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Effects of Acute Nicotine on Larval Zebrafish Exploratory Behavior in a Complex Environment

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by

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I had settled on becoming a psychology major by the end of my 1st year at Bard after having taken Neuroscience with Professor Frank Scalzo. The following year, he nurtured an idea that would later become my senior project when he insisted that the six-chamber complex environment would "make a big splash" in the pond of zebrafish research. Without his patience, encouragement, and critical guidance these last four years, the present project would have remained the idle fantasy of a naïve psychology major.

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Abstract

The larval zebrafish is emerging as a useful model to assess neurobehavioral toxicity. A variety of behavioral assays have been developed to characterize normal behavior and the acute and chronic effects of a variety of compounds. To date, such behavioral assays have been limited to relatively simple behavioral measures (e.g., swimming activity in a single well). The present experiment describes methodology to assess exploratory behavior in 5 days-post-fertilization (5 dpf) larval zebrafish using a six-chamber, complex well-plate. In addition, the effect of acute nicotine exposure on exploratory activity in this complex environment was examined. Five dpf TU strain larvae were studied. Larvae were treated with either 0, 16.25μM or 48.25μM nicotine and were observed for 15 minutes. General Locomotor Activity, Zone Preference, Thigmotaxis (outer zone preference), Thigmotaxis Path Type, Chamber Transitions, and Latency to enter the Center Zone were measured using a Noldus tracking system. These results demonstrate (1) the utility of this novel testing methodology, (2) that a low and high dose of nicotine increased exploratory behavior in a complex environment and (3) dose-dependent behavioral changes due to nicotine treatment, suggesting altered control of a specific type of exploratory behavior as compared to a general increase in behavioral activation. These results while inconsistent with the current literature on anxiety-driven behavior in other animal models may be explained by the intrinsic properties of larval zebrafish behavioral phenotypes and molecular and cellular differences in nicotinic receptor function.

Introduction

Nicotine is a fascinating substance of study; it has been studied for its addictive potential, but also for its potential neuroprotective and cognitive benefits—for patients with schizophrenia and Parkinson's disease. Many of the mechanisms by which nicotine affects cognitive abilities, physiology, genetic expression, and behavior are unknown. The use of electronic cigarettes (E-Cigarettes), which allow for the inhalation of vaporized solution of nicotine and other chemicals, has increased by nearly 800% from 2011 to 2014 (Arrazola et al. 2015). This has set a precedent for gaining further insight into how nicotine affects cognition, physiology, and behavior. Nicotine works through the activation and desensitization of nicotinic acetylcholine receptors. These receptors are spread throughout the entire central nervous system affecting pathways for GABA (Petzold et al. 2009), dopamine (Klee et al. 2011), norepinephrine (Klee et al. 2011), serotonin (Papke et al. 2012), and glutamate (Papke et al. 2012). The ubiquitous affects of nicotine on neurotransmission throughout the brain make it a difficult substance of study. Common reasons for smoking among those who range from infrequent to frequent smokers include the ability to reduce stress, increase cognitive function, habitual smoking and pleasure (Hendricks & Brandon 2008). Because of the vast effect of nicotine on neurotransmission within the CNS it is plausible to think that these benefits of smoking (i.e. anxiolytic & cognitive benefit) can be molecularly and behavioral differentiated through the use of animal models. Research using mammalian model systems (e.g. rats and mice) has demonstrated through genetic and behavioral assays potential correlates of nicotine-induced benefit and nicotine-induced detriment to the activation and desensitization of nicotinic receptors (Picciotto, Addy, Mineur, Brunzell 2008). For example, activation of the cholinergic-dopaminergic reward pathway through binding

of nicotine to nicotinic acetylcholine receptors (nAChR) located on dopaminergic neurons has been linked to the addictive properties of nicotine (McGranahan et al. 2011).

The neural pathways that nicotine effects in humans and rodents been are conserved in zebrafish (Champagne et al. 2010; Richendrfer et al. 2012; Stewart et al. 2015). Therefore zebrafish provide a high-throughput means of examining the neural pathways of nicotine exposure as an extension of previous research conducted in mammalian models. The zebrafish model affords the capability for 3D tracking (Cachat et al. 2011) of behavior. Furthermore, they can exhibit a large variety of behavioral phenotypes compared to rodent models. Nicotine has been a recent drug of interest in both mammalian and invertebrate models of anxiety as it has been shown to increase exploratory behavior at low doses, have an anxiolytic, or anxiety-relieving, effect at higher doses, and an anxiogenic, or anxiety generating, effect when chronically administered (Stewart et al. 2015; Lee 1985). This differential response to nicotine in animals has also been demonstrated in humans (Haller et al. 2013; Connors et al. 2013; Belzung & Philippot 2007).

Only recently has the adult zebrafish been used as a model for testing both anxiety and the effects of nicotine using anxiety-testing paradigms (Levin et al. 2005; Levin et al. 2007, Bencan & Levin 2008). Many of the behavioral assays used to test anxiety in mice such as the open field test and the light-dark test—which will be explained later on—have been translated to fit the zebrafish model. The validity of these designs in zebrafish have been confirmed through the testing of pharmacological compounds previously tested in mammalian models and commonly prescribed in humans (Stewart et a 2012; Richendrfer et al. 2012; Champagne et al. 2010; Blaser, Chadwick & McGinnis 2010). However, the use of zebrafish in the testing of anxiety and anxiolytic and anxiogenic drugs has been limited to adult zebrafish. Only in recent

years have larval zebrafish been used to test anxiety-related behaviors and nicotine (Petzold et al. 2009; Schnorr et al. 2012). These studies have shown that the larval zebrafish may be a viable candidate in the screening of pharmacological compounds that affect anxiety, and that many of the behavioral traits exhibited in mammalian and adult zebrafish models are conserved in larval zebrafish (Schnorr et al. 2012). While there has been much researched published on anxiety and animal models, much still has yet to be discovered about phenotypes of anxiety-related behavior. It has been contested whether the current behavioral assays have the validity to be able to translate into human pathological anxiety disorders, and whether the behavioral endpoints commonly investigated are demonstrative of anxiety-related behaviors, or are rather misinterpretations of behaviors motivated by stress, fear, or intrinsic behavioral properties of the animal model. As it has become commonplace to use these behavioral markers of anxiety, it is possible that what the current literature on anxiety has been describing is not in fact anxiety-related behavior but rather activation of general exploratory behaviors. (Richendrfer et al. 2012; Haller et al. 2013; Champagne et al. 2010; Blaser et al. 2010; Belzung & Philippot 2007; Stewart et al. 2012). This is especially a concern in relatively simple behavioral assays in which an animal is studied within a single arena. When investigating a drug such as nicotine that impacts multiple neuronal pathways, using a single arena to investigate the behavioral alterations due to drug treatment may lead to an increase likelihood to misinterpret different types of behavior.

2.1 Nicotine and Nicotinic Acetylcholine Receptors

The main mechanism of action of nicotine within the CNS is as a molecule that binds to nAChRs (see fig 2). As was stated previously, these receptors can be found throughout the brain, but the receptors to which nicotine has the highest binding affinity to are concentrated in the dopaminergic reward pathway. nAChRs can be categorized as ligand-gated ion channels, which

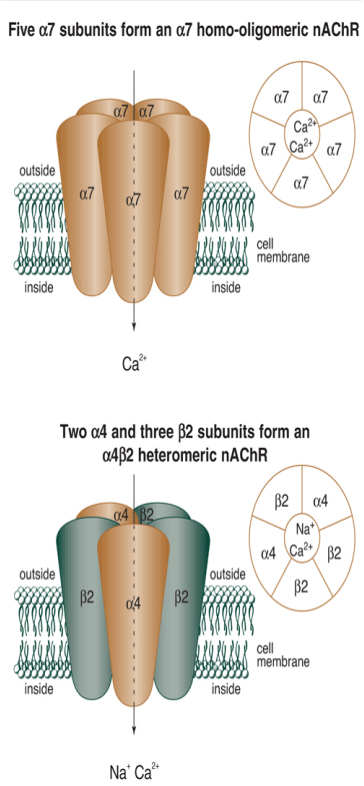


Figure 1 Illustrations of both homomeric (top) and heteromeric (bottom) nicotinic acetylcholine receptors. Reprinted from (Davis & de Fiebre 2006)

Subunits	Type	Example nAChRs Compositions
α_9	Neuronal	
α_{10}	Neuronal	
α_7	**Neuronal**	**α_7**
α_8	Neuronal	
α_2	Neuronal	
α_3	**Neuronal**	**$\alpha_3\beta_4$**
α_4	**Neuronal**	**$\alpha_4\beta_2$**
α_5	**Neuronal**	**$\alpha_4\beta_2$ α_5**
α_6	**Neuronal**	**$\alpha_4\beta_2$**
β_2	**Neuronal**	**$\alpha_6\beta_2$, $\alpha_6\beta_2$**
β_4	**Neuronal**	**$\alpha_3\beta_4$**
β_3	Neuronal	
β_5	Neuronal	
α_1	Muscle	$\alpha_1\beta_1\delta\gamma$, $\alpha_1\beta_1\delta\varepsilon$
β_1	Muscle	$\alpha_1\beta_1\delta\gamma$, $\alpha_1\beta_1\delta\varepsilon$
δ	Muscle	$\alpha_1\beta_1\delta\gamma$, $\alpha_1\beta_1\delta\varepsilon$
γ	Muscle	$\alpha_1\beta_1\delta\gamma$
ε	Muscle	$\alpha_1\beta_1\delta\varepsilon$

Table 1 Table of different subunits of nAChRs, type of subunit, and example nAChR compositions. Bolded lines indicate known subunits to which nicotine typically binds.

means that these receptors act as pores within the neuronal membrane, such that once an agonist, such as nicotine, binds to the receptor surface, the pore opens allowing the influx of ions that can activate a variety of mechanisms thereafter having different effects. Neuronal nAChRs are pentamers of both α (α 2- α 10) and β (β 2- β 4) subunits (see table 1), different combinations of these subunits, determine the type of ligand that most efficaciously binds to the receptor, as well as the function of the receptor itself as an ion channel. Nicotine acts as a partial receptor agonists (only partially activates the receptor) at all heteromeric nAChRs, but is a full agonist (fully activates the receptor) for the α7 nAChR. Furthermore, among the heteromeric nAChRs, nicotine binds most tightly to $\alpha_4\beta_2$ receptor, found most commonly on neurons within the dopaminergic reward pathway, and least tightly to muscle-type receptors (Papke et al. 2012). While the α7 nicotinic receptor activation has been implicated in cognitive benefits and neuroprotection in both Parkinson's and Alzheimer's disease (Klee et al. 2011; Dome et al. 2010), activation of the $\alpha_4\beta_2$ nAChR has been implicated in the reinforcement of nicotine addiction and regulation of anxiety (Anderson & Brunzell 2012). The

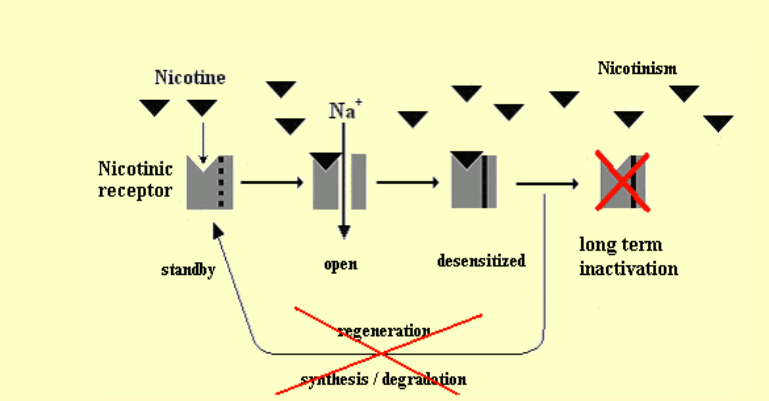


Figure 2 Illustration of the different potential states of nAChR due to nicotine. Reprinted from ("The Metabolism of Nicotine")

effects of nicotine are further complicated by its role in the desensitization of nAChRs (see figure 2). Desensitization causes the receptor to become "inactive." However, desensitization occurs in only some nAChRs, and receptors can become desensitized after acute exposure to nicotine (Picciotto et al. 2008). The activation and desensitization of nicotinic receptors have implicated in the behavioral alterations due to nicotine and the extent of activation and desensitization of nAChRs depend on the regimen of nicotine exposure (Picciotto 2003). The alteration of nAChRs by nicotine in the mesocorticolimbic system, specifically the cholinergic-dopaminergic pathway, has been implicated to be responsible for a number of behavioral changes due to nicotine exposure.

2.2 Cholinergic-Dopaminergic Reward Pathway

The Cholinergic-Dopaminergic Reward pathway (see fig 3) is a neuronal pathway that is conserved in both humans and other mammalian models such as rats and mice (Jerlhag & Engel 2011). While a homologous reward pathway of humans and mammals has yet to be identified in zebrafish, some evidence suggests that nicotine similarly plays a role in the regulation of the reward pathway in zebrafish as it does in mammals (Petzold et al. 2009). In mice, $\alpha_4\beta_2$ nAChRs have been observed to be active on dopaminergic neurons on substrates within the reward pathway, the ventral tegmental area (VTA) and nucleus accumbens (NAc), such that expression of $\alpha_4\beta_2$ nAChRs on dopaminergic neurons is necessary for the observation of anxiolytic responses observed after nicotine dosing (McGranahan et al. 2011). Specifically, desensitization

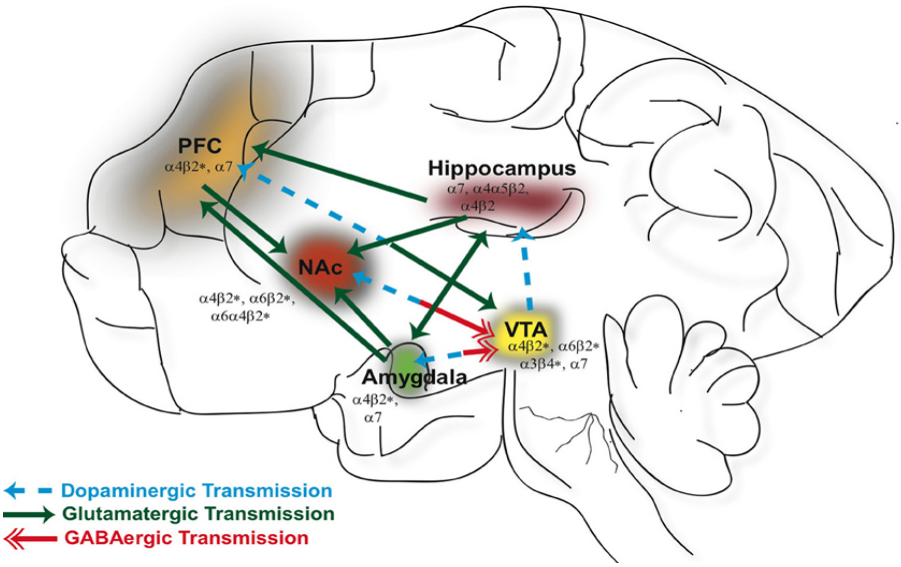


Figure 3 Illustration of the presence of different nAChRs in the human brain region. The ventral tegmental area (VTA), the NAc (Nucleus Accumbens), and the PFC (prefrontal cortex) are major substrates within the reward pathway. Hippocampus and Amygdala are involved in memory and regulation of mood, respectively. Reprinted from (Feduccia, Chatterjee, & Bartlett 2012)

the β_2 subunit of nAChRs of has been implicated in generating anxiolytic behavior in mice (Anderson & Brunzell 2012). Furthermore, although the reward pathway is commonly referred to as the dopaminergic-reward pathway, GABAergic and glutamatergic transmissions play a central role in the regulation of dopaminergic transmissions and as nAChRs are present on GABAergic, glutamatergic, and dopaminergic neurons (Pistillo, Clementi, Zoli, & Gotti 2015), nicotine exposure can have a profound effect on specific locomotor behaviors regulated by the dopaminergic-cholinergic pathway.

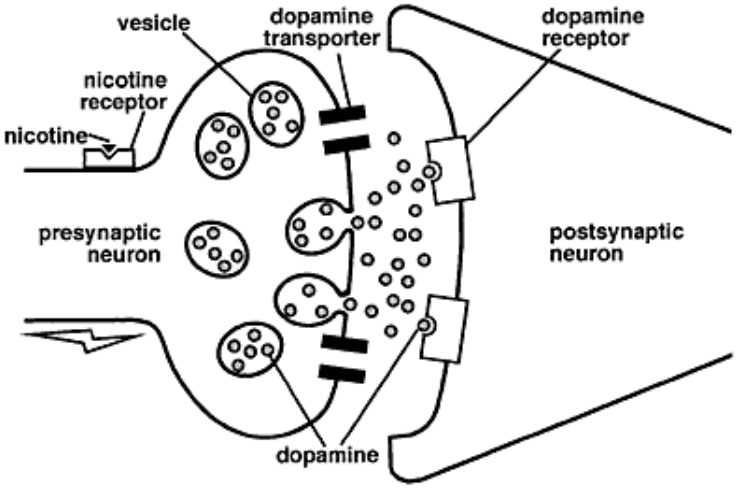


Figure 4 Illustration of the effects of nicotine binding to an nAChR on a dopaminergic neuron. Reprinted from ("Drugs Change the Way Neurons Communicate")

2.3 The Effect of Nicotine on Rodent Behavior

Despite years of research, the behavioral effects of nicotine on rodents are still not well understood. Rats dosed with nicotine behave differently across different strains (Shoaib et al. 1997) gender (Torres et

al. 2009), and ages (Levin et al. 2007). Furthermore, reduction in fear and anxiety and increase in exploratory behavior in a novel environment has been shown to be dose-dependent, where higher doses cause ataxia and decrease exploration in a novel environment, lower doses increase locomotor activity (Clarke & Kumar 1983). This effect can be most accurately described as an inverted-U dose response curve, where locomotor activity rises as dosage concentration increases up until a certain point where the dosage causes a decrease in locomotor activity. With regards to nicotine's anxiolytic effect in rats, a primary behavioral measure of anxiety has been thigmotaxis—the tendency for an animal to remain along the border of an enclosed, open field arena (Cohen et al. 2009). Increased dopaminergic and noradrenergic and decreased serotonergic neuronal activity may be responsible for the observed dose-dependent behavioral response to acute exposure to nicotine in rats (Lee 1985). Furthermore, it has been suggested that dopaminergic transmissions (see fig 4) can influence the exhibition of anxiety-like behavior such that activation of D1 and D2 receptors resulted in an anxiogenic response in rats, but the stimulation of only D1 or D2 receptors did not lead to an anxiogenic response (Simon, Dupuis, & Costenin 1994). Moreover, acute nicotine administration in rats via intra-central amygdala (CeA) injection induced an anxiogenic response, but blocking D1 and D2 receptors in the NAc and VTA by administering a D1 and D2 receptor antagonist after nicotine administration antagonist reduced the anxiogenic response (Zarrindast 2012, 2013). Although other measures have been used to determine anxiety levels, thigmotactic behavior appears to be the most common marker of anxiety across animal models, and is attenuated by dosing with anxiolytic compounds, such as nicotine, or amplified by anxiogenic compounds. Furthermore, nicotinic receptor antagonists such as mecamylamine have been shown to reverse the changes in nicotine-induced locomotor behavior in rats (Clarke & Kumar 1983). The behavioral repertoire of both rats and mice have

been translated and adopted to suite zebrafish (Champagne et al. 2010), a relatively high-throughput animal model recently developed to study the effect of nicotine on behavior.

2.4 The Effect of Nicotine on Adult Zebrafish Behavior

A variety of research has been conducted on anxiolytic compounds such as nicotine in adult zebrafish, using thigmotaxis as the primary measure of anxiety-driven behavior (Blaser et al. 2010; Levin et al. 2007). These studies have found that nicotine, indeed, has an anxiolytic effect, in that overall thigmotactic behavior is reduced after both acute and chronic exposure to nicotine. Similar to studies on their mammalian counterparts, adult zebrafish demonstrate an attenuation of the anxiolytic effects of nicotine when also treated with nAChR antagonist, mecamylamine (Levin et al. 2005). Akin to thigmotaxis, tank dwelling can serve as a demonstration of anxiety-driven behavior in adult zebrafish such that anxiety causes zebrafish to dwell in the bottom two-thirds of the test tank, and following treatment with nicotine, zebrafish spend more time in the top 1/3 of the tank (see fig 7). Furthermore, anxiety-driven behavior has been linked to involve both α7 and $\alpha_4\beta_2$ nAChR such that treatment of adult zebrafish with nicotine in addition to either α7 or $\alpha_4\beta_2$ receptor antagonists will attenuate anxiolytic responses (tank dwelling in the upper third portion and an increase in swimming speed) (Bencan & Levin 2008). However, further research is necessary to determine whether the activation of these receptors result in the observed anxiolytic responses. Previous research has speculated that α7 receptor activation is linked to cognitive improvement, whereas $\alpha_4\beta_2$ receptor activation may be related to the reward pathway (Albuquerque, Pereira, Alkondon, Rogers 2008), it is possible that the behavioral measurements employed in Bencan and Levin's study (2008) were not specific enough to distinguish between anxiety-driven behavior and changes in cognitive faculties. Furthermore, while Levin's studies focus on acute exposure to nicotine, Stewart et al. (2015)

found that chronic nicotine exposure generates an anxiogenic behavioral response. Stewart's study utilized the same novel tank dive test as in Levin's studies and found that after the fourth day of exposure to low-dose nicotine, adult zebrafish began to exhibit typical anxiety-driven behavior (i.e. tank dwelling, freezing bouts, and erratic movements). Similar to adult zebrafish, larval zebrafish provide a potential model for the investigation of behavioral phenotypes linked to nicotine exposure.

2.5 The Effect of Nicotine on Larval Zebrafish Behavior

Research using larval zebrafish provides the benefit of high-throughput capabilities, which makes it an appealing animal model for drug screening tests and other behavioral assays. Previous research that have studied the anxiety-driven behaviors in larval zebrafish have used light-dark paradigm to promote anxiety (Schnorr et al. 2012) (see fig 6). This study provided a framework for a thigmotaxis assay for the screening of drugs, and anecdotally provided evidence that anxiolytic responses were observed in larval zebrafish given anxiolytic compounds. Through the small area that is needed to work with larval zebrafish, it is easier to design mazes and open fields that can more accurately test whether a behavior is anxiety-driven or being influenced by a cognitive impairment/improvement or alterations in locomotor control. Furthermore, larval zebrafish develop both nicotinic and dopaminergic neurons and respective receptors within the first few hours post-fertilization. Complete set of dopaminergic neurons can be detected in larval zebrafish by 8 days post-fertilization (Klee et al. 2011). Therefore, larval zebrafish should work as a model organism for the study of the effects of nicotine on behavior and dopaminergic pathways. Furthermore, larval zebrafish seem to demonstrate a similar dose-response curve as do rats and adult zebrafish such that it follows an inverted-U shape, where doses higher than 100μM reduce activity and there is a peak in activity around 50μM (Petzold et al. 2009). Potential

confounds in using larval zebrafish is the difference in strain, gender, and age that were apparent in the mammalian models of the effects of nicotine on behavior. There has been research that suggests that locomotor activity is age dependent (Colwill & Creton 2011) within the larval zebrafish age group such that larval zebrafish naturally demonstrate higher thigmotactic behavior than their adult counterparts, and that larval zebrafish become significantly more active once they reach 6 or 7 days post-fertilization (dpf) relative to 4 or 5 dpf zebrafish. However, larvae begin to exhibit more complex behavior at 5 dpf (Colwill & Creton 2011). Therefore, it is possible that these effects that were observed in the investigating of the effect of nicotine on rats will be conserved in zebrafish.

2.6 Anxiety

Current animal based models of anxiety have attributed behaviors such as thigmotaxis as representative of anxiety. For the most part, these studies have not differentiated anxiety from a fear or stress response. While fear is the response to a present or imminent threat or danger, anxiety is a fearful response to an upcoming or expected threat or danger. Although, homologous neural structures that are responsible for the fear and anxiety response in humans have been found in lower-order species such as rodents and fish (Belzung & Philippot 2007), current behavioral assays rely on exploratory-driven anxiety-related paradigms in which an animal is placed into a testing arena that has two general components a safe and a risky component. An anxious reaction is thereby operationalized as the tendency to remain in the safe component as opposed to the risky component. Therefore, an animal that has less anxiety would be more likely to explore the risky component of the testing apparatus. Validation of these models has been based on the efficacy of common human treatments of anxiety such as selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines (Cachat et al. 2010), and tests on genetic

knockout strains of animals (McGrahanan et al. 2011). However, studies on animal anxiety using traditional behavioral assays such as the open-field test or the elevated-plus maze, limit their behavioral measures to traits such as thigmotaxis, freezing, increased latency to transition to the risky component. While these behaviors have been noted in human studies of anxiety (Kallai et al. 2007), in animal studies presence or absence of these behaviors may also be misconstrued merely as a fear response or as an increase in exploratory behavior unrelated to the anxiety state of the animal. One of the ways in correcting for potential misinterpretation of animal behavior models of anxiety is through the development of new behavioral models of anxiety.

2.7 Neurotransmitter Pathways Involved in Anxiety

A variety of neurotransmitters have been implicated to regulate anxiety including, but not limited to, dopamine, serotonin, GABA, and glutamate. The main treatments for anxiety in humans are SSRIs, which inhibit the reuptake of serotonin within the brain, thus resulting in the further stimulation of serotonergic receptors. The typical pharmacological compounds used in animal models of anxiety are SSRIs, benzodiazepines, MAOIs, and nicotine. The behavioral changes due to the administration of these drugs are examined in the following:

2.7.1 Anxiety: Humans

Anxiety in humans is correlated with dysregulation in the amygdala, subcortical hippocampus, habenula, prefrontal and cingulate cortex (Belzung & Philippot 2007; Reslle r & Mayberg 2007). These structures not only control the cognitive responses to anxiety, but also somatic and behavioral responses (Lang, Davis, & Ohman 2000). The primary neurotransmitters implicated in the activation of these responses are GABA, glutamate, serotonin and dopamine (Bishop 2007). Drugs prescribed to treat anxiety in humans affect these neurotransmitter pathways benzodiazepines (GABA), MAOIs (serotonin, dopamine), SSRIs (serotonin). Many of

these drugs have been used in the study of anxiety using animal models. However, it is unknown whether animals can experience anxiety in the same manner that humans can (Belzung & Philippot 2007). Nevertheless, animal models have been used to attempt to understand the behavior and genetic responses to anxiety.

2.7.2 Anxiety: Rodents

Rodents have been the most popular model to study anxiety-like behaviors and clinical treatments for anxiety. The most typical behaviors measured in rodents as markers of anxiety-related behavior include, but are not limited to thigmotaxis, erratic/avoidant behavior, freezing, decreased tendency to interact with conspecifics. Similar neurotransmitters and neural substrates have been implicated in rodent anxiety as human anxiety (Lee 1985; Zarrindast 2010, 2011, 2012, 2013). However, it has been debated whether current rodent models of anxiety are able to correctly encapsulate pathological anxiety (Belzung & Philippot 2007). While part of this skepticism is based on question of whether animals can experience anxiety in the human sense, part is based on the simplicity of the current behavioral assays used in testing anxiety in generating and differentiating anxiety-like behaviors from exploratory behavior. Similar issues arise from other animal models such as the zebrafish, but the zebrafish have the potential to exhibit a more complex variety of behaviors that may provide deeper insight into anxiety-related behaviors in animal models.

2.7.3 Anxiety: Adult Zebrafish

As of recent, the adult zebrafish has been used as a model of anxiety. Although the neural substrates that control anxiety-related behaviors in zebrafish are different than that in mammals, homologous structures have been implicated (see fig 5). The habenula, which is thought to control motor and cognitive aspects of fear-like behavior (Stewart et al. 2013), is regulated by

dopaminergic and serotonergic neurons (Amo et al. 2010). Pharmacological studies have demonstrated that dopaminergic, serotonergic, and GABAergic transmission has been implicated in the regulation of anxiety-like behavior (Herculano & Maximino 2014; Champagne et al. 2010; Maximino et al. 2010). The most commonly measured anxiety-related behaviors in adult zebrafish are top and bottom preference, thigmotaxis, light and dark preference, erratic movement, and freezing. Furthermore, the ability of the adult zebrafish model for 3D tracking of behavior allows for a more complex classification of anxiety-behaviors and therefore yields the potential to better differentiate anxiety-related behaviors from exploratory or fear motivated behaviors. Similar to the adult zebrafish, the larval zebrafish is a viable candidate for future studies of anxiety-related behaviors, as zebrafish larvae exhibit a similar behavioral repertoire to rodent and adult zebrafish in measuring anxiety-related behavior.

2.7.4 Anxiety: Larval Zebrafish

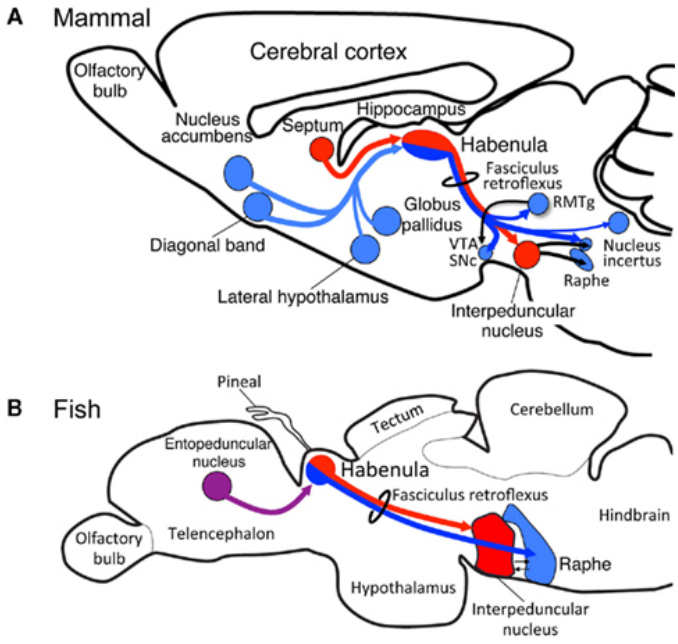


Figure 5 Schematic of the sagittal brain sections of A) rat and B) zebrafish habenular pathways. Red and blue represent medial and lateral circuits, respectively. Reprinted from (Aizawa, Amo, & Okamoto 2011)

While there have been few studies on zebrafish larvae and anxiety-related behavior. Current research has shown that zebrafish larvae can exhibit anxiety-related behavioral phenotypes such as thigmotaxis (Schnorr et al. 2012), scototaxis (Steenbergen et al. 2010), erratic and freezing behavior (Kalueff et al. 2013). Although the larval zebrafish have do not have a fully established CNS until approximately 8 dpf (Herculano &

Maximino 2014), larvae are still capable of exhibiting anxiety-like behavior from 5 dpf (Richendfrer et al. 2012). The testing of anxiolytic compounds has confirmed similar behavioral effects on larvae as in rats and adult zebrafish (Richendrfer et al. 2012). The results of these studies have suggested that the neurotransmitter pathways regulating anxiety-like behavior in humans, rodents, and adult zebrafish seem to be conserved in larval zebrafish, thereby validating their use as an animal model of anxiety. While rodents, adult zebrafish, and larval zebrafish have the potential for expressing anxiety-related behaviors, interpretation of these behaviors are limited by the behavioral assays used to study anxiety.

2.8 Different Behavioral Models of Anxiety

The most common behavioral assays for testing anxiety are the open field test and the elevated-plus maze test (Haller, Aliczki, & Pelczer 2013). The open field test measures anxiety mainly through thigmotaxis, the tendency to move along the outer areas of the open field. The elevated-plus maze measures anxiety through the tendency of an animal to stay within the closed arms of the plus maze and their avoidance of the open arms. These behavioral assays have been mainly implemented in rodent models of anxiety. The following two behavioral assays: the light-dark test and the novel tank test are recent behavioral assays that have been established in adult zebrafish as valid models to measure anxiety-related behaviors.

2.8.1 Light-Dark Test

In both adult and larval zebrafish the light-dark test has been shown as a novel way to measure anxiety. Adult zebrafish demonstrate an innate avoidance of the light chamber and a preference for the dark chamber (Stewart et al. 2010). Larval zebrafish demonstrate an innate avoidance of the dark chamber and a preference for the light chamber (Steenbergen, Richardson & Champagne 2011). The testing of anxiolytic compounds in juvenile zebrafish have been

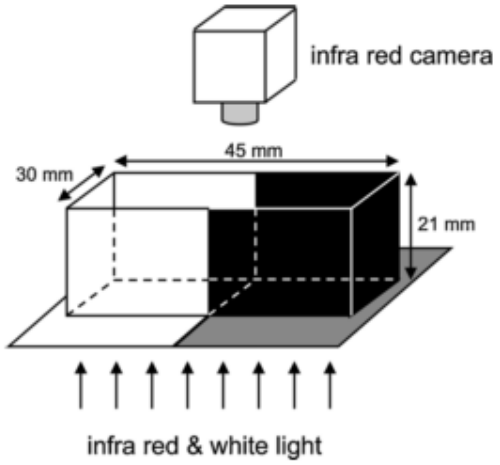


Figure 6 Schematic of light-dark test apparatus . Reprinted from (Steenbergen, Richardson, & Champagne 2010)

shown to alter this innate preference (Steenbergen, Richardson, & Champagne 2011) such that anxiolytic compounds increase the amount of time spent in the innately avoided chambers (light for adult zebrafish; dark for larval zebrafish). This behavioral assay provides a unique measure of anxiety-related behaviors as it draws on innate avoidance that may be more likely to draw on anxiety processes than fear processes. It has further been suggested that the light-dark test through pharmacological testing that different neural processes are modeled in the light-dark test as opposed to the open field tests (Maximino et al. 2010). Furthermore, the novel tank test, an adaptation of the open field test for adult zebrafish has been utilized to demonstrate that the adult zebrafish is a capable organism for modeling anxiety-related behaviors.

2.8.2 Novel Tank Test

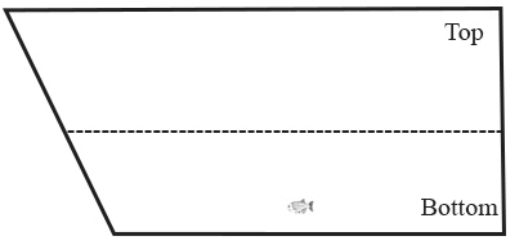


Figure 7 Schematic of Novel-Tank Test apparatus, dotted line differentiates top from bottom portion of the tank

The novel tank test works on a similar paradigm as the open field test, but instead of inner and outer zones, the tank is split into top and bottom zones. Adult zebrafish have a preference to swim near the bottom of the tank and avoid the top of the tank. Preference to swim within the bottom section of the tank has been suggested to

be an anxiety response in adult zebrafish (Levin, Bencan, & Cerutti 2006; Stewart et al. 2010). The testing of anxiolytic compounds on adult zebrafish in the novel tank test has been shown to cause similar effects as rodents in the open field test. Therefore, this research suggests that adult zebrafish may be used as a test of anxiety-related behaviors. However, the novel tank test faces similar confounds in interpreting behavior as in the open field test for rodents. An increase in exploratory behavior due to drug administration could be mistaken for a reduction in anxiety-like behavior. Only recently has 3D tracking of adult zebrafish within the novel tank test have been used to attempt to quantify more complex behaviors that may be able to differentiate these behaviors and further characterize anxiety-related behaviors (Cachat et al. 2011). The utility of the zebrafish to enable 3D tracking of behavior opens the possibility for the analysis of more complex behaviors than capable with rodent models that are restricted to 2D tracking.

2.9 Utility of Using an Open Field Test in Measuring Anxiety

The open field test utilizes the innate avoidance of novel open spaces to measure anxiety. Thigmotaxis, or the preference for the borders of an open field, is the main behavioral endpoint measured to determine anxiety levels within animals (see fig 8). Potential problems in measuring thigmotaxis in rats (Bouwknecht & Paylor 2008) have been translated into larval zebrafish models as exemplified in the study conducted to test thigmotaxis in larval zebrafish (Schnorr et al. 2012), such that thigmotactic behavior should not be calculated including low-moving or unmoving animals. To correct for time spent not moving, thigmotaxis should be measured as both a ratio of time spent in the outer zone and total duration of the trial and a ratio of distanced moved in the outer zone and total distanced moved during the trial. These two variables can give a more accurate description of thigmotactic behavior separate from individual preferences or immobility in subjects. The testing apparatus used in a study conducted by Champagne and

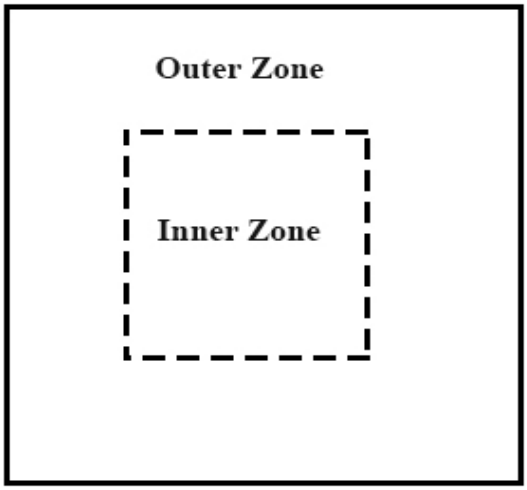


Figure 8 Schematic of an open field test for rodents, solid line represents physical borders of the testing arena while dotted lines represent virtual "inner zone"

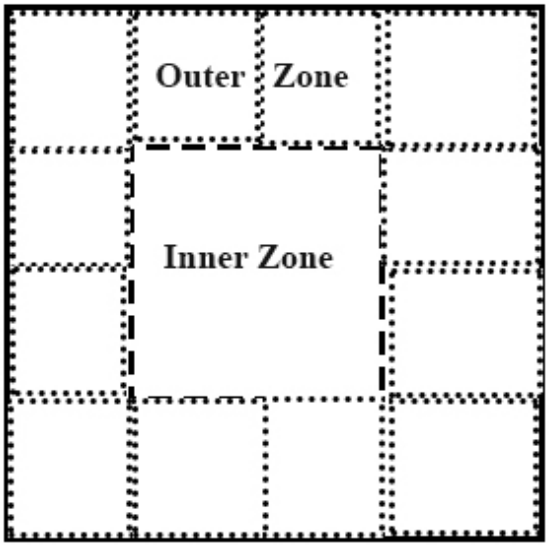


Figure 1 Schematic of open field test for adult zebrafish, outer borders represent physical boundaries of the testing arena while the dotted lines represent virtual "zones", the "inner zone" represent a virtual zone composed of the single box in the middle

colleagues (2010), where the open field was divided into several virtual square zones, and locomotor activity was measured across each zone provides greater complexity to the open field (see fig 9). This design allows for the benefit of both the open field and chambered tests such that analyzing locomotor behavior in each zone may be able to provide information on how thigmotaxic behavior changes over time. Coupled with pharmacological treatments, this type design may provide insight into how different substances may (in)activate different behavioral phenotypes. This is especially useful when investigating the behavioral effects of a substance such as nicotine and complex anxiety-related behaviors.

2.10 Anxiety and Nicotine

Acute nicotine treatment has been shown to decrease anxiety in humans (Rose, Ananda & Jarvik 2002), rodents (Lee 1985), adult zebrafish (Levin et al 2007). In zebrafish larvae there is scant research on the anxiety reducing effects of acute nicotine. Chronic nicotine, however, seems to have an anxiogenic effect on adult zebrafish (Stewart et al. 2015). Nicotine affects dopaminergic, noradrenergic, and serotonergic, GABAergic, and glutamtergic pathways in areas implicated in the modulation of anxiety. Acute

nicotine administered to rodents decreased serotonin turn-over rate, increased dopaminergic and noradrenergic activity (Lee 1985). The effect of nicotine on behavioral responses has been shown to be dose-dependent such that higher doses are anxiolytic, while lower doses increase exploratory behavior (Lee 1985). However, may affect anxiety differently than common prescription anxiolytics as they indirectly affect these neurotransmitters through activation and desensitization nAChRs (Picciotto 2003). Furthermore, as nicotine has a widespread affect of neurotransmitter pathways in different neural substrates, it is likely that nicotine can modulate different types of behavior. Therefore, in behavioral studies using nicotine it is important to be able to differentiate anxiety-related behaviors from exploratory behaviors and potential cognitive affects of nicotine that may be influencing the interpretation of behaviors in animal models.

2.11 Confounds in Interpreting Anxiety-related Behavior

Behavioral models developed for rodents and adult zebrafish have attempted to encapsulate anxiety-related behaviors. Different behavioral endpoints have been measured to attempt to differentiate anxiety-related behaviors from the activation of other behavioral phenotypes. However, many of the traditional models such as the open-field test/novel-tank test, are still limited in the types of behaviors that can be measured to differentiate activation of anxiety-related behaviors from normal exploratory responses. The current behavioral assays for larval zebrafish are currently limited to analyzing behavior in a single arena. In the evaluation of anxiety-related behaviors this does not suffice as, it increases the possibility of misinterpreting behavioral endpoints associated with anxiety. While these behavioral assays and behavioral endpoints have been partially validated by the testing of anxiolytic and anxiogenic compounds, it remains unclear whether these assays induce enough anxiety to produce anxiety-related behaviors (Blaser et al. 2010), model pathological anxiety (Prut & Belzung 2003) and whether

these behavioral endpoints are sufficient to characterize anxiety within these animal models (Simon et al. 1994; Prut & Belzung 2003). Therefore the present study attempts to outline a novel behavioral assay that may be able to provide a deeper understanding of effects of acute nicotine on exploratory behavior in larval zebrafish.

This project aims to: 1) determine the validity of a new 6-chamber complex environment as a new behavioral assay for testing exploratory and anxiety-related behavior in larval zebrafish; 2) propose a refinement of currently accepted behavioral endpoints of anxiety-related behavior such as thigmotaxis; 3) support the use of larval zebrafish as an animal model for future anxiety research; 4) determine whether the effects of nicotine on larval zebrafish can be 5) correlated with anxiety-related behavior or 6) whether nicotine explicitly activates exploratory behavior.

Methods

3.1 Fish Husbandry

Male and female adult zebrafish (danio rerio) of Tu strain were used to obtain fertilized zebrafish eggs for testing. Fish were kept on a 14 h light: 10 h dark cycle. Water and air temperature were maintained at 28°C. Male breeders were kept in tanks with maximum of 2 fish while females were kept in tanks with a maximum of 4 fish. Fish were fed twice daily—once with dry food and the once with brine shrimp.

Zebrafish eggs were obtained by mating predetermined pairs of males and females. Matings were set up overnight and embryo collection and processing was conducted the day after approximately 1 hour after the onset of lights. Matings pairs were set up on alternate days to ensure that no pair was set up on consecutive days.

Embryos were first collected and then cleaned using a bleach solution, washed out, and then transferred to a petri dishes filled with sterilized rack water (egg water) (60μg/mL Instant

Ocean (Spectrum Brands)). Embryos were housed at a temperature of 28°C under the same dark-light cycle as the adult zebrafish. Upon 5 dpf, zebrafish were moved to the testing location and were allowed to acclimate to the new room for at least 1 hour prior to testing.

3.2 Testing Apparatus: Two 6-Chamber Complex Environments

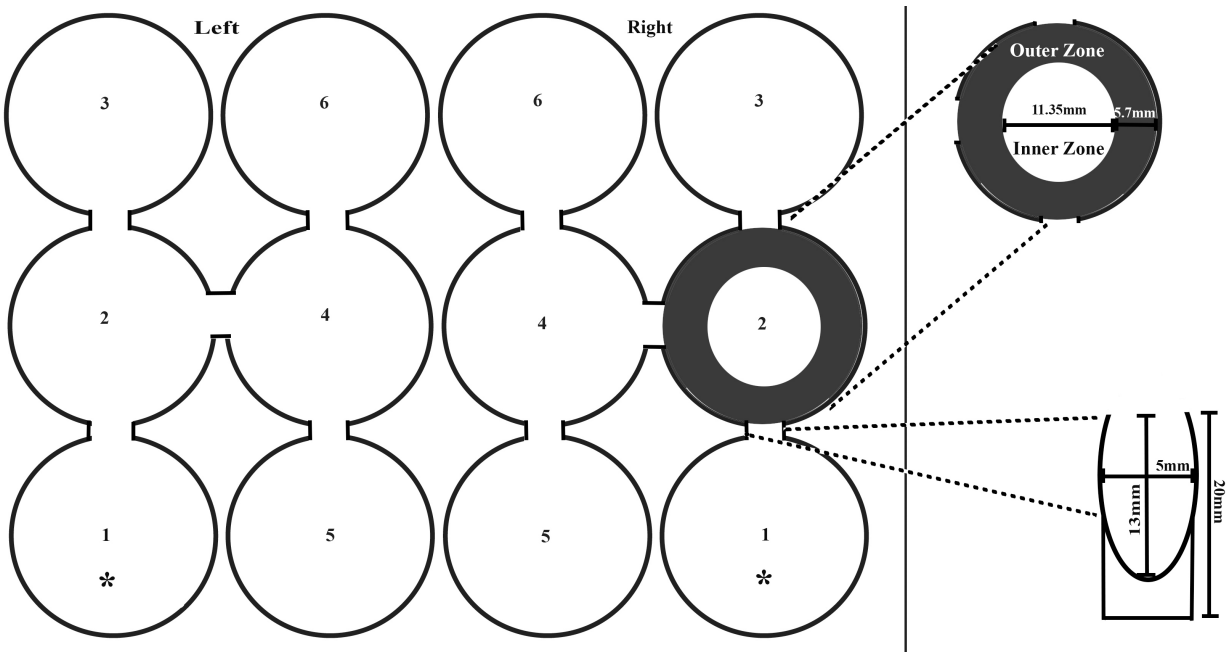


Figure 10 Schematic of two 6-Chamber Complex Environments. A single 12-well plate creates two testing arenas; outer zone and inner zone are delineated as well as the depth and width of the transition portion. "*" Indicates the start zone in both testing arenas.

A 12-well plate (Corning Inc., Corning, New York) (well diameter: 22.7mm) was made into the 6-chamber complex environment by melting the area in between wells with a soldering iron to create a leak-proof channel for movement. The 12-well plate was shaped to form 2 6-chamber complex environments (see fig 10). The environment was designed to measure the locomotor behavior of a single zebrafish larvae within a single environment. Using suggestions by (Schnorr et al. 2012) the 12-well plate was chosen to serve as the testing apparatus as the length of the delineated inner and outer zones of each chamber was larger than the length of an average larval zebrafish. Inner and outer zones were determined such that the spatial area of the

inner zone would be approximately equivalent to the other zone thereby ruling out potential biases in zone preference due to differences in zone size.

3.3 Experimental Procedure

Zebrafish were allowed to acclimate to the testing room's temperature for at least 1 hour prior to testing with the lights on while still in the petri dish. The testing apparatus was moved to the well-plate holder and filled with the testing solution (egg water, 16.25μM nicotine, or 48.75μM nicotine). 48.75μM of nicotine were decided based on observed maximum locomotor activity response to acute exposure to ~50μM nicotine in a study conducted by Petzold and colleagues (2009). 16.25μM was chosen because it was a threefold reduction in concentration of the high dose of nicotine. Zebrafish were then transferred to the testing arena (one per environment) through a plastic pipette. Minimal maneuvering of the zebrafish larvae was attempted to reduce potential stress induced by aspirating and transfer. Larvae were placed in the same relative position within the border zone of the well. Upon placement in the well, the room lights were turned off and the automated video-tracking began. All larvae were tracked using Ethovision XT 8.5 (Noldus, Wageningen, Netherlands) (see fig 11). The testing apparatus was lit by infrared LEDs (890nm)

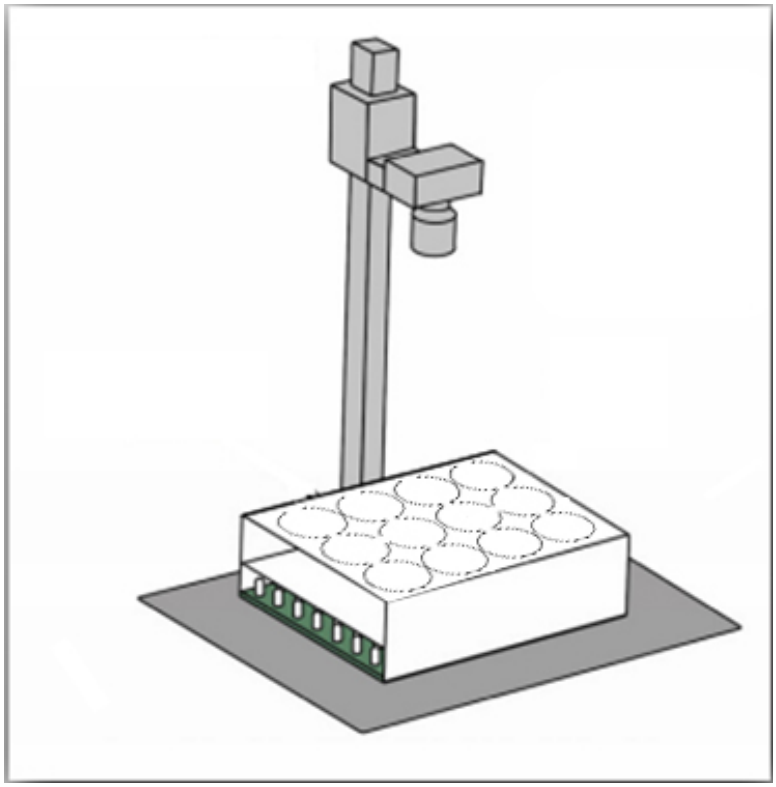


Figure 11 Schematic of experimental set-up. LEDs backlight the testing apparatus, while a camera fitted with an infrared lens tracks larval zebrafish movement. Adapted from (Ahmad & Richardson 2013)

(Jameco, Bellemont, CA) and recording using an Ikegami digital camera (model no. ICD-49) (Ikegami Tsushinki Co., Ltd., Tokyo, Japan) with a Nikon Micro-Nikkor 55mm lens attachment (Nikon Co., Tokyo, Japan). Maximum darkness was ensured through the use of infrared detection methods. Larvae were tracked for 15 minutes and then removed from the testing arena and placed in a separate petri dish for later euthanasia through tricaine overdose and disposal. No larvae were used twice for any of the experiments. When changing the solution within the testing arena, the well-plate was removed from the holder and the well-plate was thoroughly rinsed and dried before it was refilled with the new solution. The same volume of solution was maintained throughout the trials to prevent any effect of water levels on the ability of the larvae to transition from chamber to chamber.

3.4 Dosing and Drug Administration

Nicotine was chosen for its demonstrated anxiolytic properties in humans and rodents, as well as zebrafish (Levin et al. 2005). A low dose and high dose of nicotine were used to attempt to determine whether there was a dose-dependent effect of nicotine on thigmotaxic and exploratory behavior within the testing environment. Nicotine solutions were prepared by dissolving 23.5mg of nicotine di-tartrate salt (Sigma Aldrich, St. Louis, MO) (MW 498) in 50mL of sterile egg water to achieve an 1mM of nicotine di-tartrate stock solution, the stock solution of nicotine was then further diluted in egg water to achieve a 16.25μM and 48.75μM dose of free base nicotine. Larvae were exposed to the drug solution throughout the trial period (15min). Excess egg water from the petri dish was minimized during transfer of the larvae to the testing arena to prevent gross changes in the concentration of the drug solution.

3.5 Behavioral Endpoints

All locomotor activity was automatically recorded and video files were saved for later editing (to fill in missing simples or incorrect tracking). The behavioral endpoints reported in this study include, total distanced moved (mm), zone preference (measured as time spent and distance moved), thigmotaxis as described in Schnorr et al. (2012): ratio between total distanced moved in outer zone of a given chamber and total distanced moved within the same chamber and the ratio of the total time spent in the outer zone of a given chamber and the total time spent within that same chamber, thigmotaxis type (see fig 3) as described in Creed & Miller (1990), latency to first enter center zone(s), and frequency of chamber transitions. As this experiment employs a novel 6-chamber complex environment, zone preference, thigmotaxis, latency to enter the center zone, and chamber transitions were measured across the 6 chambers that may have been explored by the larvae. Furthermore, to investigate the temporal changes in behavior, general locomotor activity was analyzed within 1-minute bins (total 15 bins), and chamber transitions were analyzed within 5-minute bins (total 3 bins).

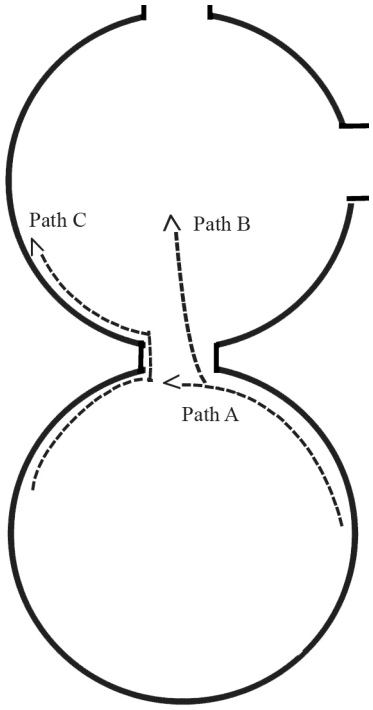


Figure 12 Illustration of potential types of thigmotaxis within the 6-chamber complex environment. Path A represents passive thigmotaxis; path B presents weakly active thigmotaxis, path C represents active thigmotaxis

3.6 Statistical Analysis

Statistical analyses were performed using SPSS (version 23). Total distanced moved was analyzed using a one-way ANOVA for comparisons of the effect of nicotine treatment. Zone preference was analyzed using student's t-tests. Thigmotaxis measures (time spent & distance

moved) and thigmotaxis type were analyzed using one-way ANOVAs. Overall chamber transitions were analyzed using a one-way ANOVA. Frequency of chamber transitions per chamber was analyzed using a MANOVA. Latency to enter the center zone data was analyzed using one-way ANOVAs and student's t-tests. Time binned data (for general locomotor activity & chamber transitions) was analyzed using a mixed ANOVA. Tukey HSD post-hoc tests were run to determine group-specific effects of drug treatment. Simple effects tests were run with bonferroni corrections to further interpret significant interactions. Graphs were created using excel and data was presented as means ± SEM. The criterion for significance was set at a probability of 5%.

Results

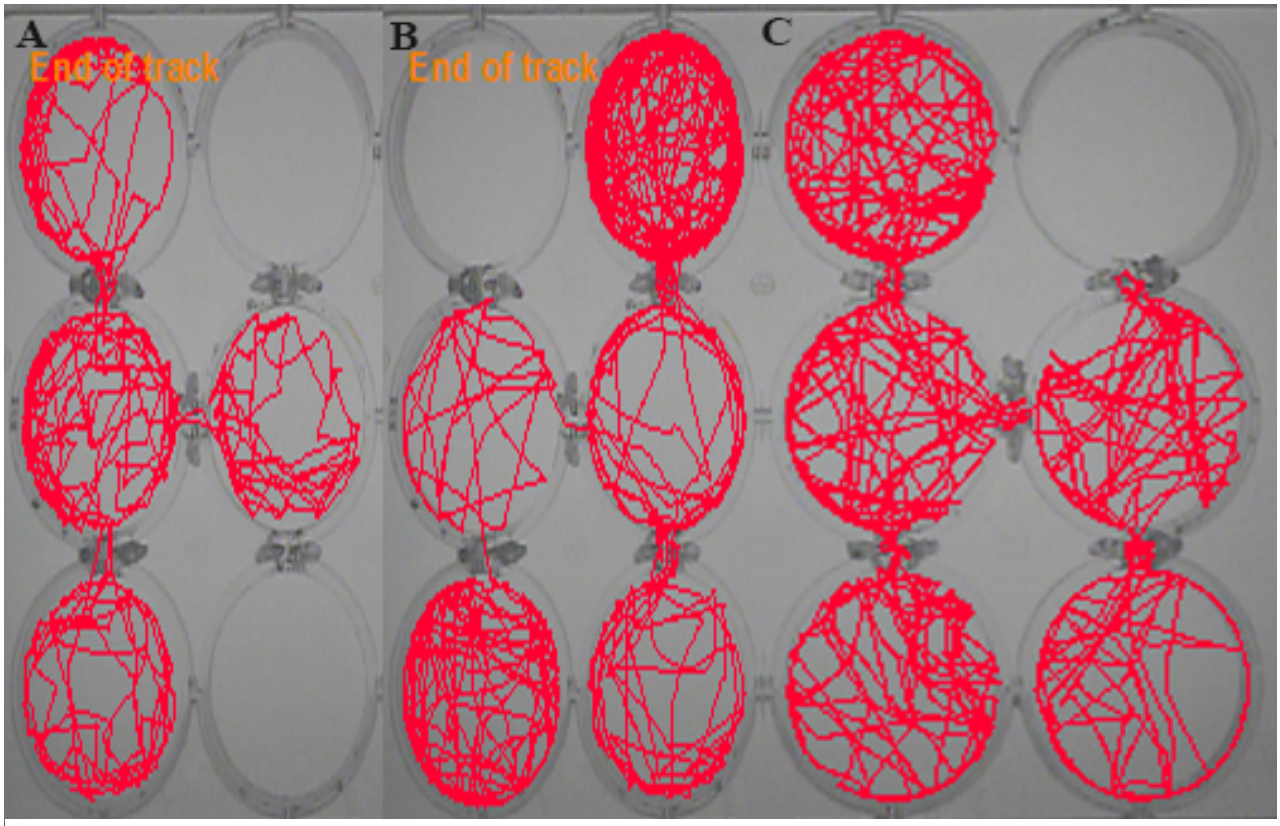


Figure 13 Sample tracks from experiment. A) Track from CON; B) Track from a 16.25μM nicotine treated larvae; and C) track from a 48.75μM treated larvae. Tracks shown outline the movement of a larvae throughout the 15-minute trial duration

4.1. General Locomotor Activity

Locomotor activity was assessed by examining total distance moved for the duration of the 15-min trial and by time using one-minute bins.

4.1A Overall Locomotor Activity

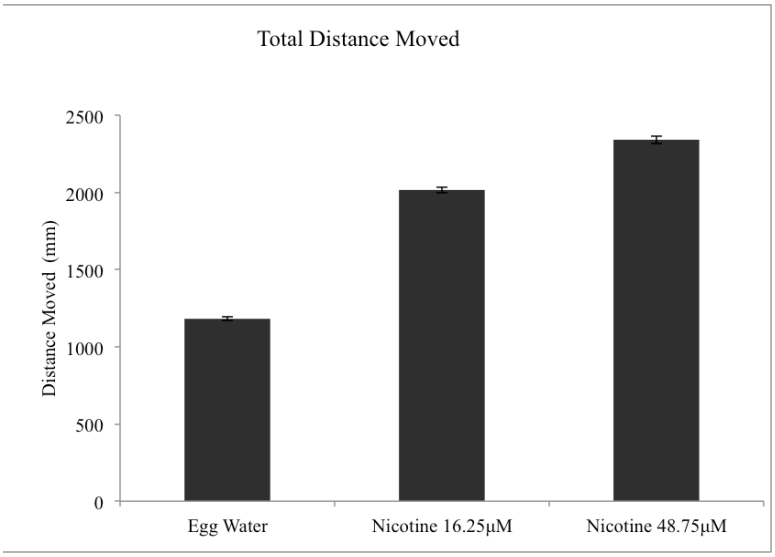


Figure 14 General locomotor activity was measured as the total distance moved throughout the 15 minute trial. Bars represent mean±SEM for each treatment condition

There was an effect of treatment condition on general locomotor activity (see fig 14), a one-way ANOVA $F(2,148)=18.11$, $p<0.05$ revealed that larvae treated with 16.25μM of nicotine (LNIC) (2017.32±129.5) moved significantly more than egg water treated larvae (CON) (1182.25±93.2). Similarly, larvae treated with 48.75μM of nicotine (HNIC) (2342.75±181.98) moved significantly more than CON (1182.25±93.2), $p<0.05$. There was no significant difference observed between LNIC and HNIC. These results demonstrate that acute nicotine treatment increases locomotor activity in larval zebrafish.

4.1B General Locomotor Activity Over Time

Total distance moved was measured in larvae over one-minute bins (see fig 15). Mixed ANOVA revealed a significant main effect of time $F(14,143)=40.353$, $p<.05$, and a significant main effect of treatment $F(2,143)=17.72$, $p<.05$. CON moved less than both the LNIC and HNIC, $p<0.05$ and $p<0.05$, respectively. There were no observed differences between the two nicotine conditions. There was a significant time by treatment condition interaction

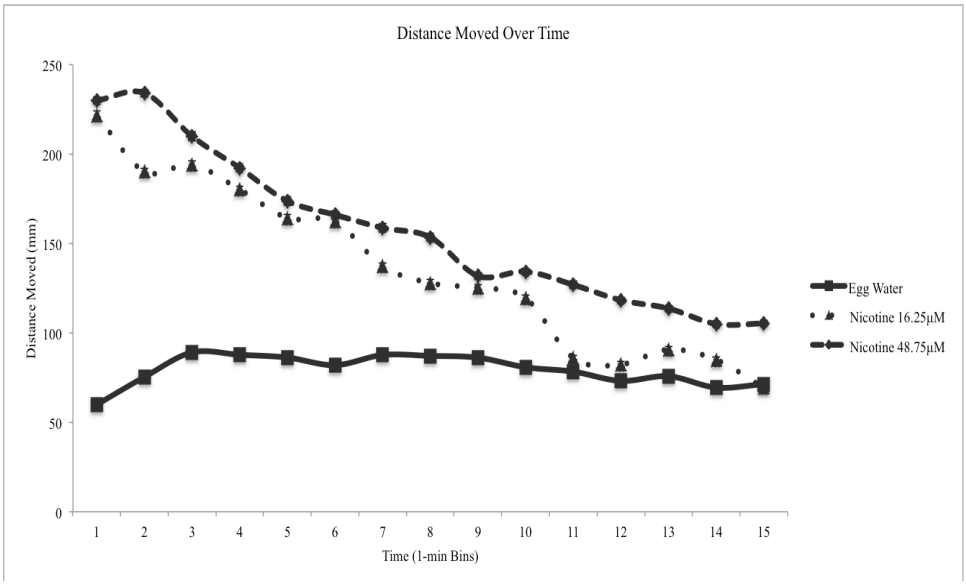


Figure 12. General locomotor activity over 15 1-minute bins (15 minutes). Data points represent average distanced moved within the time bin, mean±SEM.

F(28,143)=10.47, $p<0.05$. Simple effects tests comparing the first and last time bin revealed that CON distance moved did not change over time (1st minute: 59.918±14.23) (15th minute: 71.356±9.26), but both nicotine treatment groups distance moved decreased significantly LNIC (1st minute: 219.918±14.37) (15th minute: 69.29±9.36) $p<0.05$, and HNIC (1st minute: 230.07±14.23) (15th minute: 105.31±9.26), $p<0.05$.

Comparing the distanced moved of larvae treatment groups at the first and last minute of the trial showed that at the 1st minute CON (59.918±14.23) moved significantly less than LNIC (219.918±14.37) and HNIC (230.07±14.23), $p<0.05$ and $p<0.05$, respectively. LNIC did not significantly differ from HNIC. At the 15th minute CON (71.356±9.26) did not significantly differ from LNIC (219.918±14.37), but CON and LNIC moved significantly less than HNIC (230.07±14.23), $p=0.032$ and $p=0.021$, respectively. These results suggest that both low dose and high dose nicotine cause larvae to move more within the first few minutes of the test compared to CON, but locomotor activity decreases over time in both nicotine treatment groups. Lastly, by the end of the trial HNIC exhibited more locomotor activity than both CON and LNIC.

4.1C Summary of General Locomotor Activity

Nicotine, regardless of the strength of the dose, increased larvae locomotor activity. Furthermore, nicotine increased larval activity during the beginning of the trial relative to CON, but by the end of the trial, LNIC demonstrated the same amount of general activity as CON. Moreover, HNIC increased general activity in larvae relative to LNIC and CON by the end of the trial. The activity of both LNIC and HNIC decreased over the duration of the trial. In sum, nicotine seems to increased general locomotor activity overall, but only HNIC increased general locomotor activity from the beginning to the end of the trial.

4.2 Zone Preference: Distance Moved & Time Spent (DM & TS)

Inner versus Outer Zone Preference (IOZP) was measured by both distanced moved (DM) and time spent (TS) in outer and inner zones of each chamber. Overall zone preference was measured by averaging the IOZP across each of the six chambers. The purpose of analyzing zone preference using both distanced moved and time spent in each zone parameters is to determine the validity of both of these measures as compared to previous literature and the consistency between the two measures.

4.2.1A Zone Preference: Distance Moved (DM)

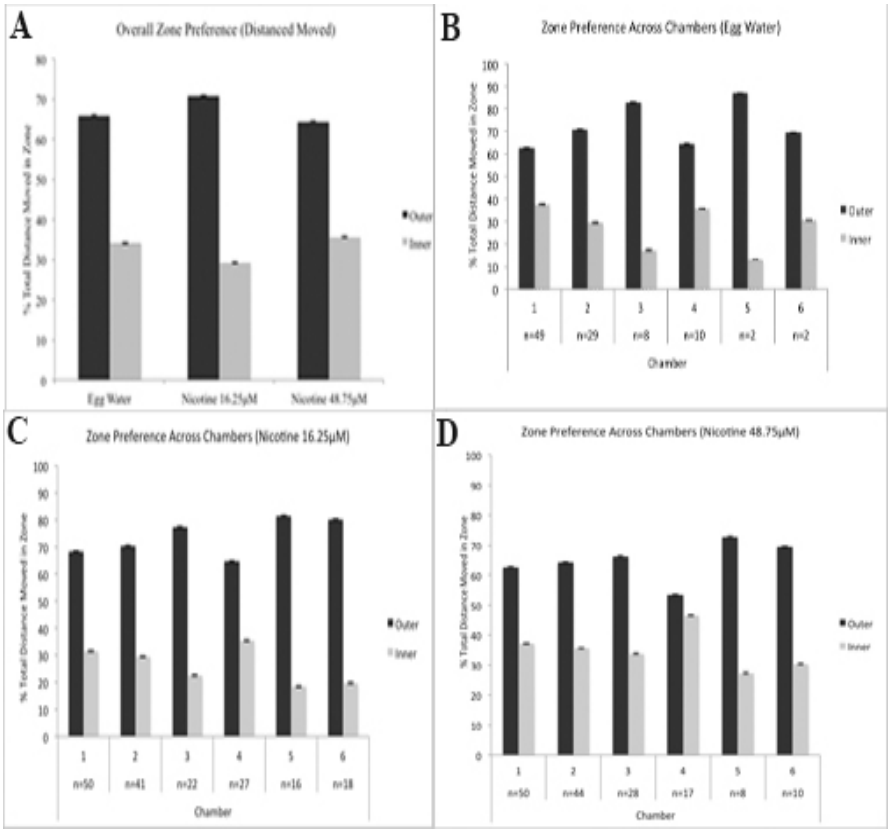
IOZP was calculated as the percent of the total distance spent in either the outer or inner zone throughout the 15-minute trial.

$$IOZP\ (\%\ Distanced\ Moved\ in\ Zone) = \frac{Distanced\ Moved\ in\ Outer\ or\ Inner\ Zone}{Total\ Distanced\ Moved\ in\ Chamber} \times 100$$

4.2.1B Overall Zone Preference

All treatment groups demonstrated a significant preference for the outer zone throughout the 15-minute trial (see fig 16A). CON exhibited a preference for the outer zone (65.88±1.78) compared to the inner zone (34.12±1.78), $t(48)=8.91$, $p<0.05$. LNIC exhibited an outer zone

preference (70.75±1.33) compared to the inner zone (29.25±1.33), t(49)=15.53, $p<0.05$. Finally, HNIC showed an outer zone preference (64.35±1.42) compared to the inner zone (35.64±1.42), t(49)=10.10, $p<0.05$. This data suggests nicotine at both high or low doses did not have an effect on outer zone preference.



Chamber	Outer Zone			Inner Zone			Significance			Zone Preferred		
	Egg Water	Nicotine 16.25μM	Nicotine 48.75μM	Egg Water	Nicotine 16.25μM	Nicotine 48.75μM	Egg Water	Nicotine 16.25μM	Nicotine 48.75μM	Egg Water	Nicotine 16.25μM	Nicotine 48.75μM
1	62.51±1.9	68.47±1.5	62.77±1.7	37.48±1.9	31.52±1.5	37.23±1.7	t(48)=6.3 p<0.05	t(49)=12.0 p<0.05	t(49)=7.3 p<0.05	O	O	O
2	70.56±2.7	70.44±2.1	64.31±2.3	29.43±2.7	29.55±2.1	35.69±2.3	t(28)=7.5 p<0.05	t(40)=9.4 p<0.05	t(43)=6.1 p<0.05	O	O	O
3	82.75±5.8	77.37±2.8	66.24±2.3	17.24±5.8	22.62±2.8	33.76±2.3	t(7)=5.6 p<0.05	t(21)=9.5 p<0.05	t(27)=6.8 p<0.05	O	O	O
4	64.34±3.9	64.65±2.8	53.45±1.7	35.66±3.9	35.35±2.8	46.55±1.7	t(9)=3.6 p<0.05	t(26)=5.1 p<0.05	ns	O	O	N
5	86.88±1.2	81.51±3.0	72.68±6.6	13.12±1.2	18.49±3.0	27.32±6.6	*	t(15)=10.3 p<0.05	t(7)=3.4 p<0.05	*	O	O
6	69.42±3.8	80.21±3.2	69.56±4.1	30.58±3.8	19.79±3.2	30.44±4.1	*	t(17)=9.3 p<0.05	t(9)=4.7 p<0.05	*	O	O
Overall	65.88±1.7	70.75±1.3	64.35±1.4	34.12±1.7	29.25±1.3	35.64±1.4	t(48)=8.9 p<0.05	t(49)=15.5 p<0.05	t(49)=10.1 p<0.05	O	O	O

Figure 16 & Table 2 A) Overall Zone Preference; B) Zone Preference of controls; C) Zone Preference of low nicotine group; D) Zone Preference of high nicotine group, data presented as mean±SEM. Table represents means±SEM for outer and inner zone preference in all chambers "*" Indicates not enough fish entered the chamber for an accurate measure of zone preference. Discrepancies between degrees of freedom between total time spent and total distanced moved measures of zone preference can be attributed to low/unmoving larvae within the chamber. N=no observed preference, O=preference for outer zone, I=preference for inner zone

4.2.1C IOZP: Per Chamber

Across all treatment groups, larvae demonstrated a higher proportion of outer zone preference across the 6 chambers of the testing arena (see table 2). Chambers in which not enough subjects entered (n<5) were excluded from statistical analysis. CON larvae demonstrated an outer zone preference in chambers 1, 2, 3 and 4

(see fig 16B). LNIC larvae similarly exhibited an outer zone preference in all chambers 1-6 (see fig 16C). HNIC larvae demonstrated an outer zone preference in chambers 1, 2, 3, 5, and 6 (see fig 16D). Together this data suggests that outer zone preference is generally conserved in nicotine treated subjects.

4.2.1D Summary of Zone Preference (DM)

Nicotine at either high or low doses does not seem to change the outer zone preference of larval zebrafish. The effect of nicotine on zone preference will further examined in the analysis of thigmotaxis. In order to determine whether this finding is reliable, zone preference as measured by time spent in outer and inner zones was analyzed as well.

4.2.2A Zone Preference: Time Spent (TS)

Zone preference was also determined by the amount of time each subject spent within either the outer zone or the inner zone. Time spent was reported as a percentage of the time spent in the chamber.

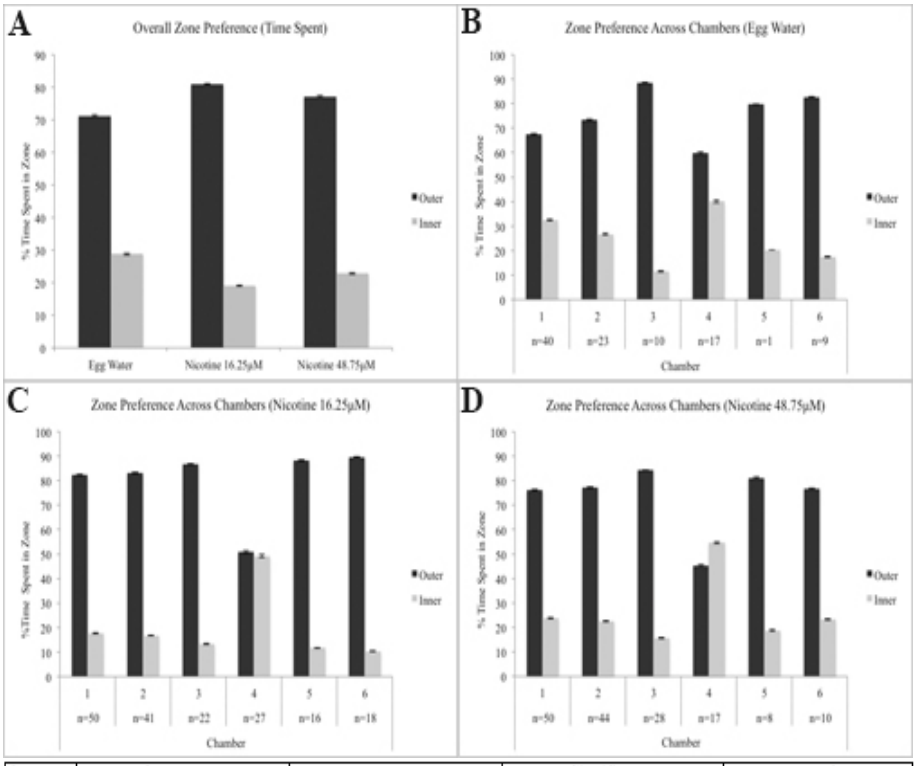
$$IOZP\ (\%\ Time\ Spent\ in\ Zone) = \frac{Time\ Spent\ in\ Outer\ or\ Inner\ Zone}{Total\ Time\ Spent\ in\ Chamber} \times 100$$

4.2.2B Overall Zone Preference

All treatment groups demonstrated a significant preference for the outer zone throughout the 15-minute trial (see fig 17A). CON exhibited a preference for the outer zone (71.22±2.27) compared to the inner zone (28.78±2.27), $t(48)=9.34$, $p<0.05$. LNIC exhibited an outer zone preference (80.89±1.35) compared to the inner zone (19.10±1.35), $t(49)=22.80$, $p<0.05$. Finally, HNIC exhibited an outer zone preference (77.17±1.72) compared to the inner zone (22.83±1.72), $t(49)=15.78$, $p<0.05$. This confirms the findings from the zone preference (DM) (section 4.2.1A-D) that subjects prefer the outer zone between treatment groups.

4.2.2C IOZP: Per Chamber

Larvae across all treatment groups demonstrated a higher outer zone preference across the 6 chambers of the testing arena (see table 2). Chambers in which not enough subjects entered (n<5) were excluded from statistical analysis. CON demonstrated a higher outer zone preference in chambers 1, 2, 3, and 6 (see fig 17B). LNIC demonstrated a higher outer zone preference in chambers 1, 2, 3, 5, and 6 (see fig 17C) HNIC demonstrated a higher outer zone preference in chambers 1, 2, 3, 5, and 6 (see fig 17D). While this data does not exactly match zone preference (TDM), it is generally consistent with the observation that



Chamber	Outer Zone			Inner Zone			Significance			Zone Preferred		
	Egg Water	Nicotine 16.25μM	Nicotine 48.75μM	Egg Water	Nicotine 16.25μM	Nicotine 48.75μM	Egg Water	Nicotine 16.25μM	Nicotine 48.75μM	Egg Water	Nicotine 16.25μM	Nicotine 48.75μM
1	67.46±2.5	82.29±1.7	76.19±2.1	32.54±2.5	17.71±1.7	23.81±2.1	t(39)=6.9 p<0.05	t(49)=18.1 p<0.05	t(49)=7.3 p<0.05	O	O	O
2	73.34±4.1	83.21±1.7	77.36±2.0	26.66±4.1	16.79±1.7	22.64±2.0	t(22)=5.6 p<0.05	t(40)=18.5 p<0.05	t(43)=6.1 p<0.05	O	O	O
3	88.48±2.3	86.70±2.3	84.34±4.7	11.52±2.3	13.30±2.3	15.66±4.7	t(9)=12.0 p<0.05	t(21)=15.6 p<0.05	t(27)=6.8 p<0.05	O	O	O
4	59.90±6.9	50.86±6.1	45.33±4.7	40.10±6.9	49.14±6.1	54.37±4.7	ns	ns	ns	N	N	N
5	79.71±	81.51±2.3	81.20±5.3	20.29±	11.74±2.3	18.79±5.3	*	t(15)=16.5 p<0.05	t(7)=5.8 p<0.05	*	O	O
6	82.60±2.9	89.62±1.9	76.72±4.9	17.40±2.9	10.37±1.9	23.27±4.9	t(8)=11.2 p<0.05	t(17)=19.9 p<0.05	t(9)=5.45 p<0.05	O	O	O
Overall	71.22±2.2	80.89±1.3	77.17±1.7	28.78±2.2	19.10±1.3	22.83±1.7	t(48)=9.3 p<0.05	t(49)=22.8 p<0.05	t(49)=15.7 p<0.05	O	O	O

Figure 17 & Table 3 A) Overall Zone Preference; B) Zone Preference of controls; C) Zone Preference of low nicotine group; D) Zone Preference of high nicotine group, data presented as mean±SEM. Table represents means±SEM for outer and inner zone preference in all chambers "*" Indicates not enough fish entered the chamber for an accurate measure of zone preference. Discrepancies between degrees of freedom between total time spent and total distanced moved measures of zone preference can be attributed to low/unmoving larvae within the chamber. N=no observed preference, O=preference for outer zone, I=preference for inner zone

larvae treated acutely with nicotine still prefer the outer zone to the inner zone.

4.2.2D Summary of Zone Preference (TS)

Larval zone preference as measured by the time spent in the outer and inner arena confirms that larvae prefer the outer zone of a chamber even when treated with low dose or high dose nicotine. These results are mostly consistent with the findings from section 4.2.1A-D. Outer zone preference (thigmotaxis) will be compared between groups to determine to what extent nicotine effects IOZP.

4.3 Thigmotaxis

Thigmotaxis (outer zone preference) was measured as percent total distance moved and percent total time spent in the outer zone across all treatment groups. Overall and per chamber thigmotaxis was analyzed.

4.3.1A Thigmotaxis (DM)

$$Thigmotaxis\ (\%\ Distanced\ Moved\ in\ Outer\ Zone) = \frac{Distanced\ Moved\ in\ Outer\ Zone}{Total\ Distanced\ Moved\ in\ Chamber} \times 100$$

4.3.1B Overall Thigmotaxis

Overall thigmotaxis was calculated by averaging the amount of thigmotaxis observed (% total distance moved in the outer zone) across all chambers (see fig 18). ANOVA revealed a significant main effect of treatment on thigmotaxis $F(2,146)=4.84$, $p=0.009$. There were no observed significant differences between CON and LNIC or HNIC, but

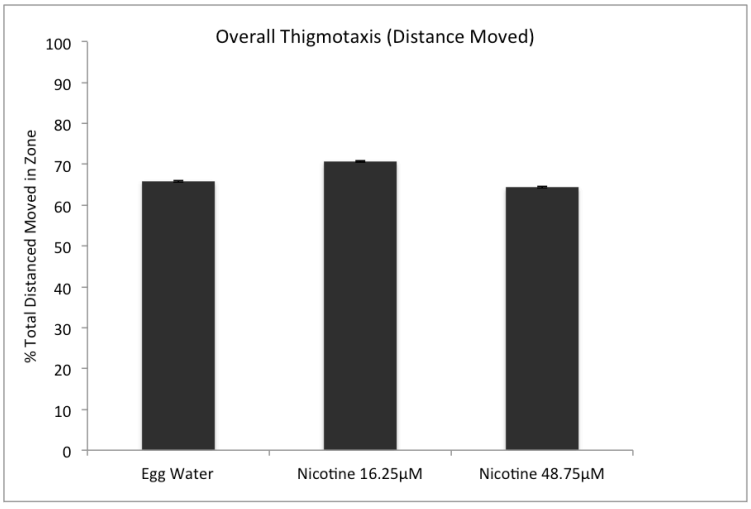
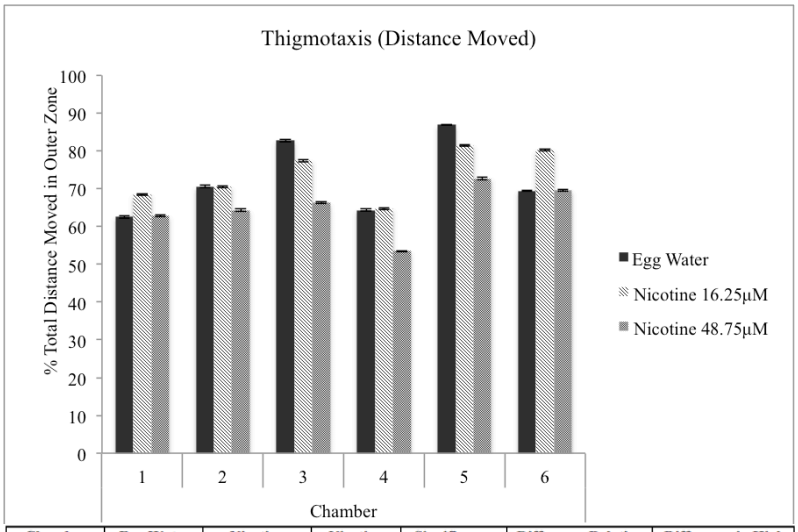


Figure 18 Overall thigmotaxis depicted as mean±SEM across all 6 chambers of the testing arena

LNIC larvae exhibited significantly more thigmotaxic behavior than HNIC larvae.

4.3.1C Thigmotaxis: Per Chamber



Chamber	Egg Water	Nicotine 16.25µM	Nicotine 48.75µM	Significance	Difference Relative to Control		Difference in High Dose Nicotine Relative to Low Dose Nicotine
					Nicotine 16.25µM	Nicotine 48.75µM	
1	62.51±1.96	68.47±1.53	62.77±1.73	F(2,146)=3.72 p=0.026	+	/	/
2	70.56±2.73	70.44±2.15	64.31±2.32	ns	/	/	/
3	82.76±5.82	77.37±2.86	66.24±2.37	F(2,55)=6.79 p=0.002	/	-	-
4	64.34±3.97	64.65±2.82	53.45±1.73	F(2,51)=4.69 p=0.013	/	/	-
5	86.88±1.22	81.51±3.05	72.68±6.67	ns	/	/	/
6	69.42±3.88	80.20±3.22	69.56±4.12	ns	/	/	/
Overall	65.88±1.78	70.513±1.33	64.35±1.42	F(2,146)=4.84 p=0.009	/	/	-

Figure 19 & Table 4 Thigmotaxis (DM) within each chamber across treatment groups. Bars represent mean±SEM. Table shows mean±SEM of thigmotaxis for each chamber across treatment groups, ns indicate non significance; "+" indicates significantly greater than; "-" indicates significantly less than; and "/" indicates non-significance

Thigmotaxis was also examined across individual chambers of the maze between all treatment groups (see fig 19 and table 4). In chamber 1, LNIC increased thigmotaxic behavior relative to CON whereas HNIC showed no difference from CON. There was no observed significant difference between treatment groups in the 2nd chamber. In the 3rd chamber HNIC decreased thigmotaxic behavior relative to both LNIC and CON. Within the 4th chamber, HNIC larvae exhibited less thigmotaxis relative to LNIC larvae, but not CON. There were no significant differences in thigmotaxis behavior in the 5th or 6th chambers.

4.3.1D Summary of Thigmotaxis (DM)

Within this 6-chamber complex environment, thigmotaxis, which is typically used as a measure of anxiety-like behavior, appeared to be affected by nicotine treatment, such that LNIC

treated larvae exhibit higher thigmotaxis than HNIC larvae (chambers 3, 4, and overall), and CON (chamber 1). Furthermore, HNIC larvae demonstrate decrease thigmotaxis than CON (chamber 3). This suggests that HNIC potentially decreases thigmotaxic behavior, while LNIC increases thigmotaxic behavior. Thigmotaxis as measured by time spent in the outer zone of a given chamber was measured to determine the consistency of the thigmotaxis (DM) results.

4.3.2A Thigmotaxis (TS)

$$Thigmotaxis\ (\%\ Time\ Spent\ in\ Outer\ Zone) = \frac{Time\ Spent\ in\ Outer\ Zone}{Time\ Spent\ in\ Chamber} \times 100$$

4.3.2B Overall Thigmotaxis

Overall thigmotaxis was measured as the amount of time spent in the outer zone across all chambers within the testing apparatus (see fig 20). There was a significant effect of nicotine treatment on thigmotaxis $F(2,148)=7.193$, $p=0.001$. Post hoc tests revealed that CON (71.21±2.27) larvae exhibited significantly less thigmotaxis than the LNIC (80.89±1.35) larvae $p=0.001$, but not the HNIC larvae (77.16±1.72). These results suggests that LNIC increases thigmotaxic behavior overall within the 6-chamber complex environment.

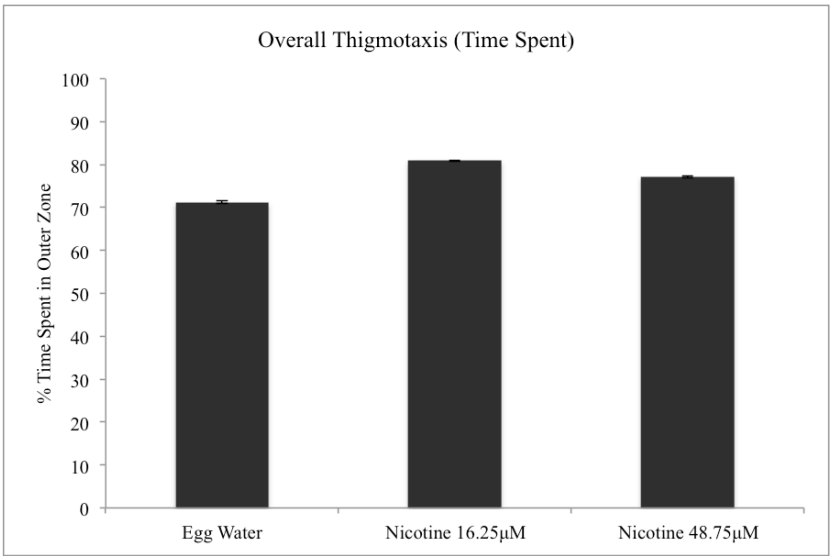
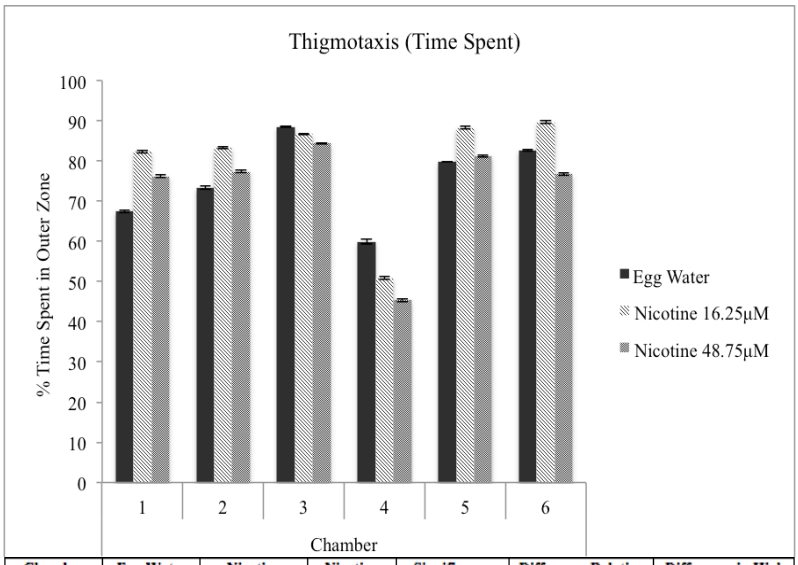


Figure 20 Overall thigmotaxis depicted as mean±SEM across all 6 chambers of the testing arena

4.3.2C Thigmotaxis: Per Chamber

Thigmotaxis per chamber was also examined (see fig 21 and table 5). Within the 1st chamber both LNIC and HNIC larvae exhibited more thigmotaxis relative to CON. There was no



Chamber	Egg Water	Nicotine 16.25µM	Nicotine 48.75µM	Significance	Difference Relative to Control		Difference in High Dose Nicotine Relative to Low Dose Nicotine
					Nicotine 16.25µM	Nicotine 48.75µM	
1	67.45±2.5	82.28±1.78	76.18±2.19	F(2,139)=11.42 p <0.05	+	+	/
2	73.33±4.15	83.21±1.79	77.36±2.03	F(2,107)=3.79 p=0.026	+	/	/
3	88.48±3.2	86.69±2.35	84.33±1.71	ns	/	/	/
4	59.9±6.96	50.86±6.15	45.32±4.75	ns	/	/	/
5	79.71±*	88.25±2.31	81.2±5.36	ns	/	/	/
6	82.59±2.91	89.62±1.98	76.72±4.91	F(2,36)=4.739 p=0.015	/	/	-
Overall	71.21±2.27	80.89±1.35	77.16±1.72	F(2,148)=7.193 p=0.001	+	/	/

Figure 21 & Table 5 Thigmotaxis (TS) within each chamber across treatment groups. Bars represent mean±SEM. Table shows mean±SEM of thigmotaxis for each chamber across treatment groups, ns indicate non significance; "+" indicates significantly greater than; "-" indicates significantly less than; and "/" indicates non-significance

observed difference in thigmotaxis between the two nicotine treatments. In the 2nd chamber, only LNIC larvae exhibited higher thigmotaxis relative to CON. Again, there were no observed differences in thigmotaxis between the two nicotine treatments. There was no effect of nicotine treatment on thigmotaxis within the 3rd, 4th, and 5th chambers. Lastly, there was a significant effect of nicotine treatment on thigmotaxis within the 6th chamber such that LNIC larvae exhibited significantly more thigmotaxis than HNIC, but not CON. There were no observed differences in thigmotaxis between both nicotine treatments and CON.

4.3.2D Summary of Thigmotaxis (TS)

Thigmotaxic behavior as measured by time spent in the outer zone did not fully corroborate the results from thigmotaxis (DM) (section 4.3.1A-D). LNIC larvae exhibited more thigmotaxis than CON both overall and within chambers 1 and 2. HNIC larvae demonstrated more thigmotaxis than CON in the 1st chamber, and demonstrated less thigmotaxis than LNIC

larvae in the 6th chamber. While the statistical analyses did not demonstrate a significant anxiolytic effect of HNIC the average amount of thigmotaxis expressed by HNIC larvae is generally lower than both LNIC and CON (see table 5). It is possible that there were not enough larvae that entered the further away chambers (3-5) to demonstrate a significant result. Nevertheless, this data demonstrates that low dose nicotine increased thigmotaxic behavior in larvae, while high dose nicotine does not seem to have a significant effect on thigmotaxis as measured by time spent in the outer zone of a chamber.

4.4. Thigmotaxis Type

The frequency of occurrence of three different types of thigmotaxis (active, passive, weakly active) was measured (see fig 22). An ANOVA revealed that nicotine treatment had a significant effect on active $F(2,148)=9.43$, $p<0.05$ and weakly active $F(2,148)=19.41$, $p<0.05$ thigmotaxis, but not passive thigmotaxis. For active thigmotaxis, post hoc tests revealed that CON (0.39±0.10) and HNIC (0.92±0.16) larvae exhibited less active thigmotaxis than LNIC (1.58±0.27) larvae, $p<0.05$ and $p=0.045$, respectively. Furthermore, CON (0.92±0.22) exhibited significantly less weakly active thigmotaxis than both LNIC (5.14±0.67) and HNIC (3.4±0.43) larvae, $p<0.05$ and $p=0.001$, respectively. LNIC larvae exhibited significantly more weakly active thigmotaxis than HNIC

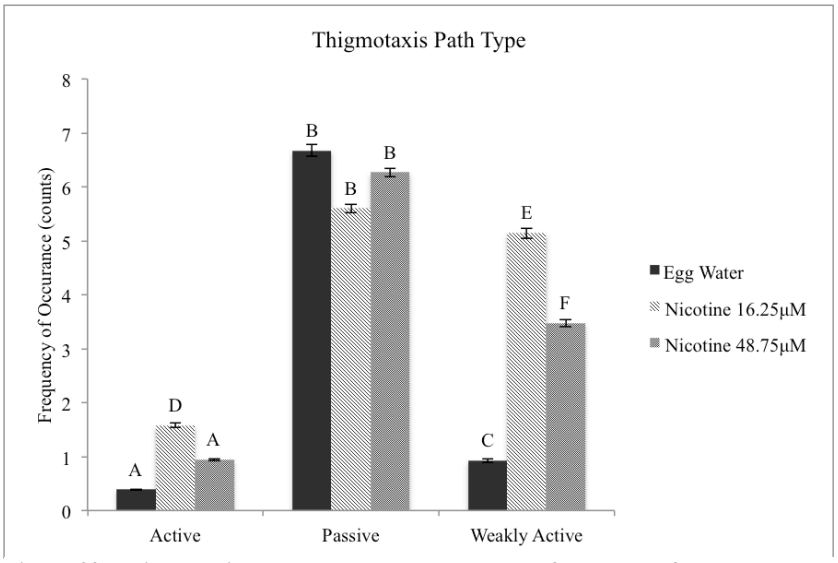


Figure 22. Thigmotaxis path types were measured as frequency of occurrence. Bars represent manually scored data, mean±SEM. Common letters denote non-significance.

larvae, p=0.030. These results demonstrate that nicotine greatly increases active and weakly active thigmotaxic behavior, but has no effect on passive thigmotaxic behavior.

4.4A Summary of Thigmotaxis Path Type

These results suggest that LNIC and HNIC increased the tendency of the larvae to engage in both active and weakly active thigmotaxis, while having no effect on the frequency of passive thigmotaxis. Specifically, LNIC increased active and weakly thigmotaxic behavior relative to both HNIC and CON. HNIC only increased weakly active thigmotaxis relative to CON. Active thigmotaxis is a more accurate descriptor of anxiety within animals, while passive thigmotaxis does not describe anxiety-like behavior. Weakly active thigmotaxis can be interpreted as anxiety-like behavior, but is demonstrative of a weaker anxiety level in the larvae. Taken this way, then it seems that LNIC and to a lesser extent HNIC increases anxiety in larval zebrafish.

4.5. Chamber Transitions

Chamber transitions were measured as the number of times larvae moved from one chamber to an adjacent chamber. Chamber transitions were measured overall, per chamber, and overall transitions over one minute time bins.

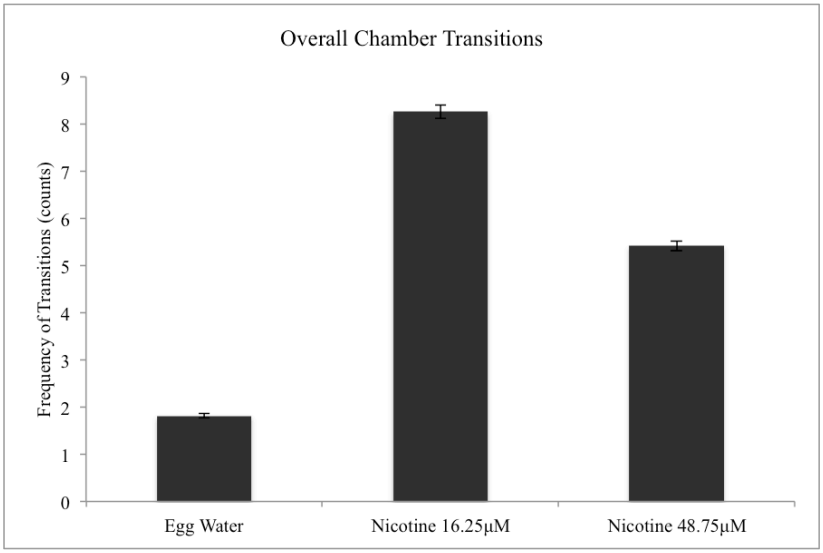
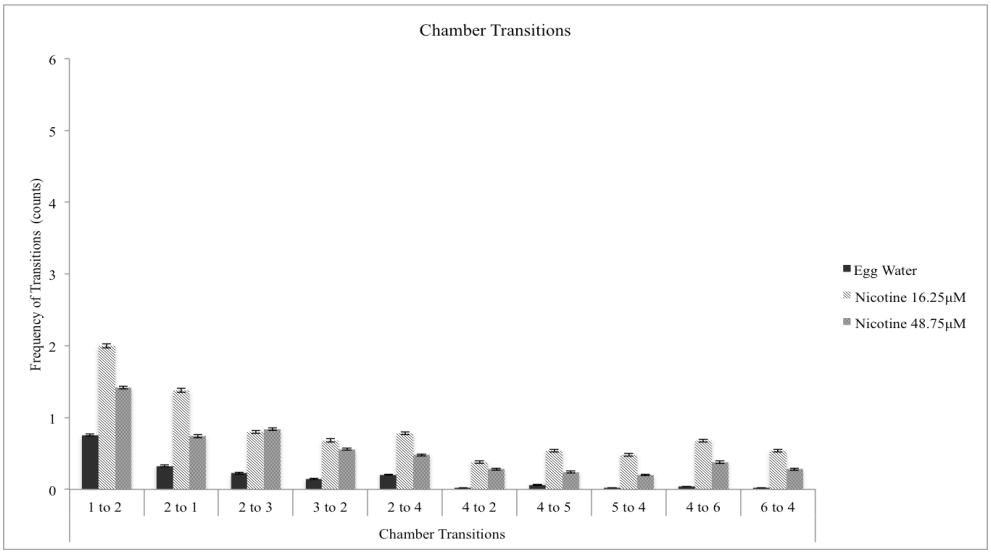


Figure 23 Overall chamber transitions represented as mean±SEM averaged across all 6 chambers.

4.5A Overall Chamber Transitions

Overall chamber transitions were measured across the treatment conditions (see fig 23). An ANOVA revealed a significant effect of nicotine treatment on the frequency of

chamber transitions F(2,148)=19.18, $p<0.05$, such that both LNIC (8.26±0.98) and HNIC (5.42±0.731) larvae transitioned significantly higher than CON (1.81±0.315) larvae, $p<0.05$ and $p=0.002$, respectively. Furthermore, LNIC larvae transition significantly more than the HNIC larvae, $p=0.019$.



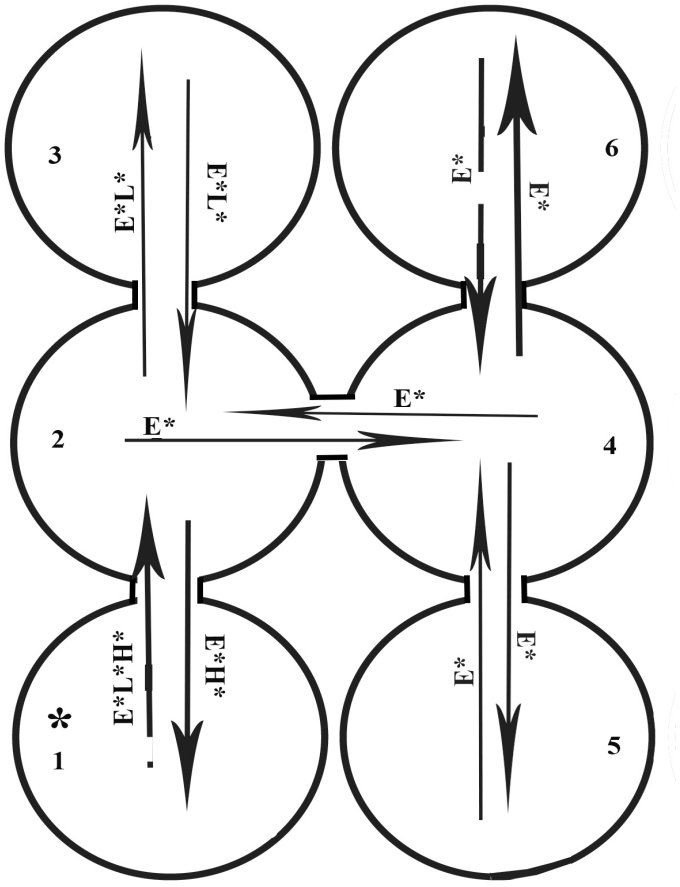


Figure 24. Chamber transitions MANOVA depicted, letters with "*" denote significance. Letters E (CON), L (LNIC) and H (HNIC) denote treatment conditions.

This suggests that a LNIC motivates more chamber transitions in larval zebrafish relative to CON and HNIC treatment.

4.5B Chamber Transitions: Per Chamber

Chamber transitions per chamber were calculated based on the frequency of transitions across chambers. Transitions in and out of a given chamber were measured separately. i.e chamber transition from chamber 1 to chamber 2 was measured as different from a

chamber transition from chamber 2 to chamber 1 (see fig 24). A MANOVA revealed a significant effect of nicotine treatment on chamber transitions Wilks' Lambda=0.690, $F(20,274)=2.793$ $p<0.05$. Specifically, CON transitioned from chambers 1-2 significantly less than both LNIC and HNIC. Furthermore, LNIC transitioned from chambers 1-2 significantly more than HNIC. LNIC transitioned from chambers 2-1 significantly more than both CON and HNIC. CON transitioned from chambers 2-3 and chambers 3-2 significantly less than both LNIC and HNIC. CON transitioned from chambers 2-4, 4-2, 4-5, 5-4, 4-6, and 6-4 significantly less than LNIC, but not HNIC. These results suggests that nicotine at a lower dose (16.25μM) increases larvae tendency to transition from chamber to chamber, while a higher dose of nicotine (48.75μM) also increases chamber transitions, does so less than the lower dose. The pattern of the data suggests that larvae treated with nicotine are more likely to transition to the other half of the testing chamber than controls.

4.5C Chamber Transitions over Time

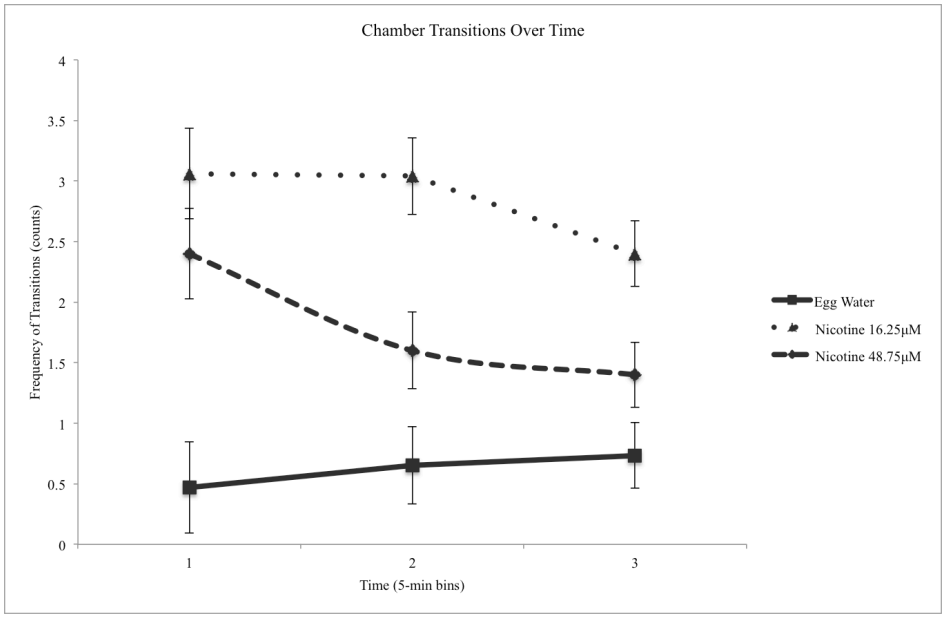


Figure 25 Chamber Transitions over time, data points represent overall chamber transitions at 5-min bins (total 15 mins) represented as means±SEM

Overall chamber transitions were analyzed over 5 minute bins for 15 minutes (see fig 25). Five minute bins were chosen over one minute bins because within one-minute bins, there were not enough chamber transitions

made across all groups, thus skewing the data. 5-minute time bins was the minimum amount of time that ensured homogeneity of variances. A mixed ANOVA revealed a significant effect of condition $F(2,146)=19.38$, $p<0.05$. This effect was such that CON (0.28±0.03) larvae transitioned significantly less than both LNIC (0.57±0.02) and HNIC (0.36±0.01) larvae, $p<0.05$ and $p=0.002$, respectively. Furthermore, LNIC larvae transitioned significantly more than HNIC larvae, $p=0.009$. There was also a significant interaction of time and condition $F(4,292)=2.415$, $p=0.049$. Simple effects test further analyzed this interaction and found that within the five minutes, CON (0.45±0.377) larvae transitioned significantly less than LNIC (3.06±0.374) and HNIC (2.40±0.374) larvae. Within the last five minutes, LNIC larvae (2.40±0.27) transitioned significantly more than CON (0.78±0.27) larvae, $p=0.001$. HNIC (1.40±0.27) larvae did not show any difference in transition frequency than CON, but did transition less than LNIC.

There was no significant change in frequency of transitions in CON or LNIC larvae from the first five minutes to the last five minutes. HNIC larvae transitioned significantly less within the last five minutes than the first five minutes of the trial, $p=0.023$.

4.5D Summary of Chamber Transitions

These results demonstrate that both LNIC and HNIC increased chamber transition frequency relative to CON. Furthermore, LNIC and HNIC larvae transitioned significantly more relative to CON across all chambers. The frequency of zone transitions did not change significantly over the duration of the trial in CON and LNIC larvae, but significantly decreased in HNIC larvae. Furthermore, both LNIC and HNIC larvae transitioned more than CON within the first 5 minutes of the trial, and that LNIC larvae transitioned more than CON and HNIC larvae in the last 5 minutes of the trial.

4.6 Latency to Center

Latency to enter the center zone was measured both overall and between chambers 1-3 and 4-6. The reason for analyzing latency between chambers 1-3 and 4-6 is because there were not enough occurrences of center zone entry to measure the latency changes over one minute bins, so examining the differences between chambers 1-3 and 4-6 are to provide an estimate of difference in time, as larvae in all treatment conditions generally did not enter the 4-6 chambers until the latter half of the trial.

4.6A Overall Latency to Center

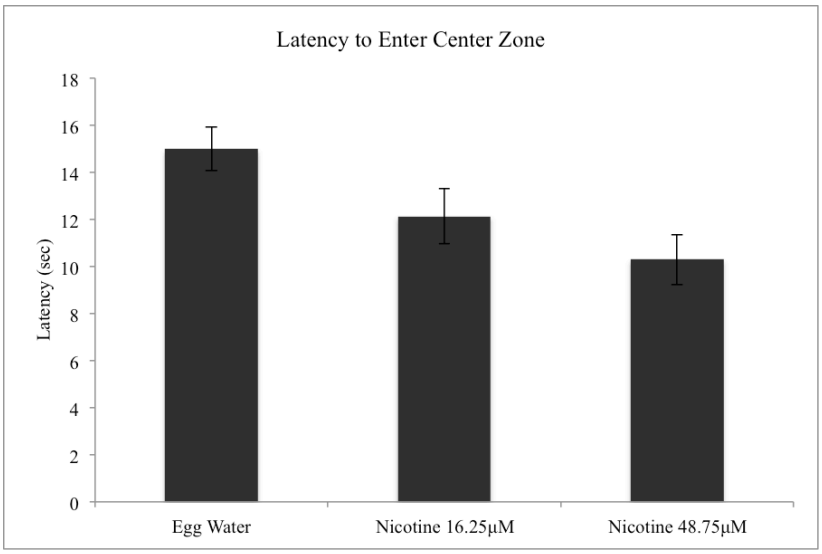


Figure 26 Latency to enter center zone averaged across each chamber. Bars represent mean±SEM

Overall latency to enter the center zone was measured averaged across all chambers within the testing arena for each treatment condition (see fig 26). An ANOVA revealed that nicotine treatment had a significant effect on latency to enter the center zone $F(2,147)=5.825$, $p=0.004$. This effect was such that both LNIC (11.42±1.11) and HNIC (10.29±0.99) larvae exhibited a shorter latency to enter the center zone than CON (14.98±0.89) larvae, $p=0.037$ and $p=0.004$, respectively. There was no observed difference in latency to enter the center between the two nicotine treated groups. These results suggest that nicotine treatment reduced the latency for the larvae to enter the center of the chamber.

4.6B Latency to Center: Between Chambers

Latency to enter the center zone within two sets of chambers, 1-3 and 4-6, was analyzed in order to estimate how latency to enter the center zone changed over time. Typically, larvae, independent of treatment, would transition to chambers 4-6 during the latter half of the trial compared to the chambers 1-3. (see fig 27). An ANOVA revealed that within chambers 1-3 there was a significant effect of nicotine treatment on latency to enter the center zone $F(2,146)=4.26$, $p=0.016$. This effect was such that HNIC larvae (10.22±1.06) had a significantly shorter latency to enter the center zone than CON (14.6±0.925) larvae. LNIC (12.13±1.16) larvae did not significantly differ from either HNIC or CON. Furthermore, latency to enter chambers 4-6 was significantly affected by nicotine treatment $F(2,49)=7.32$, $p=0.002$. Both LNIC (4.86±1.04) and HNIC (5.99±1.6) larvae had a significantly shorter latency to enter the center zone than CON (14.79±3.52), $p=0.001$ and $p=0.009$, respectively. There was no observed difference between the two nicotine treatment groups. Student's t-test revealed that there was no significant difference in the CON's latency to enter the center zone between the chambers 1-3 (9.55±1.65) and the chambers 4-6 (14.79±3.52). There was a significant difference in latency to enter the center in LNIC larvae within the chambers 1-3 (8.29±1.22) and 4-6 (4.869±1.04), $t(24)=2.57$, $p=0.017$.

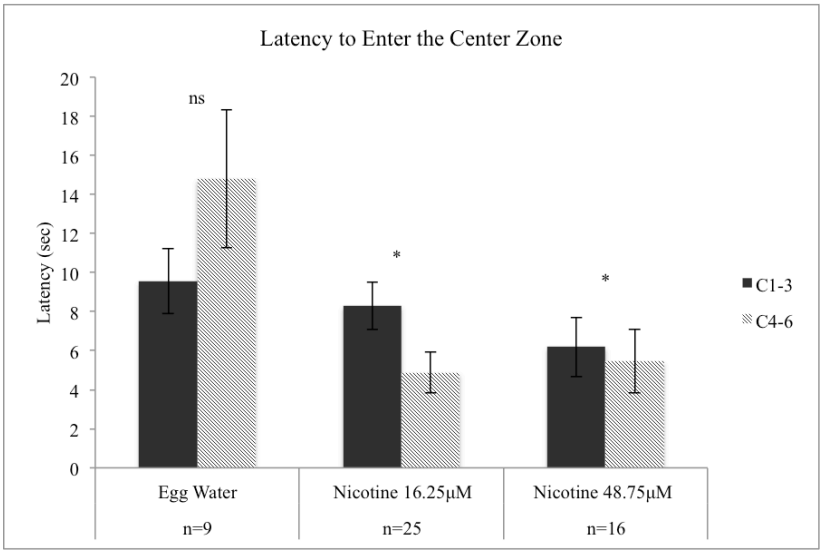


Figure 27 Latency to enter the center zone in chambers 1-3 and chambers 4-6, "*" indicate significance at the p=0.05 level, ns indicates non-significance. Bars are represented as mean±SEM

Lastly, there was no significant difference in the HNIC larvae in the chambers 1-3 (6.19±1.52) and 4-6 (5.44±1.62). These results suggest that LNIC reduces latency to enter the center zone, but is not reduced after treatment of HNIC.

4.6C Summary of Latency to Center

These results suggest that nicotine treatment had a significant effect on the latency to enter the center zone in larvae. Specifically, within the chambers further away from the initial chamber that the larvae were placed in, LNIC and HNIC larvae demonstrated a significant decrease in latency to enter the center relative to chambers 1-3, while CON did not show a difference in latency to enter the center zone.

Discussion

5.1 Interpretation of Present Results

Overall these results demonstrate that the novel 6-chamber complex environment can be used to evaluate a wide variety of larval zebrafish behavior and has potential use for examining behavioral phenotypes expression due to acute drug exposure, including but not limited to, general locomotor activity, thigmotaxis and thigmotaxis types, latency to enter zones, chamber transitions. The novelty of this testing apparatus is the ability to measure chamber transitions within a 6-chamber complex environment, this can allow for the analysis of not only overall effects of drugs on exploratory behavior, but also time-dependent drug responses. Furthermore, this testing apparatus accounts for possible biases in turning behavior as the left and right environments are mirror images of each other. Furthermore, this testing apparatus accounts for vertical movement to some extent because in order to transition to a different chamber, larvae have to reach a swim up a 7mm above from the bottom of the chamber. This is more valuable than studying an animal within a single well or chamber because it provides a more complex

analysis of behavior and reduces the likelihood of mistakenly labeling behaviors (i.e. labeling increased exploratory behavior as reduction in anxiety). Furthermore, this novel environment is capable of examining the larval zebrafish response to repeated novelty within a single trial, as each chamber that the larvae may transition to is different from the one it previously occupied. The ability to measure myriad behavioral endpoints with the 6-chamber complex environment coupled with appropriate drug dosing procedure opens the possibility of a powerful tool for drug screening and further analysis of the effects of drugs on behavior. This study demonstrated that the effects of acute nicotine on larval zebrafish are complex and behavioral complexities not previously reported in larval zebrafish.

Across most behavioral endpoints used in this study, nicotine treated larvae exhibited a dose-dependent behavioral response as seen in previous studies in larval zebrafish (Petzold et al. 2009). Furthermore, larval zebrafish exhibit an innate preference for the outer zone of a chamber when placed in a novel environment (Steenbergen et al. 2012). LNIC increased thigmotaxis significantly more relative to both HNIC and CON both overall and within some chambers of the testing apparatus. However, within some specific chambers, HNIC reduced thigmotaxic behavior in the larvae relative to both CON and LNIC. Furthermore LNIC larvae engaged in more active and weakly active thigmotaxic behavior than both CON and HNIC. Although HNIC also exhibited more weakly active thigmotaxis than CON larvae, this may be due to the effect of nicotine on exploratory behavior, as both HNIC and LNIC larvae transitioned to different chambers more than CON larvae. This suggests that while LNIC elicits an anxiogenic HNIC elicits an anxiolytic response. In light of the increase of locomotor activity in both nicotine treated groups, the differential changes in thigmotaxic behavior due to LNIC and HNIC cannot be attributed to a difference in general locomotor activity. By using distance moved and time

spent measures of thigmotaxis, changes in thigmotaxic behavior across all three conditions cannot be attributed to increase in general locomotor activity as the effects of nicotine treatment on the two measures of thigmotaxis were generally conserved.

Nevertheless, nicotine treatment increased locomotor activity early on in the trial, but then began to decrease throughout the duration of the trial. In this case, the LNIC demonstrated a decrease in locomotor activity by the last minute of the trial while the HNIC maintained a more locomotor activity than both the CON and LNIC larvae. This suggests that the HNIC motivated more locomotor activity than LNIC. However, this result is further complicated when looking at the chamber transition results.

Larvae treated with LNIC demonstrated an increased amount of transitions than both the CON and HNIC. These results fit in with the discrepancies between the LNIC and HNIC general locomotor activity when viewed in the context of thigmotaxic behavior. As LNIC not only exhibited more thigmotaxis, but also more active and weakly active thigmotaxis than both CON and HNIC, LNIC larvae would demonstrate more transitions as they were following the wall more tightly than the egg water treated or the higher dose nicotine treated, therefore reducing the time that they spent in the center well and increasing the probability of transitioning to a different chamber.

Previous literature has demonstrated that thigmotaxis is a valid measure of anxiety-driven behavior such that increased thigmotaxis demonstrates increased anxiety while decreased thigmotaxis demonstrates decreased anxiety. Furthermore, studies have shown that nicotine reduces anxiety in adult zebrafish (Levin et al. 2007). However, the data from this study suggests that LNIC caused an increased in thigmotaxis. Therefore, this data would suggest that LNIC increases anxiety. While this may be the case, it is also possible that thigmotaxis is not as a

reliable measure of anxiety in larval zebrafish. This assertion substantiated is by the latency to enter the center one arena.

The reason for why thigmotaxic behavior is a marker of anxiety is based on that an animal, when in the center of a space, is more open to predation and other forms of harm, therefore the animal moves towards the borders of its environment to attempt to escape harm. When given an anxiolytic drug such as diazepam or other anxiolytics commonly prescribed to humans, the animal is more likely to move and spend time in the center of their environment. While the LNIC larvae demonstrated higher thigmotaxis than CON and HNIC, both LNIC and HNIC larvae exhibited a shorter latency to enter the center zone than the CON. This suggests that when first entering a chamber, larvae treated with nicotine are quicker to enter the center zone than control fish. While this may be attributed to a general increase in locomotor activity, when examining the between chamber differences (1-3 as opposed to 4-6), the latency to enter the center zone of the LNIC and HNIC larvae significantly decreased when in chambers 4-6 than 1-3, while CON exhibited the same latency to enter the center zone in chambers 1-3 as 4-6. This suggests that nicotine treated larvae were more willing to enter the center zone of a new environment than controls, potentially demonstrating a reduction in anxiety, in spite of increased thigmotaxis.

The motivation behind analyzing the differences in latency between chambers 1-3 and chambers 4-6 was to estimate whether there was an effect of time on latency to transition. Assuming that latency within chambers 1-3 represented the latency within the first half of the trial, while latency within chambers 4-6 represented the latter half of the trial, then there is a time-dependent effect of nicotine on the latency to enter the center zone in larval zebrafish. The evidence for a time-dependent effect is supported by the time by condition interactions observed

in both the general locomotor activity and chamber transition time binned data. Another explanation for the observed effect of time is that nicotine reduced the larval zebrafish reaction to novelty. As chambers 1-3 include the initial chamber that the larvae are placed into, and chambers 4-6 are entirely novel chambers, it is possible that both LNIC and HNIC cause the larvae to be less fearful of novel environments.

Another way of interpreting the present results is by distinguishing between anxiolytic and exploratory behavior. LNIC larvae demonstrated increased locomotor activity, thigmotaxis, active thigmotaxis path taking, chamber transitions, and decreased latency to enter the center far away chambers. Together, these results suggest that LNIC increases exploratory behavior, but does not affect anxiety. In comparison, HNIC larvae demonstrated increase locomotor activity, slight decrease in thigmotaxis, no increase in active thigmotaxis path taking, increased chamber transitions, and decreased latency to enter the center of far away chambers. This suggests that HNIC increases exploratory behavior, but has anxiolytic effect on zebrafish larvae. Furthermore, as nicotinic receptors are mainly located on dopaminergic neurons, it is possible that nicotine mainly effects locomotor behavior, this would be evidence by this study because both low dose and high dose nicotine treated larvae exhibited and increase in locomotor behavior relative to the CON. Nicotine has been shown to have some effect on serotonin, but it has been unclear whether the effect is downstream of the primary activation of nicotinic receptors, or if there are nicotinic receptors on serotonergic neurons. In the former case, it is possible that the increase in exploratory behavior followed by the reinforcement that no harm is occurring causes a change in serotonin levels, while in the latter case nicotine would have a direct impact on the modulation of serotonin thereby expressing a direct effect on serotonin-mediated behaviors such as anxiety. As

the commonly prescribed drugs for pathological anxiety are compounds that mainly affect serotonin levels, it is unclear the mechanism that nicotine reduces anxiety—even in humans.

5.2 Limitations and Future Directions

During the execution of this study it was difficult to test the larvae at the same time of day, which may have affected the locomotor behavior of the larvae, as they tend to be more active during the early morning than in the afternoon (MacPhail et al. 2009). Furthermore, a reoccurring issue during this experiment was that for CON larvae, not enough subjects would enter the farther away cambers (chambers 4-6); therefore future modifications of this complex environment might employ 3 chambers rather than 6. Due to time issues, this experiment was unable to report the effects of nicotinic receptor antagonist, mecamylamine, on the behavioral endpoints measured in this study. Preliminary observations suggest that mecamylamine attenuates the nicotine-induced behaviors of high dose nicotine (not yet tested in low-dose nicotine). The use of nicotinic receptor antagonists may enable further characterization of exploratory versus anxiety-related behaviors. Future studies using the 6-chamber complex environment can combine different anxiety-based assays, such as the light-dark test to further examine behavioral phenotypes associated with anxiety, therefore removing the reliance on thigmotaxis and other common behavioral traits to demonstrate anxiety-driven behavior. Furthermore, in order to fully evaluate the utility of this 6-chamber complex environment for future behavioral testing, other compounds, perhaps better understand drugs need to be tested and compared to previous studies to see whether the behavioral changes are consistent. One possibility for examining anxiety-driven behavior is using an anxiogenic such as caffeine, and a commonly prescribed anxiolytic such as diazepam. Although it would be interesting to investigate fluoxetine, another commonly prescribed anxiolytic, that has been shown to be

efficacious in treating pathological anxiety, but has been confounded within the literature as the current behavioral assays available are unable to detect an anxiolytic effect of fluoxetine on their subjects using the common behavioral endpoints such as thigmotaxis. To further understand the cellular and molecular differences of nicotine, it would be interesting to quantify the neurotransmitters—GABA, dopamine, and serotonin—as well as their metabolites to see whether there are differences in neurotransmitter levels that can be attributed to specific doses of nicotine. Furthermore, if the 6-chamber complex environment is used to determine behavioral phenotypes that are linked to the desensitization of a nicotinic receptor, quantifying the neurotransmitter levels may give an idea as to on what type of neuron the desensitization is occurring. In regards to the desensitization of nAChRs is that it is possible that the methodology used in this study, immersion rather than knockout procedure allowed for the desensitization of nicotine, which can partially explain the time dependent effects of nicotine. A study that would examine how larval zebrafish exposed to a wash-out treatment schedule would be interesting to see if the same time-dependent effects of nicotine on general locomotor activity and latency to enter the center of the arena would be observed.

Nicotine plays a complicated role at the cellular level of nicotinic receptors. While it was previously thought that activation of these receptors by nicotine is the reason for the observed behavioral (addiction) and affective (anxiety) changes, recent research has suggested that these behavioral and affective changes can be attributed to the desensitization of nicotinic receptors by nicotine. One study found that rats exhibited anxiolytic behavior after a low dose administration of nicotine, and showed that the desensitization of the β_2 subunit of the nicotinic receptors found within the ventral tegmental area and nucleus accumbens was responsible for this change in anxiety-driven behavior, while activation of the nicotinic receptors contributed to the observed

addiction (Anderson & Brunzell 2012). It is not yet clear the relationship between dose strength and extent of desensitization, but this research demonstrates, that the effect of nicotine is not clear-cut due to its ability to quickly desensitize nicotinic receptors.

The results of this experiment demonstrate not only dose-dependent behavioral changes due to nicotine, but also results that are inconsistent with the current literature on anxiety-driven behavior in rodents and adult zebrafish. While these inconsistent observations may be due to intrinsic properties of larval zebrafish behavioral phenotypes and possibly molecular and cellular differences in nicotinic receptor function, they can still be resolved by examining the data within the context of other behavioral endpoints that were measured in this study. This study suggests that the larval zebrafish has a vast repertoire of behavioral phenotypes that have not yet been explored due to the limitations of current behavioral assays, but also the effect of different nicotine doses on the aforementioned behaviors. The move towards the characterization of more complex behaviors in larval zebrafish is especially important as the use of nicotine products rises. The 6-chamber complex environment provides a novel behavioral assay to further understand the effects of nicotine on behavior and has the potential to be used for the screening of novel drugs in larval zebrafish.

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