NORWEGIAN UNIVERSITY OF SCIENCE AND TECHNOLOGY

MASTER'S THESIS

Developing real-time tracking software & setup for studying active avoidance conditioning in zebrafish

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ABSTRACT

The cognitive capabilities of fish have historically been underestimated and, in many respects, they are comparable to birds and mammals. Currently, an increasing number of behavioural neuroscience laboratories have started using zebrafish as their main animal model. Although the zebrafish brain is substantially more primitive than that of mammals, basic brain anatomy is highly conserved across vertebrates. Furthermore, fish have a sophisticated behavioural repertoire that can allow quantification of functional changes in the brain induced by environmental manipulations. Zebrafish have been mostly used so far in developmental biology, genetics, pharmacology and toxicology. Currently, researchers are extending their use in behavioural neuroscience, focusing in particular on early developmental stages (one to three weeks old). At this developmental stage, their small size and transparency allows single and multi-photon imaging in order to visualize the brain activity. However, investigation on associative conditioning in larvae/juvenile zebrafish is limited and the mechanism remains poorly understood. Historically, most of the behaviour studies relied on manual quantification of fish behaviour, which is sensitive to human errors. To achieve a reproducible and high throughput quantification of fish behaviour, fundamental in behavioural studies, a fully automated setup is required.

The aim of this master thesis is to develop hardware and software for a setup that allows monitoring and quantification of zebrafish behaviour during active avoidance conditioning assays. In order to establish this learning protocol, I first developed a microcontroller based hardware platform and later used the C++ language to implement multi-threaded software able to simultaneously track the position of six fish in real time. The real time tracking allowed me to administer an aversive stimulus to individual animals based on their position. The setup developed in this master thesis proposes a systematic and fully automated approach for the investigation of zebrafish behaviour.

Using this setup, I have tested the learning response of three groups of zebrafish, from one to three weeks old. My results indicate that zebrafish can perform active avoidance already at the one-week-old larval stage. The learning performance of animals improves across developmental stages, resulting in a faster and more stable acquisition of conditioned behaviour. Moreover, thanks to the high spatial and temporal resolution in the quantification of fish behaviour, I have investigated how parameters such as baseline swim pattern, average velocity and thigmotaxis correlate with the learning performance of the fish.

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Chapter 1

Introduction

1.1) Studying associative learning

1.1.1) Synopsis

Learning which situation is potentially dangerous or profitable is a challenge every human being experiences since childhood, and the ability to predict threatening situations and avoid them is essential for surviving in the animal kingdom. Evolution has preserved this capability, across animal species in order to promote survival of the species against everyday life threats (Maren 2001). In 1898, Edward Lee Thorndike tried for the first time to explain the process of association in the animal's mind, in particular he correlated the behaviour of an animal with a stimulus following shortly after that behaviour (Thorndike 1998). In his manuscript, Thorndike wrote: "… the connection of a certain act with a certain situation and a resultant pleasure, and this general type of association is found normally throughout the animal´s life normally." (Thorndike 1998). A complementary approach to the same phenomenon was adopted by Ivan Petrovich Pavlov in 1927 who highlighted the relationship established between two stimuli experienced close in time (Maren 2001, Fanselow and Wassum 2016).

These two classifications of learning arose at the beginning of the 20th century and gave rise to two main approaches in modern neuroscience to the study of associative learning:

- Pavlovian conditioning: from the name of Pavlov who first investigated the association established among stimuli experienced closely in time, also called "Classical Conditioning".
- Instrumental conditioning: so called since the behaviour is instrumental to get an outcome, it is caused by relationships between the environment and the behaviour of the animal, also called "Operant Conditioning".

For both approaches the idea behind is similar: the association is formed when the subject experiences in a temporal sequence a neutral stimulus, rapidly followed by a biologically relevant stimulus. In both cases the only way to identify if there was or was not learning is to observe a change in behaviour due to experience (Fanselow and Wassum 2016). These two conditioning protocols approach the same concept but from two different points of view (Ciccarelli S.K. 2008). Operant conditioning involves a voluntary response from the animal and

results in an increase in the likelihood of a behaviour already occurring in the animal. On the contrary, classical conditioning involves an involuntary organism response to a stimulus and it results in a creation of a new response to a stimulus that was not present before. Operant conditioning uses consequences to form an association; classical conditioning uses the previous stimuli in forming association.

The concept of Classical (and Instrumental) conditioning has been further developed in 1957 by Fester and Skinner with the introduction of the concept of "Reinforcement" (Ferster, Skinner et al. 1957). In their work Skinner and Fester identified three classes of environment responses that can follow a specific behaviour (Ferster, Skinner et al. 1957, Morgan 2010):

- Neutral response: a response that does not change the probability of a behaviour to happen.
- Reinforcers: environment responses that increase the probability of a behaviour to be repeated. Reinforcers can be negative, such as removal of an unpleasant stimulus, or positive, i.e. administration of a pleasant stimulus.
- Punishers: responses from the environment, such as aversive events, that decrease the likelihood of a behaviour.

The concept of conditioning introduced by Pavlov (1927), Thorndike (1998) and Skinner (1950) was further investigated by Jerzy Konorski (1967). The latter used for the first time the conditioned behaviour approach to shed light on the brain mechanisms of behaviour. He postulated that the outcome behaviour was the result of integration and process of the sensory systems inputs (Fanselow 2010, Srebro 2013).

However, there is not a dedicated Pavlovian learning system, indeed conditioning processes are embedded in the neural system and evolved independently for all the systems in which they are involved (Fanselow and Wassum 2016).

The two most studied types of conditioning are fear conditioning (involving punishers) and appetitive conditioning (involving reinforcers)(Fanselow and Wassum 2016). It is important to highlight that there is no clear boundary between positive and negative reinforcers (Baron and Galizio 2005), therefore it can be very difficult and misleading to segregate positive from negative reinforcers since behaviours classified as positive reinforcers response also embed elements of punishment (Perone 2003). More in detail, the use of a positive reinforcer requires the previous absence of the unconditioned stimulus. A clarifying example is the use of food as an appetitive stimulus: to be effective as a positive reinforcer it requires that the animal is first food deprived, which can be considered as a punisher (Perone 2003, Baron and Galizio 2005). Therefore, it is important to keep in mind the relationship between the stimulus and the

context of the environment when designing an aversive or appetitive protocol. One example is the experiment performed by Scripture (1895), in which the author placed a frog in a beaker and increase the temperature of 0.0002 °C per second. After two and a half hours the frog was found dead without making any movement or reaction. The author reported: "He had been boiled without noticing it" (Perone 2003). To better understand the relation between stimulus property and its environmental context, it is relevant to compare the findings of two studies, one by Souza (Das Graças De Souza, Alves De Moraes et al. 1984)and one by Sizemore (Sizemore and Maxwell 1985). Both studied rat as animal model and shock as punishment, but Sizemore studied a punishment conditioning protocol while Souza investigated avoidance paradigm (figure 2). In his study Sizemore highlighted that a shock of 0.3 to 0.4 mA was enough to elicit an aversive response (Sizemore and Maxwell 1985). Surprisingly, in the study of Souza the minimum shock intensity leading to sustained avoidance behaviour was of the order of 1 mA (Souza 1984). This discrepancy highlight how a stimulus is evaluated based on its context and not only based on it characteristics (Perone 2003).

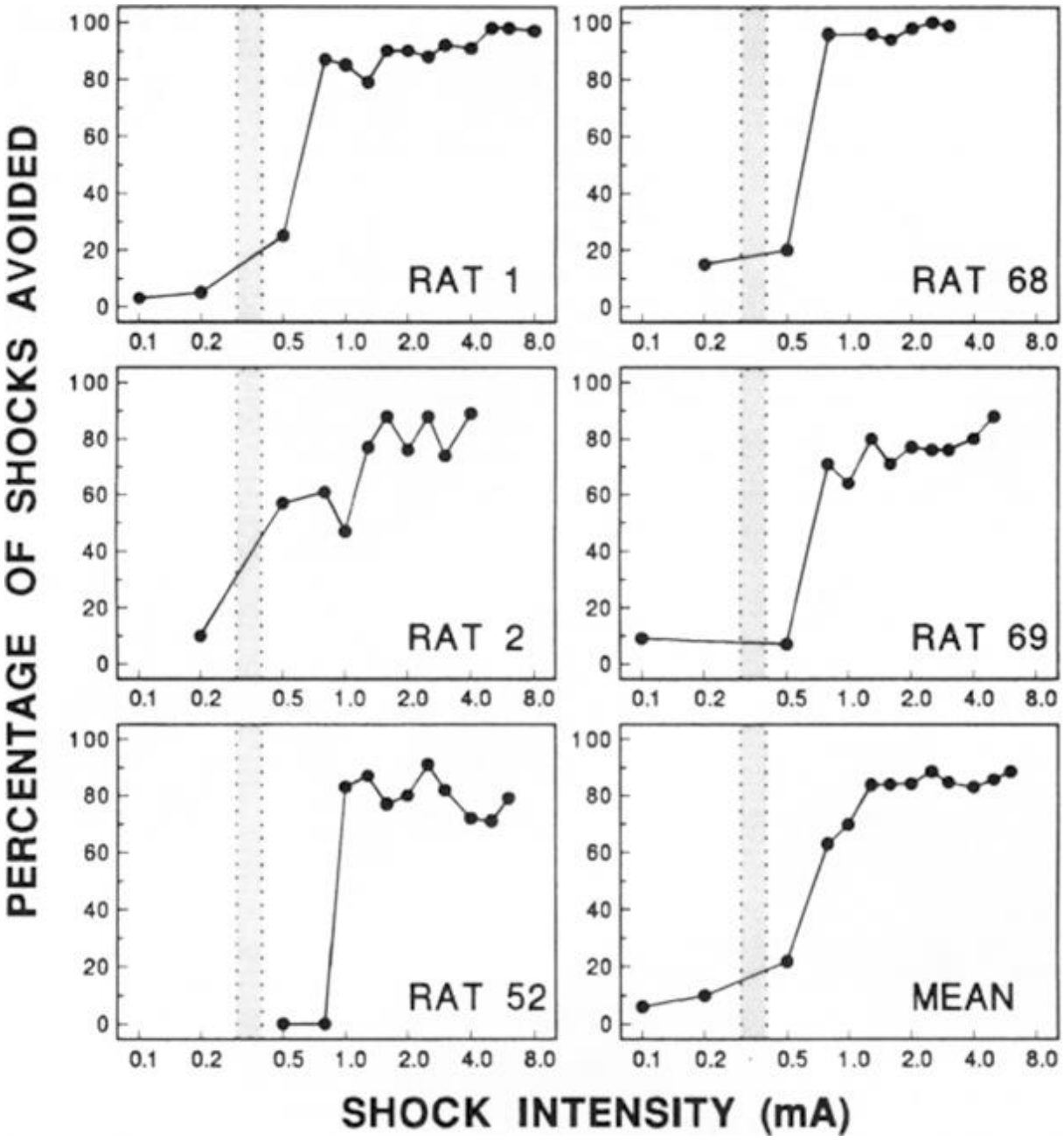


Figure 1) Performance in shock avoidance protocol as function of shock intensity presented in the study of Souza (1984). The shock intensity is represented in a logarithmic scale while the performance of the rat is quantified as percentage of shock avoided. The shaded bars represent the range of shock intensities that succeeded in supressing rat behaviour in the punishment procedure of Sizemore (1985). As shown, a shock of 0.3 mA is aversive in a punishment paradigm, but is not aversive in an avoidance paradigm (Perone 2003).

1.1.2) Fear conditioning:

Fear conditioning has been broadly investigated, mostly because it leads to a rapid and stronger response in the animal. Humans, like other mammals, have to learn to respond properly to environmental cues which signal threats for survival (such as predator) or they have to learn to associate specific environmental cues with events necessary for survival such as food, water or sexual partner (Nasser and McNally 2013). Fear conditioned response has been much more investigated than appetitive conditioned response in animals and in humans. This bias towards fear conditioning paradigm is likely related to the higher complexity of the appetitive response compared to the aversive one. For instance, as discussed above, a food reward to be effective required the animal to be food deprived (Andreatta and Pauli 2015). Moreover fear conditioning protocol is experimentally easier to control and more efficient since it is a more direct reflex than the appetitive conditioned response, the latter involving a higher cognitive processing of information (Fanselow and Wassum 2016).

1.1.3) Neural circuits involved in fear conditioning

The Amygdala is an evolutionary conserved structure which lies in the heart of the neural circuitry involved in fear conditioning (Swanson and Petrovich 1998). The amygdala can be divided in four nuclei: lateral nucleus (LA), basolateral nucleus (BLA), basomedial nucleus (BMA) and central nucleus (CeA) of amygdala (Goosens and Maren 2001). In particular, the basolateral nucleus (BLA) is defined the hub of the fear circuit (Fanselow and LeDoux 1999). Each of these nuclei receive distinct sensory inputs (figure 2) and each of the nucleus is involved in different functions. For instance, auditory inputs from geniculate nucleus of the thalamus terminate in the lateral nucleus of Amygdala whereas contextual information processed in the hippocampus terminate in the lateral and basal amygdala nuclei (Goosens and Maren 2001). This input segregation in the Amygdala highlight how different nuclei are involved in different conditioned responses (Killcross, Robbins et al. 1997). In particular, two main sub-circuits can be distinguished in the amygdala:

- The basolateral complex of amygdala, including lateral and basal nuclei, which represents the primary sensory interface of amygdala (Maren 2001). The lateral nucleus is also involved the expression of extinction of fear memory, mediated by medial prefrontal cortex (Maren and Quirk 2004).
- The central nucleus of amygdala, which receives inputs from both lateral and basal nuclei of amygdala. It modulates the behavioural and endocrine response related to fear and anxiety, due to its connections with brainstem, hypothalamus and basal forebrain

(Kalin, Shelton et al. 2004). Moreover, similarity to the lateral nucleus, it receive inputs from medial prefrontal cortex and is involved in modulating the expression of extinction of fear memory (Maren and Quirk 2004).

This segregation in stimuli processing is fundamental in regards to the investigation of conditioned behaviour since different conditioning protocol can involve different brain circuits. For example, it is known that rats with complete lesions of the central nucleus can still perform instrumental punishment conditioning, whereas showing impairment of the Pavlovian conditioned response (Killcross, Robbins et al. 1997). Furthermore, inactivation of BLA before animal training prevents the acquisition of the fear conditioned behaviour, suggesting the importance of this nuclei in fear memory acquisition (Goosens and Maren 2001). More complex is the role of the CeA, whose inactivation generates a deficit more in the expression of a learned fear response than in learning the association (Maren 2001, Maren and Quirk 2004). Finally, it is important to highlight that fear responses are driven by overlap and redundancy in the circuitry of amygdala circuitry (figure 3) (Goosens and Maren 2001). Finally, is clear from the literature that amygdala is necessary for acquisition and expression of learned fear memories in Pavlovian conditioning, but not for all form of aversive memory (Maren and Quirk 2004).

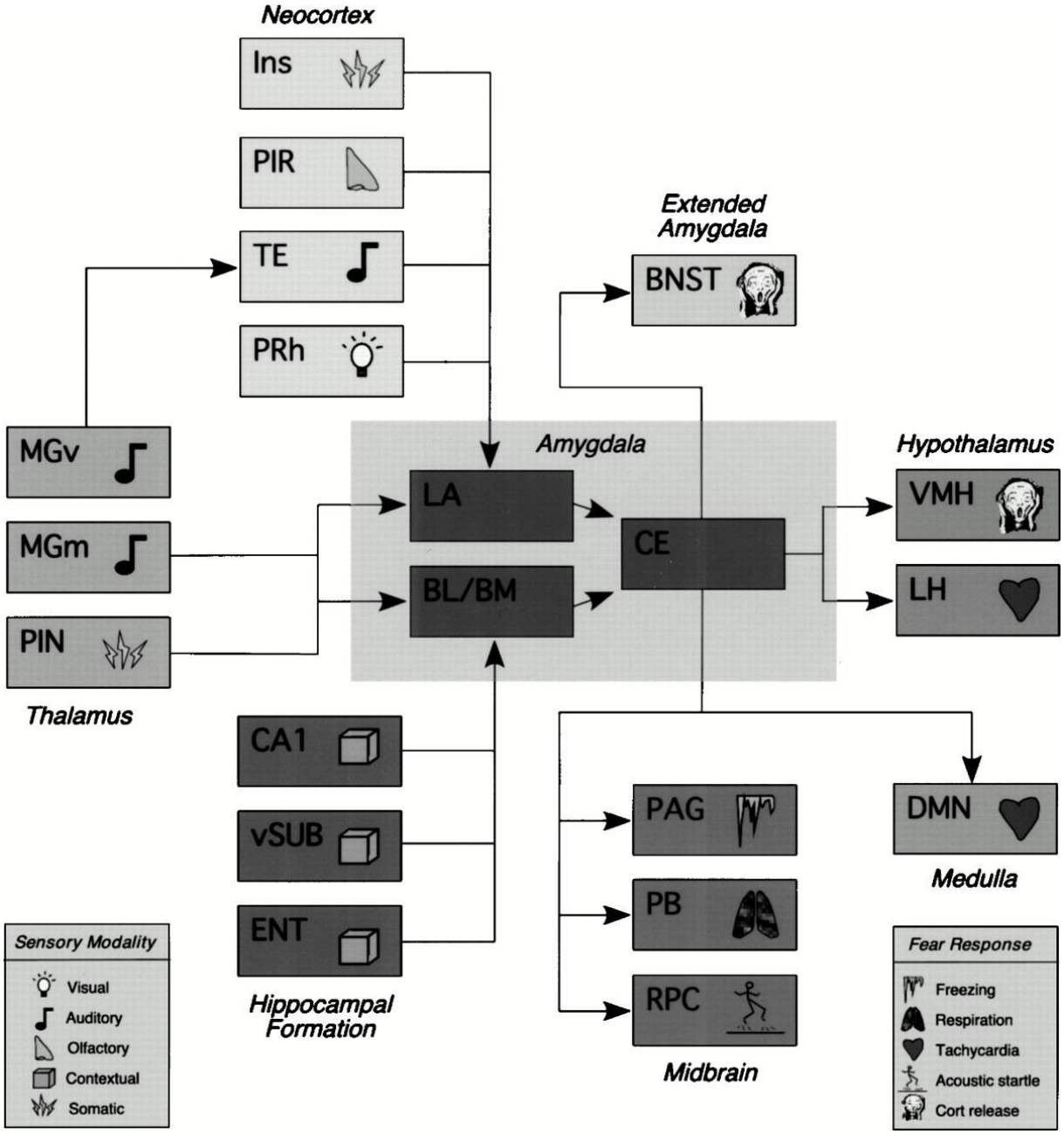


Figure 2) Schematic representation of fear conditioning circuits in the brain. The Basolateral Amygdala (BLA), including lateral amygdala (LA) and basal amygdala (BM/BL), receives and integrates sensory inputs from thalamus, hippocampal formation and neocortex. The central nucleus of amygdala (CE) is the main output nucleus and project mostly to midbrain and hypothalamus modulating the fear response of the organism. MGm/Mgv: medial geniculate nucleus medial/ventral; PIN: posterior intralaminar nucleus; vSUB: ventral subiculum; ENT: entorhinal cortex; INS: insular cortex; TE: primary auditory cortex; PRh: perirhinal cortex; PIR: piriform cortex; PAG: periaqueductal gray; RPC: nucleus reticularis pontis caudalis; LH: lateral hypothalamus; VMH: ventromedial hypothalamus; DMN: dorsal motor nucleus of the vagus; PB: parabrachial nucleus; PVN: paraventricular nucleus of the hypothalamus; BNST: bed nucleus of the stria terminalis.(Maren 2001)

1.1.4) Molecular mechanisms of learning and memory

The idea that learning and memory were embedded in the brain at a single cell level was proposed by Daniel Hebb in the book "Organization of behaviour" in 1949. The author proposed that when two neurons, connected via synapses, happens to be coactive, they increase the connection strength and the connection will stay stable in time. Hebb linked the process of synaptic plasticity with the neurobiology of learning and memory (Maren and Quirk 2004). This long-lasting increase in connections strength between two neurons is defined Long-Term-Potential (LTP), whereas the decrease in strength of these connections due to asynchrony is defined as Long-Term-Depression (LTD) (Ganguly and Poo 2013). With the introduction of electrophysiological recording in 1939 (Cole and Curtis 1939), it was finally possible to test the hypothesis of Hebb. The correlation between LTP in Amygdala and Fear conditioning was showed initially by injection of NMDA-receptors antagonist in the amygdala that impaired fear conditioned responses acquisition and amygdaloid LTP (Maren 1999); these result linked conditional fear response acquisition with LTP induced by NMDA-receptors (Maren 2001). Furthermore, investigation of the Ventral Angular Bundle (VAB), carrying projection from hippocampal formation to basolateral amygdala, linked LTP NMDA-mediated in the basolateral amygdala due to excitation from the hippocampal formation; in detail, this LTP has a critical role in processing of contextual stimuli during acquisition of conditional fear response (figure 3) (Maren and Fanselow 1995). On the other hand, the role of LTD is more blurred. The LTD may be involved in process as extinction, involving form of inhibitory learning (Maren 2001).

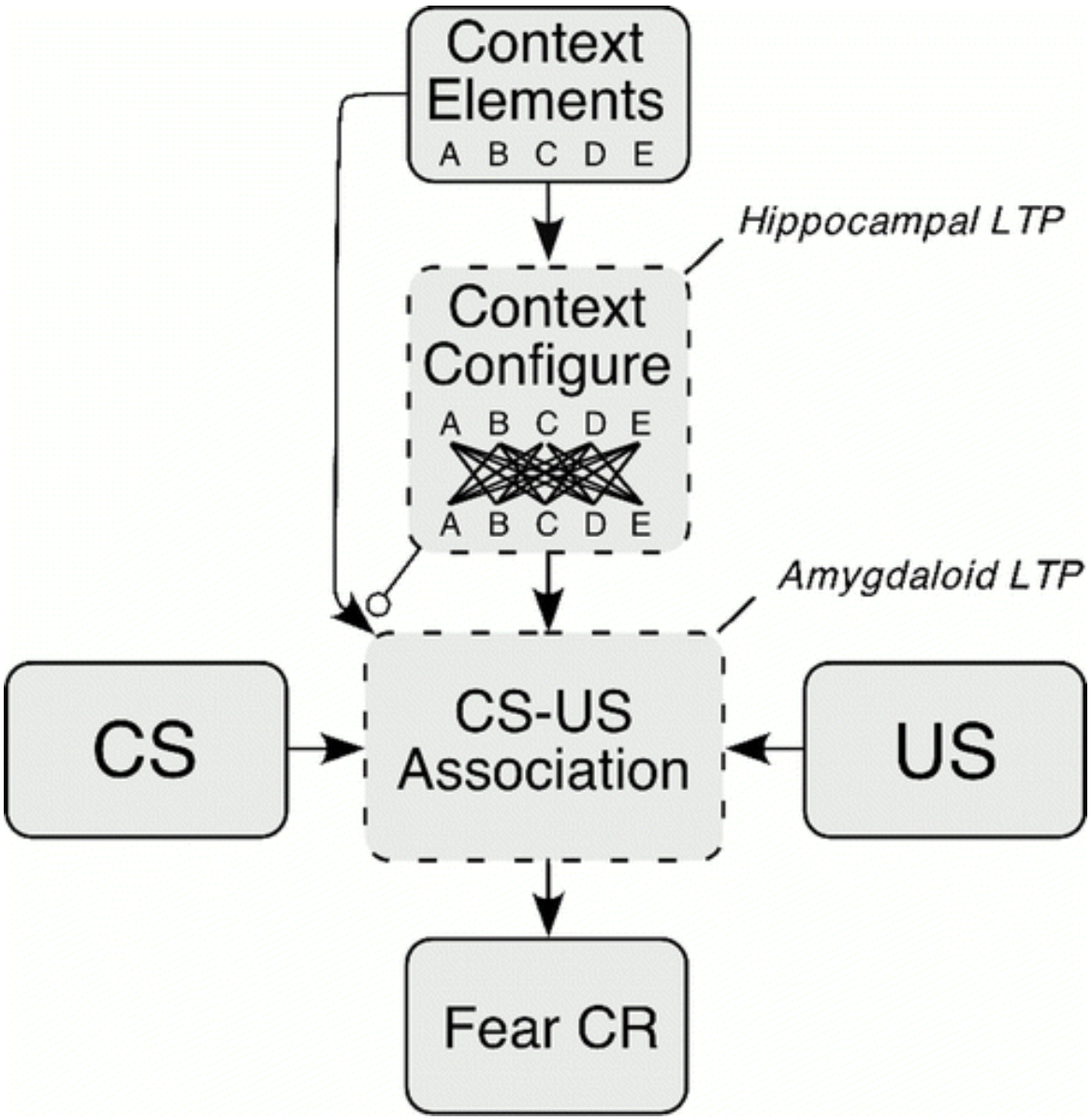


Figure 3) schematic representation of the role of hippocampal and amygdaloid LTP in a fear conditioning learning paradigm. The hippocampal signalling mediates the contextual representation of the stimuli and the hippocampal LTP is the mechanism underlying the association between context and the stimulus. Moreover, the association between conditioned and unconditioned stimulus (CS-US) is encoded in the basolateral amygdala and LTP, also in this case, is considered the mechanism driving this association between afferent primary sensory fibres and amygdaloid circuitry (Maren 2001).

1.1.5) Molecular mechanism of LTP and LTD

The first people to illustrate Long Term Potentiation (LTP) in synaptic connectivity between two neurons were by Bliss and Lømo, in 1973 (Bliss and Lomo 1973). LTP can be induced only if the post synaptic neurons fire within a time window of 20 ms since arise of the Excitatory Post Synaptic Potential (EPSP). LTD, on the other hand, can be induced by postsynaptic neuron firing in a time window of 20 ms before the onset of EPSP (Bi and Poo 1998).

The most commonly studied LTP and LTD are related with NMDA(N-methyl-D-aspartate) receptors in the hippocampus, due to anatomical simplicity and accessibility. In particular, NMDA receptors satisfy the requirements for LTP and LTD since they require pre-synaptic activity (glutamate release) and post-synaptic depolarization (Whitlock J. R. 2007). The activity of NMDA receptors is strictly correlated with the activity of AMPA receptors, since they drive the initial depolarization of the neuron necessary to activate NMDA receptors. It has been observed an increase in AMPA receptors at the post-synaptic terminals (driving a faster depolarization) following LTP, and a decreasing in the number of post-synaptic AMPA receptor following LTD (figure 4) (Whitlock J. R. 2007, Fleming and England 2010).

Is important to mention that LTP and LTD are not the only mechanism involved, a number of molecular mechanism, including neurotransmitters and intracellular signalling events contribute to this process (Johansen, Cain et al. 2011).

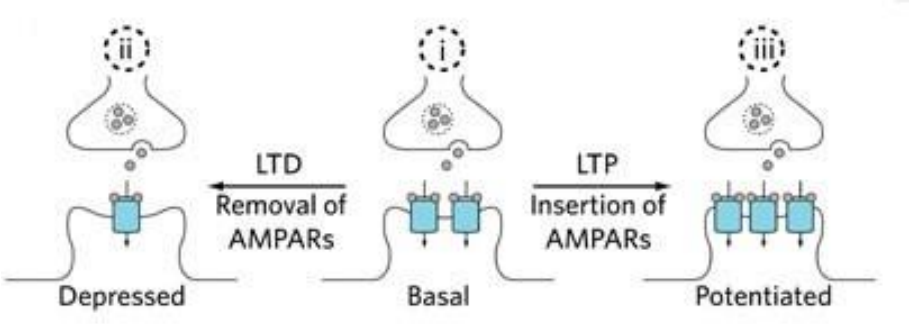


Figure 4) Graphic representation of the modulation of post-synaptic AMPA receptors due to Hebbian plasticity. LTP and LTD trigger the insertion and removal, respectively, of AMPA receptors at the postsynaptic terminals. In detail, LTD leads to a reduction in AMPA receptors at the post- synaptic membrane resulting in a slower onset of the Excitatory Post Synaptic Potential (EPSP). On the other hand, LTP induce an increase in post-synaptic AMPA receptors, facilitating the onset of EPSP (Fleming and England 2010).

1.1.6) Extinction of conditioned behaviour

Pairing conditioned and unconditioned stimulus leads to an association between the two stimuli. Nevertheless, when the conditioned stimulus is experienced alone the conditioned response decreases until it disappears (figure 5). This phenomenon is defined as extinction. Extinction of the fear memory involves the modulation of the amygdala circuitry, by prefrontal cortex and hippocampus inputs, resulting in a reduce associative plasticity in the lateral Amygdala (Maren and Quirk 2004). Furthermore, it is important to note that the association CS-US does not disappear since the conditioned response can be recalled (renewal) by new experience of the paired CS-US (Fanselow and Wassum 2016). During extinction, the association US-CS is not forgotten, but rather a new association between CS - "no US" is stored (Bouton 1993).

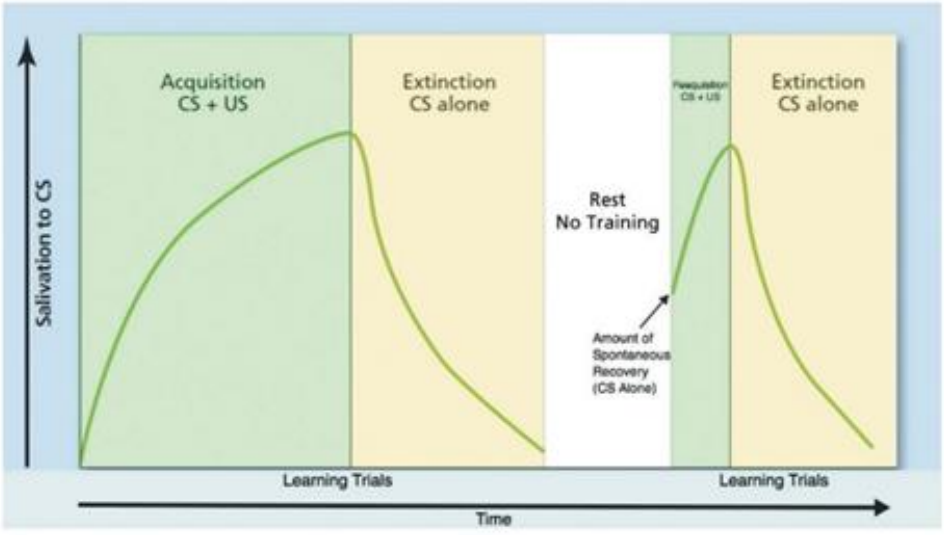


Figure 5) Conditioned response performance during learning trials. Conditioned and unconditioned stimuli are paired during the acquisition phase leading to a conditioned response in the animal. When the conditioned stimulus (CS) is not paired anymore with the unconditioned stimulus (US) there is decreasing in conditioned performance due to extinction. After a resting period it is clear how the association is recovered as soon as the CS and US are paired again; the increasing in performance is faster during the second acquisition period, showing that the association was not forget during the resting period (Ciccarelli 2008).

1.2) Zebrafish as a model

1.2.1) Zebrafish as a model for system neuroscience

"Models are used to represent complex problems in simplified forms" (Levin E.D. 2009)
In research there are three different model types that can be used:

- In vitro;
- In silico;
- In vivo;

Each of this models has advantages and disadvantages, mostly based on the investigation that is to be performed. For example, in silico models are suitable to simulate the dynamics of an artificial neural network. On the other hand, in vitro models are useful to study the effects of specific compounds on a cell culture. Finally, in vivo models are used to study the behavioural response of an animal model to a specific stimulus. As might be expected, the most common experimental model used in behavioural neuroscience is the *in vivo* animal models. Using animal models allows researchers to observe and study the link between neural processes and animal behaviour, which can then be associated with the functioning of human brain (Levin E.D. 2009).

Even though rodents and primates has been the most successfully used animal models in neuroscience research, zebrafish has become increasingly popular during the last 20 years (Levin E.D. 2009, Garcia, Noyes et al. 2016). Zebrafish is a powerful animal model for the investigation of neural circuits function. Due to its transparent at larval stages that allows single and multi-photon imaging and also allows a wide range of genetic approach to study the effect on behaviour of silenced/activated area of the brain (Friedrich, Jacobson et al. 2010). Furthermore, in 2013 the whole genome of zebrafish has been sequenced, supporting the high degree of similarity that zebrafish share with humans (Bellipanni, Cappello et al. 2016).
Zebrafish have also practical advantages over mammalians models. First of all, the maintenance costs are significantly lower than those for mammalians models. They fertilize externally and producing an average clutch of 100-200 eggs per day, easy to collect and raise which makes them easy to breed. The generation time of zebrafish is comparable to the one of mouse and longer than the one of invertebrate as drosophila (Yoshihara 2009).

In addition to all these major building plan "the bauplan" of the vertebrate brain is well preserved also in zebrafish. This important evolutionary conservation of brain structures allows neuroscientists to link their findings across vertebrates. There are many similarities between mammalians and zebrafish apart for some differences in development. The major difference

being the developmental process governing the formation of the ventricles (figure 6). In mammals, brain ventricles arise after evagination in forebrain development, whereas in zebrafish they arise from eversion process in forebrain development (Butler A.B. 2005). Importantly, the major areas of vertebrate brain that are involved in learning, are also well preserved in zebrafish such as the dorso-medial telencephalon-Dm (basolateral amygdala homologue) and zebrafish dorso-lateral telencephalon-Dl (hippocampus homologue).

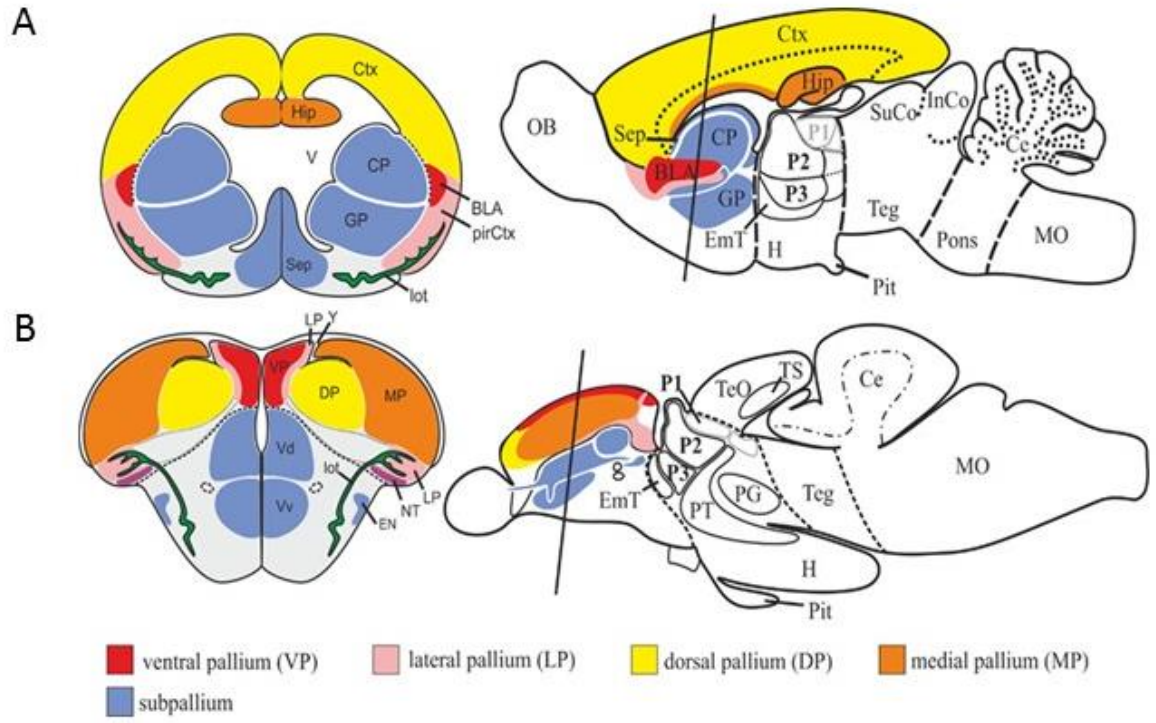


Figure 6) Schematic topological correspondence between rodent (A) and cyprinid (zebrafish/goldfish)(B). in rodent brain the ventricles are centrally located and surrounded by two hemispheres while in zebrafish the ventricles, with a T-shape, dorsally covers the two lobes. Topological correspondences are: MP: mammalian hippocampus; DP: mammalian isocortex; VP: mammalian basolateral amygdala; VL: piriform cortex. (Mueller 2012)

1.2.2) Zebrafish as a model for learning and memory

In the last 15-20 years zebrafish become increasingly popular for behavioural studies. These studies have demonstrated that zebrafish has sophisticated sensory and motor systems that allow them to perform sensory-motor tasks, such as opto-motor response (Orger, Smear et al. 2000, Kubo, Hablitzel et al. 2014, Portugues, Haesemeyer et al. 2015), opto-kinetic response (Neuhauss 2003, Fleisch and Neuhauss 2006, Huang and Neuhauss 2008, Mueller and Neuhauss 2010), olfactory behaviours (Braubach, Wood et al. 2009, Koide, Miyasaka et al. 2009, Morin, de Souza Silva et al. 2013)), acoustic startle response (Burgess and Granato 2007, Burgess, Johnson et al. 2009, Lacoste, Schoppik et al. 2015). Moreover, adult zebrafish have been used to investigate more complex behaviours such as associative learning (Sison and Gerlai 2010, Roberts, Bill et al. 2013, Blaser and Vira 2014, Aoki, Tsuboi et al. 2015), spatial learning task (Williams, White et al. 2002, Karnik and Gerlai 2012, Naderi, Jamwal et al. 2016) and active avoidance conditioning (Pradel, Schachner et al. 1999, Xu, Scott-Scheiern et al. 2007, Aoki, Kinoshita et al. 2013). Nevertheless, zebrafish researchers are still developing behavioural protocol to investigate neural circuit underlying processes such as learning and memory (Blaser and Vira 2014).

Studies on goldfish have used spatial learning task (Broglio, Rodriguez et al. 2010) and fear conditioning (Portavella, Torres et al. 2004), discovering an important role of the lateral telencephalic pallia (LP) in emotional memory and the engagement of medial telencephalic pallia (MP) in spatial or temporal memory. These structures resemble the roles of Amygdala and Hippocampus, respectively, in the human brain. This can be supported by a recent study on zebrafish (von Trotha, Vernier et al. 2014) where the author used both a drug addiction protocol and a light avoidance paradigm to investigate the role of dorsolateral (Dl) and dorsomedial (Dm) telencephalon of zebrafish. The results have shown that during acute drug injections, both Dm and Dl are activated (resemble the drug-active structures, Hippocampus-amygdala, in humans). However, during drug-conditioned motivational behaviour only Dm is involved, resembling the role of humans' basolateral amygdala (BLA) in motivated behaviour. It is important to mention that fear conditioned response, both in zebrafish and mammals, is sensitive to NMDA-receptor agonist, linking the memory phenomenon to the LTP at a single cell level (Blank, Guerim et al. 2009).

Furthermore, in the last few years researchers have highlighted the crucial role of the lateral Habenula during avoidance learning in zebrafish. It has been shown how larval zebrafish learn to avoid a light cue, paired with an electric shook during conditioning trials. This behaviour fails in larval zebrafish with the disruption of circuitry involving Habenula (Lee,

Mathuru et al. 2010). Habenula is a highly conserved structure in evolution, connecting nuclei in the telencephalon to the brain stem nuclei, as interpeduncular nucleus, raphe nuclei or ventral tegmental area (Okamoto, Agetsuma et al. 2012). By analysing the neural connectivity and the molecular characteristics of the habenula's neurons, Amo (Amo, Aizawa et al. 2010) has identified the dorsal and ventral habenula in zebrafish as the homologous of the mammalian medial and lateral habenula. Moreover, lesion of lateral Habenula has been analysed in Pavlovian conditioning paradigm resulting in a freezing response in lesioned-fish, whereas non lesioned-fish response consists in swimming away from the cue after a sufficient training (Mathuru and Jesuthasan 2013).

In particular, in zebrafish the pathway between dorsal habenula, equivalent of medial part of mammalian habenula, and the interpeduncular nucleus has been shown to be important in modulation of the fear responses due to experience (Agetsuma, Aizawa et al. 2010).

Ventral Habenula has been shown to have connections with serotonergic neurons in the medial raphe nuclei, where inactivation of this pathway impaired active avoidance conditioning, whereas fear response induced by classical fear conditioning is not affected (Amo, Fredes et al. 2014). Furthermore, acquisition and extinction of conditioned behaviour is mediated also by neurons in the cerebellum (Aizenberg and Schuman 2011), showing how complex learning and memory processes are.

Most of the attention in the past few years has been focused especially on adult zebrafish behaviour (Amo, Fredes et al. 2014). Recently, it was proposed that juvenile zebrafish at three weeks can indeed learn by classical and operant conditioning (Valente, Huang et al. 2012). These results are a major breakthrough, since at this juvenile stage zebrafish are still transparent and it is therefore possible to measure their brain activity relatively easily. However, no follow up studies have highlighted the feasibility and robustness of this approach.

The aim of this master thesis is to implement a behavioural setup to perform and optimize a learning assay based on active avoidance conditioning in larvae – juvenile zebrafish. Moreover, I decided to focus on one to three weeks old zebrafish to investigate more in detail the ontogeny of operant learning behaviour.

Chapter 2

Materials and Methods

2.1) Behavioural tracking system

2.1.1) Programming language

To made a weighted and accurate choice of which programming language was the most efficient to implement the software, four requirements were used to guide the selection:

- *Has to be an open source language;*

This was a fundamental hallmark for the candidate language that allow me to download and edit the source code of the libraries that I will use to implement the software.

- *Has to be standardized;*

This property is necessary to be able to use libraries of functions implemented by other developers and will allow upgrading of the software.

- *Has to be cross platform compatible;*

To not be limited in using only a specific platform the candidate language has to be cross-platform compatible; we have the possibility then to move the software across platform (windows, iOS, Linux, etc.) without being limited by the compatibility of the language used.

- *Has to be a compiled language, not an interpreted language;*

For my application it is really important that the processing of all the information is as fast as possible to allow the software to process information in real-time. Therefore, I chose a compiled language, who directly translate the code into machine native language by a compiler without step through an interpreter program that slow down consistently the execution of the code.
Keeping in account all these properties the chosen programming language was C++.

C++ programming language

C++ is based on Object Oriented Programming (OOP) paradigm and it represent the object-oriented version of the C language, a structured and procedural programming paradigm. The main feature of Object-oriented Programming paradigms is the presence of entities called “objects” that models object of the real word and that own proper attributes and are accessible by “methods”. This paradigm allows the code to be flexible and reusable, generating different blocks of code (“objects”) that can be processed and combined (by methods and functions) differently to achieve the purpose of the software without redundancy in the code. Moreover,

using this programming paradigm allows the programmer to abstract the code to a high-level language, feature, this one, that will allow future users of the software to understand easily the code, modify it and reuse it without an extensive knowledge of programming theory.
Many are the advantages given by C++ language:

- It is a ISO-standardized language: ISO/IEC JTC1 SC22 WG21 N 3092;
- It is a compiled language;
- It has a wide open source library support;
- Cross-Platform compatible:

  since C++ is the most frequently used programming language in the world, it has a wide range of compilers that run on different platforms;
- It is upwards compatible with C;

  This property allows the programmer to switch between C++ and C language and gives the possibility to choose between an Object-Oriented programming paradigm (C++) or a procedural parading (C). With this degree of freedom, the programmer is able to optimize the code based on the function he is implementing; for instance, all the computational effort can be optimize using the C code, since it allows a higher level of control over the resource allocation for computation, while it is more efficient to implement and control the Graphic User Interface (GUI) using the C++ language.

2.1.2) Tracking algorithm

To implement this algorithm, I used the OpenCV 3.0 library, an open source computer vision and machine learning software library (http://opencv.org accessed the 03/09/2015). This library is C++ compatible and is really useful for video processing and analysis: it implements many function for face detection, motion tracking or background subtraction that I have used to optimize and implement my tracking algorithm. Particularly, one OpenCV function was relevant for the implementation of the tracking algorithm: cv::createBackgroundSubstractorMOG2 (KaewTraKulPong and Bowden 2002, Zivkovic 2004). This function creates an object that processes each single frame giving as an output the foreground detected. This object takes as inputs two parameters: the number of frames taken into account as history and the threshold value that define how fast a pixel become background in case of constant value along consecutive frames. (KaewTraKulPong and Bowden 2002, Zivkovic 2004). A reliable estimation of these parameters was done by Lech M. et al. (2014) in the paper “Examining Quality of Hand Segmentation Based on Gaussian Mixture Models”:

they conclude that the precision of the methods can be improved by the number of the frames taken into account for the history of the image up to 300 frames (Lech, Dalka et al. 2014). Consequently, I decide to set the history´s frames to 300. Regarding the second parameter required for the function I decide to use the default value (10) since the luminosity of the background in the image is very stable and there was no significance improvement by changing it.

2.1.3) Arduino Due

A microcontroller was used to control stimulus delivery in real-time. Microcontrollers are small programmable devices, which operate exactly like a computer does. Microcontroller boards are equipped with a processor, a memory space of the order of KB, some ports to communicate with other devices (usually USB ports) and, last but not least, they can be equipped with many different sensors for input and output signals. I select the Arduino DUE board (figure 7) to implement and control the reward delivery system in my setup.

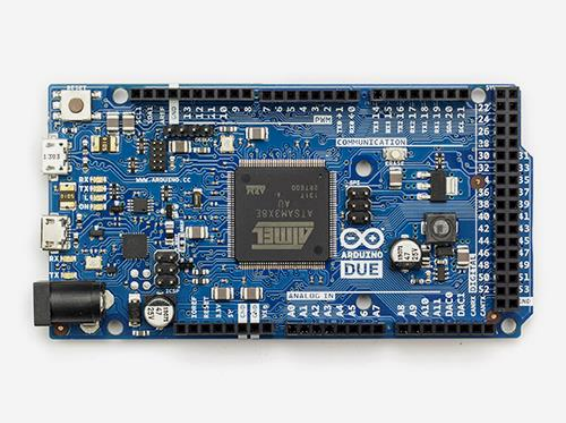


Figure 7) Arduino Due board (https://www.arduino.cc)

Arduino "is an open-source prototyping platform based on easy-to-use hardware and software" (https://www.arduino.cc accessed 24/08/2015). In particular, Arduino DUE is a prototyping board mounted a 32-bit ARM microcontroller equipped with 54 input\output digital pins, 12 analogic input pins and 2 DAC (Digital-to-Analog Converter) input\output pins. The advantages of using an Arduino board are the following:

-The price of each single board is very low compared to other microcontrollers, less than 50$.

-Is cross-platform compatible, while most microcontroller are limited to Windows.

-Simple and open source programming environment.

Using the Arduino Due board, allowed me to control the delivery of stimulus to the fish just communicating to the board via USB simple commands which trigger precompiled functions uploaded in the microcontroller processor.

2.1.4) Shock delivery system

To administrate an aversive stimulus to the fish, during the conditioning sessions, I built a simple circuit for each individual fish that receives a digital input (3,3V) from the Arduino and applies 18 V to each fish separately. Each single circuit is made by an integrated circuit, an Operational-Amplifier (Opamp), connected in a non-inverted amplified configuration (figure 8). The Voltage at the output is given by the relation:

$$\text{Voltage output} = \text{Voltage input}\ (1 + \frac{Resistance\ 2}{Resistance\ 1})$$

A gain factor of 10 was used and the output Voltage was modulated by reference voltage of the Opamp. This allowed me to vary the applied voltage flexibly. The Opamp used are TLE2142 EXCALIBUR LOW-NOISE HIGH-SPEED PRECISION OPERATIONAL AMPLIFIERS from Texas Instrument. (append datasheet in the appendix). All the circuits are mounted on a prototyping board.

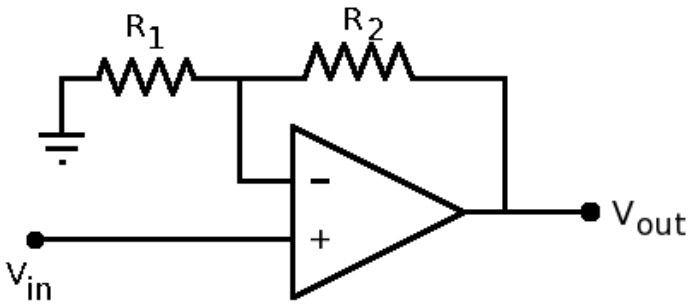


Figure 8) Non-inverting amplifier Opamp circuit.

2.1.5) Visual stimuli presentation

Visual stimulus is presented to the fish using a horizontally positioned LCD monitor. The LCD monitor give the flexibility of changing the visual stimulus, control its timing and synchronize with other electronic systems, which can be useful features for the future development of the setup. In this project, the stimulus was presented form the bottom of the fish tank. The stimulus I used was a simple pattern of a divided arena half red, half dark grey (Figure 9). A red zone, used as aversive zone during the conditioning of the fish, and a grey zone, representing the “safe zone”.

Figure 9) Pattern presented at the bottom of each single fish arena.

2.1.6) Camera used for imaging

The high resolution camera used to perform the recordings is a Manta 235B. This camera was relatively cheap (around 800 €), high resolution (1920x1280) and can flexibly record high frame rates (up to 100 Hz)

2.2) Behavioural arena parameters

2.2.1) Arena

As arena I decided to use Gosselin square Petri dishes 120mm x 120mm x 15.8 mm. the edges of each arena was covered with red electric tape to avoid social interaction with neighbour fishes.

2.2.2) Electrodes

Tungsten wire electrodes were used to administrate a mild electric shock to the fish. The inert tungsten was more effective then silver electrodes which showed toxic effects in water. However, tungsten wire showed a fast decay in conductivity due to the accumulation of ions over one of the electrodes, generating a coating layer. That phenomenon increases the resistance of the circuitry and decrease the current flow, as described by the Ohm law:

Voltage=(Resistance) x (current Intensity)

The time scale of this phenomenon is of the order of few seconds under constant voltage gradient. To avoid this phenomenon, I provide to the fish a current pulse of 10 ms of duration, delivered with a frequency of 1.33 Hz. Practically, every 740 ms a current pulse with an amplitude of 1.2 mA and 10ms of duration is delivered (figure 10). This wave shape of current allows the electrode not to be oxidised.

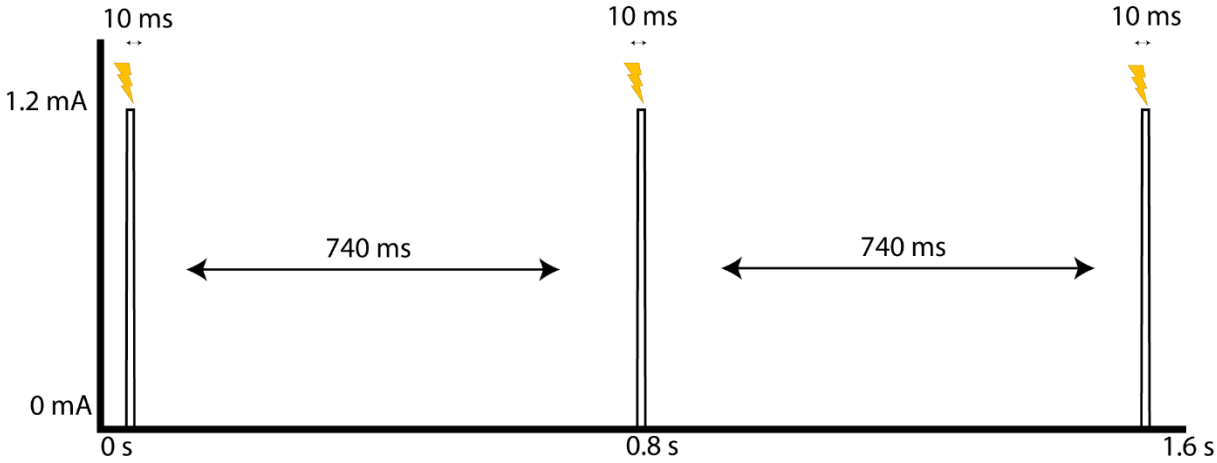


Figure 10) Graphic representation of the aversive stimulus delivered to the fish during the conditioning sessions. Each current pulse has a duration of 10 ms and an amplitude of 1.2 mA. A pulse of current is delivered every 740 ms resulting in a delivery frequency of 1.33 Hz. The aversive stimulus is administrated to the fish only when is located inside the red compartment of the arena used as conditional stimulus.

2.2.3) Water

I used artificial fish water prepared with 1.2d of marine salt in 20l of H2O purified with Reverse Osmosis technology (RO Water). The ideal temperature for zebrafish is around 27 °C so I fill the arena every morning with new RO water at the temperature of 28°. The LCD screen used for visual stimuli presentation also was warm enough to keep the water temperature not lower than 26 °C. The temperature was measured at the end of the day to check if the water was in the physiological range for the fishes and it was always in the range of 26 °C.

2.3) Experimental animals

Experiments were performed with wild type zebrafish (Danio rerio). Zebrafish eggs were produced by natural spawning collected the morning after fertilization before 12:00. To ensure optimal conditions, larvae were kept in petri dishes containing egg water with renewal of 75% of the water on a daily basis, in an incubator at 28.0°C. After 4dpf, the fish were transferred to a 3L tank in a Tecniplast ZebTec Multilinking System, under the following conditions: 28.0°C, 700mSiemens, pH 7.0. They were maintained at a 14:10 hour light/dark cycle and received a normal diet of dry feed (SDS100 up to 7dpf and SDS 200 up to 1 month, Tecnilab BMI, the Netherlands) two times a day and one droplet of Artemia nauplii (Grade 0, Platinum Label, Argent Laboratories, Redmond, USA) from a disposable pipette once a day. The fish were transported from the fish facility to the behavioural setup in the morning between 10:00 and 11:00, placed directly in the arena and the experiment was started immediately.

The fish were grouped based on their age; three groups have been defined:

- one week old fish (5-10 days): 41 fish.
- two weeks old fish (11-17 days): 30 fish.
- three weeks old fish (18-24 days): 42 fish.

To assign a fish to one of the groups, I used a tolerance of three days: a fish was included in one of the groups if the age was at most 3 days different from the group age.

2.4) Behavioural protocol

All the experiments were conducted during daytime, between 09:00 and 20:00, in a room isolated from the rest of the lab. There was no external illumination except the light coming from the LCD screen below the arenas. The setup allows training of six fishes in parallel in six different arenas. The fishes were transported from the fish facility to the room were the experiments are performed using six different flacon tubes; this transportation was always done after the morning food delivery to the fish, as programmed by the feeding routine in the fish facility.

Training protocol:

Once the fishes were placed in the arenas, the training protocol could start and it consist of:

1) 3h of baseline recording;

   I decided to record so long baseline for two reasons:

   - To give enough time to the fish to habituate to a new environment, to the visual pattern presented on the bottom of the arena and to the visual presence of the two electrodes;
   - To analyse how the behaviour of the fish change in relation to the time spent in the arena. This protocol also provide me sufficient baseline data to compute statistical probabilities of fish behaviour, while defining the learning rules

2) 30 minutes of conditioning period;

   The fish receive a mild electric shock when located in the red zone of the arena. The shock duration is 10 milliseconds and is repeated at 1.33 Hz as long as the fish stay in the red zone. The voltage applied across the electrode is 16 V, and the current distributed over the entire zone, around 72 cm^2, is 1.4 mA.

3) 30 minutes of testing period;

   The fish doesn´t receive an aversive stimulus when they enter the red zone of the arena. The visual pattern is still shown to the fish. 30 minutes is a time where short term memory can be tested.

4) 30 minutes of a second conditioning period;

   This period of conditioning is performed to reinforce the learning in the fish.

5) 30 minutes of a second testing (reinforcement) period; The fish doesn´t receive an aversive stimulus when they enter the red zone of the arena. The visual pattern is still shown to the fish. 30 minutes is a time where reinforcement can be tested.

6) 45 minutes of testing extinction of aversive response;

   This testing period is performed to analyse how long this memory is retained by the fish. This period of recording test the performance until one hour and a quarter after the last conditioning period of the fish.

7) 30 minutes of testing period mirroring the pattern presented to the fish;

   This last recording is performed to analyse if the negative reward administrate to the fish is associated with the specific half of the arena or if it is associated to the red colour presented in the conditioned zone of the arena.

After the complete protocol get executed the fishes are sacrificed by overdose of anaesthesia by MS222.

Figure 11 shows a graphic representation of the behavioural protocol described above.

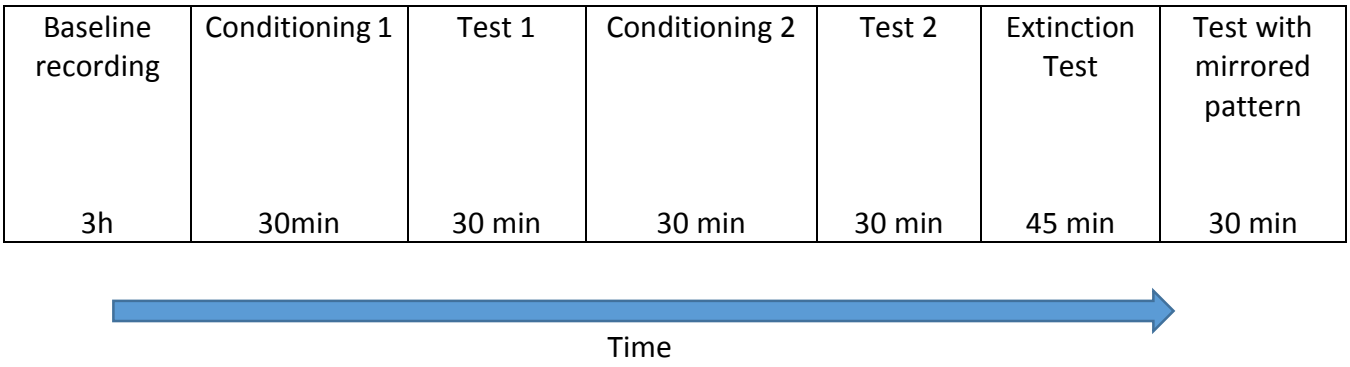


Figure 11) Graphic representation of the training and test sessions performed in each single protocol.

2.5) Associated analysis

The analysis of the data collected were performed using a custom written code in the development environment of Matlab R2014b.

2.5.1) Exploratory behaviour

For each single fish the physical boundary between conditioned and unconditioned part of the arena was manually selected by the user, before the experiment started. This value is used in the analysis to have a more precise evaluation of the fish performance.

To quantify the discrete probability distribution of the exploratory behaviour of each fish the arena was divided in 792 sectors (27x27). For each of them was quantified the number of frame in which the fish was present in the sector, normalized by the total amount of frame recorded.

2.5.2) Thigmotaxis index

To calculate the Thigmotaxis index the arena has been divided equally in two zones: one defined as centre zone and one defined as periphery. Both of them cover 50% of the surface of the arena (figure 12).

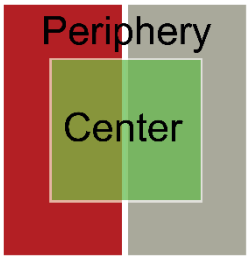


Figure 12) Scheme for the zone of the training arena for centre and periphery zones used for the quantification of thigmotaxis.

2.5.3) Learning curve

The sequence of sessions taken into account to define a learning curve: conditioning 1, test 1, conditioning 2, test 2.
Each of this session has been divided, non-linearly, in five time windows: minute 1, 2 to 5 minutes, 5 to 10 minutes, 10 to 20 minutes and 20 to 30 minutes. The learning curve is defined as the median of the learning index values for each of these time windows in each session.

2.5.4) Size quantification of the fish

To estimate the size of the fish the video recorded in each experiment has been analysed at the end of all the experiments using a custom made algorithm in Matlab R2014b. The function used was vision.ForegroundDetector; a built-in function of Matlab.

2.5.5) Learning index

The learning index has been defined as follow:

$$\frac{(\,\%\text{ time in red during baseline } -\ \%\text{ time in red in session alanysed})}{(\,\%\text{ time in red during baseline})}$$

$$\%\text{ time in red during baseline} = -\frac{(\text{ time in red during baseline session })}{(\text{ total time of baseline session})}$$

$$\%\text{ time in red during session} = -\frac{(\text{ time in red during the analysed session })}{(\text{ total time of analysed session})}$$

The learning index smaller than -1 has been grouped and set to -1 since to make the index symmetric between 1 (perfect learners) and -1 (increase preference toward the conditioned compartment). 0 value represent no changes in preference.

To evaluate the performance during the switched pattern session the learning index has been modified ad calculated as follow:

$$-\frac{(\%\ \text{time in red during baseline} - \%\ \text{time in the half arena paired with the CS})}{(\%\ \text{time in red during baseline})}$$

This index calculates the aversive response of the fish toward the compartment of the arena coupled with the administration of the aversive stimulus. This index qualitatively represents if the fish avoid the space location coupled with the aversive stimulus (value below 0) or if the aversive response is coupled with the red pattern coupled with the aversive stimulus (value above 0). Threshold value of the learning index to distinguish between learner and non-learners fish is 0.3 and is the same in both cases presented above.

2.5.6) Statistical analysis

The statistical analysis was performed using built-in function of Matlab R2014b:

-Ranksum: perform a two-side Wilcoxon rank sum test; a nonparametric test for two populations when samples are independent. The test compares the medians of the two populations.

-Signrank: perform a two-side Wilcoxon signed rank test; a nonparametric test for two populations when the observations are paired. Briefly the statistic in this test is performed on the median of the difference between the two population.

The ranksum test was used to quantify a statistical difference between two different population of fish: among the three different groups of zebrafish analysed in this study.
The signrank test was used to investigate significant differences intra-group since it is a paired test and keep into account the evolution of a parameter for each single fish.

Chapter 3

Results

3.1) Development of software and hardware for studying learning behaviour in juvenile zebrafish

3.1.1) Software implemented

3.1.1.1) Programming language

The languages taken into account to implement the software were Python, C++ and Matlab. Among these languages I selected C++ since it offers the best compromise between level of abstraction of the syntax and efficiency of the code. Indeed, Python offers a very user friendly syntax, but it is an interpreted language which increases the time of execution of the code; Matlab has a broad offer of library and tools already implemented in the software but has a relatively slow acquisition frequency for USB signals which makes this language not suitable for my application.

3.1.1.2) Algorithm for real time tracking of zebrafish behaviour

The first idea taken into account was to implement a background-subtraction algorithm to subtract from every frame a constant background defined in the beginning of the recording. Unfortunately, there are some limitations with this approach:

-Very sensitive to external noise in the image due to illumination flickering or small drift between the camera and the arena.

-The background subtracted is fixed and calculated in the beginning of the recording. This approach doesn´t allow changes of the pattern presented or correction of errors, as can be a drift of the field of view of the camera, that can happen during the recording.

To overcome these limitations, I used a class of algorithm called “Adaptive Background Gaussian Mixture Model for foreground segmentation” (KaewTraKulPong and Bowden 2002, Power and Schoonees 2002, Zivkovic 2004, Lech, Dalka et al. 2014).
The principle behind this algorithm is the following: the intensity value of each pixel in the image is modelled by an adaptive parametric mixture model of N, typically three or five, Gaussian distribution, as shown in figure 1. (KaewTraKulPong and Bowden 2002, Power and

Schoonees 2002)The image has to be modelled at least by three Gaussian distributions because the algorithm models at any time point both background, with two Gaussian in this case, and foreground, with one (KaewTraKulPong and Bowden 2002, Power and Schoonees 2002, Zivkovic 2004). Once the image has been modelled the posterior probability is estimated, to define the current state of the pixel: background or foreground. (Power and Schoonees 2002)The likelihood, defined as posterior probability, that the current pixel arises from one of the mixtures is calculated from the Bayes´s theorem (Power and Schoonees 2002). Once the current state of the pixel is estimated, to decide if it represents background or foreground it is compared with each single Gaussian distribution defined before and if it doesn't match any of this distribution it is classified as foreground, otherwise it is classified as background (KaewTraKulPong and Bowden 2002, Power and Schoonees 2002, Zivkovic 2004).

The implementation of this algorithm is described more in detail in the methods. In summary, the foreground is detected in the analysed image, it is eroded to filter out all the noisy pixel detected as foreground and the coordinates of the area of the fish detected are extracted (figure 13). As a control two criteria has to be satisfied by the area detected:

- The size of the detected zebrafish has to be between a physiological range for the developmental stage of the analysed fish.
- The distance between zebrafish detected in two consecutive frame has to be smaller than a threshold defined by the physiological maximum velocity for the zebrafish taken into account.

The result of this new tracking algorithm has proven to be very stable and reliable even in conditions of weak contrast between the fish and the surrounding environment as clearly shown by figure 1. Moreover, the acquisition frequency of 15 fps allows me to accurately track the fish in time and detect the smallest movements. An example two minutes' pathway for one juvenile is shown in figure 14.

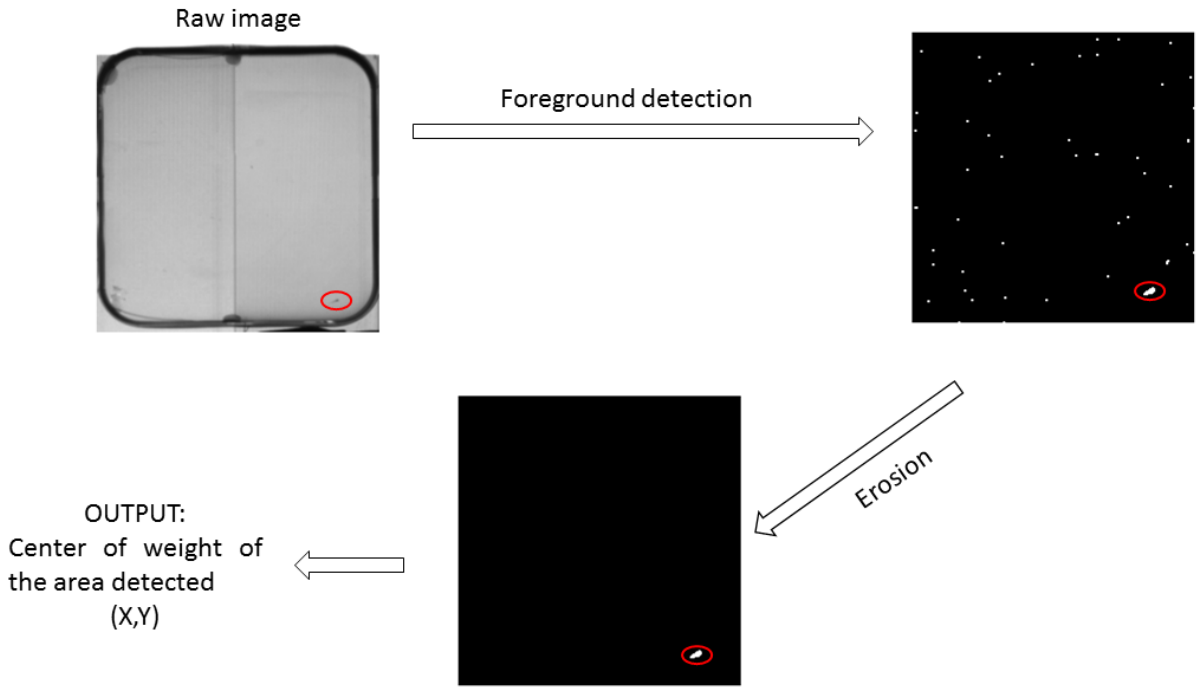


Figure 13) Example processing of a raw image: the foreground is detected by the tracking algorithm and is eroded to reduce the noise due to flickering in the intensity of background pixels. The coordinates of the biggest area detected, which match the size range defined by the user, are extracted and processed by the software.

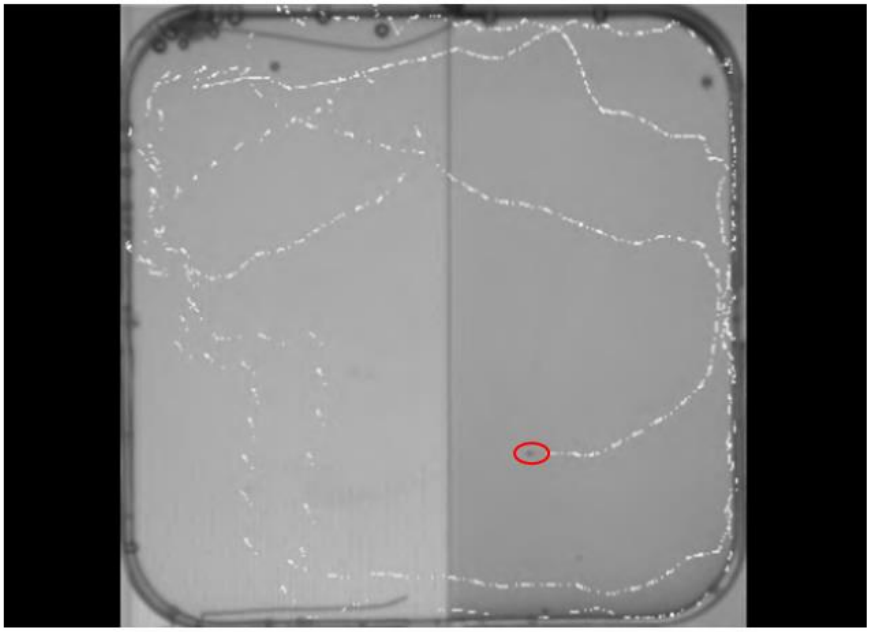


Figure 14) Example of two minutes raw trace superimposed to the raw image detected by the camera at the end of the two minutes. Dashed white lines shows the detected positions of fish during 2 min, recorded ad 15 frames per second. Note that tacking algorithm can handle low contrast as well as air bubbles in the behavioural arena.

3.1.1.3) Software architecture

The software was designed on a multi-thread base. A thread is a sequence of instructions in the code of an application that can be executed in parallel and independently from other portions of the code. Each process that runs into the application is handled independently in a different thread and all the thread are connected to process the information in parallel. With this approach I´ve designed a software that is able to process independently the recording and training of six different fish simultaneously. The advantages of this approach is that instead of processing one big image where all the fish were recorded, six smaller images are processed simultaneously by different thread; this approach reduce significantly the execution time of the code and hence increase the recording/tracking frequency. Moreover, since six fish can be trained at the same time, this approach six fold reduces the time that is necessary to train a certain number of fish. Last but not least, in this approach all six fish are exposed to the same environmental condition so the result will have a higher reliability than if each single fish was trained independently. In summary, the user can select how many fish to train in each single experiment, from one to six. For each fish the user has to define a specific region of interest, corresponding with the image of the specific arena in image, and each of this region of interest is processed simultaneously but independently from the others (figure 16).

3.1.1.4) Software user interface

The software user interface has been developed with a modular architecture (figure 15). There is a bottom buttons line that allows the user to select the module of interest that will be shown in a column on the left of the camera image. I have chosen this approach to integrate my software for tracking and conditioning zebrafish with a previous software used in the Yaksi-lab mainly for controlling CMOS camera and synchronizing stimulus administration with signal recording, via an Arduino.

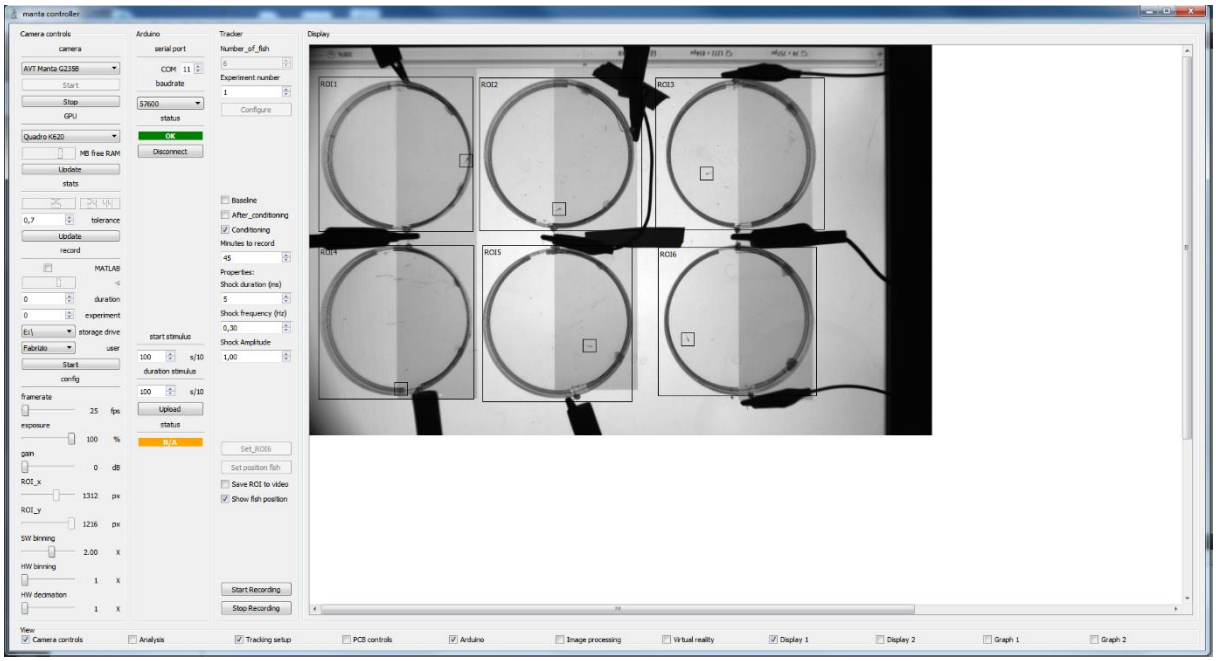


Figure 15) Software interface during conditioning of six zebrafish in a round shape arena. The six region of interest (ROI) are highlighted on the live image detected from the camera and the fish position is on-line plotted as a square box over the fish.

3.1.1.5) Software output data file

The software has been programmed to save a ".txt" file with all the settings of the camera tuned by the user, information of a performed protocol (baseline recording, conditioning, time recorded, stimulus parameters, etc.), the ROI dimension (width x height) and at every iteration over a frame the software will append to this text file a string containing: time passed from the last position saved, x and y coordinate of the fish detected and as well as the training parameters such as the amplitude/duration of aversive stimulus and whether it has been administrate to the fish or not. This rather small sized (in the order of 1 MB) ".txt" file will be used for off-line processing of the fish behaviour.

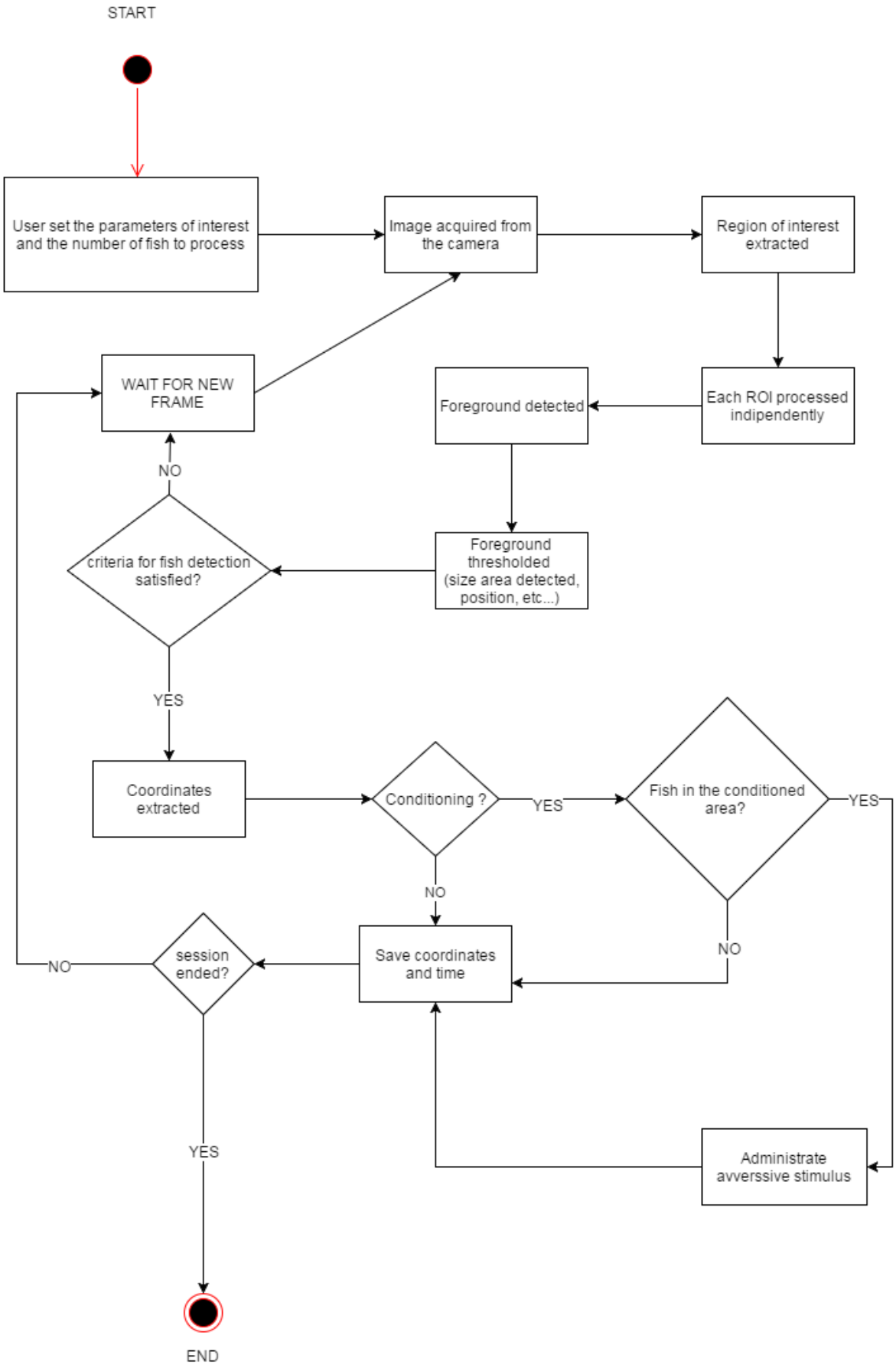

START
User set the parameters of interest and the number of fish to process
Image acquired from the camera
Region of interest extracted
Each ROI processed indipendently
Foreground detected
Foreground thresholded (size area detected, position, etc...)
criteria for fish detection satisfied?
NO
WAIT FOR NEW FRAME
YES
Coordinates extracted
Conditioning ?
YES
Fish in the conditioned area?
YES
NO
Save coordinates and time
NO
session ended?
NO
YES
Administrate avverssive stimulus
END

Figure 16) Flow chart diagram describing the information flow in the software for one single region of interest. All the diamond boxes represent conditional statement and based on the verifying of a specific condition the output can be different. As shown in the flow chart the user has to selects the number of fish to process and their region of interest, the protocol to perform ad set up all the settings requested by the software. The software process automatically each region of interest independently, will detect the fish coordinates and administrate or not the aversive stimulus to the animal based on the settings selected by the user. Once the frame is processed the software will save the relevant information into a txt file and wait for the next frame to process or end the execution of the code if the ending condition is verified.

3.1.2) Hardware implementation of the training environment

Figure 17 shows the final setup that was used for training larvae and juvenile zebrafish. An LCD Monitor is used to present a visual stimulus flexibly with any arbitrary pattern from the bottom of the arena. Moreover, laying the arena on the monitor allows me to keep the temperature of the water at 26°C, close to the optimal temperature for zebrafish. Each sub region of the arena has a dedicated electrical circuit for aversive stimulus delivery and all the six circuits are controlled by an Arduino Due that receive serial inputs by the software. The camera is situated at 1,3m above the arena to be able to image all the six arena at the same time.

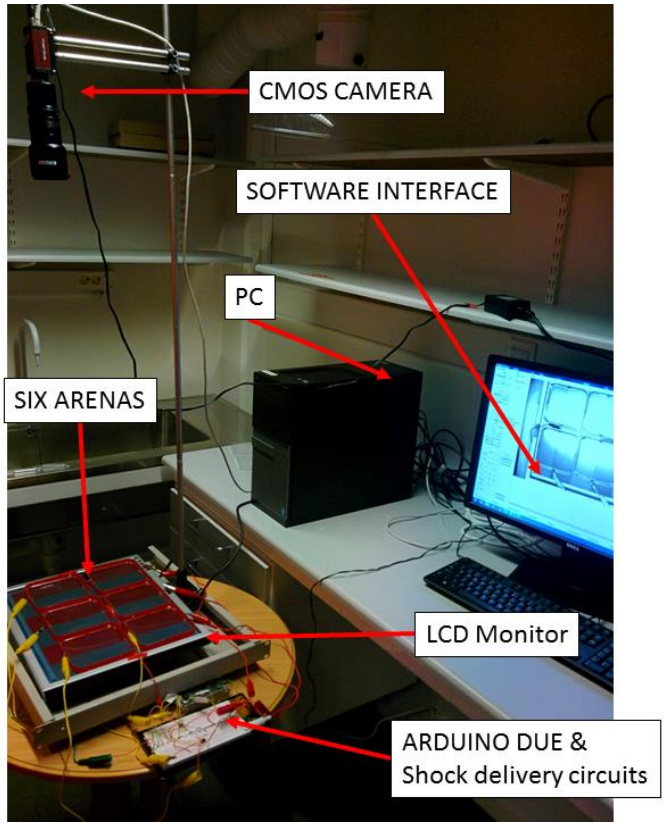


Figure 17) Hardware implemented to perform active avoidance conditioning in larvae and juvenile zebrafish. The six arenas are located on an LCD monitor, which present visual stimuli on the bottom of each arena. The circuits for aversive stimulus administration are controlled by an Arduino due connected to the computer via a USB port and is controlled by the software via serial communication. The camera is located above the six arenas at 1.3 meters of distance to include the entire LCD screen in the field of view of the camera.

3.2) Baseline behaviour

Once the setup was implemented and all hardware/software components were optimized, I started collecting data on larvae/juvenile freely behaving wild type zebrafish. In order to follow developmental changes in zebrafish behaviour, three different age groups have been selected:

- Group 1: one week old zebrafish;
- Group 2: two weeks old zebrafish;
- Group 3: three weeks old zebrafish.

First I tested the baseline behaviour of zebrafish in the experimental setup, in order to follow its response to novel environment as well as identifying potential biases of the behavioural arena. Since it is known that zebrafish respond to a novel environment with increasing stress levels (Stewart, Cachat et al. 2010, Wong, Elegante et al. 2010, Rosemberg, Rico et al. 2011, Schnorr, Steenbergen et al. 2012, Ibrahim, Mussulini et al. 2014). I decided to expose the zebrafish to this new behavioural environment for a habituation period, baseline, of three hours, before any training protocol.

During this baseline period, I investigated the changes of several parameters, such as average swim velocity or Thigmotaxis index, to evaluate the habituation to the new environment and the stress level of every single fish. This approach allowed me to explore how the behaviour of each fish developed/changed over the adaptation period and estimated their potential stress levels. During the three hours of baseline recordings the fish is exposed to the same visual pattern that is used during the conditioning session but no rewards/punishments are administrated to the fish. The baseline period of three hours has been divided in three shorter periods of one hour each (figure 18A). Later I use these periods to quantify the changes in fish behaviour.

3.2.1) Size differences across the three groups

Since the development of zebrafish is correlated with an increase in size, the first investigated parameter was the size of each recorded fish. I observed no significant difference in size between one and two weeks old fish, while there is a significant increase in size between the three weeks old group and the two younger groups (figure 18B). It is important to highlight how the standard deviation increase among three weeks old fishes, meaning that there is a broad variability in size of fish at that stage compared to the two other groups of fish. Moreover, the

lower limit for all the three groups is constant and around 1 cm^2 while the upper limit increases significantly between two and three weeks old fish.

3.2.2) Average swim velocity

The first investigated parameter to estimate the stress response of the fish to the new environment was the change in average swim velocity of each fish. I have defined three different time windows in the baseline period to evaluate how swim speed evolve/change as the fish habituate to its new environment (figure 18C).

The first important finding is that for all the three investigated age groups there is no significant changes in average swim velocity across the three hours recording period. To further investigate this aspect, I defined six time windows of half hour length each, but also at this time scale there was no significant changes in average swim velocity for each group along the baseline period (data not shown). These results show that the swim velocity does not significantly change during the baseline period.

Moreover, I investigated how the developmental stage affect the average swim velocity of zebrafish: in average the older fish, three weeks old, swim significantly faster than the one week old fish. In particularly, the one and two weeks old fish show no significance difference in average swim velocity, while three weeks old fish swim significantly faster than the one week old group along the entire three hours period of baseline. This difference in average swim velocity is reduced between two and three weeks old fish. Specifically, the three weeks old group swim significantly faster than the two weeks group only during the first hour of baseline recording. It is also interesting to note that two weeks old zebrafish show a significant increase in velocity after the first hour recording in the baseline and then the average swim velocity is stable across the last two recorded hours.

3.2.3) Thigmotaxis

The second parameter investigated during the baseline period is the Thigmotaxis index. This index represent the percentage of time spent by the fish in the centre of the arena and has been widely used, especially in mouse and rat behaviour, as a stress indicator (Simons 1994). In brief, a stressed animal prefers to spent more time close to the border of the arena than in the centre of the arena (Simon, Dupuis et al. 1994, Schnorr, Steenbergen et al. 2012)). I calculated the Thigmotaxis index, for each single fish, quantifying the percentage of time spent in the centre of the arena; mean and standard deviation are highlighted for each group (figure 18D).

It is important to highlight that for one week and three week old animals I observed no preferences between centre or periphery of the arena along the baseline period. In contrast, the two weeks old fish spent significantly more time in the centre of the arena during the first time of baseline period. As the animals further habituate to the arena, I observed no significant difference between the three age group, during the second and third hour of baseline recording. It is important to highlight, while on average there is no significant preference between centre of periphery of the arena, the variability across individual animals is very high and this result in high standard deviation value for all groups in all the periods.

These results suggest that while individual animals have different Thigmotaxis preferences, I observed no strong changes of this behaviour during the habituation period.

3.2.4) Baseline biases of zebrafish towards different zones of the behavioural arena

Finally, to be able to objectively estimate the learning performance, I have investigated if there is any bias of zebrafish toward one of the two side of the arena during the baseline period. To estimate this baseline behavioural bias, I have quantified the percentage of time that every fish spent in the "red side" of the arena, the one that will be associated with the aversive stimulus during the conditioning sessions (figure 18E).

Two and three weeks old fish shows a significant preference, during exploratory behaviour, toward the darker compartment of the arena, while the one week old group tend to explore equally both compartments. These results are in accord with previous finding highlighting the preferences, in larvae zebrafish, for brighter environment compared to juvenile/adult zebrafish (Schnorr, Steenbergen et al. 2012, Cheng, Krishnan et al. 2016). Furthermore, one and three weeks old fish doesn´t show significant changes among the three hours of baseline recording while the two weeks old group show a significant increase in preference toward the red compartment of the arena after the first hour of recording. In addition, one week old zebrafish show a broad behavioural variability, variability reduced in two and three weeks old groups.

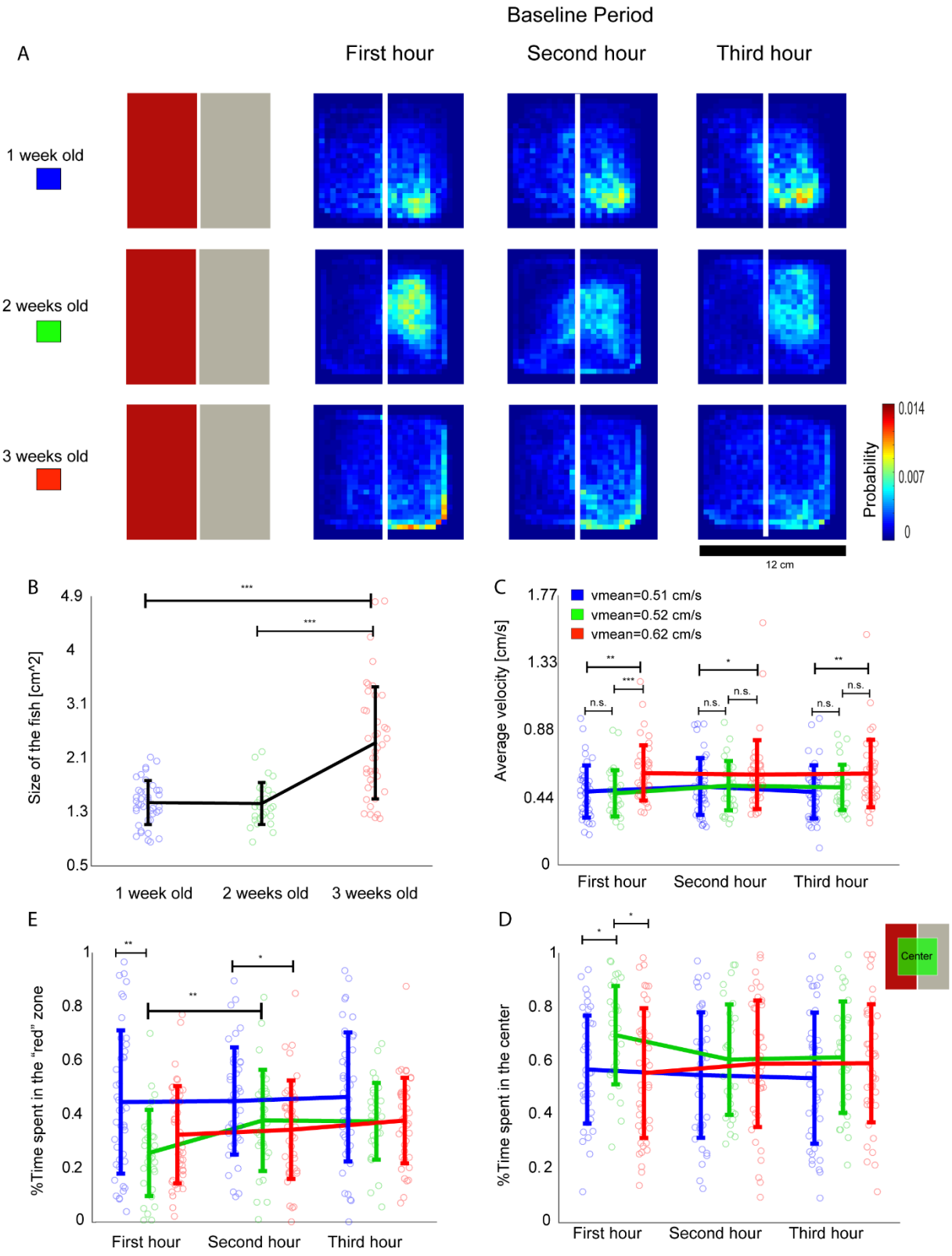


Figure 18) Investigation of three hour baseline recording for the three age group selected to perform the active avoidance conditioning protocol. (A) Average heat map for each group during the three defined time windows, of one hour each, during the baseline recording. The boundary between red and grey compartment of the arena is highlighted in white and superimposed to each heat map. (B, C, D, E) The three groups have been colour coded to simplifying the visualization of differences among them: blue for one week old fish, green for two weeks old fish and red for the three weeks old group. For each graph the value of each single fish is showed as a shade dot and the mean and standard deviation for each group have been highlighted for each of the groups. (B) Plot of the size of each single fish for each group investigated; Three weeks old

*fish are significantly bigger (*** p<0.001) than the two younger group. (C) Average mean velocity trend across the three time windows defined in the baseline period. Three weeks old fish swim significantly faster than one week old animal (** p<0.01; * p<0.5) and they swim significantly faster than two weeks old animal only during the first hour of baseline recording (*** p<0.001). There are no significant differences between one and two weeks old animals. (D) To investigate the Thigmotaxis index, representing the preferences for each fish toward the centre or the periphery of the arena, the arena has been divided in two areas: Centre and Periphery. Each area covers 50% of the total surface of the arena (12x12 cm^2). There are no significant differences between one and three weeks old fish, while two weeks old fish show a significant higher preference for the centre of the arena during the first hour recorded compared to the two other groups (* p<0.05). (E) To determine if there were any bias toward one of the two side of the arena during the three hours of baseline I have calculated the percentage of time spent into the red compartment of the arena. The graph shows for two weeks old fish a significant increase in time spent in the red zone of the arena between the first and the second hour of baseline recorded (**p<0.01). No significant differences are highlighted between two and three weeks old fish. Regarding one week old animals they spent significantly more time in the red area of the arena compared to two weeks old animals only during the first hour recorded (**p<0.01) and significantly more time than three weeks old animal only during the second hour recorded (*p<0.05).*

3.3) Studying learning performance in zebrafish across development

3.3.1) Description of the training protocol

The protocol designed to perform active avoidance conditioning is composed by six sessions (figure 19A):

1. 3h baseline recording session where the fish is exposed to the new arena for the first time;
2. first conditioning session of thirty minutes, where the brief aversive stimulus (1 mA for 10 ms at 1.33 Hz) is administrated in a closed loop configuration, only when the fish is located in the red compartment of the arena;
3. test session of 30 minutes where no aversive stimulus is administrated to the fish but only the position of the fish is recorded (Test 1);
4. second conditioning session of 30 minutes;
5. second test session of 30 minutes (Test 2);
6. longer additional test session of 45 minutes (Extinction test).

All these sessions are performed without any human interference or delay between consecutive sessions and in all the sessions the pattern is always showed on the bottom of the arena for all the fish.

I have decided to perform two different sessions of conditioning of half hour each, instead of a longer single conditioning session, in order to maximize the learning (Kermen, Sultan et al. 2010), and minimizing the stress accumulated by the fish. Figure 19A shows the behaviour of an example fish from the three weeks old group during learning; the heat map encoding the positon of the fish are plotted for each session. These maps suggest that already after the first conditioning session the fish can avoid the part of the arena paired with the aversive stimulus. This avoidance behaviour is preserved until the end of the protocol, but is important to highlight how during the last testing period the fish already start to explore again the red compartment of the arena implying a re-learning/extinction process taking place.

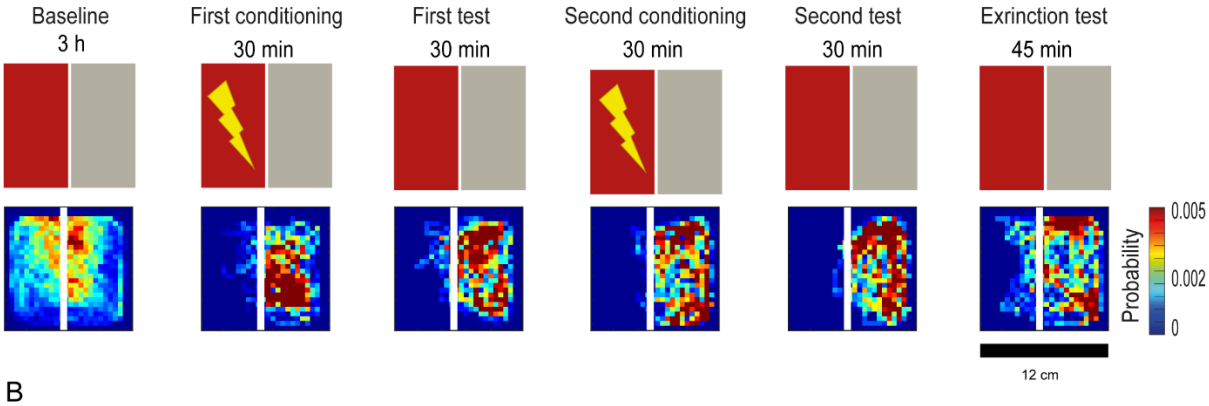
3.3.2) Quantifying the learning performance using a learning index

The behavioural assay is designed to test if zebrafish can perform active avoidance conditioning and to investigate the ontogeny of it.

To investigate the learning performance of each fish I have defined a learning index (figure 19B). This index is defined to normalize the learning performance of each fish taking into account the initial baseline bias (during the first three hours) of the zebrafish for the red

compartment of the arena (figure 18E). Hence learning index is calculated as the **difference** for the ratio of time spent in the red zone during the baseline period and test period **divided** by ratio of time spend in red zone during baseline. This learning index will have value 1 in case of perfect learning, where animals completely learn to avoid the red compartment of the arena during the test session. This index will have a negative value in case of increase preference for the red side of the arena during the test session compared to the baseline period while it will have a value of zero in case of no differences for time spent in the red area between baseline and test session.

A



B

$$\text{Learning Index} = \frac{\%\ T\ \text{baseline} - \%\ T\ \text{session}}{\%\ T\ \text{baseline}}$$

$$\%T\ \text{baseline} = \frac{T\ \text{in red zone during baseline}}{T\ \text{total of baseline}}$$

$$\%T\ \text{session} = \frac{T\ \text{in red zone during session of interest}}{T\ \text{total of the session of interest}}$$

Figure 19) (A) Graphic representation of the protocol used to perform active avoidance conditioning in the three age group of zebrafish. The aversive stimulus is administrated to the fish only during the two conditioning sessions and only when the fish is located in the red compartment of the arena. The aversive stimulus is a 1mA shock with a duration of 10 milliseconds delivered with a frequency of 1.33 Hz. There is no delay between two consecutive sessions. The heat maps showed are examples, from each session, of one fish selected among the three weeks old group. (B) Formula used to calculate the learning index for each fish. This formula takes into account the biases observed during the baseline recording and normalize the learning index based on that bias. A learning index equal to zero means no learning, equal to one means perfect learning and negative learning index means no learning with increase preference for the conditioned area of the arena.

3.3.2.1) Studying the increase of learning performance across development

In order to investigate the ontogeny of learning, all the three age groups of fish (one, two and three weeks old) were trained using the behavioural assay described above. To determine if there were any changes in the preference of the fish for the conditioned part of the arena, I first quantified the percentage of time, in each session, that every single fish spent in the conditioned compartment of the arena (Figure 20A).

- One-week old fish: This age group of zebrafish showed a significant decrease in percentage of time spent in the conditioned compartment when the entire conditioning protocol was performed. This avoidance behaviour can be observed starting from test 1 session. In test 2 session I observed a further decrease in preference for the red side. Moreover, between test 2 and the extinction test session there is a significant increase in time spent in the red side of the arena. It is worth noting the broad behavioural variability among all the fish in this group.

- Two-weeks old fish: This age group of zebrafish showed a significant decrease in percentage of time spent in the conditioned compartment at the end of the entire protocol. This group of zebrafish displayed avoidance learning behaviour already at test 1 session. Furthermore, they do not show a significant decreasing in preference for the red side between test session 1 and 2.

- Three-weeks old fish: This age group showed a significant decrease in time spent in the red compartment of the arena at the end of the protocol. This avoidance behaviour is significant already after the first conditioning session, during test 1 session. There is no significant decrease of preferences for the red zone after the second conditioning session, between test 1 and test 2. In addition, I observed a significant increase in preference toward the red side of the arena between test 2 and extinction test session.

To evaluate the learning performance of each fish I calculated the learning index during the three sessions: test 1, test 2 and extinction test (figure 20E).

In order to define the success levels in learning performance of individual zebrafish, I used the statistics of zebrafish behaviour during the long baseline period prior to the training protocol. Comparing the statistics for six different thirty-minutes periods during the baseline

period, I identified that learning index of 0.3 is the learning level that can be obtained purely by chance of fish swimming during 30min of recording. I use this value from now on as the border for defining success for learning.

Using this definition of successful learning performance, I studied learning at 3 different ages of zebrafish. My results suggest that the majority of zebrafish can successfully perform active avoidance learning at every tested age. Moreover, I observed that the ratio of learners gradually increased from 54% (at 1 week), 72% (at 2 week), 76% (as 3 week), as the animals develop (Figure 20B). Moreover, I also observed that even without this threshold for defining success over all, learning indices of all recorded fish (learners and non-learners) significantly increased as the animals develop from one to three weeks.

Moreover, I observed that the animals' learning performance gets significantly better with more conditioning sessions (figure 20B-D). While the learning performance for animals from all age groups gets better across training sessions, three weeks old fish can still perform significantly better than younger fish. The data collected and described above show that larvae/juvenile zebrafish can perform active avoidance learning and that the performance increase across developmental stage.

Finally, I have quantified the avoidance performance on a time window of 45 minutes (extinction test session) just after the second test session, without any additional conditioning session performed in between (figure 20D). A significant percentage of fish, in all investigated groups, retain a strong avoidance behaviour toward the red compartment of the arena. Percentage, this one, that increase across developmental stage according with previous findings.

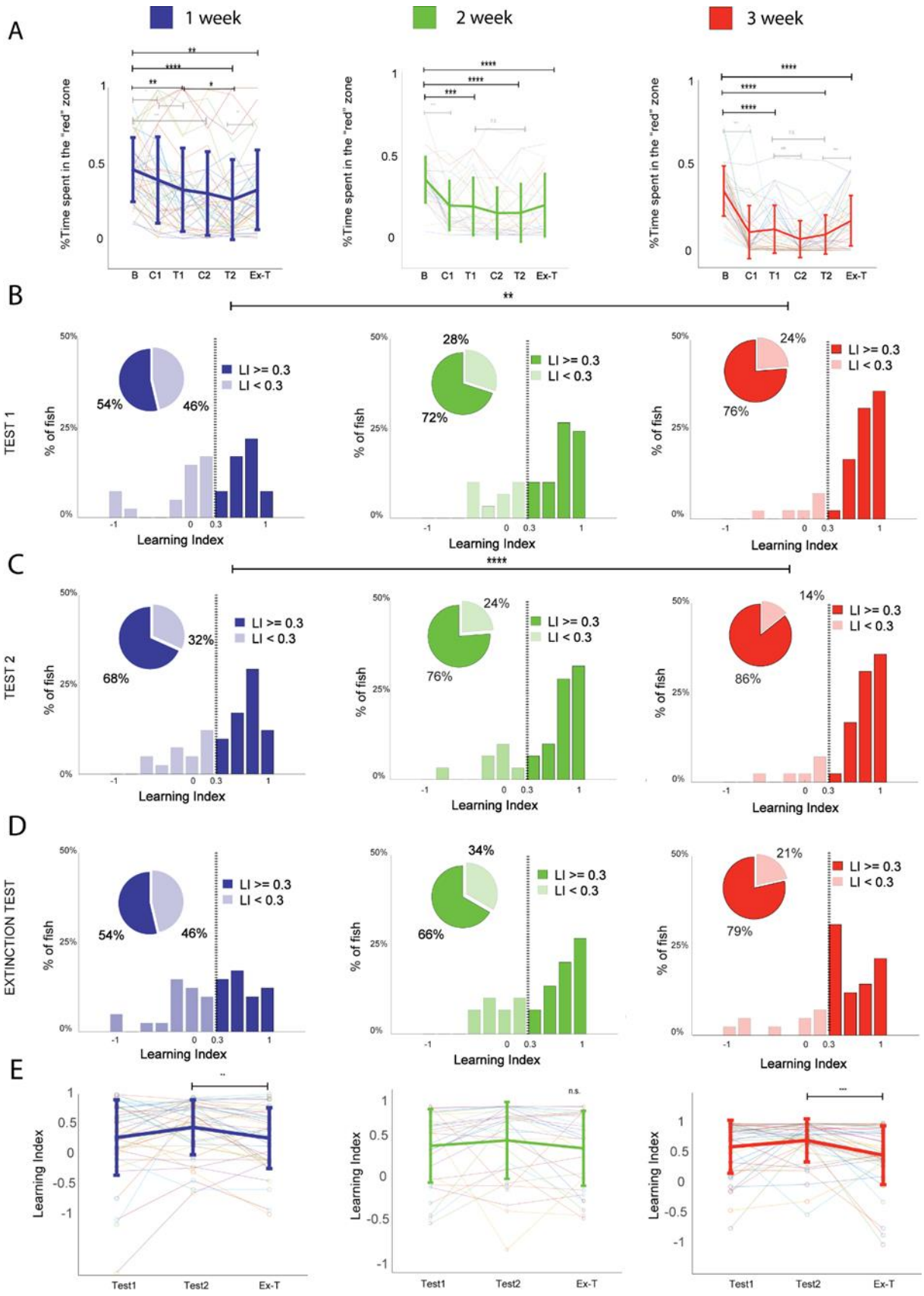


Figure 20) Learning performances of each age group, calculated over the three testing session defined in the protocol. (A) Percentage of time spent in the red compartment of the area across all the protocol session for the three different group of zebrafish. One week old fish show a significant decrease in time spent in the conditioned side between baseline and test 1

*(**p<0.01) and between test 1 and test 2 session (*p<0.05); they show also a significant increase in time spent in the red area between test 2 and extinction test session(**p<0.01), but the time spent in red during extinction test session is still significantly smaller that the baseline percentage (**p<0.01). The two older group, two and three weeks old animals, show a similar pattern: there is a significant decrease in time spent in the red side of the arena during the test 1 session (***p<0.001 for two weeks and ****p<0.0001 for three weeks old fish); they do not show significant differences between test 1 and test 2 sessions; for both group the test 2 and extinction test sessions (Ex-T) show significant smaller preferences for the red area of the arena in respect at the baseline period (****p<0.001). (B, C, D) Histogram showing the Learning Index distribution for each age group for the three different test session: (B) test 1, (C) test 2, (D) extinction test. The threshold value (0.3) has been defined as mean + one standard deviation of the Learning indexes calculated over six time windows, of half hour each, during the baseline period without any conditioning session performed. For all the three group the mean of this Learning indexes was 0 with a standard deviation always of 0.3. (B) Histograms representing the distribution of the Learning indexes during the test 1 session: three weeks old fish perform significantly better than one week old animals (**p<0.01). There are no significant differences between one and two weeks old fish and between two and three weeks old fish. (C) Histograms representing the distribution of the Learning indexes during the test 2 session: three weeks old fish perform significantly better than one week old animals (****p<0.0001). (D) . Histograms representing the distribution of the Learning indexes during the extinction test session: there is no significant differences among the three groups. (E) in the plot are showed the trend for the Learning indexes across the three test session for the three different age group of fish. Evolution of Learning index for each fish; mean and the standard deviation for each group are highlighted. For all the three groups there is no significant increase in the learning index value between test 1 and test 2 session. For one week old and three weeks old animals there is a significant decrease in Learning index value between test 2 and extinction test session (** p<0.01 for one week old and *** p<0.001 for three weeks old animals).*

3.2.2.2) Studying the temporal dynamics of learning performance across development

Once I determined that larvae/juvenile zebrafish can perform active avoidance conditioning, I investigated the temporal dynamics of this learning process from the first conditioning session until the second test period to answer the question: Can older zebrafish learn faster as they develop?

To investigate the temporal dynamics of learning I have divided non-linearly each session period in 5 time windows (Figure 21B). This approach allows me to build a learning curve and to investigate the differences for the speed of learning between different ages (figure 21A). In particularly, having shorter time windows in the beginning of each session allows me to precisely investigate the dynamics of the onset of avoidance behaviour.
For all the three groups the learning curve has common properties:

- Positive slope during the conditioning period;
- A maximum peak just after the conditioning period;
- Negative slope during the testing period;

While these three properties are common for all the three learning curve, I observed differences for every age group are in the temporal evolution of the learning curve:

- One week old fish: this group learns significantly slower than both older groups during the conditioning sessions and the learning indices decay significantly faster than two and three weeks old animal during the testing sessions. Is important to highlight that after the first minutes of the second conditioning session there is no significant increase of the learning index, in contrast with the two older group. In addition, during the second conditioning session the median learning index value increases significantly faster than during the first conditioning session.
- Two weeks old fish: this age group perform significantly faster during both conditioning sessions and the learning index decay slower during both testing period compared to the one week old group. On the other hand, they perform significantly slower than three weeks old fish during both conditioning sessions and the learning index decay faster during both testing sessions than the three weeks old group. In addition, during the second conditioning session the median learning index increase significantly faster than during the first conditioning session.

- Three weeks old fish: this group of fish perform significantly faster than both younger groups during the conditioning sessions. In addition, the decay rate of the learning index is significantly smaller than that of younger fish groups, indicating a longer retention of the avoidance behaviour during the test sessions. It is important to mention that after the first minute of the first session of conditioning already more than 50% of the fish has a learning index above 0.5. Furthermore, after the first minute of the second conditioning session there is a significant increase in the median of the Learning indexes population, while it decayed around 0.6 at the end of the first testing period. In addition, during the second conditioning session the median learning index increase significantly faster than during the first conditioning session.

These results show significant differences in the temporal evolution of the avoidance response (learning curve) across the three age groups. In brief, older fish shows a significant increase in avoidance behaviour earlier, in the conditioning session, than the younger fish. In addition, for all the three groups the avoidance performance increase significantly faster during the second conditioning session, compared to the first conditioning session. Furthermore, the decay rate of the avoidance performance becomes significantly slower in more developed animals, suggesting a better memory retention in older animals than in younger ones.

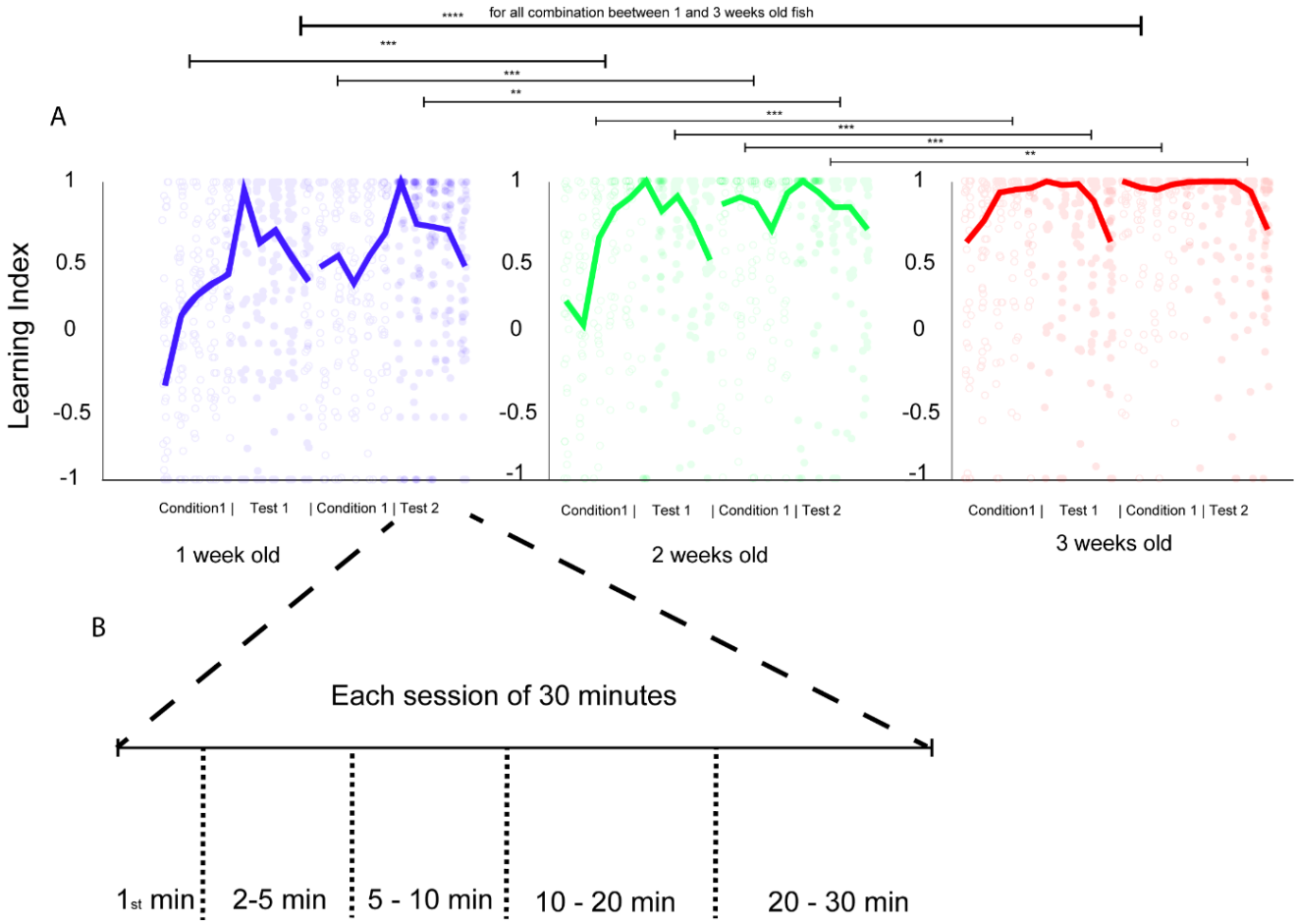


Figure 21)(A) Learning curves describing the evolution of the learning index from the beginning of the first conditioning session till the end of the second test session. The learning curve describe the median value of the learning indexes for each of the five time windows defined for each session. Single Learning indexes are plotted as filled dots, for test sessions, and empty dots, for conditioning session. (B) Each session analyse has been divided non-linearly; five time windows have been defined: the first minute of the session, from minute two to minute five, from minute five to minute 10, from minute 10 to minute 20 and the last time window covers the last 10 minutes. With this approach is possible to visualize the evolution of the Learning indexes highlighting the temporal dynamic during the first then minute of the session.
(A*) Three weeks old animals perform significantly faster than one week old fish during both session of conditioning (**** p<0.0001) and the decay rate during the test sessions is significantly smaller for three weeks old animal than the one week old (**** p<0.0001). The three weeks old group perform significantly faster than the two weeks old group during conditioning session (*** p<0.001) with a smaller decay rate during the test session (*** p<0.001 for test 1 session and **p<0.01 for test 2 session). This age group performs significantly faster during the second conditioning session than during the first conditioning session (**p<0.01). Two weeks old animals perform significantly faster than one week old fish during the conditioning session (***p<0.001); during the test 1 session there is no difference in the decay rate of the Learning index but during the test 2 session the decay rate is smaller for two weeks old fish than for one week old fish (**p<0.01). During the second conditioning session two weeks old fish perform significantly faster than the first conditioning session (** p<0.01). One week old animal perform significantly slower than the two older group as described above and the decay rate is significantly faster during the test session; as the other two groups, they perform significantly faster during the second conditioning session than during the first conditioning session (**p<0.01). Furthermore, only three weeks old animals show a significant increase in Learning Index after the first minute of the conditioning 2 session (**** p<0.0001).*

3.2.2.3) Qualitative analysis of extinction of avoidance performance

Speaking about active avoidance conditioning, an important phenomenon to keep in mind is the extinction of the aversive response. To investigate this phenomenon, I have quantified the avoidance performance on a time window of 45 minutes (extinction test session) just after the second test session, without any additional conditioning session performed in between (figure 22A). A significant percentage of fish, in all investigated groups, retain a strong avoidance behaviour toward the red compartment of the arena. Percentage, this one, that increase across developmental stage according with previous findings.

3.2.2.4) Testing whether the learning is associated with location/space or with colour

To further investigate this avoidance response, I decided to decouple the spatial component from the colour component of the presented pattern to the fish by performing an additional test session, where avoided red colour pattern switched locations, mirroring the pattern previously showed. This test session of 30 minutes was performed after the extinction test session without any delay and no aversive stimulus was administrated to the fish. The performance of this session was possible since the avoidance response was retained, in all groups of fish, during the extinction test session (figure 22A).

To evaluate the performance over this session the learning index was calculated with the following formula:

$$-\frac{(\%\ \text{time in red during baseline} - \%\ \text{time in the half arenapaired with the CS})}{(\%\ \text{time in red during baseline})}$$

The threshold used to distinguish learners and non-learners is constant during all experiments. This new learning index allows me to evaluate if the avoidance response performed by the fish correlates with the red colour, coupled with the aversive stimulus during the conditioning, or if it correlates with the compartment of the arena where the aversive stimulus was administrated. In detail, this index will be positive in case the fish will avoid the red pattern presented over the spatial localization of the aversive stimuli; negative in the opposite case.

I quantified the behavioural response of the fish and a significant percentage of fish showed a learning index above threshold (figure 22B).

These results indicate an avoidance response of the fish toward the red pattern more than toward the spatial location coupled with the aversive stimulus.

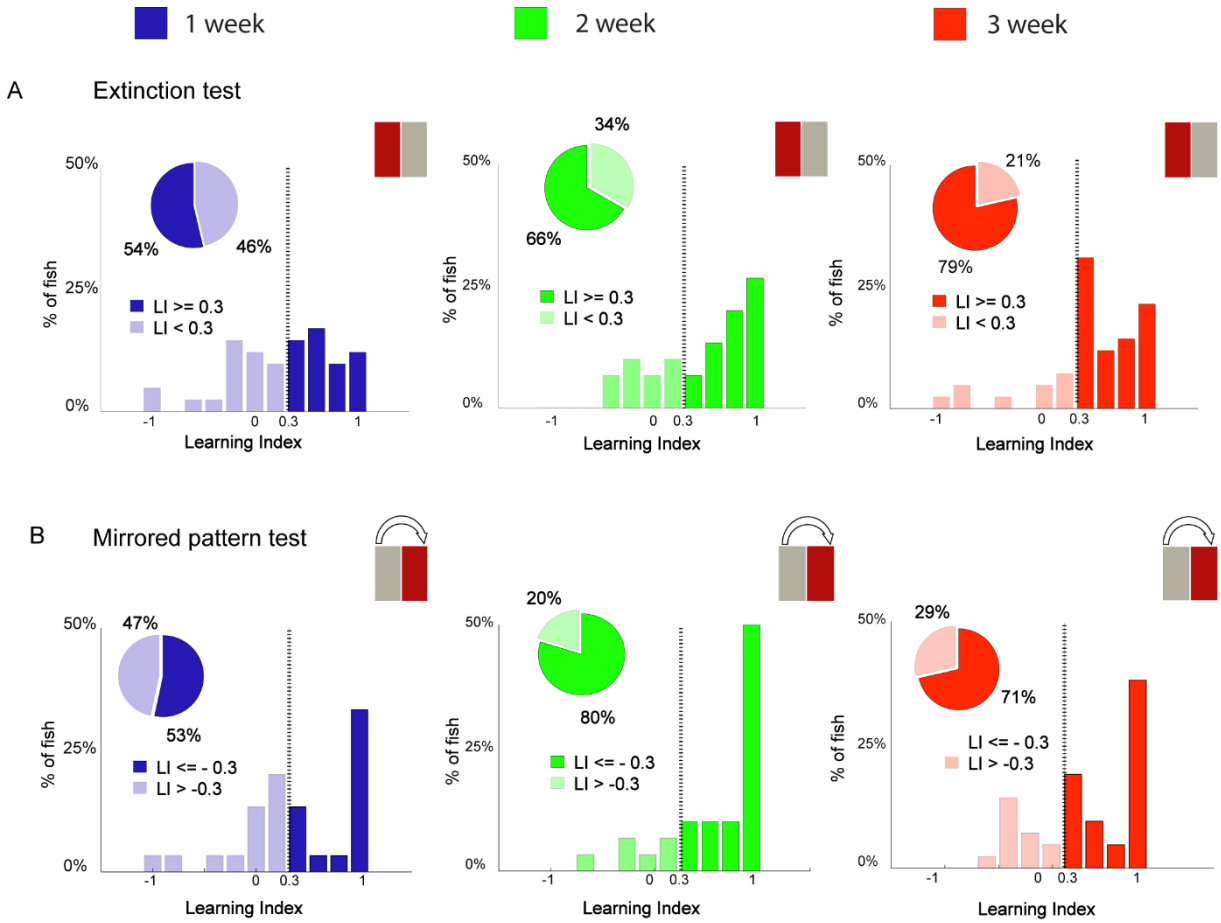


Figure 22)(A) Histograms showing the distribution of the Learning index for the three age group during extinction test session, same as showed in figure 20D. (B) Histograms showing the distribution of the Learning index, calculated over a new test session of 30 minutes, where the pattern presented on the bottom of the arena was mirrored. The learning index defined for this session has been calculated as:

$$-\frac{(\%\ time\ in\ red\ during\ baseline\ -\ \%\ time\ in\ the\ half\ arena\ used\ for\ conditioning\ during\ the\ protocol)}{(\%\ time\ in\ red\ \ during\ baseline)}$$

This new learning index will have value 1 if the fish avoid the red colour more than the side of the arena toward which has been conditioned during the protocol, and value -1 if the fish avoid the arena location toward which he was conditioned more than the red pattern. As shows in the histograms, after mirroring the pattern there is a significant number of fish that avoid the red patter in the arena rather than the side of the arena toward which they have been conditioned during the protocol. No aversive stimuli are delivered during this last session of testing.

3.2.2.5) Identification of factors affecting the learning performance

My results suggest that animals' learning performance significantly increases by age and older animals not only learn better but also faster and they retain these memories longer. However, at all developmental stages I observed a considerably high variability across animals of the same age. This variability can potentially be explained by several factors, such as initial stress levels of individual animals, perceived level of unconditioned stimulus, or simply the differences in the speed animals maturate or develop.

First, I evaluated the potential stress levels of the animals and how they change during my experiments. The two evaluated parameters are average swim velocity and the Thigmotaxis index (figure 23A). For all the three groups analysed after the first conditioning session, there is a significant decrease in average swim velocity. This decrease is still significant at the end of the third test session. However, the Thigmotaxis index did not significantly change in juvenile zebrafish, while it did change for larvae zebrafish.

Together, these findings suggest that the learning protocol can indeed affect the natural behaviour of the fish. Later I tested whether the variability in animals' baseline behaviours can indeed explain animals learning performance. The parameters taken into account are:

- Thigmotaxis index (figure 23B).
- average swim velocity during baseline (figure 23C).
- average velocity peak after aversive stimulus administration (figure 23D): defined as the average peak in velocity during 200 ms after the administration of the second aversive stimulus after the fish entered the red compartment of the arena.
- size of the fish (figure 23E).

Among all the investigated parameters I observed no significant effects over the learning performances of the fish within the age group. The two parameters that show a trend (perceive shock intensity and fish size), were non-significant, potentially due to the low sample size.

These results demonstrated how variable the behavioural responses of zebrafish are and highlight the importance of experimental design which can reduce this variability and increase the throughput.

In summary, all these results suggest that despite the individual variability, the young zebrafish can perform active avoidance tasks and animals' performance significantly increased by age. I observed a variability in animals' performances within age groups which can partly be explained by the differences in animals' sizes as well as the perceived intensity of the mild

electric shock used for training. Nevertheless, more experiments might be necessary to see the significance of these effects.

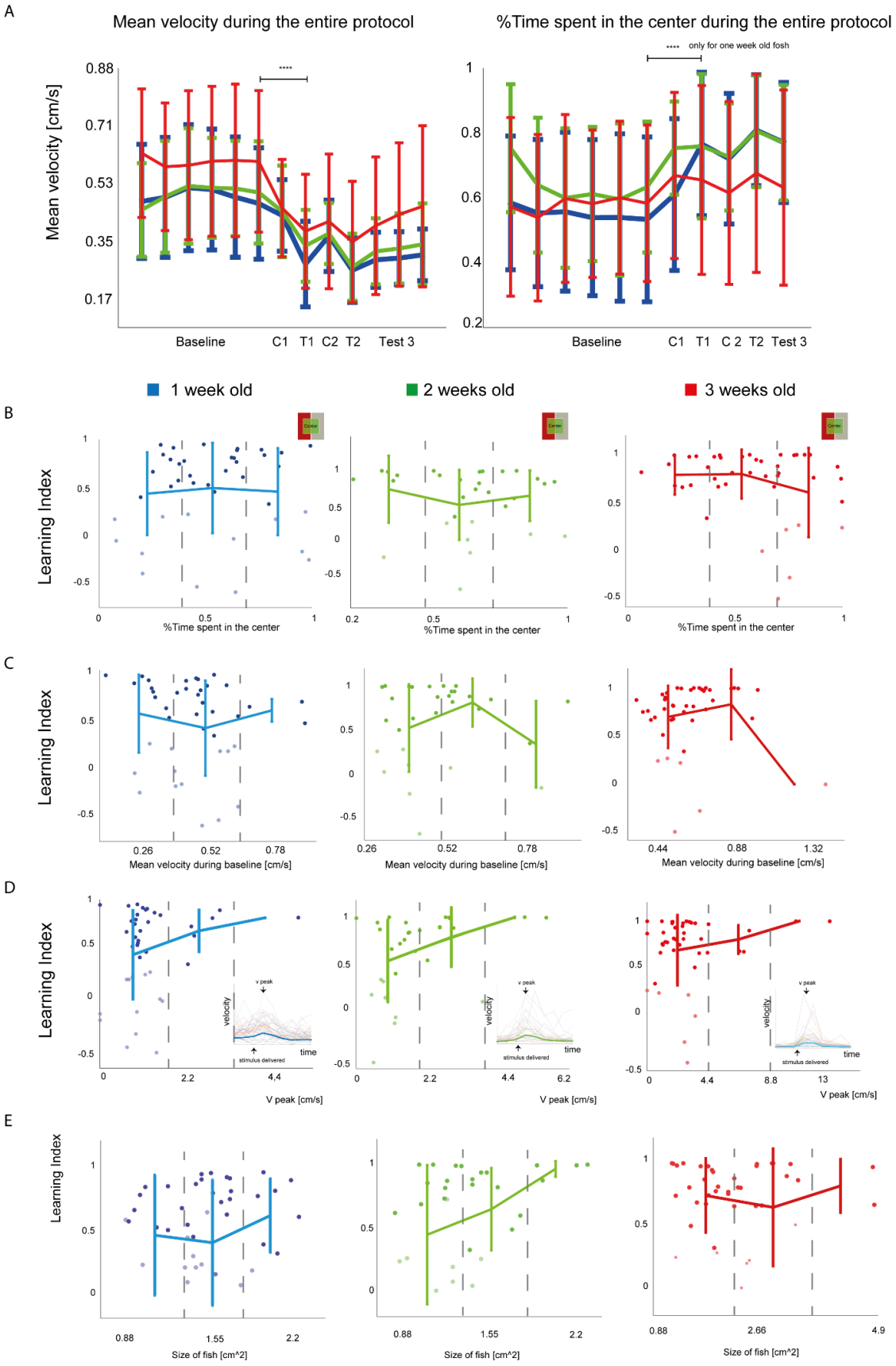


*Figure 23) (A) Average swim velocity and Thigmotaxis trend over the entire protocol session. After the first conditioning session there is a significant decrease in average swim velocity for all the three group of fish (****p<0.0001). All the three group show this decreasing in average swim velocity and ant the end of the protocol, during extinction test session, the average swim velocity is still significantly lower than the velocity during the baseline period (****p<0.0001). The Thigmotaxis index does not significantly change for the two and three weeks old fish; it increases significantly only for one week old fish after the first conditioning session of the protocol (****p<0.0001).*
(B, C, D, E) Correlation between different behavioural parameters and learning performance in the three group analysed colour coded coherently with the previous figures presented. (B) Modulation of the learning performances by the Thigmotaxis index calculated over the baseline period. There is no significant effect of this parameter over the learning performance. (C) Effect of the average swim velocity, in the baseline session, over the learning performances of the zebrafish. In none of the three groups there is a significant effect of the velocity on the learning performance. (D) Modulation of the learning performance due to stimulus perception of the aversive stimulus. To estimate how intense is the aversive stimulus perceived by the fish I used the average velocity peak just after the second aversive stimulus was administrate to the fish every time he entered the red compartment of the arena. There is no significant correlation, but there is a clear trend in the distribution: the more the swim faster after receiving the second shock the better they perform. (E) Correlation between the size of the fish and the learning performance. There are no significant effects for all of the three group, but the two weeks old fish show a trend in which the bigger they are the better they perform.

Chapter 4

Discussion

4.1) Real-time custom made tracking algorithm and comparison to previously existing tools

Since zebrafish are gaining increasing popularity as a model for behavioural neuroscience (Kalueff, Stewart et al. 2012), a video-tracking software is an essential methodological tool to be able to quantify and investigate the behavioural response of the animal. A popular software tool used in past studies is the EthoVision XT7 (Cachat, Stewart et al. 2011, Kalueff, Stewart et al. 2012, Stewart, Gerlai et al. 2015), developed and sold by Noldus Information Technology. Due to the high cost of this software, researchers have developed custom made tracking algorithms. Cario, in 2011, implemented a script, in Matlab, to automatically measure movement of larval zebrafish in a multiwell plate (Cario, Farrell et al. 2011). Unfortunately, in this approach the video is processed post-recording. This is a common problem in this class of algorithm due to the big amount of computational resources requested for such operations. Another approach was proposed by Cheng (2016) with a custom made tracking algorithm, written in Python using the open source library OpenCv. This approach only allows a real-time tracking of the fish at an acquisition rate of one frame per second and only in specific background conditions with high illumination intensities (Cheng, Krishnan et al. 2016).

To overcome these limitations, I implemented a brand new tracking algorithm, using OpenCv library, with a multi-thread approach allowing fast computations. The algorithm processes the image of every single section of the behavioural arena concurrently and independently reducing the execution time of the code and allowing a tracking of the fish's swim path at fifteen frames per second. Moreover, the algorithm has been optimized to work efficiently in my experimental conditions of low brightness, resulting in high spatial and temporal tracking resolution. Due to this high speed tracking, the aversive stimulus administration was very precise in time, only a few milliseconds after the fish crossed the boundary of the conditioned zone. Additionally, this novel algorithm has proven to be very robust to external noise in various experimental conditions, such as drastic changes in the luminosity of the room or unexpected drifts in the relative position between camera and arena. This robustness is achieved due to a dynamic background subtraction algorithm that I

implemented, allowing the tracking algorithm to quickly adapt to changes in the image background. All these allowed, an accurate tracking of up to six zebrafish larvae effectively from five days old age up to adulthood.

4.2) Modularity of Software-Hardware interactions allow easy adjustments of stimulus parameters

Having a high temporal and spatial tracking of the fish position in the arena allows me to have a fine control over the stimulus administration to the animal. In detail, the software controls a LCD monitor for visual stimuli presentation and a microcontroller (ARDUINO DUE) which activates the circuit designated for the delivery of the aversive stimulus. In this project I decided to follow a modular approach in order to develop the setup used to perform active avoidance learning. This modularity allowed changing the aversive stimulus parameters and hardware relatively easily.

4.3) Learning performance

Zebrafish, mostly adults, have been broadly used to investigate complex behaviours such as associative learning (Sison and Gerlai 2010, Roberts, Bill et al. 2013, Blaser and Vira 2014, Aoki, Tsuboi et al. 2015), spatial learning task (Williams, White et al. 2002, Karnik and Gerlai 2012, Naderi, Jamwal et al. 2016) or olfactory behaviours (Braubach, Wood et al. 2009). Nevertheless, zebrafish researchers are still developing behavioural protocols to investigate neural circuit underlying processes like learning and memory (Blaser and Vira 2014). Furthermore, previous studies showed that, just as mammals, adult zebrafish are able to actively avoid stimuli that were previously paired with noxious stimuli (active avoidance conditioning). However, the investigation of active avoidance response in larvae/juvenile zebrafish is limited and not well understood.

The ontogeny of active avoidance conditioning in zebrafish, aged from one week to eight weeks, has been recently reported (Valente, Huang et al. 2012). In that study, the author used a protocol very similar to the one I presented, where the fish is freely swimming in an arena of 6 x 6 cm wide and 2.5 cm deep. In their study a black and white chess pattern is then presented at the bottom, covering one half of the arena. Fish were conditioned during thirty minutes, to avoid the chess halve using electric shocks, delivered when the fish was above that pattern. The chess pattern was used only in larvae/juvenile zebrafish while in case of adult zebrafish a binary pattern presented at the bottom of the arena, divided the arena in two halves, a red section and a grey section. The performance index used in this study, taking into account

only the first five minutes of the test period, is defined as the percentage of frames, spent by the fish, in the non-conditioned side of the arena. They did not observe any innate preferences toward any of the cues used in the protocol for all the ages of zebrafish.
Moreover, this study suggests no significant learning for young zebrafish larva (1-2 weeks), on average. They noticed however, a broad variability in the learning performances of these two age-groups. Indeed, around 20% of the larvae were learning the task, showing a positive performance index, whereas the other 80% were not. They claim that this variability could arise from the ability, of some of the larvae, to perform the task, but they do not discuss if such a performance index can be related to an actual aversive response or if it is just chance preference toward one of the side of the arena.

In contrast with these negative results, my data showed that larvae of 1 and 2 weeks can perform active avoidance conditioning and this behaviour is retained in a time window of 1 hour after the protocol is performed. After the first conditioning session already 54% of one week and 72% of two weeks old zebrafish can be classified as significant learners (76% in case of three weeks old). Moreover, this avoidance behaviour is acquired faster and efficiently by older zebrafish. To quantify the learning performance, I have defined a similar learning index to the one used by Valente (2012) and I decided to take into account also the baseline preferences toward the conditioned halve of the arena. This approach allows me to normalize the learning performance of each age since during the baseline period, especially for older animals (two and three weeks old zebrafish), which display an innate preference toward the grey side of the arena. In detail, this normalization was necessary since there is a broad variability among all the fish, and especially in case of the one week old, in the time spent in the red compartment of the arena during baseline.

Several factors might explain why my results showed much better learning performance for zebrafish larvae and juveniles:

1) Intensity/frequency of the aversive electric shock.

In the study performed by Valente (2012) the stimulus used as an unconditioned aversive stimulus, an electric shock administrated to the fish at 1Hz, has been used for all zebrafish regardless of age. Moreover, is it difficult to quantify the intensity of this stimulus since the author quantifies the shock as 9V over 6cm. From these details it is difficult to quantify the amount of current delivered to the fish. Indeed, the current levels are the major variable of the aversive stimulus, since it is strictly related with the type of electrode used and the conductivity

of the water. In this thesis I optimized a minimum aversive stimulus to reduce the stress levels for the fish while still providing an avoidance response, measured by the change of fish swimming speed on stimulus delivery. Consequently, the noxious stimulus administrated to the fish has been a 1.2 mA current pulse, with a duration of 10 ms, administrated with a frequency of 1.33 Hz. Compared to the aversive stimulus used by Valente (2012), I have used a seven-time shorter stimulus duration but administrated 1.3 times more often. It is possible that the aversive stimulus administrate to the fish was too noxious in Valente study for small and fragile one-two weeks old larvae and as a consequence, this strong stimulus can result in disrupting the fish behaviour, as documented in rats by Souza (1984). In his study Souza identified a minimum shock intensity necessary to performance and retain avoidance response, increasing stimulus intensity did not significantly increase the learning performance of the animals, on the contrary disrupting learning ant high intensity. All together these results highlight the importance of aversive stimulus parameters matching the right age to maximize the learning performance while reducing the stress for the fish especially at early developmental stage, one two and three weeks old.

2) Visual/Spatial pattern used to perform conditioning.

In the study of Valente (2012) a black and white chess pattern is presented to the fish. They suggest that at 7 dpf the visual system of larvae zebrafish is not mature enough to fully distinguish the two pattern presented in their study. To simplify the task and for practical reason (a homogeneous pattern result in a more stable trace of the fish behaviour by the tracking algorithm), I chose to conditioned the fish toward a red uniform patter instead of a chess pattern, to clearly highlight the boundary between conditioned (red) and unconditioned (grey) compartment of the arena. Using this pattern resulted in a bias preference toward the grey section of the arena especially in two and three weeks old zebrafish. This bias is taken into account in the calculation of the learning index. It is also worth to note that low illumination intensities that are used to present the visual patterns ensured that zebrafish do not experience intense light illumination from the bottom of the tank, which is a rather unnatural condition for fish (Cheng, Krishnan et al. 2016, Cordova, Dos Santos et al. 2016)

3) Genetic Background of zebrafish strain.

Differences in behaviour between different strains of Wild Type zebrafish, AB and TU strain, was previously shown (Vignet, Begout et al. 2013). To be able to compare the result

from my study with the result obtained by Valente (2012) I have used the same strain of zebrafish: AB Wild Type zebrafish. Indeed, this parameter will be important in the future when comparing the results from zebrafish with different genetic ablations perturbing different components of brain networks involved in learning.

4) Quantification of the aversive response.

In the study performed by Valente (2012) the performance index was calculated only on a 5 minutes time windows at the end of the conditioning session, without taking into account the baseline behaviour of the single animal. In my study I notice a broad variability, especially in one week old zebrafish, in preference toward the red compartment of the arena. To take into account this bias I have normalized the learning index based on the baseline preference of the animal toward the conditioned compartment of the arena. This allows me to highlight the learned avoidance response independently from the innate bias of animals toward one of the sides of the arena. For instance, a fish with a strong bias against the red pattern will avoid the conditioned compartment easier than a fish with a strong baseline bias toward that compartment. It will be interesting to perform the same protocol presented in this thesis using different colours as visual cues to investigate if there is an innate preference toward a specific colour or if some of them can lead to a stronger avoidance response.

5) Developmental stage of the tested zebrafish.

In accordance with Valente's findings for older zebrafish (starting from three weeks old), my results demonstrate that the learning performance improves significantly with development starting form 1 weeks old. Indeed, three weeks old fish perform significantly better than one and two weeks old fish. Learning performance increased from 54% (at 1 week), 72% (at 2 week), 76% (at 3 week). It is important to note that in Valente´s study the avoidance response was very weak in three weeks old animals, showing an average performance index around 0.1, while my results suggest that avoidance learning in three weeks old zebrafish is very strong and stable with a median learning index close to 1.

These findings, together with the results of Valente (2012), clearly demonstrate that young zebrafish are able to learn and remember an association between a visual stimulus (colour) and a noxious stimulus. The result of this thesis highlight the necessity of optimizing the aversive stimulus based on the developmental stage of the fish, resulting in reduced stress

and optimal learning performance. It will be interesting to extend this analysis to older zebrafish, up to six weeks old, to investigate how this learning evolves further in development.

4.4) Temporal dynamics of learning performance and memory retention/extinction

The first aspect highlighted by this analysis is the significant increase of learning performance across conditioning sessions only for one week old zebrafish. Two and three weeks old zebrafish showed, already during the first conditioning session, a high learning index and no significant increase in performance was observed in consecutive conditioning sessions.

To further investigate developmental differences in learning of avoidance behaviour I focused on the temporal evolution of the learning and the extinction of this behaviour. To highlight the temporal dynamics of this response I have divided, non-linearly, the conditioning and test sessions in five different time windows. This approach allows me to highlight the onset of the avoidance response in the early phase of conditioning and trace the extinction of this phenomenon during the test session.

My results revealed that the older zebrafish learn much faster. Specifically, three weeks old zebrafish already show after only 1 minute of conditioning a median learning index of 0.5, which increase up to 0.8 in the next 5 minute and is stable for both the conditioning sessions. Furthermore, analysing the second conditioning session is clear how for two and three weeks old fish the avoidance behaviour is recalled after the first few experiences of the aversive stimulus, showing that the association to conditioned stimulus is already established. The association CS-US is retained during the test session despite the small decrease in avoidance performance during the test period, which is likely due to mild extinction of learning. Such trends were not observed in one week old zebrafish, which have to go through the entire conditioning session to increase the performance of the aversive response. These findings also highlight a longer avoidance behaviour retention in older animals, whereas one week old zebrafish after few minutes from the end of the conditioning session show already a significant decrease in avoidance response. Three weeks old zebrafish retain very prominent avoidance behaviour up to 20 minutes from the end of the conditioning period.

Finally, a test session, of 45 minutes, at the end of the protocol highlights how a significant percentage of fish in all the three analysed groups retain an aversive conditioned behaviour up to one hour and a quarter from the end of the last conditioning session. In accord with the previous considerations, the percentage of fish showing this retained behaviour increases across developmental stages. Moreover, since it has been shown that adult zebrafish

can retrieve avoidance response two days after the conditioning session(Pradel, Schachner et al. 1999), it will be interesting to test memory retention with this protocol over such time scales.

4.5) Decoupling memory consolidation from extinction of memory

It will be necessary in future investigations to decouple the two phenomena of memory consolidation and extinction of the memories. In the protocol performed in this master thesis the pattern used for conditioning the fish was always presented to the animal also during the session between two conditioning session. This continuous exposure to the CS, in absence of the US, can lead to two different processes: reconsolidation or extinction of the memories. These two phenomena occur concurrently when the CS is experienced alone, with no US. After short experience of the CS alone reconsolidation seems to be prevalent, while if the exposure to the CS alone is prolonged than the extinction phenomenon seems to be prevalent (Tronson and Taylor 2007). Though these two phenomena happen concurrently, evidence suggest that they are driven by different molecular mechanism. For example, injection of protein synthesis inhibitors in the basolateral amygdala disrupt reconsolidation but not extinction (Tronson and Taylor 2007). To be able to test memory consolidation it will be very important that the fish is exposed to the CS only when it is paired with the US and a test session, with only CS presented after a longer gap where neither CS nor US are presented (such as a black chamber with no visual cues).

4.6) Mirrored pattern test for decoupling place learning and visual colour learning

Once the avoidance learning protocol was performed I investigated if the aversive response of the fish was coupled with the spatial location of the aversive stimulus or with the red visual pattern coupled with the aversive stimulus. To decouple these two components, I ran a final test session, following the initial training and test sessions without any delay. In this additional session the fish were exposed to the same visual pattern used during the protocol but its spatial location was mirrored. This test allows me to qualitatively estimate which of these two components (spatial or visual) of the conditioned stimulus was associated stronger with the unconditioned stimulus. The Results suggest that all ages of zebrafish associate the red visual pattern coupled with the aversive stimulus more than the spatial location of it. These results are in accord with the findings of Valente (2012) who shows that the aversive response of the fish was strictly related to the pattern presented at the bottom of the arena and coupled with the aversive stimulus. Nevertheless, it will be necessary to further investigate this association to

design a better experimental protocol to decouple the spatial component from the visual component of the conditioned stimulus.

4.6) Major factors that are correlated with learning performance

In final analysis, I investigated the relations between different behavioural parameters and the learning performance of the fish. This analysis has been possible thanks to the high temporal and spatial resolution of the tracing of the fish behaviour and of the stimulus delivery. I focused on different parameters as described above in the thesis, but in general I observed that the learning performance is affected by two major factors:

1) Size of fish

   It correlates with developmental stages of the fish. In accord with what I discussed above increases in size correlate with increase in learning performance. Especially at these younger stages, zebrafish size directly correlates with its maturity. Zebrafish juveniles display a large range of sizes at these early stages. It is therefore safe to assume that the variability that we see in animals' performance can partly be explained by the variability in animals' size.

2) Intensity of the perceived aversive stimulus.

   This is mostly related with practical difficulties for delivering exactly the same aversive stimulus to every fish. Due to the fixed location of our electrode, the current flowing through the arena will not be homogeneously distributed among the arena, but will flow through the lowest resistivity path. This inhomogeneity in the current flow will determine a different intensity of the aversive stimulus related to the position of the fish in the arena as well as the size of the fish. In order to ensure a relatively constant current flow across the arena, I abandoned the initial circular dish and adopted the square shaped arena, which keeps the distance between the electrodes constant. This square arena allowed a more homogeneous delivery of the aversive electric shock. Moreover, initial test experiments provide me with the most optimal electric shock duration and current intensity, which generates sufficient discomfort to fish without inducing too much stress. This minimal stimulation protocol was crucial in obtaining superior learning performances, compared to previous studies. In future studies it will be important to be able to control the aversive stimulus administrate to the fish more precisely.

In addition, I also quantified estimators of animals' stress such as the Thigmotaxis index or mean swim velocity during the baseline period. Surprisingly these parameters have very little variance and did not correlate with the learning performance of the fish. This small variability in such estimators of stress levels displays the robustness and gentleness in the way animals were handled and treated during this behavioural experiments.

It will be useful to perform a multivariate regression analysis over these parameters to quantify the effect of each parameter of their combinations for estimating the learning performance of individual animals. However, such analysis will perform better with bigger sample size, which requires future experimentation of similar kinds.

Finally, it is worth to note that environmental conditions such as, water temperature, previous handling of the animal, illumination intensity, arena shape, quality of used electrodes were all shown to be important for animal wellbeing and hence learning performance. Throughout the early optimization period these parameters were carefully adjusted for ensuring animals comfort and wellbeing.

In summary, this thesis demonstrated that juvenile zebrafish can perform avoidance learning and their learning performance improves both with age and animals size. It is however extremely important to note that animals wellbeing is the most crucial element for successful behavioural experiments including learning. The Animals' wellbeing can only be ensured when all aspects form environmental parameters, to handling and training protocols are carefully optimized.

4.7) Future direction

The results shown in this thesis, opposing to the general perception in past studies, highlight the capability of larvae/juvenile zebrafish to perform active avoidance conditioning. Furthermore, following the study performed in this master thesis, it will be interesting to investigate this avoidance response in older zebrafish, at least until six or eight weeks old.

These results will lead to further investigation of the neural circuitry mediating this behaviour at larval stage. Due to the broad availability of genetic tools in zebrafish and the possibility to perform live two photon imaging at larval stages, it will be possible to investigate the effects of genetic silencing of the neural activity in brain regions involved in active avoidance condition. In particular, it will be interesting to investigate further the role of habenula in fear conditioning since it has been proposed as a core structure in this behaviour (Lee, Mathuru et al. 2010). Furthermore, since habenula connects telencephalonic brain regions to several brainstem nuclei, as interpeduncular nucleus, raphe nuclei or ventral tegmental area

(Okamoto, Agetsuma et al. 2012), investigation of these pathways, such as basolateral amygdala-habenula-raphe connections (Amo, Fredes et al. 2014), can reveal the roles and links between these structures in activate avoidance learning.

Chapter 5

Conclusions

The aim of this master thesis was to investigate the aversive learning in larvae/juvenile wild type zebrafish. My results show that zebrafish can perform this type of learning as early as the one week old larval stage and animals learning performance increases across developmental stages. These results are in line with previous studies testing learning performance in zebrafish, however I observed in general better learning performance, which are likely due to the better animal handling and better experimental design, compared to past studies.

The software implemented and used in this study to investigate behavioural response of zebrafish provides a systematic and reproducible approach in investigation of fish behaviour, with high temporal and spatial resolution. Moreover, this software allows a high temporal control on the stimulus delivery, that is a crucial parameter for associative learning.

In the next set of experiments, this setup will give us the possibility to compare the learning performance of fish while modulating/perturbing the activity of selected brain regions. For instance, it is possible to activate/inhibit activity of hippocampus, amygdala or habenula in zebrafish, using specific transgenic lines specifically expressing either (opto)genetic controllers of neural activity such as Channelrhodopsin, Halorhodopsin, Botilinumtoxin or Nitorreductase. This will enable us to investigate the involvement of specific brain structures in learning and memory tasks.

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