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Applying automatic operant boxes ("Skinner boxes") for studies on behavioural flexibility in zebrafish (Danio rerio)

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Abstract

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The most successful and widely used animal models in neuroscience are rats and mice, which has been vital for the understanding of the human brain. Another model that has become more prominent during recent years is the zebrafish (Danio rerio). Due to its well-elaborated visual system and learning of aversive events, the zebrafish has become a suitable model for learning and memorizing. In this paper, the relationship between coping styles and learning in different zebrafish strains has been studied by using automatic operant boxes. The strains that were compared are the Tupfel Long Fin (TL). offspring of wild-caught zebrafish (WT) and the Spiegel danio (Spd). The results from the novel tank diving test suggest that there is a difference in coping style depending on the strain of zebrafish. The TL was characterized as a reactive (shy) fish as it had the longest cumulative duration time at the bottom of the tank. The Spd was characterized as a proactive (bold) fish as it had the longest cumulative duration time at the top of the tank. While, the WT characterized as a reactive fish as it exhibited the longest time not moving at all in the tank, indicating a high-stress response profile. An automatic operant box was used to study learning in the different strains. The purpose of the script used was for the fish to associate the feeder mechanism noise and the white light feeder with a food reward. However, no tendency of learning could be observed for any of the strains.

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Popular scientific summary

The use of animals as models in research is an important tool that has been used in the research world over the past centuries. The long-standing practice of animal models is widely used in biological research because the anatomy and physiology of humans and animals, especially in mammals, are extremely similar. The most widely used and well-established animal models in neuroscience are rats and mice, these models have been crucial to understanding a variety of research areas in neuroscience. Another model that has become more prominent in recent years is the zebrafish *(Danio rerio)*. Research has shown that there are a number of similarities between man and the physiology of zebrafish. The zebrafish has proven to be a suitable animal model for e.g. learning and memory because of its well-developed visual systems and the learning of various events. The aim of the report was to study the relationship between proactive and reactive behavior and learning in different zebrafish strains using automatic operative boxes. The strains that were compared are Tupfel Long Fin, the offspring of wild-caught zebrafish, and Spiegel danio a strain that carries a mutation in the fibroblast-derived growth factor receptor 1A gene (fgfr1a - / -).

Associative learning is when an animal has to associate a conditioned stimulus with an unconditioned stimulus. Two different types of associate learning that can be applied in research, the classic conditioning also known as the pavlovian conditioning and the operant conditioning. A pavlovian conditioning is when the stimuli presented are not dependent on the action of the animal. While the operant conditioning is dependent on the behavior of the animal as unconditioned stimuli will be presented. In this project, operant conditioning was used with a positive reinforcement. Automatic operant boxes are one of the few robust methods that have been developed to study zebrafish learning and behavior. The method has shown clear advantages as it requires minimal handling of the fish, as well as several fish, can be studied simultaneously. This aspect is extremely important as the method becomes both time-efficient and cost-effective. By minimizing the factors that cause anxiety and stress such as human handling, the results of the experiment become more reliable and reproducible.

Psychosocial factors are another important aspect to consider when looking at learning ability in animal models. There have been many studies showing that individuals of the same species can differ in capacity. This ability differs depending on various factors such as genotype, prior exposure, and the environment. The two stress coping styles described in the zebrafish are proactive (bold) and reactive (shy) coping. A proactive animal will use a so-called “feed-forward” process, which means that their behavior will be based on previous exposure and experience. They often have low behavioral flexibility and a low physiological stress response profile. The reactive animal, on the other hand, is characterized by high behavioral flexibility and a high physiological stress response profile. This means they will take time to learn all parts of the environment because of their cautious behavior. The most widely used behavioral test to study individual variation in stress management is the novel tank diving test. A proactive fish will spend larger parts of the test in the upper part of the tank, closer to the water surface. This is because of its boldness while a reactive fish will spend larger parts of the test in the lower part of the tank.

The main findings from this study suggest that there is a difference in coping style depending on the strain of zebrafish. However, there was no tenancy of learning for any of the strains in the automatic operant box. This could have been caused by several factors but it was probably because of the human handling that was involved in the experiment.

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1. Introduction

1.1 The use of animal models in neuroscience

The use of animals as models in research is a significant tool that has been used in the field of science for the past century, and has contributed to a better understanding of existing human and animal diseases[1]. The long-standing practice of animal models is widely used in biological research as the anatomy and physiology between humans and animals, especially in mammals is extremely similar [2]. In the field of science, complex processes are often referred to as models. The use of these models is primarily based on a function of its fidelity, in fields as mathematics and physics, the accuracy of the model is used as a function that corresponds to the terms of predicted findings. Animals used in research are also referred to as models, however, fidelity as a function is hard to apply in biological research especially in neuroscience so the term validity is used instead. The reasoning for this, is that there are two complex processes involved in neuroscience; behavioral psychology and neurochemistry which are different processes that correspond to each other. The most successful and widely used animal models in neuroscience are rats and mice, which has been vital for the understanding of the human brain[3]. Another model that has become more prominent during recent years is the zebrafish *(Danio rerio)* a tropical freshwater fish that belong to the family Cyprinidae, it can be found in South Asia primarily in rivers located in India, Bangladesh, and Nepal[4]. The use of zebrafish as a model was introduced in the 1980s by George Streisinger to study genetics[5].

1.2 Visual system and associative learning

1.2.1 Visual system

Research has shown that there are various similarities between human physiology and genetics when comparing it to the zebrafish. According to these studies, around 70 % of the human genes have been found in the zebrafish [6 7]. The zebrafish has during the last decade become a representative vertebrate model in the field of neuroscience. One of the factors is the similarities between the visual system of the zebrafish and other vertebrate models[8]. In addition to this, the zebrafish has the ability to present visual behavior earlier than other vertebrates due to its possession of a duplex retina which anatomy continues to develop three days post-fertilization. This results in a unique opportunity to compare the visual physiology and behavior to the anatomical development of the retinal system [8]. Due to its well-elaborated visual system and learning of aversive events, the zebrafish has become a suitable model for learning and memory[9] The retina structure is comparable in all vertebrates as the structure is well conserved. Although, they do not have similar processing of visual information as their brain anatomy varies. The processing of visual information in all the regions of the brain is not well studied in the zebrafish. Although, it is known that they do not possess a cortex and most of their visual information is processed in the optic tectum. While, in mammals it is only a small portion of the visual information that is processed in the superior colliculus a brain structure that is homologous to the optic tectum. The larger portion of the visual information in mammals is processed in the cortical structures, which the teleosts do not possess [10].

1.2.2 Associative learning

Associative learning is when an animal has to associate a conditioned stimulus with an unconditioned stimulus. Two types of associate learning that can be applied, the classic conditioning also known as the pavlovian conditioning and the operant conditioning. The pavlovian conditioning is when the stimuli presented are not dependent on the action of the animal. While the operant conditioning is dependent on the behavior of the animal as unconditioned stimuli will be presented. In this project operant conditioning is used with a positive reinforcement. Positive reinforcement learning is when there will be no consequences followed for an incorrect response. The correct response will be followed by a positive unconditioned stimulus [11]. The zebrafish has to use it associative memory to acquaintance a certain task with a reward. There have been numerous studies on this type of learning and memory of zebrafish and its cognitive abilities. Due to its well elaborated visual system and learning of aversive events, the zebrafish has become a suitable model for learning and memory, as zebrafish are capable of complex learning due to their advanced sensory and motor system, which has been demonstrated in various tasks such as avoidance learning, spatial alternation task and visual discrimination learning [12]. Although, the zebrafish has proven to be an established model for genetic and development purposes, there are few studies on the role of neurotransmitters behind their cognitive abilities. Studies conducted on rodents exhibits that the dopaminergic agonists and antagonists are extremely important for different types of learning and memory. The same results can be seen when studying the effects of the dopamine receptors in zebrafish which was comparable to mammals. The effect of the D1 and D2 receptors have an important role in the associative memory in zebrafish. The D1 receptor has a role in the acquisition of memory while the D2 receptor has a role in memory retention[13]. The medial pallium of the dorsal telencephalon is the correspondent structure to the amygdala homolog structure in mammals. These structures have an important role in associative learning and memory. Both structures contain the endocannabinoid receptor CB1, studies show that the endocannabinoid system has a prominent role in the storage and retaining of memory[14]. There probably are several other neurotransmitters involved in the memory and learning in zebrafish that have not been studied as cognitive functions are complex and composed of several brain regions.

1.3 Coping style in zebrafish

Psychosocial factors are other important aspects to consider when looking at the learning ability in animal models. There have been numerous of studies showing that individuals of the same species may differ in coping style, this ability differs depending on various factors such as; genotype, previous experiences, environment and various other factors [15,16]. Knowledge on individual stress coping styles and mechanisms controlling these divergent phenotypes are important for our understanding of cognitive processes and stress related effects on behavior and physiology. There have been several standardized methods developed to study stress coping styles in animal models. One prominent method is behavioral testing, in particular on zebrafish as they have proven to be a suitable model for complex behavioral studies [17]. The two divergent stress coping styles that have been described in the zebrafish are proactive (bold) and reactive (shy) coping. Depending on the coping style differences in stress responses profiles and behavioral flexibility are expected. A proactive animal will be using a so-called feed-forward process meaning, that their behavior will be based on previous exposure and experience. They often have low behavior flexibility and a physiological stress response profile dominated by sympathetic activation. By contrast, the reactive animal is characterized by a high behavior flexibility and a more pronounced elevation of plasma glucocorticoids, i.e. cortisol in teleosts and humans. Reactive animals are likely to take their time in learning every part of the environment due to their cautious nature. This divergence in coping style, therefore, leads to differences in learning. A proactive animal is more likely to act based on previously experienced environmental conditions while a reactive fish is more likely to react to environmental cues and adapt to them [18,19]. Novel tank diving test is the most widely used behavioral test "anxiety-like" behavior is often used to study individual variation in coping styles. The individual fish is placed in a tank and a video tracking system is used to analyze its activity. Each tank is divided into three zones labeled as zone 1 (bottom zone), zone 2 (middle zone) and zone 3 (surface zone). A proactive fish is more likely to have a longer duration time in zone 3, which is the top part of the tank, due to its "risk-taking" nature. While a reactive fish will have a longer duration time in zone 1, the bottom part of the tank, due to its "cautious" nature. The individual variation in coping style is also reflected at the organization of the endogenous opioid system. Previous studies using rodents show that the dopamine D2 receptor is an important measurement for stress coping. This can also be seen in zebrafish as the proactive fish have shown to have a significantly higher gene expression of dopamine D2 receptors (*drd2a* and *drd2b)*. Another receptor involved in the reward network as well as in emotional responses is the delta opioid receptor, a higher expression of delta opioid receptors (*oprd1b*) were found in proactive zebrafish compared to a reactive zebrafish[20].

1.4 Replicability crisis in experiments involving zebrafish

Despite the remarkable similarities to human physiology and genetics, there have not been many robust and reliable methods developed to assess the full potential of zebrafish as a model in behavioral studies. The already existing methods according to scientists are difficult to replicate and reproduce, which they refer to as a replicability crisis [21] [22]. Research shows that around 50% of scientists have problems to reproduce their experiment and 70% can not replicate another scientist's experiment. This crisis can clearly be seen in experiments involving zebrafish. A huge factor in the inability to replicate and reproduce an experiment involving zebrafish is human handling, which causes stress and behavioral disturbance in zebrafish. As the handling of the fish varies depending on the circumstances and the method used, this makes it hard to conduct behavioral studies using zebrafish as a model. This is of course seen in other animal models such rodents but it is much more prominent in the zebrafish. This is due to the fact that zebrafish is less domesticated than rodents, even the lab strain of the zebrafish is less domesticated then the lab strains of rats and mice. It is hard to generalize the coping style for a certain group of individuals, because stress handling is extremely individual based. A variation in coping style depending on the anxiety level will be seen not just between strains but also within the same strain. One way to minimize the anxiety level caused by human handling is multiple exposures [21]. This can be done by implementing habution session to minimize negative aspects of the task [23]. By implementing a habution time before the actual experiment involving human handling it may reduce the stress-physiology induced in the fish. However, this must be conducted very carefully as it might lead to the opposite effect by inducing high fear and stress if not handled well [21].

1.5 The use of automatic operant boxes

Automatic operant boxes ("Skinner boxes") are one of the few robust methods that have been developed to study the learning and behavior of zebrafish. The method has shown clear advantages as it requires minimal handling of the fish, as well as several fish, can be studied at the same time. This aspect is extremely important as the method becomes both efficient and cost-effective. By minimizing the factors causing anxiety and stress, the results of the experiments become more reliable and homogenous. This automatic operant boxes are a great alternative to previous behavioral tests that were based on human observations. Interval recording and scoring systems was previously used as observation tools and were based on human observations, and has shown to be quite accurate if the person is trained well[24]. However, this could cause potential bias in results. Hence, the clear advantage of automatic operant boxes is that the method becomes standardized resulting in experiments that can be both replicated and reproduced[25]. One of several variables that can be observed by using automatic operant boxes is learning. The zebrafish has proven to have great ability to learn and memorize[26]. Discrimination learning can be performed in automatic operant boxes to test the learning and associative memory of zebrafish. This report will in particular focus on visual discrimination only using positive reinforcement as a food reward will be used as stimuli.

1.6 Aim

This project aims to study the relationship between proactive and reactive behavior and reversible learning in different zebrafish strains by using automatic operant boxes.

2. Materials and Methods

2.1 Animals and animal maintenance

All of the zebrafish used in this study were adults, bred in Uppsala University. They were maintained according to Uppsala University guidelines for animal welfare, ethical permit 5.8.18-10125/2018. The fish were fed three times a day with both dry feed (Sparos, Olhão, Portugal) and rotifers in the morning and afternoon and with only dry feed at lunch. The fish were separated according to strains. Each strain was put together in a tank of the size (20 L x 23 W x 13 H cm) that was connected to a rack system (Aquaneering, San Diego, USA) while they were not being tested individually. Each tank also contained artificial plants to mimic a natural habitat. The room and water temperature were maintained at 27-29 ° C, around 8.5 pH and conductivity 480.3 mS/cm.

2.2 Instruments and material

2.2.1 Electronic p-chip

The p-Chips (Pharmaseq, USA) that are used to tag the fish have a size of 500 x 500 micrometer and 100 micrometers thick. Each p-Chips provides a unique series of numbers (ID). The photocells in the p-Chip provide power to the electronic circuits when it's illuminated by light. Each unique p-chip has an antenna that transmits the identification number when sensing pulsation, provided from the light in the p-chips reader. There is a variation of the magnetic field surrounding each chip that is detected by the coli in the p-chip reader. The coli will analyze the chip and decode it to a serial number[27].The p-Chip reader was connected to a computer that read the identification number of the p-chip and display it on the computer. The p-Chips reader consists of a power regulator, a USB 2.0 microcontroller, air coil pickup that is connected to a radio-frequency receiver, laser diode with programmable laser drive, a Field-Programmable Gate Array and an optical focusing module. A red laser light emits ranging between 5-60 mW of optical power at 660 nm when placed to the scan the p-chip, the identification number was determined by the radio-frequency signals [27].

2.2.2 Ethovision

Ethovision (Noldus, Netherlands) is a video tracking system used to analyze animal behavior. A video camera is used to distinguish the fish from its background and track its movements. The video recording is displayed on the computer by a frame grabber. The inbuilt software will then analyze each frame and distinguish the animal from the background. When the software distinguish the animal from the background, the required data can be extracted from the software [28].When running experiments in several arenas, the user can assign each arena position and measurements before starting the experiment. By predefining the arena settings, the software will not be able to video track the undefined areas in the arena. This will minimize interferences in the experiment. A signal from the video camera makes it possible to display the arena set up on the computer screen so that the user can draw the experimental setup and measurements they want to include in the video track. The arenas can also be calibrated to obtain an accurate video image. After running all the trials, the required video files data can be acquisitioned [28]. The tracking of the object will be displayed on the computer and any errors done in the tracking can be detected and retracted manually in the software.

2.2.3 Zantiks- an automatic operant system

The Zantiks AD (Zantiks, Cambridge, UK) is an automatic operant box that can be used to study numerous different behaviors, including learning in zebrafish. The fish are placed in chambers of size (14 L x 20 W x 15 H cm) which can be placed in the Zantiks units. Each fish can be tracked using an infrared backlight that the chamber is placed on and an integrated infrared camera is also placed above the chamber to record the experiment. Each unit is connected through a LAN cable using a wireless router which can be accessed through the computer by searching for the IP address on the browser. Each IP address will give the user access to the control setting for the respective unit. Several units can be assessed through the same computer[25].

In this experiment, visual discrimination will be used as stimuli. The stimuli are in the form of color light and can be seen in the LED screen which the chamber is placed on. Depending on the experimental setup required, different sample scripts are available that can be modified into the desired function. The system has the ability to coordinate the 2D position of the fish by using the x and y coordinates to locate the fish. This is done by using the infrared camera that is placed above the chamber and the infrared backlight that the chamber is placed upon[25]. All activity can be monitored during the experiment by using the live camera in the control settings for each unit. Variables such as, stimuli, feeding machine, color light, trials and length of the experiment are according to the selected pre-modified script[25].All data results will be saved automatically after each trial in the form of a CSV file that can be accessed through excel.

3. Experimental procedures

3.1 Separation of sexes

The three strains of zebrafish, TL, WT and Spd were obtained and separated based on sex. This was done by examining the body shape of the fish. Female zebrafish have a more rounded body shape while the male zebrafish has a slender shape. The fishes that were assumed to be females where set aside in separated tanks of size (20 L x 23 W x 13 H cm) based on strains, WT (n=18), TL (n=17), Spd (n=16). All tanks were filled with 6 liters of water from the zebrafish rearing systems.

3.2 Identification tagging

The fish was transformed with a net from the home tank and placed under a microscope and injection containing a small p-Chip with a size of 500 x 500 x 100 μm is used to tag the fish. The chip is inserted in the fish with the injection into the trunk's dorsal muscle. Each fish was weighed on a scale and the length of the fish was also measured. The inserted p-Chip was then scanned by a p-chip reader that was connected to a computer where the unique identification number was displayed. Along with the identification number, strain, length, as well as weight were documented for every individual fish. This procedure was done for all the fish (n=51). The fish were then placed in three different tanks with the size (20 L x 23 W x 13 H cm) according to their strain (n_{Spd}=16, n_{TL}= 17, n_{WT}=18). The three tanks contained artificial plants to minimize aggressiveness.

3.3 Novel tank diving test

All fish (n=51) were allowed to recover for eleven days to completely heal from the injection before the novel tank diving test. There was a shelf with three rows and two tanks with a size of (8 L x 21 W x 12 H cm) placed on each row, in total each trial contained six arenas. An integrated IR camera was placed in front of the shelf and an IR board was placed behind the shelf. All the tanks had three white lines on them to identify the three different zones in the tank *(see figure 1A)*. The video recording was displayed on the computer by pressing frame grabber. Each tank was then adjusted to fit within the IR board and calibrated with the correct measurements of the tank. Each individual arena was then modified by using the drawing option to fit the experimental set up that could be seen through the video camera connected to the computer. After adjusting the set-up, two of each strain was put randomly into the tanks that were filled with 4-liter water and the video recording started when the last fish was put in. After the test the length and weight for each fish was measured. The whole procedure applied can be seen in *figure 1B*. The detection setting was set to 12 minutes per trial, in total 9 trials where recorded. After all the recordings were done the video files data could be acquisitioned. After acquisition, the data all the video files that had an error in their tracking was retraced manually. The desired data point was preselected before the recording; Distanced moved center point (s), Velocity center point means (cm/s), Movement not moving/ center point cumulative duration (s), Movement moving at the bottom/ center point cumulative duration (s), Movement moving at the top/ center point cumulative duration (s). Each fish was removed from the arena with a net and the p-chip on the fish was then quickly scanned with the p-chip reader and placed in its home tank. This data points were extracted from Ethovision and analyzed in Graphpad Prism 8 and Jamovi.

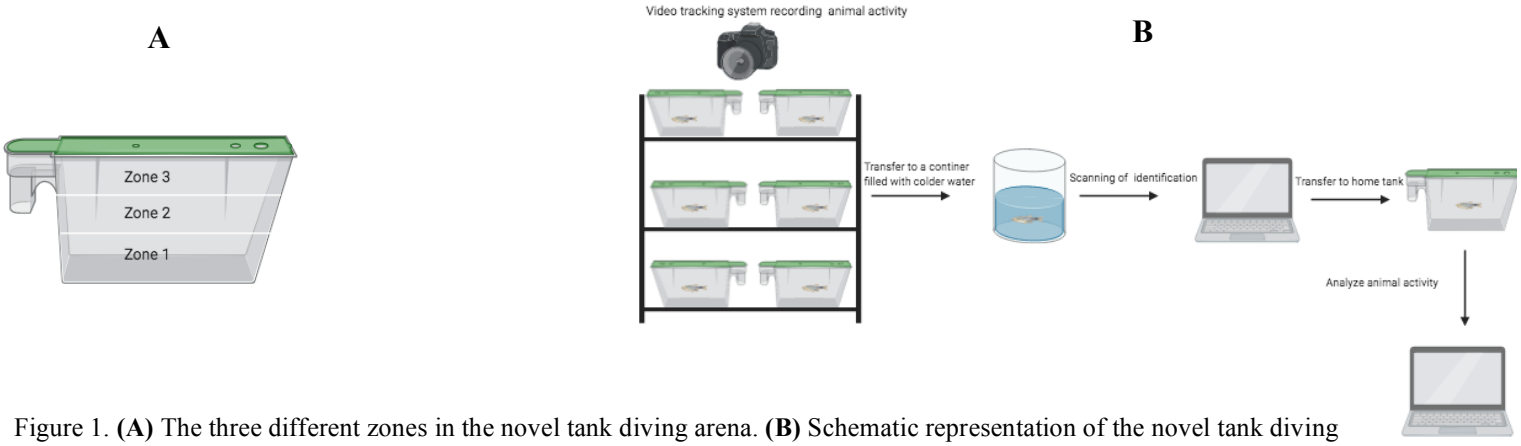

Figure 1. **(A)** The three different zones in the novel tank diving arena. **(B)** Schematic representation of the novel tank diving test.

3.4 Automatic operant box

3.4.1. Habituation

All the fish were habituated to the arena for 20 minutes/day over five days in prior to any experiment. Each fish was placed in the arena with a net from the home tank and placed under a microscope. Thereafter, the fish was placed under the p-chip reader and the individual identification number was displayed on the computer. The fish was quickly placed into the Zantiks arena (Fig.2) with a wet paper towel. Four arenas could be observed at the same time each containing one fish. All the arenas were filled with ~1.5 Liter water. During this habituation time, the fish was supposed to get used to the experiment. As well as trying to reduce the stress induced by human handling.

3.4.2. Randomization

The arena placement of the fish was made randomly to avoid bias. Fish was randomly selected from each strain and then scanned for its identification number. An equal amount of placement in each arena was tried to be obtained for all strains to minimize the arena effect and to further randomize the arena placement.

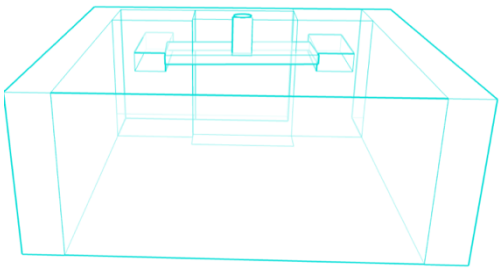

Figure 2. Schematic sketch of the Zantiks arena.

3.4.3 Shaping step

Each Zantiks unit was connected through a LAN cable using a wireless router which was accessed through a computer by searching for the IP address on the browser, to enable the control setting were all four arenas could be observed using a live camera. For this phase the script pav_choice was used *(see appendix)*. Each individual fish (n=36) was moved from the home tank with a net and placed in a container filled with water for five seconds. The fish was then placed on a wet towel and scanned with the p-chip reader. When the identification number was displayed on the screen the fish was quickly put in the Zantiks arenas that were filled with 1.5-liter water. The identification number, arena position, and strain for each fish were documented. The fish was then quickly put back into its home tank. The script pav_choice was defined to run for 20 minutes consisting of 20 trials were the initiator light stays on for 20 seconds and the feeder for 20 seconds. The fish had to associate the feeder mechanism noise and the white light with a food reward. The conditioned stimulus was presented as a white light and was followed by an unconditioned food stimulus. The fish must swim into the white light to be rewarded with the food. The criteria set for this particular script was 80% correct trials, two days in a row meaning that the fish has to be rewarded food 16 out of 20 trials to move on to the next script. When the 20 min trials were over a CSV file and video recording could be saved. The fish were then removed from the Zantiks arenas with a net into the home tank. This method was also conducted in a different way day 13, 14, and 15. The fish was moved from the home tank with a net directly to the Zantiks arena and the experiment started. After the 20-minute session was over the fish was removed with a net from the Zantiks arena and placed on a wet towel. The p-chip on the fish was scanned and when the identification was displayed on the computer screen the fish was quickly put back into its home tank. The script pav_choice was also used during these three days.

3.5 Data analysis

Statistical analyses were performed using GraphPad Prisma 8 to analyze the output parameters from Ethovision and Zantiks. The output parameters that were observed from ethovison are: duration time moving at the top, duration time moving at the bottom, duration time not moving, frequency in each zone. All strains and their paired parameters were assessed by one –way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. The Zantiks output parameters assessed are the total trials and correct trials. Group analyses were done using ANOVA followed by Tukey's multiple comparisons test. The p-value system used is GP with a significance level 0.05 and confidence level 95 %. The GP system refers all p values less than 0.0001 as $p < 0.0001$. The length and weight of the fish before the novel tank diving test was summarized by using Jamovi.

4. Results

4.1 Novel tank diving test

The novel tank diving test is used to behaviorally characterize in zebrafish. The result from the novel tank diving test shows a significant diverge between the three strains of zebrafish in several parameters. Interestingly, there is a significant difference in the cumulative duration at the bottom of the tank (zone 1). The Spd significantly differs from the TL ($p < 0.0001$), similarly the WT significantly differs from the TL ($p < 0.0001$). However, there is no significant difference in cumulative duration at the bottom between the Spd and the TL (Fig.3A). Similar results can be seen when observing the cumulative duration at the top of the tank (zone 3). Furthermore, here is no significant difference between the Spd and WT, however, the Spd significantly differs from the TL ($p < 0.0001$) as well as the WT significantly differs from the TL ($p = 0.0003$) (Fig.3B). When looking at the cumulative duration for not moving at bottom (zone 1), WT had the longest duration while the Spd had the shortest. However, there is no significant difference between the three strains (Fig.3C). Overall the TL spends most of its time moving at the top of the tank, while the TL spends it moving at the bottom of the tank.

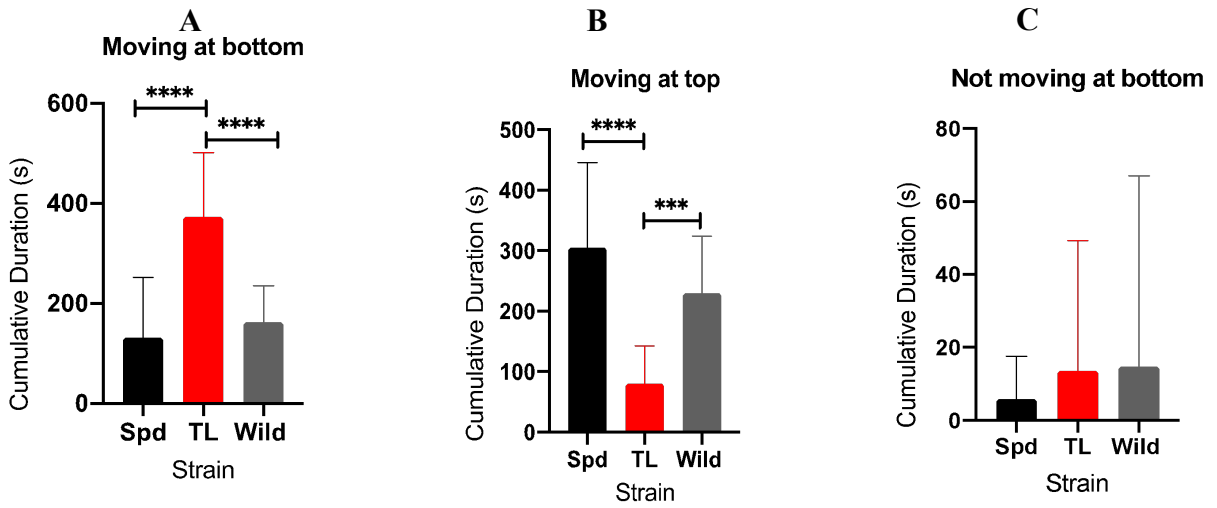

Figure 3. The cumulative duration (s) exhibited in each zone from the novel tank diving test. **(A)** The cumulative duration time at the bottom of the tank (zone 1) for each strain. **(B)** The cumulative duration time at the top of the tank (zone 3) for each strain. **(C)** The cumulative duration time exhibited not moving at the bottom of the tank (zone 1).

The p-value system used is GP: ***= $0.0001 \geq p \leq 0.001$,****= $p < 0.0001$. (Tukey's multiple comparisons test)

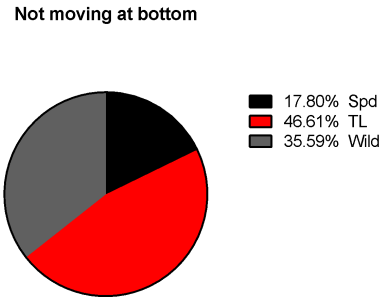

Figure 4. Percentage duration time not moving at all in the bottom for the three strains.

The frequencies in each zone also differ depending on the strain. There no significant difference between the TL and WT in zone 1, while there is a significant difference when comparing the Spd to TL and WT (p= 0.0278, p= 0.0179) (Fig.5A). However, in zone 2 there is a significant difference between TL and WT (p= 0.0056) (Fig.5B). Lastly, when looking at the frequency in zone 3, Spd and WT significantly differ from the TL (p =0.0034, p= 0.0014), while there was no significant difference for the frequency in zone 3 for the Spd and WT (Fig.5C).

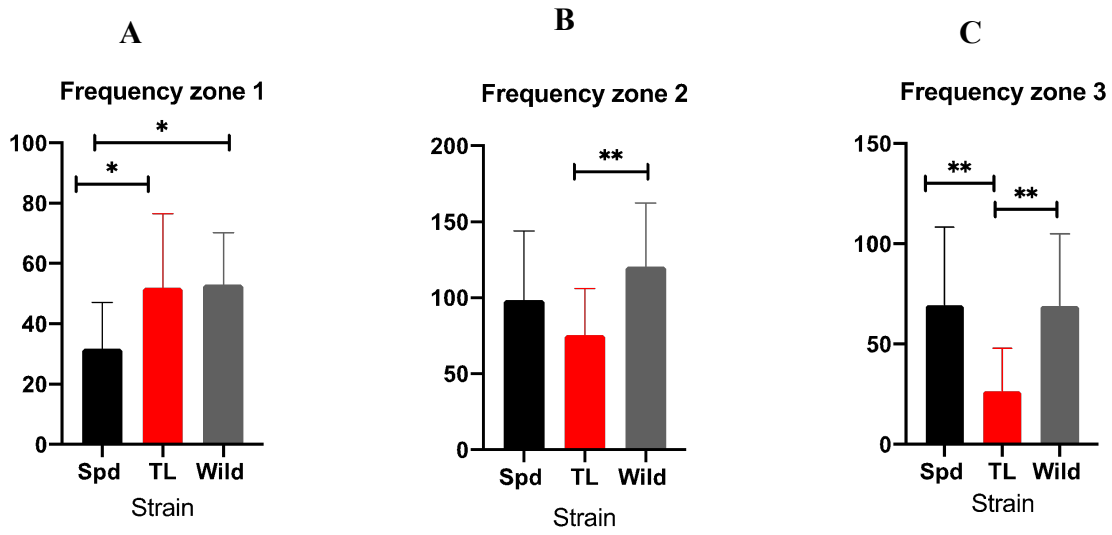

Figure 5. The frequency in each zone form the novel tank diving test. **(A)** The frequency at the bottom of the tank (zone 1). **(B)** The frequency at the middle part of the tank (zone 2). **(C)** The frequency at the top of the tank (zone 3).

The p -value system used is GP: * = $0.01 \geq p \leq 0.05$, **= $0.001 \geq p \leq 0.01$ (Tukey's multiple comparisons test)

Other parameters that were observed are the distance moved in the arena as well as the mean velocity for each strain. However, the three strains do not indicate on any significant difference in velocity between them (Fig.6A). Equivalent results can be seen in the box plot when comparing the mean distance moved in the arena for each strain, there is no indication on a significant difference in the distanced moved in the arena between them (Fig.6B).

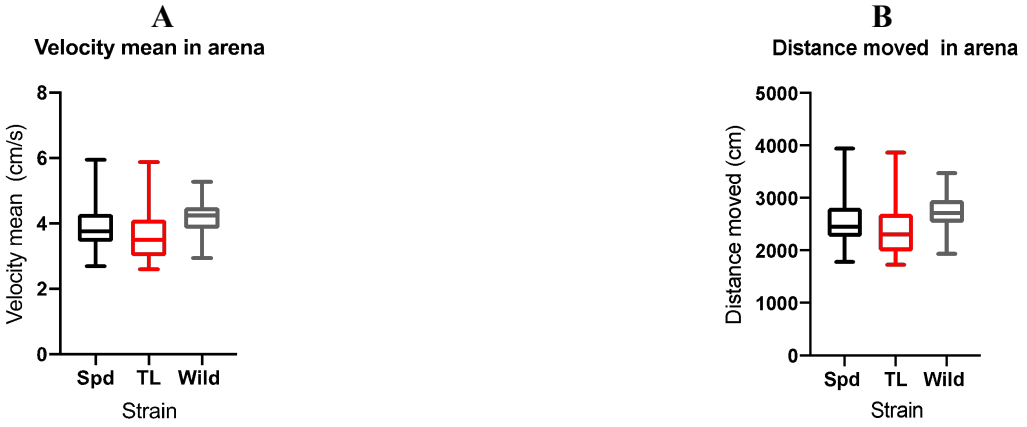

Figure 6. The velocity and distances moved in the novel tank diving test. **(A)** The mean velocity (cm/s) for each strain is displayed. **(B)** The distanced moved (cm) in the arena for each strain can be observed.

4.2 Zantiks

The mean percentage of correct trials for a period of 12 days for each strain is displayed in *figure 7*. The maximum and minimum correct trial s can be seen for each strain displayed in *figure 8* and *table 1.* The maximum percentage correct trial s, for the Spd was 85% and 50 % for both TL and WT (Table.1). The Spd strain had the most correct trial s compared to the other two strains (Fig.8) with a mean value of 11.93% (Table.1). The mean value for the TL (3.948 %) and WT (3.707%) was relatively the same (Table.1). Spd is significantly different from TL ($p<0.0001$) and WT ($p<0.0001$). There is no significant difference between TL and WT. There were only two fish that passed the criteria of 80% correct trials, both of them are Spd (Fig.9). There were not any fish that reach the required criteria in the three-day period were the p-chip identification was done after the experiment (Fig.10).

Table.1 Descriptive statistic of the percentage of correct trials, maximum and minimum correct trials reached from the Zantiks experiment.

Strain	Spd	TL	WT
Minimum correct trials	0.000 %	0.000 %	0.000 %
Maximum correct trials	85.00 %	50.00 %	50.00 %
Mean correct trial	11.93 %	3.948 %	3.707 %
Std. Deviation	17.21	8.277	8.622
Std. Error of Mean	1.498	0.5214	0.7112

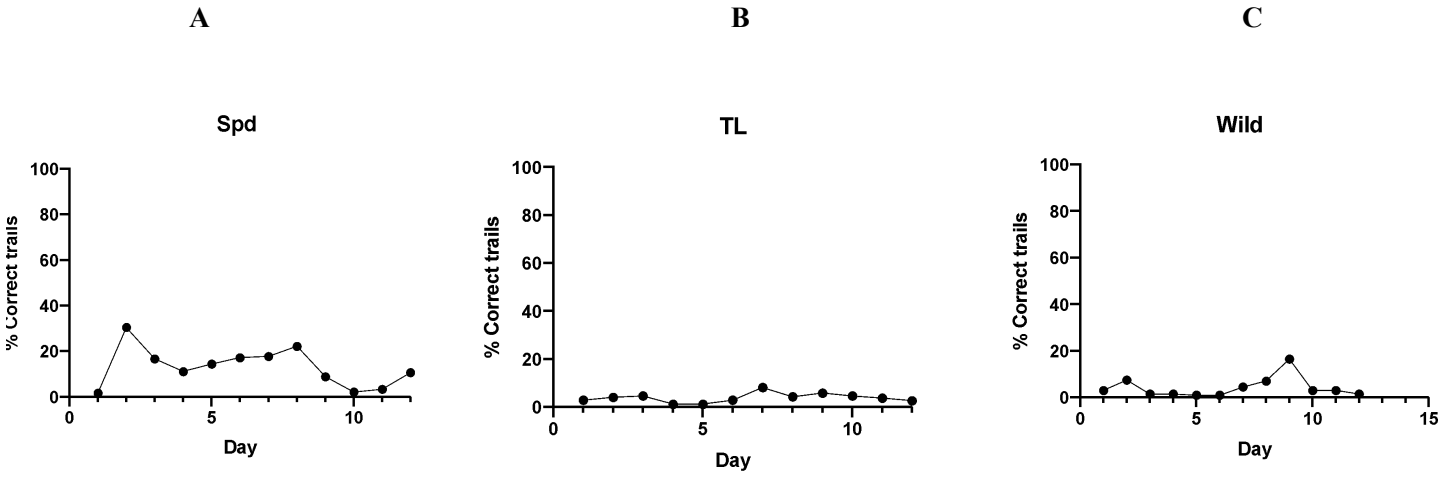

Figure 7. The mean percentage of correct trials for a period of 12 days for each strain. **(A)** The mean percentage correct trials for the Spd over a 12-day period. **(B)** The mean percentage correct trials for the TL over a 12-day period. **(C)** Mean percentage correct trials for the Wild over a 12-day period.

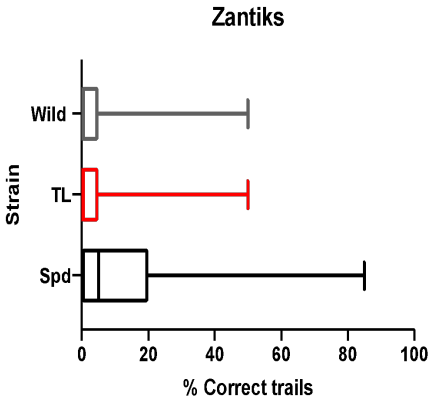

Figure.8 The total median percentage of correct trials as well as the upper and lower quartile can be observed for each strain.

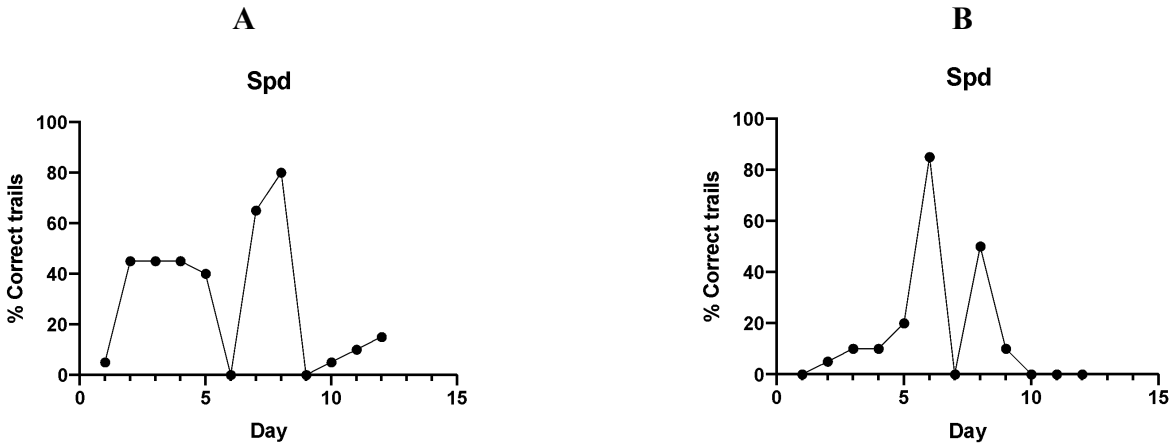

Figure 9. The mean percentage of correct trials for a period of 12-days for the two Spd that passed the required criteria of 80 % correct trials. (A) The Spd passed the criteria after 7th day of experiment. **(B)** The Spd passed the criteria at the 6th day of experiment.

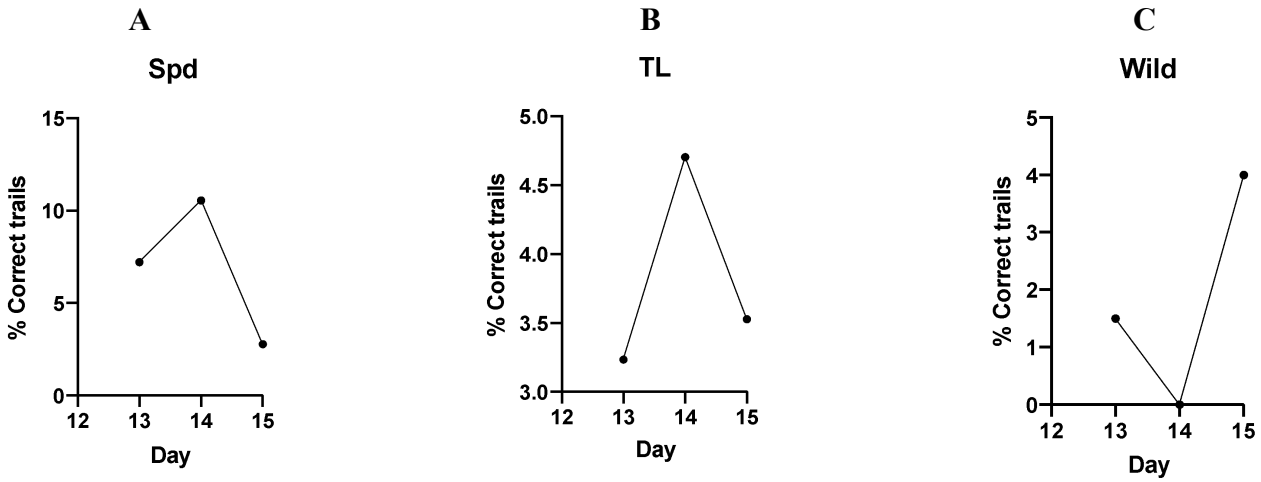

Figure 10. The mean percentage of correct trials for a period of three days for each strain with minimized human handling. **(A)** The mean percentage of correct trials for the Spd during a 3 day period. **(B)** The mean percentage of correct trials for the TL during a 3 day. **(C)** The mean percentage of correct trials for the Spd during a 3 day.

5. Discussion

5.1 Behavioral characterization of zebrafish

The main findings from this study suggest that there is a difference in coping style depending on the strain of zebrafish. Several parameters were measured by conducting a novel tank diving test to determine the coping style for each strain. These parameters showed that there is a significant difference in movement and frequency depending on the zone of the tank. The Spd and WT had a significantly lower cumulative duration time in the bottom zone compared to the TL (Fig.3A). A similar result could be concluded from the movement at the top of the tank were TL had the lowest cumulative duration and the Spd had the longest duration time While, no statistically significance could be found between Spd and WT (Fig.3B). The frequency in each zone also varied, surprisingly both the TL and WT had roughly the same frequency in zone 1 (Fig.5A), but a significant difference in zone 2 (Fig.5B). As expected, the TL had the least frequency in zone 3 while there was no significance between Spd and WT (Fig.5C). By only comparing the movement at the top and bottom part of the arena, the coping style of the TL could easily be concluded to a reactive (shy) coping. A reactive fish as mentioned earlier is characterized by high behavior flexibility and high physiological stress response profile due to their cautious nature. This can clearly be seen for the TL as it has the longest duration time in zone 1 and the shortest in zone 3. As predicted from the duration times at each zone it also has the highest frequency in zone 1 compared to the other two strains. However, there seems to be no significant difference between the frequency in zone 2 between the TL and the Spd making it the only parameter that the Spd and TL do not significantly differ in. An explanation to this is probably the high flexibility in the TL as it is trying to adapt cautiously to the tank. A high physiological stress response profile is another factor that can be seen for the TL which exhibited the longest duration time by not moving at all at the bottom (Fig.4). This is a typical behavior seen when a fish is under a lot of stress. The opposite results were seen for the Spd as it had the shortest duration time at the bottom of the tank and the longest duration time at the top of the tank. As well as it had the lowest frequency in zone 1 and the highest in zone 3. All these parameters indicate a proactive fish, which often have low behavior flexibility and low physiological stress response profile. This can be further concluded by looking at the duration time for not moving at the bottom, were the Spd exhibits the shortest duration time indicating that it has a low-stress response (Fig.4). However, the coping style for the WT was a bit harder to conclude as it seems to have the characteristics of both a proactive and reactive coping style. The WT has the same behavior as the Spd with a shorter duration time at the bottom compared to the TL and long duration time in the top of the tank. By only observing these parameters it seems as though, the WT displays more of a proactive behavioral profile. However, the WT seems to have a high frequency in all three zones and a significant difference can be seen in zone 1 between the WT and Spd (Fig.5A). It also moves the longest distance in the arena (Fig.6B). This indicates that the WT probably has high flexibility as its frequency in all zones is relatively high. This can also be observed in nature when looking at migration where proactive fish do better in stable conditions while reactive ones are more successful during migration as they adapt to the new environment [16]. The WT also seems to have a long duration time not moving in the tank compared to the to the Spd indicating a more pronounced stress response (Fig.4). This leads to the conclusion that the WT probably displays a more reactive coping style.

Previous research that has used the novel tank diving test on the Spd and WT presents similar results as this project. WT according to these studies demonstrates more anxiety and less bold behavior than the Spd making it a reactive fish. The Spd is bolder than the WT and was compared to the AB strains which is a known proactive (bold) strain, similar behavior was observed [29]. Additionally, is important to point out that the Spd is a strain carrying a mutation in the fibroblast-derived growth factor receptor 1A gene (*fgfr1a-/-*). This mutation of the gen is the reason for the bold behavior exhibited. According to research this modification of the genetic locus, *fgfr1a,* is the component behind numerous behavioral traits seen in the Spd such as an increased aggregation, boldness, and risk-taking [30]. An important factor to consider when determining the coping style in zebrafish is that the result is dependent on the strain, sex, and the behavioral test used. The most common behavioral test used to characterize the coping style of zebrafish is the novel tank diving test, shelter test, and the scototaxis test. Studies have shown that these tests do not necessarily give the same results. When comparing both sex and coping style of three strains Spd, WT, and AB by using the three different behavioral test a difference in results could be seen. In the shelter test, the male AB had higher activity than the male Spd but their duration time and frequency in the open area did not indicate any difference in boldness. The same test was conducted in the novel tank diving test, here the results was reversed the male Spd had higher activity than the male AB. The Spd also displayed a more risk-taking behavior having a higher frequency and movement at the top of the tank then the male AB. When comparing the difference in sex using the novel tank diving test, the male Spd appeared to be bolder than the female. While in the scototaxis test no significant difference could be found between the sexes. However, the outcomes of the WT was quite the same despite sex and test, they showed a similar result regardless method. The WT displayed a lower activity level in all tests as well as no difference between the male and female WT could be detected [29]. These differences in test and sex should be taken into consideration as the sex of the zebrafish that was used in this paper was only determined by visual inspection and was never confirmed by dissection.

5.2 Visual learning in automatic operant boxes

The results from the Zantiks experiments show that there is no tendency of learning in any of the strains. The criteria to succeed the script was set to 80% correct trials two days in a row. As seen in the results, the Spd had the highest mean percentage correct trials at 11.9%, which is roughly three times the results observed for the TL and WT that respectively had 3.9 % and 3.7 % (Tabel.1). The Spd was the only strain that had any fish that passed the criteria but no tendency of learning could be seen (Fig.8, Fig.9). The Spd that had the highest percentage of a fish passing at 85 % correct trials while both the TL and WT had a maximum at 50 % correct trials (Table.1). In the first 12 days after the habitation time, the fish was scanned before they were put in the Zantiks arena. There was no tendency of learning within those 12 days (Fig.7). The reasoning for this was thought to be the human handling which is involved when scanning the p-chip. This resulted in a modification of the method where the scanning of the p-chip was done after the experiment. This was conducted during a 3-day session but the same results could be seen for these trials as no tendency of learning could be observed (Fig.10).The used script that was supposed to have the fish associate the feeder mechanism noise and the white light with a food reward, by understandig this the fish would have formed an associate memory and learned that the noise “equals” food . The fish had to swim into the white light to trigger the feeder mechanism so it could be rewarded with food. Looking at previous research a variation in result can be seen. In some studies, it only took the fish 6 days to associate the feeder mechanism noise and the white light feeder with a food reward [25]. While another study used Pavlovian conditioning, a method using a light stimulus with food

or animated shoal as a reward. The fish had to trigger the light stimulus to be rewarded with food, this took the fish 3 days to reach 74 % correct trials. In this same study, they also observed visual discrimination learning in zebrafish. The fish was presented two color stimuli were one stimulus was set as the correct one and the other incorrect. A tendency of increased learning could be observed for the first 10 days [31]. By comparing other studies to the result presented in this paper, no progress of learning could be further concluded in our case. However, it is important to mention that the criteria set in other studies are quite unclear. There is no specification on the set criteria to pass a script. In the current study, the criteria were set to 80% correct trials two days in a row. The reasoning behind this was to ensure that there was an actual learning ability and not a coincidence of passing the criteria. Surprisingly, this was not the case for other studies as they only mentioned the percentage correct trials that were needed and not how many times the fish had to pass this criterion.

5.3 Memory Retention

There are rarely any studies on memory retention in zebrafish. This made it hard to conclude if the reasoning behind the fish not learning could be because the time interval between each session may have been too long. Another factor as mentioned earlier that also could have caused that no progress in learning was observed could be the human handling involved in the scanning of the p-chip. This of course was taken into consideration before starting the actual experiment by implementing a habution time involving human handling. Both the TL and WT were characterized to be reactive fish, resulting in that they have a high physiological stress response profile. The human handling might have caused a higher stress response in the TL and WT compared to the Spd. Hence, why their coping style may be a factor as to why there is such a difference in performing compared to the Spd who roughly performed three times better. The result seen between the Spd and the other two strains is significantly higher. This can be an indication that the Spd has a faster learning ability then the two other strains. Although, it does not come as a surprise as the boldness in the Spd probably is an advantage in the short run. Memory retention and the formation of memories are important for an animal. By forming a memory of food locations and potential threats the animal will be able to survive for a longer period. There has been numerous research on the memory retention of mammals but the research done on fish is less [32]. Even though having a better understanding of the cognitive abilities in the zebrafish would lead to it being a prominent animal model in screening anxiolytic drugs [33]. A reason for the few studies that have been done on fish is because of their small brain size. A fish brain has an average size of 1/15 compared to a bird with a similar body size [34]. However, despite its small brain size the fish has as mentioned earlier shown to have a great learning ability which has been observed in several behavioral tests. A memory test that has been widely used in other animal models such as rats and mice is the one-trial memory test where the episodic like memory is used. The animal is exposed to a pair of identical objects and a pair of non-identical objects. The measurement in this test is based on the tendency to explore a novel object if the animal has more tendency to the novel object it is considered to be a memory performance. This test has also been applied to the zebrafish where they first were being exposed to one of the objects. After familiarization with the object, a novel object was presented in a different sector of the tank. In this particular experiment, the fish spent more time in the sector with the novel object. Indicating that the fish probably recognized the first object, hence it shows curiosity to the novel object. This test was also performed during three different time intervals (2 h, 6h, 24h) to observe memory retention. The result showed that the time interval did not make any

significant difference, indicating that there is no memory decay in object recognition over a 24 h time interval [35].Comparing the one-trial memory test to the Zantiks experiment the learning or recognition span of a zebrafish is quite broad. Although, the memory types in the one-trial memory and the Zantiks are different, a comparison can be drawn. The one-trial memory test was simply based on the recognition, while the Zantiks experiment was based on learning and recognition as well as a food reward was given. The Zantiks experiment should have led to a stronger response because of the positive reinforcement as well as the fish not being fed during any other time outside of the experiment. Surprisingly, it seems that the hunger and multiple exposure did not lead to the response expected. This may be due to the fact that the time interval between each session might have been longer than 24 h since they were randomly selected during the 6 h experimental time per day. However, that should not out way the hunger and memory formation that should be quite adamant. Another reason for the change in method except to minimize human handling was to have more trials within a day for a fish that passed the criteria. Implementing more trials per day would have clarified if there was an actual learning and not a coincidence of passing the required criteria. This can be seen in another experiment where guppies were used to observe their ability to revisable learning and the effect that brain size has on it. In the study they had one group with small-brained guppies and one group with large-brained guppies. The experiment set up was similar to the one in this paper where they have a color stimulus with a food reward. The results for the two groups had no significant difference. Unlike my experiment, they had a criterion of 80% correct trials per session and they had to pass six consecutive sessions in a row within a day[34]. A similar method construction for my experiment combined with minimizing human handling might have resulted in a tendency of learning.

6. Conclusion

6.1 Behavioral characterization of zebrafish

In conclusion, differences in coping styles can be found between different zebrafish strains in the novel tank diving test. The difference in the behavioral profile can be seen as the strains display a different amount of flexibility and physiological stress response profiles. Although, it is important to highlight that the behavioral test used in this paper is the novel tank diving test and that the behavioral characterization of the zebrafish can vary depending on test, sex, and strain. Another factor that is of importance is that the sex of the fish used in this paper was based on visual observation of external characters and has not been confirmed by dissection. Further studies that were supposed to be done in this project that could be interesting to look in to in the future is the different level of monoamine neurotransmitters in the brain. To further understand the role of different neurotransmitters would be a good way to confirm that the characterization of the zebrafish is accurate. This could be done by dissecting the brain for each fish and using HPLC or qPCR analyses to confirm the levels of different monoamine such as dopamine and serotonin in the brain.

6.2 Learning in zebrafish

There was no tenancy of learning for any of the strains in the Zantiks experiment. This could have been caused by several factors but is probably because of the human handling that was involved before the actual experiment. This may have induced anxiety and fear in the fish resulting in that they were not performing as well as they could have. This was however changed the last three days of the experiment as the human handling was implanted after the experiment. Unfortunately, this could not be further looked into due to the circumstances of spring 2020. This method minimizing human handling, if it was possible, would have been a greater strategy to use from the first day. The reason for not doing this was to know which individual fish that being tested. By knowing this before the experiment the score from the previous day could be looked up before the experiment so that the correct script could be chosen for each fish. There were five different scripts with different levels of learning tasks that had been prepared. Unfortunately, only the first level was used in this project. Another factor that could have caused that they were not learning is the time interval between each trial as the memory retention in zebrafish is quite unknown is hard to tell if the lack of performing is due to a short memory span. There have not been many studies done on all the brain regions of the zebrafish that are involved in learning and memorizing. Although, the zebrafish has proven to be an established model for genetic and developmental there are few studies on the role of neurotransmitters behind their cognitive abilities. The zebrafish is comparable to rodents in many different behavioral aspects e.g., spatial alternation task, and visual discrimination learning, due to its well-elaborated visual system and the learning of aversive events exhibited by the zebrafish. The results shown in various tasks such as avoidance learning, spatial alternation task, and visual discrimination learning is comparable to rodents. This suggests a good face validity of the associative learning performance. The effects of the dopamine receptors in zebrafish are also comparable to other mammals suggesting good construct validity. However, for the zebrafish to be able to compare with other well-used animal models such as rats and mice there have to be further studies done on the neurobiological mechanism behind visual learning and associative memory. This to fully understand the learning and memory retention in the zebrafish. The understanding of the this would result in the zebrafish being an exceptional model that can be used in screening for a different types of neurological disorders. It will also be an efficient model to screen new drug substances as well as being a great model to facilitate the development of anxiolytic drugs and novel psychotherapeutic drugs. The zebrafish as a model in learning would overall be an exceptional addition to the drug and medical industry.

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Appendix

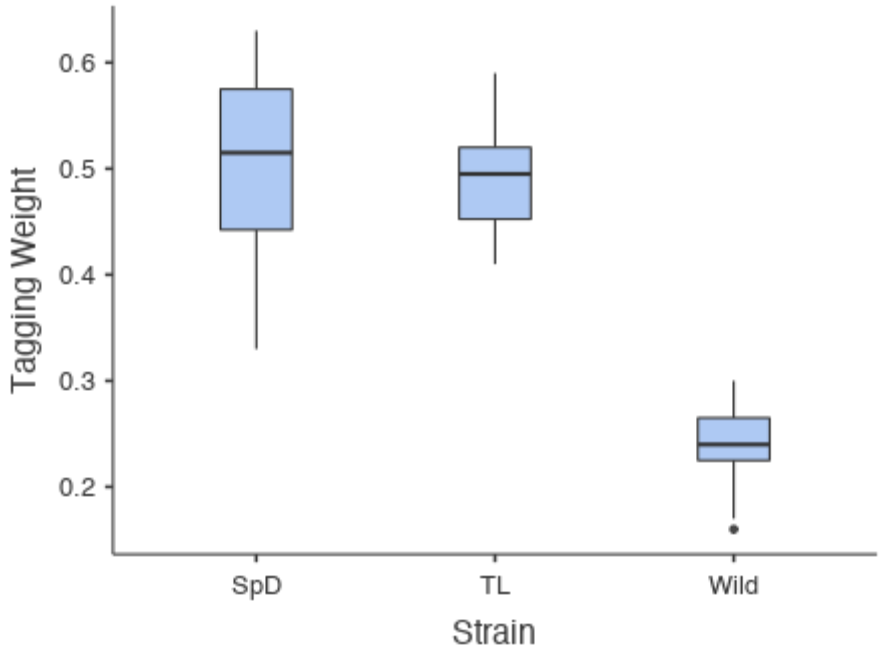
The script used for the Zantiks experiment:

```
NCLUDE zsys

DEFINE CIRCLE 1
DEFINE SQUARE 2

# define experiment requirements
DEFINE FEED_TIME 20
DEFINE LIGHT_TIME 20
DEFINE INTERTRIAL_TIME 60
DEFINE HABITUATION_TIME 0#300
DEFINE TRIALS 20

# define the animal model tracking requirments (dependent on animal size)
SET(DETECTOR_THRESHOLD,2)
SET(SEARCH_DISTANCE,50)
SET(SEARCH_STEP,7)
SET(FILTER_RADIUS,15)

# define auto reference tracking requirements
SET(AUTOREF_MODE,1)
SET(AUTOREF_TIMEOUT,10)

# set light stimuli coordinates on the screen
SETLIGHT(LIGHT7,SQUARE,150,500,140)

SET(COUNTER1,COUNTER_ZERO)
SET(COUNTER2,COUNTER_ZERO)

# load detector asset
LOAD(DETECTORS,"fullyzoned_5hole.bmp")


ACTION MAIN

   LIGHTS(ALL,OFF)

   LOGCREATE("RUNTIME|TEXT:|TEXT:|TEXT:TRIAL")
   LOGAPPEND("TEXT:TIME_OF_FEED|TEXT:FEEDER_VISIT")
   LOGAPPEND("TEXT:TIME_OF_FIRST_VISIT")
```

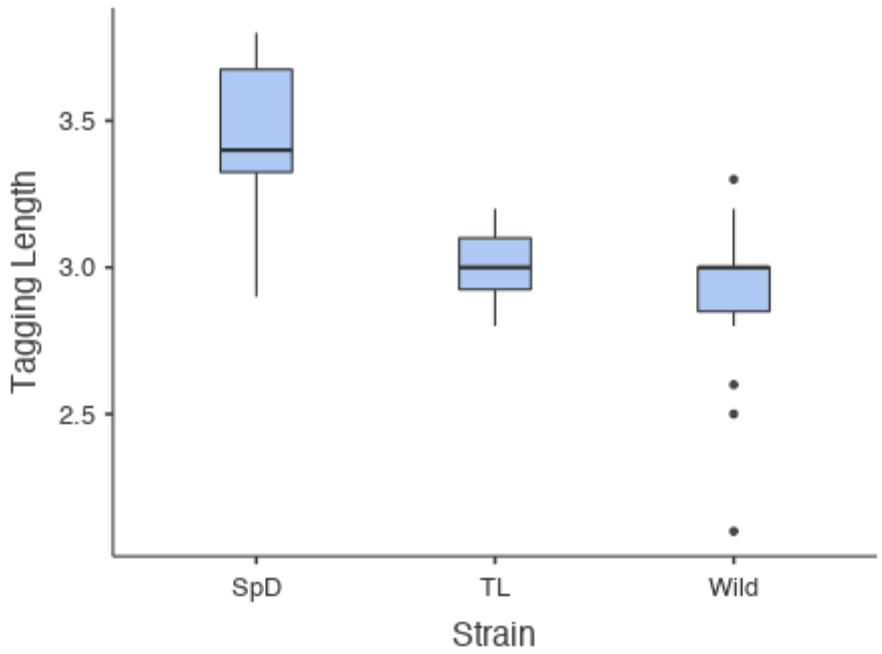
```
   LOGAPPEND("TEXT:FEEDER_ENTRIES")
   LOGAPPEND("TEXT:TIME_AT_FEEDER")
   LOGRUN()

   AUTOREFERENCE()
        WAIT(HABITUATION_TIME)              #300
        VIDEO(1200,"Pavlovian2choice")

   LOGDATA(DATA_SNAPSHOT,"BEGIN")
   INVOKE(INITIATOR,TRIALS)                          #30

        LOGDATA(DATA_SNAPSHOT,"AFTER")
        LOGDATA(DATA_SELECT,"BEGIN")
        LOGDATA(DATA_DELTA,"AFTER")

        LOGCREATE("TEXT:|TEXT:|TEXT:|TEXT:---SUMMARY---")
   LOGRUN()

        LOGCREATE("TEXT:|TEXT:|TEXT:|TEXT:TOTAL_TRIALS")

   LOGAPPEND("TEXT:|TEXT:TOTAL_FEEDER_TRIGGERS")
   LOGAPPEND("TEXT:TOTAL_ARENA_DISTANCE")
   LOGRUN()

   LOGCREATE("TEXT:|TEXT:|TEXT:|COUNTER1|TEXT:")
   LOGAPPEND("COUNTER2|ARENA_DISTANCES:A1")
        LOGRUN()

        LOGCREATE("TEXT:")
   LOGRUN()

COMPLETE


ACTION INITIATOR

   SET(COUNTER1,COUNTER_INC)
        LOGFIELD(2,COUNTER1)
   LOGFIELD(3,RUNTIME)
   LOGFIELD(4,"OMISSION")
        FEEDER(1)
   LIGHTS(LIGHT7,WHITE)
        LOGDATA(DATA_SNAPSHOT,"BEGINfeed")
   DETECTOR(DETECTOR7,AT_FEEDER)
```

```
        WAIT(LIGHT_TIME)                #20

        LOGDATA(DATA_SNAPSHOT,"AFTERfeed")
        LOGDATA(DATA_SELECT,"BEGINfeed")
        LOGDATA(DATA_DELTA,"AFTERfeed")

        LOGFIELD(COMMIT)

   LOGCREATE("TEXT:|TEXT:|TEXT:|COUNTER1")
   LOGAPPEND("TEXT:|TEXT:|TEXT:|TEXT")
   LOGAPPEND("ZONE_COUNTERS:Z7|ZONE_TIMERS:Z7")
   LOGRUN()

        LIGHTS(ALL,OFF)
   WAIT(INTERTRIAL_TIME)                #60

COMPLETE


ACTION AT_FEEDER

   LOGFIELD(5,RUNTIME)
   LOGFIELD(4,"TRIGGERED")
        SET(COUNTER2,COUNTER_INC)
   WAIT(FEED_TIME)                #20
        LIGHTS(ALL,OFF)

COMPLETE
```

Length and weight after the p-chip tagging for each strain:

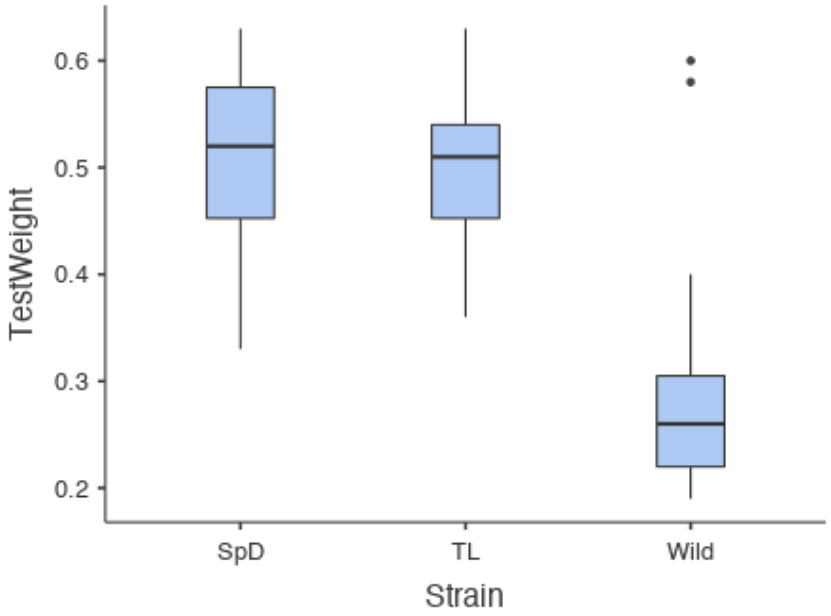


A.1 The weight of each strain after the p-Chip tagging them.

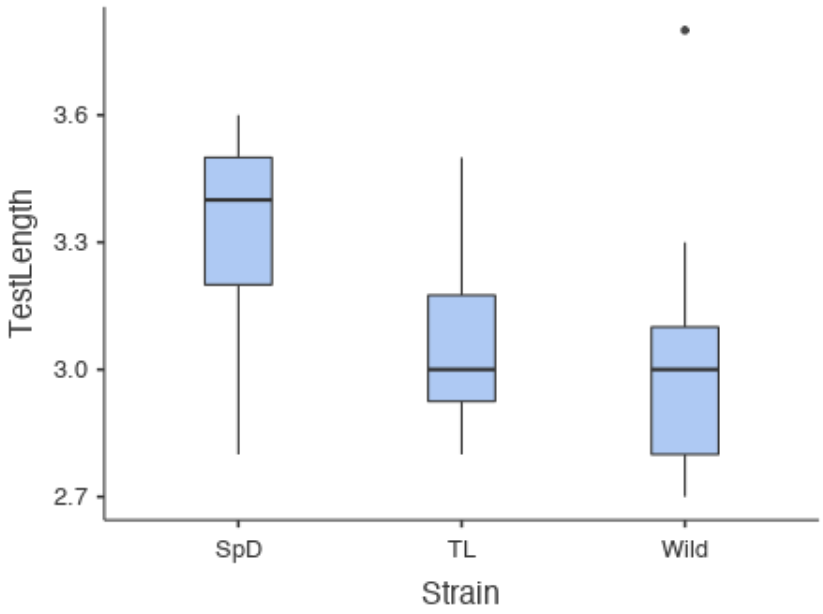


A.2 The length of each strain after the p-chip tagging.

Length and weight after the novel tank diving test for each strain:



A3. The weight of each strain after the novel tank diving test.



A4. The length of each strain after the novel tank diving test.