A SIMPLE AUTOMATED SYSTEM FOR APPETITIVE CONDITIONING OF ZEBRAFISH IN THEIR HOME TANKS AND STUDYING UNDERLYING NEURAL ACTIVATION

by

Neil H. Merovitch

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

at

Dalhousie University
Halifax, Nova Scotia
August 2016

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Zebrafish are emerging as a novel model for studying learning and memory. However, the number of behavioural paradigms which minimize handling stress and are suited to their social nature is limited. We developed an automated learning paradigm to condition groups of adult and juvenile zebrafish in their home tanks. Fish consistently learned to associate an auditory stimulus with the presentation of food and showed robust conditioned responses as early as the 5th trial. Memory of the association persisted for at least 2 days after training, when fish were tested either as groups or as individuals. This retention in juveniles was associated with increased immunoreactivity to phosphorylated ERK, a marker of neural activity, in the dorsolateral telencephalon. This simple paradigm permits scalable conditioning of zebrafish with minimal intervention, reducing variability and labour-intensiveness. In addition, these results support the use of phosphorylated ERK to examine the neural correlates of learning and memory.

LIST OF ABBREVIATIONS USED

ΔAIC	Change in Akaike's information criterion
°C	Degrees Celsius
%	Percent
3D	Three-dimensional
ANOVA	Analysis of variance
BSA	Bovine serum albumin
cm	Centimeter
CS	Conditioned stimulus
CUBIC	Clear, unobstructed brain imaging cocktails and computational analysis
dpf	Days post-fertilization
ERK	Extracellular receptor kinase
FM	Frequency modulated
fps	Frames per Second
h	Hour
hrs	Hours
Hz	Hertz
LED	Light-emitting diode
L	Litre
μm	Micrometer
mg	Milligram
mm	Millimetre
min	Minute

pERK	Phosphorylated ERK
PFA	Paraformaldehyde
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with triton
ROI's	Regions of interest
sec	Seconds
s.e.m.	Standard error of mean
US	Unconditioned stimulus

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere appreciation to my supervisors Dr. Alan Fine and Dr. Roger Croll for their support, guidance and patience over these past five years. Also, thank you to my committee members Drs. Patrice Cote, Simon Gadbois, Paivi Torkkeli, Steven Barnes and Alexander Quinn for their helpful suggestions, feedback and discussions.

I would also like to extend my appreciation to my fellow lab members, both past and present, Drs. Ulli Hoeger and Yi-ling Hu for helping me get settled in the lab and answering my numerous questions, Matt Stoyek for his assistance with the fish and helping me to become a more critical thinker, Jillian Doyle for her assistance with the behavioural experiments, encouragement and baked goods, Arnaud Gaudin, Alexia Scaros and Tatjana Golovin for their enthusiasm about research and support, Matthew MacDougall and Borbala Podor for their help with my attempts at live imaging and taking care of the fish, Juyang Long, Emma Finlayson-Trick, Charlotte Nauss and Stephanie Shewchuk for their assistance with data collection and weekend fish care. Also, thank you to the other members of the Croll and Fine labs for providing a stimulating work environment and encouragement. I would also like to acknowledge Dr. Russell Wyeth for his help with analysis in Matlab and statistics and Alexander Goroshkov for his technical assistance.

In addition, thank you to Drs. Arunika Gunawardena and Marty Leonard for sharing their passion about science and teaching me that nothing is impossible if you put your mind to it. I would also like to express my gratitude to Setareh Lahsaee, Megan Crosby and Emma Neilson for their assistance and patience throughout their time in the lab.

Finally, I would like to thank my family for their encouragement and support throughout this endeavor.

CHAPTER 1 INTRODUCTION

1.1 Learning and Memory

Learning and memory are fundamental cognitive processes found throughout the animal kingdom, from unicellular organisms to vertebrates. The capacity to encode, store and retrieve details of previous events is essential for an organism to survive and adapt to its environment. Learning can be defined as the acquisition of a change in behaviour that is dependent on experience, whereas memory refers to the ability to retrieve this learned information (Abel and Lattal, 2001; Gerlai, 2011). A great deal of our knowledge regarding these processes and the underlying mechanisms has come from human case studies and animal models (Wright and Watkins, 1987; Milner et al., 1998). However, choosing an appropriate animal model involves balancing factors such as simplicity, homology and expense. The simplicity of invertebrates has made them useful tools for understanding the underlying genetics and molecular substrates (Agranoff et al., 1999). Some widely used invertebrate models, such as *C. elegans,* are restricted by their primitive nervous system, lack of homologous brain structures, and behavioural responses (Hughes, 2013). On the other hand, rodents are more closely related to humans; however, they have large complex neural circuits, are costly to maintain, nocturnal and produce offspring requiring parental care (Lonstein et al., 2002; Lieschke and Currie, 2007; Fadool and Dowling, 2008; Friedrich et al., 2013). These reasons have led to increased interest in zebrafish (*Danio rerio*) as a novel intermediate animal model (Avdesh et al., 2012).

1.2 Zebrafish as a Model

Zebrafish offer numerous advantages for the study of learning and memory. These

include ease and efficiency of animal husbandry (Patton and Zon, 2001; Spence et al., 2008); ready availability of molecular tools to dissect underlying mechanisms; homology of genes with mammals (including humans); and similarity of basic developmental, morphological, and physiological processes shared across the vertebrates (Bally-Cuif and Vernier, 2010). Zebrafish also have a rich repertoire of behaviours, which they execute using relatively simple neuronal circuits and may therefore possess further advantages for reductionist approaches to understanding underlying brain mechanisms (Roberts et al., 2013). Furthermore, the small size and relative transparency of zebrafish, particularly at early developmental stages and in non-pigmented mutant strains, renders them particularly suitable for powerful methods of optical imaging of electrical activity and optogenetic activation or inhibition of specific sets of neurons (Sumbre and de Polavieja, 2014). A final and increasingly important incentive for studies of learning and memory in zebrafish comes from the use of high-throughput screens to test for pharmacological and genetic effects on cognition (Gerlai, 2010; Miscevic et al., 2012).

1.3 Developing Learning Paradigms

A comprehensive appraisal of an animal's ability to learn and remember will require multiple assays employing different sensory modalities, behavioural responses and forms of learning. One of the most studied forms of learning is associative learning, which underlies many experience-dependent adaptive behaviours (LaLumiere and W. Kalivas, 2007). This type of learning allows animals to form meaningful associations between two or more stimuli or events. For instance, in the simplest form of associative learning, classical conditioning, temporal pairing of a neutral stimulus (conditioned stimulus, CS) with an appetitive or aversive stimulus (unconditioned stimulus, US)

results in the formation of a conditioned response elicited by the previously neutral CS (Gerlai, 2011).

The development of appropriate associative learning paradigms for zebrafish is contingent upon the careful selection of stimuli and measurement of behavioural responses. Most learning and behavioural studies in zebrafish repurpose rodent maze designs and paradigms, despite clear distinctions between innate behaviours and natural environments of rodents and fish. Therefore, paradigms that are more compatible with the natural behaviour and ecology of zebrafish may reveal new insights about their cognitive capabilities (Gerlai, 2011).

In case of associative learning, the process of choosing appropriate conditioned stimuli requires knowledge of the sensory and perceptual capacities of zebrafish in each modality. For instance, zebrafish have been shown to have a complex visual system and rely heavily on their sense of vision (Fadool and Dowling, 2008). Moreover, zebrafish are sensitive to auditory and olfactory cues, such as amino acids or alarm pheromones (Suboski et al., 1990; Steele et al., 1991; Cervi et al., 2012). These stimuli have the potential to be used in conditioning paradigms as long as they can be properly paired with other reinforcing stimuli. Caution is needed in determining the strength of stimuli, however, in order to ensure that they fall within the detection range of zebrafish.

In addition to sensory threshold and perceptual capabilities of zebrafish, evolution may favor certain innate biases; thus making careful selection of conditioned stimuli even more crucial (Spence et al., 2008). Innate biases result in stronger or weaker responses to certain stimuli (Basolo, 2000). For example, although zebrafish are capable of colour discrimination, there are contradictory reports on their innate colour biases. In one study,

zebrafish were reported to have an innate red colour bias when foraging for food (Spence and Smith, 2008). In contrast, Colwill et al. (2005) found a significant baseline bias toward purple in a colour discrimination paradigm. However, the discrepancies between these studies could have resulted from differences in the environment in which fish were raised (Spence and Smith, 2008).

Also, there is limited literature on appropriate unconditioned stimuli for zebrafish. Food has been widely used as a positive reinforcer in associative conditioning; however, the motivational value of food depends on physiology of the animal model. Feeding behaviour in zebrafish does not resemble that of rodents. In contrast to rodents that require a constant supply of nutrients, zebrafish are found to be satiated quickly after a number of trials, limiting the effectiveness of food as a rewarding stimulus (Gerlai, 2011).

The choice of appropriate reinforcers in learning paradigms also requires knowledge about the species specific behaviours which predispose animals to perceive certain stimuli as rewarding. An example of such behaviour in zebrafish is shoaling. Shoaling is an innate tendency of zebrafish to swim in close proximity to one another (Engeszer et al., 2007a). As a result, sight of conspecifics is an alternative choice of reward for individual zebrafish (Al-Imari and Gerlai, 2008).

1.4 Adult Associative Learning Paradigms

A variety of conditioning paradigms for adult zebrafish have been described in recent years, with each paradigm possessing particular strengths and limitations. For example, several aversive conditioning paradigms have been developed, including ones that employ electric shock associated with changes in lighting of observation tanks

(Gleason et al., 1977; Pradel et al., 1999; Xu et al., 2007; Agetsuma et al., 2012). Generally, such use of aversive electrical shocks is highly effective (Blank et al., 2009), but may also have direct effects on patterns of electrophysiological activity in aquatic animals, potentially confounding further studies of associated brain mechanisms or function (Gerlai, 2011). Appetitive paradigms, including the association of food with a particular location or with discrete visual or olfactory cues (Braubach et al., 2009; Sison and Gerlai, 2010; Chacon and Luchiari, 2014), avoid delivery of external electrical stimuli, but the use of food as a reward can be complicated by satiation, as mentioned above, limiting the rate of training.

One major issue common to all these paradigms is that they require special mazes or observation tanks. Periods of acclimation are therefore needed before behavioural assays can be performed (Sison and Gerlai, 2010). Handling during transfer to such special apparatus, including netting, has been shown to increase cortisol levels significantly (Ramsay et al., 2009), potentially confounding analysis.

Isolation has been shown to increase stress hormone levels and acclimatization period in zebrafish, suggesting that group training may be more compatible with their social nature (Kalueff et al., 2014). Therefore, even with long periods of habitation to their new training environment, responses of isolated fish may not fully correspond with their behaviour in the home tank where they are normally maintained.

Furthermore, most existing paradigms are time- and labour-intensive, requiring large numbers of pairings usually performed manually. Requirements for such extended acclimation and manual execution of experiments reduce the usefulness of such paradigms for high-throughput screening (Gerlai, 2010). These issues could be overcome

through automation for precise and reliable delivery of the conditioned stimuli with food, preventing inconsistencies or disruptions in the temporal contiguity of pairings.

1.5 Spatial Learning in Zebrafish

In addition to classical conditioning, other forms of learning such as spatial learning have been observed in zebrafish. Spatial learning requires animals to learn the relationships among environmental cues and construct a map of their environment; hence spatial learning may be considered as a more complex form of associative learning (O'Keefe and Nadel, 1979). Spatial learning paradigms such as the Morris water maze (Morris, 1984) or radial arm maze (Olton et al., 1977) are well established in rodent models. Some studies have found that zebrafish are capable of using external environmental cues to navigate towards food or a conspecific reward (proximity to a group of zebrafish) in a maze or tank (Braubach et al., 2009; Sison and Gerlai, 2010; Karnik and Gerlai, 2012). These studies suggest that zebrafish are also capable of higher, more complex forms of learning.

1.6 Social Effects on Learning

Social behaviour is also known to play a key role in the way zebrafish learn. Groups of five adult zebrafish in an avoidance learning paradigm were found to learn faster than individuals, though single fish still performed better than pairs (Gleason et al., 1977). In addition, an increased response to an appetitive olfactory stimulus has been observed in groups of four fish which decreased in groups of two, six, or eight compared to individual fish (Steele et al., 1991). These experiments suggest that the type of task and environment may affect the optimal group size for faster learning. It has also been suggested that social facilitation may be involved in group conditioning. Mixing naïve

fish with conditioned fish has shown that the mixed group not only demonstrated the conditioned response but also naïve fish tested individually acquired the conditioned response, suggesting the transmission of conditioned response from trained fish to naïve individuals (Suboski et al., 1990). These results imply that fish can learn by observing the behaviour of other fish (Brown and Laland, 2003) and further reinforce the value of group training as it reduces variability between animals and shortens training.

1.7 Learning in Developing Zebrafish

Despite strong evidence showing that adult zebrafish can be conditioned, the cognitive capabilities of developing zebrafish are relatively unexplored, and is not clear when they start to exhibit conditioned responses (Spence et al., 2008; Roberts et al., 2013). Measuring behaviour in juvenile and larval is also more challenging due to their limited mobility. These smaller fish have trouble navigating in the water column as their swim bladder, which controls buoyancy, is not fully functional until approximately 30 days post-fertilization (dpf) (Robertson et al., 2007; Winata et al., 2009). Also, until the first week larva depend on the yolk sac for feeding, making it difficult to train them with food rewards. As a result, the number of learning paradigms in larvae and juvenile zebrafish is limited (Roberts et al., 2013). Studies in juvenile fish have shown robust conditioned responses to visual cues paired with electric shock, beginning at 3 weeks and reaching the same performance level as adults by 6 weeks (Lee et al., 2010; Valente et al., 2012). Juveniles 3-4 weeks in age could not learn a spatial learning task in which fish have to alternate between two sides of a tank to get food. However, fish 6-8 weeks old reliably learned the task as well as or better than adults (Williams et al., 2002). It is not clear whether the lack of learning is due to the limited cognitive capacity or limited

mobility of younger fish (Spence et al., 2008). A simpler paradigm using innate behaviours such as feeding may be more successful with juvenile zebrafish.

1.8 Functional Mapping

Understanding the circuits responsible for learning and memory has been the goal of many neurobehavioral studies. The application of zebrafish as a reliable neurobiological animal model for human neurological diseases relies on accurate determination of homologous regions between zebrafish and mammalian brains. The hippocampus is necessary for formation and consolidation of certain types of memory in mammals (Jarrard, 1993; Broadbent et al., 2004). However, it is thought that new memories are then transferred to the cortex over time and established as long-term memories (Jarrard, 1993; Frankland et al., 2004; Maviel et al., 2004; Frankland and Bontempi, 2005; Teixeira et al., 2006). In addition, a subset of cortical neurons has been shown to activate soon after learning (Yasuda and Mayford, 2006; Lesburguères et al., 2011; Tse et al., 2011).

The networks underlying learning and memory in zebrafish are not well understood. Historically, the zebrafish brain was seen as a primitive structure with minimal anatomical (e.g., lamination) or functional divisions compared to mammals (Aoki et al., 2013; Key, 2015). However, more recent developmental, behavioural, ablation and electrophysiological studies collectively suggest that the telencephalon in zebrafish contains discrete regions including those involved in learning and memory. The dorsal part of the telencephalon is known as the pallium and the ventral area is described as the subpallium (Figure 1.1) (Ganz et al., 2014). Several studies have also suggested that the dorsolateral part of the pallium is functionally homologous to the hippocampus

and that the medial pallium corresponds to the amygdala which is involved in emotional conditioning (Rodríguez et al., 2002; Portavella et al., 2004; Salas et al., 2006; Broglio et al., 2010). Hippocampal-like sharp waves in anterior dorsolateral pallium have been observed in addition to pharmacological similarities to the rodent hippocampus (Vargas et al., 2012). More recently, it has been suggested that the central zone of the dorsal telencephalon is homologous to the mammalian isocortex (Mueller and Wullimann, 2009; Mueller et al., 2011; Northcutt, 2011).

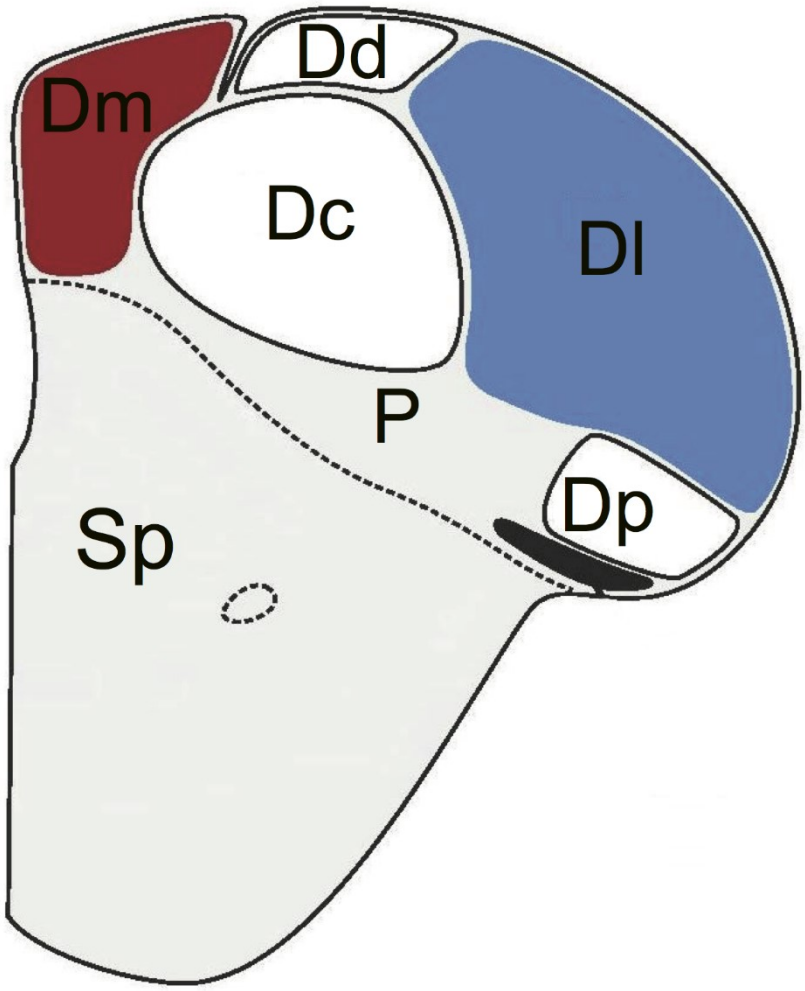

Figure 1.1. Coronal section of adult zebrafish illustrating telencephalic subdivisions. The telencephalon is divided into a dorsal section (pallium; P) and a ventral section (subpallium; Sp). The pallium is divided into a medial section (Dm), lateral section (Dl), central zone (Dc), posterior zone (Dp) and dorsal region of dorsal telencephalon (Dd). Used with permission from Mueller et al., (2011).

1.8.1 Visualizing Calcium Activity

Calcium imaging in adult zebrafish has revealed localised activity in the central zone of the dorsal telencephalon during the retrieval of an active avoidance association. Ablations of this central region inhibited retrieval of long-term memory, but did not affect learning (Aoki et al., 2013). This study supports previous studies suggesting that the central zone may have some similar functions to that of the mammalian cortex. This is the only report of long-term memory visualization *in vivo* and has yet to be confirmed.

Some types of associative learning are not telencephalon-dependent. Cerebellar-dependent learning has also been observed in larva that were classically conditioned to associate light with a tactile stimulus. This type of learning was associated with increased calcium activity in the cerebellum. However, ablation of the cerebellum only affected acquisition and extinction and not retention of the memory (Aizenberg and Schuman, 2011). These results suggest that this associative memory may be stored in other regions, possibly in dorsal telencephalon.

1.8.2 Alternative Visualization Techniques

Although calcium imaging allows for visualization of brain activity during certain learning tasks, it is restricted to partially immobilised fish. Furthermore, there are relatively few learning paradigms suited for restrained zebrafish. In addition, immobilisation limits behavioural responses which are useful indicators for detecting learned responses. One alternative to live calcium imaging is CaMPARI (calcium-modulated photoactivatable ratiometric integrator), which allows visualization of neural activity in freely swimming fish. However, this technique is limited primarily to larval

stages or transparent lines, due to the extent of light penetration which is required for activation of CaMPARI indicator (Fosque et al., 2015).

Neural activity can be also be detected by examining phosphorylation of activated extracellular receptor kinases (ERKs) and expression of immediate early genes (IEGs) such as c-Fos (Chatterjee et al., 2015; Randlett et al., 2015). Calcium influx caused by membrane depolarization triggers a mitogen-activated protein kinase (MAPK) cascade that ultimately results in phosphorylation of ERKs. ERKs are serine/threonine kinases with downstream targets including transcription factors such as CREB and Elk and c-Fos that in turn regulate downstream transcription of a number of genes involved in protein synthesis, long term plasticity and memory formation (Thomas and Huganir, 2004; Randlett et al., 2015). Phosphorylated ERK (pERK) has some advantages compared to c-Fos, as it has higher temporal resolution and reaches a detectable level in the cytoplasm within 5 minutes (min) of neural activity (Ji et al., 1999; Dai et al., 2002; Cancedda et al., 2003; Gao and Ji, 2009). In comparison, c-Fos protein expression peaks between 1-2 hours (hrs) after acute stimulation (Kovács, 1998; Okuyama et al., 2011).

Several studies in rodents have demonstrated the involvement of ERK in cascades underlying long-term memory. For instance, retrieval of a fear conditioning association was found to be ERK dependent with up-regulation of pERK in rat hippocampus 1 hour (h) after training (Atkins et al., 1998). Others have also shown that inhibition of ERK impaired spatial learning and memory (Blum et al., 1999; Selcher et al., 1999). These studies collectively provide evidence for the involvement of ERK in hippocampal-dependent acquisition and memory retrieval in rodents.

In zebrafish, pERK has been used to examine neuronal activity in larvae. Up-regulation of pERK was observed in response to short periods of light-pulse stimulation, hunting and feeding and exposure to noxious stimuli, such as electric shock. Moreover, the pattern of pERK immunoreactivity overlapped activity map obtained by calcium imaging (Randlett et al., 2015). These findings provided evidence that pERK is a reliable indicator of neuronal activity in response to a wide range of external stimulations in zebrafish brain. Despite the high temporal resolution of pERK for labeling active neurons and its involvement in memory cascades, no studies have previously used pERK to study neural activity underlying telencephalic dependent learning and memory in zebrafish.

1.9 Objectives

Given the usefulness of zebrafish as a novel model for studying learning and memory and the demand for behavioural paradigms compatible with their ecology, this study had the following objectives:

1. Develop an automated, easily reconfigurable appetitive system that can be used to condition groups of both adult and juvenile zebrafish in their home tanks.
2. Examine the efficiency of the developed paradigm to characterize the rate of acquisition and duration of memory retention for auditory conditioning in groups of both adult and juvenile zebrafish.
3. Determine if fish removed from a group after conditioning could also demonstrate the learned response when tested for retention individually, in order to test the effectiveness of group conditioning.

4. Examine whether alterations in neural activity in the telencephalon are associated with memory retrieval, using pERK immunostaining.

CHAPTER 2 MATERIALS AND METHODS

2.1 ANIMALS

Wild-type adult zebrafish, 3.5-4.0 cm in length were obtained from PetSmart (Bedford, NS, CAN). AB strain juvenile zebrafish, 49 and 30 days post-fertilization (dpf), 10-14 mm and 7.8-10 mm in length, respectively were provided by the Faculty of Medicine, Zebrafish Core Facility (Dalhousie University, Halifax, NS, CAN). Adult and juvenile fish were housed as mixed-gender groups of five fish in 3 litre (L) and 1.5 L plastic tanks (Pentair Aquatic Eco-Systems, Apopkoka, FL, USA), respectively, beginning at least two days prior to experimentation. The fish were maintained on a 14:10 hour (h) 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 L. Each tank was provided with a water flow of 13-14 L/h while on the recirculating zebrafish housing system. Adult fish were normally fed twice daily using 300-500 µm pellets of Golden Pearl Reef Diet (Brine Shrimp Direct, Ogden, UT, USA). Juvenile zebrafish were fed once daily using 200-500 µm GEMMA Micro Food (Skretting, Westbrook, ME, USA). All experiments were conducted in accordance with the Canadian Council on Animal Care standards and guidelines.

2.2 EXPERIMENTAL APPARATUS

For training and testing, each home tank of five fish was moved to a specialised 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 room lights or light-emitting diode (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 either a dedicated 40 L reservoir for adults or the recirculating zebrafish housing system (Pentair Aquatic Eco-Systems, Apopkoka, FL, USA) for juvenile experiments.

A micro controller (Arduino Uno, Arduino, Ivrea, ITA) with an associated motor control board (shield) (Product ID: 1438), auditory wave shield (Product ID: 94) and DS1307 real time clock (Product ID: 264) from Adafruit, New York, NY, USA was used to control automatic feeders and to present the auditory stimulus. 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), Motorshield (Adafruit, 2016) and waveHC (Adafruit, 2015). 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 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, 4 or 2 mg of food for adult, 49 dpf and 30 dfp fish, respectively. A white plastic divider was placed at the level of the water, 6.5 cm from the front of the 3 L, to keep the dispensed food floating near the feeder.

Auditory stimuli were presented to the fish using an 8 Ohm bone conduction sound transducer (Product ID: 1674) (Adafruit, New York, NY, USA) which was

centered laterally and vertically underneath the outflow at the back of each tank, (Figure 2.1). The auditory conditioned stimulus consisted of frequency modulated (FM) half second (sec) ascending and descending tone sweep between 100 and 1000 hertz (Hz) (Sweep Tone Generator, http://www.audiocheck.net), amplified to half of the rated output power of the wave shield (0.125 Watts). This auditory stimulus was selected based on previous evidence showing maximum sensitivity to this range of frequencies (Cervi et al., 2012). To indicate when the auditory stimulus was administered, a 5 mm red LED (Digi-Key, Thief River Falls, MN, USA) was placed on the lid of each tank, partially occluded by heat shrink tubing to allow detection by video recording equipment (see below) but not by the fish.

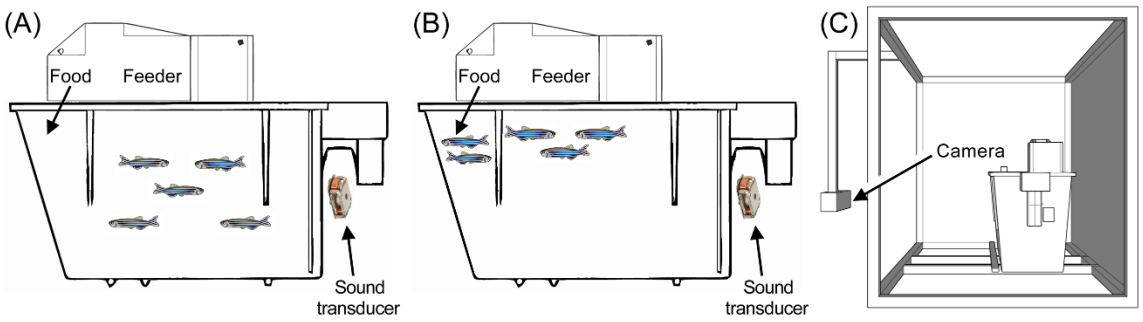

Figure 2.1. Behavioural apparatus for auditory conditioning in home tanks.

Panel A and B illustrate the positions of control and experimental fish, respectively, during the presentation of conditioned stimulus. Bone conduction sound transducers were used for the presentation of the auditory stimulus. Food pellets were dispensed by the automatic feeder located above the tank. Panel C illustrates the position of camera relative to the tank.

2.3 Conditioning

Training consisted of 10 sessions during the light period on each of two consecutive days. Inter-trial intervals of 34-108 minutes (min) were selected from those produced using a random time generator (Random Time Generator, http://www.random.org). Conditioning was performed by playing the FM tone sweep for a 20-sec 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 auditory inter-stimulus interval except for the last trial in which it was administered 17-54 min later.

After the completion of training for adults, the feeders, plastic dividers and sound transducers were removed from each tank. These tanks were then moved back to the racks on which they were regularly maintained, and normal care was resumed until animals were tested for memory retention. Tanks with juvenile fish were left on the training/testing rack for the duration of experiments.

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 were tested individually. For adult group testing, the entire tanks of five fish were moved from the maintenance racks back to the observation arenas, and the upper divider that prevented dispersion of the food was replaced as a visual landmark at one end of the tank. The sound transducer was also reattached to the tank. 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 the sound transducer. All adult 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 auditory stimulus for 20 sec without the food reward. Each group or individual fish was given only a single probe trial at 2, 4, 8, 16 or 32 days after training.

2.5 Data Collection and Analysis

A single camera was centered 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/sec and were then trimmed to 40 sec clips (VirtualDub, http://www.virtualdub.org) covering the 20 sec immediately before exposure to the auditory or visual conditioned stimulus and the 20 sec 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 in preliminary experiments, they were excluded from further analysis and only the positional values were examined.

The average vertical (Y) and horizontal (X) positions of the fish in each tank were calculated for the 20 sec **before** the presentation of the conditioned stimulus and compared to average coordinates **during** presentation of the stimulus. These coordinates were measured relative to the food source as the common origin. 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). These horizontal and vertical positions were combined into a single measure using the following equation $d = \sqrt{(X^2 + Y^2)}$ corresponding to the distance from the food source. The distances during presentation of the conditioned stimulus (d during) were then subtracted from the distances before the stimulus (d before). This subtraction was also performed independently for vertical and horizontal positions (X before – X during and Y before – Y during). Positive subtraction 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. However, adult fish exhibited a substantial latency in

responding to the conditioned stimulus and therefore average positions were only calculated during the last 10 sec of the 20 sec stimulus presentation. Juvenile fish showed a shorter latency and therefore average positions were calculated during the last 15 sec of the 20 sec stimulus presentation.

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 the tanks (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's Information Criterion (ΔAIC) were also examined to determine which model (reduced vs. full) better fitted the data, with ΔAIC=0 denoting no difference in the two models (Burnham and Anderson, 2004). Conclusions paralleled those from the log-likelihood test P-values, with full models showing ΔAIC values >10 over the reduced models for all adult acquisition tests. Juvenile ΔAIC values were <10 (1.5-7.4) with the exception of vertical position for 49 fish. 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 and juveniles, respectively. 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 and Weisberg, 2011), ggplot (Wickham, 2009), sjplot (Lüdecke, 2016), plotly (Sievert et al., 2016). P-values are reported in text but for full statistical analyses see Appendix C (adult data), Appendix D (49 dpf juvenile data) and Appendix E (30 dpf juvenile data).

2.6 IMMUNOHISTOCHEMISTRY

Juvenile zebrafish 30 dpf (at the start of training) were euthanized immediately following the presentation of the auditory sweep during 2-day probe trials by immersion in cold (< 4°C) 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hartfield, PA, USA) in phosphate buffered saline (PBS: 50 mM Na_2HPO_4, 140 mM NaCl, pH 7.4) for approximately 5 min. Fish were then decapitated and the heads fixed in 4% fresh PFA overnight at 4°C. The next day the heads were rinsed in PBS and dissected to isolate the brain which was then post fixed in 4% PFA for 1-2 hrs at room temperature. Brains were washed three times (5-10 min each) in PBS and transferred to PBS-T containing 0.1% Triton X-100 (X100; Sigma-Aldrich), 1% bovine serum albumin (BSA; A9576; Sigma-Aldrich), and 2% dimethylsulfoxide (D128-1; Fisher Chemical) for 48 hrs at 4°C. Samples were then incubated with gentle agitation for 5-7 days at 4°C with the primary antibody against pERK (Cell Signaling, 4370), in order to localise neuronal activity underlying associative memory. The primary antibody was diluted 1:400 in PBS-T. This antibody has previously been used as an indicator of neuronal activity in zebrafish larvae (Randlett et al., 2015) and adult olfactory bulbs (Yabuki et al., 2016) and sensory neurons (Hussain et al., 2013). Also, specificity of the antibody for pERK in zebrafish was previously established by Western blot using the immunogen (Hussain et al., 2013). As a control for non-specific staining by the secondary antibody, brains were processed without the primary antibody. These control brains exhibited no immunoreactivity.

Brains were rinsed three times (20 min each) in PBS-T and incubated with a donkey anti-rabbit secondary antibody conjugated to AlexaFluor 488 (Life Technologies, Burlington, Ontario, CAN) for 5-7 days at 4°C with gentle agitation. The secondary

antibody was diluted in a solution of PBS-T with a final concentration of 1:100. Brain were then washed in PBS and immersed in Scale CUBIC-1 clearing solution (Susaki et al., 2014) for 2 days at 4°C.

Brains were mounted as whole-mounts of the telencephalon with the dorsal surface facing up on glass slides, in wells cut out of 3 layers of electrical tape adhered to the slide. Wells were then filled with gelvatol mounting medium (Center for Biologic Imaging, 2016) to prevent movement. Slides were finally sealed with a coverslip using nail polish and kept at 4°C prior to imaging.

2.7 Microcopy and Image Analysis

Samples were viewed using a Zeiss LSM 510 laser scanning confocal microscope with a 25X oil immersion objective (Carl Zeiss Inc., Thornwood, NY, USA). Image stacks were captured using Zeiss Zen 2009 software at an interval of 0.89 μm to a maximum depth of 129.36 μm and at a resolution of 1024 x 1024 pixels. Other settings including scan speed, laser intensity, pinhole size, gain and digital offset were maintained among all samples to ensure consistency and to allow direct unbiased comparisons between preparations (Forero and Hidalgo, 2011).

Confocal stacks were viewed and processed using Vaa3D (Peng et al., 2010, 2014a, 2014b) and Fiji (Schindelin et al., 2012) with the BoneJ Particle Analyser plugin (Doube et al., 2010) and Analyze Particles. Simple adaptive thresholding was performed on all images stacks in Vaa3D, as it provided more reliable segmentation of neurons than global thresholding in Fiji. Adaptive thresholding assigns values to different regions of an image based on the intensity, controlling for variation, whereas global thresholding assigns the same value to the whole image regardless of intensity (Forero and Hidalgo, 2011). Stacks

were then converted to 8-bit binaries in Fiji and cell counting was performed using Particle Analyser. These 3D cell counts were divided by the surface area of the maximum intensity projections, to account for variation in brain size, creating a cell density index (cells per μm^2). In order to further localize immunoreactivity within the dorsal telencephalon, regions of interest (ROIs) were created in each hemisphere with the aid of a zebrafish atlas (Wullimann et al., 1996). Hemispheres were split into lateral and medial halves; these were then subdivided into anterior and posterior regions creating 4 ROIs (quadrants). Quantification was performed by measuring the immunoreactive area of the ROIs in the maximum intensity projection (using Analyze Particles). In order to control for brain size, these values were then divided by the surface area of the telencephalon, resulting in an immunoreactive area (%) (Renno et al., 2014). Immunoreactive area (%) was used instead of the cell density index for regional analysis, as Particle Analyser did not support ROIs. Also, other cell counting plugins could not accurately resolve overlapping neurons. A Welch two sample t-test and two-way ANOVA (with conditioning and quadrant factors) were then conducted in R (R Core Team, 2016) for the resulting cell density index and immunoreactive area (%) values, respectively. Post-hoc, Welch two sample t-tests were conducted to identify regional differences in immunoreactive area (%) between the brains of control and experimental fish.

CHAPTER 3 RESULTS

3.1 Auditory Conditioning and Memory Retention of Adult Fish

3.1.1 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 second (sec) period before presentation of the FM tone sweep, with the mean position of the fish being near the center of the tank (Figure 3.1A, Supplemental Movie 1). During training, the control groups, which were presented food with variable delays following the FM sweep, continued a similar swimming pattern in the 20 sec period that the auditory stimulus was presented. In contrast, the experimental fish, which were presented with a food reward directly after each FM sweep, increasingly spent more time near the feeding location during the presentation of the auditory stimulus as training progressed (Figure 3.1B, Supplemental Movie 2).

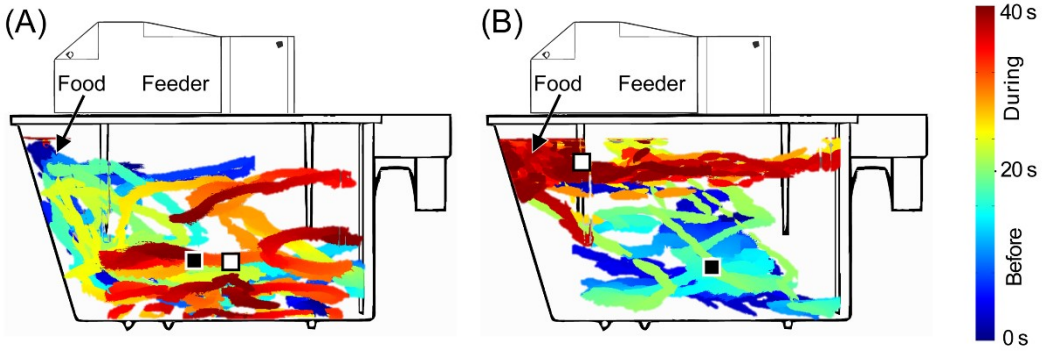

Figure 3.1. Instantaneous positions of five zebrafish in a control (A) and experimental tank (B) during trial 20.

Time interval from 20 sec before and 20 sec during the presentation of auditory stimulus. The colour scale refers to the time (s) when corresponding areas were occupied by the five fish. The mean positions of the group before, and during the last 10 sec of, the auditory stimulus are indicated by black and white squares, respectively. Mean movement in the control tank was 2.10 cm away from the food source and mean movement in the experimental tank was 10.11 cm toward the food source

Figure 3.2A shows this progressive movement of experimental (but not control) fish away from their initial mean position near the centre of the tank and closer to the corner in which food was presented as training proceeded. Analysis of linear mixed effects models confirmed a significant interaction between conditioning and training trial ($\chi^2(1)= 39.45$, $p<0.001$). The experimental and control group scores appeared to begin to diverge after only a few trials and bootstrapped confidence intervals suggested that by the 5-8th training trial the experimental groups were moving consistently toward the food source during the presentation of the auditory stimulus. The fish at trial 11 (first trial of the second day) showed no apparent forgetting from the previous day (Figure 3.2A).

Separate analyses of horizontal and vertical components of the movements each showed significant interactions between condition and training trial (vertical: $\chi^2(1)= 25.528$, $p<0.001$, Figure 3.3A; horizontal: $\chi^2(1)= 32.471$, $p<0.001$, Figure 3.4A) suggesting fish learned to adjust both their depth and horizontal position in the tank in response to the conditioning auditory stimulus.

3.1.2 Memory Retention in Groups of Fish

In order to examine whether the memory for the association between the auditory stimulus and the food reward was retained after the acquisition period, we tested the groups of fish for their responses to the auditory stimulus alone with probe trials at 2 and 16 days following training (represented as circles in Figure 3.2B). A two-way ANOVA on the movement of fish towards the feeding location, revealed a significant overall effect of conditioning ($p=0.001$) and a significant interaction between conditioning and day of retention ($p=0.007$) but no significant effect of retention day ($p>0.05$). Therefore, our data indicate that the memory is retained for at least 2 days and that there is a decline in

the strength of the memory over 16 days. An analysis of only the vertical component of movement showed a significant effect of conditioning (two-way ANOVA, $p=0.020$) but no effect of retention day or interaction between conditioning and retention day (both $p>0.05$; Figure 3.3B). Finally, analysis of the horizontal components of the movement indicated no effect of retention day (two-way ANOVA, $p>0.05$; Figure 3.4B), but a significant effect of conditioning ($p=0.001$) and a significant effect of interaction between condition and day of retention ($p=0.007$), thus supporting the analysis of the vertical movements of the fish.

3.1.3 Memory Retention in Individual Fish

We considered the possibility that only dominant fish in each group actually learned the conditioned association, with subordinate fish merely following in the shoal. To determine whether fish trained in groups actually learned and retained memories of the conditioned associations and to attempt a better determination of the duration of memory retention, we performed additional probe trials using single fish at 2, 4, 8, 16 and 32 days post training. An analysis of movement towards the feeding location showed a significant overall effect of conditioning (two-way ANOVA, $p=0.002$; represented as triangles in Figure 3.2B), but no effect of retention day or interaction between retention day and condition (both $p>0.05$). Two-way ANOVAs on the vertical and horizontal data also showed a significant effect of conditioning ($p<0.001$; Figure 3.3B & $p=0.038$; Figure 3.4B, respectively) but no effect of retention day or interaction between retention day and conditioning (all $p>0.05$). Therefore, the fish showed retention of memory during the period of 2-32 days but the data did not permit definitive assessment of the time course of memory decline during this period.

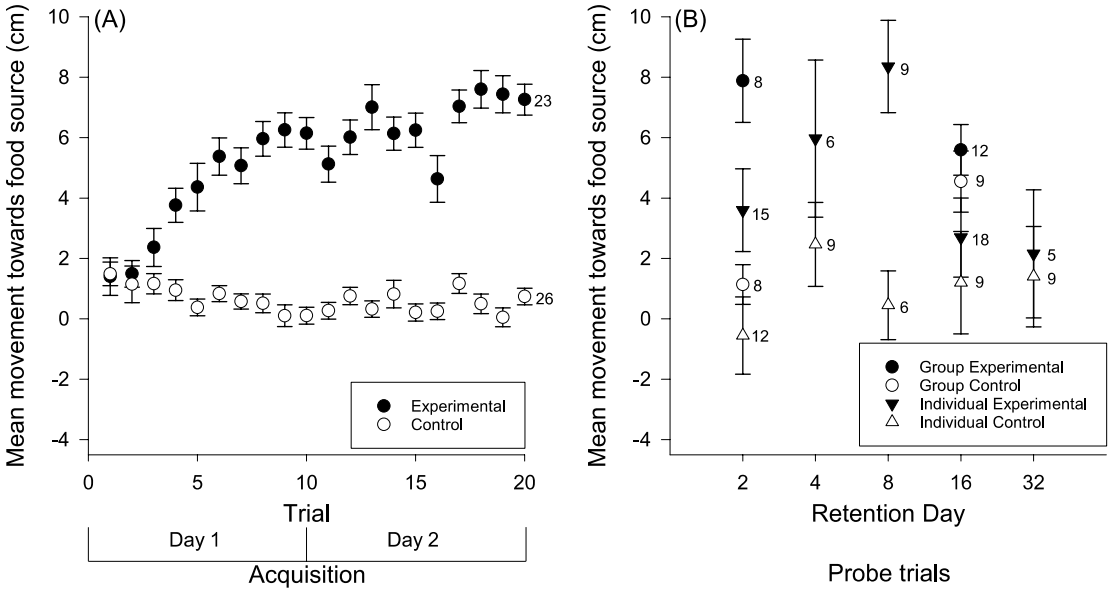

Figure 3.2. Adult zebrafish in the experimental group moved away from their initial positions towards the food source as a result of conditioning to the auditory stimulus. This response increased throughout the training trials. Zebrafish in the control group did not move toward the food source in response to the auditory stimulus (A). When tested for retention, trained groups and individuals moved closer to the food source compared to controls (B). Data points are mean distance from the food source before the FM tone sweep minus mean distance from the food source during the FM tone sweep. Numbers beside data points represent replicates for individuals (single fish) and groups (each containing 5 fish) in each condition. Error bars = ± s.e.m.

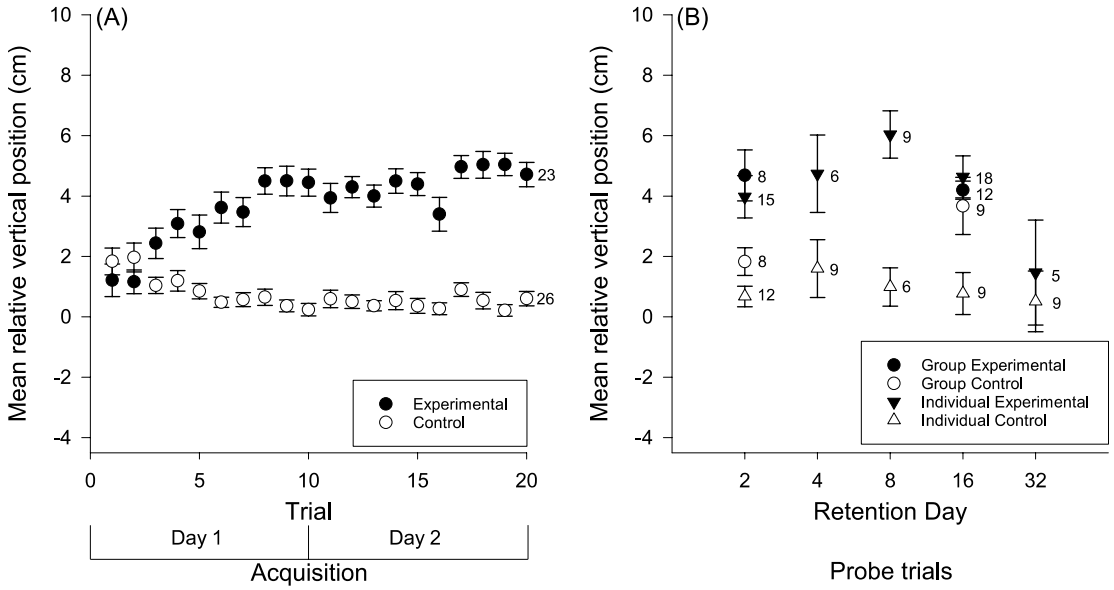

Figure 3.3. Adult zebrafish in the experimental group moved vertically from their initial positions towards the surface as a result of conditioning to the auditory stimulus. This response increased throughout the training trials. Zebrafish in the control group did not move toward the surface in response to the auditory stimulus (A). When tested for retention, trained groups and individuals moved closer to the surface compared to controls (B). Data points are mean vertical position before the FM tone sweep minus mean vertical position during the FM tone sweep. Numbers beside data points represent replicates for individuals (single fish) and groups (each containing 5 fish) in each condition. Error bars = ± s.e.m.

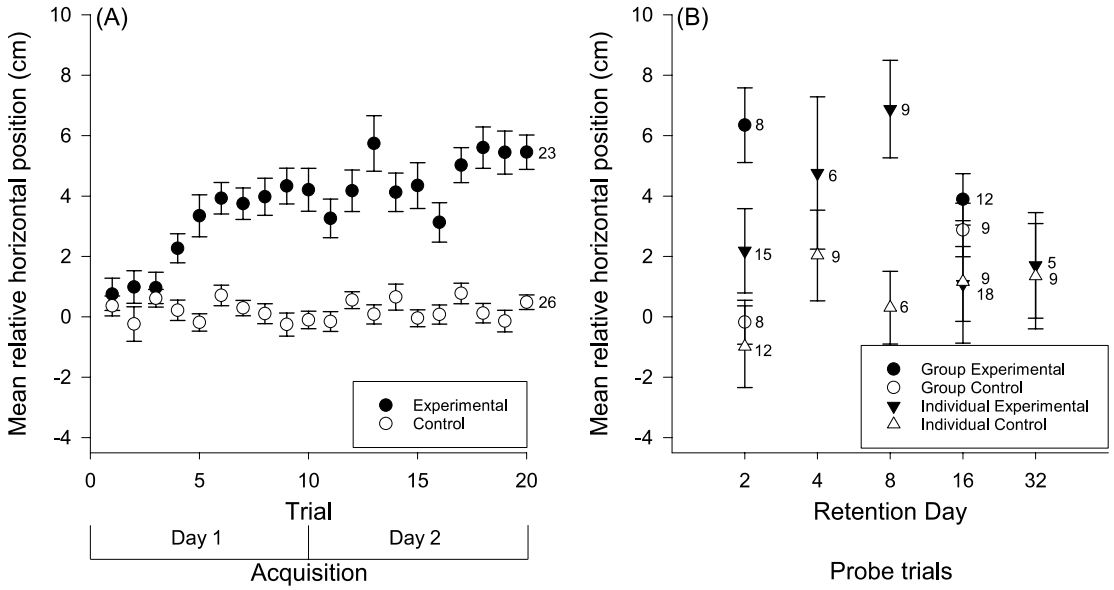

Figure 3.4. Adult zebrafish in the experimental group moved laterally from their initial positions towards the food source as a result of conditioning to the auditory stimulus. This response increased throughout the training trials. Zebrafish in the control group did not move laterally towards the food source in response to the auditory stimulus (A). When tested for retention, trained groups and individuals moved closer, laterally, towards the food source compared to controls (B). Data points are mean horizontal position before the FM tone sweep minus mean horizontal position during the FM tone sweep. Numbers beside data points represent replicates for individuals (single fish) and groups (each containing 5 fish) in each condition. Error bars = ± s.e.m.

3.2 Auditory Conditioning and Memory Retention of Juvenile Fish (49 dpf)

Next, we examined whether the paradigm that was effective in rapidly conditioning adult fish was also applicable to juvenile fish.

3.2.1 Acquisition of Appetitive Conditioning

Similar to the behaviour observed during conditioning of adult fish, the juvenile fish (49 dpf) also swam closer to the food source during the presentation of the auditory stimulus that was paired with food. Figure 3.5A shows the movement of experimental fish towards the corner of the tank in which food was presented as training proceeded. Analysis of linear mixed effects models confirmed a significant interaction between conditioning and training trial ($\chi^2(1)= 9.4213$, $p=0.002$). Bootstrapped confidence intervals again suggested that by the 10-13th training trial, the experimental groups were moving consistently closer to the food source during the presentation of the auditory stimulus. Separate analyses of horizontal and vertical components of the movements each indicated significant interactions between condition and training trial (vertical: $\chi^2(1)= 16.048$, $p<0.001$, Figure 3.6A; horizontal: $\chi^2(1)= 7.1794$, $p=0.007$, Figure 3.7A) suggesting fish learned to adjust both their depth and horizontal position in the tank in response to the conditioning auditory stimulus.

3.2.2 Memory Retention for Groups of Fish

We again examined the retention of the memories for the paired associations by testing groups of juvenile fish (49 dpf) for their responses to the auditory stimulus alone with probe trials 2 days after training (Figure 3.5B) and found that the 2 day experimental group was significantly different from the control group (Welch two sample t-test,

$p<0.046$), indicating that the experimental groups retained the memory of the association between the auditory stimulus and the presentation of food. An analysis of the vertical component of the data indicated a significant difference between the control and the experimental data (Welch two sample t-test, $p=0.002$; Figure 3.6B). However, analysis of the horizontal data showed no significant difference between the control and experimental values (Welch two sample t-test, $p>0.05$; Figure 3.7B).

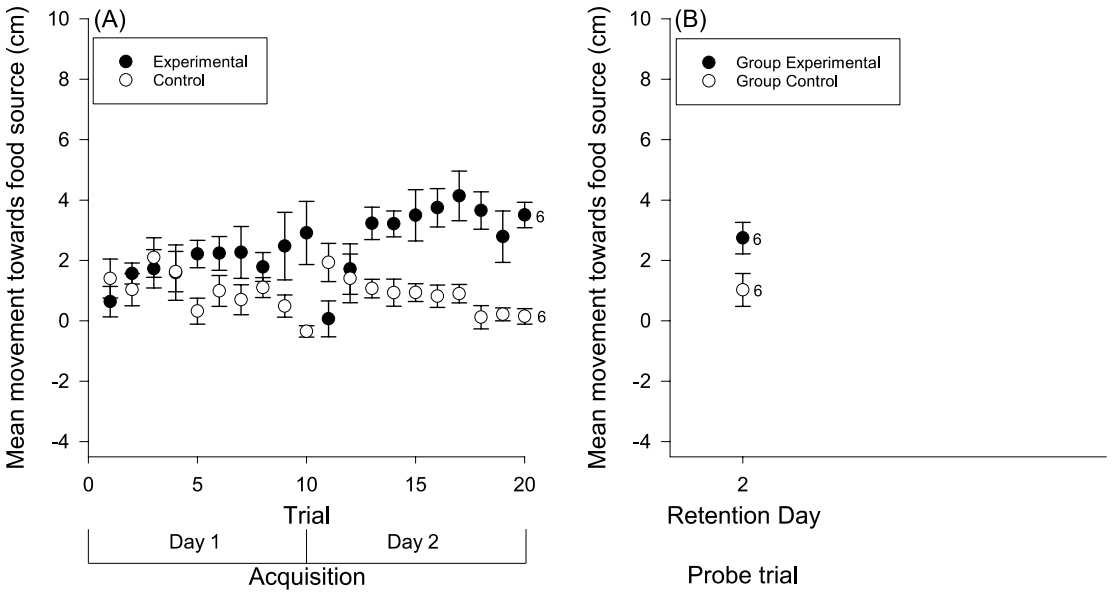

Figure 3.5. Juvenile zebrafish (49 dpf) in the experimental group moved away from their initial positions towards the food source as a result of conditioning to the auditory stimulus.

This response increased throughout the training trials. Zebrafish in the control group did not move toward the food source in response to the auditory stimulus (A). When tested for retention in groups after 2 days, trained fish moved closer to the food source compared to controls (B). Data points are mean distance from the food source before the FM tone sweep minus mean distance from the food source during the FM tone sweep. Numbers beside data points represent replicates for groups of 5 fish in each condition. Error bars = ± s.e.m.

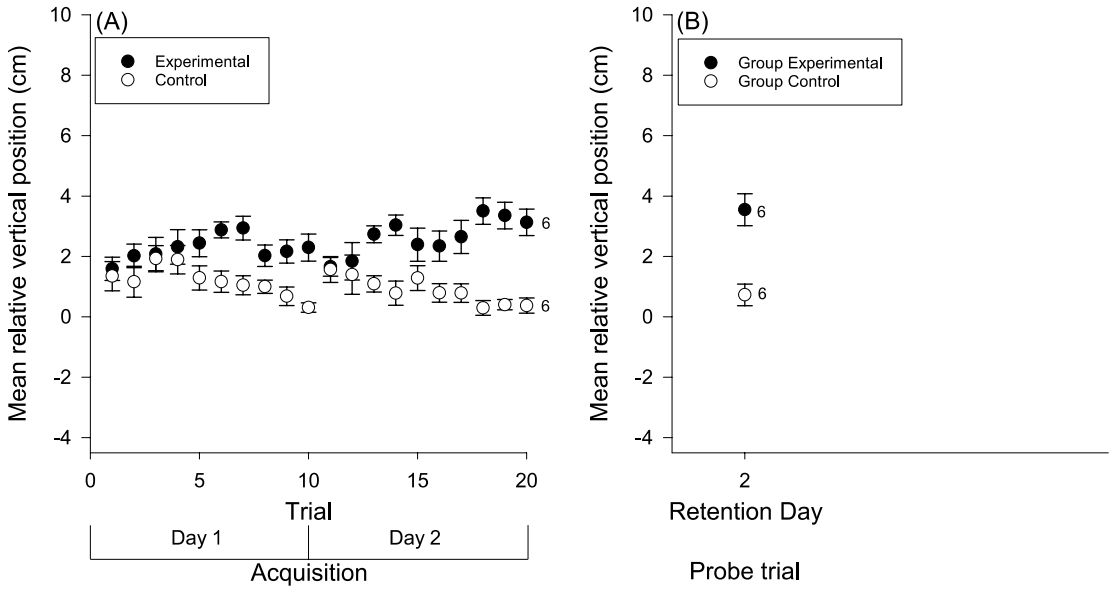

Figure 3.6. Juvenile zebrafish (49 dpf) in the experimental group moved vertically from their initial positions towards the surface as a result of conditioning to the auditory stimulus.

This response increased throughout the training trials. Zebrafish in the control group did not move toward the surface in response to the auditory stimulus (A). When tested for retention in groups after 2 days, trained fish moved closer to the surface compared to controls (B). Data points are mean vertical position before the FM tone sweep minus mean vertical position during the FM tone sweep. Numbers beside data points represent replicates for groups of 5 fish in each condition. Error bars = ± s.e.m.

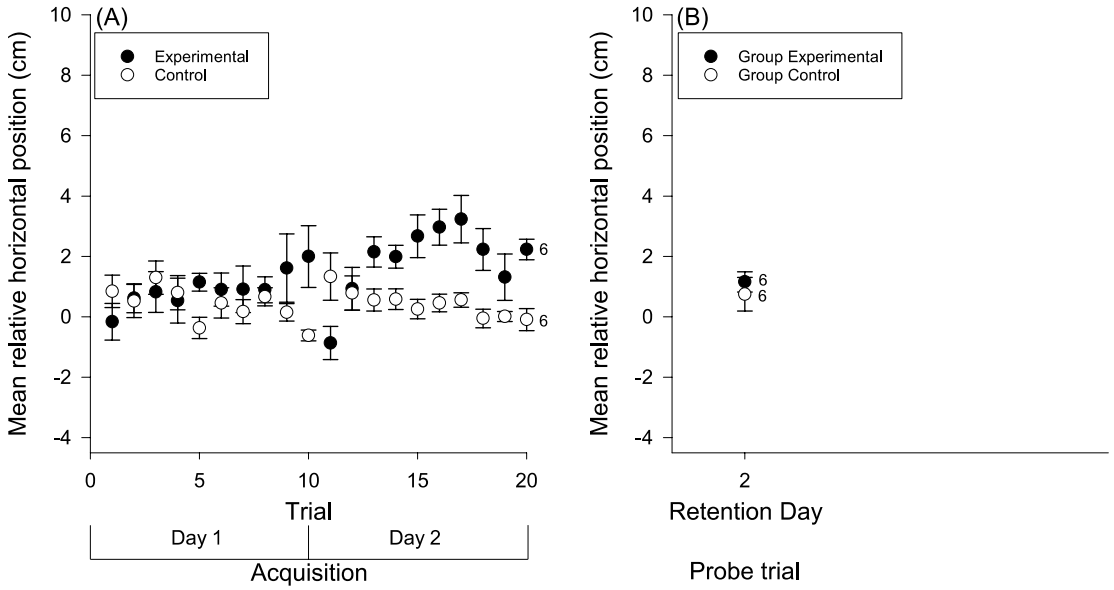

Figure 3.7. Juvenile zebrafish (49 dpf) in the experimental group moved laterally from their initial positions towards the food source as a result of conditioning to the auditory stimulus.

This response increased throughout the training trials. Zebrafish in the control group did not move toward the food source in response to the auditory stimulus (A). When tested for retention in groups after 2 days, trained fish did not move closer, laterally, to the food source compared to controls (B). Data points are mean horizontal position before the FM tone sweep minus mean horizontal position during the FM tone sweep. Numbers beside data points represent replicates for groups of 5 fish in each condition. Error bars = ± s.e.m.

3.3 Auditory Conditioning and Memory Retention of Younger Juvenile Fish (30 dpf)

Finally, we examined whether younger juvenile zebrafish could also learn the association.

3.3.1 Acquisition of Appetitive Conditioning

As with older juveniles, 30 dpf fish came to swim closer to the food source during the presentation of an auditory stimulus that was paired with food. Figure 3.8A 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 ($\chi^2(1)= 5.1038$, $p=0.024$). Bootstrapped confidence intervals suggested that by the 8-10th training trial, the experimental groups were moving consistently toward the food source during the presentation of the auditory stimulus. Analysis of the vertical component of movement showed no significant interaction between conditioning and training trials ($\chi^2(1)= 3.4757$, $p>0.05$, Figure 3.9A). Analysis of horizontal components of the movements each showed significant interactions between conditioning and training trials ($\chi^2(1)= 5.2002$, $p=0.023$, Figure 3.10A) suggesting fish learned to adjust their horizontal position in the tank in response to the conditioning auditory stimulus.

3.3.2 Memory Retention for Groups of Fish

To examine whether the association between the auditory stimulus and the food reward was retained after training in the 30 dpf juvenile fish, we tested the groups of fish for their responses to the auditory stimulus alone with probe trials 2 days after training

(Figure 3.8B). A Welch two sample t-test on the movement of fish towards the feeding location revealed a significant difference between the experimental and control group ($p<0.001$) indicating that experimental groups moved closer to the food source compared to controls. Separate analysis of the vertical and horizontal components of movement each showed significant differences between control and experimental groups (vertical: $p<0.001$, Figure 3.9B; horizontal: $p<0.001$, Figure 3.10B).

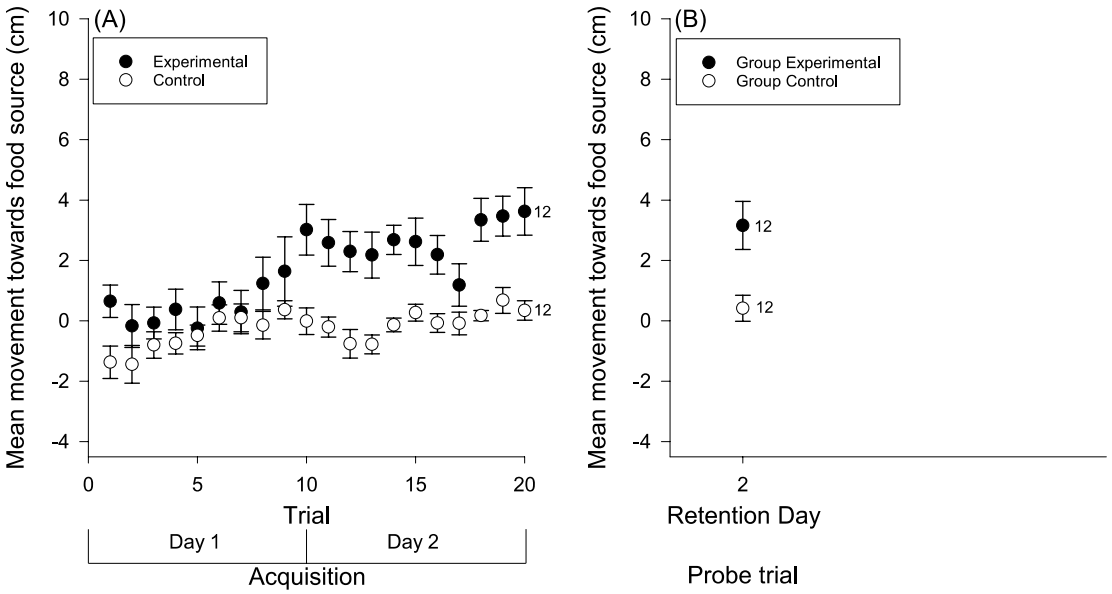

Figure 3.8. Juvenile zebrafish (30 dpf) in the experimental group moved away from their initial positions towards the food source as a result of conditioning to the auditory stimulus.

This response increased throughout the training trials. Zebrafish in the control group did not move toward the food source in response to the auditory stimulus (A). When tested for retention in groups after 2 days, trained fish moved closer to the food source compared to controls (B). Data points are mean distance from the food source before the FM tone sweep minus mean distance from the food source during the FM tone sweep. Numbers beside data points represent replicates for groups of 5 fish in each condition. Error bars = ± s.e.m.

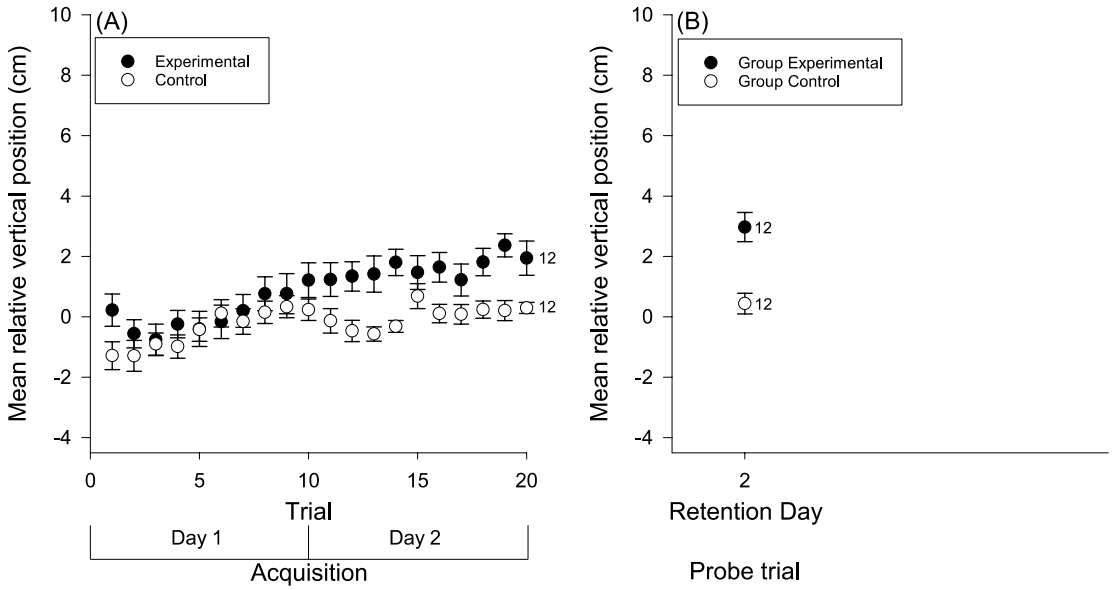

Figure 3.9. Juvenile zebrafish (30 dpf) in the experimental group moved vertically from their initial positions towards the surface as a result of conditioning to the auditory stimulus.

This response increased throughout the training trials. Zebrafish in the control group did not move toward the surface in response to the auditory stimulus (A). When tested for retention in groups after 2 days, trained fish moved closer to the surface compared to controls (B). Data points are mean vertical position before the FM tone sweep minus mean vertical position during the FM tone sweep. Numbers beside data points represent replicates for groups of 5 fish in each condition. Error bars = ± s.e.m.

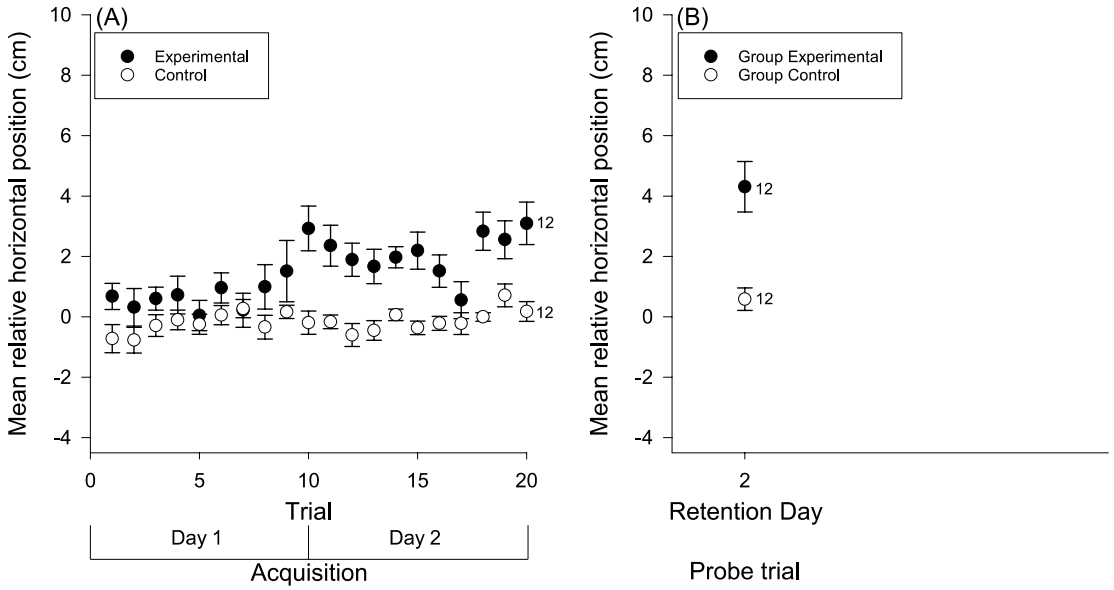

Figure 3.10. Juvenile zebrafish (30 dpf) in the experimental group moved laterally from their initial positions towards the food source as a result of conditioning to the auditory stimulus.

This response increased throughout the training trials. Zebrafish in the control group did not move toward the food source in response to the auditory stimulus (A). When tested for retention in groups after 2 days, trained fish moveed closer, laterally, to the food source compared to controls (B). Data points are mean horizontal position before the FM tone sweep minus mean horizontal position during the FM tone sweep. Numbers beside data points represent replicates for groups of 5 fish in each condition. Error bars = ± s.e.m.

3.4 Characterization of Phosphorylated ERK Immunoreactivity

Brains of juvenile zebrafish (30 dpf) which were fixed immediately following the 2-day probe trial were used to assess pERK immunoreactivity in the telencephalon. Maximum intensity projections obtained from the confocal image stacks were used to qualitatively assess differences in the staining pattern between experimental and control brains. In both groups, strong immunoreactivity was observed in the telencephalon. However minimal immunostaining was observed elsewhere in the brain, the tectum and medulla (data not shown).

In addition, a similar qualitative pattern of cell distribution was observed in both control and experimental brains. Phosphorylated ERK immunoreactive cells appeared to be predominantly located along the anterolateral borders of the telencephalon, and became more diffused towards the midline and posterior regions (Figure 3.11).

In order to quantitatively analyze if there was an effect of conditioning on the number of pERK immunoreactive cells, cell density index (number of cells per μm^2) was measured in the entire dorsal telencephalon (pallium). A Welch two sample t-test revealed significantly higher cell density index values in the experimental brains compared to controls ($p=0.004$; Figure 3.12), suggesting an effect of conditioning. A two-way ANOVA for immunoreactive area (%) (percentage of the total telencephalic area covered by pERK immunoreactive cells), also revealed a significant overall effect of conditioning ($p=0.011$) (Figure 3.13). In addition, a significant effect of dorsal pallium quadrant ($p<0.001$) and interaction between conditioning and quadrant were also found ($p=0.013$) This suggests that the difference in pERK immunoreactivity is not homogeneous throughout the dorsal pallium.

Next, in order to localize the difference in immunoreactive area (%) to specific regions, post-hoc tests were performed for each quadrant. Analysis of the anterior and posterior quadrants of the lateral dorsal pallium showed significantly higher immunoreactivity in experimental fish compared to controls (Welch two sample t-test, $p=0.020$ & $p=0.035$, respectively) (Figure 3.13). Comparisons of the medial region showed no effect of conditioning in the anterior and posterior quadrants (Welch two sample t-test, both $p> 0.05$). This suggests that the difference in pERK immunoreactivity is predominantly restricted to the lateral regions of the dorsal pallium.

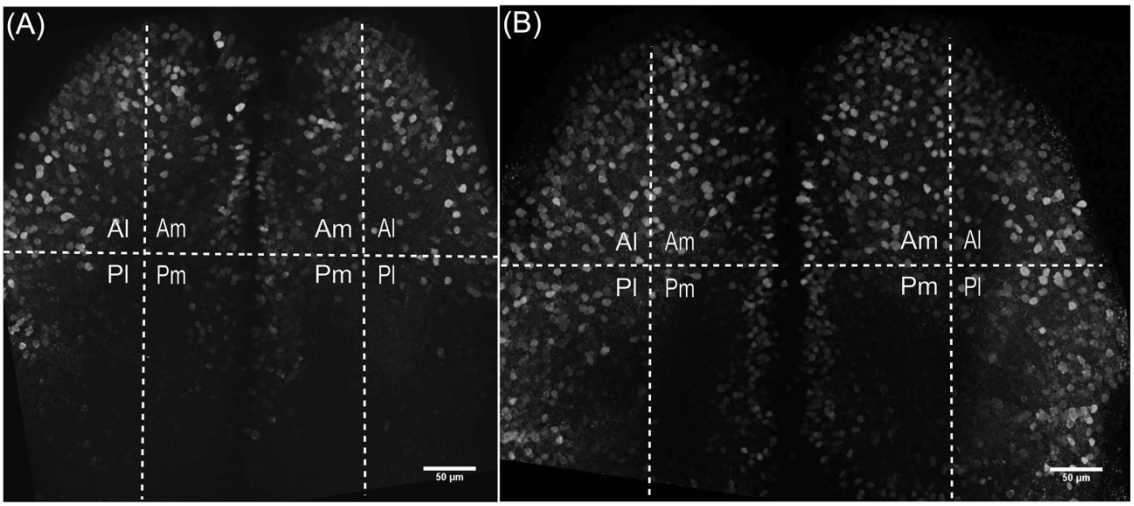

Figure 3.11. Representative pERK immunoreactivity (maximum intensity projection) in the dorsal telencephalon of a control (A) and experimental (B) juvenile zebrafish (30 dpf) when tested for retention after 2 days.

Anterior lateral (AL), posterior lateral (Pl), anterior medial (Am) and posterior medial (Pm) ROIs are indicated by labeled white dashed lines, scale bars = 50 µm.

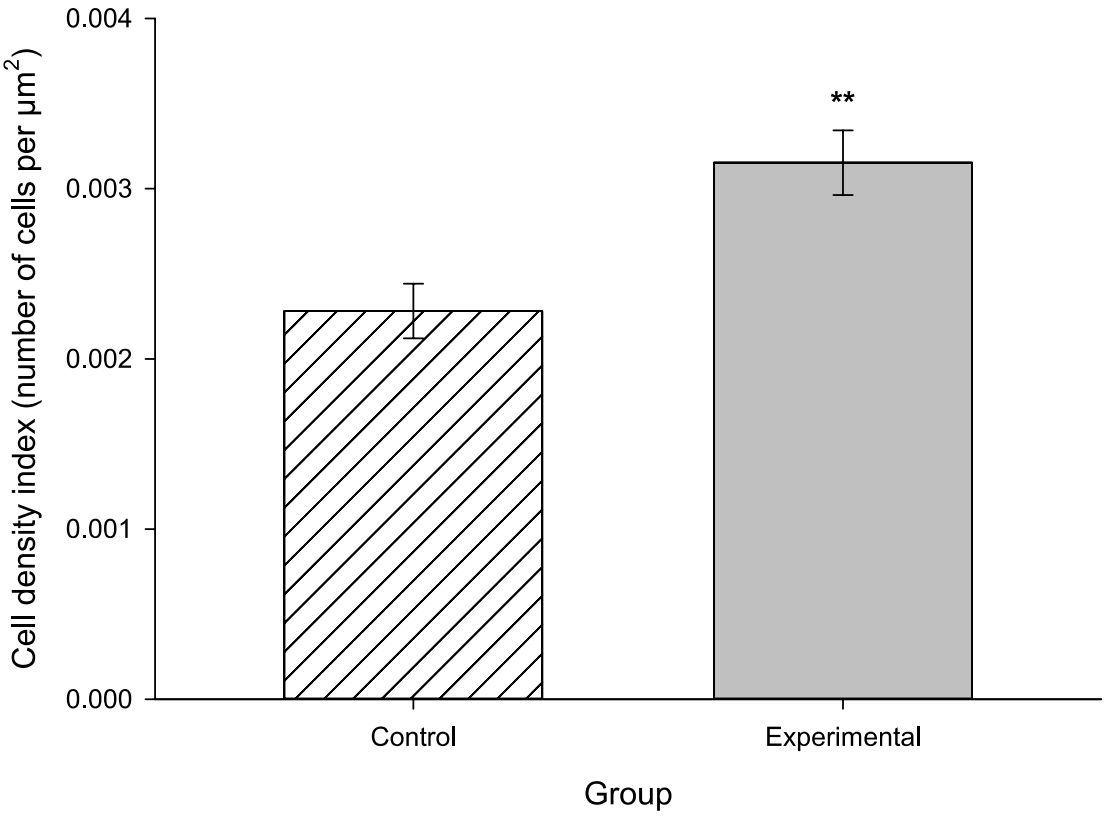

Figure 3.12. Mean pERK immunoreactive cell density index values (number of cells per μm^2) in the whole dorsal pallium.

Density index values of pERK immunoreactive cells in the dorsal pallium were significantly higher in experimental juvenile zebrafish (30 dpf) than control fish (*p=0.004), when tested for retention after 2 days. Control n=7, experimental n=9 and error bars = ± s.e.m.

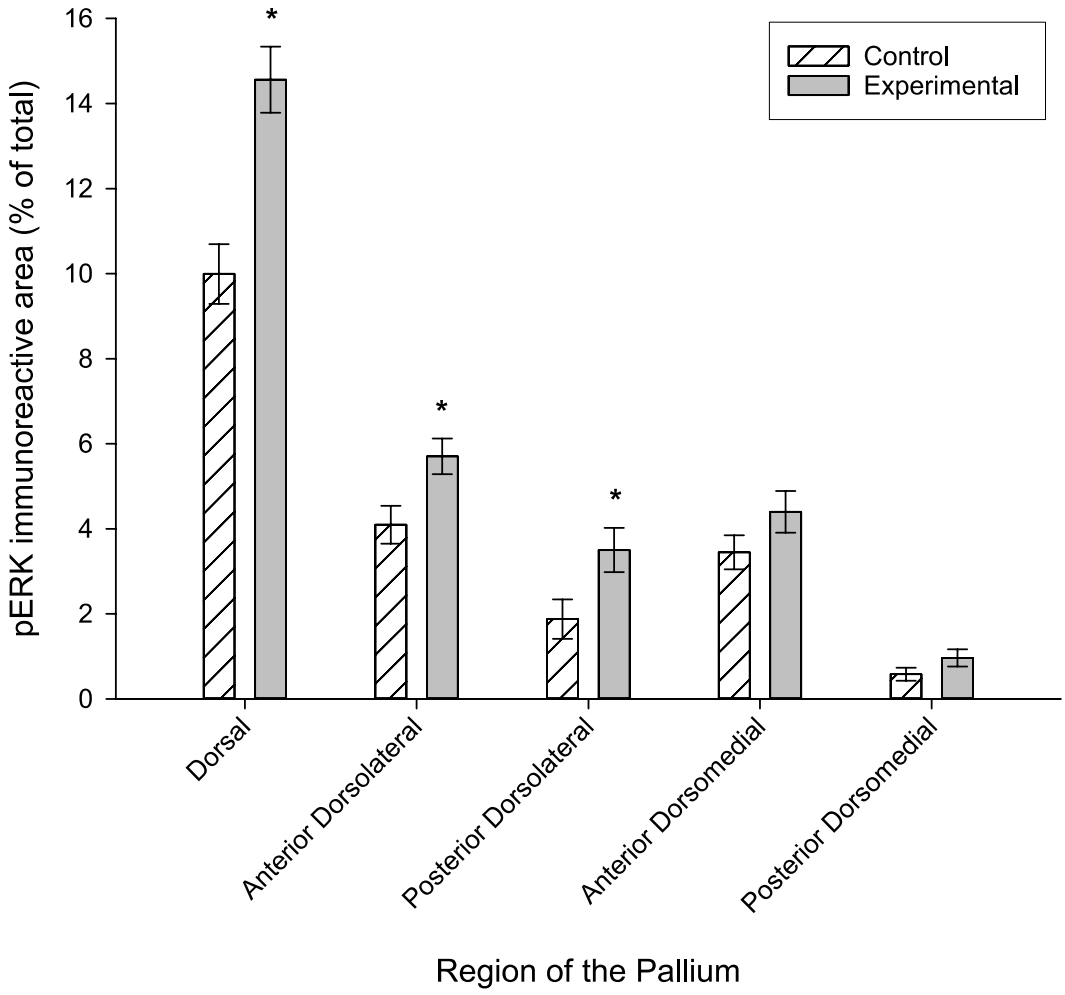

Figure 3.13. Mean percentage of the total telencephalic area covered by pERK immunoreactivity in the whole dorsal pallium, as well as the lateral and medial quadrants of the anterior and posterior dorsal pallium.

Immunoreactivity to pERK was significantly higher in whole dorsal pallium of conditioned juvenile zebrafish (30 dpf) than controls when tested for retention after 2 days (*p=0.011). This increase in immunoreactivity was localised to the anterior and posterior quadrants of the lateral dorsal pallium (*p= 0.020 & *p=0.035, respectively). Immunoreactivity was not significantly different in the anterior and posterior quadrants

of the medial dorsal pallium (both $p > 0.05$). Control n=7, experimental n=9 and error bars = ± s.e.m.

CHAPTER 4 DISCUSSION

In this study, we developed an automated appetitive paradigm to condition zebrafish in their home tanks. Our results demonstrated that fish could rapidly learn to associate an auditory stimulus, consisting of continuous 100-1000-100 Hz FM sweeps, with the presentation of food. Groups of zebrafish navigated towards the food source upon presentation of the conditioned stimulus. The strength of association increased over the course of 20 acquisition trials, suggesting fish progressively improved in their ability to anticipate the presentation of food as an unconditioned stimulus. In addition, individual and groups of zebrafish were capable of retaining and retrieving the associative memory for at least 2 days post training indicating that they formed a robust long-term memory of the association. Finally, conditioned juvenile zebrafish (30 dpf) showed increased pERK immunoreactivity in the dorsolateral pallium after probe trials, suggesting that this area may be involved in memory retrieval.

4.1 CONDITIONING

A question naturally arises regarding what was learned by the fish in this study. We observed little or no response by the control fish to the FM sweeps as illustrated by their consistent, near-zero movements toward the food source, thereby suggesting that these stimuli were neutral in the absence of any training. In contrast to the naïve (at the beginning of training) and control fish, zebrafish that were trained to associate the auditory stimulus with food rose to the surface in response to the conditioned stimuli (Figures 3.3, 3.6, 3.9), thus mimicking the innate consummatory behavior of these surface-feeding fish (Suriyampola et al., 2016). We therefore propose that this robust

conditioned response to a previously neutral stimulus represents a form of classical conditioning.

In addition to classical conditioning, which resulted in fish travelling upwards to the surface, the fish also travelled laterally towards the feeder in anticipation of food (Figures 3.4, 3.7, 3.10). This response suggests that they might also learn to associate food with the specific location at which the food was dispensed. This finding is consistent with previous studies that showed zebrafish are capable of forming concurrent double associations both between salient cues and a food reward, and between the reward and its location (Braubach et al., 2009; Karnik and Gerlai, 2012). It is not clear, however, whether zebrafish formed a cognitive map of their environment (O'Keefe and Nadel, 1979) or simply formed associations between a specific landmark (such as the plastic divider near the feeder) and the location of the food source (Karnik and Gerlai, 2012). Additional work is needed to determine whether there was a spatial component to the learned response. The conditioned response to go toward one side of the tank was, however, consistently weaker than the response to swim toward the surface, although caution must also be exercised regarding the interpretation of this difference. More salient landmarks, clearly indicating the end of the tank from which the food was dispensed, might be expected to improve the lateral component of the learned response significantly (Williams et al., 2002; Gerlai, 2011).

Together, these findings suggest that the responses described in this study are the result of classical conditioning and that a more complex form of learning may also be involved. The ability of fish to anticipate both the timing of food availability and the

location of the food would provide a substantial advantage for fish competing for limited resources in their natural environment (Engeszer et al., 2007).

4.2 Rate of Acquisition

A number of associative paradigms have been developed to condition adult zebrafish with between 1 to 40 pairing trials administered over the course of 1 to 5 days using aversive stimuli, such as electrical shocks (Xu et al., 2007; Blank et al., 2009; Agetsuma et al., 2012) or disturbances in the water (Morin et al., 2013). Appetitive paradigms, which minimize undesired stress-induced reactions (Gerlai, 2011), often require large numbers of pairings, ranging from 20 to 400 trials for up to 8 days (Colwill et al., 2005; Braubach et al., 2009; Sison and Gerlai, 2010; Mueller and Neuhauss, 2012; Chacon and Luchiari, 2014). In addition, one appetitive paradigm has shown that juveniles can be conditioned within 28 trials over 2 days (Williams et al., 2002). However, as learning is task and age dependent, it is unclear if juveniles can be conditioned in a shorter period of time.

The appetitive paradigm described in this paper conditioned groups of fish in less than 2 days with 10 trials delivered per day. A significant conditioned response was observed in both adults and juveniles by 5-13 pairings. Acquisition of the vertical component was not significant for the 30 dpf fish, most likely as the swim bladder is not fully functional at this age (Robertson et al., 2007; Winata et al., 2009) and the smaller sample size compared to the adult experiments. The purpose of this study was to determine whether zebrafish could be conditioned in their home tanks, without stress of handling and with the use of an auditory stimulus. The conditioned stimulus was chosen simply based on the limits of what zebrafish were known to be able to detect (Cervi et al.,

2012) but not cause large startle responses (data not shown). Also, these results were obtained using somewhat arbitrary inter-trial intervals and food amounts. Optimization of these parameters is likely to improve the rates of learning even further (Morin et al., 2013).

We suggest that the rapidity of learning demonstrated in our studies is the consequence of both monitoring natural food-finding responses normally exhibited by zebrafish, and the use of home tanks to eliminate stressors, which are inherent features of many other paradigms involving zebrafish. Other paradigms often involve transferring fish into new experimental tanks, inducing handling stress (Ramsay et al., 2009) and necessitating long acclimation periods (Sison and Gerlai, 2011). We minimized stress by conducting experiments in home tanks using the same or equivalent water temperature, composition and flow as in conventional facilities where zebrafish are reared and/or maintained. Setting up new experiments and testing fish after long retention periods involves only the moving of whole tanks with pre-existing groups of fish from the maintenance racks to the observation racks, followed by overnight acclimation.

4.3 Memory Retention

Despite the growing number of learning paradigms for zebrafish, few studies have examined memory retention beyond 2-3 days after training. For instance, there is evidence that zebrafish can maintain associative memory of food with either a visual (Al-Imari and Gerlai, 2008) or olfactory (Braubach et al., 2009) stimulus in place preference paradigms for 1 and 2 days, respectively. Another study observed retention of an aversive association between a visual stimulus and shock after 3 days (Xu and Goetz, 2012).

Evidence does exist, however, for spatial memories lasting for 10 days (Williams et al., 2002).

Our study provides further evidence for long-term memory in zebrafish. Two-way ANOVAs in adults indicated significant main effects during retention periods ranging from 2 to 32 days post training and demonstrated memories persisting for at least 2 days. A significant interaction for groups suggests that memory declined between 2 and 16 days. However, as there was no interaction for retentions in individual fish, presumably due to low statistical power, we were unable to determine the exact time course of retention. As with other aspects of this study, future optimization of procedures will likely result in more definitive retention curves. For example, initial conditioning of fish to high performance criteria, rather than simply to arbitrary numbers of training trials, will probably provide more accurate estimates of maximum duration of memory retention in these animals.

We also demonstrated a novel aspect of memory retention in zebrafish. When animals behave as members of social groups, it can be unclear, without detailed analyses (Seeley and Buhrman, 1999; Nagy et al., 2010), how individuals contribute to the behaviour of the group as a whole (here, a shoal of zebrafish). Do all or most fish in the shoal learn the conditioned associations or do only a few fish learn and the rest of the shoal follows those leaders? It was also possible that outside the context of the shoal, no individual fish would exhibit memories of the conditioning acquired as a group. Here we exploited the efficiency of quickly training groups of fish to demonstrate unambiguous memory retention by individuals. These results are consistent with the previous studies emphasizing the transmission of learned responses from a group to individuals and

effectiveness of group training (Suboski et al., 1990; Brown and Laland, 2003). It should be possible in the future to correlate such measures of individual performance during retention with changes in brain activity, to elucidate neural substrates of learning and memory (Sumbre and de Polavieja, 2014). However, correlating the performance of individual fish with brain activity was not possible in the current study as juveniles were tested only in groups. Individual testing could also provide a foundation for genetic and pharmacological screens for factors affecting long-term memory consolidation and retrieval.

4.4 Advantages of Automation

Though automated systems offer conspicuous advantages over paradigms utilizing manual pairings, few automated paradigms have yet been developed for zebrafish (Mueller and Neuhauss, 2012; Cerutti et al., 2013; Manabe et al., 2013). Automation offers a more controlled environment by minimizing possible confounds such as the presence of experimenters who could potentially act as predictors for the food reward (Mueller and Neuhauss, 2012), and by providing more precise and consistent delivery of the food reward. Our automated feeding system also overcame a potential problem of satiation (Sison and Gerlai, 2010), as food was dispensed in small amounts throughout conditioning. The feeder can dispense a wide variety of commercially available fish food of varying sizes, making it suitable for different fish of different ages, as demonstrated here with both adult and juvenile fish.

Importantly, automation also offers the opportunity to easily scale operations up to efficiently condition large number of animals. The automated apparatus described here is relatively inexpensive, easily constructed, and can be added to existing tanks without

modifications. In addition, the apparatus has the capacity to present a wide variety of tones and coloured lights as conditioned stimuli (Doyle et al., 2016). Durations and intervals can be easily programmed in Arduino sketches. Thus, the paradigms can be used not only to test cognitive abilities of fish in high-throughput screens but also to test for fine sensory discrimination in studies of psychophysics.

In addition to describing an easily constructed and inexpensive apparatus to produce conditioning, we also demonstrated that behavioural responses in zebrafish can be reliably measured by analysing movement in two dimensions using inexpensive cameras and subsequent analyses through ImageJ and Matlab. These are readily available and cost effective alternatives to commercially available tracking software packages used in many other conditioning paradigms (Al-Imari and Gerlai, 2008; Sison and Gerlai, 2010, 2011; Karnik and Gerlai, 2012; Chacon and Luchiari, 2014).

4.5 Neural Correlates of Learning

One approach to understanding the neural correlates of learning and memory is to create a functional map of the brain using a diverse range of learning paradigms. This map would allow the different stages of memory formation to be linked to corresponding brain regions. Given the established use of pERK as an indicator of neural activity in zebrafish (Randlett et al., 2015), we used pERK to identify brain regions that are active during memory retrieval in free-swimming fish. To our knowledge, this is the first reported use of ERK phosphorylation in zebrafish to characterize the regions associated with memory.

Significantly stronger pERK immunoreactivity was observed in the dorsal pallium of conditioned fish compared to control, suggesting a possible link between retrieval of

the auditory conditioned response and increased brain activity. Moreover, regional analysis revealed significantly higher immunoreactivity in lateral regions of the pallium in conditioned fish. These results suggest that dorsolateral pallium is activated and involved in retrieval of the conditioned association.

These findings are in line with other studies that have shown similarities between subdivisions of the pallium in zebrafish and mammalian hippocampus and isocortex. These similarities have been previously proposed on the basis of developmental morphogenesis (Mueller et al., 2011). In addition, ablation of goldfish medial and lateral pallium has been shown to impair acquisition and retention of an avoidance task, respectively (Portavella et al., 2004). Localised activity, covering a small focal area, close to the center of dorsal telencephalon has been demonstrated during retrieval of an active avoidance association in adult zebrafish using calcium imaging. Furthermore, ablation of the activated area impaired retrieval of the long-term memory, suggesting its functional homology to mammalian isocortex (Aoki et al., 2013).Collectively the presented evidence suggests that subdivisions of dorsal pallium are involved in retrieval of aversive and appetitive associative memories. However, as the boundaries between these subdivisions are not clear, it is difficult to draw direct links between anatomical divisions and their functions.

The increased immunoreactivity observed in the dorsolateral pallium of appetitively conditioned fish may be a direct result of enhanced activity due to the retrieval of the learned association. However, it is possible that ERK phosphorylation could have been triggered by sensory stimulation such as conditioned stimulus alone or behaviours such as motor responses. Although pERK can serve as a useful activity marker, phosphorylation

of ERK is dependent on the temporal pattern of neural firing and may not inclusively represent all neural activity (Randlett et al., 2015). To eliminate potential confounds, it would be necessary to also examine phosphorylation of ERK in response to the auditory stimulus, feeding and random swimming. In addition, to better resolve the activated areas of interest, background immunoreactivity could be reduced through double labeling with an antibody against total ERK. This would allow the background signal to be subtracted, giving a clearer indication of activated areas (Randlett et al., 2015).

The observed involvement of dorsolateral pallium in memory retention needs to be confirmed with more evidence, as the accurate identification of activated brain nuclei was limited by lack of physical and histological landmarks. In order to determine if the selected ROIs align correctly with the pallium sub-divisions (lateral and medial pallium), additional neuronal markers would be needed. Also, although brain activity was studied only during retention, it is possible that the activated areas are involved in acquisition as well as consolidation and retention. In spite of these limitations, this study provides a useful preliminary framework for future neurobehavioural studies in zebrafish.

4.6 Conclusions

With the increasing use of zebrafish as models for the study of learning and memory, it becomes important to establish paradigms to test a fuller range of this animal's behavioural repertoire. Demand for high-throughput screening further requires efficient training procedures that can produce robust learning and long-lasting memories. The appetitive paradigm described here meets these demands by quickly and reliably conditioning zebrafish to associate a neutral auditory stimulus with food in under 20 trials over less than 2 days. The paradigm, with our inexpensive automated apparatus, can

easily be run using groups of either adult or juvenile fish in their home tanks, thus eliminating the need for specialised tanks and extended periods of acclimation. After training, fish can be removed from the observation arenas and moved back to maintenance racks while awaiting probe trials for memory retention. In this way, we have shown that fish trained in groups can learn individually, demonstrating long-term memories lasting at least 2 days. In addition, retention of the learned response was associated with increased neural activity in the entire dorsal telencephalon as well as regions roughly overlapping the lateral pallium, previously reported as being involved in memory retention in zebrafish.

The results of this study have significant implications for increasing our understanding of the neural basis of learning and memory. Neural activity can be mapped at various stages during learning and memory retention to identify nuclei involved in these processes. Furthermore, this paradigm can be used for rapid, high-throughput screening of compounds modifying these processes.

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APPENDIX A: SUPPLEMENTAL MOVIES

Supplemental Movie 1. Representative video of acquisition trial 20 for auditory 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, indicated by the red light, fish did not exhibit any changes in their swimming behaviour.

Supplemental Movie 2. Representative video of acquisition trial 20 for auditory 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, indicated by the red light, fish moved towards the food source (upper-left corner of the tank) in anticipation of the food reward.

APPENDIX B: ARDUINO SKETCHES FOR CONDITIONING

A.1.1: AUDITORY TRAINING DAY ONE

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

int Led = 7;

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

SdReader card;    // This object holds the information for the card
FatVolume vol;    // This holds the information for the partition on the card
FatReader root;   // This holds the information for the filesystem on the card
FatReader f;      // This holds the information for the file we're playing
WaveHC wave;      // This is the only wave (audio) object, since we will only play one at
a time

void setup() {
  Serial.begin(9600);   // set up Serial library at 9600 bps for debugging
  setSyncProvider(RTC.get);   // the function to get the time from the RTC

    Alarm.alarmRepeat(9,45,0, SoundOn);  // Set Alarm For 9:45AM Every Day
    Alarm.alarmRepeat(9,45,20, Feeder);  // Set Alarm For 9:45:20AM Every Day
    Alarm.alarmRepeat(10,19,0, SoundOn);  // Set Alarm For 10:19AM Every Day
    Alarm.alarmRepeat(10,19,20, Feeder);  // Set Alarm For 10:19:20AM Every Day
```

```
  Alarm.alarmRepeat(11,28,0, SoundOn);  // Set Alarm For 11:28AM Every Day
  Alarm.alarmRepeat(11,28,20, Feeder);  // Set Alarm For 11:28:20AM Every Day
  Alarm.alarmRepeat(12,20,0, SoundOn);  // 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, SoundOn);  // Set Alarm For 13:48PM Every Day
  Alarm.alarmRepeat(13,48,20, Feeder);  // Set Alarm For 13:48:20PM Every Day
  Alarm.alarmRepeat(14,35,0, SoundOn);  // Set Alarm For 14:35PM Every Day
  Alarm.alarmRepeat(14,35,20, Feeder);  // Set Alarm For 14:35:20PM Every Day
  Alarm.alarmRepeat(16,23,0, SoundOn);  // Set Alarm For 16:23OM Every Day
  Alarm.alarmRepeat(16,23,20, Feeder);  // Set Alarm For 16:23:20PM Every Day
  Alarm.alarmRepeat(17,59,0, SoundOn);  // Set Alarm For 17:59PM Every Day
  Alarm.alarmRepeat(17,59,20, Feeder);  // Set Alarm For 17:59:20PM Every Day
  Alarm.alarmRepeat(19,10,0, SoundOn);  // Set Alarm For 19:10PM Every Day
  Alarm.alarmRepeat(19,10,20, Feeder);  // Set Alarm For 19:10:20PM Every Day
  Alarm.alarmRepeat(20,22,0, SoundOn);  // Set Alarm For 20:22PM Every Day
  Alarm.alarmRepeat(20,22,20, Feeder);  // Set Alarm For 20:22:20PM Every Day

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

// Set the output pins for the DAC control. This pins are defined in the library
pinMode(2, OUTPUT);
pinMode(3, OUTPUT);
pinMode(4, OUTPUT);
pinMode(5, OUTPUT);
pinMode(Led, OUTPUT);

//  if (!card.init(true)) { //play with 4 MHz spi if 8MHz isn't working for you
if (!card.init()) {         //play with 8 MHz spi (default faster!)
  putstring_nl("Card init. failed!");  // Something went wrong, lets print out why
  while(1);                            // then 'halt' - do nothing!
```

```
  }

  // enable optimize read - some cards may timeout. Disable if you're having problems
  card.partialBlockRead(true);

  // Now we will look for a FAT partition!
  uint8_t part;
  for (part = 0; part < 5; part++) {     // we have up to 5 slots to look in
    if (vol.init(card, part))
      break;                             // we found one, lets bail
  }
  if (part == 5) {                       // if we ended up not finding one  :(
    putstring_nl("No valid FAT partition!");
    while(1);                            // then 'halt' - do nothing!
  }

  // Lets tell the user about what we found
  putstring("Using partition ");
  Serial.print(part, DEC);
  putstring(", type is FAT");
  Serial.println(vol.fatType(),DEC);     // FAT16 or FAT32?

  // Try to open the root directory
  if (!root.openRoot(vol)) {
    putstring_nl("Can't open root dir!"); // Something went wrong,
    while(1);                            // then 'halt' - do nothing!
  }
}

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

```
}

void SoundOn() {
  digitalWrite(Led, HIGH);   // turn the LED on (HIGH is the voltage level)
  playcomplete("Tone.WAV");
}

void Feeder() {
  digitalWrite(Led, LOW);    // turn the LED off by making the voltage LOW
  Motor->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE,
DOUBLE, INTERLEAVE, MICROSTEP)
  Motor->release();
}

void playcomplete(char *name) {
 // call our helper to find and play this name
 playfile(name);
 while (wave.isplaying) {
 // do nothing while its playing
 }
 // now its done playing
}

void playfile(char *name) {
 // see if the wave object is currently doing something
 if (wave.isplaying) {// already playing something, so stop it!
   wave.stop(); // stop it
 }
 // look in the root directory and open the file
 if (!f.open(root, name)) {
   putstring("Couldn't open file "); Serial.print(name); return;
```

```
  }
  // OK read the file and turn it into a wave object
  if (!wave.create(f)) {
    putstring_nl("Not a valid WAV"); return;
  }

  // ok time to play! start playback
  wave.play();
}
```

A.1.2: AUDITORY TRAINING DAY TWO

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

int Led = 7;

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

SdReader card;    // This object holds the information for the card
FatVolume vol;    // This holds the information for the partition on the card
FatReader root;   // This holds the information for the filesystem on the card
FatReader f;      // This holds the information for the file we're playing
WaveHC wave;      // This is the only wave (audio) object, since we will only play one at
a time

void setup() {
  Serial.begin(9600);   // set up Serial library at 9600 bps for debugging
  setSyncProvider(RTC.get);   // the function to get the time from the RTC

    Alarm.alarmRepeat(10,06,0, SoundOn);  // Set Alarm For 10:06AM Every Day
    Alarm.alarmRepeat(10,06,20, Feeder);  // Set Alarm For 10:06:20AM Every Day
    Alarm.alarmRepeat(10,58,0, SoundOn);  // Set Alarm For 10:58AM Every Day
    Alarm.alarmRepeat(10,58,20, Feeder);  // Set Alarm For 10:58:20AM Every Day
    Alarm.alarmRepeat(11,46,0, SoundOn);  // Set Alarm For 11:46AM Every Day
```

```
  Alarm.alarmRepeat(11,46,20, Feeder);  // Set Alarm For 11:46:20AM Every Day
  Alarm.alarmRepeat(12,34,0, SoundOn);  // 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, SoundOn);  // Set Alarm For 13:54PM Every Day
  Alarm.alarmRepeat(13,54,20, Feeder);  // Set Alarm For 13:54:20PM Every Day
  Alarm.alarmRepeat(14,38,0, SoundOn);  // Set Alarm For 14:38PM Every Day
  Alarm.alarmRepeat(14,38,20, Feeder);  // Set Alarm For 14:38:20PM Every Day
  Alarm.alarmRepeat(15,52,0, SoundOn);  // Set Alarm For 15:52PM Every Day
  Alarm.alarmRepeat(15,52,20, Feeder);  // Set Alarm For 15:52:20PM Every Day
  Alarm.alarmRepeat(17,42,0, SoundOn);  // Set Alarm For 17:42PM Every Day
  Alarm.alarmRepeat(17,42,20, Feeder);  // Set Alarm For 17:42:20PM Every Day
  Alarm.alarmRepeat(19,20,0, SoundOn);  // Set Alarm For 19:20PM Every Day
  Alarm.alarmRepeat(19,20,20, Feeder);  // Set Alarm For 19:20:20PM Every Day
  Alarm.alarmRepeat(20,06,0, SoundOn);  // Set Alarm For 20:06PM Every Day
  Alarm.alarmRepeat(20,06,20, Feeder);  // Set Alarm For 20:06:20PM Every Day

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

// Set the output pins for the DAC control. This pins are defined in the library
pinMode(2, OUTPUT);
pinMode(3, OUTPUT);
pinMode(4, OUTPUT);
pinMode(5, OUTPUT);
pinMode(Led, OUTPUT);

//  if (!card.init(true)) { //play with 4 MHz spi if 8MHz isn't working for you
if (!card.init()) {         //play with 8 MHz spi (default faster!)
  putstring_nl("Card init. failed!");  // Something went wrong, lets print out why
  while(1);                            // then 'halt' - do nothing!
}
```

```
  // enable optimize read - some cards may timeout. Disable if you're having problems
  card.partialBlockRead(true);

  // Now we will look for a FAT partition!
  uint8_t part;
  for (part = 0; part < 5; part++) {     // we have up to 5 slots to look in
    if (vol.init(card, part))
      break;                             // we found one, lets bail
  }
  if (part == 5) {                       // if we ended up not finding one  :(
    putstring_nl("No valid FAT partition!");
    while(1);                            // then 'halt' - do nothing!
  }

  // Lets tell the user about what we found
  putstring("Using partition ");
  Serial.print(part, DEC);
  putstring(", type is FAT");
  Serial.println(vol.fatType(),DEC);     // FAT16 or FAT32?

  // Try to open the root directory
  if (!root.openRoot(vol)) {
    putstring_nl("Can't open root dir!"); // Something went wrong,
    while(1);                            // then 'halt' - do nothing!
  }
}

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

```
void SoundOn() {
  digitalWrite(Led, HIGH);   // turn the LED on (HIGH is the voltage level)
  playcomplete("Tone.WAV");
}

void Feeder() {
  digitalWrite(Led, LOW);    // turn the LED off by making the voltage LOW
  Motor->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE,
DOUBLE, INTERLEAVE, MICROSTEP)
  Motor->release();
}

void playcomplete(char *name) {
 // call our helper to find and play this name
 playfile(name);
 while (wave.isplaying) {
 // do nothing while its playing
 }
 // now its done playing
}

void playfile(char *name) {
 // see if the wave object is currently doing something
 if (wave.isplaying) {// already playing something, so stop it!
   wave.stop(); // stop it
 }
 // look in the root directory and open the file
 if (!f.open(root, name)) {
```

A.2.1: AUDITORY CONTROL DAY ONE

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

int Led = 7;

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

SdReader card;    // This object holds the information for the card
FatVolume vol;    // This holds the information for the partition on the card
FatReader root;   // This holds the information for the filesystem on the card
FatReader f;      // This holds the information for the file we're playing
WaveHC wave;      // This is the only wave (audio) object, since we will only play one at
a time

void setup() {
  Serial.begin(9600);   // set up Serial library at 9600 bps for debugging
  setSyncProvider(RTC.get);   // the function to get the time from the RTC

Alarm.alarmRepeat(9,45,0, SoundOn);  // Set Alarm For 9:45AM Every Day
    Alarm.alarmRepeat(10,02,20, Feeder);  // Set Alarm For 9:45:20AM Every Day
    Alarm.alarmRepeat(10,19,0, SoundOn);  // Set Alarm For 10:19AM Every Day
    Alarm.alarmRepeat(10,53,30, Feeder);  // Set Alarm For 10:19:20AM Every Day
    Alarm.alarmRepeat(11,28,0, SoundOn);  // Set Alarm For 11:28AM Every Day
```

```
  Alarm.alarmRepeat(11,54,20, Feeder);  // Set Alarm For 11:28:20AM Every Day
  Alarm.alarmRepeat(12,20,0, SoundOn);  // Set Alarm For 12:20PM Every Day
  Alarm.alarmRepeat(13,02,20, Feeder);  // Set Alarm For 12:20PM Every Day
  Alarm.alarmRepeat(13,48,0, SoundOn);  // Set Alarm For 13:48PM Every Day
  Alarm.alarmRepeat(14,11,20, Feeder);  // Set Alarm For 13:48:20PM Every Day
  Alarm.alarmRepeat(14,35,0, SoundOn);  // Set Alarm For 14:35PM Every Day
  Alarm.alarmRepeat(15,29,20, Feeder);  // Set Alarm For 14:35:20PM Every Day
  Alarm.alarmRepeat(16,23,0, SoundOn);  // Set Alarm For 16:23OM Every Day
  Alarm.alarmRepeat(17,11,20, Feeder);  // Set Alarm For 16:23:20PM Every Day
  Alarm.alarmRepeat(17,59,0, SoundOn);  // Set Alarm For 17:59PM Every Day
  Alarm.alarmRepeat(18,35,30, Feeder);  // Set Alarm For 17:59:20PM Every Day
  Alarm.alarmRepeat(19,10,0, SoundOn);  // Set Alarm For 19:10PM Every Day
  Alarm.alarmRepeat(19,46,20, Feeder);  // Set Alarm For 19:10:20PM Every Day
  Alarm.alarmRepeat(20,22,0, SoundOn);  // Set Alarm For 20:22PM Every Day
  Alarm.alarmRepeat(20,54,20, Feeder);  // Set Alarm For 20:22:20PM Every Day

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

// Set the output pins for the DAC control. This pins are defined in the library
pinMode(2, OUTPUT);
pinMode(3, OUTPUT);
pinMode(4, OUTPUT);
pinMode(5, OUTPUT);
pinMode(Led, OUTPUT);

//  if (!card.init(true)) { //play with 4 MHz spi if 8MHz isn't working for you
if (!card.init()) {         //play with 8 MHz spi (default faster!)
  putstring_nl("Card init. failed!");  // Something went wrong, lets print out why
  while(1);                            // then 'halt' - do nothing!
}
```

```
  // enable optimize read - some cards may timeout. Disable if you're having problems
  card.partialBlockRead(true);

  // Now we will look for a FAT partition!
  uint8_t part;
  for (part = 0; part < 5; part++) {     // we have up to 5 slots to look in
    if (vol.init(card, part))
      break;                             // we found one, lets bail
  }
  if (part == 5) {                       // if we ended up not finding one  :(
    putstring_nl("No valid FAT partition!");
    while(1);                            // then 'halt' - do nothing!
  }

  // Lets tell the user about what we found
  putstring("Using partition ");
  Serial.print(part, DEC);
  putstring(", type is FAT");
  Serial.println(vol.fatType(),DEC);     // FAT16 or FAT32?

  // Try to open the root directory
  if (!root.openRoot(vol)) {
    putstring_nl("Can't open root dir!"); // Something went wrong,
    while(1);                            // then 'halt' - do nothing!
  }
}

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

```
void SoundOn() {
  digitalWrite(Led, HIGH);   // turn the LED on (HIGH is the voltage level)
  playcomplete("Tone.WAV");
}

void Feeder() {
  digitalWrite(Led, LOW);    // turn the LED off by making the voltage LOW
  Motor->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE,
DOUBLE, INTERLEAVE, MICROSTEP)
  Motor->release();
}

void playcomplete(char *name) {
 // call our helper to find and play this name
 playfile(name);
 while (wave.isplaying) {
 // do nothing while its playing
 }
 // now its done playing
}

void playfile(char *name) {
 // see if the wave object is currently doing something
 if (wave.isplaying) {// already playing something, so stop it!
   wave.stop(); // stop it
 }
 // look in the root directory and open the file
 if (!f.open(root, name)) {
```

A.2.2: AUDITORY CONTROL DAY TWO

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

int Led = 7;

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

SdReader card;    // This object holds the information for the card
FatVolume vol;    // This holds the information for the partition on the card
FatReader root;   // This holds the information for the filesystem on the card
FatReader f;      // This holds the information for the file we're playing
WaveHC wave;      // This is the only wave (audio) object, since we will only play one at
a time

void setup() {
  Serial.begin(9600);   // set up Serial library at 9600 bps for debugging
  setSyncProvider(RTC.get);   // the function to get the time from the RTC

    Alarm.alarmRepeat(10,06,0, SoundOn);  // Set Alarm For 10:06AM Every Day
    Alarm.alarmRepeat(10,26,20, Feeder);  // Set Alarm For 10:26:20AM Every Day
    Alarm.alarmRepeat(10,58,0, SoundOn);  // Set Alarm For 10:58AM Every Day
    Alarm.alarmRepeat(11,22,20, Feeder);  // Set Alarm For 11:22:20AM Every Day
    Alarm.alarmRepeat(11,46,0, SoundOn);  // Set Alarm For 11:46AM Every Day
```

```
  Alarm.alarmRepeat(12,10,20, Feeder);  // Set Alarm For 12:10:20AM Every Day
  Alarm.alarmRepeat(12,34,0, SoundOn);  // 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, SoundOn);  // Set Alarm For 13:54PM Every Day
  Alarm.alarmRepeat(14,16,20, Feeder);  // Set Alarm For 14:16:20PM Every Day
  Alarm.alarmRepeat(14,38,0, SoundOn);  // Set Alarm For 14:38PM Every Day
  Alarm.alarmRepeat(15,15,20, Feeder);  // Set Alarm For 15:15:20PM Every Day
  Alarm.alarmRepeat(15,52,0, SoundOn);  // Set Alarm For 15:52PM Every Day
  Alarm.alarmRepeat(16,47,20, Feeder);  // Set Alarm For 16:47:20PM Every Day
  Alarm.alarmRepeat(17,42,0, SoundOn);  // Set Alarm For 17:42PM Every Day
  Alarm.alarmRepeat(18,31,20, Feeder);  // Set Alarm For 18:31:20PM Every Day
  Alarm.alarmRepeat(19,20,0, SoundOn);  // Set Alarm For 19:20PM Every Day
  Alarm.alarmRepeat(19,43,20, Feeder);  // Set Alarm For 19:43:20PM Every Day
  Alarm.alarmRepeat(20,06,0, SoundOn);  // Set Alarm For 20:06PM Every Day
  Alarm.alarmRepeat(20,36,20, Feeder);  // Set Alarm For 20:36:20PM Every Day

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

// Set the output pins for the DAC control. This pins are defined in the library
pinMode(2, OUTPUT);
pinMode(3, OUTPUT);
pinMode(4, OUTPUT);
pinMode(5, OUTPUT);
pinMode(Led, OUTPUT);

//  if (!card.init(true)) { //play with 4 MHz spi if 8MHz isn't working for you
if (!card.init()) {         //play with 8 MHz spi (default faster!)
  putstring_nl("Card init. failed!");  // Something went wrong, lets print out why
  while(1);                            // then 'halt' - do nothing!
}
```

```
  // enable optimize read - some cards may timeout. Disable if you're having problems
  card.partialBlockRead(true);

  // Now we will look for a FAT partition!
  uint8_t part;
  for (part = 0; part < 5; part++) {     // we have up to 5 slots to look in
    if (vol.init(card, part))
      break;                             // we found one, lets bail
  }
  if (part == 5) {                       // if we ended up not finding one  :(
    putstring_nl("No valid FAT partition!");
    while(1);                            // then 'halt' - do nothing!
  }

  // Lets tell the user about what we found
  putstring("Using partition ");
  Serial.print(part, DEC);
  putstring(", type is FAT");
  Serial.println(vol.fatType(),DEC);     // FAT16 or FAT32?

  // Try to open the root directory
  if (!root.openRoot(vol)) {
    putstring_nl("Can't open root dir!"); // Something went wrong,
    while(1);                            // then 'halt' - do nothing!
  }
}

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

```
void SoundOn() {
  digitalWrite(Led, HIGH);   // turn the LED on (HIGH is the voltage level)
  playcomplete("Tone.WAV");
}

void Feeder() {
  digitalWrite(Led, LOW);    // turn the LED off by making the voltage LOW
  Motor->step(100, BACKWARD, DOUBLE); //Steps, Direction, Step Type (SINGLE,
DOUBLE, INTERLEAVE, MICROSTEP)
  Motor->release();
}

void playcomplete(char *name) {
 // call our helper to find and play this name
 playfile(name);
 while (wave.isplaying) {
 // do nothing while its playing
 }
 // now its done playing
}

void playfile(char *name) {
 // see if the wave object is currently doing something
 if (wave.isplaying) {// already playing something, so stop it!
   wave.stop(); // stop it
 }
 // look in the root directory and open the file
 if (!f.open(root, name)) {
```

A.3: AUDITORY RETENTIONS

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

int Led = 7;

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

SdReader card;    // This object holds the information for the card
FatVolume vol;    // This holds the information for the partition on the card
FatReader root;   // This holds the information for the filesystem on the card
FatReader f;      // This holds the information for the file we're playing
WaveHC wave;      // This is the only wave (audio) object, since we will only play one at
a time

void setup() {
  Serial.begin(9600);   // set up Serial library at 9600 bps for debugging
  setSyncProvider(RTC.get);   // the function to get the time from the RTC

    Alarm.alarmRepeat(15,1,0, SoundOn);  // Set Alarm For 15:1AM Every Day

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

```
// Set the output pins for the DAC control. This pins are defined in the library
pinMode(2, OUTPUT);
pinMode(3, OUTPUT);
pinMode(4, OUTPUT);
pinMode(5, OUTPUT);
pinMode(Led, OUTPUT);

//  if (!card.init(true)) { //play with 4 MHz spi if 8MHz isn't working for you
if (!card.init()) {         //play with 8 MHz spi (default faster!)
  putstring_nl("Card init. failed!");  // Something went wrong, lets print out why
  while(1);                            // then 'halt' - do nothing!
}

// enable optimize read - some cards may timeout. Disable if you're having problems
card.partialBlockRead(true);

// Now we will look for a FAT partition!
uint8_t part;
for (part = 0; part < 5; part++) {     // we have up to 5 slots to look in
  if (vol.init(card, part))
    break;                             // we found one, lets bail
}
if (part == 5) {                       // if we ended up not finding one  :(
  putstring_nl("No valid FAT partition!");
  while(1);                            // then 'halt' - do nothing!
}

// Lets tell the user about what we found
putstring("Using partition ");
Serial.print(part, DEC);
putstring(", type is FAT");
```

```
  Serial.println(vol.fatType(),DEC);     // FAT16 or FAT32?

  // Try to open the root directory
  if (!root.openRoot(vol)) {
    putstring_nl("Can't open root dir!"); // Something went wrong,
    while(1);                           // then 'halt' - do nothing!
  }
}

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

void SoundOn() {
   digitalWrite(Led, HIGH);   // turn the LED on (HIGH is the voltage level)
   playcomplete("Tone.WAV");
}

void playcomplete(char *name) {
  // call our helper to find and play this name
  playfile(name);
  while (wave.isplaying) {
  // do nothing while its playing
  }
  // now its done playing
}

void playfile(char *name) {
  // see if the wave object is currently doing something
  if (wave.isplaying) {// already playing something, so stop it!
    wave.stop(); // stop it
```

```
  }
  // look in the root directory and open the file
  if (!f.open(root, name)) {
    putstring("Couldn't open file "); Serial.print(name); return;
  }
  // OK read the file and turn it into a wave object
  if (!wave.create(f)) {
    putstring_nl("Not a valid WAV"); return;
  }

  // ok time to play! start playback
  wave.play();
}
```

APPENDIX C: STATISTICAL ANALYSES FOR ADULT ZEBRAFISH

C.1: ADULT - HORIZONTAL MOVEMENT

C.1.1: Acquisition – Linear Mixed Effects Model

```
Adult   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 4369.4 4403.6 -2177.7   4355.4
mod.dfs5  8 4338.9 4378.0 -2161.4   4322.9 32.471      1   1.21e-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: 4336.2

Scaled residuals:
Min      1Q  Median      3Q     Max
-3.8488 -0.5619  0.0027  0.5546  5.0646

Random effects:
Groups   Name        Variance Std.Dev. Corr
Tank     (Intercept) 1.25287  1.11932
TrialN      0.00568  0.07537  -0.06
Residual             4.22077  2.05445
Number of obs: 980, groups:  Tank, 49

Fixed effects:
Estimate Std. Error t value
(Intercept)       0.157766   0.288475   0.547
ConditionE        1.353893   0.421059   3.215
TrialN            0.003524   0.021508   0.164
ConditionE:TrialN 0.208660   0.031393   6.647

Correlation of Fixed Effects:
(Intr) CndtnE TrialN
ConditionE  -0.685
TrialN      -0.443  0.304
CndtnE:TrlN  0.304 -0.443 -0.685
```

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure
Chisq Df Pr(>Chisq)
Condition 47.264 1 6.203e-12 ***
TrialN 41.945 1 9.388e-11 ***
Condition:TrialN 44.179 1 2.997e-11 ***

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

C.1.2: Group Retention – Two-Way ANOVA

Adult dX
Df Sum Sq Mean Sq F value Pr(>F)
Condition 1 108.42 108.42 13.396 0.000873 ***
DayN 1 0.15 0.15 0.018 0.893192
Condition:DayN 1 68.31 68.31 8.441 0.006503 **
Residuals 33 267.08 8.09

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

C.1.3: Individual Retention – Two-Way ANOVA

Adult dX
Df Sum Sq Mean Sq F value Pr(>F)
Condition 1 114.8 114.78 4.421 0.0384 *
DayN 4 176.7 44.19 1.702 0.1568
Condition:DayN 4 117.2 29.31 1.129 0.3483
Residuals 88 2284.9 25.97

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

C.2: ADULT - VERTICAL MOVEMENT

C.2.1: Acquisition – Linear Mixed Effects Model

```
Adult   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 3763.7 3797.9 -1874.8   3749.7
mod.dfs5  8 3740.1 3779.2 -1862.1   3724.1 25.528      1  4.359e-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: 3738

Scaled residuals:
Min      1Q  Median      3Q     Max
-3.3131 -0.5642 -0.0675  0.4891  3.8236

Random effects:
Groups   Name        Variance Std.Dev. Corr
Tank     (Intercept) 2.53516  1.5922
TrialN      0.01428  0.1195   -0.84
Residual             2.17834  1.4759
Number of obs: 980, groups:  Tank, 49

Fixed effects:
Estimate Std. Error t value
(Intercept)        1.27617    0.33998   3.754
ConditionE         0.81670    0.49623   1.646
TrialN            -0.05470    0.02599  -2.105
ConditionE:TrialN  0.21501    0.03793   5.669

Correlation of Fixed Effects:
(Intr) CndtnE TrialN
ConditionE  -0.685
TrialN      -0.843  0.577
CndtnE:TrlN  0.577 -0.843 -0.685
Analysis of Deviance Table (Type II Wald chisquare tests)
```

Response: Measure
Chisq Df Pr(>Chisq)
Condition 142.4570 1 < 2.2e-16 ***
TrialN 5.9626 1 0.01461 *
Condition:TrialN 32.1335 1 1.439e-08 ***

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

C.2.2: Group Retention – Two-Way ANOVA

Adult dY
Df Sum Sq Mean Sq F value Pr(>F)
Condition 1 23.20 23.20 5.949 0.0203 *
DayN 1 3.33 3.33 0.854 0.3621
Condition:DayN 1 12.24 12.24 3.138 0.0857 .
Residuals 33 128.69 3.90

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

C.2.3: Individual Retention – Two-Way ANOVA

Adult dY
Df Sum Sq Mean Sq F value Pr(>F)
Condition 1 300.6 300.55 43.695 2.85e-09 ***
DayN 4 47.6 11.90 1.729 0.151
Condition:DayN 4 30.6 7.64 1.111 0.356
Residuals 88 605.3 6.88

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

C.3: Adult – Movement Towards Food

C.3.1: Acquisition – Linear Mixed Effects Model

```
Adult   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 4332.5 4366.7 -2159.3   4318.5
mod.dfs5  8 4295.1 4334.2 -2139.5   4279.1 39.45      1  3.365e-10 ***
---
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: 4291.4

Scaled residuals:
Min      1Q  Median      3Q     Max
-4.1610 -0.5614 -0.0206  0.5174  4.4706

Random effects:
Groups   Name        Variance Std.Dev. Corr
Tank     (Intercept) 3.18609  1.7850
TrialN      0.01305  0.1142   -0.72
Residual             3.91780  1.9793
Number of obs: 980, groups:  Tank, 49

Fixed effects:
Estimate Std. Error t value
(Intercept)        0.96302    0.39377   2.446
ConditionE         1.56494    0.57475   2.723
TrialN            -0.03318    0.02699  -1.229
ConditionE:TrialN  0.30039    0.03940   7.625

Correlation of Fixed Effects:
(Intr) CndtnE TrialN
ConditionE  -0.685
TrialN      -0.756  0.518
CndtnE:TrlN  0.518 -0.756 -0.685
Analysis of Deviance Table (Type II Wald chisquare tests)
```

Response: Measure
Chisq Df Pr(>Chisq)
Condition 168.532 1 < 2.2e-16 ***
TrialN 30.071 1 4.166e-08 ***
Condition:TrialN 58.136 1 2.446e-14 ***

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

C.3.2: Group Retention – Two-Way ANOVA

Adult dD
Df Sum Sq Mean Sq F value Pr(>F)
Condition 1 117.04 117.04 13.010 0.00101 **
DayN 1 1.36 1.36 0.151 0.70021
Condition:DayN 1 73.07 73.07 8.122 0.00748 **
Residuals 33 296.89 9.00

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

C.3.3: Individual Retention – Two-Way ANOVA

Adult dD
Df Sum Sq Mean Sq F value Pr(>F)
Condition 1 266.2 266.17 10.725 0.00151 **
DayN 4 172.1 43.01 1.733 0.14983
Condition:DayN 4 121.0 30.26 1.219 0.30845
Residuals 88 2184.1 24.82

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

APPENDIX D: STATISTICAL ANALYSES FOR JUVENILE ZEBRAFISH (49 DPF)

D.1: JUVENILE (49 DPF) - HORIZONTAL MOVEMENT

D.1.1: Acquisition – Linear Mixed Effects Model

Juvenile (49 dpf) 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 7 832.19 856.56 -409.10 818.19
mod.dfs5 8 827.01 854.86 -405.51 811.01 7.1794 1 0.007374 **

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: 822.5

Scaled residuals:
Min 1Q Median 3Q Max
-3.00097 -0.47572 -0.03762 0.50838 2.88206

Random effects:
Groups Name Variance Std.Dev. Corr
Tank (Intercept) 1.197829 1.09445
TrialN 0.005594 0.07479 -0.83
Residual 1.502983 1.22596
Number of obs: 240, groups: Tank, 12

Fixed effects:
Estimate Std. Error t value
(Intercept) 0.70229 0.50368 1.394
ConditionE -0.54650 0.71231 -0.767
TrialN -0.02735 0.03618 -0.756
ConditionE:TrialN 0.14643 0.05117 2.862

Correlation of Fixed Effects:
(Intr) CndtnE TrialN
ConditionE -0.707

TrialN -0.838 0.593
CndtnE:TrlN 0.593 -0.838 -0.707
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure
Chisq Df Pr(>Chisq)
Condition 8.9351 1 0.002797 **
TrialN 3.2141 1 0.073006 .
Condition:TrialN 8.1900 1 0.004212 **

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

D.1.2: Group Retention – Two-Way ANOVA

Juvenile (49 dpf) dX
data: dX.Control and dX.Experimental
t = -0.62814, df = 8.2215, p-value = 0.547
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-1.900382 1.083716
sample estimates:
mean of x mean of y
0.7466667 1.1550000

D.2: JUVENILE (49 DPF) - VERTICAL MOVEMENT

D.2.1: Acquisition – Linear Mixed Effects Model

Juvenile (49 dpf) 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 7 621.26 645.62 -303.63 607.26
mod.dfs5 8 607.21 635.06 -295.61 591.21 16.048 1 6.176e-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: 606.2

Scaled residuals:
Min 1Q Median 3Q Max
-3.04527 -0.60284 -0.03616 0.62326 2.89600

Random effects:
Groups Name Variance Std.Dev. Corr
Tank (Intercept) 0.3696534 0.60799
TrialN 0.0004155 0.02038 0.22
Residual 0.5991292 0.77403
Number of obs: 240, groups: Tank, 12

Fixed effects:
Estimate Std. Error t value
(Intercept) 1.60139 0.28837 5.553
ConditionE 0.27420 0.40781 0.672
TrialN -0.05462 0.01481 -3.687
ConditionE:TrialN 0.11102 0.02095 5.300

Correlation of Fixed Effects:
 (Intr) CndtnE TrialN
ConditionE -0.707
TrialN -0.263 0.186
CndtnE:TrlN 0.186 -0.263 -0.707
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure
Chisq Df Pr(>Chisq)
Condition 4.5906 1 0.03215 *
TrialN 0.0073 1 0.93189
Condition:TrialN 28.0883 1 1.159e-07 ***

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

D.2.2: Group Retention – Two-Way ANOVA

Juvenile (49 dpf) dY
data: dY.Control and dY.Experimental
t = -4.422, df = 8.7781, p-value = 0.001771
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-4.275775 -1.374225
sample estimates:
mean of x mean of y
0.7266667 3.5516667

D.3: JUVENILE (49 DPF) - MOVEMENT TOWARDS FOOD

D.3.1: Acquisition – Linear Mixed Effects Model

Juvenile (49 dpf) 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 7 847.06 871.42 -416.53 833.06
mod.dfs5 8 839.64 867.48 -411.82 823.64 9.4213 1 0.002145 **

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: 834.1

Scaled residuals:
Min 1Q Median 3Q Max
-2.97926 -0.43232 -0.01699 0.51014 2.89656

Random effects:
Groups Name Variance Std.Dev. Corr
Tank (Intercept) 1.244204 1.11544
TrialN 0.005851 0.07649 -0.69
Residual 1.549211 1.24467
Number of obs: 240, groups: Tank, 12

Fixed effects:
Estimate Std. Error t value
(Intercept) 1.40876 0.51292 2.747
ConditionE -0.33881 0.72538 -0.467
TrialN -0.04915 0.03692 -1.331
ConditionE:TrialN 0.18034 0.05222 3.453

Correlation of Fixed Effects:
(Intr) CndtnE TrialN
ConditionE -0.707
TrialN -0.737 0.521
CndtnE:TrlN 0.521 -0.737 -0.707
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Measure
Chisq Df Pr(>Chisq)
Condition 9.4232 1 0.0021426 **
TrialN 2.4677 1 0.1162077
Condition:TrialN 11.9264 1 0.0005535 ***

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

D.3.2: Group Retention – Two-Way ANOVA

Juvenile (49 dpf) dD
data: dD.Control and dD.Experimental
t = -2.2714, df = 9.9867, p-value = 0.04649
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-3.40753129 -0.03246871
sample estimates:
mean of x mean of y
1.02 2.74

APPENDIX E: STATISTICAL ANALYSES FOR JUVENILE ZEBRAFISH (30 DPF)

E.1: JUVENILE (30 DPF) - HORIZONTAL MOVEMENT

E.1.1: Acquisition – Linear Mixed Effects Model

Juvenile (30 dpf) dX
Data: dfs
Models:
mod.dfs3: dX ~ Condition + TrialN + (TrialN | Tank)
mod.dfs5: dX ~ Condition * TrialN + (TrialN | Tank)
Df AIC BIC logLik deviance Chisq Chi Df Pr(>Chisq)
mod.dfs3 7 1778.1 1807.3 -882.05 1764.1
mod.dfs5 8 1774.9 1808.3 -879.45 1758.9 5.2002 1 0.02258 *

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

Linear mixed model fit by REML ['lmerMod']
Formula: dX ~ Condition * TrialN + (TrialN | Tank)
Data: dfs

REML criterion at convergence: 1771.1

Scaled residuals:
Min 1Q Median 3Q Max
-3.2365 -0.5082 -0.0225 0.4944 3.5203

Random effects:
Groups Name Variance Std.Dev. Corr
Tank (Intercept) 1.877660 1.37028
TrialN 0.006477 0.08048 -0.74
Residual 1.938785 1.39240
Number of obs: 480, groups: Tank, 24

Fixed effects:
Estimate Std. Error t value
(Intercept) -0.45784 0.43742 -1.047
ConditionE 0.68387 0.61860 1.105
TrialN 0.02810 0.02798 1.004
ConditionE:TrialN 0.09128 0.03957 2.307

Correlation of Fixed Effects:
 (Intr) CndtnE TrialN
ConditionE -0.707

TrialN -0.763 0.539
CndtnE:TrlN 0.539 -0.763 -0.707

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: dX
Chisq Df Pr(>Chisq)
Condition 19.6453 1 9.323e-06 ***
TrialN 13.8948 1 0.0001933 ***
Condition:TrialN 5.3227 1 0.0210492 *

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

E.1.2: Group Retention – Two-Way ANOVA

Juvenile (30 dpf) dX
data: dX.Control and dX.Experimental
t = -3.0036, df = 15.197, p-value = 0.008805
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-4.687910 -0.798763
sample estimates:
mean of x mean of y
0.4137572 3.1570938

E.2: JUVENILE (30 DPF) - VERTICAL MOVEMENT

E.2.1: Acquisition – Linear Mixed Effects Model

Juvenile (30 dpf) dY
Data: dfs
Models:
mod.dfs3: dY ~ Condition + TrialN + (TrialN | Tank)
mod.dfs5: dY ~ Condition * TrialN + (TrialN | Tank)
Df AIC BIC logLik deviance Chisq Chi Df Pr(>Chisq)
mod.dfs3 7 1566.8 1596.0 -776.38 1552.8
mod.dfs5 8 1565.3 1598.7 -774.65 1549.3 3.4757 1 0.06228 .

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

Linear mixed model fit by REML ['lmerMod']
Formula: dY ~ Condition * TrialN + (TrialN | Tank)
Data: dfs

REML criterion at convergence: 1561

Scaled residuals:
Min 1Q Median 3Q Max
-3.2236 -0.5718 -0.0156 0.5383 3.2161

Random effects:
Groups Name Variance Std.Dev. Corr
Tank (Intercept) 2.399560 1.54905
TrialN 0.008804 0.09383 -0.76
Residual 1.177019 1.08490
Number of obs: 480, groups: Tank, 24

Fixed effects:
Estimate Std. Error t value
(Intercept) -0.91416 0.47024 -1.944
ConditionE 0.25020 0.66502 0.376
TrialN 0.06763 0.02968 2.278
ConditionE:TrialN 0.07773 0.04198 1.852

Correlation of Fixed Effects:
 (Intr) CndtnE TrialN
ConditionE -0.707
TrialN -0.766 0.542
CndtnE:TrlN 0.542 -0.766 -0.707

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: dY
Chisq Df Pr(>Chisq)
Condition 7.8129 1 0.005187 **
TrialN 25.7424 1 3.902e-07 ***
Condition:TrialN 3.4283 1 0.064089 .

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

E.2.2: Group Retention – Two-Way ANOVA

Juvenile (30 dpf) dY
data: dY.Control and dY.Experimental
t = 4.2646, df = 19.712, p-value = 0.0003897
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
1.293635 3.775351
sample estimates:
mean of x mean of y
2.9714668 0.4369738

E.3: Juvenile (30 dpf) - Movement Movement Towards Food

E.3.1: Acquisition – Linear Mixed Effects Model

Juvenile (30 dpf) dD
Data: dfs
Models:
mod.dfs3: dD ~ Condition + TrialN + (TrialN | Tank)
mod.dfs5: dD ~ Condition * TrialN + (TrialN | Tank)
Df AIC BIC logLik deviance Chisq Chi Df Pr(>Chisq)
mod.dfs3 7 1878.7 1907.9 -932.36 1864.7
mod.dfs5 8 1875.6 1909.0 -929.81 1859.6 5.1038 1 0.02387 *

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

Linear mixed model fit by REML ['lmerMod']
Formula: dD ~ Condition * TrialN + (TrialN | Tank)
Data: dfs

REML criterion at convergence: 1869.6

Scaled residuals:
Min 1Q Median 3Q Max
-2.8835 -0.5553 -0.0088 0.5187 3.4885

Random effects:
Groups Name Variance Std.Dev. Corr
Tank (Intercept) 3.60130 1.898
TrialN 0.01299 0.114 -0.76
Residual 2.30451 1.518
Number of obs: 480, groups: Tank, 24

Fixed effects:
Estimate Std. Error t value
(Intercept) -0.95779 0.58442 -1.639
ConditionE 0.66918 0.82650 0.810
TrialN 0.06721 0.03703 1.815
ConditionE:TrialN 0.11956 0.05236 2.283

Correlation of Fixed Effects:
(Intr) CndtnE TrialN
ConditionE -0.707
TrialN -0.777 0.549
CndtnE:TrlN 0.549 -0.777 -0.707

Analysis of Deviance Table (Type II Wald chisquare tests)

Response: dD
Chisq Df Pr(>Chisq)
Condition 16.8460 1 4.054e-05 ***
TrialN 23.5253 1 1.233e-06 ***
Condition:TrialN 5.2131 1 0.02242 *

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

E.3.2: Group Retention – Two-Way ANOVA

Juvenile (30 dpf) dD
data: dD.Control and dD.Experimental
t = -4.1055, df = 16.962, p-value = 0.000741
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-5.641725 -1.811062
sample estimates:
mean of x mean of y
0.5818747 4.3082680

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