Appetitive Learning Paradigm for Zebrafish (*Danio rerio*) in their Home Tanks Utilising Visual or Olfactory Cues

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
August 2017

I would like to dedicate this thesis to my fiancé, Anthony Edmonds for all his love and support but also his assistance with apparatus modifications, visits to the hardware store, mathematical equations and grammatical corrections.

I would also like to dedicate this thesis to my sister, Christina Doyle for all her moral support and help with fish tracking, coding and corrections.

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ABSTRACT

A visual or olfactory stimulus (green light or phenylethyl alcohol) was presented to groups of adult zebrafish in their home tanks. An automatic feeder dispensed food immediately after the conditioned stimuli (CS), or at variable delays for controls. Fish showed anticipatory movement towards the food dispensing area after as few as 7-10 trials, learning that the CS was a predictor of food presentation at the water surface. Memories of the conditioned association persisted at least 2 days after training when fish were again presented with the CS. Control fish, for which the visual or olfactory stimuli were unpaired with food, showed no response when exposed to the CS. This simple, low-cost, automated system permits scalable conditioning of zebrafish with minimal human intervention, greatly reducing both variability and labour-intensiveness. It will be useful for studies of the neural basis of learning and memory, and for high-throughput screening of compounds modifying those processes.

LIST OF ABBREVIATIONS USED

ΔAIC	Change in Akaike information criterion
°C	Degrees Celsius
%	Percent
3D	Three-dimensional
ANOVA	Analysis of variance
cm	Centimeter
CS	Conditioned stimulus
dpf	Days post-fertilisation
ERK	Extracellular signal-regulated kinase
FM	Frequency modulated
fps	Frames per Second
h	Hour
hrs	Hours
Hz	Hertz
hpf	Hours post-fertilisation
LED	Light-emitting diode
L	Litre
mg	Milligram
mL	Millilitre
mm	Millimetre
min	Minute

pERK	Phosphorylated extracellular signal-related kinase
psu	Practical Salinity Units
ROI	Region of Interest
sec	Seconds
S.E.M	Standard error of mean
UCS	Unconditioned Stimulus
UV	Ultraviolet

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge the funding that made this research possible: the Alexander Graham Bell Canada Graduate Scholarship-Master's from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Nova Scotia Graduate Entrance Scholarship from the Government of Nova Scotia.

This project would not have been possible without the support and encouragement of my supervisors, Dr. Roger Croll and Dr. Alan Fine. I'm especially grateful for their guidance and allowing me use of their labs, equipment, and time.

Acknowledgement and thanks also goes to my committee members, Drs. Younes Anini, Robert Rose, Simon Gadbois, Patrice Côté, Päivi Torkkeli, and Steven Barnes, for their feedback and assistance in this project.

I am thankful for all those who helped me with lab techniques and technical concerns: Dr. Matthew Stoyek, Dr. Arnaud Gaudin and Len Edmonds. I also wish to extend my thanks and appreciation to my lab-mates, Neil Merovitch, Alexia Scaros, Anna Semaniakou and Griffin Beach for their support and helpful comments.

Thanks are also due to everyone who assisted me with data collection: Juyang Long, Emma Finlayson-Trick, Charlotte Nauss, Setareh Lahsaee, Megan Crosby, Emma Neilson, Stephanie Shewchuk, Remington Manchester, Nicole Pickett. Special thanks to Omar Sickander for his assistance with the olfactory experiments.

I would also like to express my gratitude to my family, for their love and continued support in all my endeavours.

CHAPTER 1: INTRODUCTION

1.1 ZEBRAFISH AS A MODEL ORGANISM

Zebrafish are providing increasing opportunities as models for scientific research. They share similar developmental and physiological processes and homologous genes with higher vertebrates, making for a less complex model to study more complicated mammalian systems (Bally-Cuif & Vernier 2010). Zebrafish are also small, have a high fecundity and are easy to maintain, making them well suited to high-throughput studies (Sumbre & de Polavieja 2014; Traver et al. 2003; Lee et al. 2007; Laughlin et al. 2008; Lawson 2002; Patton & Zon 2002). Larval zebrafish and some mutant strains are largely transparent, allowing their internal structures to be visible (White et al. 2008), also making them an excellent choice for *in vivo* optical imaging of electrical activity and optogenetic activation or inhibition of sets of neurons (Sumbre & de Polavieja 2014).

In addition to the benefits of various strains and general maintenance, zebrafish also possess a wide range of well documented behaviours, which in combination with the aforementioned characteristics make them suitable for studies in which behaviour is altered (Kalueff et al. 2013). In fact, zebrafish have become a popular model for examining the effects of various drugs on behaviour. For example, zebrafish are used to study addiction and withdrawal with alcohol or cocaine and also as model to examine the effects of hallucinogens on neurochemistry (Gerlai et al. 2009; Gerlai et al. 2000; Lockwood et al. 2004; Tran et al. 2016; Darland & Dowling 2001; López Patiño et al. 2008; Grossman et al. 2010; Kyzar et al. 2012; Cachat et al. 2013). Studies that use zebrafish as a behavioural model often rely on learning paradigms to examine how

certain substances or conditions affect the fish's ability to learn and remember associations (Chacon & Luchiari 2014; Levin & Chen 2004).

1.2 IMPORTANCE OF LEARNING PARADIGMS

Using a variety of learning paradigms, it is possible to determine the basic abilities of zebrafish to learn associations. For example, several studies on the visual capabilities of zebrafish have determined that they could detect and distinguish between different colours and shapes (Colwill et al. 2005; Fetsko 2003). In one study, fish were successfully trained to associate one colour or shape with food and could correctly identify it when given a choice between the familiar and the unfamiliar colour or shape (Colwill et al. 2005). Similarly, several olfactory experiments used learning paradigms to determine that zebrafish and other species can discriminate between certain amino acids. Catfish were conditioned to associate L-amino acids with food and then exposed to an unconditioned amino acid. Fish exhibited increased food searching behaviour when exposed to the conditioned amino acid but not when exposed to the unconditioned ones; however, the catfish required multiple pairings to distinguish between similar amino acids (Miklavc & Valentinčič 2012; Valentinčič et al. 1994; Valentinčič et al. 2000).

In addition to imparting important information about the detection and discrimination abilities of animals, learning paradigms can help examine the genetic effects on cognition. Zebrafish are already used as models for studying the cognitive decline associated with age (Yu et al. 2006; Paquet et al. 2010). The use of transgenic strains, like those that approximate the cellular aggregates found in Alzheimer's Disease

and other tauopathies, allow experimenters to use zebrafish as models of disease states (Kalueff et al. 2014; Rubinstein 2003; Newman et al. 2014; Gerlai 2012; Paquet et al. 2010). There are even models in development for other cognitive disorders such as schizophrenia and autism spectrum disorder (Gerlai 2012; Tropepe & Sive 2003; Stewart et al. 2014). These models could be used in conjunction with learning paradigms to examine the effects that these diseases have on the ability to learn and retain associations.

Learning paradigms can also be used to explore the underlying processes governing learning and memory. Most of these learning paradigms, developed for use with rodents, have provided important insight into the regions of the brain and the neurotransmitters involved in mammalian learning and memory retrieval (Jarrard 1993; Morris 2008; McClelland et al. 1995; Owen et al. 1997; Hyman et al. 2006; Myhrer 2003; Johansen et al. 2011; Blokland 1996; Ammassari-Teule & Caprioli 1985; Maurice et al. 1996). Learning paradigms for zebrafish have also been successful in examining the underlying mechanisms of learning. For instance, experiments have found that nicotine exposure improved zebrafish performance at learning tasks when compared to a control group (Eddins et al. 2009; Levin et al. 2006). Learning paradigms have also been used extensively to examine the retrieval of learned associations in zebrafish. Several studies have determined that MK-801, an N-methyl-d-aspartate (NMDA) receptor antagonist, can block retrieval of a learned association when administered after conditioning (Blank et al. 2009; Sison & Gerlai 2011). Some studies suggest that the formation of memories can also be blocked by MK-801 exposure prior to training (Cognato et al. 2012), but that

finding is contentious, as other studies have been unsuccessful at blocking memory formation with pre-exposure to MK-801 (Dix et al. 2010; Castellano et al. 2001). Regardless, the demonstrated role of MK-801 suggests that memory retrieval is glutamate mediated, a finding that is consistent with studies of other vertebrates (Sweatt 2010). Other studies further explored the role of the cholinergic system by examining induced learning impairments in zebrafish caused by the anti-nausea medication, scopolamine, a cholinergic blocker. Using the zebrafish, they found that these impairments could be prevented by pre-treatment with the flavonol, quercetin or the cholinesterase inhibitor, physostigmine, indicating the importance of acetylcholine in learning (Kim et al. 2010; Richetti et al. 2011). To more effectively explore learning and the various methods by which it can be effected in zebrafish, these types of studies require simple but robust learning paradigms.

1.3 FACTORS TO CONSIDER WHEN USING ZEBRAFISH FOR LEARNING STUDIES

Various factors must be taken into account when adapting learning paradigms for zebrafish. One of the most important is selection of the unconditioned stimulus (UCS). As with conditioning in other animals, paradigms use either an aversive or attractive stimulus. Several studies use changes in tank lighting, as a conditioned stimulus (CS), paired with electric shock, as an aversive UCS. Such a pairing is highly effective but has undesirable consequences because unlike electric shock applied to mammals, it is difficult to localize in an aqueous milieu and must be applied to an entire tank (Pradel et al. 1999; Gleason et al. 1977; Agetsuma et al. 2012; Blank et al. 2009). A generalized electric shock may disrupt normal physiological processes and thereby

confound elicited behavioural responses and analyses (Gerlai 2011). Conversely, studies that use positive reinforcement usually consist of a food reward. One major drawback of using a food reward is that fish may become satiated and therefore may ignore the UCS upon repeated presentation (Sison & Gerlai 2011; Al-Imari & Gerlai 2008). This factor must be taken into account when determining the amount of food reward and the number of pairings per day.

Another major factor to consider is the apparatus in which the fish are conditioned. Introducing fish to a specialized apparatus needed for certain paradigms requires long periods of acclimation. Furthermore, the increased handling necessary to transport fish has been shown to cause increased cortisol levels, which could change behaviour and interfere with results (Ramsay et al. 2009). Zebrafish are shoaling animals and often exhibit anxious behaviours when isolated (Engeszer et al. 2007), so testing fish individually may also contribute to higher stress levels (Sison & Gerlai 2011; Braubach et al. 2009; Blank et al. 2009; Al-Imari & Gerlai 2008). Therefore, a paradigm that conditions zebrafish in groups may garner a truer response from the fish, with the added advantage of training more fish in a shorter period of time, making it beneficial for high-throughput screening (Braubach et al. 2011; Wyeth et al. 2011).

1.4 EXPLORING SENSORY MODALITIES

Many paradigms focus on the ability of the zebrafish to navigate using visible cues because they are highly visual animals (Easter & Nicola 1996; Neuhauss 2010), but other paradigms focus on olfaction (Doyle et al. 2017; Valentinčič et al. 2000; Braubach et al. 2009), and our lab has recently developed a robust, auditory appetitive learning

paradigm for conditioning zebrafish (Doyle et al. 2017; Merovitch 2016; Merovitch et al. 2016).

The auditory conditioning paradigm presented by Doyle et al (2017) and Merovitch (2016) was robust, very effective and suitable for high throughput. Groups of five fish were conditioned in their home tank, which reduced handling stress common to other paradigms (See Section 1.3 – Factors to consider when using zebrafish for learning studies). Continual frequency modulated (FM) tone sweeps (100-1000-100 Hz) acted as the CS and were paired with a food reward (UCS) ten times daily for two days. The entire system was automated to minimize experimenter intervention and ensure precise amounts of food are presented. A camera recorded the behaviour before and during presentation of the CS, then software was used to track horizontal and vertical positions of the fish. From those data, the net movement of the fish towards the food source was calculated and those scores were then compared to the movement of a control group which experienced an auditory cue unpaired with the food reward. The experimental group exhibited fast, efficient learning by the 5th pairing of the auditory stimulus and food reward.

The experiment also examined the ability of the fish to retain the learned association. Probe trials were conducted with groups of fish at 2 and 16 days after conditioning, then with individual fish at 2, 4, 8, 16 and 32 days after conditioning. The fish retained the association for at least two days when tested in groups or as individuals, and then the strength of the association declined over the next month. **Due**

to the success of the auditory paradigm, the aim of this thesis is to determine whether the paradigm could be adapted to use visual or olfactory cues.

1.5 VISUAL LEARNING PARADIGM

Zebrafish possess excellent vision systems, so a visible conditioned cue should represent a particularly salient stimulus (Fleisch & Neuhauss 2006; Easter & Nicola 1996; Neuhauss 2010). Therefore, Chapter 2 will focus on adaptation of the paradigm to use a visual cue. The factors that were considered when choosing the visual stimulus will be fully discussed in Section 2.1.3 – Factors to consider when using visual paradigms.

1.5.1 Objectives

- Adapt an existing auditory learning paradigm (Doyle et al. 2017; Merovitch 2016) to use a salient visual cue (green LED) as the conditioned stimulus.
- Use the adapted paradigm to examine the rate of acquisition of the association.
- Determine if zebrafish retain the memory of the association in groups with probe trials at 2 and 16 days, but also as individuals at 2, 4, 8, 16 and 32 days.

1.6 OLFACTORY LEARNING PARADIGM

Chapter 3 focuses on adapting the paradigm to use an olfactory cue as the CS. Using olfactory cues, especially in an aqueous environment, presents unique challenges not encountered with visual or auditory stimuli. It is necessary for the fish to encounter

the olfactory cue in a concentration that is above their threshold of detection. Due to the imprecise nature of odourant dispersal, many hyper-concentrated regions may exist temporarily, even if the final mixed concentration is not above the detection threshold of the zebrafish. It is also necessary to achieve washout, lowering the concentration of odour below the detection threshold subsequent to each pairing. When a paradigm involves multiple pairings, it is particularly important to achieve washout well before subsequent pairings. Due to these specific requirements, olfactory paradigms require a greater volume of water than other paradigms. All of the obstacles associated with olfactory stimuli will be fully discussed in Section 3.1.3 – Technical problems with olfactory paradigms.

1.6.1 Objectives

- Develop a robust olfactory paradigm to condition groups of adult zebrafish in their home tanks.
- Determine the rate of acquisition of the learned association.
- Examine the duration of the learned association by performing probe trials 6-7 days and 13-14 days after training.

CHAPTER 2: VISUAL LEARNING PARADIGM

2.1 VISUAL INTRODUCTION

2.1.1 Zebrafish as a model for vision

Vision is critical for the survival of zebrafish; it is used for food detection, predator avoidance and identification of conspecifics (other zebrafish) (Easter & Nicola 1996). An obvious indicator of the importance vision is the disproportionately large eye size in zebrafish larvae (Fleisch & Neuhauss 2006). The zebrafish visual system develops very early, so that the fish can evade predators and capture food (Neuhauss 2010). For example, the visual startle response develops by 70 hours post fertilisation (hpf), and by 72 hpf the eye and lens are emmetropic, meaning that they gain the ability to focus (Easter & Nicola 1996; Neuhauss 2010).

Zebrafish are a popular visual model because their visual system is similar in organization to higher vertebrates but is much less complex (Bilotta & Saszik 2001). In addition, the zebrafish retina possesses similar cell types and layering as other vertebrate retinas (Hitchcock & Raymond 2004; Fleisch & Neuhauss 2006). The zebrafish retina is capable of regeneration after injury, even in adult animals, which makes it popular for studies examining ocular damage (Fleisch & Neuhauss 2006; Hitchcock & Raymond 2004).

2.1.2 Assessing function through behaviour

As stated in Chapter 1, transgenic and mutant zebrafish strains are extensively used for research on a wide variety of topics. Several strains exist that emulate specific eye deficits or diseases, such as glaucoma or retinal degeneration (Stujenske et al. 2011;

Penberthy et al. 2002; Goldsmith 2001). However, the severity of the deficits may be difficult to determine because even though zebrafish rely heavily upon vision, they can navigate using other senses (Burgess et al. 2010; Ghysen & Dambly-Chaudiere 2004). Conversely, transgenic or mutant strains may be used to study another aspect of zebrafish physiology or anatomy but may have unwanted visual deficits that prevent the fish from being used in a particular paradigm (Brockerhoff et al. 1995, 1998; Neuhauss et al. 1999; Gross et al. 2005; Muto et al. 2005). For example, the sleepy (sly) mutant expresses notochord and brain defects, but it would be difficult to examine the effect of these defects on behaviour because they also possess a visual deficit that affects their mobility (Neuhauss 2003). Learning paradigms that rely on vision can be useful in determining the fish's visual acuity, as fish with severe visual deficits will not be able to see a light cue or successfully associate a shape with an unconditioned stimulus (UCS).

2.1.3 Factors to consider when using visual paradigms

The majority of existing learning paradigms for zebrafish focus on visual cues as either the conditioned stimulus (CS), the UCS or sometimes both (Fleisch & Neuhauss 2006; Colwill et al. 2005; Arthur & Levin 2001; Mueller & Neuhauss 2012). Choice of the UCS is important, as each has its benefits and drawbacks. Aversive stimuli, such as the sight of a predator or electric shock, are effective but can cause stress for the fish (Xu et al. 2007; Agetsuma et al. 2012; Blank et al. 2009; Gerlai et al. 2009; Gerlai 2011). Conversely, a positive stimulus, like the sight of conspecifics, is effective as a UCS, due to their shoaling nature (Spence et al. 2008); however, this stimulus would be difficult to accomplish logistically in home tank learning paradigms, as exploited in this thesis (see

Section 2.1.5 – Benefits of a home tank paradigm). Another traditional positive stimulus is food, which zebrafish usually identify visually, though complications can arise with satiation; therefore, the amount of food administered must be calculated carefully (Williams et al. 2002; Sison & Gerlai 2011).

Like the UCS, the CS can also take various forms. A very common CS is the use of an indicator light or lighted area in a tank (Blank et al. 2009; Xu et al. 2007). The light can be white but also can utilize a variety of colours. Zebrafish utilise the same visual spectrum as humans but also include the ultraviolet spectrum (Easter & Nicola 1996). They possess four distinct photopigments: ultraviolet (UV) sensitive pigment (λ_{max} 362 nm), short wavelength sensitive pigment (λ_{max} 415 nm, medium sensitive wavelength pigment (λ_{max} 480 nm) and long sensitive wavelength pigment (λ_{max} 570 nm) (Bilotta & Saszik 2001). Despite this knowledge of the eye structure, there is still debate about their capacity to visualize distinct colours (Risner et al. 2006; Fleisch & Neuhauss 2006; Neuhauss 2010; Avdesh et al. 2012; Bilotta & Saszik 2001). Regardless, they are able to differentiate between stimuli of different colours, as several paradigms use colours as the conditioned stimuli (Colwill et al. 2005; Sison & Gerlai 2011; Bilotta et al. 2005; Williams et al. 2002).

2.1.4 Current visual learning paradigms

For brevity, I will review a selection of the most common types of visual paradigms. As mentioned in the previous section, many paradigms use aversive stimuli, of which electric shock is by far the most common. An example of a cued-fear conditioning paradigm was presented by Agetsuma et al. (2012). This paradigm used a

red light to repeatedly warn fish of an impending electric shock. After training, the fish exhibited freezing behaviour upon presentation of the CS. Avoidance paradigms are also popular and most use a tank arranged like a shuttle box. One paradigm has the fish move from a lighted area to an area of darkness to avoid an electric shock. The fish were exposed to 20 to 40 pairings spread over 1 to 3 sessions, although another paradigm with similar methods presented it as a single trial avoidance paradigm (Xu et al. 2007; Blank et al. 2009).

There are also several paradigms that utilize attractive or appetitive stimuli. Sison & Gerlai (2010) present a paradigm that used a four-armed maze with a coloured card (CS) to indicate the arm with the UCS (the sight of conspecifics). The fish learned this association over the course of 16 trials (four trials per day for four days). A paradigm by Chacon & Luchiari (2014) trained fish to associate an indicator light (CS) in a specific area of the tank with the impending delivery of food (UCS) over a period of eight days. A paradigm presented by Mueller & Neuhauss (2012) is one of the few that shows full automation with UCS (food) and CS (video screen with an arbitrary visual stimulus) delivery and fish tracking. However this paradigm, like all of the others mentioned in this section, requires a specialized tank for conditioning.

2.1.5 Benefits of a home tank paradigm

As mentioned in the previous chapter, Doyle et al. (2017) and Merovitch (2016) demonstrated a robust auditory paradigm using appetitive conditioning for zebrafish. Fish showed an association between the UCS and CS by the 5^{th} pairing. Due to the effectiveness of the auditory paradigm, the goal of this chapter is to adapt the paradigm

to utilize a visual cue. Training the fish in their home tank reduces handling stress and minimizes acclimation time. It is also easier and more convenient to train fish if a specialized tank is not required. The result is a fully automated, cost-effective, appetitive paradigm that does not require a special tank and has a minimal stress impact on the fish.

2.1.6 Summary

The goal of this experiment was to create an effective learning paradigm for zebrafish in their home tanks by adapting an existing auditory paradigm (Doyle et al. 2017; Merovitch 2016) to use a salient visual cue (green LED) as the conditioned stimulus. This section also examined retention of learned association in groups with probe trials at 2 and 16 days, but also as individuals at 2, 4, 8, 16 and 32 days.

2.2 MATERIALS & METHODS

2.2.1 Animals

Wild-type adult zebrafish, 3.5-4.0 cm in length, (PetSmart, Bedford, NS, CAN), were housed as mixed-gender groups of five fish in 3 litre plastic tanks (Pentair Aquatic Eco-Systems, Apopkoka, FL, USA), beginning at least two days prior to experimentation. The fish were maintained on a 14:10 hour light: dark cycle and in municipal water (28.5°C) that had undergone reverse osmosis and was then treated with 600 mg Instant Ocean (United Pet Group, Blacksburg, VA, USA) and 26.4 mg sodium bicarbonate (Pentair Aquatic Eco-Systems, Apopkoka, FL, USA) per litre. Each tank was provided with a water flow of 13-14 litres per hour. Adult fish were normally fed twice daily using 300-500 micron pellets of Golden Pearl Reef Diet (Brine Shrimp Direct, Ogden, UT, USA). All experiments were conducted in accordance with the Canadian Council on Animal Care standards and guidelines (Protocol #: 14-132).

2.2.2 Experimental apparatus

For training and testing, each home tank containing five fish was moved from the rack on which they were routinely maintained to a specialized rack partitioned into three arenas, each containing one fish tank (Figure 2.1). Arenas were separated from one another by white corrugated plastic sheets (Coroplast, Granby, QC, CAN), and the back wall of the enclosure was covered in translucent white nylon fabric, which diffused the LED backlighting for each tank (1600 lumen LED work lights, Snap-on, Kenosha, WI, USA). While on the training/testing rack, each tank was provided with recirculating water from a dedicated 40 litre reservoir.

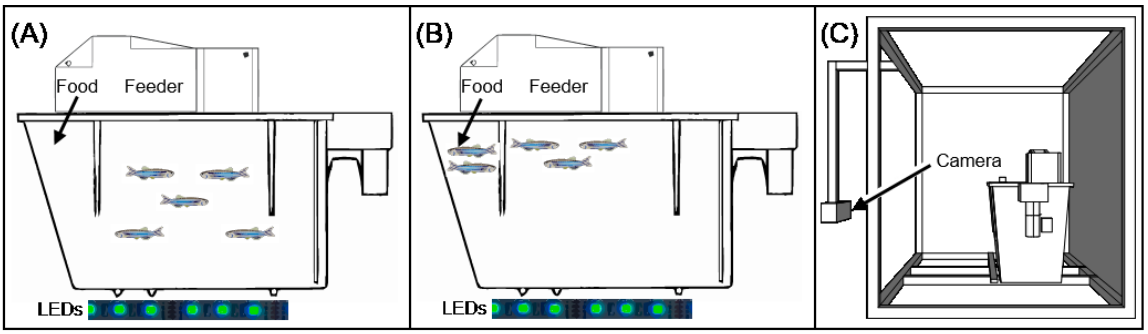

Figure 2.1: Diagram of behavioural apparatus for visual conditioning in home tanks. Panel A and B illustrate the positions of control and experimental fish respectively during the presentation of conditioned stimulus. Green LED strips were used for the presentation of the visual stimuli. Food pellets were dispensed by the automatic feeder located above the tank. Panel C illustrates the position of the camera relative to the tank.

A micro controller (Arduino Uno, Arduino, Ivrea, ITA) with an associated motor control board (shield) (Product ID: 1438), and DS1307 real time clock (Product ID: 264) from Adafruit, New York, NY, USA was used to control automatic feeders and to present visual stimuli. Arduino programs (sketches) were created in the Arduino integrated development environment (Arduino 2014) utilizing the following libraries to control the experiments: Time (Margolis 2016), TimeAlarms (Margolis 2014), and Motorshield (Adafruit 2016). See Appendix B for Arduino sketches.

An automatic feeder, produced with a 3D printer (Replicator 2, Makerbot, New York, NY, USA) using biodegradable polylactic acid thermoplastic (stereolithography file downloadable from http://crollab.physiology.dal.ca/automaticfeeder) was placed over an existing hole in the lid of each tank (Figure 2.1). Food was placed in the hopper of each feeder and could be dispensed using a stepper motor (Sparkfun, Niwot, CO, USA) which turned a 5 mm steel drill bit. The bit served as an auger to dispense approximately 10 mg of food at a time. A white plastic divider was placed at the level of the water, 6.5 cm from the front, to keep the dispensed food floating near the feeder.

The visual conditioned stimulus was presented using a 15 cm light strip with 6 RGB LEDs (Mosaic LED Flexible Light Kit, Sylvania, Danvers, MA, USA). The LED strips were placed against each tank on the support shelf, visible to both the camera and fish (Figure 2.1). The visual conditioned stimulus consisted of green illumination, and was selected based on the spectral sensitivity of zebrafish (Risner et al. 2006), with a rated output of 6.3 lumens.

2.2.3 Conditioning

Training consisted of 10 sessions during light hours on each of two consecutive days. Inter-trial intervals of 34-108 minutes were selected from those produced using a pseudorandom time generator (Random Time Generator, http://www.random.org). Conditioning was performed by illuminating green LEDs (visual conditioned stimuli) for a 20-second period. The conditioned stimulus was immediately followed by the presentation of the food reward from the automatic feeder. In trials with control fish, the unconditioned stimulus (food) did not immediately follow the conditioned stimulus, but was instead administered at the midpoint of the inter-stimulus interval, except for the last trial in which it was administered 17-54 minutes later.

After the completion of training, the feeders and plastic dividers were removed from each tank. The tanks were then moved back to the racks on which they were routinely maintained, and routine care was resumed until animals were tested for memory retention.

2.2.4 Probe trials

Probe trials to test memory retention were conducted at various times after training. Fish were either tested in the groups in which they were trained or tested individually. For group testing, the entire tank of five fish was moved from the maintenance rack back to the observation arena, and the upper divider that prevented dispersion of the food was reintroduced as a visual landmark at one end of the tank. LED strips used for visual conditioning were left adhered to the shelf throughout conditioning and testing. For testing single fish, one animal at a time was removed from

each of the maintenance tanks and transferred to a new tank equipped with the food divider and LED strip. All fish were transferred back to the observation arenas one day before testing in order to re-acclimate them to the apparatus. On the day of testing, fish were exposed to the LED stimulus to which they were conditioned for 20 seconds without the food reward to test the association. Each group or individual fish was given only a single probe trial at 2, 4, 8, 16 or 32 days after training.

2.2.5 Data collection and analysis

A single camera was centred along one side of each tank in the observation arenas such that the outflow was on the right. Experiments were video recorded either in black and white at a resolution of 640x480 pixels (HCM5748 camera from Honeywell Video Systems, Louisville, KY, USA) or in colour at a resolution of 1280x720 pixels (C930e camera from Logitech, Newark, CA, USA). Surveillance software (iSpy, http://www.ispyconnect.com or Novex, Toronto, ON, CAN) permitted recording time-stamped video files from multiple cameras simultaneously. Videos were recorded at or converted to 6 frames/second and were then trimmed to 40 second clips (VirtualDub, http://www.virtualdub.org) covering the 20 seconds immediately before exposure to the visual conditioned stimulus and the 20 second period during presentation of the conditioned stimulus.

The behaviour of groups of fish during acquisition and probe trials was analysed using a program (Wyeth et al. 2011) developed in Matlab (The Mathworks Inc., Natick, MA, USA). Average positional values for the group were generated as mean vertical and horizontal locations of the individual fish. The behaviour of single fish in probe trials was

analysed in ImageJ (Schindelin et al. 2015) using the built-in Manual Tracking plugin. We also reanalysed the tracks of individual fish from acquisition groups in three control and three experimental tanks using the Manual Tracking plugin since this plugin generated vertical and horizontal positional values for each fish in each frame and allowed for analysis of factors such as velocity and turn angle of individuals and nearest neighbour analysis for group acquisitions. However, because no significant differences were found in any of these measures, they were excluded from further analysis and only the positional values were examined.

The average vertical and horizontal positions of the fish in each tank were calculated for the 20 seconds **before** the presentation of the conditioned stimulus and compared to average coordinates **during** presentation of the stimulus. This comparison is similar to what has been previously used to analyse responses of fish to the presentation of odours (Hussain et al. 2013), and to examine effects of stress on the position of fish relative to the bottom of the tank (Tran et al. 2016). However, adult fish exhibited a substantial latency in responding to the conditioned stimulus and therefore average positions were only calculated during the last 10 seconds of the 20 second stimulus presentation. These horizontal and vertical positions were combined into a single measure using Pythagorean Theorem ($\sqrt{(x^2 + y^2)}$), corresponding to the distance from a common origin in the top left corner of the tank, near the food source. The distances during presentation of the conditioned stimulus were then subtracted from the distances before the stimulus. This subtraction was also performed individually for vertical and horizontal positions. Positive scores for vertical coordinates correspond

to upward movements towards the surface, and positive scores for horizontal coordinates correspond to a lateral movement toward the end of the tank with the food source, regardless of initial positions. Positive combined distance scores correspond to movement towards the food source.

Linear mixed-effects models were used to analyse the acquisition data. Models included conditioning treatment and trial number as fixed effects, and two random effects for tank (both intercept and by-trial slope). Log-likelihood ratio tests compared reduced models with only main effects for conditioning treatment and trial versus the full model including both main effects and the interaction between the two. Differences in Akaike Information Criterion (ΔAIC) were also examined (Burnham & Anderson 2002). Conclusions paralleled those from the log-likelihood test P-values, with full models showing ΔAIC values >10 over the reduced models for all acquisition tests. In all cases, residual plots showed no major deviations from normality or homoscedasticity. Two-way full factorial analyses of variance (ANOVAs; with conditioning and probe time factors) and Welch two sample t-tests were conducted for the probe trials in adults. All analyses were performed in R (R Core Team 2016) with the help of the following packages: nlme (Pinheiro J et al. 2016), effects (Fox 2003), car (Fox & Weisberg 2011), ggplot (Wickham 2009), sjplot (Lüdecke 2015), plotly (Sievert et al. 2016). P-values are reported in text but for full statistical analyses see Appendix C.

2.3 VISUAL CONDITIONING RESULTS

2.3.1 Acquisition of appetitive conditioning

Both experimental and control fish were observed to swim over much of the depth and length of the tank during the 20 s period before presentation of the LED illumination, with the mean position of the fish being near the centre of the tank. During training, the control groups, which were presented food with variable delays following the LED illumination, continued a similar swimming pattern in the 20 s period that the auditory stimulus was presented (Fig. 2.2A, Supp. Movie 1). In contrast, the experimental fish, which were presented with a food reward directly after each LED illumination, increasingly spent more time near the feeding location during the presentation of the auditory stimulus as training progressed (Fig. 2.2B, Supp. Movie 2). Hence, on average, the fish moved closer to the food source during presentation of the visual stimulus. As with auditory conditioning, fish came to swim closer to the food source during the presentation of a visual stimulus that was paired with food. Figure 2.3A shows this progressive tendency of fish in the conditioning treatment (but not those in the control treatment) to swim closer to the corner of the tank in which food was presented as training progressed. Analysis of linear mixed effects models confirmed a significant interaction between conditioning and training trial ($\chi^2(1)= 31.755$, $p<0.001$). Bootstrapped confidence intervals suggested that by the 7-10th training trial, the experimental groups were moving consistently toward the food source during the presentation of the visual stimulus. Separate analyses of vertical and horizontal components of the movements each showed significant interactions between

conditioning and training trial (horizontal: $\chi^2(1)= 15.798$, $p<0.001$, Figure 2.4A; vertical: $\chi^2(1)= 28.233$, $p<0.001$, Figure 2.5A) suggesting fish learned to adjust both their depth and horizontal position in the tank in response to the conditioning visual stimulus.

2.3.2 Memory retention for groups of fish

To examine whether the association between the visual stimulus and the food reward was retained after training, we tested the groups of fish for their responses to the visual stimulus alone with probe trials at 2 and 16 days after training (Figure 2.3B). A two-way ANOVA on the movement of fish towards the feeding location revealed a significant effect of conditioning ($p=0.001$) but no significant effect of retention day or interaction between retention day and condition (both $p>0.05$). A two-way ANOVA of the horizontal data indicated a significant effect of condition ($p=0.038$) and a significant effect of day of retention ($p=0.039$; Figure 2.4B) but no interaction between retention day and condition ($p>0.05$). An analysis of the vertical components also showed a significant effect of conditioning (two-way ANOVA, $p<0.001$; Figure 2.5B) but no effect of retention day or interaction between retention day and condition (both $p>0.05$).

2.3.3 Memory retention for individual fish

Probe trials were performed from 2-32 days post training to determine whether fish trained in groups also retained memories for the conditioned associations with a visual stimulus when tested individually. An analysis on the movement towards the feeding location showed no significant effects of condition, day of retention or any interaction between them (two-way ANOVA, all $p>0.05$; Figure 2.3B). Analysis of the horizontal data showed no significant effects of condition, day of retention or any

interaction between them (two-way ANOVA, all $p>0.05$; Figure 2.4B). A two-way ANOVA of the vertical data indicated a significant effect of condition ($p=0.012$; Figure 2.5B) but no effect of retention day or interaction between retention day and condition (both $p>0.05$). This weaker retention is probably due to a less robust conditioning than what was seen with the auditory conditioning.

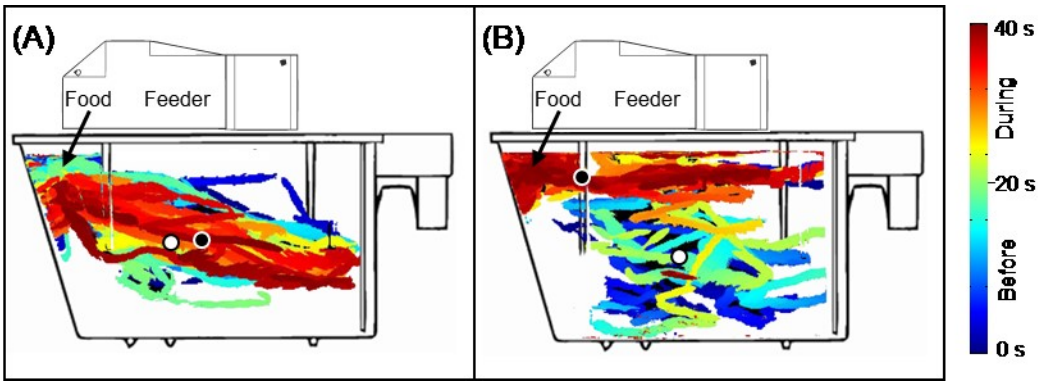

Figure 2.2: Panels A and B illustrate the position of control and experimental zebrafish respectively during trial 20, 20 s before and during the presentation of visual stimulus. The colour scale refers to the time (s) that corresponding areas were occupied by groups of moving fish. The average positions of the fish before and during the last 10 s of the visual stimulus are indicated by black and white circles respectively. The average distance traveled laterally and vertically in the control tank are 3.48 cm and 0.37 cm respectively. The average distance traveled laterally and vertically in the experimental tank are 6.79 cm and 6.23 cm respectively.

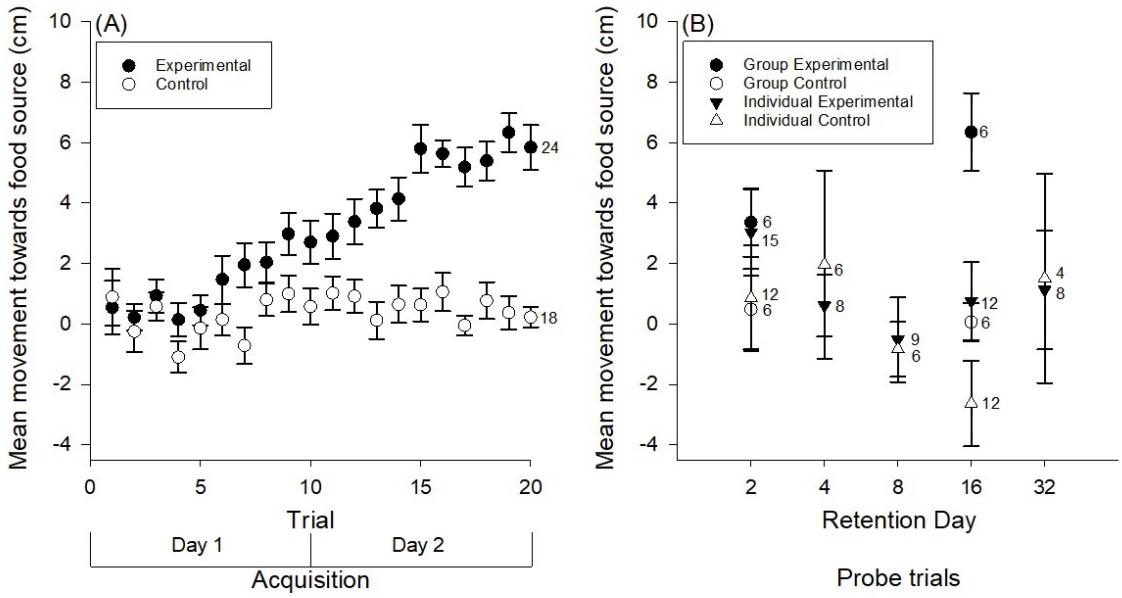

Figure 2.3: Movement of adult zebrafish during acquisition and retention of a visual appetitive paradigm. (A) Zebrafish in the experimental group moved towards the food source from their initial positions as a result of conditioning to the visual stimulus. This response increased throughout the training trials. Zebrafish in the control group did not move toward the food source in response to the visual stimulus. (B) When tested for retention, trained groups moved closer to the food source when compared to controls. Individual fish did not move closer to the food source, when compared with controls. Data points are mean distance from the food source before LED illumination minus mean distance from the food source during LED illumination. Numbers beside data points represent replicates for individuals (single fish) and groups (each containing 5 fish) in each condition. Error bars = ± S.E.M.

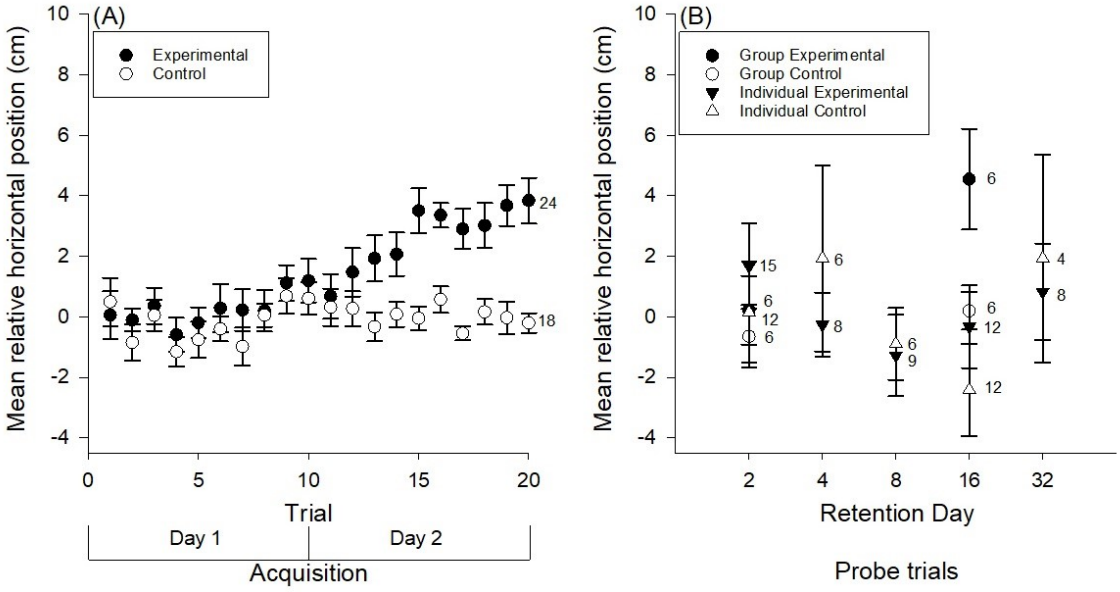

Figure 2.4: Horizontal movements of adult zebrafish during acquisition and retention of a visual appetitive paradigm. (A) Adult zebrafish in the experimental group moved laterally from their initial positions towards the food source as a result of conditioning to the visual stimulus. This response increased throughout the training trials. Zebrafish in the control group did not move laterally towards the food source in response to the visual stimulus. (B) When tested for retention on various days, trained groups moved closer, laterally, towards the food source compared to controls. The individual fish did not move closer to the food source when compared with the controls. Data points are mean horizontal position before the LED illumination sweep minus mean horizontal position during the LED illumination. Numbers beside data points represent replicates for individuals (single fish) or groups (each containing 5 fish) in each condition. Error bars = ± S.E.M.

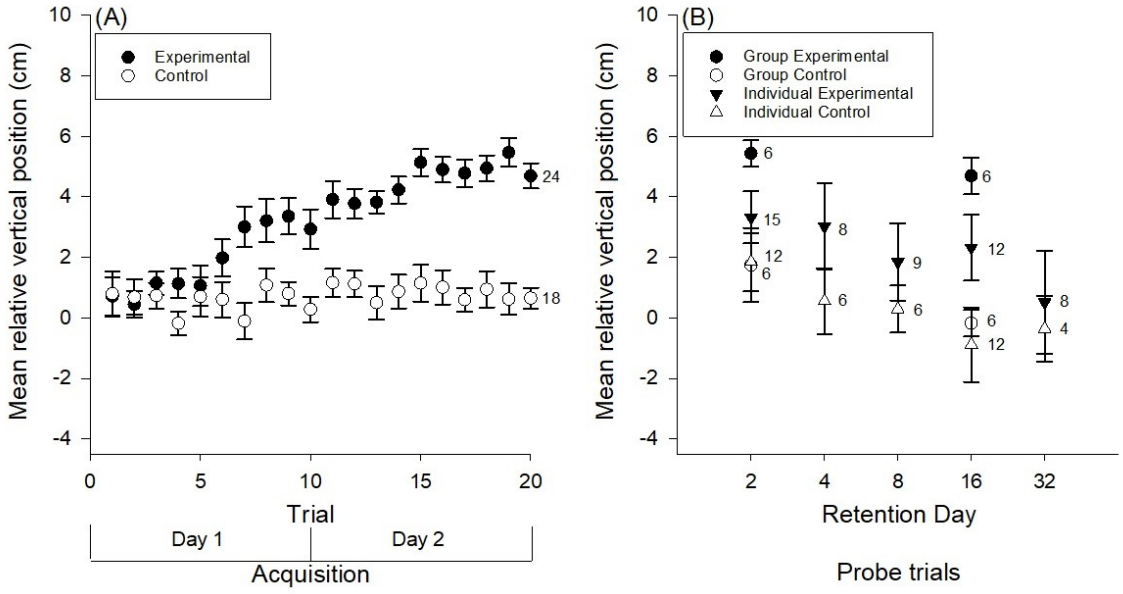

Figure 2.5: Vertical movements of adult zebrafish during acquisition and retention of a visual appetitive paradigm. (A) Adult zebrafish in the experimental group moved vertically from their initial positions towards the surface as a result of conditioning to the visual stimulus. This response increased throughout the training trials. Zebrafish in the control group did not move vertically towards the food source in response to the visual stimulus. (B) When the fish were tested for retention on various days, both trained groups and individuals moved more towards the surface compared to controls. Data points are mean vertical position before the LED illumination minus mean vertical position during LED illumination. Numbers beside data points represent replicates for individuals (single fish) or groups (each containing 5 fish) in each condition. Error bars = ± S.E.M.

2.4 SUMMARY

The auditory learning paradigm presented in Doyle et al. (2017) and Merovitch (2016) was successfully adapted to use a salient visual cue (green LED) as the conditioned stimulus. Results showed that by the 7-10th pairing, fish were consistently moving toward the feeding location after presentation of the conditioned stimulus (green LED). Groups of fish tested for memory retention showed a significant effect of conditioning at least 2 days after training. Individual fish tested for retention moved towards the surface but not laterally towards the food location for at least 2 days after training.

CHAPTER 3: OLFACTORY LEARNING PARADIGM

3.1 INTRODUCTION

3.1.1 Zebrafish are important models for olfaction

The sense of smell plays an integral part in the survival of all vertebrates (Doty 1976; Hara 1994; Stoddart 1980). Traditional models, such as mice and rats, have been extensively studied, examining the role of olfaction in various aspects of animal behaviour, including danger detection, food location and various social cues (Bowers & Alexander 1967; Kinney & Antill 1996; Yang & Crawley 2009). However, fully understanding the physiology and anatomy of the rodent olfactory system can be challenging, due to its complexity. The rodent olfactory bulb contains 1800 – 2000 glomeruli, and the mouse genome possesses approximately 1200 olfactory receptor genes. (Potter et al. 2001; Mombaerts 2006; Oliva et al. 2008; Jones et al. 2008; Schaefer et al. 2001; Buck & Axel 1991). Zebrafish are a comparable vertebrate alternative, as they possess an olfactory system similar in organization but much simpler than that found in many mammals, making it better suited for many olfactory experiments (Baier & Korsching 1994). Zebrafish possess only approximately 140 glomeruli, which have been characterized (Braubach et al. 2012; Braubach et al. 2013), and the regions of the olfactory bulb have been mapped. These factors make zebrafish especially useful for studies of physiology (Braubach et al. 2012; Friedrich & Korsching 1998; Li et al. 2005). Insight into the underlying function of these regions can be gained by examining the behavioural responses of zebrafish towards odours.

3.1.2 Assessing function through behaviour

Zebrafish have a rich and well-documented behavioural repertoire that can be used to examine the effects that physiological changes have on function (Spence et al. 2008; Moretz et al. 2007; Kalueff et al. 2013). They display specific behaviours in response to odours, and certain odours have been found to activate specific areas in the olfactory bulb (Friedrich & Korsching 1997; Friedrich & Korsching 1998; Stensmyr & Maderspacher 2012). For instance, feeding responses in zebrafish are elicited by amino acids, which activate the lateral glomeruli of the olfactory bulb (Friedrich & Korsching 1997; Michel & Derbidge 1997). Bile acids are responsible for most social responses and activate areas in the medial bulb (Friedrich & Korsching 1998; Hamdani & Døving 2007). Exposure to pheromones such as the alarm substance, *Schreckstoff*, activates a unique area of the zebrafish medial bulb (Stensmyr & Maderspacher 2012; Speedie & Gerlai 2008; Jesuthasan & Mathuru 2008; Hamdani & Døving 2007). The effects of a single odourant exposure on zebrafish physiology thus are well documented, but exploring the effects of various odourants on zebrafish becomes difficult when moving from single exposure to the repeated exposures needed for a conditioning paradigm. As discussed in Chapter 1, learning paradigms can provide useful insight into how animals interact with their environments, and olfactory paradigms have previously been used to study fish behaviour.

3.1.3 Technical problems with olfactory paradigms

Creating an effective olfactory learning paradigm can be difficult for several reasons. Working with odourants is inherently challenging because temporal and spatial

distribution of odourants are more difficult to control than auditory or visual stimuli. Olfactory conditioning paradigms that utilize multiple pairings also require large volumes of water to dilute odourants between trials. The flow rate and odourant concentration must be chosen to balance the effective threshold and rate of wash out. The odourant must be sufficiently salient to be detected during pairing but must subsequently be diluted below the effective threshold before the next pairing.

Choosing an appropriate odourant is also important when using an olfactory paradigm. Amino acids are a common odourant for use in odour exposure studies involving fish (Valentinčič et al. 2000; Valentinčič & Caprio 1994; Valentinčič et al. 2005). Zebrafish can also be conditioned using amino acids but show an innate preference for them due to an association with food (Braubach et al. 2009; Lipton & Rosenberg 1994; Koide et al. 2009). To investigate the acquisition rate, conditioned behaviour must be sufficiently distinct from innate behaviour; therefore, the conditioned stimulus must be initially neutral to the fish (Braubach et al. 2009). A more suitable odourant for this study is phenylethyl alcohol (PEA), which has been shown to be initially neutral to zebrafish (Harden et al. 2006). All of the factors mentioned in this section must be carefully examined when developing an olfactory paradigm.

3.1.4 Current olfactory paradigms

An olfactory paradigm developed in our lab in 2009 showed successful conditioning of adult zebrafish (Braubach et al. 2009). This experiment featured single fish acclimated in a large cylindrical tank. Fish were exposed to 12 pairings of PEA or an amino acid with a food reward per day for five days. At the end of conditioning,

zebrafish showed increased appetitive swimming behaviour when presented with the odourant and restricted their swimming to the immediate area of food reward. An examination of memory found the fish retained the association up to 48 hours later. To ensure that conditioning was olfactory and not gustatory, the nares of fish were occluded before testing. The anosmic fish did not exhibit the same association, which confirmed that the olfactory conditioning was occurring.

Unfortunately, this paradigm's use of individual animals makes it difficult to implement on a large scale. In 2011 the paradigm was successfully adapted to train groups of fish; however, further issues remain unaddressed (Braubach et al. 2011; Wyeth et al. 2011). One of the issues is that the paradigm requires a specialized tank that is unfamiliar to the fish, thereby necessitating an acclimation period. In addition, the large water requirement (360L/tank/hour) makes it difficult to run more than one tank simultaneously (Braubach et al. 2009). While this paradigm has proven that groups of zebrafish can be conditioned using an olfactory cue, it suffers from limitations that make it unsuitable for high-throughput training.

3.1.5 Benefits of a home tank olfactory paradigm

Recently, Doyle et al (2017: see also Chapter 2) developed an appetitive conditioning paradigm for zebrafish in their home tanks (Doyle et al. 2017; Merovitch 2016). A major advantage of this paradigm was that the fish were conditioned in the same tanks in which they were housed, which greatly reduces handling stress and in turn reduces acclimation time. The paradigm used auditory cues paired with a food reward ten times daily for two days. Fish showed a robust association between the

stimulus and the food reward after two days of training and retained the association for at least another two days. In this chapter, the paradigm was adapted to use olfactory cues, while also addressing the drawbacks of other olfactory paradigms. The result was a high-throughput, cost-effective, appetitive paradigm with reasonable water requirements and a minimal stress impact on the fish.

3.1.6 Summary

The goal of this chapter was to adapt the auditory learning paradigm presented in Doyle et al (2017) and Merovitch (2016) to use a salient olfactory cue (PEA) as the conditioned stimulus. The rate of acquisition will be examined, in addition to the retention of the learned association at one and two weeks post training. An effective olfactory conditioning paradigm will be useful for studying the physiological aspects of learning and the olfaction process.

3.2 MATERIALS AND METHODS

3.2.1 Animals

AB zebrafish (both wild type and unscreened UAS:GCamp zebrafish) (Muto et al. 2013) were obtained from the Zebrafish Core Facility, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada and housed as mixed-gender groups of five fish in 3 L tanks (Pentair Aquatic Eco-Systems, Apopkoka, FL, USA), beginning at least one day prior to experimentation. The fish were maintained on a 14:10 hour light/dark cycle in treated municipal water (28°C, pH: 7.3 and salinity: 0.20 psu) with a flow of approximately 13-14 L per hour per tank. All experiments were conducted in accordance with the Canadian Council on Animal Care standards and guidelines (Dalhousie Protocol 14-132).

3.2.2 Experimental apparatus

For conditioning, the home tank was moved to one of the eight specialised observation arenas (See figure 3.1). Each arena contained a camera (C930e camera from Logitech, Newark, CA, USA), centred along one side of each tank, which was used to observe the fish. To provide contrast for the video, the long side of the tank, opposite the camera, was covered with opaque white tape. In addition, the opaque side prevented the fish from being distracted and provided a landmark. The end of the tank, opposite the drain, was covered with alternating green and red stripes on the diagonal to use as a visual cue for the location of food delivery. Each arena also contains a water inlet (See 3.2.3 Water delivery).

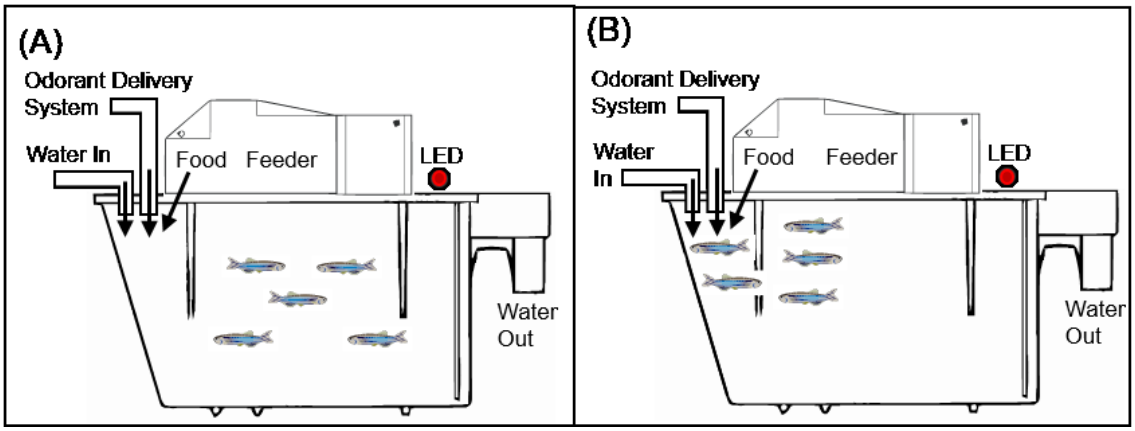

Figure 3.1: Diagram of behavioural apparatus for olfactory conditioning in home tanks. Panel A and B illustrate the positions on control and experimental fish respectively during the presentation of conditioned stimulus. A tube delivered phenylethyl alcohol (PEA) to the tank. A red LED indicated to the camera when the odourant was being delivered. Food pellets were dispensed by the automatic feeder located above the tank.

A 3D printed automatic feeder, controlled by an Arduino microprocessor (See Section 2.1.2) was placed over an existing hole in the lid of each tank (stereolithography file downloadable from http://crollab.physiology.dal.ca/automaticfeeder) (Fig. 3.1). Food was dispensed using a stepper motor and a 5 mm steel drill bit, which served as an auger to dispense approximately 10 mg of food at a time. Programs (sketches) were created in the Arduino software (Arduino 2014) utilizing the following libraries to control the experiments: Time (Margolis 2016), TimeAlarms (Margolis 2014) and Motorshield (Adafruit 2016) (See Appendix B for Arduino sketches). A white polylactic acid divider was placed at the level of the water, 8 cm from the front of the tank, to keep the dispensed food floating near the feeder.

The odorant was dispensed using a syringe pump (Model 200, KD Scientific, Holliston, Massachusetts, USA) adapted to hold eight 20 mL syringes (Becton Dickinson, Franklin Lakes, New Jersey, USA). Each syringe was connected to polyethylene tubing (PE No. 160, Becton Dickinson) using an 18 gauge needle (Becton Dickinson). Each tube was placed through a hole in the lid of the tanks by the water inflow, near the front of the tanks. An LED light partially wrapped in heat shrink tubing was adhered to the lid so that it was only visible to the camera. The light indicated when the odourant was being delivered.

3.2.3 Water delivery

For approximately 9 hours during the 14 hour daylight period, the tanks were maintained by a fresh water, flow-through system (13-14 L per hour) to prevent odourant from contaminating the main recirculating system. Municipal water (28°C) was

run through a charcoal filter (FC200, Rainfresh, Richmond Hill, ON, CAN) and into a 200 L reservoir at ground level. Water was then treated with 600 mg Instant Ocean (United Pet Group, Blacksburg, VA, USA), 26.4 mg sodium bicarbonate (Pen-tair Aquatic Eco-Systems, Apopkoka, FL, USA) and 0.1 ml dechlorinator/conditioner (Aquasafe Plus, PetSmart, NS, CAN) per litre. An ActiveAQUA Submersible Pump 1000 (Hydrofarm, CA, USA) then transferred the water into a 40 L polyethylene reservoir on the top of the shelf of the racks housing the experiment. A hose (12.7 mm or 1/2") connected the reservoir to a ball valve (12.7 mm or ½"), which in turn connected to a manifold with outflows to the individual tanks. Tubing (3.2 mm or 1/8") connected each outflow to a tank via a Y-connector (3.2 mm or 1/8"), which allowed easy delivery of either recirculating system water or fresh flow-through water to each tank. During conditioning, the outflow of each tank was directed to a floor drain using two 1" (25.4 mm) PVC ball valves (Home Depot, Halifax, NS, CAN) to prevent contamination of the system water with odourant.

Overnight, when the fish were not being exposed to odourants, the tanks were switched back to the recirculating system water, and the outflows drained back into the recirculation system.

3.2.4 Conditioning

Training consisted of 10 sessions during daylight hours on each of two consecutive days. Inter-trial intervals of 40-60 minutes were selected from those produced using a pseudorandom time generator (Random Time Generator, http://www.random.org). Conditioning was performed by introducing an odourant to

the tank. Twenty seconds after the beginning of odourant delivery, the conditioned stimulus was followed by the presentation of the food reward from the automatic feeder. In trials with control fish, the unconditioned stimulus (food) did not immediately follow the conditioned stimulus, but was instead administered at the midpoint of the inter-stimulus interval, except for the last trial in which it was administered at a time 15-34 minutes later as determined by the random time generator. Of the eight tanks run simultaneously, four were designated experimental and four control.

Phenylethyl alcohol (PEA) was used for the odourant because it has previously been shown to be a neutral stimulus for zebrafish (Braubach et al. 2009; Harden et al. 2006). During each of the 20 sessions, 1.5 mL of $2x10^{-4}$ M PEA was delivered to the tank. In preliminary experiments, 1.5 mL of dye was injected into the tanks to assess the dispersion of odourant. After injection, a single bolus of dye was visible for 20 s when it broke into concentrated swirls that were observed for approximately 120 s before what appeared to be homogeneous dispersion throughout the tank. During the dye experiment, all fish in the tank encountered the concentrated dye within the 20 s (See Supp. Movie 3). Assuming that the dye dispersed similarly to PEA odourant and was evenly distributed by about 120 s after injection, each fish would be expected to encounter a concentrated area. After full dispersion in the tank, the concentration of PEA was calculated to be $1x10^{-7}$ M, which is below the functional dosage for zebrafish (Harden et al., 2006). The continuous water inflow further diluted the odourant during the inter-trial period. See Appendix E for a mathematical analysis of odourant dilution.

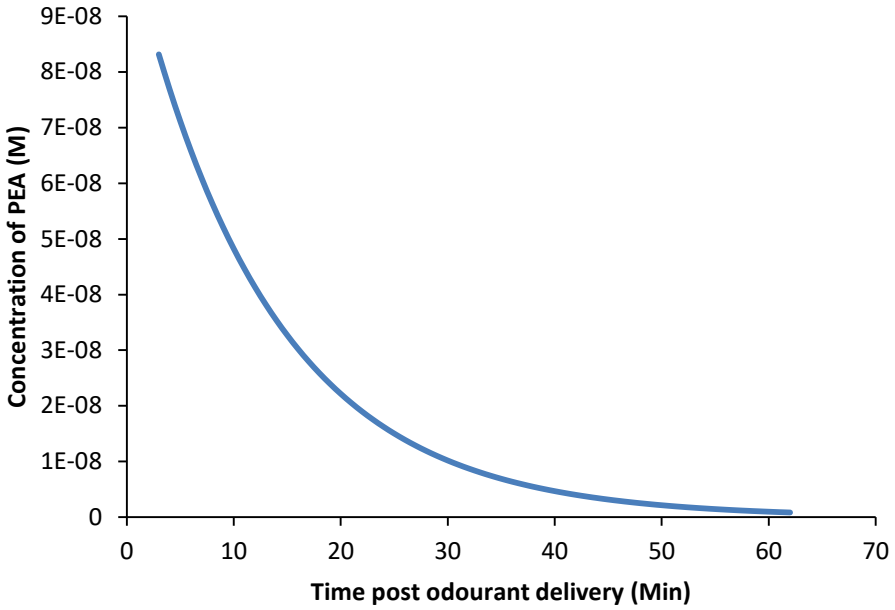

Figure 3.2: Theoretical calculation of phenylethyl alcohol (PEA) dilution delivery to the tank. The equation ($C= 1.05*10^{-7}$ M x $e^{(-t/12.86)}$) assumes the odourant is homogenously distributed in the tank, which occurs approximately 2 min after delivery to the tank (See Appendix E for calculations).

After the completion of training on the second day, the feeders, indicator lights and odourant dispensing tubes were removed from the lid and tanks were then moved back to the racks on which they were routinely maintained, until animals were tested for memory retention.

3.2.5 Water only trial

To ensure that the fish were responding specifically to the PEA and not to the water turbulence that the odourant delivery creates, each tank was exposed to a water-only trial after training. Instead of odourant, 1.5mL of reservoir water was delivered to the tank using the same delivery method. The responses of fish were recorded and behaviour analyzed using the same method as the acquisition videos.

3.2.6 Probe trials

Probe trials to test memory retention were conducted 6-7 and 13-14 days after training. Each entire tank of five previously trained fish was moved from the maintenance rack back to the observation arena and switched over to the flow-through water system at least one hour before testing. The feeders, indicator lights and odourant tubes were placed back on the lids of the tanks. Fish were exposed to the stimulus to which they were conditioned for 20 seconds without the food reward to test whether the fish retained a conditioned association between the CS and the UCS.

3.2.7 Data collection and analysis

Experiments were video recorded in colour at a resolution of 1280x720 pixels. Surveillance software (iSpy, http://www.ispyconnect.com) permitted recording time-stamped video files from all eight cameras simultaneously. Videos were recorded at 6

frames/second and were then trimmed to 40 second clips (VirtualDub, http://www.virtualdub.org) covering the 20 seconds immediately before exposure to the olfactory conditioned stimulus and the 20 second period during presentation of the conditioned stimulus. The food (UCS) was dispensed immediately after the video concluded at 40 s.

The behaviour of groups of fish during acquisition and probe trials was analysed using a program (Wyeth et al. 2011) developed in Matlab (The Mathworks Inc., Natick, MA, USA). Average positional values for the group were generated as mean vertical and horizontal locations of the individual fish. The average vertical and horizontal positions of the fish in each tank were calculated for the 20 seconds **before** the presentation of the conditioned stimulus and compared to average coordinates **during** presentation of the stimulus. However, the fish in the olfactory paradigm exhibited a different response time than fish in the visual paradigm. In order to examine when the fish were responding, the positions of the fish in all the tanks were averaged together on trial 20 (see Figure 3.3). The fish showed a spike in response to the stimulus for a duration of 5 seconds, from the 25th second to the 30th second of the video. The positions of fish during this 5 second window were compared to the positions during the entire 20 second period before stimulus delivery. These horizontal and vertical positions were combined into a single measure using Pythagorean Theorem ($\sqrt{(x^2 + y^2)}$), corresponding to the distance from a common origin in the top left corner of the tank, near the food source. The distances during presentation of the conditioned stimulus

were then subtracted from the distances before the stimulus to produce a measure of movement relative to the location of food presentation.

Analysis was again performed independently for changes in vertical and horizontal position. Positive scores for vertical coordinates correspond to upward movements towards the surface, and positive scores for horizontal coordinates correspond to a lateral movement toward the end of the tank with the food source, regardless of initial positions. Positive combined distance scores correspond to movement towards the food source. Statistical analysis was performed using the same methods as the visual paradigm (See Section 2.2.5 – Data collection and analysis). For full statistical analyses see Appendix D.

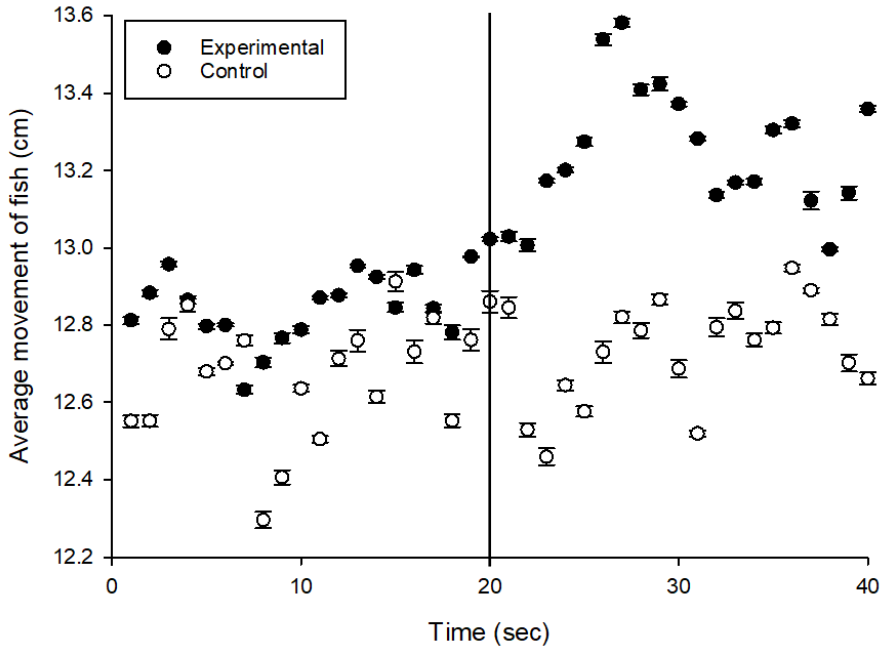

Figure 3.3: Average position of groups (n=44) in experimental and control tanks over the last pairing trial (Trial 20). The trial begins at 0 s and the odourant is delivered at 20 s. Experimental fish showed a spike in activity approximately 5 s after odourant delivery for a duration of about 5 s.

3.3 RESULTS

3.3.1 Acquisition of appetitive conditioning

With training, the fish swam closer to the food source during the presentation of an olfactory stimulus that was paired with food, although the association was weaker than the visual conditioning. Figures 3.5A and 3.6A show a weak tendency of fish in the conditioning treatment (but not those in the control treatment) to swim closer to the corner of the tank in which food was presented as training progressed. Analyses of vertical and horizontal components of the movements each showed significant interactions between conditioning and training trials (horizontal: $\chi^2(1)= 5.5545$, $p<0.05$, Fig. 3.5A; vertical: $\chi^2(1)=6.3594$, $p<0.01$, Fig. 3.6A) suggesting that fish learned to adjust both their depth and horizontal position, albeit slightly, in the tank in response to the conditioned olfactory stimulus. Analysis of linear mixed effects models showed no significant interaction between conditioning and training trials ($\chi^2(1)= 1.9669$, $p>0.05$, Fig 3.4A) in the combined measure, mean movement towards food. The effect of training was too small to determine during which trial they begin to form an association.

3.3.2 Response to water only trial

A comparison of the experimental and control response to the water only trial revealed no significant difference in both mean movement towards food and vertical movement (T-Test: both $p>0.05$, Figs. 3.4A & 3.6A). Analysis of the horizontal movement indicated a significant difference between the experimental and control groups (T-Test: $p<0.05$, Fig. 3.5A), although this result was not due to an elevated control group but by a control group that was below the original baseline.

3.3.3 Memory retention

Groups of fish were tested for their responses to the olfactory stimulus alone with probe trials one and two weeks after training to examine whether the association between the olfactory stimulus and the food reward was retained. A two-way ANOVA on the mean movement of fish towards the feeding location revealed a significant effect retention day ($p<0.05$, Fig. 3.4B) but no significant effect of condition or interaction between retention day and condition (both $p>0.05$). Independent analyses on the horizontal and vertical data indicated no significant effect of condition, retention day or interaction between retention day and condition (Two-way ANOVA, all $p>0.05$, Figs. 3.5B & 3.6B).

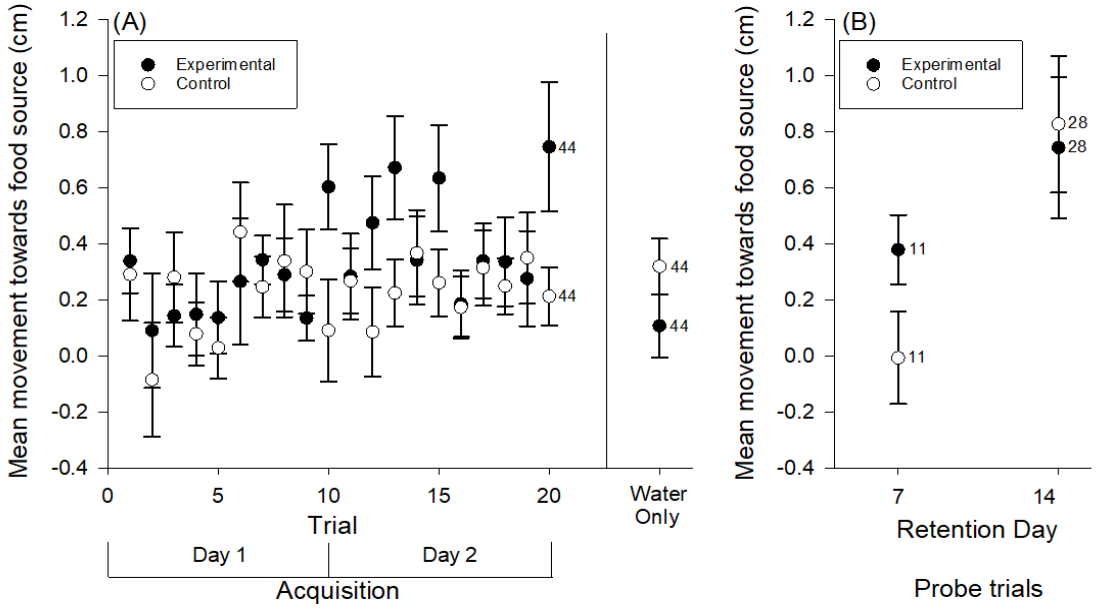

Figure 3.4: Movement of adult zebrafish during acquisition and retention of an olfactory appetitive paradigm. (A) Zebrafish in the experimental group showed small movements towards the food source from their initial positions as a result of conditioning to the olfactory stimulus, although the difference was not significant. This response increased slightly throughout the training trials. Zebrafish in the control group did not move toward the food source in response to the olfactory stimulus. The water only trial showed no significant response by either the control or experimental fish. (B) When tested for retention, some trained groups moved closer to the food source when compared to controls. Data points are mean distance from the food source before PEA delivery minus mean distance from the food source during PEA delivery. Numbers beside data points represent replicates for groups (each containing 5 fish) in each condition. Error bars = ± S.E.M.

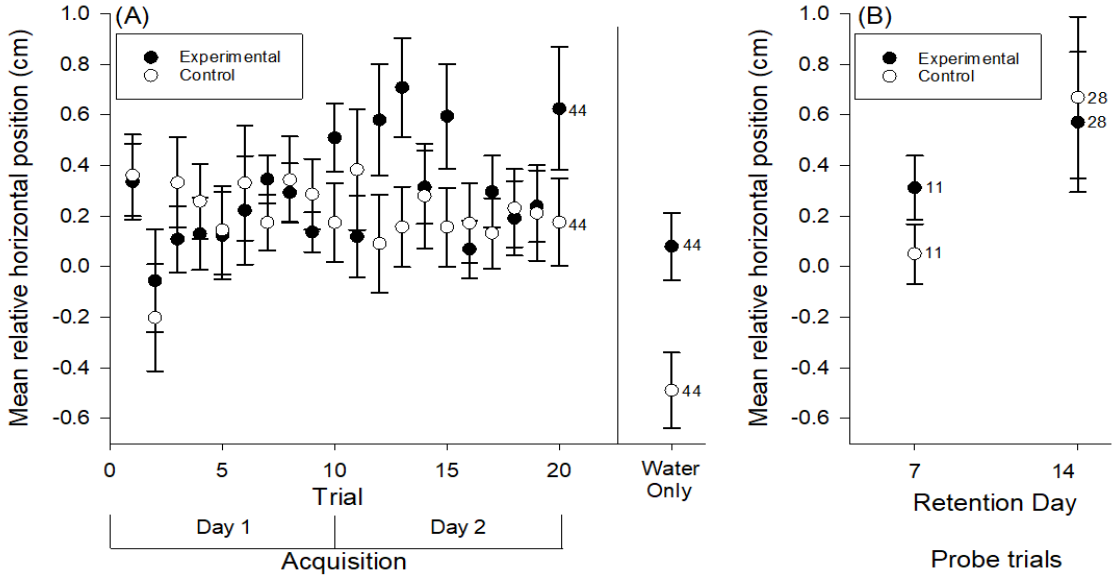

Figure 3.5: Horizontal movements of adult zebrafish during acquisition and retention of an olfactory appetitive paradigm. (A) Adult zebrafish in the experimental group moved laterally from their initial positions slightly towards the food source as a result of conditioning to the olfactory stimulus. This response increased throughout the training trials. Zebrafish in the control group did not move laterally towards the food source in response to the olfactory stimulus. The water only trial showed a significant response by either the control or experimental fish, it appears to be caused by the control group moving below baseline. (B) When tested for retention on various days, some trained groups moved closer, laterally, towards the food source compared to controls, although not significantly. Data points are mean horizontal position before the PEA delivery sweep minus mean horizontal position during the PEA delivery. Numbers beside data points represent replicates for groups (each containing 5 fish) in each condition. Error bars = ± S.E.M.

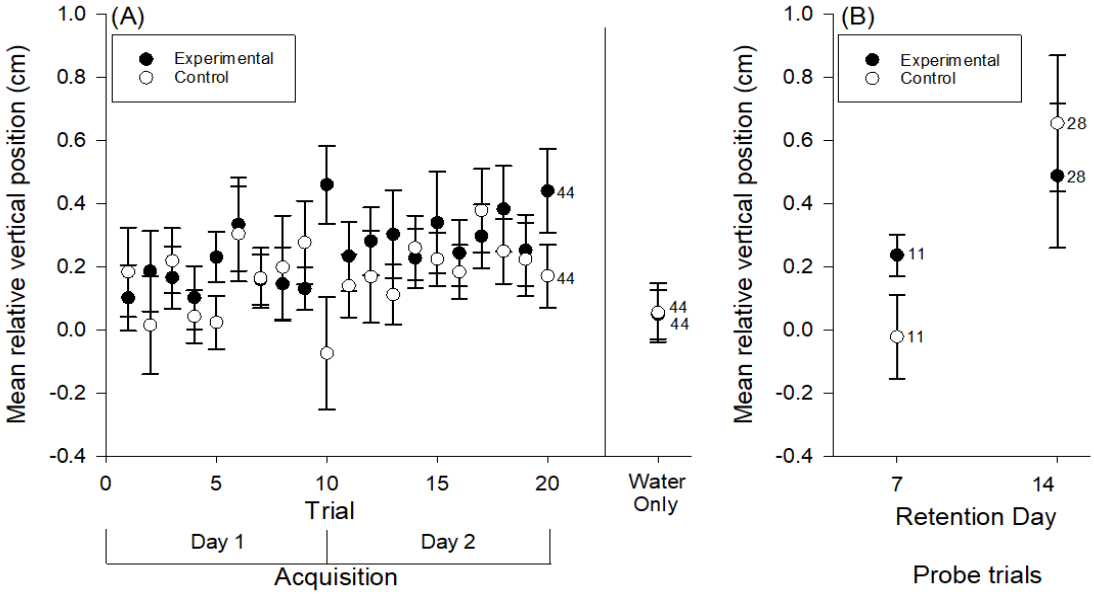

Figure 3.6: Vertical movements of adult zebrafish during acquisition and retention of an olfactory appetitive paradigm. (A) Adult zebrafish in the experimental group moved vertically from their initial positions slightly towards the surface as a result of conditioning to the olfactory stimulus. This response increased throughout the training trials. Zebrafish in the control group did not move vertically towards the food source in response to the olfactory stimulus. The water only trial showed no significant response by either the control or experimental fish. (B) When the fish were tested for retention on various days they showed slight movement towards the surface compared to controls, although not significantly. Data points are mean vertical position before the PEA delivery minus mean vertical position during PEA delivery. Numbers beside data points represent replicates for groups (each containing 5 fish) in each condition. Error bars = ± S.E.M.

3.4 SUMMARY

The auditory learning paradigm presented in Doyle et al (2017) and Merovitch (2016) was adapted to use an initially neutral, salient olfactory cue (PEA) as the conditioned stimulus. The results showed that though the fish seem to move towards the food source in the lateral and horizontal directions, the small response in the data makes it difficult to determine by which pairing the fish have learned the association. Groups of fish tested for memory retention at one and two weeks showed a slight effect of retention day when examining the combined measure, mean movement towards food.

CHAPTER 4: DISCUSSION

4.1 SUMMARY

This thesis presents two adaptations of a previously described automated appetitive auditory paradigm (Doyle et al. 2017; Merovitch 2016; Merovitch et al. 2016).

4.1.1 Visual learning paradigm

This visual paradigm possesses several improvements over previous visual paradigms. It is an efficent, fully automated paradigm that is easily adaptable to any conventional fish rack, and the programmable nature of the LED light strip makes it simple to alter the light stimulus. Potential possibilities include different colours, light intensities and spatiotemporal patterns of illumination.

Conditioning: The fish formed an association between the illumination of the green LED (conditioned stimulus, CS) and the food reward (unconditioned stimulus, UCS). In as few as 7-10 pairings, the fish moved consistently to the feeding area upon the presentation of the CS.

Retention: Groups of fish, tested for memory retention at 2 and 16 days, retained the association for at least 2 days. Individual fish, tested for memory retention at 2, 4, 8, 16 and 32 days, showed significant vertical movement towards the feeding area at 2 days, which suggested that they retain the association with food but not the exact lateral location.

4.1.2 Olfactory learning paradigm

In addition to the general improvements mentioned with the visual paradigm, such as automation and adaptability, the olfactory paradigm conditioned fish to form an

association with a fraction of the water usage of previously described paradigms (Braubach et al. 2009). The lower water requirements of the current paradigm make it compatible for use in standard zebrafish facilities and lessen the need for a specialised testing area.

Conditioning: Fish formed a weak association between the PEA (CS) and the food reward (UCS). The fish moved towards the food location after presentation of the odourant, although the response appears less pronounced than the response of the fish trained with the visual paradigm.

Retention: When examining the combined measure, mean movement towards food, the retention data indicated significance in retention duration, but the response was too weak to determine how long they remember the association.

4.2 CONDITIONING

Initially, it was necessary to determine if conditioning occurred and whether the association formed was between the food and the intended CS. In both paradigms, naïve fish at the beginning of training exhibited similar movements before and during the presentation of the CS (Figs. 2.3A & 3.4A), which indicated that the respective stimuli (green LED and PEA) were innately neutral to the fish. At the end of the acquisition period, fish in the control groups showed no significant difference in behaviour from the naïve fish, which indicated that no association was formed between the CS and the UCS (Figs 2.3A & 3.4A). At the end of training, only the fish in the experimental groups move towards the feeding area when presented with the CS. These

results suggest that the fish learn to associate previously neutral stimuli with a food reward, which indicates that classical conditioning occurred.

As discussed in Section 3.1.3 - Technical Problems with Olfactory Paradigms, the olfactory paradigm presented unique challenges with regard to CS presentation. For instance, it was necessary to ensure that the fish were responding to the odour itself and not to the changes in water flow caused by the odourant delivery. Therefore, the fish were exposed to a water-only trial at the end of training to gauge the fish's response to changes in water flow (see Section 3.2.5 – Water Only Trial). The fish showed no significant change in behaviour before and during the delivery of the water, thus indicating that the fish were responding to the odour and not the changes in water flow caused by the odourant delivery (Fig 3.4A). These findings are consistent with those presented by Braubach et al 2009 (see Section 3.1.4 – Current olfactory paradigms) which showed that fish with occluded nares could not form an association between an odourant and a food reward. Therefore, it can be concluded that the fish were associating the smell of the odour with the food, not changes in water flow caused by delivery.

My results clearly show that fish can form associations between either a visual or olfactory stimulus and a food reward as exhibited by reliable changes in their swimming patterns in the automated paradigms that I developed. A question arises, however, whether the fish actually develop a spatial map of the tank or if the association is purely with landmarks used for navigation. The fish rose to the surface in anticipation of food when presented with the CS. This association is probably rooted in the innate surface

feeding behaviours of zebrafish (Spence et al. 2008). The association between the CS and the lateral position of the food seems to be weaker. The weaker lateral association also occurred with the auditory paradigm presented in Doyle et al. (2017) and Merovitch (2016). In an effort to provide more salient visual landmarks for the fish in the olfactory paradigm, red and green stripes were affixed to the tank on the end where food was dispensed. The fish did show a weak association with the side of the tank where the food was dispensed. This response was similar to the vertical response of the fish, perhaps indicating that although weak, the fish form an association with the landmarks, as other studies that suggest they are capable of learning such associations (Karnik & Gerlai 2012; Arthur & Levin 2001; Ruhl et al. 2014). Another explanation for the lack of evidence of an association in the lateral direction relates to the small size of the tank. The size constraint of the tank may limit the natural circling behaviour exhibited by zebrafish when they are anticipating food (Kalueff et al. 2013). Other studies conducted in a larger testing apparatus have found that zebrafish will circle in the area of expected food after the delivery of the CS (Braubach et al. 2009; Kalueff et al. 2013). The fish may be associating the food with the specific location but circle the area instead of remaining stationary near the feeding site. The weak lateral response still suggests that there is an association and therefore incorporating that measurement into the analysis provides a more accurate representation of the fish's behaviour.

4.3 RATE OF AQUISITION

A review of other studies presented a wide range of acquisition rates. Studies using aversive stimuli such as water turbulence or administering electric shock, have

used 1 to 40 pairings presented over 1 to 5 days (Agetsuma et al. 2012; Xu et al. 2007; Blank et al. 2009; Morin et al. 2013). In contrast, previous appetitive paradigms use 20 to 400 trials for up to 8 days (Sison & Gerlai 2011; Braubach et al. 2009; Chacon & Luchiari 2014; Colwill et al. 2005; Mueller & Neuhauss 2012). These findings suggest that acquisition rates vary widely between paradigms and sensory modalities and that fish may take longer to form appetitive associations when compared to aversive paradigms. However, based on the results from Doyle et al (2017) and this thesis, the fish appear to form appetitive associations relatively quickly.

Fish trained with the visual paradigm began to exhibit an association between the CS (LED) and the unconditioned stimulus (food reward) by the 7^{th} to 10^{th} pairings on the first day of training. This rate of learning is similar to the one observed in the previously described auditory paradigm, which showed learning by the 5^{th} pairing (Doyle et al. 2017; Merovitch 2016). The olfactory paradigm showed that the fish formed a weak association between the presentation of odourant and the food reward over two days of training. The weaker association may be due to the imprecise nature of PEA diffusion. Each fish encounters the odourant at a slightly different time, and the concentration varies depending on the location in the tank, which may contribute to delay in forming the association.

Comparison of acquisition rates between modalities can be problematic for several reasons. Firstly, the intensities of the conditioned stimuli are not directly comparable. One challenge in designing a learning paradigm is in choosing the intensity of the CS. Several studies have been conducted to examine the effect of stimulus

intensity on the reaction of zebrafish (Avdesh et al. 2012; Wolman et al. 2011; Carvan et al. 2004; Bilotta et al. 2005). The intensities of the stimuli were chosen to fall within the detection range known for zebrafish but such that they did not evoke a startle response. In the case of the olfactory stimulus, we also had to factor in the time until the odourant fell below the effective threshold. In all three sensory modalities (auditory, visual and olfactory) the fish formed some association between the CS and the UCS by the end of the two day (20 trials) training period. The auditory paradigm began to show an association by the 5th pairing; however, the strong pairing may be due to the nature of the stimulus itself. The sound was applied via a surface transducer attached to the side of the tank, which evenly administered the sound instantaneously to the whole tank. In the visual paradigm, the LED light strip that provided the stimulus was adhered adjacent to the tank as not to interfere with the videotaping of the fish. It is possible that the fish in certain areas of the tank did not immediately see the light, therefore resulting in a delayed response from some fish in the group. Similar limitations also apply to the olfactory paradigm. The olfactory stimulus presents the greatest challenge to implementation. As seen in the dye trial video (See Supp. Movie 3) each fish in the group was exposed to the odourant at slightly different times, which may be the reason this paradigm also showed the most delayed acquisition. Although the fish were exposed to PEA at a concentration above their effective threshold, it is possible that the stimulus was not salient enough to form a strong association.

An additional point to consider is that each sensory system may have a different neural substrate for learning. Most of the current studies on zebrafish examine brain

regions associated with learning generally and not learning specific to a sensory stimulus (Rodríguez et al., 2002; Portavella et al., 2004; Salas et al., 2006; Broglio et al., 2010; Mueller and Wullimann, 2009; Mueller et al., 2011; Northcutt, 2011). Therefore, it is unknown if different types of learning utilize different areas of the zebrafish brain or different mechanisms. Consequently, it is also unknown if association via one sensory modality may be inherently easier for zebrafish to acquire than via another sensory modality.

4.4 RETENTION

One goal of this study was to examine the memory retention of these two paradigms at various periods after training. There are very few studies that have examined memory retention beyond 2–3 days after training. Zebrafish can remember the association between a visual or olfactory cue and food for 1 to 2 days (Al-Imari & Gerlai 2008; Braubach et al. 2009). Memory of an association between a visual stimulus and an electric shock can persist for 3 days (Xu & Goetz 2012) and one study has suggested that zebrafish can retain spatial memories for up to 10 days (Williams et al. 2002).

For the visual paradigm, the individual fish did not exhibit movement towards the food in the lateral direction when tested for retention, but they did retain the vertical association for at least 2 days (Fig 2.5). When groups of fish were tested for retention at 2 days they showed a general movement towards the feeding location. The paradigms were demonstrably effective when testing retention on individuals or groups as required for an experiment design.

When tested, the fish showed significance in retention duration but due to the weak association, it is difficult to quantify how long they retained the association. Solutions to this issue are addressed in Section 4.6.2 – Future Studies.

4.5 FUTURE IMPROVEMENTS

4.5.1 Improvements in Visual Paradigm

As discussed in Section 4.3 – Rate of Acquisition, one possible reason that fish quickly formed associations during the auditory learning paradigm was the intensity and salience of the conditioned stimulus. Accordingly, increasing the intensity of the light may improve rate of acquisition and retention of association for the visual paradigm. It was also postulated that the light's position made it difficult for some fish to see immediately upon illumination. Moving the light to a more visible location or the addition of more light strips may help the fish to form the association more quickly. Presenting light of modulating intensity or colour may also increase the salience of the visual stimulus.

4.5.2 Improvements in Olfactory Paradigm

Due to the small responses in the olfactory data, various improvements could be implemented. In preliminary experiments, the volume and concentration of the odourant were varied to ensure that the fish encounter PEA at a concentration above the zebrafish effective threshold determined by other studies (Harden et al. 2006; Braubach et al. 2009), while also ensuring washout and dissipation to below threshold However, it is still possible that the odourant is not salient enough for the fish to form a strong association. Therefore, the concentration could be increased two fold to ensure

the stimulus is salient enough. The fish do form a weak association, but it is possible that the association may become stronger with more training. Also it may take longer for zebrafish to form association with olfactory cues versus visual or auditory cues. Therefore, an additional day of training could be added with an extra 10 trials, for a total of 30 trials over three days.

Further refinements to the olfactory paradigm may yield even greater scalability and reduced labour intensiveness. Adapting the olfactory paradigm to utilize recirculated water would be beneficial for several reasons. Firstly, this would make the water requirements less onerous by removing the need to drain off the odourant contaminated water. As discussed in section 3.2.3 - Water Delivery, the olfactory paradigm uses 13-14 litres of water per tank per hour, so approximately 252 L were required to train one group of fish to criterion over two days. For comparison, the paradigm presented by Braubach et al., (2009) used 360 litres per tank per hour, so approximately 16000 L were required to train one fish to criterion over five days. The significant reduction in water usage makes the olfactory paradigm easier to replicate, although this is still a large water requirement for some systems to tolerate. If the water could instead be reconditioned and then returned to the existing system, there could be further reductions in the requirements for water supply. This olfactory paradigm utilized a flow-through system to ensure no contamination of the main water supply; however, this precaution was extremely conservative. The PEA added to the tanks was already diluted below threshold by the time it was homogeneously diffused in the tank. The concentration of PEA would be negligible if the entire volume of water in the whole

recirculation system was used. Also, methods of treating the outflow to break down the odourant should be investigated, as this would allow reconditioned water to be recirculated into the main water supply without any odourant contamination.

This olfactory paradigm can be adapted for use with any liquid odourants, so the water reconditioning would need to be adapted specifically to neutralize the odourant being used. For example, amino acids could be easily used with a recirculation system because they will degrade in any system with a bio-filter (Barker 1981). Using recirculating water would also make adapting the paradigm to a traditional rack system easier due to the reduced space requirements for water reservoirs. Additionally, having all of the water delivered from the main supply could allow for better water quality and more consistent control of water temperature.

4.6 IMPLICATIONS

4.6.1 Applications of these paradigms

Both of these paradigms will have many beneficial applications. They can be easily adapted into high-throughput screens, which can be used to examine genetic and pharmacological effects on learning and memory. As discussed in Chapter 1: Introduction, these paradigms may prove useful when examining the neural substrates of behaviours.

4.6.2 Juveniles

A future goal will be to further adapt the olfactory paradigm for use with juvenile zebrafish. The auditory paradigm has been successfully adapted for use on younger animals (Doyle et al. 2017). Juveniles (49 dpf) can successfully learn to associate an FM

tone sweep with food reward by the 10th to 13th pairing, and they can remember the association for at least two days. Merovitch (2016) showed that 30 dpf fish began to show an association between the 8th and 10th pairing and retained the memory for at least 2 days after training. Therefore, the olfactory paradigm will first adapted for use on 49 dpf fish and if successful will be further adapted for 30 dpf fish.

4.6.3 Physiological study

Another potential future study could be an examination of neural activity during memory retrieval. One method of studying neural activity is by examining the phosphorylation of activated extracellular kinases (ERKs). When the cell's membrane depolarises, it triggers an influx of calcium, which in turn triggers a mitogen-activated protein kinase (MAPK) cascade, which results in phosphorylation of ERKs (pERK) (Randlett et al. 2015). Several studies have examined the role of ERK in the retrieval of memories in rodents (Atkins et al. 1998; Selcher et al. 1999; Blum et al. 1999), and ERK has also been used as a general indicator of neural activity in larval zebrafish by using immunohistochemistry to stain against pERK (Randlett et al. 2015). Merovitch (2016) presented an examination of neural activity in the zebrafish brain during memory retrieval of a learned auditory association. This study determined that pERK immunoreactivity increases in certain regions of the brain when memory retrieval is occurring. The goal would be to apply the same technique to examine neural activity with fish that have undergone conditioning with the visual or olfactory paradigms. In fact, several studies have already used pERK to examine activity in the olfactory bulb

when the animal is exposed to an odour (Biechl et al. 2016; Mirich et al. 2004; Hussain et al. 2013).

A further study could explore how olfactory discrimination abilities develop as zebrafish age. The zebrafish olfactory system begins development before hatching at about 22 hours post fertilisation (hpf), and by 48 hpf rudimentary proto-glomeruli have developed (Whitlock & Westerfield 1998; Miyasaka et al. 2007). Over the course of development, the glomeruli begin to differentiate into smaller, more numerous glomeruli. As mentioned in Section 3.1 – Olfactory Introduction, certain odourants, like amino acids, elicit activity in certain glomeruli. However, when the fish are developing, these glomeruli may still be amalgamated and therefore may be unable to differentiate between two similar odours. The goal of this experiment would be to determine at what age the fish develop the ability to discriminate between amino acids. At 30 dpf the olfactory paradigm could be used to condition the fish to a specific amino acid, but during the inter-trial period, a second amino acid will be introduced to the tank. This could determine if the fish can discriminate between the two amino acids and successfully associate the correct amino acid with a food reward. This could also be paralleled with a physiological study of the differentiation of the glomeruli in the olfactory bulb.

CHAPTER 5: CONCLUSIONS

In conclusion, this thesis presents two successful home tank learning paradigms for zebrafish using a visual or olfactory cue. The visual paradigm conditioned fish to associate a light cue with a food reward in 7-10 pairings and retain the memory of this association for at least two days. The olfactory paradigm, although weaker than the visual paradigm, still showed formation of an association over two days and some indication of retention.

Both these paradigms eliminate the need for a specialised apparatus, which in turn increases efficiency and reduces handling stress on the fish. This simple, low-cost, automated system permits scalable conditioning of zebrafish with minimal human intervention, greatly reducing both variability and labour-intensiveness. It will be useful for studies of the neural basis of learning and memory, and for high-throughput screening of compounds modifying those processes.

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APPENDIX A: SUPPLEMENTAL MOVIES

Supplemental movies can be accessed from Dalspace (dalspace.library.dal.ca)

Supplemental Movie 1: Representative video of acquisition trial 20 for visual conditioning in a control tank. Fish exhibit normal swimming behaviour in the 20 seconds before the presentation of conditioned stimulus. During the 20 second presentation of the conditioned stimulus, green light (bottom of tank), the fish did not exhibit any changes in their swimming behaviour.

Supplemental Movie 2: Representative video of acquisition trial 20 for visual conditioning in an experimental tank. Fish exhibit normal swimming behaviour in the 20 seconds before the presentation of conditioned stimulus. During the 20 second presentation of the conditioned stimulus, green light (bottom of tank), fish moved towards the food source (upper-left corner of the tank) in anticipation of the food reward.

Supplemental Movie 3: Demonstration of odourant delivery to tank during olfactory conditioning. Blue food dye is substituted for phenylethyl alcohol to provide a visual representation of odourant dispersion and how fish encounter areas of high concentration.

APPENDIX B: ARDUINO SKETCHES FOR CONDITIONING AND RETENTION

B.1: VISUAL CONDITIONING DAY ONE

```
#include <Time.h>
#include <TimeAlarms.h>
#include <DS1307RTC.h>
#include <Wire.h>
#include <Adafruit_MotorShield.h>
#include "utility/Adafruit_PWMServoDriver.h"

Adafruit_MotorShield AFMS = Adafruit_MotorShield();

Adafruit_StepperMotor *Motor = AFMS.getStepper(200, 1);

#define REDPIN 6
#define GREENPIN 9
#define BLUEPIN 11

void setup() {
  Serial.begin(9600);
  while (!Serial) ; // wait until Arduino Serial Monitor opens
  setSyncProvider(RTC.get);   // the function to get the time from the RTC
  Alarm.alarmRepeat(9,45,0, LightOn);  // Set Alarm For 9:45AM Every Day
  Alarm.alarmRepeat(9,45,20, LightOff);  // Set Alarm For 9:45:20AM Every Day
  Alarm.alarmRepeat(9,45,20, Feeder);  // Set Alarm For 9:45:20AM Every Day
  Alarm.alarmRepeat(10,19,0, LightOn);  // Set Alarm For 10:19AM Every Day
  Alarm.alarmRepeat(10,19,20, LightOff);  // Set Alarm For 10:19:20AM Every Day
  Alarm.alarmRepeat(10,19,20, Feeder);  // Set Alarm For 10:19:20AM Every Day
  Alarm.alarmRepeat(11,28,0, LightOn);  // Set Alarm For 11:28AM Every Day
  Alarm.alarmRepeat(11,28,20, LightOff);  // Set Alarm For 11:28:20AM Every Day
  Alarm.alarmRepeat(11,28,20, Feeder);  // Set Alarm For 11:28:20AM Every Day
  Alarm.alarmRepeat(12,20,0, LightOn);  // Set Alarm For 12:20PM Every Day
  Alarm.alarmRepeat(12,20,20, LightOff);  // Set Alarm For 12:20PM Every Day
  Alarm.alarmRepeat(12,20,20, Feeder);  // Set Alarm For 12:20PM Every Day
  Alarm.alarmRepeat(13,48,0, LightOn);  // Set Alarm For 13:48PM Every Day
  Alarm.alarmRepeat(13,48,20, LightOff);  // Set Alarm For 13:48:20PM Every Day
  Alarm.alarmRepeat(13,48,20, Feeder);  // Set Alarm For 13:48:20PM Every Day
  Alarm.alarmRepeat(14,35,0, LightOn);  // Set Alarm For 14:35PM Every Day
  Alarm.alarmRepeat(14,35,20, LightOff);  // Set Alarm For 14:35:20PM Every Day
  Alarm.alarmRepeat(14,35,20, Feeder);  // Set Alarm For 14:35:20PM Every Day
  Alarm.alarmRepeat(16,23,0, LightOn);  // Set Alarm For 16:23OM Every Day
```

```
  Alarm.alarmRepeat(16,23,20, LightOff);  // Set Alarm For 16:23:20PM Every Day
  Alarm.alarmRepeat(16,23,20, Feeder);  // Set Alarm For 16:23:20PM Every Day
  Alarm.alarmRepeat(17,59,0, LightOn);  // Set Alarm For 17:59PM Every Day
  Alarm.alarmRepeat(17,59,20, LightOff);  // Set Alarm For 17:59:20PM Every Day
  Alarm.alarmRepeat(17,59,20, Feeder);  // Set Alarm For 17:59:20PM Every Day
  Alarm.alarmRepeat(19,10,0, LightOn);  // Set Alarm For 19:10PM Every Day
  Alarm.alarmRepeat(19,10,20, LightOff);  // Set Alarm For 19:10:20PM Every Day
  Alarm.alarmRepeat(19,10,20, Feeder);  // Set Alarm For 19:10:20PM Every Day
  Alarm.alarmRepeat(20,22,0, LightOn);  // Set Alarm For 20:22PM Every Day
  Alarm.alarmRepeat(20,22,20, LightOff);  // Set Alarm For 20:22:20PM Every Day
  Alarm.alarmRepeat(20,22,20, Feeder);  // Set Alarm For 20:22:20PM Every Day

  pinMode(REDPIN, OUTPUT);
  pinMode(GREENPIN, OUTPUT);
  pinMode(BLUEPIN, OUTPUT);

  AFMS.begin(); // Start the bottom shield
  Motor->setSpeed(500); // Speed in RPM
}

void loop() {
 Alarm.delay(1000); // wait one second between clock display
}

void LightOn() {
 analogWrite(REDPIN, 255);
 analogWrite(GREENPIN, 255);
 analogWrite(BLUEPIN, 0);
}

void LightOff() {
 analogWrite(REDPIN, 0);
 analogWrite(GREENPIN, 0);
 analogWrite(BLUEPIN, 0);
}

void Feeder() {
 Motor->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
 Motor->release();

}
```

B.2: VISUAL CONDITIONING DAY TWO

```
#include <Time.h>
#include <TimeAlarms.h>
#include <DS1307RTC.h>
#include <Wire.h>
#include <Adafruit_MotorShield.h>
#include "utility/Adafruit_PWMServoDriver.h"

Adafruit_MotorShield AFMS = Adafruit_MotorShield();

Adafruit_StepperMotor *Motor = AFMS.getStepper(200, 1);

#define REDPIN 6
#define GREENPIN 9
#define BLUEPIN 11

void setup() {
   Serial.begin(9600);
   while (!Serial) ; // wait until Arduino Serial MOnitor opens
   setSyncProvider(RTC.get);   // the functiOn to get the time from the RTC
  Alarm.alarmRepeat(10,06,0, LightOn);  // Set Alarm For 10:06AM Every Day
   Alarm.alarmRepeat(10,06,20, LightOff);  // Set Alarm For 10:06:20AM Every Day
   Alarm.alarmRepeat(10,06,20, Feeder);  // Set Alarm For 10:06:20AM Every Day
   Alarm.alarmRepeat(10,58,0, LightOn);  // Set Alarm For 10:58AM Every Day
   Alarm.alarmRepeat(10,58,20, LightOff);  // Set Alarm For 10:58:20AM Every Day
   Alarm.alarmRepeat(10,58,20, Feeder);  // Set Alarm For 10:58:20AM Every Day
   Alarm.alarmRepeat(11,46,0, LightOn);  // Set Alarm For 11:46AM Every Day
   Alarm.alarmRepeat(11,46,20, LightOff);  // Set Alarm For 11:46:20AM Every Day
   Alarm.alarmRepeat(11,46,20, Feeder);  // Set Alarm For 11:46:20AM Every Day
   Alarm.alarmRepeat(12,34,0, LightOn);  // Set Alarm For 12:34PM Every Day
   Alarm.alarmRepeat(12,34,20, LightOff);  // Set Alarm For 12:34PM Every Day
   Alarm.alarmRepeat(12,34,20, Feeder);  // Set Alarm For 12:34PM Every Day
   Alarm.alarmRepeat(13,54,0, LightOn);  // Set Alarm For 13:54PM Every Day
   Alarm.alarmRepeat(13,54,20, LightOff);  // Set Alarm For 13:54:20PM Every Day
   Alarm.alarmRepeat(13,54,20, Feeder);  // Set Alarm For 13:54:20PM Every Day
   Alarm.alarmRepeat(14,38,0, LightOn);  // Set Alarm For 14:38PM Every Day
   Alarm.alarmRepeat(14,38,20, LightOff);  // Set Alarm For 14:38:20PM Every Day
   Alarm.alarmRepeat(14,38,20, Feeder);  // Set Alarm For 14:38:20PM Every Day
   Alarm.alarmRepeat(15,52,0, LightOn);  // Set Alarm For 15:52PM Every Day
   Alarm.alarmRepeat(15,52,20, LightOff);  // Set Alarm For 15:52:20PM Every Day
   Alarm.alarmRepeat(15,52,20, Feeder);  // Set Alarm For 15:52:20PM Every Day
```

```
  Alarm.alarmRepeat(17,42,0, LightOn);  // Set Alarm For 17:42PM Every Day
  Alarm.alarmRepeat(17,42,20, LightOff);  // Set Alarm For 17:42:20PM Every Day
  Alarm.alarmRepeat(17,42,20, Feeder);  // Set Alarm For 17:42:20PM Every Day
  Alarm.alarmRepeat(19,20,0, LightOn);  // Set Alarm For 19:20PM Every Day
  Alarm.alarmRepeat(19,20,20, LightOff);  // Set Alarm For 19:20:20PM Every Day
  Alarm.alarmRepeat(19,20,20, Feeder);  // Set Alarm For 19:20:20PM Every Day
  Alarm.alarmRepeat(20,06,0, LightOn);  // Set Alarm For 20:06PM Every Day
  Alarm.alarmRepeat(20,06,20, LightOff);  // Set Alarm For 20:06:20PM Every Day
  Alarm.alarmRepeat(20,06,20, Feeder);  // Set Alarm For 20:06:20PM Every Day

  pinMode(REDPIN, OUTPUT);
  pinMode(GREENPIN, OUTPUT);
  pinMode(BLUEPIN, OUTPUT);

  AFMS.begin(); // Start the bottom shield
  Motor->setSpeed(500); // Speed in RPM
}

void loop() {
 Alarm.delay(1000); // wait one second between clock display
}

void LightOn() {
 analogWrite(REDPIN, 255);
 analogWrite(GREENPIN, 255);
 analogWrite(BLUEPIN, 0);
}

void LightOff() {
 analogWrite(REDPIN, 0);
 analogWrite(GREENPIN, 0);
 analogWrite(BLUEPIN, 0);
}

void Feeder() {
 Motor->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
 Motor->release();


}
```

B.3: VISUAL CONTROL DAY ONE

```
#include <Time.h>
#include <TimeAlarms.h>
#include <DS1307RTC.h>
#include <Wire.h>
#include <Adafruit_MotorShield.h>
#include "utility/Adafruit_PWMServoDriver.h"

Adafruit_MotorShield AFMS = Adafruit_MotorShield();

Adafruit_StepperMotor *Motor = AFMS.getStepper(200, 1);

#define REDPIN 6
#define GREENPIN 9
#define BLUEPIN 11

void setup() {
  Serial.begin(9600);
  while (!Serial) ; // wait until Arduino Serial Monitor opens
  setSyncProvider(RTC.get);   // the function to get the time from the RTC
  Alarm.alarmRepeat(9,45,0, LightOn);  // Set Alarm For 9:45AM Every Day
  Alarm.alarmRepeat(9,45,20, LightOff);  // Set Alarm For 9:45:20AM Every Day
  Alarm.alarmRepeat(10,02,20, Feeder);  // Set Alarm For 10:02:20AM Every Day
  Alarm.alarmRepeat(10,19,0, LightOn);  // Set Alarm For 10:19AM Every Day
  Alarm.alarmRepeat(10,19,20, LightOff);  // Set Alarm For 10:19:20AM Every Day
  Alarm.alarmRepeat(10,53,20, Feeder);  // Set Alarm For 10:52:20AM Every Day
  Alarm.alarmRepeat(11,28,0, LightOn);  // Set Alarm For 11:28AM Every Day
  Alarm.alarmRepeat(11,28,20, LightOff);  // Set Alarm For 11:28:20AM Every Day
  Alarm.alarmRepeat(11,54,20, Feeder);  // Set Alarm For 11:54:20AM Every Day
  Alarm.alarmRepeat(12,20,0, LightOn);  // Set Alarm For 12:20PM Every Day
  Alarm.alarmRepeat(12,20,20, LightOff);  // Set Alarm For 12:20:20PM Every Day
  Alarm.alarmRepeat(13,02,20, Feeder);  // Set Alarm For 13:02:20PM Every Day
  Alarm.alarmRepeat(13,48,0, LightOn);  // Set Alarm For 13:48PM Every Day
  Alarm.alarmRepeat(13,48,20, LightOff);  // Set Alarm For 13:48:20PM Every Day
  Alarm.alarmRepeat(14,11,20, Feeder);  // Set Alarm For 14:11:20PM Every Day
  Alarm.alarmRepeat(14,35,0, LightOn);  // Set Alarm For 14:35PM Every Day
  Alarm.alarmRepeat(14,35,20, LightOff);  // Set Alarm For 14:35:20PM Every Day
  Alarm.alarmRepeat(15,29,20, Feeder);  // Set Alarm For 15:29:20PM Every Day
  Alarm.alarmRepeat(16,23,0, LightOn);  // Set Alarm For 16:23PM Every Day
  Alarm.alarmRepeat(16,23,20, LightOff);  // Set Alarm For 16:23:20PM Every Day
  Alarm.alarmRepeat(17,11,20, Feeder);  // Set Alarm For 17:11:20PM Every Day
```

```
  Alarm.alarmRepeat(17,59,0, LightOn);  // Set Alarm For 17:59PM Every Day
  Alarm.alarmRepeat(17,59,20, LightOff);  // Set Alarm For 17:59:20PM Every Day
  Alarm.alarmRepeat(18,35,20, Feeder);  // Set Alarm For 18:35:20PM Every Day
  Alarm.alarmRepeat(19,10,0, LightOn);  // Set Alarm For 19:10PM Every Day
  Alarm.alarmRepeat(19,10,20, LightOff);  // Set Alarm For 19:10:20PM Every Day
  Alarm.alarmRepeat(19,46,20, Feeder);  // Set Alarm For 19:46:20PM Every Day
  Alarm.alarmRepeat(20,22,0, LightOn);  // Set Alarm For 20:22PM Every Day
  Alarm.alarmRepeat(20,22,20, LightOff);  // Set Alarm For 20:22:20PM Every Day
  Alarm.alarmRepeat(20,54,20, Feeder);  // Set Alarm For 20:54:20PM Every Day

  pinMode(REDPIN, OUTPUT);
  pinMode(GREENPIN, OUTPUT);
  pinMode(BLUEPIN, OUTPUT);

  AFMS.begin(); // Start the bottom shield
  Motor->setSpeed(500); // Speed in RPM
}

void loop() {
 Alarm.delay(1000); // wait one second between clock display
}

void LightOn() {
 analogWrite(REDPIN, 255);
 analogWrite(GREENPIN, 255);
 analogWrite(BLUEPIN, 0);
}

void LightOff() {
 analogWrite(REDPIN, 0);
 analogWrite(GREENPIN, 0);
 analogWrite(BLUEPIN, 0);
}

void Feeder() {
 Motor->step(25, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
 Motor->release();


}
```

B.4: VISUAL CONTROL DAY TWO

```
#include <Time.h>
#include <TimeAlarms.h>
#include <DS1307RTC.h>
#include <Wire.h>
#include <Adafruit_MotorShield.h>
#include "utility/Adafruit_PWMServoDriver.h"

Adafruit_MotorShield AFMS = Adafruit_MotorShield();

Adafruit_StepperMotor *Motor = AFMS.getStepper(200, 1);

#define REDPIN 6
#define GREENPIN 9
#define BLUEPIN 11

void setup() {
  Serial.begin(9600);
  while (!Serial) ; // wait until Arduino Serial Monitor opens
  setSyncProvider(RTC.get);   // the function to get the time from the RTC
  Alarm.alarmRepeat(10,06,0, LightOn);  // Set Alarm For 10:06AM Every Day
  Alarm.alarmRepeat(10,06,20, LightOff);  // Set Alarm For 10:06:20AM Every Day
  Alarm.alarmRepeat(10,26,20, Feeder);  // Set Alarm For 10:26:20AM Every Day
  Alarm.alarmRepeat(10,58,0, LightOn);  // Set Alarm For 10:58AM Every Day
  Alarm.alarmRepeat(10,58,20, LightOff);  // Set Alarm For 10:58:20AM Every Day
  Alarm.alarmRepeat(11,22,20, Feeder);  // Set Alarm For 11:22:20AM Every Day
  Alarm.alarmRepeat(11,46,0, LightOn);  // Set Alarm For 11:46AM Every Day
  Alarm.alarmRepeat(11,46,20, LightOff);  // Set Alarm For 11:46:20AM Every Day
  Alarm.alarmRepeat(12,10,20, Feeder);  // Set Alarm For 12:10:20AM Every Day
  Alarm.alarmRepeat(12,34,0, LightOn);  // Set Alarm For 12:34PM Every Day
  Alarm.alarmRepeat(12,34,20, LightOff);  // Set Alarm For 12:34PM Every Day
  Alarm.alarmRepeat(13,14,20, Feeder);  // Set Alarm For 13:14PM Every Day
  Alarm.alarmRepeat(13,54,0, LightOn);  // Set Alarm For 13:54PM Every Day
  Alarm.alarmRepeat(13,54,20, LightOff);  // Set Alarm For 13:54:20PM Every Day
  Alarm.alarmRepeat(14,16,20, Feeder);  // Set Alarm For 14:16:20PM Every Day
  Alarm.alarmRepeat(14,38,0, LightOn);  // Set Alarm For 14:38PM Every Day
  Alarm.alarmRepeat(14,38,20, LightOff);  // Set Alarm For 14:38:20PM Every Day
  Alarm.alarmRepeat(15,15,20, Feeder);  // Set Alarm For 15:15:20PM Every Day
  Alarm.alarmRepeat(15,52,0, LightOn);  // Set Alarm For 15:52PM Every Day
  Alarm.alarmRepeat(15,52,20, LightOff);  // Set Alarm For 15:52:20PM Every Day
  Alarm.alarmRepeat(16,47,20, Feeder);  // Set Alarm For 16:47:20PM Every Day
```

```
  Alarm.alarmRepeat(17,42,0, LightOn);  // Set Alarm For 17:42PM Every Day
  Alarm.alarmRepeat(17,42,20, LightOff);  // Set Alarm For 17:42:20PM Every Day
  Alarm.alarmRepeat(18,31,20, Feeder);  // Set Alarm For 18:31:20PM Every Day
  Alarm.alarmRepeat(19,20,0, LightOn);  // Set Alarm For 19:20PM Every Day
  Alarm.alarmRepeat(19,20,20, LightOff);  // Set Alarm For 19:20:20PM Every Day
  Alarm.alarmRepeat(19,43,20, Feeder);  // Set Alarm For 19:43:20PM Every Day
  Alarm.alarmRepeat(20,06,0, LightOn);  // Set Alarm For 20:06PM Every Day
  Alarm.alarmRepeat(20,06,20, LightOff);  // Set Alarm For 20:06:20PM Every Day
  Alarm.alarmRepeat(20,36,20, Feeder);  // Set Alarm For 20:36:20PM Every Day

  pinMode(REDPIN, OUTPUT);
  pinMode(GREENPIN, OUTPUT);
  pinMode(BLUEPIN, OUTPUT);

  AFMS.begin(); // Start the bottom shield
  Motor->setSpeed(500); // Speed in RPM
}

void loop() {
 Alarm.delay(1000); // wait one second between clock display
}

void LightOn() {
 analogWrite(REDPIN, 255);
 analogWrite(GREENPIN, 255);
 analogWrite(BLUEPIN, 0);
}

void LightOff() {
 analogWrite(REDPIN, 0);
 analogWrite(GREENPIN, 0);
 analogWrite(BLUEPIN, 0);
}

void Feeder() {
 Motor->step(25, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
 Motor->release();


}
```

B.5: VISUAL RETENTION

```
#include <Time.h>
#include <TimeAlarms.h>
#include <DS1307RTC.h>
#include <Wire.h>
#include <Adafruit_MotorShield.h>
#include "utility/Adafruit_PWMServoDriver.h"

Adafruit_MotorShield AFMS = Adafruit_MotorShield();

Adafruit_StepperMotor *Motor = AFMS.getStepper(200, 1);

#define REDPIN 6
#define GREENPIN 9
#define BLUEPIN 11

void setup() {
   Serial.begin(9600);
   while (!Serial) ; // wait until Arduino Serial MOnitor opens
   setSyncProvider(RTC.get);   // the functiOn to get the time from the RTC
  Alarm.alarmRepeat(15,1,0, LightOn);  // Set Alarm For 15:1AM Every Day
   Alarm.alarmRepeat(15,1,20, LightOff);  // Set Alarm For 15:1:20AM Every Day

   pinMode(REDPIN, OUTPUT);
   pinMode(GREENPIN, OUTPUT);
   pinMode(BLUEPIN, OUTPUT);

   AFMS.begin(); // Start the bottom shield
   Motor->setSpeed(500); // Speed in RPM
}

void loop() {
  Alarm.delay(1000); // wait one second between clock display
}

void LightOn() {
  analogWrite(REDPIN, 255);
  analogWrite(GREENPIN, 255);
  analogWrite(BLUEPIN, 0);
}
```

```
void LightOff() {
  analogWrite(REDPIN, 0);
  analogWrite(GREENPIN, 0);
  analogWrite(BLUEPIN, 0);
}

}
```

B.6: OLFACTORY CONDITIONING DAY ONE/ TWO (CONDITIONING & CONTROL)

```
#include <Time.h>
#include <TimeAlarms.h>
#include <DS1307RTC.h>
#include <Wire.h>
#include <Adafruit_MotorShield.h>
#include "utility/Adafruit_PWMServoDriver.h"

Adafruit_MotorShield AFMS = Adafruit_MotorShield();
Adafruit_StepperMotor *stepper1 = AFMS.getStepper(200, 1);
Adafruit_StepperMotor *stepper2 = AFMS.getStepper(200, 2);


void setup() {

  pinMode(10, OUTPUT);
  pinMode(9, OUTPUT);
  digitalWrite(9, HIGH);

  AFMS.begin(); // Start the bottom shield
  stepper1->setSpeed(500); // Speed in RPM
  stepper2->setSpeed(500); // Speed in RPM



Serial.begin(9600);
  while (!Serial) ; // wait until Arduino Serial Monitor opens
  //setTime(9,59,0,9,31,14); // set time to Wednesday 15:18:00pm April 16 2014
  setSyncProvider(RTC.get);   // the function to get the time from the RTC
    Alarm.alarmRepeat(8,7,0, PumpOn);  // Set Alarm For 9:45AM Every Day
    Alarm.alarmRepeat(8,7,20, Feeder1);  // Set Alarm For 9:45:20AM Every DayFeeder2
    Alarm.alarmRepeat(8,20,0, Feeder2);  // Set Alarm For 9:45:20AM Every Day

    Alarm.alarmRepeat(8,40,0, PumpOn);  // Set Alarm For 9:45AM Every Day
    Alarm.alarmRepeat(8,40,20, Feeder1);  // Set Alarm For 9:45:20AM Every DayFeeder2
    Alarm.alarmRepeat(9,5,0, Feeder2);  // Set Alarm For 9:45:20AM Every Day

    Alarm.alarmRepeat(9,30,0, PumpOn);  // Set Alarm For 10:19AM Every Day
    Alarm.alarmRepeat(9,30,20, Feeder1);  // Set Alarm For 10:19:20AM Every Day
    Alarm.alarmRepeat(9,50,0, Feeder2);  // Set Alarm For 10:19:20AM Every Day
```

```
  Alarm.alarmRepeat(10,15,0, PumpOn);  // Set Alarm For 11:28AM Every Day
  Alarm.alarmRepeat(10,15,20, Feeder1);  // Set Alarm For 11:28:20AM Every Day
  Alarm.alarmRepeat(10,35,0, Feeder2);  // Set Alarm For 11:28:20AM Every Day

  Alarm.alarmRepeat(10,50,0, PumpOn);  // Set Alarm For 12:20PM Every Day
  Alarm.alarmRepeat(10,50,20, Feeder1);  // Set Alarm For 12:20PM Every Day
  Alarm.alarmRepeat(11,15,0, Feeder2);  // Set Alarm For 12:20PM Every Day

  Alarm.alarmRepeat(11,50,0, PumpOn);  // Set Alarm For 13:48PM Every Day
  Alarm.alarmRepeat(11,50,20, Feeder1);  // Set Alarm For 13:48:20PM Every Day
  Alarm.alarmRepeat(12,10,0, Feeder2);  // Set Alarm For 13:48:20PM Every Day

  Alarm.alarmRepeat(12,30,0, PumpOn);  // Set Alarm For 14:35PM Every Day
  Alarm.alarmRepeat(12,30,20, Feeder1);  // Set Alarm For 14:35:20PM Every Day
  Alarm.alarmRepeat(12,52,0, Feeder2);  // Set Alarm For 14:35:20PM Every Day

  Alarm.alarmRepeat(13,15,0, PumpOn);  // Set Alarm For 16:23OM Every Day
  Alarm.alarmRepeat(13,15,20, Feeder1);  // Set Alarm For 16:23:20PM Every Day
  Alarm.alarmRepeat(13,45,0, Feeder2);  // Set Alarm For 16:23:20PM Every Day

  Alarm.alarmRepeat(14,5,0, PumpOn);  // Set Alarm For 17:59PM Every Day
  Alarm.alarmRepeat(14,5,20, Feeder1);  // Set Alarm For 17:59:20PM Every Day
  Alarm.alarmRepeat(14,35,0, Feeder2);  // Set Alarm For 17:59:20PM Every Day

  Alarm.alarmRepeat(15,0,0, PumpOn);  // Set Alarm For 19:10PM Every Day
  Alarm.alarmRepeat(15,0,20, Feeder1);  // Set Alarm For 19:10:20PM Every Day
  Alarm.alarmRepeat(15,20,0, Feeder2);  // Set Alarm For 19:10:20PM Every Day

}

void loop() {
 Alarm.delay(1000); // wait one second between clock display

}

void PumpOn() {
 digitalWrite(10, HIGH);
 digitalWrite(9, LOW);
 delay(100);
 digitalWrite(9, HIGH);
 delay(6900);
 digitalWrite(10, LOW);
```

```
  digitalWrite(9, LOW);
delay(100);
  digitalWrite(9, HIGH);
}



void Feeder1() {
  stepper1->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
  stepper1->release();
}
void Feeder2() {
  stepper2->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
  stepper2->release();
}
```

B.7: WATER ONLY TRIAL

```
#include <Time.h>
#include <TimeAlarms.h>
#include <DS1307RTC.h>
#include <Wire.h>
#include <Adafruit_MotorShield.h>
#include "utility/Adafruit_PWMServoDriver.h"

Adafruit_MotorShield AFMS = Adafruit_MotorShield();
Adafruit_StepperMotor *stepper1 = AFMS.getStepper(200, 1);
Adafruit_StepperMotor *stepper2 = AFMS.getStepper(200, 2);


void setup() {

  pinMode(10, OUTPUT);
  pinMode(9, OUTPUT);
  digitalWrite(9, HIGH);

  AFMS.begin(); // Start the bottom shield
  stepper1->setSpeed(500); // Speed in RPM
  stepper2->setSpeed(500); // Speed in RPM



Serial.begin(9600);
  while (!Serial) ; // wait until Arduino Serial Monitor opens
  //setTime(9,59,0,9,31,14); // set time to Wednesday 15:18:00pm April 16 2014
  setSyncProvider(RTC.get);   // the function to get the time from the RTC
    Alarm.alarmRepeat(8,7,0, PumpOn);  // Set Alarm For 9:45AM Every Day


}

void loop() {
  Alarm.delay(1000); // wait one second between clock display

}

void PumpOn() {
  digitalWrite(10, HIGH);
```

```
  digitalWrite(9, LOW);
  delay(100);
  digitalWrite(9, HIGH);
  delay(6900);
  digitalWrite(10, LOW);
  digitalWrite(9, LOW);
delay(100);
  digitalWrite(9, HIGH);
}



void Feeder1() {
  stepper1->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
  stepper1->release();
}
void Feeder2() {
  stepper2->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
  stepper2->release();
}
```

B.8: OLFACTORY RETENTION

```
#include <Time.h>
#include <TimeAlarms.h>
#include <DS1307RTC.h>
#include <Wire.h>
#include <Adafruit_MotorShield.h>
#include "utility/Adafruit_PWMServoDriver.h"

Adafruit_MotorShield AFMS = Adafruit_MotorShield();
Adafruit_StepperMotor *stepper1 = AFMS.getStepper(200, 1);
Adafruit_StepperMotor *stepper2 = AFMS.getStepper(200, 2);


void setup() {

  pinMode(10, OUTPUT);
  pinMode(9, OUTPUT);
  digitalWrite(9, HIGH);

  AFMS.begin(); // Start the bottom shield
  stepper1->setSpeed(500); // Speed in RPM
  stepper2->setSpeed(500); // Speed in RPM



Serial.begin(9600);
  while (!Serial) ; // wait until Arduino Serial Monitor opens
  //setTime(9,59,0,9,31,14); // set time to Wednesday 15:18:00pm April 16 2014
  setSyncProvider(RTC.get);   // the function to get the time from the RTC
    Alarm.alarmRepeat(8,7,0, PumpOn);  // Set Alarm For 9:45AM Every Day
    Alarm.alarmRepeat(8,40,0, PumpOn);  // Set Alarm For 9:45AM Every Day

}

void loop() {
  Alarm.delay(1000); // wait one second between clock display

}

void PumpOn() {
  digitalWrite(10, HIGH);
```

```
  digitalWrite(9, LOW);
  delay(100);
  digitalWrite(9, HIGH);
  delay(6900);
  digitalWrite(10, LOW);
  digitalWrite(9, LOW);
delay(100);
  digitalWrite(9, HIGH);
}



void Feeder1() {
  stepper1->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
  stepper1->release();
}
void Feeder2() {
  stepper2->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE, DOUBLE,
INTERLEAVE, MICROSTEP)
  stepper2->release();
}
```

APPENDIX C: STATISTICAL ANALYSIS FOR VISUAL PARADIGM

C.1: VISUAL ACQUISITION – LINEAR MIXED EFFECTS MODEL – MOVEMENT TOWARDS FOOD

Visual dD
refitting model(s) with ML (instead of REML)
Data: dfs
Models:
mod.dfs3: Measure ~ Condition + TrialN + (TrialN | Tank)
mod.dfs5: Measure ~ Condition * TrialN + (TrialN | Tank)

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
mod.dfs3	**7**	**4051.5**	**4084.6**	**-2018.7**	**4037.5**			
mod.dfs5	**8**	**4021.7**	**4059.6**	**-2002.8**	**4005.7**	**31.755**	**1**	**1.749e-08 *** **

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Linear mixed model fit by REML ['lmerMod']
Formula: Measure ~ Condition * TrialN + (TrialN | Tank)
Data: dfs

REML criterion at convergence: 4016.5

Scaled residuals:

Min	1Q	Median	3Q	Max
-4.1367	-0.5832	-0.0825	0.5558	4.3496

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
Tank	(Intercept)	1.91916	1.385	
	TrialN	0.01368	0.117	-0.30
Residual		6.00896	2.451	

Number of obs: 839, groups: Tank, 42

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	0.06524	0.42280	0.154
ConditionE	-0.59856	0.55925	-1.070
TrialN	0.02918	0.03553	0.821
ConditionE:TrialN	0.31596	0.04700	6.723

Correlation of Fixed Effects:

	(Intr)	CndtnE	TrialN
ConditionE	-0.756		
TrialN	-0.532	0.402	
CndtnE:TrlN	0.402	-0.532	-0.756

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure

	Chisq	Df	Pr(>Chisq)
Condition	8.7433	1	0.003107 **
TrialN	81.3192	1	< 2.2e-16 ***
Condition:TrialN	45.1943	1	1.784e-11 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

C.2: VISUAL ACQUISITION – LINEAR MIXED EFFECTS MODEL – VERTICAL MOVEMENT

Visual dY
refitting model(s) with ML (instead of REML)
Data: dfs
Models:
mod.dfs3: Measure ~ Condition + TrialN + (TrialN | Tank)
mod.dfs5: Measure ~ Condition * TrialN + (TrialN | Tank)

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
mod.dfs3	7	3656.8	3690.0	-1821.4	3642.8			
mod.dfs5	8	3630.6	3668.5	-1807.3	3614.6	28.233	1	**1.076e-07 *** **

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Linear mixed model fit by REML ['lmerMod']
Formula: Measure ~ Condition * TrialN + (TrialN | Tank)
Data: dfs

REML criterion at convergence: 3626.1

Scaled residuals:

Min	1Q	Median	3Q	Max
-3.6004	-0.5462	-0.0139	0.5338	3.4332

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
Tank	(Intercept)	3.09189	1.7584	
	TrialN	0.01066	0.1033	-0.58
Residual		3.65380	1.9115	

Number of obs: 839, groups: Tank, 42

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	0.51428	0.46437	1.107
ConditionE	-0.04497	0.61426	-0.073
TrialN	0.01759	0.02996	0.587
ConditionE:TrialN	0.24541	0.03963	6.192

Correlation of Fixed Effects:

	(Intr)	CndtnE	TrialN
ConditionE	-0.756		
TrialN	-0.650	0.491	
CndtnE:TrlN	0.491	-0.650	-0.756

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure

	Chisq	Df	Pr(>Chisq)
Condition	26.977	1	2.058e-07 ***
TrialN	64.756	1	8.477e-16 ***
Condition:TrialN	38.342	1	5.937e-10 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

C.3: VISUAL ACQUISITION – LINEAR MIXED EFFECTS MODEL – HORIZONTAL MOVEMENT

Visual dX
refitting model(s) with ML (instead of REML)
Data: dfs
Models:
mod.dfs3: Measure ~ Condition + TrialN + (TrialN | Tank)
mod.dfs5: Measure ~ Condition * TrialN + (TrialN | Tank)

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
mod.dfs3	7	3962.8	3995.9	-1974.4	3948.8			
mod.dfs5	8	3949.0	3986.9	-1966.5	3933.0	15.798	1	**7.048e-05 *** **

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Linear mixed model fit by REML ['lmerMod']
Formula: Measure ~ Condition * TrialN + (TrialN | Tank)
Data: dfs

REML criterion at convergence: 3943.8

Scaled residuals:

Min	1Q	Median	3Q	Max
-3.8945	-0.5757	-0.0350	0.5641	4.6594

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
Tank	(Intercept)	1.92618	1.3879	
	TrialN	0.01671	0.1292	-0.38
Residual		5.45141	2.3348	

Number of obs: 839, groups: Tank, 42

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	-0.35632	0.41528	-0.858
ConditionE	-0.65661	0.54930	-1.195
TrialN	0.02411	0.03720	0.648
ConditionE:TrialN	0.21030	0.04921	4.274

Correlation of Fixed Effects:

	(Intr)	CndtnE	TrialN
ConditionE	-0.756		
TrialN	-0.555	0.420	
CndtnE:TrlN	0.420	-0.555	-0.756

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure

	Chisq	Df	Pr(>Chisq)
Condition	2.0013	1	0.1572
TrialN	35.1094	1	3.117e-09 ***
Condition:TrialN	18.2659	1	1.921e-05 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

C.4: VISUAL GROUP RETENTION – TWO-WAY ANOVA – MOVEMENT TOWARDS FOOD

> # aov independent samples tests

VISUAL

> varname="dD"	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Condition	1	125.72	125.72	16.476	0.000613 ***
DayN	1	9.87	9.87	1.294	0.268787
Condition:DayN	1	17.50	17.50	2.294	0.145531
Residuals	20	152.60	7.63		

C.5: VISUAL GROUP RETENTION – TWO-WAY ANOVA – VERTICAL MOVEMENT

> varname="dY"	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Condition	1	109.74	109.74	32.436	1.42e-05 ***
DayN	1	10.61	10.61	3.137	0.0918 .
Condition:DayN	1	2.11	2.11	0.623	0.4393
Residuals	20	67.66	3.38		

C.6: VISUAL GROUP RETENTION – TWO-WAY ANOVA – HORIZONTAL MOVEMENT

> varname="dX"	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Condition	1	40.75	40.75	4.936	0.0380 *
DayN	1	40.10	40.10	4.858	0.0394 *
Condition:DayN	1	18.29	18.29	2.216	0.1522
Residuals	20	165.10	8.25		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

C.7: VISUAL INDIVIDUAL RETENTION – TWO-WAY ANOVA – MOVEMENT TOWARDS FOOD

varname="dD"	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Condition	1	46.9	46.85	1.751	0.189
DayN	4	142.2	35.55	1.329	0.266
Condition:DayN	4	66.4	16.60	0.620	0.649
Residuals	82	2194.4	26.76		

C.8: VISUAL INDIVIDUAL RETENTION – TWO-WAY ANOVA – VERTICAL MOVEMENT

varname="dY"	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Condition	1	88.6	88.61	6.639	0.0118 *
DayN	4	78.9	19.73	1.478	0.2163
Condition:DayN	4	15.4	3.85	0.289	0.8845
Residuals	82	1094.5	13.35		

C.9 : VISUAL INDIVIDUAL RETENTION – TWO-WAY ANOVA – HORIZONTAL MOVEMENT

varname="dX"	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Condition	1	8.2	8.232	0.315	0.576
DayN	4	111.5	27.887	1.067	0.378
Condition:DayN	4	56.4	14.102	0.539	0.707
Residuals	82	2143.9	26.145		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

APPENDIX D: STASTISTICAL ANALYSIS FOR OLFACTORY PARADIGM

D.1: OLFACTORY ACQUISITION – LINEAR MIXED EFFECTS MODEL – MOVEMENT TOWARDS FOOD

Olfactory dD
refitting model(s) with ML (instead of REML)
Data: dfs
Models:
Mod.dfs3: Measure ~ Condition + TrialN + (TrialN | Tank)
Mod.dfs5: Measure ~ Condition * TrialN + (TrialN | Tank)

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
mod.dfs3	7	4331.9	4370.2	-2158.9	4317.9			
mod.dfs5	8	4331.9	4375.7	-2158.0	4315.9	1.9669	1	0.1608

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Linear mixed model fit by REML t-tests use
Satterthwaite approximations to degrees of
freedom [lmerMod]
Formula: Measure ~ Condition * TrialN + (TrialN | Tank)
Data: dfs

REML criterion at convergence: 4342.3

Scaled residuals:

Min	1Q	Median	3Q	Max
-8.6836	-0.4021	-0.0906	0.2646	7.7377

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
Tank	(Intercept)	0.1002759	0.31666	
	TrialN	0.0007043	0.02654	-0.77
Residual		0.6457511	0.80359	

Number of obs: 1760, groups: Tank, 44

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	2.028e-01	7.380e-02	8.510e+01	2.748	0.00732 **
ConditionE	2.131e-03	7.959e-02	1.670e+03	0.027	0.97865
TrialN	4.687e-05	6.171e-03	8.490e+01	0.008	0.99396
ConditionE:TrialN	9.315e-03	6.644e-03	1.670e+03	1.402	0.16109

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

	(Intr)	CndtnE	TrialN
ConditionE	-0.539		
TrialN	-0.833	0.472	
CndtnE:TrlN	0.473	-0.877	-0.538

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure

	Chisq	Df	Pr(>Chisq)
Condition	6.8049	1	**0.009091 ****
TrialN	0.8184	1	0.365656
Condition:TrialN	1.9657	1	0.160905

Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

D.2: OLFACTORY ACQUISITION – LINEAR MIXED EFFECTS MODEL – VERTICAL MOVEMENT

Olfactory dY
refitting model(s) with ML (instead of REML)
Data: dfs
Models:
Mod.dfs.3: Measure ~ Condition + TrialN + (TrialN | Tank)
Mod.dfs.5: Measure ~ Condition * TrialN + (TrialN | Tank)

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
Mod.dfs3	7	3639.9	3678.2	-1813.0	3625.9			
Mod.dfs5	8	3635.5	3679.3	-1809.8	3619.5	6.3594	1	**0.01168 ***

Signif. codes:
0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Linear mixed model fit by REML t-tests use
Satterthwaite approximations to degrees of
freedom [lmerMod]
Formula: Measure ~ Condition * TrialN + (TrialN | Tank)
Data: dfs

REML criterion at convergence: 3648.4

Scaled residuals:

Min	1Q	Median	3Q	Max
-6.3024	-0.3322	0.0484	0.3439	7.8136

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
Tank	(Intercept)	4.032e-03	0.06350	
	TrialN	6.512e-05	0.00807	1.00
Residual		4.458e-01	0.66770	

Number of obs: 1760, groups: Tank, 44

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	8.324e-02	4.773e-02	5.523e+02	1.744	0.08173
ConditionE	-1.932e-01	6.613e-02	1.713e+03	-2.922	0.00353 **
TrialN	5.728e-03	4.089e-03	3.093e+02	1.401	0.16222
ConditionE:TrialN	-1.392e-02	5.520e-03	1.713e+03	-2.522	0.01176 *

Signif. codes:

0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

	(Intr)	CndtnE	TrialN
ConditionE	-0.693		
TrialN	-0.760	0.592	
CndtnE:TrlN	0.607	-0.877	-0.675

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure

	Chisq	Df	Pr(>Chisq)
Condition	113.6815	1	< 2e-16 ***
TrialN	0.1670	1	0.68278
Condition:TrialN	6.3601	1	0.01167 *

Signif. codes:

0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

D.3: OLFACTORY ACQUISITION – LINEAR MIXED EFFECTS MODEL – HORIZONTAL MOVEMENT

Olfactory dX (Unbinned)

refitting model(s) with ML (instead of REML)

Data: dfs

Models:

mod.dfs3: Measure ~ Condition + TrialN + (TrialN | Tank)

mod.dfs5: Measure ~ Condition * TrialN + (TrialN | Tank)

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)
mod.dfs3	7	4798.0	4836.3	-2392.0	4784.0			
Mod.dfs5	8	4794.4	4838.2	-2389.2	4778.4	5.5545	1	**0.01843 ***

Signif. codes:

0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Linear mixed model fit by REML t-tests use

Satterthwaite approximations to degrees of freedom [lmerMod]

Formula:

Measure ~ Condition * TrialN + (TrialN | Tank)

Data: dfs

REML criterion at convergence: 4804.6

Scaled residuals:

Min	1Q	Median	3Q	Max
-7.7465	-0.2798	0.0646	0.3499	7.4203

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
Tank	(Intercept)	0.0512245	0.22633	
	TrialN	0.0002735	0.01654	-0.65
Residual		0.8596503	0.92717	

Number of obs: 1760, groups: Tank, 44

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	-2.328e-01	7.335e-02	1.150e+02	-3.173	0.00193 **
ConditionE	6.259e-02	9.183e-02	1.670e+03	0.682	0.49560
TrialN	7.882e-03	5.966e-03	1.231e+02	1.321	0.18889
ConditionE:TrialN	-1.807e-02	7.665e-03	1.670e+03	-2.357	0.01852 *

Signif. codes:

0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

	(Intr)	CndtnE	TrialN
ConditionE	-0.626		
TrialN	-0.832	0.563	
CndtnE:TrlN	0.549	-0.877	-0.642

> print(Anova(mod.dfs5))

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure

	Chisq	Df	Pr(>Chisq)
Condition	8.2748	1	**0.00402 ****
TrialN	0.0636	1	0.80097
Condition:TrialN	5.5570	1	**0.01841 ***

Signif. codes:

0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

D.4: OLFACTORY WATER TRIAL – T-TEST – MOVEMENT TOWARDS FOOD

Table Analyzed	Water Trial - dD
Column B	Experimental
vs.	vs.
Column A	Control
Unpaired t test	
P value	0.1637
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.405 df=86
How big is the difference?	
Mean ± SEM of column A	0.3189 ± 0.09996, n=44
Mean ± SEM of column B	0.1075 ± 0.1124, n=44
Difference between means	-0.2114 ± 0.1505
95% confidence interval	-0.5105 to 0.08773
R squared (eta squared)	0.02243
F test to compare variances	
F, DFn, Dfd	1.265, 43, 43
P value	0.4433
P value summary	ns
Significantly different (P < 0.05)?	No

D.5: OLFACTORY WATER TRIAL – T-TEST – VERTICAL MOVEMENT

Table Analyzed	Water Trial - dY
Column B	Experimental
vs.	vs.
Column A	Control
Unpaired t test	
P value	0.9587
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.0519 df=86
How big is the difference?	
Mean ± SEM of column A	0.05484 ± 0.09354, n=44
Mean ± SEM of column B	0.04855 ± 0.07713, n=44
Difference between means	-0.006292 ± 0.1212
95% confidence interval	-0.2473 to 0.2347
R squared (eta squared)	3.132e-005
F test to compare variances	
F, DFn, Dfd	1.471, 43, 43
P value	0.2100
P value summary	ns
Significantly different (P < 0.05)?	No

D.6: OLFACTORY WATER TRIAL – T-TEST – HORIZONTAL MOVEMENT

Table Analyzed	Water Trial - dX
Column B	**Experimental**
vs.	**vs.**
Column A	**Control**
Unpaired t test	
P value	**0.0441**
P value summary	*
Significantly different (P < 0.05)?	**Yes**
One- or two-tailed P value?	Two-tailed
t, df	t=2.043 df=86
How big is the difference?	
Mean ± SEM of column A	0.4891 ± 0.1495, n=44
Mean ± SEM of column B	0.07982 ± 0.1333, n=44
Difference between means	-0.4092 ± 0.2003
95% confidence interval	-0.8074 to -0.01108
R squared (eta squared)	0.0463
F test to compare variances	
F, DFn, Dfd	1.258, 43, 43
P value	0.4548
P value summary	ns
Significantly different (P < 0.05)?	No

D.7: OLFACTORY GROUP RETENTION – TWO-WAY ANOVA – MOVEMENT TOWARDS FOOD

Table Analyzed	Retention - dD				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**	
Interaction	0.8343	0.4195	ns	No	
Retention Day	5.448	0.0415	*	**Yes**	
Condition	0.3467	0.6023	ns	No	
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.869	1	0.869	F (1, 74) = 0.6591	P=0.4195
Retention Day	5.675	1	5.675	F (1, 74) = 4.304	**P=0.0415**
Condition	0.3612	1	0.3612	F (1, 74) = 0.2739	P=0.6023
Residual	97.57	74	1.318		
Number of missing values	34				

D.8: OLFACTORY GROUP RETENTION -TWO-WAY ANOVA – VERTICAL MOVEMENT

Table Analyzed	Retention - dY				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**	
Interaction	0.8773	0.4108	ns	No	
Retention Day	4.185	0.0749	ns	No	
Condition	0.04148	0.8578	ns	No	
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.7126	1	0.7126	F (1, 74) = 0.6842	P=0.4108
Retention Day	3.399	1	3.399	F (1, 74) = 3.263	P=0.0749
Condition	0.03369	1	0.03369	F (1, 74) = 0.03235	P=0.8578
Residual	77.08	74	1.042		
Number of missing values	34				

D.9: OLFACTORY GROUP RETENTION – TWO-WAY ANOVA – HORIZONTAL MOVEMENT

Table Analyzed	Retention - dX				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**	
Interaction	0.3584	0.6035	ns	No	
Retention Day	2.149	0.2056	ns	No	
Condition	0.07435	0.8129	ns	No	
ANOVA table	**SS (Type III)**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.5088	1	0.5088	F (1, 74) = 0.2721	P=0.6035
Retention Day	3.05	1	3.05	F (1, 74) = 1.631	P=0.2056
Condition	0.1056	1	0.1056	F (1, 74) = 0.05644	P=0.8129
Residual	138.4	74	1.87		
Number of missing values	34				

APPENDIX E: DILUTION CALCULATION

Assuming the solution remains well-mixed, the form of the differential equation is:

$C(t) = C_i\, e^{(-t/T)}$

Concentration (C) as a function of time (t) is equal to initial concentration (C_i) times Euler's number (e) to the power of negative time over a time constant (T).

Known:

$C_i = 1.05 * 10^{-7}$ M

V = 3 L

Q = -14 L/hr

Initial rate of solute loss is:

$Q * Co = -14$ L/hr $* 1.05*10^{-7}$ M $= -1.47 * 10^{-6}$ mol/hr

Such that the initial rate of change of concentration is:

$Q * Co / V = -1.47 *10^{-6}$ mol/hr / 3L $= -4.9*10^{-7}$ M/hr

This is equal to C'(0), or dC/dt at t=0, where dC/dt may be found using calculus:

$dC/dt = -Co/T\, e^{(-t/T)}$

Such that:

$C'(0) = -Co/T = -4.9*10^{-7}$ M/hr

T = 1.05*10-7 / $4.9*10^{-7}$ M/hr = 0.2143 hr

OR

T = 12.86 minutes

OR

T = 771.4 seconds

Concentration at any time is therefore given by:

$C(t) = 1.05*10^{-7}\ M * e^{(-t/0.2143\ hr)}$

The concentration decreases by a factor of *e* (2.718) every ~13 minutes.

Concentration will halve after -ln(0.5)*T = 0.69*12.86 = ~9 minutes.

Concentration will be below 1% of initial by -ln(0.01)T = ~1 hour.

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