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The Effects of Δ^9 -tetrahydrocannabinol (THC) on development
and hyperekplexia in embryonic zebrafish model

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Masters of Integrative Biology Thesis

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Abstract

Marijuana is one of the most commonly used illicit drugs around the world. It has gained attention as an alternative medicine to many different disorders. Though initially investigated in modern medicine for its analgesic and antiemetic properties, cannabis has been recently found to benefit numerous neurological disorders. Though there are over 100 different cannabinoids produced by the cannabis plant, Δ^9 – tetrahydrocannabinol (THC) has been shown to be the most efficacious phytocannabinoid in neurological disorders. THC has been shown in numerous previous studies to be effective at treating spasticity caused by multiple sclerosis and spinal cord injury, as well as seizures. Through binding of the endogenous cannabinoid receptor system THC is able to affect both the excitatory and inhibitory synapses. Hyperekplexia is an upper motor neuron disease characterized by hypertonia and exaggerated startle response. In Hyperekplexia, there is a decrease in inhibitory synapse caused by a loss of glycine receptors. Because previous studies have demonstrated the efficacy of THC in disorders similar to hyperekplexia, it should be investigated as a treatment for this disorder as well. This study demonstrates that in embryonic zebrafish, THC does not detrimentally affect the development of the synapses needed to complete the startle response. Additionally, this study supports the use of THC as a treatment option for the alleviation of spasticity caused by hyperekplexia.

Introduction:

Cannabis sativa was among the first domesticated plant species. It has been used and distributed across the world for many different purposes (van Backel et al. 2011). Originally domesticated in Asia for its textile use, the *Cannabis sativa* species, or more commonly known as marijuana, has become increasingly cultivated for medicinal purposes (van Backel et al. 2011). Records dating back to 2000 B.C. report on marijuana's application for the treatment of pain and beriberi, a thiamine deficiency that has neurological symptoms. In the 19th century Queen Victoria's personal physician, Sir John Russel Reynolds, praised the plant for its analgesic and muscle relaxing qualities. It was not until the 1960's that the first active compound in *Cannabis*, Δ^9 - tetrahydrocannabinol (THC), was characterized (Hagenbach et al. 2007). THC has been found to be the main psychoactive component of *Cannabis*, but it is actually only one of the more than one hundred phytocannabinoids produced by the plant.

THC, as well as other phytocannabinoids, is extremely hydrophobic. These molecules readily penetrate the central nervous system and cell membranes. It was discovered through the advent of synthetic cannabinoids, that THC elicits its effects through a receptor-driven mechanism (Baker et al. 2012, Howlett, 1985). In 1990 the cannabinoid receptor type 1 (CB₁) was cloned and the endogenous cannabinoid system (ECS) was discovered (Matsuda et al 1990). The ECS is comprised of two G-protein coupled receptors, CB₁ and CB₂, their neuromodulatory lipid ligands, and the biosynthetic machinery responsible for receptor and ligand synthesis and degradation. The CB₁ receptor has been found to be the most numerous G protein coupled receptor within the central nervous system (Baker et al., 2012, Hill et. al., 2012, Krug II, et. al., 2015, Lam et. al., 2006). These receptors are typically presynaptically localized and function as retrograde messengers to decrease synaptic output. Through activation of the G_α subunit,

cannabinoids are able to inhibit voltage-gated calcium channels and potentiate inward rectifying potassium channels (Baker et. al., 2012, Hill et al., 2012). Through activation of CB₁ receptor, cannabinoids can regulate synaptic neurotransmission, and effect numerous physiological processes including those involved in Alzheimer's, anxiety, epilepsy, multiple sclerosis (MS), Huntington's, pain perception, and nervous system function (Hill et. al., 2012). It is now believed that THC's therapeutic effects are caused by its binding of the CB₁ receptor. For this reason, it has been researched as a treatment for many neurological and muscular dysfunction disorders. Multiple clinical studies have been conducted testing the efficacy of THC and CBD (cannabinoid) containing oromucosal spray as a treatment option for spasticity caused by MS (Wade et. al., 2010, Zajicek et. al., 2003, Wade et. al., 2006, Karst et. al., 2010, Stott et. al., 2013, Hagenbach et. al., 2007, Syed et. al., 2014). These studies have found it to be a viable option for alleviating spasticity, and have shown there are no notable severe side effects. Hagenbach et. al.'s (2007) research showed that THC significantly decreased spasticity and pain in spinal cord injury victims. These previous studies and the finding that THC has neuromodulator effects through binding of the CB₁ receptor, lend support to its potential as a treatment option for the spasticity, or hypertonia, caused by hyperekplexia.

Hyperekplexia, or human startle disease, is a rare but potentially fatal neurological disorder. It is caused by a loss of function mutation in the genes that encode key components of the glycinergic synapse (Ganser et. al., 2013, Mine et. al., 2014, Chung et. al., 2010, Becker et al., 2008, and Villmann et. al., 2009). Glycine is the major inhibitory neurotransmitter in the vertebrate hindbrain and spinal cord. It functions at the synapse by binding the glycine receptor to open chloride channels that depolarize the cell, leading to inhibition of neurotransmission, or relaxation of the muscle. In the majority of hyperekplexia patients, the gene that encodes the

glycine receptor $\alpha 1$ subunit has a mutation that prevents the receptor protein from recognizing its ligand (Ganser et. al., 2013, Mine et. al., 2014, Chung et. al., 2010, Becker et al., 2008). The main symptom of hyperekplexia is an extreme startle reaction to sudden or unexpected movement, touch or noise. During the startle response, it is common to see an exaggerated head-retraction reflex, spastic jerking movements, rapid eye blinking, and the patient may fall stiffly. During these reactions, all voluntary motor control is lost (Ganser et. al., 2013, Mine et. al., 2014). Additional symptoms of this disorder include: exaggeration of reflexes, unstable walk, and intermittent apnea, in infants. The current treatment for hyperekplexia is commonly clonazepam, an anti-anxiety and anti-seizure medication (Chung et. al., 2010). This medication is part of a family of medications called benzodiazepines (BZD). These medicines work by binding allosterically, meaning not at the active site, to γ -aminobutyric acid (GABA) receptor subtype A within the central nervous system. Binding of these receptors by BZD enhances the opening of chloride channels, which produces a stronger inhibitory response, when GABA, the ligand to these receptors, binds at the synapse (Riss, J., et. al., 2008). There are currently no known cures for hyperekplexia, so it is important to continue investigating potential treatments with the hope of identifying a cure. In previous research it has been demonstrated that THC can be used to alleviate over excitation caused by MS as well as the spasticity caused by spinal cord injury (Wade et. al., 2010, Zajicek et. al., 2003, Wade et. al., 2006, Karst et. al., 2010, Stott et. al., 2013, Hagenbach et. al., 2007, Syed et. al., 2014). Previous research in similar disorders suggest it to also be a viable treatment option for hyperekplexia. In order to investigate this hypothesis, I chose the zebrafish model system, in particular the escape behavior and circuitry to understand how THC can alleviate spasticity.

Zebrafish (*Danio rerio*) are a prime model system for testing the effects of THC on development as well as investigating its potential as a treatment option for hyperekplexia. Because they are a model organism, zebrafish have a well characterized development as well as a sequenced and easily manipulated genome. Their nearly translucent body allows for immunostaining and microscopy, and they are also easily and inexpensively reared, making replication of the experimental process very simple (Kalueff et. al., 2014, Ganser et. al., 2013, Krug II et. al., 2015, Lam et. al., 2006). Juvenile zebrafish can absorb molecules like phytocannabinoids across their skin. The ECS has been found to be highly conserved (Krug II et. al., 2015). McPartland et al. (2007) found that the ECS of zebrafish serves a similar function to the mammalian system. It has also been demonstrated, by Ganser et al. (2013), that hyperekplexia can be modeled in the zebrafish using an antisense oligonucleotide morpholino sequence, or morpholino, targeting exon 4 of the *glral* gene. For this reason, the morpholino injection was used again in this study.

A morpholino is a sequence of nucleic acid that resembles RNA (ribonucleic acid) or DNA (deoxyribonucleic acid) but contains morpholine instead of either of the sugars from these other nucleic acids polymers. The sequence of bases can be constructed in the morpholino to complementarily bind a target sequence of mRNA. In doing so, translation is prevented (Ekker, S., 2000). Morpholinos have been shown to be highly efficacious at knocking down, or preventing, target proteins from being expressed throughout the entire animal (Nasevicus, A and Ekker, S., 2000). Morpholino is able to be injected into the yolk of the dividing embryo as early as the 1-2 cell stage. Such an early injection time ensure all the cells are exposed. As the embryo uses the yolk to grow and develop the cells become endowed with the morpholino which preferentially binds the target mRNA in both the cytosol and nucleus to stop the production of a

desire protein, in this case GLRA1. Because embryos are so small (about 0.7mm) injecting the yolk of the embryo is achieved using a pulled capillary tube under pressure. This is calibrated to deliver a consistent amount of morpholino into the egg without rupturing the chorion.

In summary, using zebrafish I was able to investigate the effects of THC on developing neuronal circuitry. Embryonic zebrafish were treated at different stages of the development, and the highly characterized startle response was analyzed. Then to test the potential of THC as a treatment option for hyperekplexia, I created hyperekplexic morphants, through morpholino gene manipulation techniques. Then treated those morphants with THC at the same developmental stages previously study and analyzed their startle response. I found that THC does ameliorate the spasticity caused by hyperekplexia, and found no evidence that THC is detrimental to development.

Methods:

Fish care and embryo rearing

Experiments were carried out using wild type strains of *Danio rerio*. Adults were kept on a 12hour light/12 hour dark circadian cycle, and housed at 28.5°C. Crosses were set up the night prior to embryo collection in a divided breeding tank. Dividers were lifted at 0900 hours the following morning and fertilized embryos were collected 1 hour after dividers were lifted. Embryos were then reared in petri dishes containing system water, the same water the adult fish were housed in, and kept in a 28.5°C incubator. Embryos were also kept on the same 12 hour light/12 hour dark cycle. All experiments were conducted in accordance with Kennesaw State University's Animal Care and Use committee guidelines.

Morpholino injection

Splice site-targeted morpholinos (MO) were previously designed against the 3' acceptor site of exon 4 in the glycine receptor α 1 (*glra1*) gene and tested for off-target effects (Ganser et al., 2013). For control injections, I used the standard control MO provided by Gene Tools (Gene Tools, LLC; Philomath OR). Mutant injections used MO stock solution that was heated to 65°C for 5mins and diluted into 1% (w/v) fast-green dye at a 1:1000 ratio. MOs were injected using filament-lined kwik-fil borosilicate capillary glass (World Precision Instruments, Sarasota FL) pulled on a P-97 micropipette puller (Sutter Instruments, Novato CA) to a long taper with a fine tip that was broken back to a 1 μ m diameter using forceps. Injection was achieved using a picopump injection rig, calibrated to produce a 100 μ m diameter bolus. MOs were injected into wild type embryos at the 1-2 cell state. Embryos were sorted 5-7 hours after injection so that only morphants in which the MO bolus had evenly dissipated were treated and analyzed.

THC treatments

Pharmaceutical grade THC (Sigma Aldrich Co, LLC; St. Louis MO) was administered via vaporization at 60mg/L to entire treatment groups in 50mLs of system water. A proprietary vaporization chamber was used to treat the animals (Figure 1). Embryos were placed into a sealed plexi-glass box containing a micropore air stone (Pentair, Aquatic habitats, Apopka, FL). The stone was connected by aquarium tubing to a Whisper pump in a second sealed plexi-glass box. The box that housed the Whisper pump also included a titanium nail which was heated to 185°C, optimal vaporization temperature of THC, by an industrial soldering iron. Both boxes were sealed to prevent any vapor from escaping. Embryos were exposed to THC at a constant rate for 30 minutes. For treatment embryos were separated into groups and each experimental group was

treated once at varying developmental stages. Treatment occurred at 3 hpf (hours post fertilization), 24 hpf, or at 48 hpf (see figure 2).

Behavior analysis

A high-speed camera (FASTEC IMAGING HiSpec1L, Germany) was used to record spontaneous and touch-evoked behaviors of 24hpf and 48hpf control and treatment fish. Embryos were manually dechorionated at 24hpf and imaged 1 hour after dechorionation. 24 and 48hpf embryos that were treated the day of imaging were imaged 1 hour after treatment had ended. Videos were scored by hand and data was analyzed by one-way ANOVA using Minitab Express (Minitab Inc. State College PA). Comparisons of significance are all based on $p < 0.05$.

Immunohistochemistry

Cryosectioning and antibody staining were performed as previously described in Ogino et al., 2011. 48hpf embryos were embedded in O.C.T compound (Tissue-Tek, Torrance, CA) and gradually frozen in liquid nitrogen. Samples were sectioned on a cryostat (CM-1850, Leica) and mounted on Superfrost plus slides (Fisher Scientific, Pittsburgh PA) prior to a 10 min fixation with 4% (w/v) paraformaldehyde (Sigma Aldrich Co, LLC; St. Louis MO). Anti-glutamate gated ion channel (clone NMDAR2a, mouse IgG, 1:500) and Anti-Glycine receptor (clone mAB2b, mouse IgG, 1:500) were used as primary antibodies. Alexa 488- and Alexa 568- conjugated donkey anti-mouse IgGs were used as secondary antibodies (1:1000, Life Technologies, Carlsbad, CA). Double staining with anti-glutamate and anti-glycine receptor antibodies was performed sequentially. Stained sections were mounted in Vectashield/DAPI (Vector

Laboratories, Burlingame, CA) and images were captured on a confocal microscope using a 1.4 NA 63x oil objective (Zeiss LSM 700, Jena Germany).

Quantification of synaptic staining

Images were processed using Zeiss Zen software 2009 (Zeiss, Jena Germany). Brightness and contrast were optimized, and concentration of pigment in the spinal cord was measured. These concentration measurements were analyzed against negative controls to remove background noise. Final data were compared using ANOVA analysis in Minitab Express (Minitab Inc., State College PA).

Results

Exposure to THC during development

At 24hpf zebrafish exhibit a coiling behavior in response to a stimulus. When poked the embryo will bend in one direction, usually opposite the direction the stimulus came from, until the tail touches the head, called a C-bend. This behavior is promoting the development and growth of the neural circuitry necessary for the “fight or flight” response that the fish will need to survive. While the behavior itself is highly characterized the exact timing it takes for the behavior to occur varies (Kimmel et al., 1974). Control embryos, injected with control morpholino and not further treated with THC were imaged and analyzed to provide a basis for comparison to morpholino injected THC treated fish. The average C-bend time for untreated embryos at 24hpf was found to be 174.76 (± 36.53) milliseconds. At 48hpf embryos perform the C-bend response and couple it to a rhythmic swim pattern away from the stimulus. Control embryos at 48hpf completed this behavior in 15.93 (± 0.71) milliseconds.

To test the effects of THC on the startle response, embryos were collected and separated into 3 experimental groups ($n \geq 30$). All groups were treated with 60mg/L. At 24 and 48hpf the startle response was imaged by high speed camera, and then later analyzed. Average C-bend times for treatment groups were compared to controls using ANOVA analysis. Comparisons of 24hpf groups can be seen in figure 3. C-bend times were significantly higher in 3hpf and 24hpf treated fish as compared to control. However, it can be seen in figure 4 that by 48hpf these embryos are completing the C-bend in an average time similar to the control embryo's average. Embryos treated at 48hpf were treated as controls prior to treatment. 1 hour after treatment had concluded, these embryos were imaged and compared to control. As seen in figure 4, the C-bend time was significantly higher in this treatment group.

Along with behavior, spasticity and non-responsiveness were counted in each treatment group to assay for disturbances in circuitry development after THC treatment. Figure 5 shows the percentage of each population, over all experimental replicates ($n \geq 50$), that were labeled non-responsive or spastic. Embryos that were not able to mount a response after 3 pokes were labeled as non-responsive. The spastic label was given to embryos that got stuck at the top of the C-bend for more than 1 second, or continued to bend from side to side more than once per side. It can be seen in the graph (figure 5) that control embryos had less than 15% of the population labeled as non-responsive or spastic.

Immunostaining was carried out to investigate the effects of THC on developing excitatory and inhibitory synapse. Fresh-frozen 48hpf treated and control embryos were sectioned and double labeled with antibodies recognizing the glycine receptor 1 α subunit and glutamate ion channel. Figure 9 shows magnified images taken of the spinal cord sections after immunostaining was completed. Glutamate channels appear red, and are seen at the top of this figure. Glycine

receptors appear green, in the center of this image. The bottom of figure 10 shows the glycine and glutamate channels merged together. Each colored dot, or puncta, was counted as a single channel or receptor. Negative controls in which only the secondary antibodies were applied were used to ensure only puncta of interest were being counted. The puncta were averaged among each treatment group. The densities of both glycine receptors and glutamate channels within the developing spinal cords were compared to control using ANOVA analysis. Control embryos showed a larger concentration of glutamate channels compared to glycine receptors. THC treatment groups, by comparison, showed a higher amount of both glycine and glutamate receptors within the spinal cord, though only embryos treated at 3 and 48hpf were significantly different from controls.

In summary THC exposed fish showed a marked increase in the amount of time necessary to perform the startle response, initially after treatments. However within 36 hours after treatments, they recovered and began behaving normally. When embryos were sequentially stained, THC exposed fish from 2 of the treatment groups showed significantly different density of glycine receptor and glutamate ion channel as compared to control embryos.

Hyperekplexia morphant exposure to THC

Morphant embryos were treated by vaporization at 60mg/L of THC, after which their startle responses were tested. Because of the exaggerated startle response not all embryos were responsive to the touch stimulus. Fish that were unable to respond to touch after 3 consecutive pokes, were deemed non responsive. These fish were counted and compared to control groups. Along with inability to mount a response, some spastic fish would over coil and/or get stuck in the C-bend and be unable to uncoil for several seconds. These fish were deemed spastic. At 48hpf

control fish are able to swim in a rhythmic alternating pattern, hyperekplexic fish often have spastic swim patterns where they bend to the same side consecutively or, because of over contraction, are unable to swim altogether. These fish were counted as spastic. Percentages of non-responsive and spastic fish throughout all experimental replicates can be found in figure 8 ($n \geq 50$). It can be seen that the number of spastic embryos at both 24 and 48hpf in the morphant group is greater than that of any other treatment group. Along with counts of non-responsiveness and spasticity, the average times for embryos to complete initial C-bend were again analyzed. These averages can be seen in figures 7 and 8. Hyperekplexic behavior was measured at both 24 and 48hpf. At 24hpf hyperekplexic fish completed the C-bend in $758.40(\pm 134.50)$ milliseconds, a significant difference from the $174.76 (\pm 36.53)$ milliseconds of the control group. By 48hpf these fish were able to complete the C-bend in $165.99 (\pm 26.76)$ milliseconds. Again this is significantly different from control time. When morphants were treated with THC, the data shows a decrease in the average amount of time taken to complete the C-bend, for all treatment groups at both 24 and 48hpf (figures 3 and 4); however, at 24hpf the only treatment group that is significantly different from hyperekplexic are the clutches treated at 3hpf. At 48hpf all treatments groups are significantly different from the Hyperekplexic group.

Immuno-staining was also performed on hyperekplexic experimental groups to investigate the effect of THC treatment on glycine and glutamate receptors. Figure 10 shows example images taken from each treatment group. Again the images are zoomed into the spinal cord and contrast is enhanced. Glutamate channels appear in red and to the top, and glycine receptors appear in green in the middle panels. Once more the puncta were quantified after negative controls had been utilized to remove any background staining and averaged. Untreated morpholino injected fish showed a reduced density in glycine receptor as compared to control, as well as, an increased

density of glutamate receptor puncta. All THC treatment groups had an increased density of glycine receptor in the spinal cord; however only 24hpf treated fish were significantly higher than hyperkplexic embryos. There was also a significant reduction in the amount of glutamatergic staining in the spinal cord of all treatment groups compared to untreated hyperkplexic embryos.

Based on both immunohistochemistry and behavioral observation, THC does have an effect on the spasticity caused by hyperkplexia. All treatment groups showed a decrease in the amount of time taken to perform the startle response, and the amount of spastic and nonresponsive embryos was significantly decreased in all three treatment groups. Immuno-staining of both the glycine and glutamate receptors showed a significant decrease in glutamatergic puncta, and an increase in glycinergic puncta.

Discussion

My research demonstrates that THC does not appear to detrimentally alter development. Though THC treated fish showed a markedly longer C-bend time initially after treatment (24hpf group) and even 24 hours after treatment (3hpf treatment group), by 48 hours post treatment, embryos from these treatment groups were completing the C-bend and coupled swim in similar amounts of time to controls. Also when comparing percentages of the population that behaved spastically or were unresponsive to touch stimulus, THC treatment groups were within 3% of the control populations. This suggests that the development of the excitatory and inhibitory neurons utilized by the autonomic nervous system to complete the 'fight or flight' response is occurring properly. The initial increase in behavior time that is seen in the 24 hpf and 48hpf treatment groups one hour after exposure may be due to continual binding of the CB₁ receptors throughout the nervous system. Hunault et. al.'s (2008) showed that serum concentrations of THC decreased

slowly even after one hour post exposure. This means THC is still available to bind the receptor. If CB₁ is being continually bound, and has not reached any saturation point, inward rectifying potassium channels should be continually or perhaps remaining open. Inward rectifying potassium channels differ from the common leak channels in that they push more potassium into the cell to re-establish membrane potential (Nichols and Lopatin, 1997). However, constitutively active inward rectifying potassium channels would hyperpolarize the cell, making depolarization, or activation of the synapse, delayed. The dosage of THC used in this study at 60mg/L was higher than the average dose used in previous studies, this was to show that higher dosages were not harmful to development, and to compensate for the biphasic effect of THC seen in some previous studies (Baker et al., 2012, Hill et al., 2012, Ruhl et al., 2014). It is possible that at a lower dose, the lag time seen in the behavior initially after treatment would not be seen. The effects on the embryos treated at 3hpf merits further investigation. Though the fish began behaving normally at 48hpf, there may be mechanisms that delay the development, such as a decrease in cellular respiration. During this study Dr. Adrienne King, assistant professor at Kennesaw State University, performed a respiration test on adult treated zebrafish to investigate the effects of THC on mitochondrial respiration. This preliminary data, displayed in figure 10, shows that though THC treated fish were not significantly different from controls, they were taking in more oxygen than control fish, but appeared unable to convert it to ATP. There have been previous studies that demonstrated that THC inhibited oxidation and increased oxidative stress (Wolff et al., 2015 and Fišar et al., 2014). It may be that though studies have shown the benefit of THC in many different disease states, the harm could outweigh the good (Hofmann et al., 2013, Syed, et al., 2014, Wade et al., 2009, Hill et al., 2012, Baker et al., 2012, Zajicek et al., 2003, Wade et al.,

2006, Karst et al., 2010, Stott et al., 2013, Hagenbach et al., 2007). It is important to note that there are many studies out there that support both sides of this argument.

The immunohistochemistry performed in this study demonstrated that THC does indeed have an effect on both the glycinergic and glutamatergic synapses in the spinal cord of developing embryos. Tagliaferro et al. (2006) showed that treatment with a synthetic CB₁ agonist, WIN 55,212-2, increased synaptic densities. My data supports this in that treatment with THC increased receptor densities compared to control. There have been a number of fairly recent studies investigating the activation of the cannabinoid receptor system during development (Fried and Smith, 2001, Campolongo et al., 2007). It has been shown to play a role in neural progenitor differentiation and synaptogenesis (Rubio-Araiz et al., 2008). I believe the data here support this idea as well. Exposure to THC showed an increase in both glycine receptors and glutamate ion channels within the developing spinal cord. Survey polls from previous years have deemed marijuana the number one illicit drug used during pregnancy in the western world (Philipot et al. 2016, Fried and Smith, 2001). It is for that reason that it is so important to continue investigating the developmental effects of THC and other phytocannabinoids.

THC helps to alleviate spasticity caused by hyperekplexia. As supported by previous work, (Ganser et al. 2013) the morpholino knockdown of the *glra1* α subunit did produce hyperekplexic fish that behaved spastically and were mostly non-responsive to touch stimulus. However, when treated with THC, there was a significant decrease in spastic and nonresponsive events. Hyperekplexia, though its own separate disorder, is still marked by an upper motor neuron dysfunction, just like Multiple Sclerosis (MS). There has been a lot of evidence supporting the efficacy of THC as a treatment for spasticity caused by MS and some investigating its efficacy in spinal cord injury, my research again supports the findings of these other studies (Syed et. al.,

2014, Stott et. al., 2013, Karst et. al., 2010, Wade et. al., 2006, Zajicek et. al., 2003, and Wade et. al., 2010, Hagenbach et al., 2007, Baker et al., 2012, Hofmann et al., 2013). THC/CBD oromucosal, Sativex has been tested in clinical trials and been found to alleviate spasticity in MS patients (Syed et. al., 2014, Stott et. al., 2013, Karst et. al., 2010, Wade et. al., 2006, Zajicek et. al., 2003, and Wade et. al., 2010). Klein et al. 2011, demonstrated that CBD can ameliorate the psychoactive effects of THC. And it has been proposed that the efficacy of medicinal marijuana is caused by the cocktail effect of many different phytocannabinoids working in concert (Baker et al., 2012). It would be of benefit to investigate the efficacy of a combined phytocannabinoid substance in the treatment of hyperekplexic spasticity. Along with spasticity there are also studies that support THC as an effective treatment for seizure victims (Hofmann and Frazier, 2013). These patients are commonly prescribed clonazepam, the same treatment prescribed to hyperekplexic patients. My research, and previous data from other studies support further investigating THC as a treatment option for hyperekplexia.

The immunostaining performed on in this study lends more support to the proposition of THC as a treatment for hyperekplexia. Hyperekplexia embryos showed a decreased density of glycine receptor and an increase in glutamate density. After treatment of THC all treatment groups had a decreased density of glutamate and an increased density of glycine. However, only the 24hpf fish had a significant difference from hyperekplexic morphants. If carried into the future it would be necessary to not only look at the developing spinal cord but also investigate whether THC exposure has a rescue effect on the synapse themselves. Chevalyere et. al. (2006) demonstrated that stimulation of the endogenous cannabinoid receptor system can increase synaptic plasticity and potentially rescue damaged neurons.

Conclusion

This study supports that THC exposure is not detrimental to development. After the initial effects of THC exposure have worn off, embryos completing the startle response in a similar time to controls. This study has also shown that THC can be used to ameliorate the spasticity caused by hyperekplexia. This research lends more support to the previous studies showing THC's effects on spasticity as well as lends support to as yet untested upper motor neuron disorders.

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I would like to everyone in the Ganser lab for their help and support in this study. I would like to especially thank Lisa Ganser without whom this study would have never been possible. I would also like to thank Kennesaw and the MSIB program.

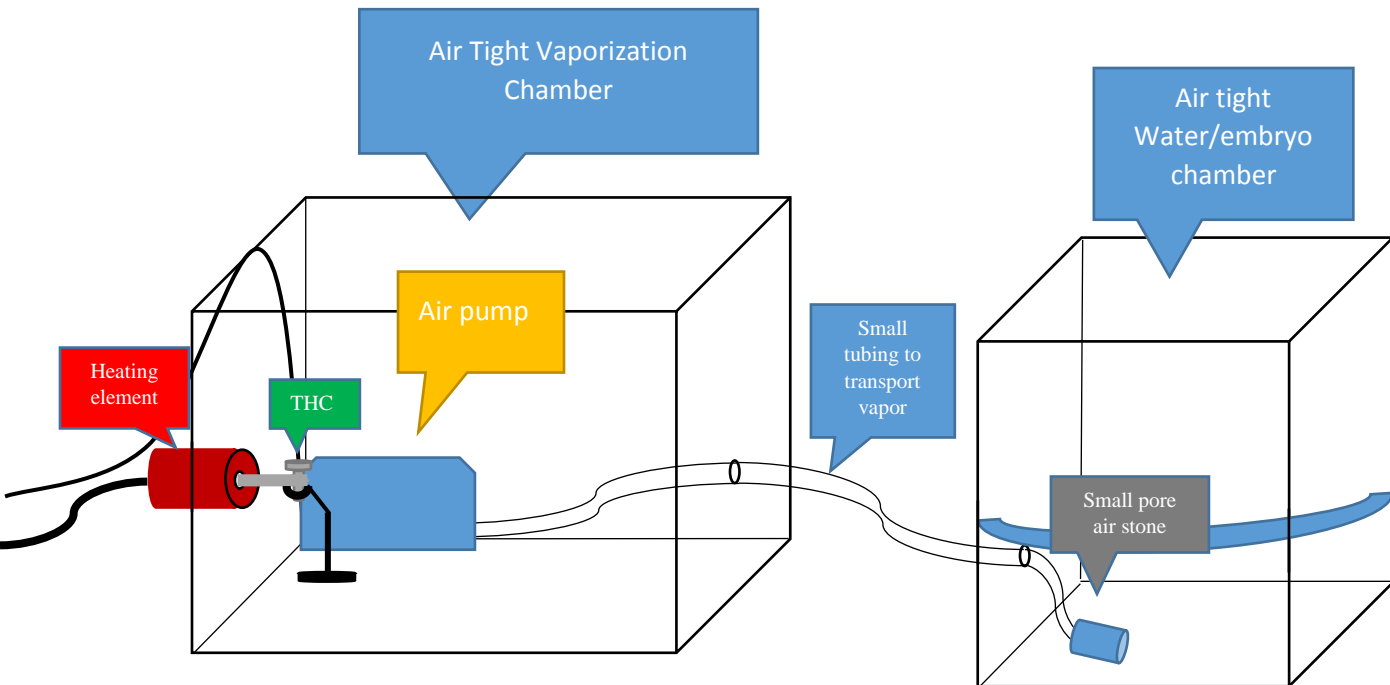


Figure 1: Schematic of vaporization chamber. This figure shows a schematic representation of the vaporization chamber invented to treat embryos. The large chamber on the left houses the THC nail, heated by the soldering iron (in red) and the Whisper air pump (marked by the yellow box). The pump is attached to the aquarium tubing which exits the left chamber and enters the right chamber. The right chamber is filled with 500ml of aquarium water. It houses the small pore air stone, and the embryos during treatment.

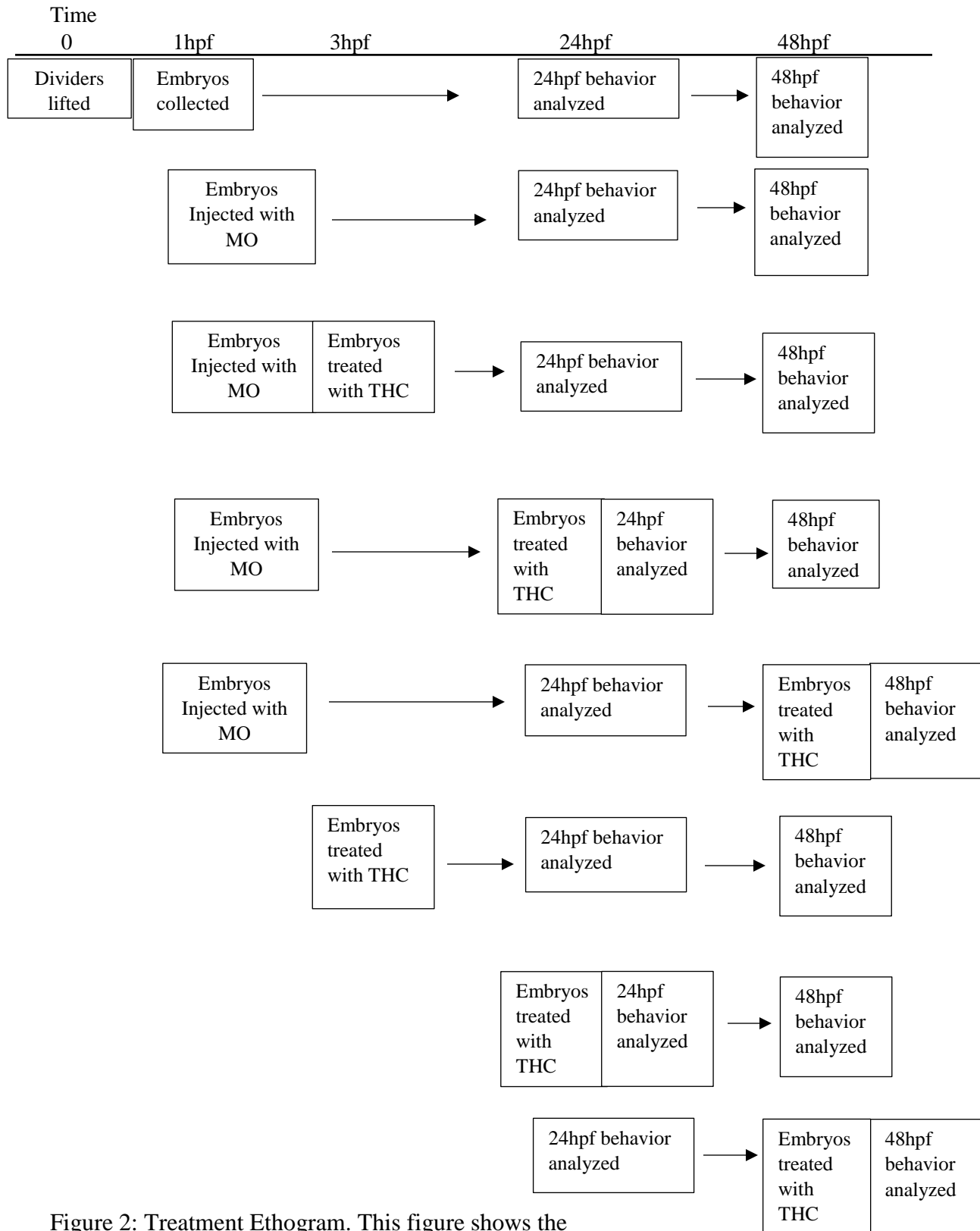


Figure 2: Treatment Ethogram. This figure shows the breakdown of each experimental step with the time given by the bar running across the top.

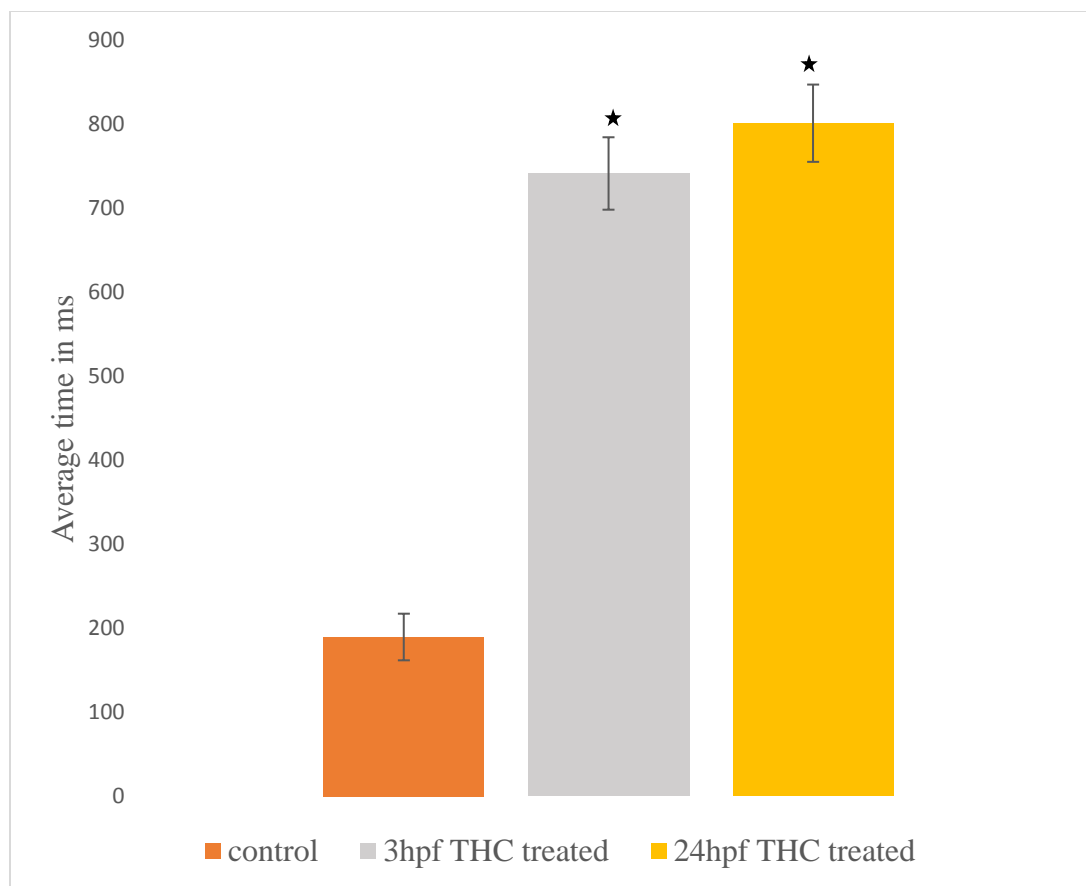


Figure 3: Escape response at 24hpf after THC treatment. This graph shows the average times taken to complete the C-bend for THC treatment group. Stars show averages that are significantly different from control average response time ($p < 0.05$). The legend can be found at the bottom of the graph. Orange is control. Grey represents the embryos treated with THC 3 hours post fertilization (hpf). Gold represents embryos treated with THC at 24hpf.

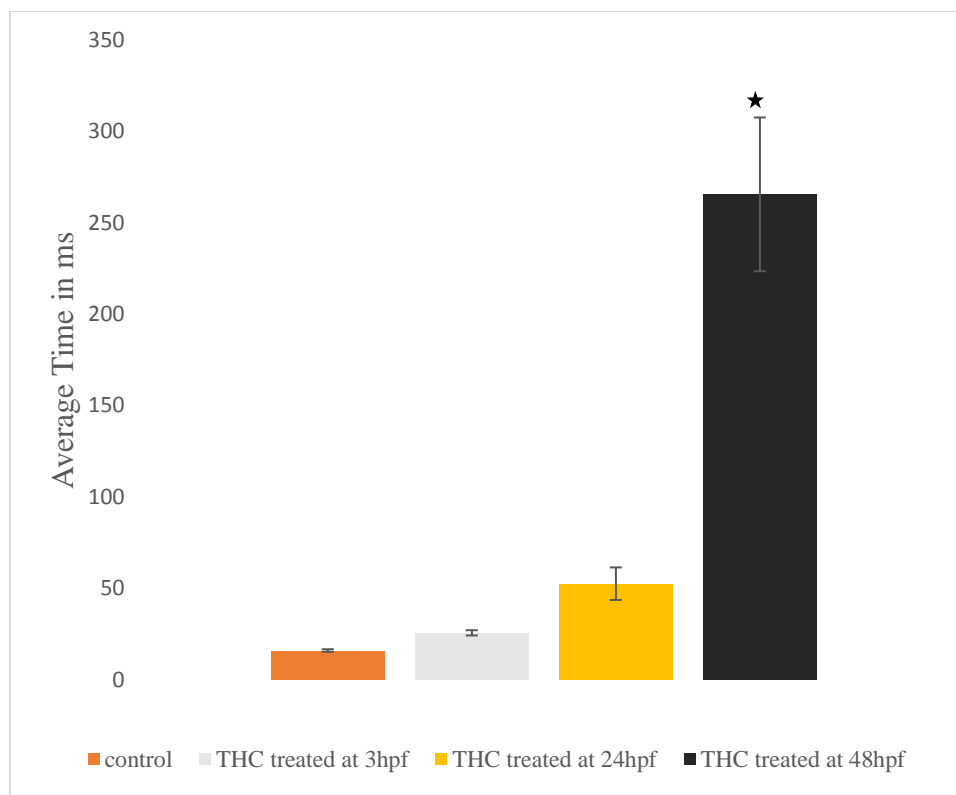


Figure 4: Escape response at 48hpf after THC treatment. This graph represents the average times taken to complete the C-bend at 48hpf after THC treatment group. Stars represent averages that are significantly different from control ($p > 0.05$). The color key is at the bottom of the graph and colors follow the previous graph with the addition of embryos treated at 48hpf in black and morpholino injected embryos treated at 48hpf in yellow. The abbreviations are as follows: MO is morpholino and COMO is control morpholino, hpf is hours post fertilization, and THC is Δ^9 -tetrahydrocannabinol.

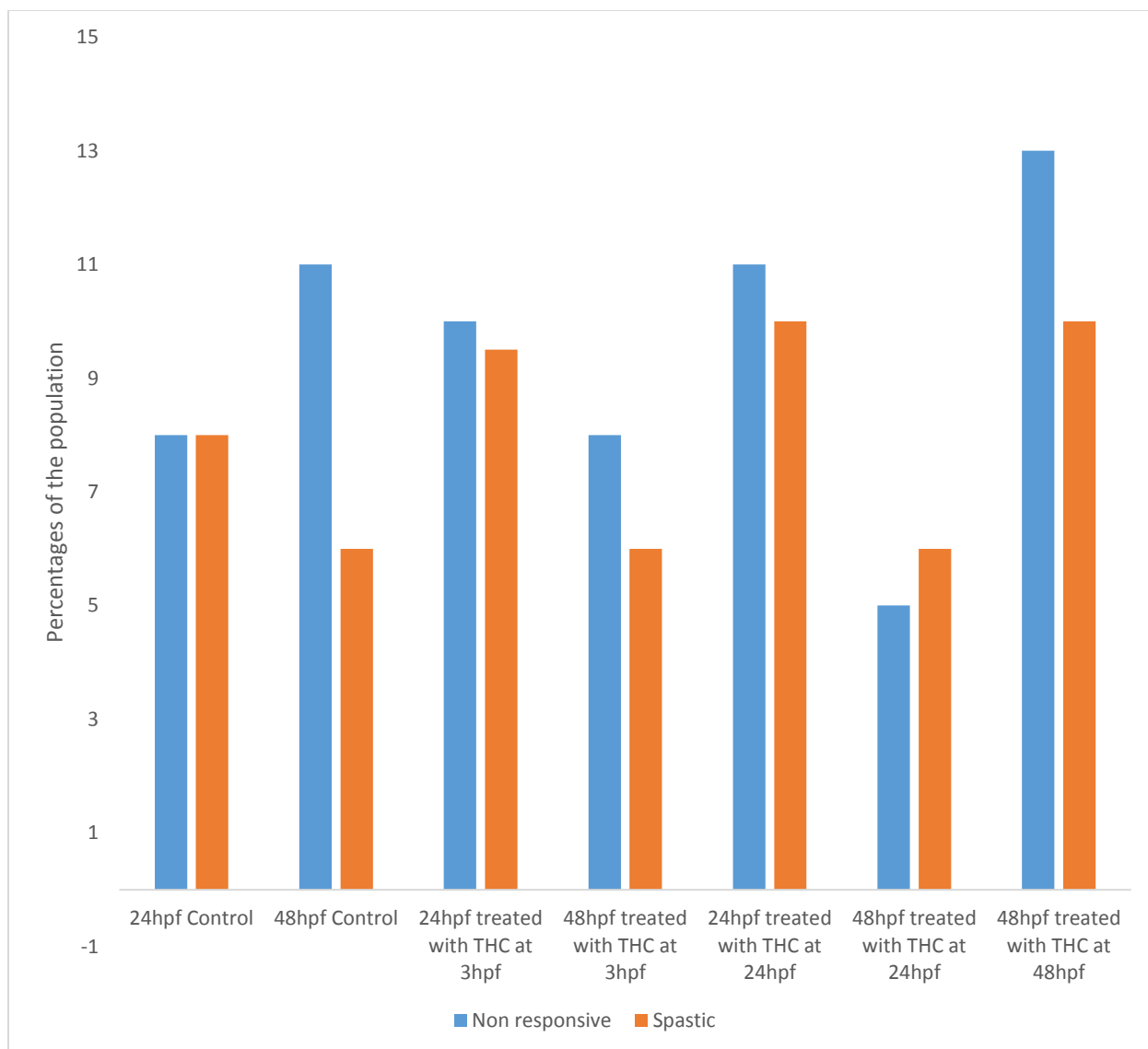


Figure 5: Spastic and non-responsive THC treatment groups. The above graph shows the spastic and non-responsive percentages across all experimental replicates for control and THC treatment groups. The y-axis lists the percentages and the x-axis lists the treatment group. Spastic labeled percentages are seen in orange and non-responsive are seen in blue.

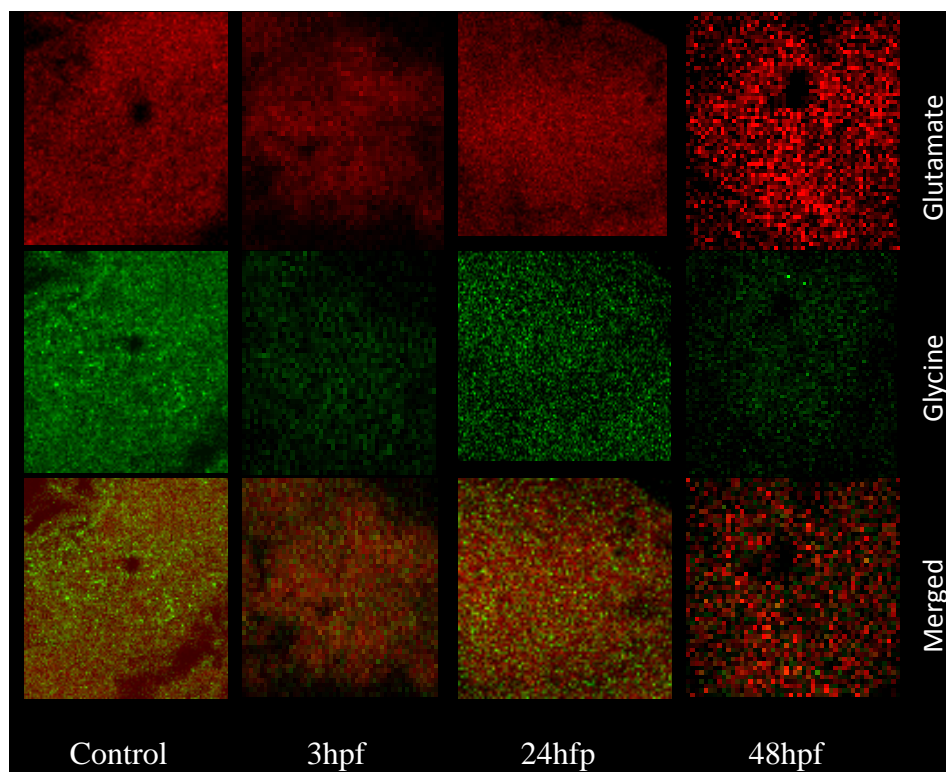


Figure 6: Glycine/glutamate expression in THC treated spinal cord sections. These are confocal images zoomed in on the spinal cord from control and THC treated embryos. Glutamate ion channels appear in red and glycine receptors appear in green. The images were taken at 40x magnification.

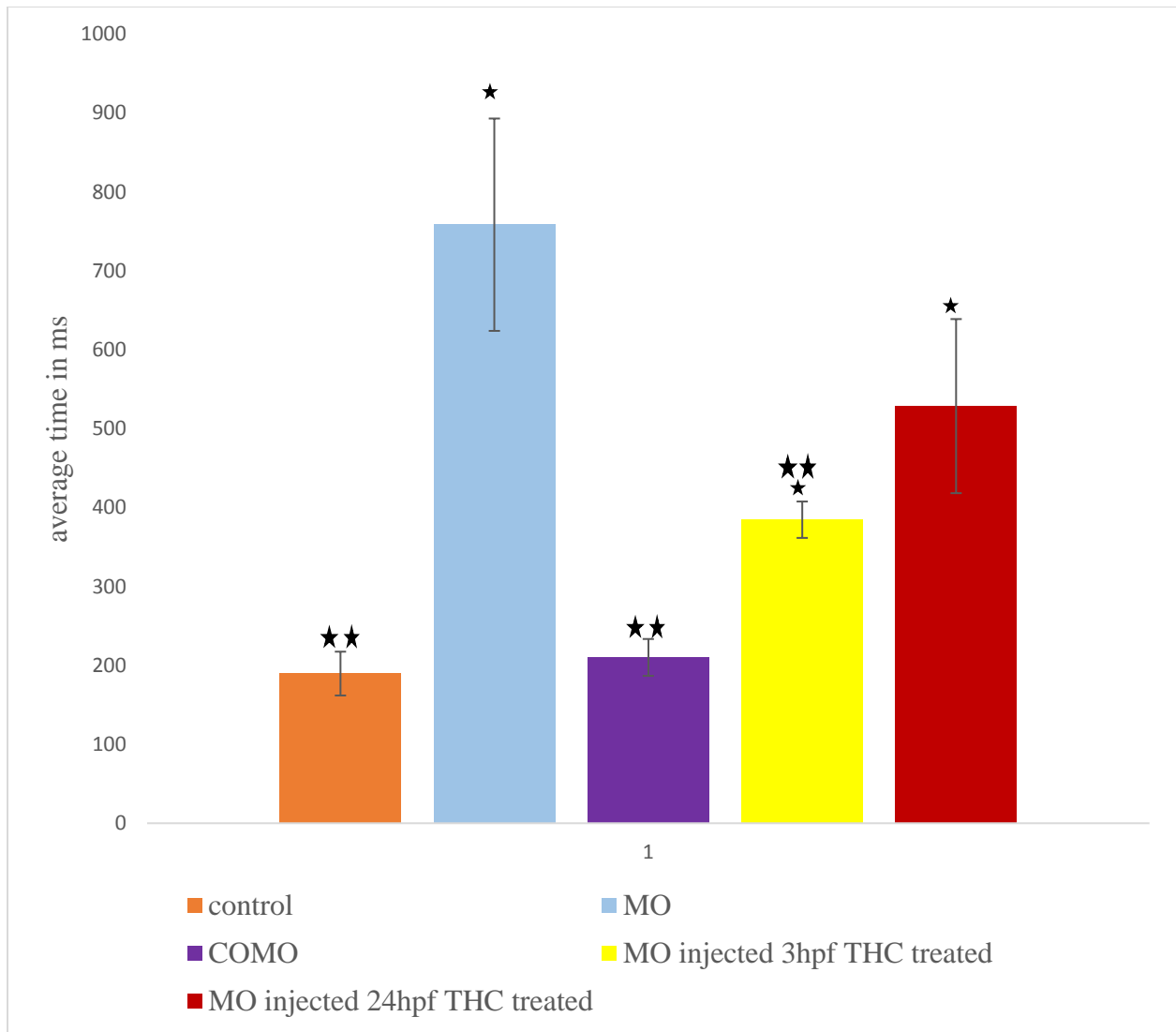


Figure 7: Escape response of Hyperekplexic embryos. This graph shows the average times taken to complete the C-bend for embryos injected with morpholino (MO) to model hyperekplexia. 1 star show averages that are significantly different from control average response time and 2 stars show averaged that are significantly different from MO($p>0.05$). The legend can be found at the bottom of the graph. Orange is control. Blue represents morpholino (MO). Purple is control morpholino (COMO). Yellow represents morpholino injected embryos treated at 3hpf, and the red bar is morpholino injected embryos treated at 24hpf.

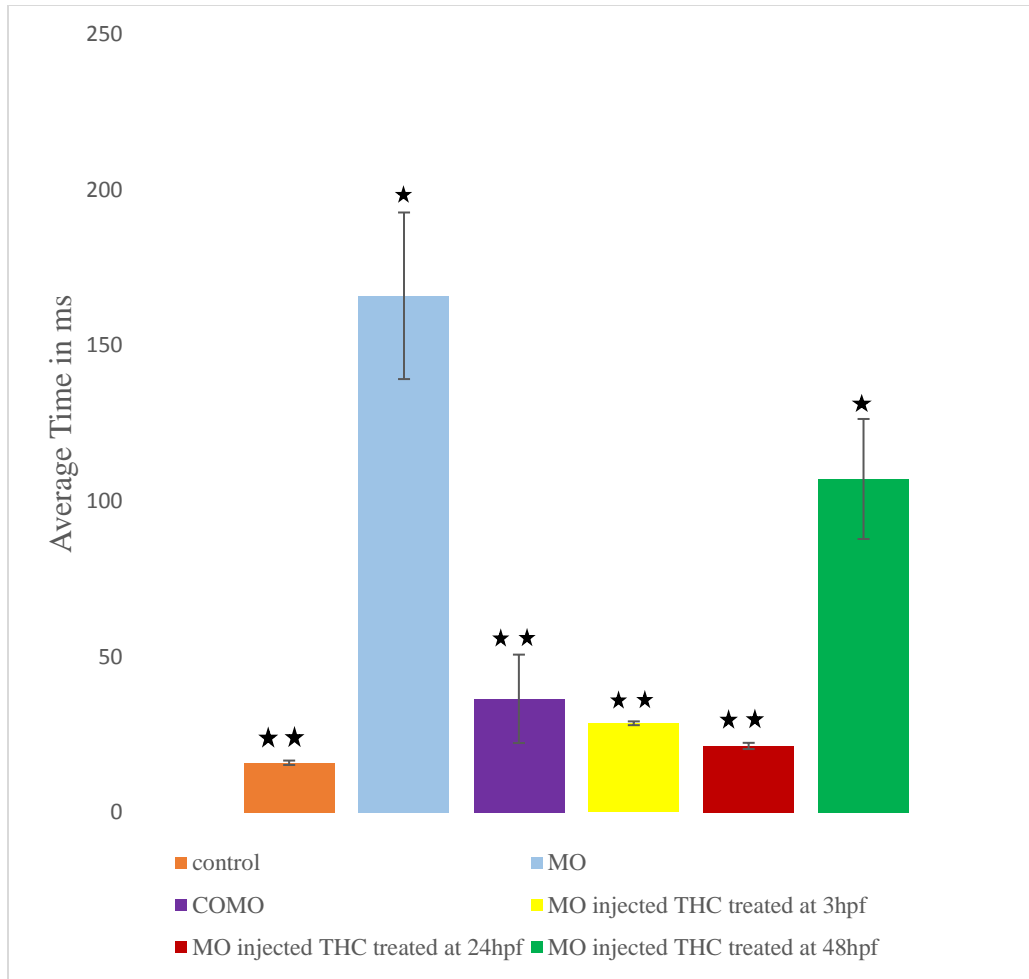


Figure 8: Escape response at 48hpf of Hyperekplexic embryos after THC treatment. This graph represents the average times taken to complete the C-bend at 48hpf after THC treatment in morpholino (MO) injected embryos. 1 stars represent averages that are significantly different from control and 2 stars represent averages significantly different from MO ($p > 0.05$). The color key is at the bottom of the graph and colors follow the previous graph with the addition of embryos treated at 48hpf in Green. The abbreviations are as follows: MO is morpholino and COMO is control morpholino, hpf is hours post fertilization, and THC is Δ^9 -tetrahydrocannabinol.

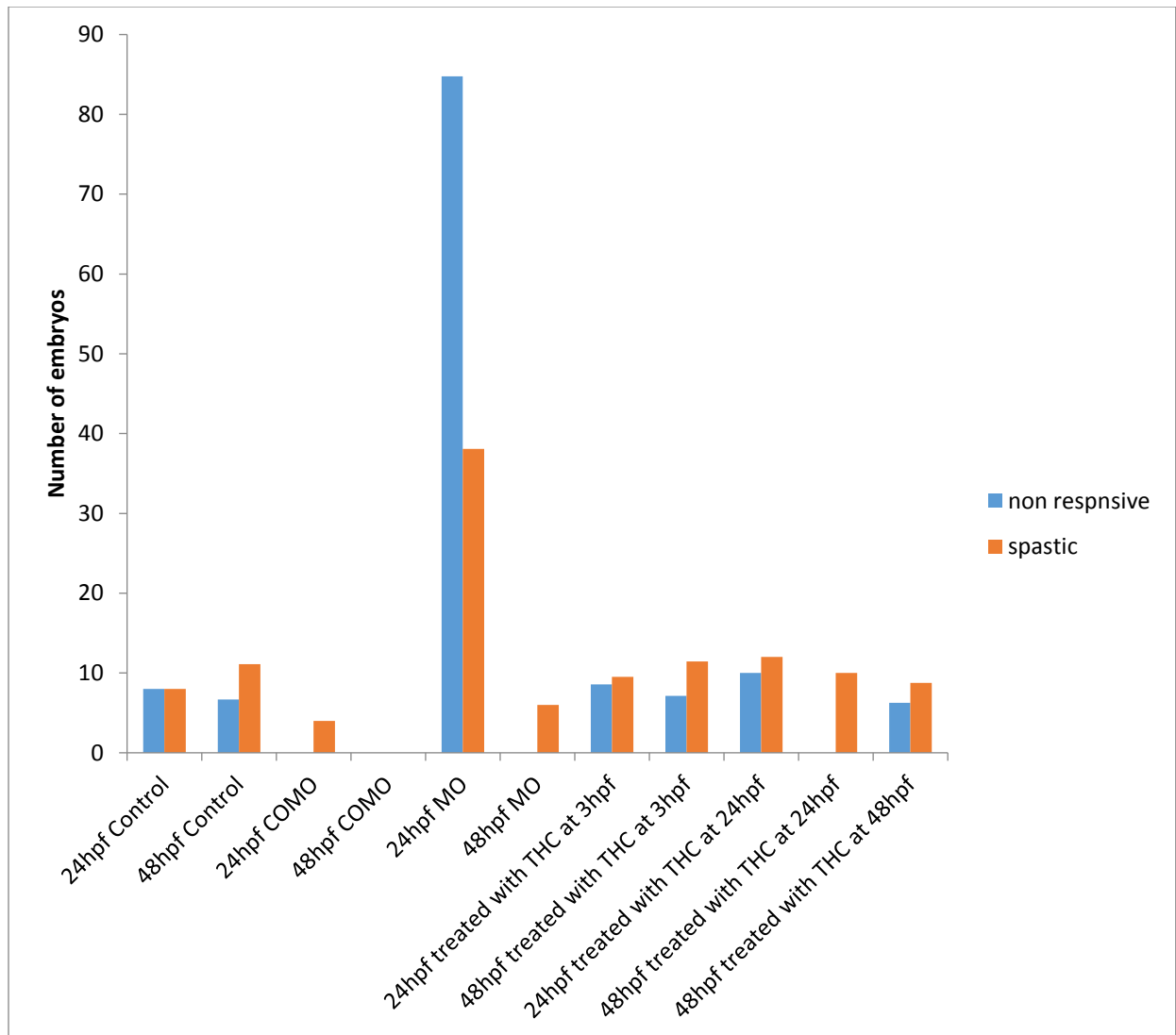


Figure 9: Spastic and non-responsive percentages of injected groups. The above graph shows the percentages of total populations, including all experimental replications that, that were not responsive to stimulus (blue) or spastic (orange). Aside from controls, all of these embryos were injected. COMO represents control morpholino and MO is morpholino injected with no treatment. Each of the THC treatment groups was injected with morpholino at 1hpf and treated at their respective treatment times. The y-axis gives the percent of the population and the x-axis names each treatment group.

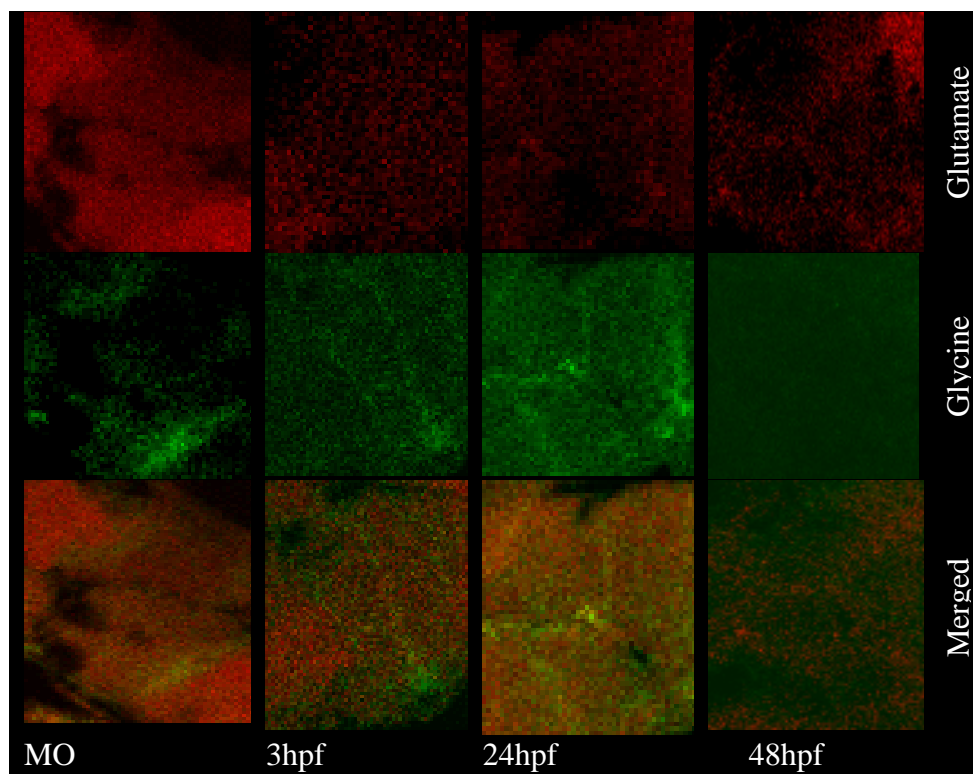


Figure 10: Glycine/glutamate expression in hyperekplexic, THC treated spinal cord sections. These are confocal images zoomed into the spinal cord from hyperekplexic embryos from each THC treatment time. MO stands for morpholino injected. Glutamate ion channels appear in red and glycine receptors appear in green.

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