetic acid, and Prime® (Seachem, Madison, GA., USA). Water temperature was maintained between 26-28°C and pH between 6.5-8.0 and was monitored daily by an animal care technician. Once daily, fish were fed a diet of commercial zebrafish food (Gemma Micro 300, Skretting Ltd., France) The lights were kept on a 12 hour light-dark cycle with lights on at 08:00 and lights off at 20:00. All animal care procedures and experimental procedures were approved by the MacEwan Animal Research Ethics Board and comply with Canadian Council of Animal Care (CCAC) guidelines for the care use of experimental animals.

**Habituation Period**

Prior to dosing and testing, fish were transferred in groups of four to 3L tanks in the testing room for habituation for 30 minutes. The purpose of this period was to permit acclimatization to the testing room, to minimize stress, and to allow them to shoal together prior to testing. After this period, individual groups were netted and placed in a dosing tank.

**Drug Administration**

Chlorpheniramine maleate was administered to experimentally naïve zebrafish through immersion at 0mg/L, 20mg/L, and 25mg/L for 10 minutes. Once daily a stock solution was created by dissolving 0.1g chlorpheniramine maleate salt (Sigma-Aldrich, St. Louis, MO., USA) in 1mL RO water. 400µL stock solution was added to 2L of RO water to obtain a concentration of 20mg/L and 500µL for 25mg/L for dosing. Zebrafish were placed in a 3L tank filled with 2L of RO water or chlorpheniramine treatment water maintained at 26-28ºC. The dosing tank was surrounded by white corrugated plastic to reduce any external visual stimuli.

**Shoaling Procedure**

Fish were tested in a white, round, non-toxic plastic arena with a diameter of 35cm. The arena was surrounded on three sides with white corrugated plastic to minimize any external

visual stimuli and overhead lighting was diffused to minimize shadows. Fresh buffered habitat water was added to the arena to a depth of 5cm and maintained at 26-28ºC. Eight groups of four fish were tested in each condition (control, 20mg/L, and 25mg/L) and each group was randomly assigned to a condition. Testing was carried out during the light portion of the circadian cycle and each fish was tested only once.

After dosing, a spawning insert with a group of four fish was gently lifted out of the dosing tank and placed in the testing arena. The insert was slowly tipped to allow fish to swim out of the insert into the arena. The insert was tipped in opposite directions each trial, either to the researcher's left or right, to minimize any visual bias. Once all fish were in the arena for 15s, behaviour was recorded for 10 minutes by an overhead camera suspended approximately 1 metre above the arena. The inter-individual distance was calculated for each shoal by averaging the distance between each fish. Behaviour was analyzed using Ethovision XT motion tracking software (version 11, Noldus, VA, USA). After testing, fish were netted and returned to the habitat.

## Results

Data were analyzed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). One trial (four fish) was excluded from analysis due to freezing, which was defined as having a velocity of <1cm/s for more than 90s. Additionally, the first minute of recorded behaviour in each trial was not included because of freezing (velocity <1cm/s). Normality was tested using the D'Agostino and Pearson omnibus normality test (alpha=0.05). Data that passed the test of normality was analyzed using 1-way ANOVA, and data which did not pass was analyzed using the Kruskal-Wallis test.

The 20mg/L (n=8) and 25mg/L (n=8) groups passed the test for normality, however the control group (n=7) did not, so data was analyzed using the Kruskal-Wallis test (alpha=0.05). We found that there was no significant difference in the inter-individual distance between groups (p=0.3614) (Figure 1), indicating that chlorpheniramine does not produce anxiolytic effects in the shoaling test at 20mg/L or 25mg/L doses.

Additionally, we evaluated whether chlorpheniramine had an effect on individual fish motility by analyzing velocity. Data were tested for normality using the D'Agostino and Pearson omnibus test normality (alpha=0.05). The control (n=28) and 25mg/L groups (n=32) passed normality, but the 20mg/L group (n=32) did not pass normality, so the Kruskal-Wallis test was used. There was no difference in mean velocity between groups (p=0.0834), suggesting that chlorpheniramine does not have any effect on motility at either dose (Figure 2).

## Discussion

In this study, we failed to find evidence to support the hypothesis that chlorpheniramine acts as an anxiolytic in zebrafish. Shoal cohesion was used as a measure of anxiety and no differences were found between fish treated with 20mg/L or 25mg/L, and controls. To our knowledge, this is the first study investigating the role of histamine antagonists on anxiety in zebrafish. We hypothesized that chlorpheniramine would act as an anxiolytic because several studies have demonstrated its usefulness in reducing anxiety in rodents (Hasenöhrl, Weth, & Huston, 1999; Miyata et al., 2011; Privou et al., 1998; Zarrindast et al., 2005). Velocity was also examined as a measure of activity and neither 20mg/L or 25mg/L of chlorpheniramine had an effect on individual fish velocity, suggesting that chlorpheniramine does not have sedating or stimulating effects.

It is possible that the shoaling test was insufficiently sensitive to detect anxiolytic effects of chlorpheniramine or that chlorpheniramine does not affect the type of anxiety tested in the shoaling test. In the novel tank diving test, 20mg/L of chlorpheniramine causes an increase in exploratory behaviour (i.e. zebrafish spend more time in top of the tank compared to the bottom), suggesting that this dose decreases anxiety (unpublished data). The novel tank diving test evokes a stronger anxiety response in zebrafish because they are introduced into a novel situation, as well as being removed from their shoal (Egan et al., 2009); whereas the shoaling response is strongly associated with predator avoidance only (Miller & Gerlai, 2007). It may be that the perceived threat in this situation elicited a response that was too weak to result in demonstrable anxiolytic effects after treatment with chlorpheniramine. As well, due to the differences between the shoaling test and the novel tank diving test, it is possible that the drug only produced pronounced effects in individual zebrafish, as individual fish become more stressed and display more robust anxiety behaviours. Conversely, zebrafish are a highly social species and research has demonstrated that testing fish in groups may be more valid than testing fish individually, because shoaling is a stable behaviour and shows less variability than behaviours of fish tested individually (Miller & Gerlai, 2007; Pagnussat et al., 2013).

Many of the studies evaluating the effects of histamine on anxiety in rats used the elevated plus maze, which resembles the novel tank diving test in that both tests are based on the willingness of an individual animal to explore its environment. The elevated plus maze can also be used to analyze emotional memory, and as chlorpheniramine has shown to affect learning and memory (Medalha, Coelho, & Mattioli, 2000; Serafim et al., 2016), this might help explain the types of anxiety chlorpheniramine acts on. Some studies have demonstrated that high doses of $H_1$ antagonists inhibit entry into the open arms of the elevated plus maze, which can be explained by

deficits in emotional memory or decreases in motivation (Orofino, Ruarte, & Alvarez, 1999; Serafim, Kishi, Canto-de-Souza, & Mattioli, 2013). Therefore, other tests assessing learning and memory in zebrafish, such as the novel object recognition test should be employed to further study the effects of chlorpheniramine on behaviour.

The comparison of zebrafish and rodent studies is further complicated by physiological differences, methodological differences, and behavioural differences. In the zebrafish brain, histaminergic neurons are clustered in one group, whereas the rat brain has five clusters (Kaslin & Panula, 2001). The limited understanding of the zebrafish histaminergic system makes it difficult to compare to the rat histaminergic system. Because the anatomy of the fish brain and the rodent brain is not homologous, the cellular distribution may not bear any significance, however this could conceivably result in subtle effects on behaviour. Administering drugs to rodents is also more complicated, as rats must be injected with the drug to ensure controlled dosage, which is a stressful procedure for animals compared to drug administration through immersion. Studies in rodents used different injection sites in the brain, which may influence the results depending on the affected brain structures (Zarrindast et al., 2005). As well, some studies used chronic administration as opposed to acute administration, which would influence the drug's effects and should also be considered. Not all behavioural tests in rats have analogous tests in zebrafish and vice versa, for example rats were all tested individually, whereas zebrafish can be tested in groups as in the shoaling test.

Given that chlorpheniramine has never been administered to zebrafish through immersion, it is possible that the doses used were not adequate for decreasing anxiety. Pilot testing indicated that 25mg/L was at the upper limit of what zebrafish could tolerate without adverse effects. We observed that at doses above 20mg/L, zebrafish had increased mobility and

exhibited decreased thigmotaxis, whereas at doses below 20mg/L, zebrafish exhibited significant thigmotaxis and bouts of freezing. Increments of 5mg/L were chosen to test zebrafish, as smaller increments did not produce observable changes in behaviour. In the novel tank diving test, we observed that 20mg/L of chlorpheniramine decreased anxiety, but not the 25mg/L dose, which suggests that at high doses zebrafish have increased sensitivity to the effects of the drug. Additionally, studies examining the effects of chlorpheniramine on memory in teleosts though intraperitoneal injections have found that different doses produce opposing effects; for example, a high dose of chlorpheniramine caused a reinforcing effect, and a low dose caused an amnesiac effect (Serra, Medalha, Bueno, & Mattioli, 2002). Further studies examining a full dose response curve for chlorpheniramine may aid in determining the range of doses at which it is most effective in zebrafish.

Another consideration when determining the effects of histamine antagonists in zebrafish is whether different histamine antagonists have multiple competing effects. It is possible that other $H_1$ antagonists may have varying effects on the $H_1$ receptor, and that other receptors ($H_2$ and $H_3$) have different roles in behaviours depending on the type of anxiety tested (i.e. predator avoidance versus neophobia). Most studies have found that the $H_1$ receptor is more involved in anxiety related behaviours compared to the $H_2$ receptor (Serafim, Kishi, Canto-de-Souza, & Mattioli, 2013; Zarrindast et al., 2008), however studies have also demonstrated that the $H_2$ receptor is involved in anxiety (Chee & Menard, 2013; Mohsen et al., 2014). The $H_3$ receptor has may also mediate anxiety-related behaviour, but results have been inconsistent (Chee & Menard, 2013; Yokoyama et al., 2009). Studies have reported varying effects of the same drug on the same behaviour, depending on the dosage and site of delivery (Chee & Menard, 2013; Gianlorenço, Serafim, Canto-de-Souza, & Mattioli, 2013). Because we chose to use immersion

rather than injection, we expect that chlorpheniramine was active on $H_1$ receptors throughout the brain, rather than in one area as in injection studies.

While the exact mechanisms are still unknown, histamine has a clear role in anxiety related behaviours in animal models and future research to further elucidate its role in behaviour is necessary. Given the advantages of the zebrafish model, continued studies investigating the histaminergic system in this species would be beneficial to understand this system and its role in anxiety disorders. Despite our nonsignificant results in the shoaling test, it is possible that this test is not suitable for observing histamine mediated behaviours. Considering that we found that chlorpheniramine decreased anxiety in the novel tank diving test, it is likely that the $H_1$ receptor is involved in anxiety related behaviours in zebrafish. Further studies using other histamine antagonists, especially $H_2$ and $H_3$ antagonists, and using other behavioural assays should be conducted to fully understand the effects of the histaminergic system on behaviour. Additional doses of chlorpheniramine should also be tested in the shoaling test as well as other behavioural assays to further understand chlorpheniramine's potential in zebrafish. While this research does not support the hypothesis that chlorpheniramine reduces anxiety in zebrafish, additional studies are necessary to determine the role of histamine on anxiety in zebrafish.

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Appendix

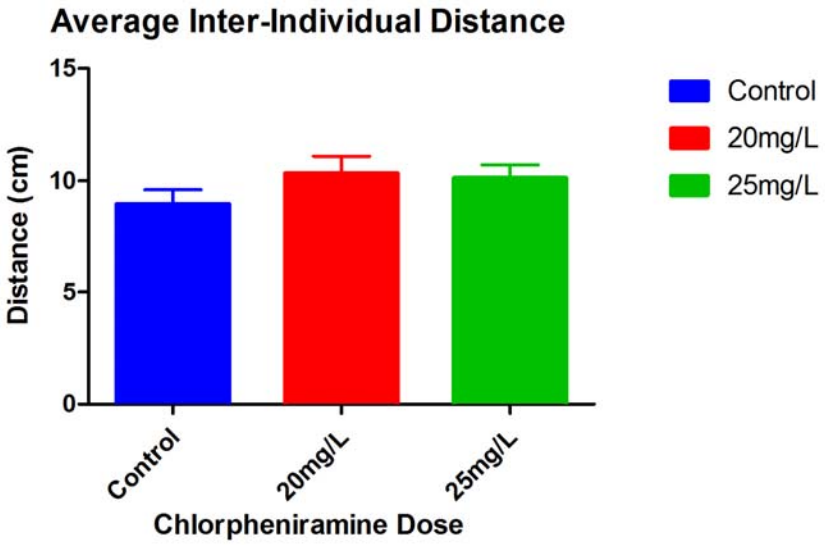


*Figure 1.* Average inter-individual distance as a measure of shoal cohesion. No differences in inter-individual distance were found between any groups (p=0.3614).

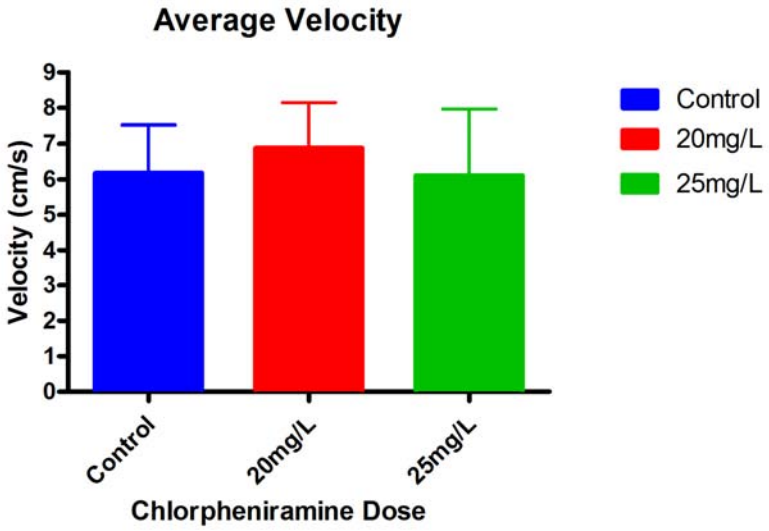


*Figure 2.* Average velocity of individual fish. No differences in velocity were found in between groups (p=0.0834).