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Biochemical and behavioural characterization of some animal models of schizophrenia in zebrafish, based on the differentiation of individual personality traits

PHD THESIS SUMMARY

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Per audacia ad astra

Over the years, one aspect that always impressed me in the people around me was courage, the courage to take risks, the courage to overcome one's condition, the courage to want more from life and the courage to follow one's passion. If I had been asked 10 years ago if I thought I would make it here, my answer would have been a resounding No, but what changed my answer was the people around me who chose to be an integral part of my life.

So, on this occasion, I would like to thank my mother, uncle, grandmother and aunt who supported me both emotionally and financially and who encouraged me to want more, to go further and not let anyone say I can't. Family is the most precious thing in my life, and I wouldn't have gotten here without the help of each and every one of them. A special mention to my cousin who has inspired me countless times to follow my passion and has always been by my side.

Thank you to my friends who often had more faith in me and my strengths than I did. They are the ones who listened to every complaint (sometimes for no real reason), celebrated every small victory, and pushed me to keep my head up. A special mention to my lab mate who has been by my side both professionally and personally and pushed me to do more.

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Everything I have achieved and where I am now is only due to these people, and where I will be in the future is also thanks to them.

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Abbreviations list and keywords

5-HNE – 5- hydroxynonenal

ADN – deoxyribonucleic acid

APA – American Psychiatric Association

ATP – Adenosine triphosphate

BDNF – brain-derived neurotrophic factor

CAT – catalase

CNV – copy number variation

COMT – catechol-O-methyltransferase

DISC1 – disrupted-in-schizophrenia

DRD2 – dopamine D2 receptor

DSM – Diagnostic and Statistical Manual of mental disorders (eng.)

GABA – gamma-aminobutyric acid

GCL – glutamate-cysteine ligase

GPx – glutathione peroxidase

GSH – glutathione

HPA – hypothalamic-pituitary-adrenal axis

LSD – lysergic acid diethylamide

LTD – long term depression

LTP – long-term potentiation

MDA – malondialdehyde

mGluR – metabotropic glutamate receptors

MP – microplastics

NMDA/R – N-methyl-D-aspartate/receptor

NRG1 – neuregulin 1

PCNA – proliferating cell nuclear antigen

PSD-95 – postsynaptic density protein 95

RCS – reactive chlorine species

RNS – reactive nitrogen species

ROS – reactive oxygen species

RSS – reactive sulfur species

sAA – salivary alpha-amylase

SAMe – S-adenosylmethionine

CNS – central nervous system

SOD – superoxide dismutase

TBARS – thiobarbituric acid reactive substances (eng.)

TNF – tumor necrosis factor

TNFIL-8 – tumor necrosis factor interleukin 8

Keywords: zebrafish, personality, schizophrenia, physiological stress, oxidative stress, behavior, individual differences, alpha-amylase

Introduction

Schizophrenia, as a disorder, is a relatively new concept compared to other pathologies and manifestations of psychiatric nature, such as melancholia, "hysteria" or madness (1).

Despite the various observations made on the etiology of this disorder, the exact causes are still unknown as this disorder is the result of the entanglement of multiple risk factors from genetics to environmental, economic, social, cultural and family history factors (2). These factors can influence the prevalence of a disorder in the general population, the onset of the disorder as well as the severity of the symptoms along with the therapeutic response (3). Psychiatric disorders can often be extremely similar in presentation, which is why there is a continuous need for new studies and new directions of research to more effectively clarify the main distinctions and similarities between the disorders (4,5) so that the diagnosis cannot be subjective and dependent on the experience of the psychiatrist.

The brain is an organ that requires a large volume of energy obtained through the oxidative phosphorylation of the mitochondria, a process that can lead to the formation of reactive oxygen species. When these reactive oxygen species are produced in excess, the body enters a state of oxidative stress where the protective mechanisms are compromised and neurons become susceptible to damage (6).

Oxidative stress is defined as an imbalance between oxidants (reactive oxygen, nitrogen and sulfur species) and antioxidant defenses (antioxidants) leading to various damages to DNA, proteins, lipids and cellular integrity. This concept was introduced for the first time in 1985 and since then it has gained special attention from the scientific community that continues to investigate the way oxidative stress is defined and its possible role in various pathologies (7).

Zebrafish, *Danio rerio*, are an indigenous species in the southern and eastern regions of Asia and are a favorite aquarium species and are also widely bred and propagated in laboratories for mutagenesis and screening in order to maintain a strictly controlled environment to ensure a stable genetic background (8). Despite the general belief that less complex animals have less complex characteristics, previous studies have shown that zebrafish behaviour covers most of the cognitive and socio-affective traits either the same or very similar to human behavior. Assessment of zebrafish behaviour includes the assessment of response to multiple types of stimuli (visual, acoustic, tactile, olfactory) (9).

Zebrafish, like other living beings, can change their behaviour depending on how well they feel in their environment. According to the American Psychiatric Association (APA), personality refers to individual differences in characteristic patterns of thinking and behavior, and when studying personality, one must understand individual differences in particular personality characteristics, such as sociability or irritability (10). In animals, personality traits refer to consistent patterns of behavior, cognition, and emotional responses that can be observed in different situations over time (11).

Although neuropsychiatric disorders, such as schizophrenia affect a relatively small percentage of the world's population, they are some of the most debilitating conditions, are not fully understood, and it is often a lengthy process of "trial and error" to identify and determine the best therapeutic option. Therefore, by continuing the study started with my dissertation thesis on schizophrenia and oxidative stress, I want to strengthen the social characteristics of zebrafish in terms of personality, cognitive learning ability, the obvious expression of social preferences and the natural tendency to stay in groups compared to the behavioral disruptions observed in schizophrenia.

Personality is a complex characteristic of humans that can predict the direction in which a potential cognitive and behavioral deficit might go but studying it in correlation with biochemical parameters is a direction little explored in humans and even less so in animal models. For this reason, another point of interest of this thesis is the evaluation of the individual and collective differences of the zebra fish that make up the unique and individual personality of each animal. Zebrafish show behavioral differences within the same group despite identical physiological conditions and developmental stages, which could make them an excellent candidate for studying behavioral disturbances associated with specific psychiatric disorders.

Furthermore, studies have shown that social isolation can lead to the release of glucocorticoids and oxytocin and altered levels of dopamine, serotonin, GABA and altered NMDAR sensitivity (12), which is why I chose to create experimental groups both socially isolated in individual aquariums, covered on the sides and in normally housed groups. Based on data from the literature, social isolation causes anxiety, depression, increases the level of aggression and decreases the preference for staying in groups. The rationale for isolation is the potential for social isolation to be sufficient for modeling psychiatric disorders if no significant differences are identified between the isolated control group and pharmaceutical models.

Scope and main objectives

Despite the advancement of technology and information available on psychiatric diseases, there are no animal models that reproduce very faithfully the symptomatology associated with schizophrenia. Models accepted by the scientific community at the moment are that of ketamine administration and that of methionine administration, which is why in this study an animal model is proposed consisting of the simultaneous administration of the two substances in traditionally housed fish (together) and in fish housed separately (social isolation).

Also, another aim of the study is to appreciate the neurotoxic effects of the administration of microplastics, especially in the context of schizophrenia modeling.

The objectives of the present study were:

1. Development of a potential robust animal model for highlighting schizophrenia-associated symptomatology
2. Analysing the social and cognitive trends of zebrafish with the development of a new methodology to test the ability to learn of zebrafish through their natural color preference and positive conditioning (food)
3. Separating fish by personality based on chosen traits and evaluating behavioral and cognitive

differences in each test between shy and bold fish

4. Evaluation of the dissociative effect of ketamine
5. Evaluation of the effects of the substances used on the tissues
6. Evaluation of the level of oxidative stress
7. Evaluation of the level of alpha-amylase in order to determine the level of physiological stress

Current state of knowledge

1. Schizophrenia

Schizophrenia is considered to be the most severe psychiatric disorder (13). Psychiatric disorders have a multifactorial constitution and etiology, being the result of the entanglement of multiple risk factors from genetics to environmental, economic, social, cultural and family history factors (2). These factors can influence the prevalence of a disorder in the general population, the onset of the disorder as well as the severity of the symptoms along with the therapeutic response (3).

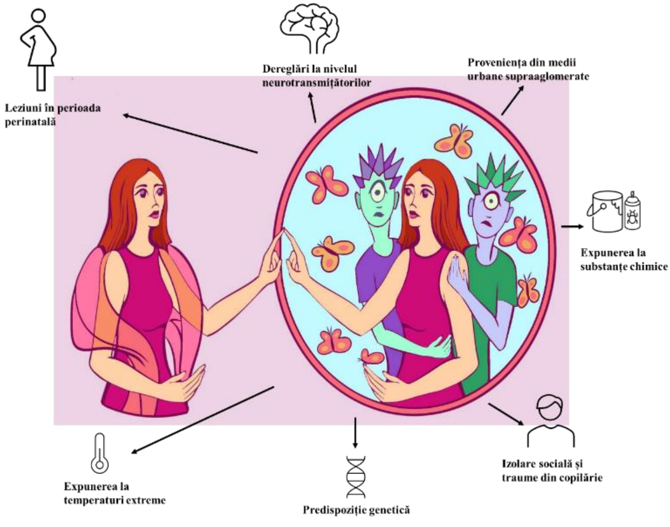


Figura 1.1 Risk factors for the development of psychotic episodes and symptomatology (original image created by Maria Hreniuc (visual artist))

Overall, both dopamine overproduction and dopamine underproduction are thought to contribute to the symptoms of schizophrenia (14,15). This connection was made by administering antipsychotic drugs in anxiolytic doses (such as diazepam) that help prevent hyperdopaminergic states in adults (16). In addition to the previously mentioned theory, the pathophysiology of schizophrenia incorporates several other theories of other neurotransmitters (such as serotonin and glutamate), but also of gamma-aminobutyric acid (GABA) (17). Other theories have been explored because giving D2 receptor blocking drugs to patients with secondary psychosis only exacerbated their condition. According to the glutamate theory, N-methyl-D-aspartate (NMDA) receptor hypofunction may contribute to psychotic episodes (18,19).

The N-methyl-D-aspartate receptor is specifically regulated by redox status. More specifically, pairs of redox-sensitive cysteine residues form disulfide bridges that can decrease NMDAR currents. In addition, there is a group of overlapping cysteine residues that are susceptible to inhibitory effects and facilitate S-nitrosylation (20). This delicate balance between disulfide bond formation and S-nitrosylation regulates NMDAR activity. It should be noted that the redox balance is also influenced by glutathione, and the hypofunction of this receptor can lead to a decrease in the amount of GSH (21).

Methionine is one of the essential sulfur-containing amino acids, it is aliphatic and plays a precursor role in the metabolism of several amino acids (cysteine, creatine, homocysteine, etc.). Methionine is considered to be neutral and the most dynamic amino acid, which through metabolism can become acidic (22). S-adenosylmethionine is a metabolite of the methionine transmethylation pathway with ATP that is involved in lipid, protein and nucleotide methylation reactions, neurotransmitters, phosphatidylcholine synthesis and intracellular RNA methylation where it plays the role of -methyl group donor. Under physiological conditions of stress, glutathione is used in an attempt to reduce cellular oxidative stress (23,24).

Moreover, SAMe is known to increase the enzyme activity of catechol-O-methyltransferase (*COMT*) whose low level has been associated with aggression in schizophrenia (25), while other studies have proposed the administration of SAMe as a possible treatment for patients missing one copy of the *COMT* gene (deletion *22q11.2*), a condition associated with an increased prevalence of developing psychosis, depression and ADHD (26,27).

Microplastics (MPs) are particles of relatively small size (< 5 mm) obtained from the degradation process of different types of plastic. In modern society, microplastics come from a variety of sources as plastic is a material intense used in industries ranging from food to agriculture, construction and cosmetics (28), and waste management methods of packaging and used materials are not always conducive and plastics end up in nature deliberately or not. Microplastics can reach the human body through several routes, such as inhalation, ingestion or translocation as can be seen in Figure 1.5. Studies using animal models on the effects of microplastics have shown that they can generate metabolic disturbances in the direction of lipid metabolism and reduction of ATP production, but could also affect the offspring, by inducing oxidative stress and decreasing the reproductive rate (29). Whereas, a study on microplastic pollution in South Korea showed that high rates of MPs in the air could lead to cardiovascular and lung diseases through chronic inhalation (30).

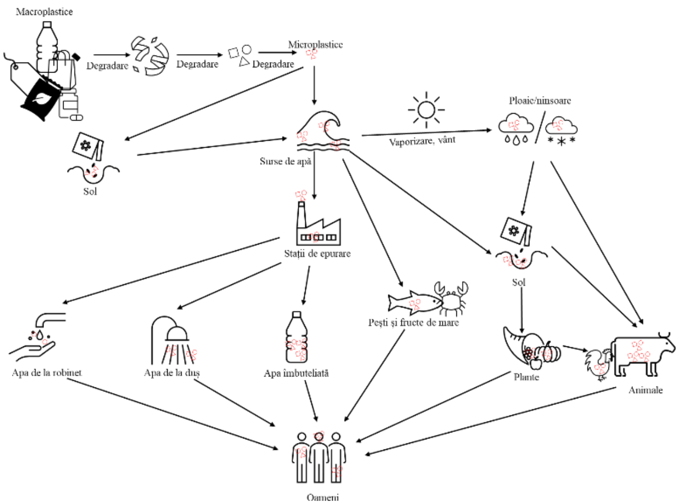


Figure 1.5 Pathways through which microplastics reach back to humans (original image).

Despite the real threat of microplastics on health, studies analyzing their harmful effects in individuals with psychiatric disorders are limited. A study by Poldermann et al. illustrated that 55% of the risk for major depressive disorder, 32% of the risk for bipolar disorder, and 23% of the risk for schizophrenia are environmental factors (31). Moreover, there are numerous studies that correlate exposure to heavy metals with the risk of developing schizophrenia, an aspect that is of particular importance since microplastics have the ability to absorb heavy metals from the environment (32). In a study carried out by our research group, we observed that microplastics, more specifically polypropylene fibers, acted as a buffer against the toxicity associated with ketamine administration and as a stimulant for methionine exposure, an aspect that could be correlated with the worsening of cognitive deficits (33).

2. Oxidative stress

The accumulation of oxidants has long been associated with oxidative stress (34). In addition to reactive oxygen species (ROS), there are also reactive nitrogen species (RNS), reactive sulfur species (RSS) and reactive chlorine species (RCS) (35). Reactive species are free radicals or not. The most common free radicals are hydroxyl, superoxide and nitric oxide, and they are produced through exposure to ionizing radiation or reactions to toxic elements in the environment.

Superoxide-dismutases (SOD) are metalloenzymes with an antioxidant and anti-inflammatory role that neutralize superoxide radicals by cleaving them into harmless normal molecules (oxygen and hydrogen peroxide)(36). An increased level of SOD does not suggest oxidative stress but suggests the activation of a compensatory mechanism to combat oxidants (37).

Glutathione peroxidases are a family of enzymes with a role in regulatory processes, synthesis functions and prevention of free radical formation (38). Glutathione peroxidase (GPx) has a role in reducing lipid peroxidation by converting peroxides to the corresponding alcohols and hydrogen peroxide to water (39).

Malondialdehyde (MDA) is a biomarker for oxidative stress for disorders of a tumor, psychiatric, pulmonary or cardiovascular nature because it is a component derived from the peroxidation of polyunsaturated fatty acids (40). As the amount of free radicals increases, more MDA is produced (41).

In an ideal scenario, SOD and GPX levels should be high to scavenge superoxide radicals and reduce hydrogen peroxide and lipid hydroperoxides, while MDA levels should be low to indicate minimal lipid peroxidation.

Studies show that in schizophrenia patients in the first psychotic episode, the levels of GSH, CAT, SOD are low and bring the body into a state of oxidative imbalance (42–47).

3. Alpha-amylase

Beginning in the 1990s, studies began to suggest that alpha-amylase may be an indicator for physiological stress, as its concentration decreases during exposure to acute stress, although later studies reported the opposite. Exposure to acute stress leads to the activation of the sympathetic nervous system which stimulates the secretion of alpha-amylase, but it is also secreted by parasympathetic stimulation which could explain the contradictory results obtained by measuring the level of salivary alpha-amylase (48). Salivary alpha-amylase is produced by acinar cells by stimulating the sympathetic and parasympathetic systems (49). The sympathetic nervous system increases the concentration of alpha-amylase in saliva, while the parasympathetic system increases the rate of saliva production without affecting its composition (50).

The correlation between salivary alpha-amylase and schizophrenia is a topic of interest to the scientific community, but current observations are not yet definitive or fully understood. Studies have explored the association between these on the basis that sAA is a biomarker of sympathetic nervous system activity. It is involved in the body's response to stress and plays an important role in regulating physiological arousal. Several studies have shown altered sAA levels in individuals with schizophrenia compared to healthy controls, suggesting a potential dysregulation of the sympathetic nervous system. Multiple studies have shown that blood alpha-amylase levels are higher in patients with schizophrenia (51,52), and that the higher the level, the more severe the psychiatric symptoms (53,54).

4. Zebrafish as animal models for psychiatric disorders

Clinical studies as well as research based on the use of animal models can illustrate important features of the etiology and pathology of psychiatric disorders. Today, animal models are an important tool in biomedical research. Among all animal models, zebrafish (*Danio rerio*) are preferred for most studies due to the advantages it brings, such as easy and cost-effective growth and reproduction conditions, central nervous system morphology, genetics and specific fertilization with *ex-utero* development (8,55–57).

They exhibit a wide spectrum of responses to visual, tactile and olfactory stimuli and are able to learn and express different types of memory and complex behaviors such as anxiety/fear, cognition, social behavior, reward-based, pain-based, sleep and neurological (58,59).

Adding to the wide repertoire of advantages is the ability of zebrafish to absorb substances by immersion through the gills and skin in the case of water-soluble substances – a method that can be used from the embryonic stage (60) by intraperitoneal injection (61) or by oral administration starting from the age of 72 hours post-fertilization (62). On the one hand, immersion is a very useful method because it does not produce any kind of stress on the animal compared to injection or oral administration, but on the other hand, intraperitoneal injection and oral administration allow better control over the dosage, a smaller amount of the drug used and an easier and more direct correlation with the results obtained in mice, for example (63).

Zebrafish, like other living beings, can change their behavior depending on how well they feel in their environment. According to APA, personality refers to individual differences in characteristic patterns of thought and behavior, and when studying personality one must understand individual differences in particular personality characteristics such as sociability or irritability (10,64).

Thus, if the fish actively swims through the whole aquarium or not, if it searches and ingests food or not, if it sits at the bottom of the aquarium or next to other fish, if it swims fast or slowly, in the case of zebra fish all these can be considered personality traits as they are unique and constant to each individual fish. Also, proactive individuals leave the "comfort zone" faster and recover faster and easier after exposure to a stressor (65). In addition, they eat more and more often than the reactive ones (66).

Studies have noted that zebrafish can display aggressive behaviour, particularly during territorial disputes or competition for food with some individuals showing more pronounced aggressive tendencies and others being more submissive. Moreover, zebrafish are known to be social animals and tend to form groups in their natural environment, but even within this situation differences in behavior have been observed within the same group, with some fish actively seeking the company of others group members and others preferring less socially active environment. Zebrafish show different levels of activity with some more active, with higher swimming speed and higher exploratory tendencies and others more sedentary. In addition, zebrafish show individual variation in response to stressors, with some fish having greater resilience and adaptability, while others are more prone to stress and show more pronounced behavioural and physiological responses (11,66,67).

Experimental part

5. Materials and methods

For this experiment, 160 adult wild-type zebrafish obtained from a local trader were used and housed under optimal conditions with repeated measurements of water parameters, maintaining a constant temperature, light:dark cycle and level of oxygen.

The experimental groups were:

Social isolation:

Group 1 – Control (C iso) (10 fish)

Group 2 – Ketamine (K iso) (10 fish)

Group 3 – Methionine (M iso) (10 fish)

Group 4 – Ketamine + methionine (K+M iso) (10 fish)

Group 5 – Control Microplastics (C MP iso) (10 fish)

Group 6 – Microplastics + Ketamine (MP+M iso) (10 fish)

Group 7 – Microplastics + Methionine (MP+M iso) (10 fish)

Group 8 – Microplastics + Ketamine + Methionine (MP+K+M iso) (10 fish)

Normal(together) groups:

Group 1` – Control (C imp) (10 fish)

Group 2`– Ketamine (K imp) (10 fish)

Group 3` – Methionine (M imp) (10 fish)

Group 4`– Ketamine + Methionine (K+M imp) (10 fish)

Group 5` – Control Microplastics (C MP) (10 fish)

Group 6` – Microplastics + Ketamine (MP+K imp) (10 fish)

Group 7` – Microplastics + Methionine (MP+M imp) (10 fish)

Group 8` – Microplastics + Ketamine + Methionine (MP+K+M imp) (10 fish)

The studies were carried out at the Faculty of Biology of the "Alexandru Ioan Cuza" University in Iasi and at the Faculty of Veterinary Medicine of the "Ion Ionescu de la Brad" University of Life Sciences in Iasi according to the approvals of the appropriate ethics commissions, no. 2533/08.09.2022 (UAIC Iasi), respectively no. 165/26.01.2022 (USV Iasi). Moreover, the studies were carried out in accordance with the provisions of European directives (2010/62/EU) and Romanian legislation (43/2014) regarding the protection of animals used for scientific or experimental purposes.

Exposure to ketamine was done over a period of 5 days. On administration days, fish were removed from their respective tanks and placed in tubes of 50 mL of 0.1% ketamine solution for 5 min. The administration of methionine was carried out for a period of 7 days. The solution had a concentration of 6.0 mM, was prepared *de novo* daily and constituted the tank water. To prepare for the co-administration of the two substances, the assigned groups were prior exposed to methionine solution for 48 hours, and on the third day the administration of ketamine was also started according to the instructions previously presented. For exposure to microplastics, polypropylene fibers (size <2 mm) were used as this is one of the most identified polymers in nature and human tissues (68), and the plastic concentration was calculated as 2 mg/L added to the amount of feed required according to the weight of the fish (4% of their weight according to studies (69)).

For the polypropylene-ketamine co-administration, exposure of fish to microplastics in food started 48 h before the start of ketamine treatment. For the combined polypropylene-methionine group, exposure to the two substances started simultaneously,

To prepare for the co-administration of the three substances, the assigned groups were exposed to 48 hours of dietary methionine and polypropylene solution, and on the third day the administration of ketamine was started according to the instructions presented previously.

The behavioral tests used were:

- Novel tank test
- Social preference test
- Aggressivity test
- Light-dark test
- Cognitive test (memory test)

The cognitive test consisted of three stages: the color preference test, the learning period and the memory test.

The color preference test was carried out according to Figure 5.5.

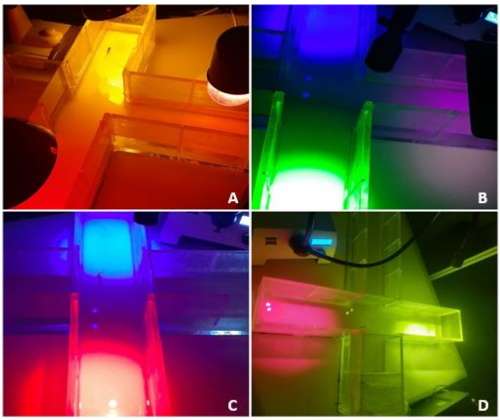


Figure 5.5 Experimental apparatus for the social preference test. A – round 1 (red vs. orange vs. yellow), B – round 2 (blue vs. purple vs. green), round 3 (red vs. blue) and round 4 (purple vs. yellow).

Testing was done in rounds in a maze with 3 equal arms and a decision point: 1. Yellow vs. orange vs. red, 2. Blue vs.

purple vs. green, 3. Blue vs. red (least favorite colors) and 4. Yellow vs. purple (my two favorite colors). Following the results obtained in the color preference test (yellow – preferred color, red – least preferred color) we decided to carry out a memory test associated with colors with a corresponding learning period. The fish spent half an hour daily in the most avoided ambient light (red), during which time they were fed, for a period of 10 days (starting with the pre-treatment period until the last day of administration) (Figure 5.6.).

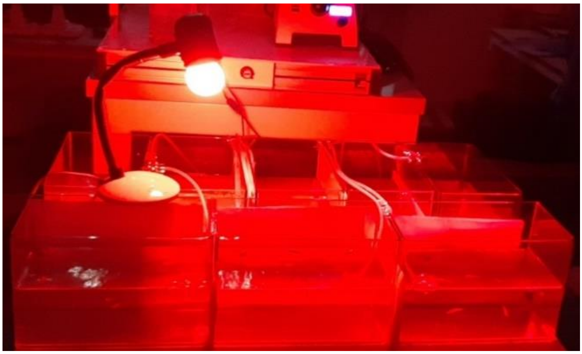


Figure 5.6 Experimental apparatus used for exposure to red light for 30 minutes during the learning period and association of food with the color red.

On the day after the completion of the learning period, the fish were subjected to the memory test (1 minute) in which one arm was illuminated in the least preferred color (red) and one arm in the most preferred color (yellow) according to Figure 5.7.

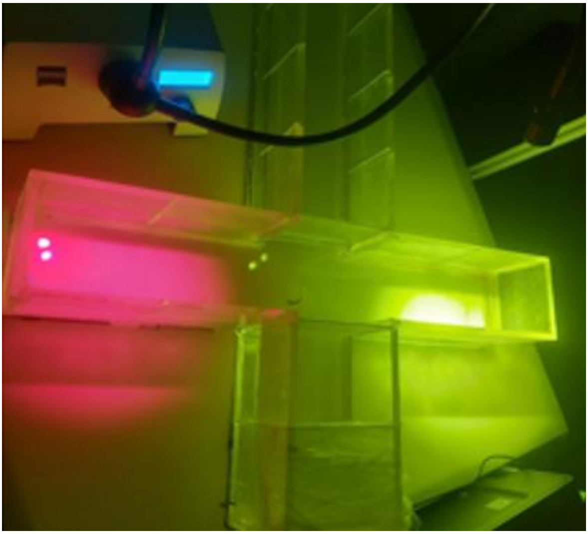


Figure 5.7 The experimental apparatus of the memory test.

For the immunohistology study of the samples, three fish from each representative group were randomly chosen at the end of the exposure period. The methodology followed is in accordance with that described in the specialized literature, so that the head was cut immediately after the death of the fish and fixed in Bouin for 48 hours. The paraffin blocks were sectioned at a thickness of 4 µm using a SLEE microtome, and then the sections stained HE and IHC with the antibodies: PCNA, TNFAI8, Cox41, BDNF, Map2, H2A, S100, GFAP, Tub2. The sectioning was done longitudinally.

For the measurement of oxidative stress biomarkers, zebrafish were euthanized by immersion in cold water according to ethical recommendations for 20 minutes and stored at -80°C until biochemical analysis. Fish were slowly thawed to -4°C and then weighed and mortared until completely homogenized. The samples were solubilized with 0.1M potassium triphosphate buffer solution (pH 7.4) and 1.15% KCl. The homogenates thus obtained were centrifuged for 15 minutes at 960 x g rpm, and the specific activities of SOD, GPX, MDA and total soluble proteins were determined from the supernatants obtained according to the manufacturer's instructions.

For measurement of alpha-amylase levels, zebrafish were euthanized by immersion in cold water according to ethical recommendations for 20 minutes and stored at -80°C until biochemical analysis. Fish were slowly thawed to -4°C and then weighed and mortared until completely homogenized. Exactly 0.1 g of tissue was weighed to which 0.9 ml of double-distilled water was added and mechanically homogenized in an ice-water bath. The homogenate was collected, left at room temperature for 15 minutes, followed by oscillation for 5 minutes and centrifugation at 3000 g for 10 minutes at room temperature. The supernatant was collected and distilled water was added for a final volume of 10 ml. The alpha-amylase level was measured using a specific kit (α-amylase activity assay kit, Elabscience, USA) according to the manufacturer's instructions.

In order to prove the existence of personality in zebra fish and to highlight the specific traits and behavioral differences, we separated the fish into the two main personality types (Shy and Bold) based on the parameters that describe their behavior.

The parameters chosen for this differentiation are:

- From the novel tank test: tracks, heatmaps, distance moved, total freeze duration and latency to first entry into upper half.
- From the social preference test: time spent in the left arm
- From the aggressivity test: swim bursts

For the tie-breaking according based on the measurable parameters, the median was calculated and according to this value, the two personalities were separated as follows::

- Median for distance moved = 1732.03. Any value below the median was considered = shy personality, any value above = bold personality.
- Median for total freezing time = 100.36; above this value = shy personality, below this value = bold personality.
- Median for latency to first entry in upper half = 0.01; values equal to or less than the median = bold personality, values above the median = shy personality.
- Median for the time spent in the left arm (arm with conspecifics) = 51.2007; above this value = shy personality, below this value = bold personality.
- Median for swim bursts = 12; above this value = bold personality, below this value = shy personality.

The final personality was decided when 4 of the 7 parameters indicate either Shy or Bold.

The idea behind the mini study is based on the notion that ketamine has dissociative effects meaning that it affects the personality in one way or another. But is this also true for zebrafish? The mini study is focused on the possibility that zebrafish can change their personality following the exposure to ketamine. The division of zebrafish into one personality or another was done based on tracks and heatmaps, and the evolution and change of personality traits following ketamine treatment was observed. As visual observations are not sufficient to support that fish personality is indeed affected by ketamine treatment, parameters corresponding to the novel aquarium test were also measured and analyzed, namely: latency to first entry into the upper half of the aquarium , time spent and number of entries in it, time spent in lower half and number of entries in lower half.

Statistical analysis of all obtained results was performed using the GraphPad Prism 10 program (USA) and were plotted as mean ± SD. The results were analyzed statistically by means of the student "t" test, uni- and bi-directional ANOVA, followed by post-hoc analysis (Tukey's test). For correlations, the Pearson test was used where a $p<0.05$ means rejecting the hypothesis that the identified correlation is due to the random selection of results. For ANOVA tests, the Brown-Forsynthe test was also applied to check for variability where a $p<0.05$ suggests failure to reject the hypothesis that variability is equal and the ANOVA test can be performed. Test results were considered significant if $p<0.05$.

6. Results

6.1. Fish separation based on personality

In order to prove the existence of personality in zebrafish and to highlight specific traits and behavioral differences, this chapter begins by separating fish into the two main personality types (Shy and Bold) based on parameters that describe their behavior.

The tracks and heatmaps used to identify the personality type based on the results of the novel tank test can be viewed in Table 6.1 in the full thesis.

6.2. Novel tank test

6.2.1. General

For latency to first upper half entry, statistical analysis showed significant differences ($F(DFn, DFd)=5.463(15, 104)$, $p<0.001$) with the individual significant differences of interest illustrated in Figure 6.1.A.

Moreover, the analysis performed on the values obtained for the time spent in the upper half also showed significant differences ($F(DFn, DFd)=1.442(15, 123)$, $p<0.001$) with the individual comparisons between groups illustrated in Figure 6.1.B.

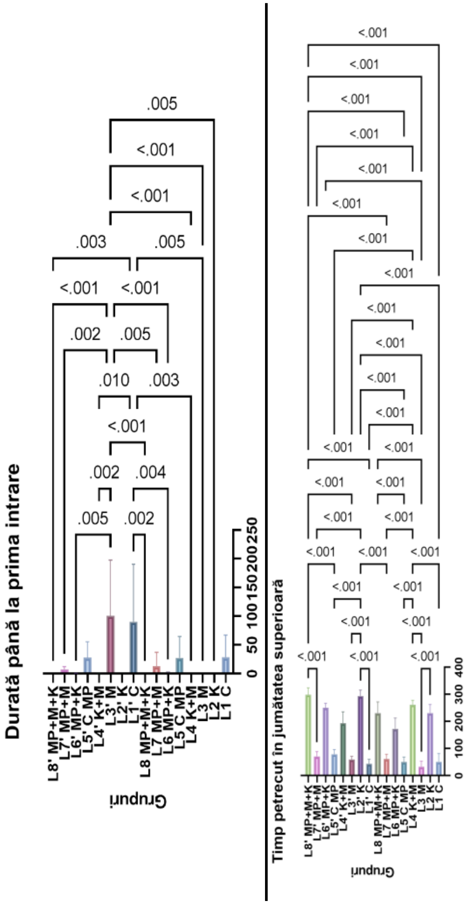


Figure 6.1 Results of the parameters time to first entry into the upper half (s)(A) and time spent in the upper half (s)(B) of the novel aquarium test. Results are illustrated as mean ±SD with a p<0.05 considered significant.

In the case of the no. of clockwise rotations, statistical analysis showed significant differences (F(DFn, DFd)=1.044(15, 123), p<0.001) with the individual significant differences of interest illustrated in Figure 6.3.A. The analysis performed on the values obtained for no. of counterclockwise rotations also showed significant differences (F(DFn, DFd)=2.127(15, 123), p=0.035) with the individual comparisons between groups illustrated in Figure 6.3.B. The analysis performed on the values obtained for the total duration of freezing also showed significant differences (F(DFn, DFd)=1.078(15, 123), p=0.007) with the individual comparisons between groups illustrated in Figure 6.3. C.

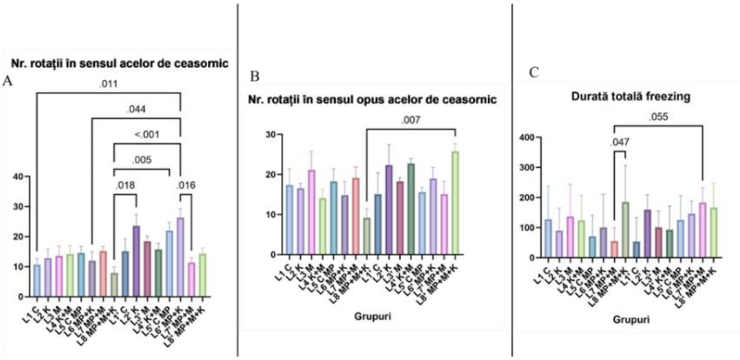


Figure 6.3 Results of parameters no. clockwise rotations (A), no. counterclockwise rotations (B) and total freezing time (C) of the new aquarium test. Results are illustrated as mean ±SD with a p<0.05 considered significant.

6.2.2. Personality

Statistical analysis of time spent in the upper half showed significant differences between groups (p<0.001), but not between personality types (p=0.438). Moreover, multiple significant differences were observed between groups based on personality, such as between shy isolated control and shy isolated ketamine group (p=0.004) or between bold together ketamine and methionine group (p< 0.001). These differences can be seen in Figure 6.6.A. Differences of p<0.05 were considered significant. Regarding the analysis based on personality and housing conditions, no significant differences were identified (p=0.069)(Figure 6.6 B), nor in the case of the analysis between personality types independent of the treatment group (p=0.104)(Figure 6.6 C)).

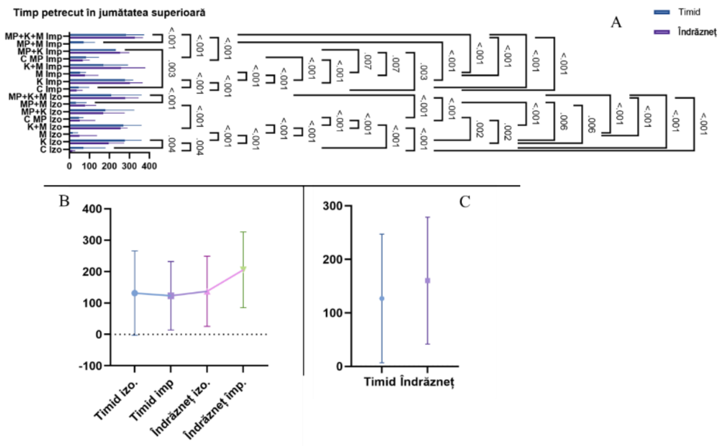


Figure 6.6 Statistical analysis of time spent in the upper half (s). A – analysis based on groups and personality, B – analysis based on personality and hosting conditions, C – analysis based on personality. Results are illustrated as mean ± SEM/SD and those with p<0.001 were considered significant).

The statistical analysis of the number of entries in the upper half showed significant differences between groups (p<0.001) and also between personality types (p=0.023). Significant differences were observed between groups based on personality. These differences can be seen in Figure 6.7.A. Differences of p<0.05 were considered significant. Regarding the analysis based on personality and housing conditions, significant differences were identified (p=0.011) between the shy fish together and the bold ones isolated (p=0.009) (Figure 6.7 B), but also in the case of the analysis between personality types independently by the treatment group (p=0.002)(Figure 6.7 C).

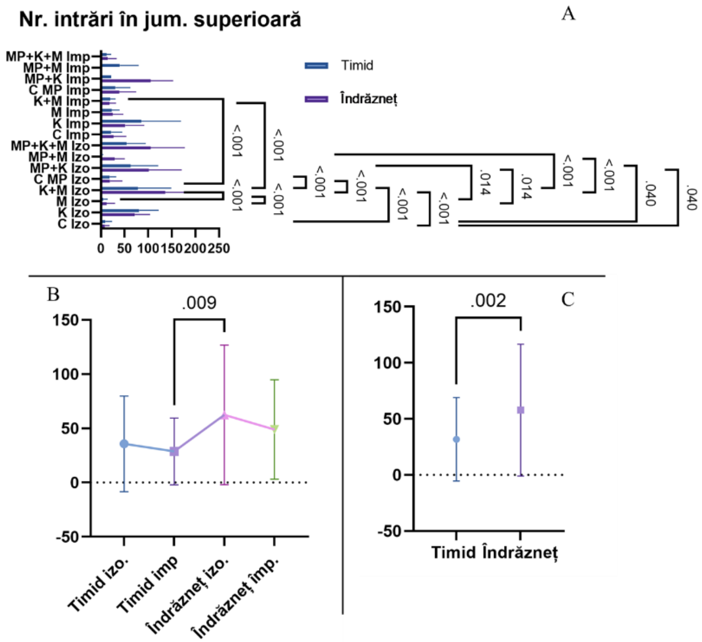


Figure 6.7 Statistical analysis of no. upper half entries. A – analysis based on groups and personality, B – analysis based on personality and hosting conditions, C – analysis based on personality. Results are depicted as mean ± SD/SEM and those with p<0.001 were considered significant).

The statistical analysis of the distance traveled showed significant differences between groups (p=0.002), but also between personality types (p<0.001). Multiple significant differences were observed between the groups based on personality. These differences can be seen in Figure 6.8.A. Regarding the analysis based on personality and housing conditions, significant differences (p<0.001) were identified between isolated shy fish and isolated bold ones (p=0.001) (Figure 6.8 B), but also in the case of analysis between personality types independently by the treatment group (p<0.001)(Figure 6.8 C).

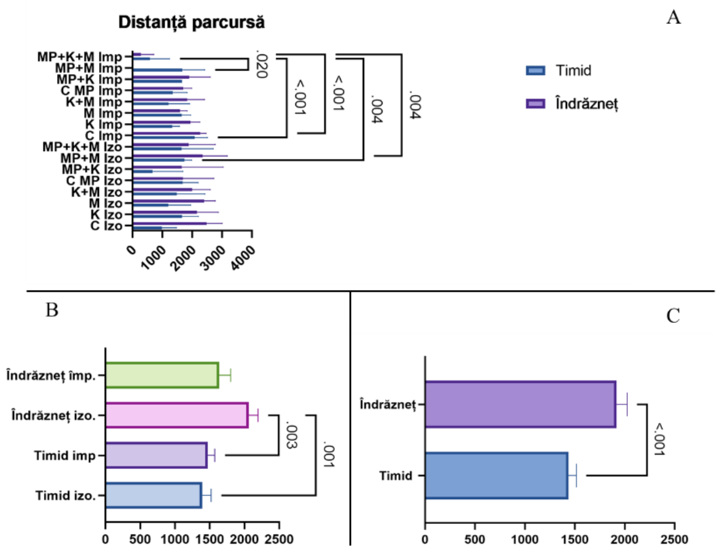


Figure 6.8 Statistical analysis of total distance traveled (mm). A – analysis based on groups and personality, B – analysis based on personality and hosting conditions, C – analysis based on personality. Results are illustrated as mean ± SEM and those with p<0.001 were considered significant).

Statistical analysis of total freezing duration showed significant differences between groups, but also between personality types (Figure 6.9.A). Significant differences were identified between isolated shy fish and those together (p=0.033), between isolated bold fish and those together (p=0.004), but also between iso shy and iso bold (p<0.001) (Figure 6.9 B), but also in the case of the analysis between personality types independent of the treatment group (p<0.001) (Figure 6.9 C).

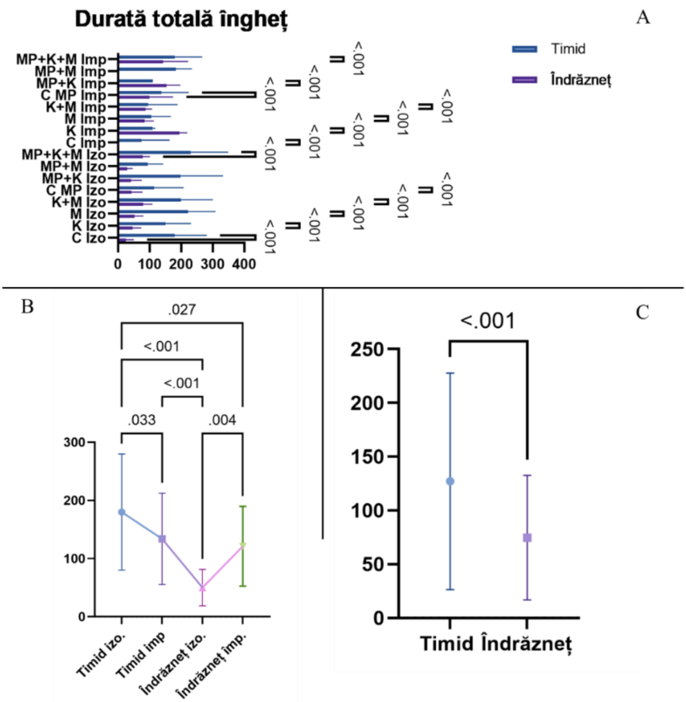


Figure 6.9 Statistical analysis of total freezing duration (s). A – analysis based on groups and personality, B – analysis based on personality and hosting conditions, C – analysis based on personality. Results are illustrated as mean ± SD and those with p<0.001 were considered significant.

6.3. Social preference test

6.3.1. General

The analysis of the values of the total time spent in the left arm showed significant differences ($p<0.001$) with significant differences both between the groups together and those isolated and within the same experimental protocol such as: L`C v L2` K ($p=0.002$) , L1` C v L3` K+M ($p=0.044$) or L3` M v L3 M ($p<0.001$). The results are illustrated in Figure 6.11.

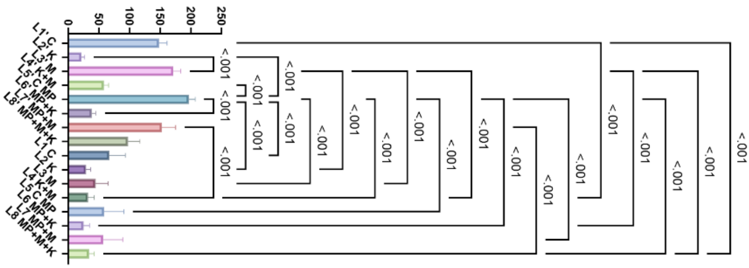


Figure 6.11 Statistical results of the parameter total time spent in the left arm (s). Results are illustrated as mean±SD and those with p<0.001 were considered significant).

Similar results were obtained for the number of entries in the left arm. Statistical analysis showed significant differences (F (DFn, DFd) = 1.256 (15, 125), $p<0.001$). In this case, the together groups had a higher number of entries in the left arm compared to the isolated groups with statistically significant differences especially between the L8` MP+M+K group and each of the isolated groups ($p<0.001$). The specific statistical results are illustrated in Figure 6.12.

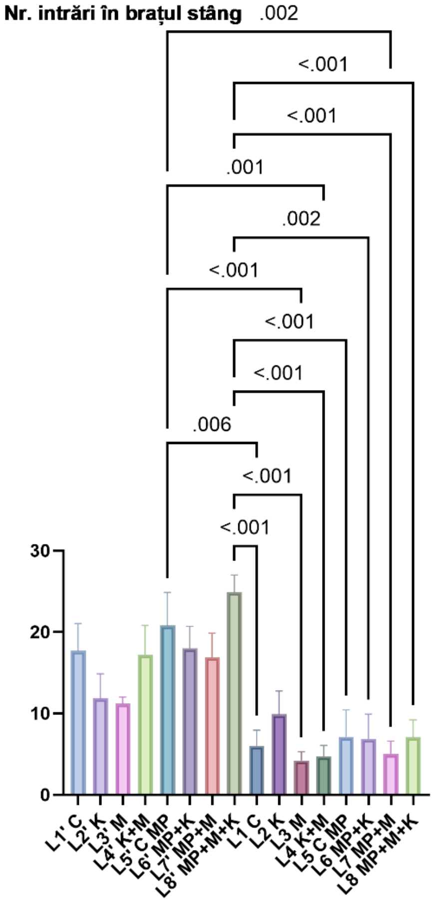


Figure 6.12 Statistical results of the number of entries parameter in the left arm. Results are illustrated as mean ± SEM and those with p<0.05 were considered significant).

6.3.2. Personality

Statistical analysis of the duration until the first entry into the left arm showed significant differences between groups (p=0.041), but not between personality types (p=0.319.) (Figure 6.15.A.). Regarding the analysis depending on the type of personality and the housing conditions, significant differences were identified (p=0.002) between the shy fish isolated and those together (p=0.001), but also between the shy fish isolated and the bold ones together (p=0.035) (Figure 6.15 B), but without differences between personality types independent of the treatment group (p=0.989) (Figure 6.15 C).

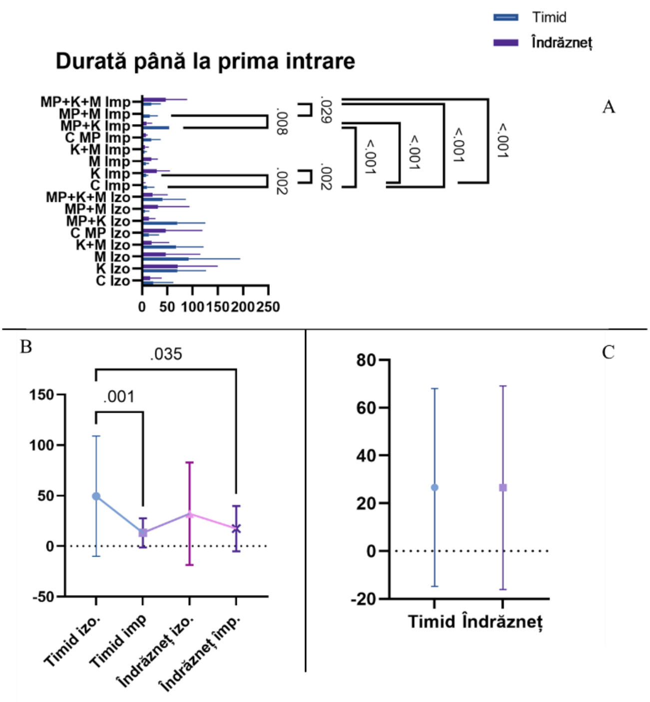


Figure 6.15 Statistical analysis of time to first entry into the left arm (s). A – analysis based on groups and personality, B – analysis based on personality and hosting conditions, C – analysis based on personality. Results are illustrated as mean ± SD and those with p<0.05 were considered significant.

Regarding the total time spent in the left arm, significant differences were observed only for the groups together (p<0.001) (Figure 6.16 A). Regarding the analysis dependent on personality type and housing conditions, significant differences were identified (p<0.001) (Figure 6.16 B), and with significant differences between personality types independent of treatment group (p=0.004)(Figure 6.16 C).

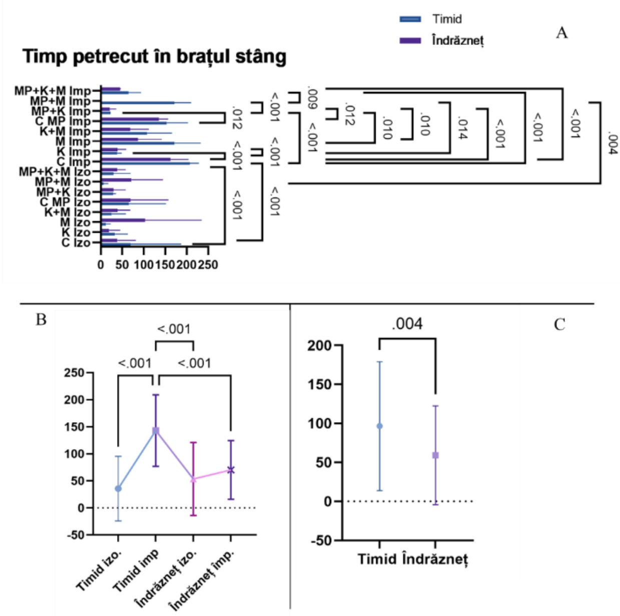


Figure 6.16 Statistical analysis of total time spent in the left arm (s). A – analysis based on groups and personality, B – analysis based on personality and hosting conditions, C – analysis based on personality. Results are illustrated as mean ± SD and those with p<0.05 were considered significant.

Statistical analysis of the number of entries in the left arm showed significant differences between groups (p<0.001), but not between personality types (p=0.176.)(Figure 6.17.A.). Regarding the analysis dependent on personality type and housing conditions, significant differences (p<0.001) were identified between the shy fish isolated and those together (p<0.001), but also between the bold iso fish and the bold imp (p<0.001) (Figure 6.17 B), but without differences between personality types independent of the treatment group (p=0.635) (Figure 6.17 C).

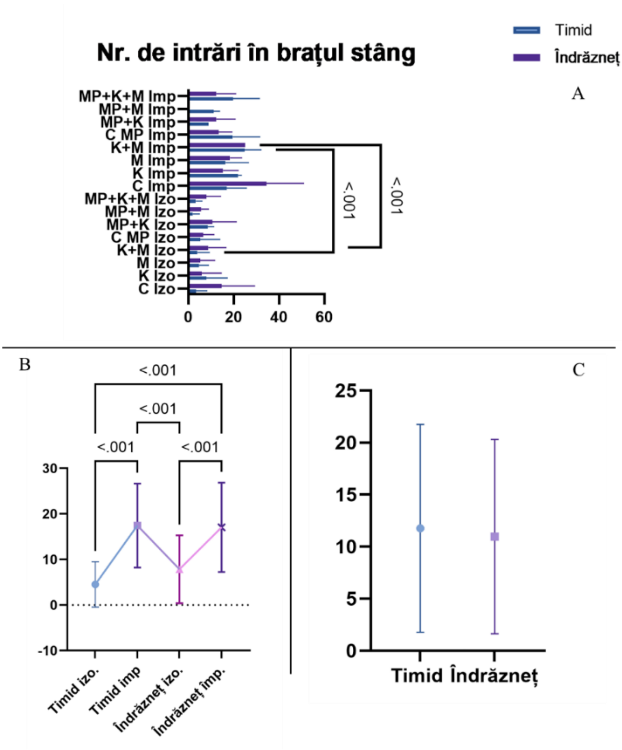


Figure 6.17 Statistical analysis of the number of entries in the left arm. A – analysis based on groups and personality, B – analysis based on personality and hosting conditions, C – analysis based on personality. Results are illustrated as mean ± SD and those with p<0.05 were considered significant).

6.4. Aggressivity test

6.4.1. General

Analysis of the time spent in the left arm showed significantly different results ($p<0.001$) with multiple individual differences. Similar results were obtained for the number of entries in the left arm ($p<0.001$). Despite the fact that there are no significant differences regarding the first entry into the left arm (even though visually there are groups that needed a longer period to make the first entry into the left arm), there are major individual differences regarding the time spent and the number of entries, for example, the isolated groups (L1-L8) entered the left arm later, spent less time there, and entered fewer times. These results can be seen in Figure 6.22.A and B.

For accelerated swimming strokes, no significant differences were identified based on duration, but they were identified based on number (F (DFn, DFd)=1.395 (15, 125), $p<0.001$) (Figure 6.23).

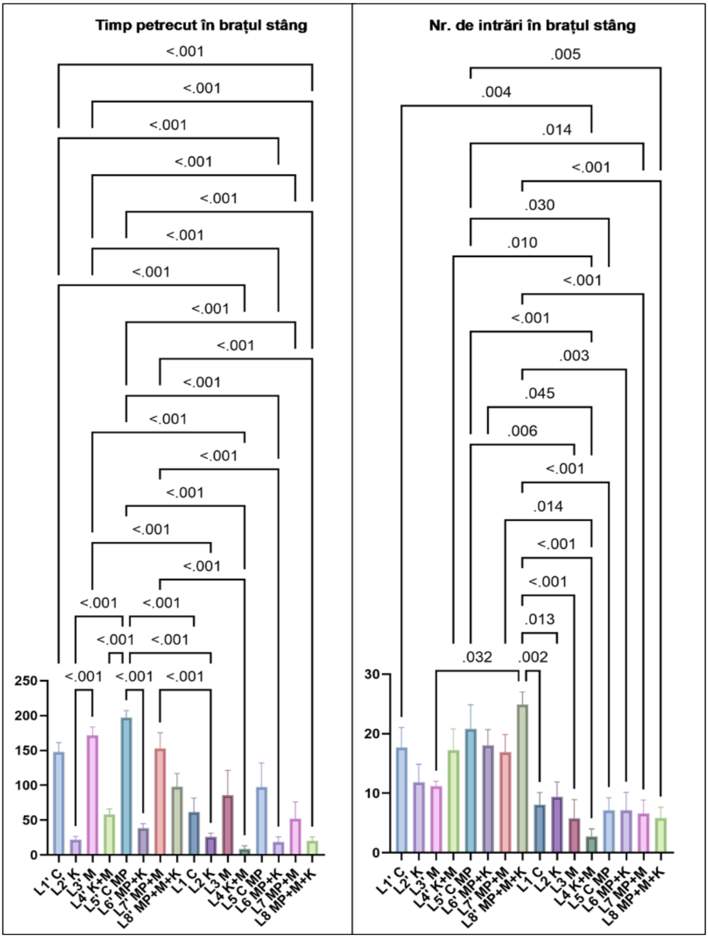


Figure 6.22 Statistical results of the parameter total time spent in the left arm (s) (A) and number of entries in the left arm (B). Results are illustrated as mean ± SD and those with p<0.05 were considered significant.

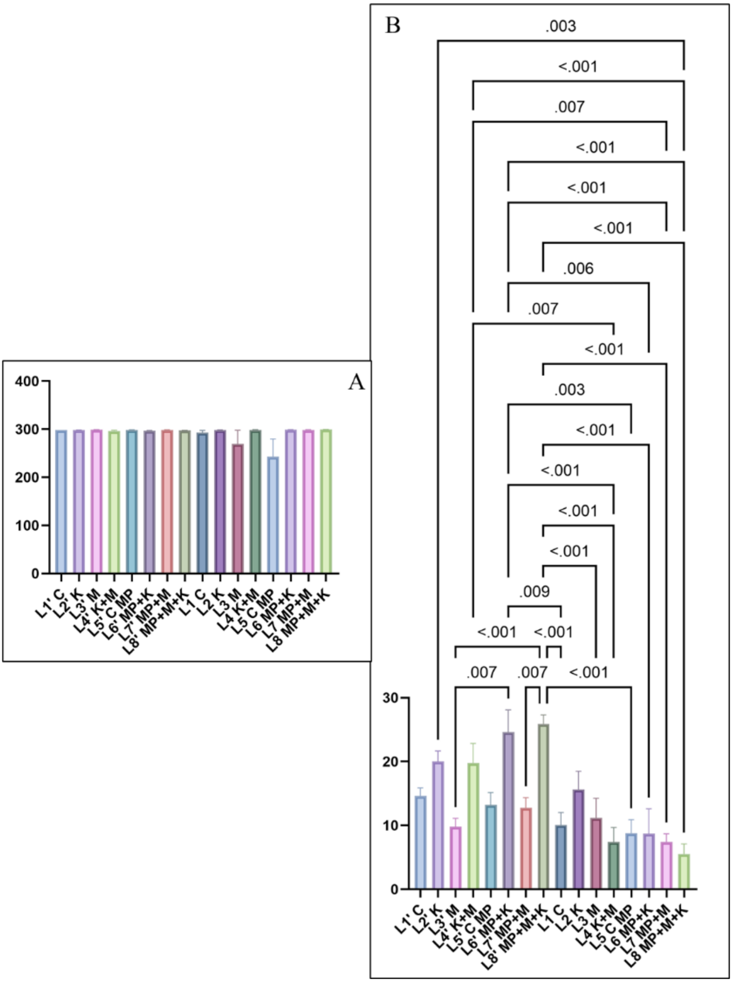


Figure 6.23 Statistical results of the parameter speeded swimming stroke duration (s) (A) and number of speeded swimming strokes (B). Results are illustrated as mean ± SD and those with p<0.05 were considered significant).

6.4.2. Personality

In the case of the total time spent in the left arm, significant differences were observed only between treatments ($p<0.001$), but without significant individual differences (Figure 6.26 A). Statistically significant differences ($p<0.001$) were identified in the analysis depending on personality type and housing conditions (Figure 6.26 B). Moreover, significant differences were observed in the independent analysis of treatment group and housing conditions ($p<0.001$) (Figure 6.26 C).

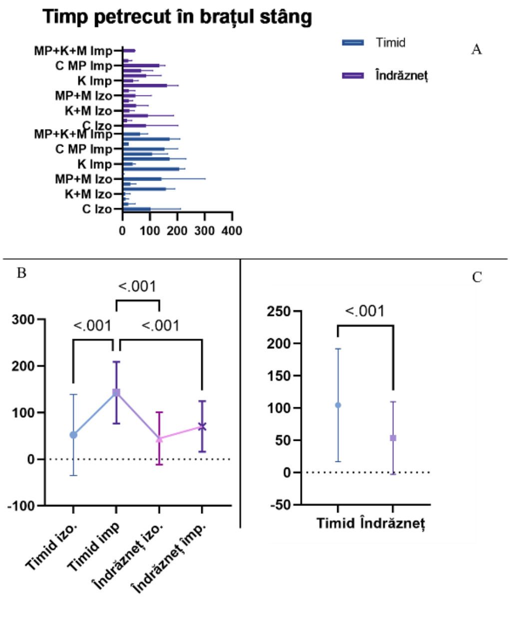


Figure 6.26 Statistical analysis of total time spent in the left arm (s). A – analysis based on groups and personality, B – analysis based on personality and hosting conditions, C – analysis based on personality. Results are illustrated as mean ± SD and those with p<0.05 were considered significant).

Statistical analysis of the number and duration of swimming bouts showed significant differences between treatment groups (p<0.001), but not between personality types (p=0.065). These differences can be seen in Figure 6.29.A. Regarding the analysis dependent on personality type and housing conditions, significant differences were identified (p<0.001) (Figure 6.29 B), although no differences were identified between personality types independent of the treatment group (p= 0.204)(Figure 6.29 C).

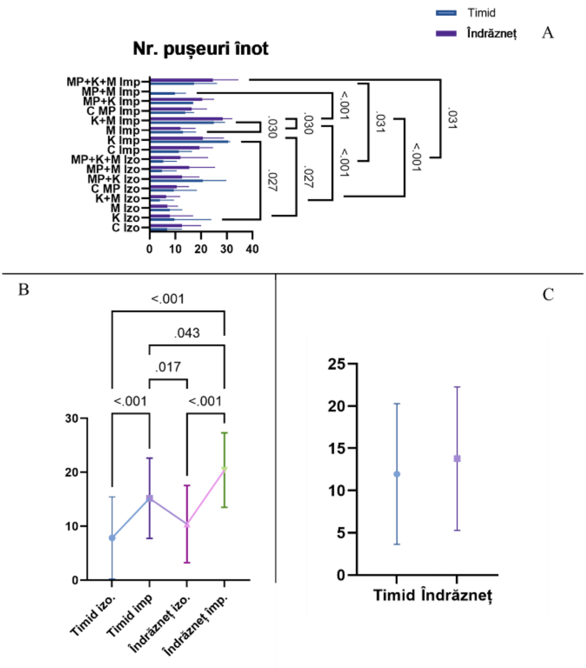


Figure 6.29 Statistical analysis of the number of swimming strokes. A – analysis based on groups and personality, B – analysis based on personality and hosting conditions, C – analysis based on personality. Results are illustrated as mean ± SD and those with p<0.05 were considered significant.

6.5. Light-dark test

6.5.1. General

In the case of the ratio of the time spent in each of the two arms, statistically significant individual results were obtained for each study group according to Figure 6.35.

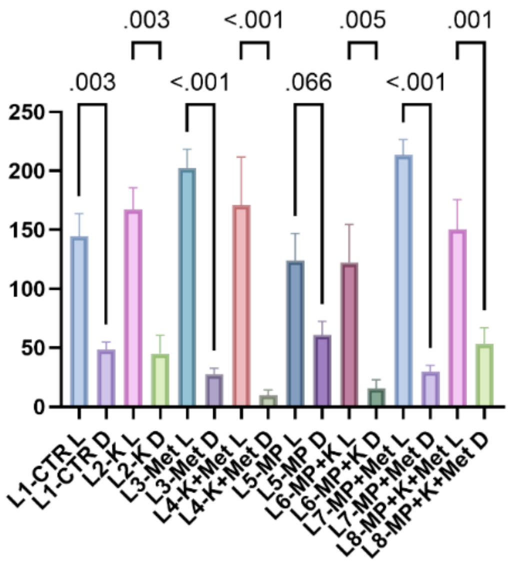


Figure 6.35 Results of analyzing the ratio of time spent in each of the two arms of the groups together. Results are illustrated as mean ± SD and those with p<0.05 were considered significant. L – in the light arm, D – in the dark arm.

Moreover, significant results were obtained between the groups and regarding the total distance traveled (Figure 6.36).

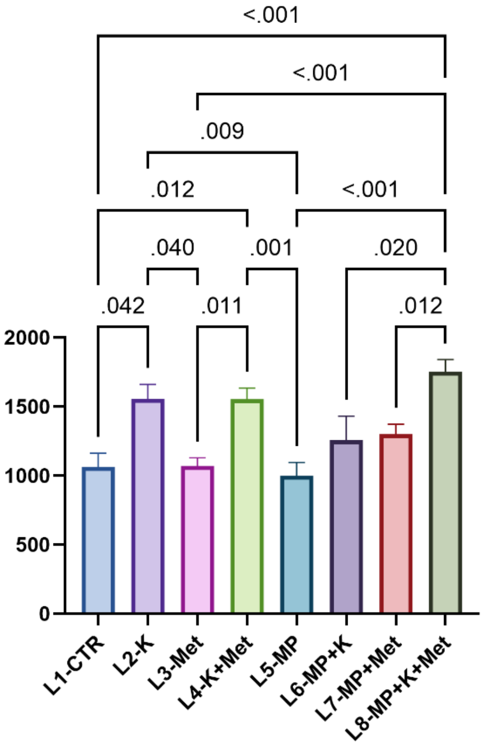


Figure 6.36 Results of analyzing the distance traveled by groups together (mm). Results are illustrated as mean ± SD and those with p<0.05 were considered significant

6.5.2. Personality

Analysis of time spent in the dark arm showed significant differences between treatment groups (p=0.001) (Figure 6.41 A). However, no significant differences were identified between personality types regarding total time spent in the dark arm (p=0.748) (Figure 6.42 B).

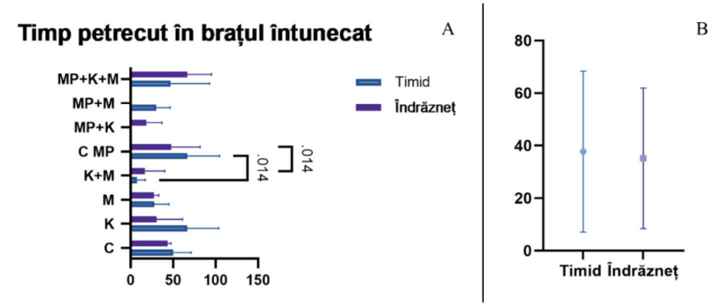


Figure 6.41 Statistical analysis of time spent in the dark arm (s). A – analysis based on groups and personality, B – analysis based on personality independent of treatment. Results are illustrated as mean ± SD and those with p<0.05 were considered significant.

For the number of entries in the dark arm, significant differences were obtained between treatment groups (p=0.007) (Figure 6.42 A), but no significant differences between personality types (p=0.677) (Figure 6.42 B).

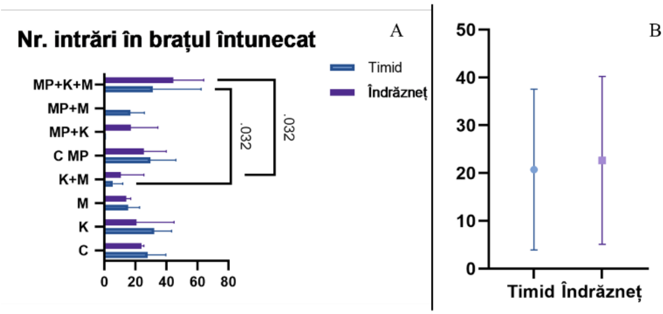


Figure 6.42 Statistical analysis of the number of dark arm entries. A – analysis based on groups and personality, B – analysis based on personality independent of treatment. Results are illustrated as mean ± SD and those with p<0.05 were considered significant.

Statistical analysis of total distance traveled showed significant differences between treatment groups (p<0.001) where shy fish in the C, M, C MP groups explored significantly less than shy fish in the MP+K+M group (p<0.001), but also bold fish from C, M, C MP groups explored the test maze significantly less than bold fish from MP+K+M group (p<0.001 and p=0.007, respectively) (Figure 6.43 A). However, no significant differences were identified between personality types independent of treatment (p=0.680) (Figure 6.43 B).

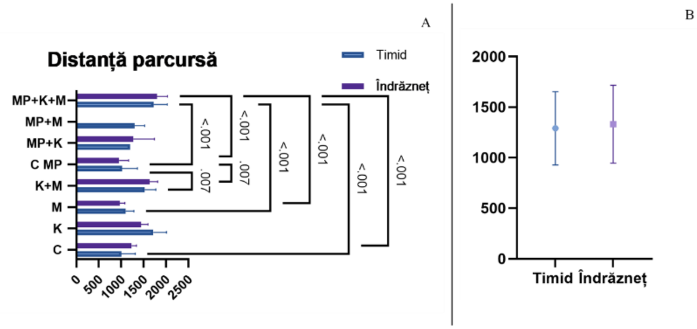


Figure 6.43 Statistical analysis of total distance traveled (mm). A – analysis based on groups and personality, B – analysis based on personality independent of treatment. Results are illustrated as mean ± SD and those with p<0.05 were considered significant.

6.6. Colour-based memory test

6.6.1. General

Based on visually observed differences, the L3 M group showed the best cognitive ability, as it had more than 50% of fish choosing the red arm the first time, followed by L5 MP, L4 M+L and L6 MP+K . These results can be viewed in Figure 6.45.

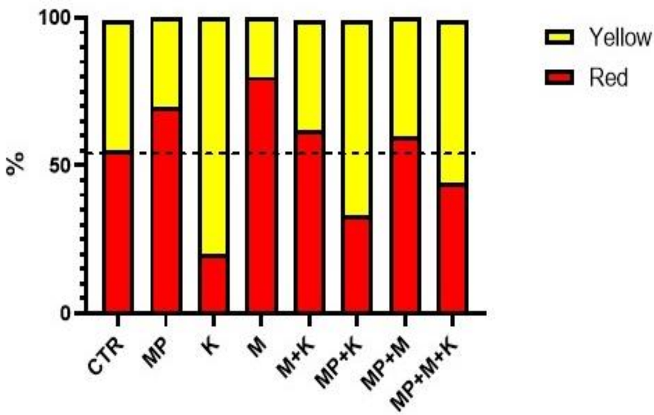


Figure 6.45 The results of the first choice made by each group. Yellow – yellow, Red - red

Regarding the latency to first entry into the red arm, there were significant differences between L1 C and L2 K, L5 MP and L2 K, L2 K and L3 M, and L2 K and L4 K+M. At the same time, significant differences were observed between L2 K and L1 C, respectively L3 M regarding the time spent in the red arm, with the latter spending significantly more time in the trained color. Moreover, significant differences were also observed regarding the number of entries into the red arm, respectively the distance traveled where the L2 K and L6 MP+K groups entered the red arm significantly less times compared to the other groups and traveled and the shortest distance. All these results can be viewed in Figure 6.46.

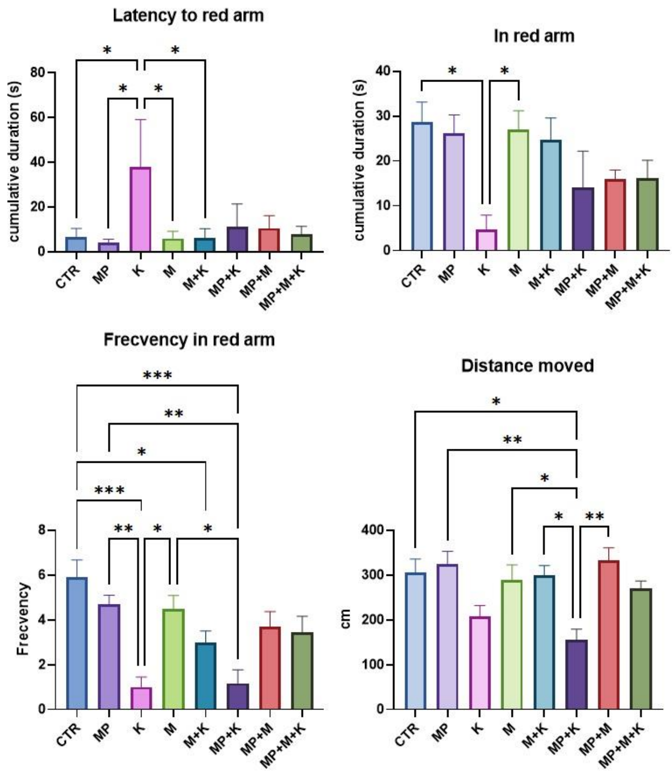


*Figure 6.46 Analysis of the results obtained in the color-based memory test of the following study parameters: time to first entry into the red arm (s), time spent in the red arm (s), number of entries into the red arm, and distance traveled (cm). Results are illustrated as mean ± SD and those with p<0.05 were considered significant. * - p<0.05, ** - p<0.01, *** - p<0.001.*

6.6.2. Personality

The first choice of fish in each treatment group differentiated on the basis of personality can be viewed in Figure 6.47.

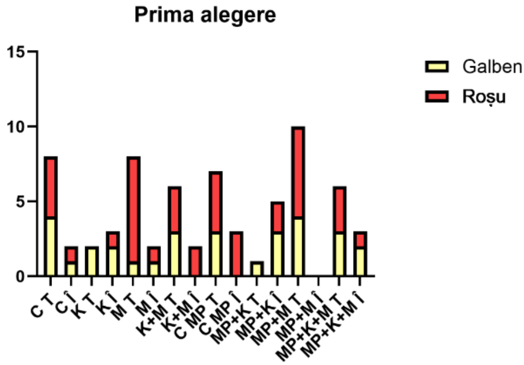


Figure 6.47 first choice of zebrafish by treatment group and personality

Differences appear regarding total time spent in the red arm where significant differences were identified between treatment groups ($p<0.001$), but no individual differences (Figure 6.50 A) and no significant differences between personality types ($p=0.432$) (Figure 6.50 B).

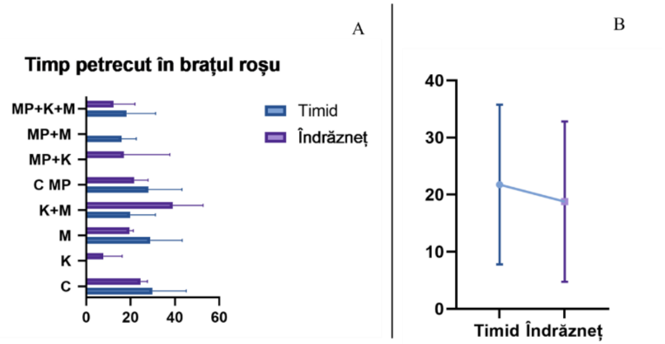


Figure 6.50 Statistical analysis of time spent in the red arm (s). A – analysis based on groups and personality, B – analysis based on personality independent of treatment. Results are illustrated as mean ± SD and those with $p<0.05$ were considered significant.

Regarding the number of entries into the red arm, the statistical analysis between treatment groups was statistically significant ($F(7, 58)=5.210$, $p<0.001$) with notable differences between bold fish in group C and those in MP+M ($p=0.003$) and those bold in group K with those in group C MP ($p=0.048$) (Figure 6.52 A). However, no significant differences were identified between personality types independent of treatment groups ($t=1.853$, $df=65$, $p=0.068$) (Figure 6.52 B).

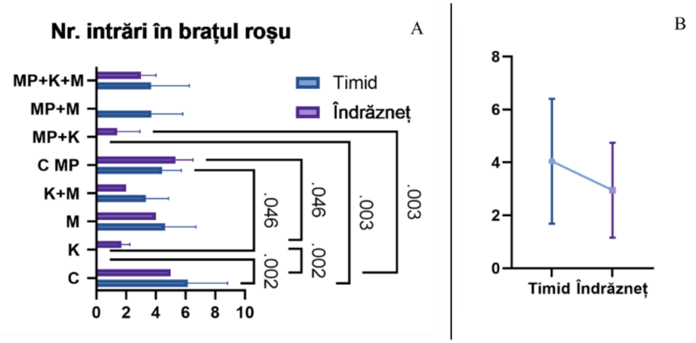


Figure 6.52 Statistical analysis of the number of entries in the red arm. A – analysis based on groups and personality, B – analysis based on personality independent of treatment. Results are illustrated as mean ± SD and those with p<0.05 were considered significant).

6.7. Ministudy on the dissociative effect of ketamine

The separation of zebrafish into one personality or another was done based on the tracks and the evolution and change of personality traits following ketamine treatment was followed.

Figure 6.54 shows the tracks and heatmaps of a group of 4 fish categorized as shy pre-treatment and bold post-treatment.

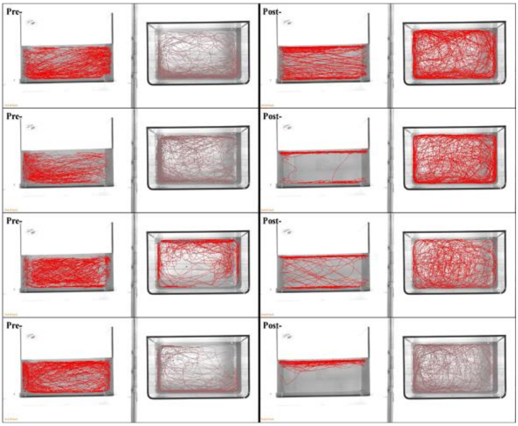


Figure 6.54 Tracks of four fish pre- and post-ketamine treatment illustrating clear behavioral changes in swimming pattern and exploratory propensity.

In Figure 6.55 heatmaps are illustrated for a better appreciation of the preference to sit by the walls/explore the aquarium of each of the fish studied.

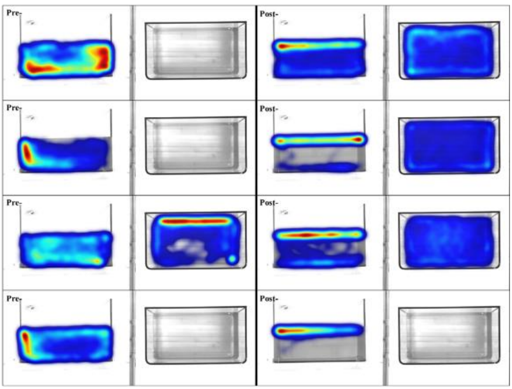


Figure 6.55 Heatmaps of four fish pre- and post-ketamine treatment illustrating clear behavioral changes in swimming pattern and exploratory propensity.

In Figure 6.56 the following parameters are presented: latency first entry into the upper half of the tank (A), time spent in the upper half (B), number of entries in the upper half (C), time spent in the lower half (D) and the number of entries in the lower half (E). Thus, statistically significant differences were obtained between before and after treatment in the case of the time spent in the upper half (p=0.002) and the number of entries in the lower half (p<0.001).

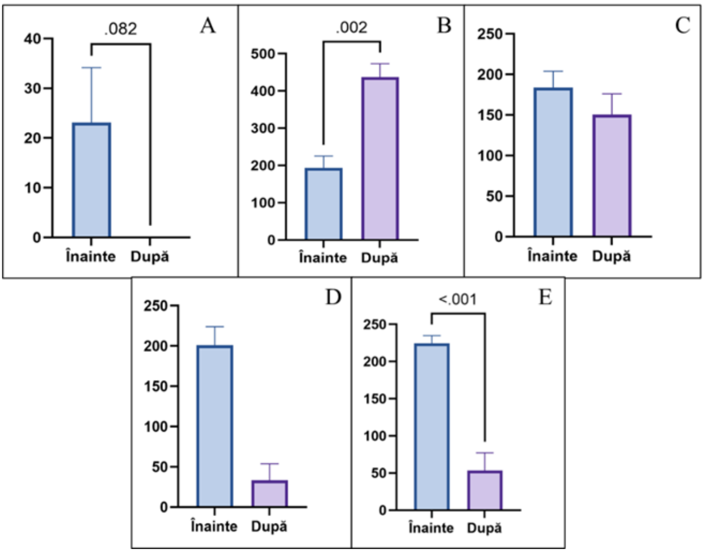


Figure 6.56 Latency to first entry in upper half (s) (A), time spent in upper half (s)(B), number of entries in upper half (C), time spent in lower half (s)(D) and the number of entries in the lower half (E). Results are illustrated as mean ± SD and those with p<0.05 were considered significant.

6.8. Immunohistochemistry

A series of histopathological changes were observed in different layers of the optic tectum of zebrafish following exposure to microplastics, ketamine, methionine and the combined ones. The severity and frequency of lesions was more pronounced in the L6 MP+K, L7 MP+M groups and more attenuated in the L8 MP+K+M and L4 M+K groups.

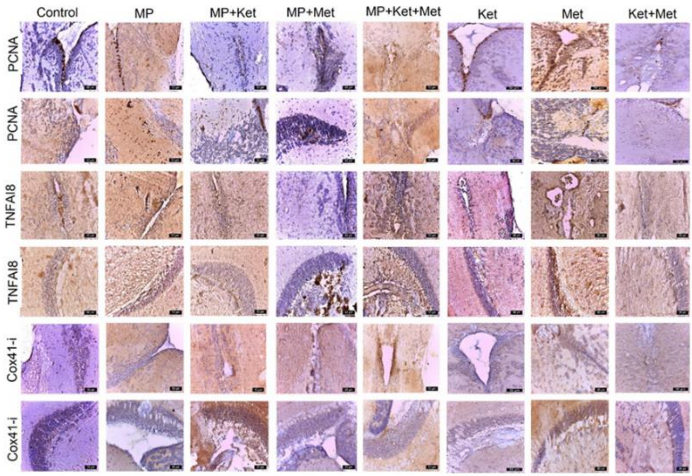


Figure 6.59 The different expressions of PCNA, TNFAI8, COX4l-I in different experimental groups and control groups. Rows 1,3 and 5 show periventricular expression, while rows 2,4 and 6 show expression in the optic tectum.

The presence of *TNFαI8* expression in the nervous system of zebrafish from the experimental groups suggests the presence of an inflammatory process generated by MP, K, M and their combination. Moreover, different levels of *COX41-I* expression were observed in the periventricular zone and optic tectum, suggesting the existence of different degrees of ATP generation dependent on hypoxia-inducible factor expression.

6.9. Oxidative stress

Moreover, regarding SOD level (Figure 6.62), significant differences were observed between: L2 K and L6 MP+K ($p<0.05$), L3 M and L6 MP+K ($p<0.05$) , L6 MP+K and L7 MP+M ($p<0.01$). Regarding the GPx level (Figure 6.62), significant differences were identified between L1 C and L8 MP+K+M ($p<0.0001$), L5 MP and L8 MP+M+K ($p<0.01$), L2 K and L8 MP+M+K ($p<0.01$), L6 MP+K and L8 MP+M+K ($p<0.01$), L7 MP+M vs.. L8 MP+M+K ($p<0.05$). And regarding MDA (Figure 6.62), significant differences were observed between L5 MP and L7 MP+M ($p<0.01$), L2 K and L7 MP+M ($p<0.05$).

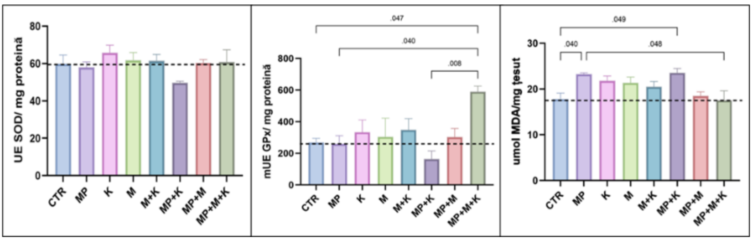


Figure 6.62 Results of oxidative stress biomarkers

6.10. Alpha-amylase activity

Alpha-amylase activity was measured for both isolated and pooled groups, and results were expressed as U/mg tissue.

In the case of this measurement, the statistical analysis was significant ($F (15, 30)=2.613$, $p=0.012$), but no statistically significant individual differences were identified (Figure 6.63).

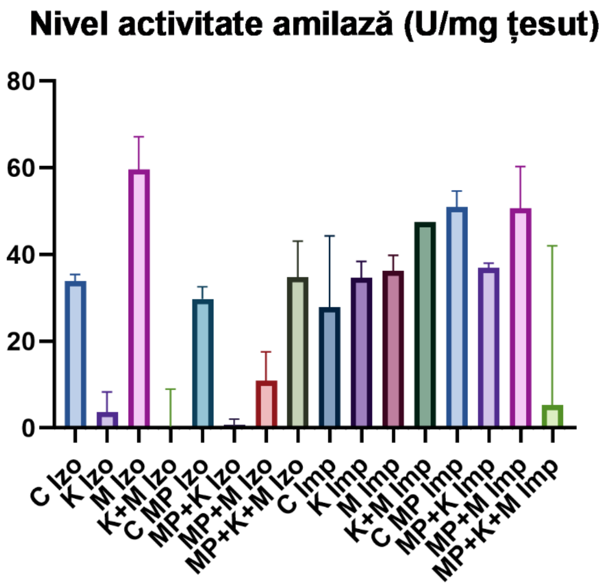


Figure 6.63 Statistical analysis of alpha-amylase activity expressed as U/mg tissue. Results are illustrated as mean ± SD and those with $p<0.05$ were considered significant.

6.10.1. Correlations between alpha-amylase activity and behavioural parameters

Markers for anxiety used in this analysis were time to first entry into the upper half of the aquarium and duration of freezing episodes. In the case of fish with an increased level of alpha-amylase, a weak positive correlation was identified (Figure 6.64

A), but stronger than in the case of the correlation between fish with a low level of alpha-amylase (Figure 6.64 B).

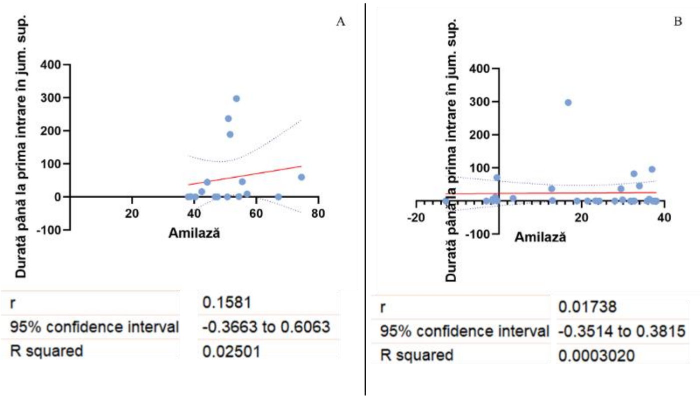


Figure 6.64 Correlation between alpha amylase level and time to first upper half entry. A – correlation between an increased level of alpha-amylase and the time until the first entry into the upper half (weakly positive correlation, r=0.1581), B – correlation between a low level of alpha-amylase and the time until the first entry into the half superior (non-existent correlation, r=0.01738).

In the case of the duration of freezing episodes, a weak negative correlation was obtained between a low alpha amylase level and the total duration of freezing episodes (Figure 6.65 A).

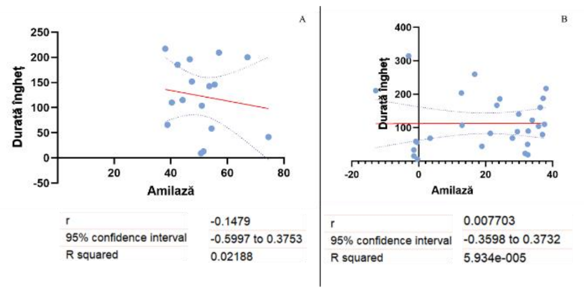
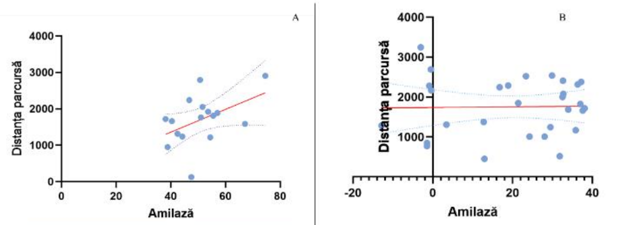


Figure 6.65 Correlation between alpha amylase level and duration of freezing episodes. A – correlation between an increased level of alpha-amylase and the duration of freezing episodes (weakly negative correlation, r=-0.1479), B – correlation between a low level of alpha-amylase and the duration of freezing episodes (non-existent correlation, r= 0.007703).

Only a moderate positive correlation was obtained between an elevated alpha-amylase level and total distance traveled (Figure 6.66 A).

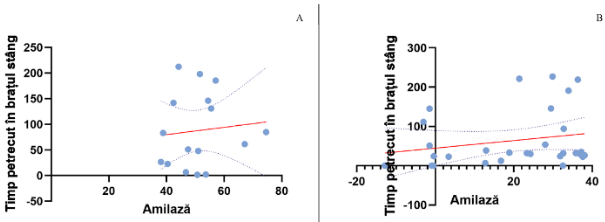


A	
r	0.4586
95% confidence interval	-0.04805 to 0.7775
R squared	0.2103

B	
r	0.01892
95% confidence interval	-0.3500 to 0.3828
R squared	0.0003579

Figure 6.66 Correlation between alpha amylase level and total distance traveled. A – correlation between an increased level of alpha-amylase and the total distance traveled (moderate positive correlation, r=0.4586), B – correlation between a low level of alpha-amylase and the total distance traveled (non-existent correlation, r=0.01892).

Only a weak positive correlation was obtained between a low amylase level and time spent in the conspecific arm (Figure 6.67 B).

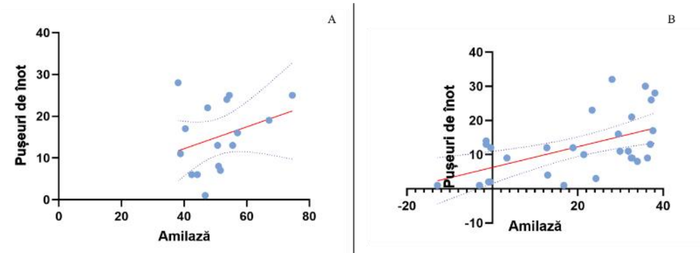


A	
r	0.09763
95% confidence interval	-0.4183 to 0.5659
R squared	0.009531

B	
r	0.2113
95% confidence interval	-0.1683 to 0.5363
R squared	0.04464

Figure 6.67 Correlation between alpha amylase level and time spent in the left arm. A – correlation between an increased level of alpha-amylase and time spent in the left arm (non-existent correlation, r=0.09763), B – correlation between a low level of alpha-amylase and time spent in the left arm (weakly positive correlation, r =0.2113).

Aggressiveness in this case was measured by means of the number of swimming strokes in the aggressiveness test. Through the analysis, we obtained a moderate positive correlation between an increased level of amylase and the number of swimming strokes (r=0.3145, R2=0.09888) (Figure 6.68 A), but also a strong moderate positive correlation between a low alpha-amylase level and the number of swimming strokes (r=0.5433, R2=0.2951) (Figure 6.68 B).



r	0.3145
95% confidence interval	-0.2147 to 0.7009
R squared	0.09888

r	0.5433
95% confidence interval	0.2207 to 0.7587
R squared	0.2951

Figure 6.68 Correlation between alpha amylase level and number of swimming strokes. A – correlation between an increased level of alpha-amylase and the number of swimming strokes (moderate positive correlation, r=0.3145), B – correlation between a low level of alpha-amylase and the number of swimming strokes (strong moderate positive correlation , r=0.5433.

7. Discussions

In animals, personality traits refer to consistent patterns of behavior, cognition, and emotional responses that can be observed in different situations over time (70). Some of these traits are: boldness (71) (the tendency to take risks or show courage in a new situation), aggression (72) (highlighted by the degree of fighting, territoriality and dominance), sociability (73) (the preference to stay in the vicinity of conspecifics or even individuals of other species), the level of activity (74) (animals can be differentiated based on activity level), exploration level (74) (tendency to explore new environments, to be more curious), neophobia/neophilia (75) (fear/curiosity for new things), anxiety (76) (the way they respond to stimuli or situations).

That being said, studies suggest the following distinction between personalities (and specific characteristics): the proactive or daring personality (increased exploratory tendency, increased level of aggression, increased level of sociability, swimming in the whole tank, less prone to anxiety, better cognitive skills , leaving the comfort zone faster) and reactive or shy personality (low exploratory tendency, low aggression, low sociability, preference to sit at the bottom of the tank, prone to anxiety, more time spent in the comfort zone (77–80).

In the context of the novel aquarium test, the latency to first entry into the upper half serves as an indicator of anxiety level. Thus, the longer this time is, the greater the anxiety of the fish. In the current study, the combined control group (L1` C) took significantly longer to enter the top half compared to almost all other groups (minus the combined methionine group) and spent significantly less time in the top half, aspects that reflect the behavior considered normal for this species and which are consistent with the data in the literature. An interesting aspect is that the isolated fish had a significantly higher level of anxiety than the groups together in all monitored parameters, except for the ketamine groups which had a lower level of anxiety. This is also illustrated in other studies investigating possible anxiolytic effects of ketamine and may be explained by the fact that ketamine increases neuroplasticity and regulates glutamate receptors which in turn increase the flow of positive thoughts and decrease anxiety and depression. Differences between socially isolated and together groups are supported by results from the literature on the level of anxiety investigated in the novel aquarium test (81).

The literature suggests that a healthy group should show anxious behavior when exposed to a new environment, manifested by a tendency to stay at the bottom of the tank, longer freezing duration, reduced exploration, and longer latency to first entry into the upper half. Based on the fact that the exposure to ketamine mimics the positive symptoms of schizophrenia, research suggests that ketamine toxicity leads to reduced anxiety manifested by shorter latency to first entry into the upper half of the tank, more time spent in it, increased exploration and clockwise rotation number increased. On the other hand, studies show that a concentration of 6.0 mM methionine should decrease anxiety and increase exploration, but without affecting locomotor activity (82). Although studies on the effects of microplastics are scarce, in particular polypropylene polymers are suggested to induce anxiety-related behaviors (83) which suggests that in the case of the new aquarium test, it could lead to swimming patterns and parameters closer to the values of the control groups. The idea of social isolation is a continuation of a previously published study obtained through a collaboration of our study group with a research group in Egypt that looked at the potential of using social isolation to show schizophrenia in male albino rats (84).

In the case of methionine, other studies have shown that exposure to it leads to reduced locomotion, reduced aggressive behavior, impaired memory and increased anxiety (85). This aspect is also observed in the present study where the methionine groups spent significantly less time in the upper half, although they entered faster, had fewer entries and more clockwise rotations.

For the control and ketamine groups together, the study results are consistent with those in the literature. In the case of methionine, there was a small improvement in the time to first entry into the upper half and the time spent there compared to the control group, but locomotor activity was also affected, as seen by decreasing the distance moved and increasing the duration of freezing (86). Multiple studies have observed that increased anxiety leads to reduced locomotion through a sustained pause in movement known as freezing episodes (87,88). Therefore, anxiety can be assessed based on the number and cumulative duration of freezing episodes and the distance moved. The fact that microplastics increased freezing time and reduced distance moved could suggest that they have an anxiogenic effect when administered over a 7-day period. However, it should be taken into account that episodes of freezing and reduced movement can also be due to the toxicity of the substances used.

Thus, we found that the latency to reach the upper half of the tank was shorter, while the time spent in the upper half was longer in the MP group compared to the untreated control group, consistent with the view that MPs have an anxiolytic effect. This finding is in contrast to previous reports showing that MP induces anxiety in zebrafish without affecting locomotor activity.

Studies have shown that subanesthetic administration of ketamine significantly alters upper half time, number of entries, and time to first upper half entry (89), whereas in the case of methionine, a concentration of 6.0 mM causes a substantial increase in locomotion and exploration in the upper half compared to an untreated control (82) which are in accordance with the results of the present study and validate them.

Differences between the together and isolated groups regardless of substance exposure are minor in most parameters, suggesting that there may be other methods of inducing anxiety/depressive mental disorders-lie symptoms that cause less distress to the animal. However, the major differences are observed in the groups that were simultaneously treated with ketamine and methionine and may represent a better approach for modeling schizophrenia, since it is a symptomatologically nonlinear disorder in the sense that it is described by both positive symptoms (mimicked by the effect of ketamine), as well as by negative symptoms (the effect of methionine) and cognitive symptoms (the effect of microplastics). These results and findings from the literature (90) suggests that the combined effects of these substances provide a robust model of complex symptomatology that cannot be fully replicated in humans, but models as close as possible can be created.

According to the studies, bold fish should have a lower anxiety level compared to shy ones, which is true for isolated microplastic groupd as well as for those together. There were no significant differences between the two personalities in the independent analysis, but shy fish together spent the least time in the top half, followed by isolated Shy, isolated Bold, and together Bold suggesting that social isolation on its own also affects behavior and fish anxiety regardless of the applied treatments.

In the case of the total distance moved, in all study groups differences are observed between shy and bold fish with significant difference between the two personalities in the independent comparison of group and experimental protocol. This supports the personality trait that shy fish tend to explore less than bold ones.

This could be explained by the fact that the fish that were socially isolated had a greater curiosity and desire to explore once they were placed in the test tank which was significantly larger and of a different shape compared to the isolation tanks. Thus, social isolation can have strong effects on mental health even in humans, including anxiety levels.

These results, however, are contrary to results in the literature which claim that social isolation can lead to increased anxiety-like behavior by staying more at the bottom of the tank, reduced exploration and altered locomotor activity.

Surprisingly, data from the literature suggest that regardless of whether ketamine administration is chronic or acute in zebrafish, it increases social preference, manifested specifically by increased time in the arm with conspecifics (91), while methionine induces social impairment in rats (90) and zebra fish (82). A large study found that social isolation is associated with increased anxiety, but that this could be reversed by administering anxiolytic substances (92). In the current study, the results are slightly different in the sense that ketamine did not have effects of facilitating and improving social preferences, and methionine did not cause social impairments but on the contrary increased the time spent in the arm with conspecifics compared to the other groups, while ketamine lowered it. Furthermore, groups that ingested microplastics showed increased social preference with the L5 MP group having the longest time spent in the conspecific arm.

On the other hand, in the personality analysis things were similar. Ketamine did not increase social preference and methionine did not cause social impairment. In isolated fish, the bold ones preferred to stay longer near conspecifics, but in shared fish, the shy ones preferred to stay more near conspecifics, they entered the arm with conspecifics faster and more times compared to the isolated ones, which supports the fact that social isolation has induced social deficiencies.

A study that looked at the social preferences of zebrafish reported that it identified a smaller proportion of fish within the same group that preferred solitude and avoided social interactions (93) which matches the traits shown for isolated fish but also for those bold together and supports the existence of distinct traits between individuals of the same species and group.

In the case of the personality-based analysis where isolated shy fish swam significantly faster in the mirror arm compared to together, and isolated bold fish swam faster in the mirror arm compared to bold and shy together. Regarding time spent in the arm of interest, fish together of both personalities spent significantly more time in the corresponding arm compared to the corresponding isolated groups. In terms of accelerated swimming bouts, the bold fish together had the most accelerated swimming bouts, while the isolated shy ones had the fewest with statistically significant differences between all four categorizations (Shy iso., Bold Izo., Timid imp., Bold imp.) with the mention that the bold izo. they had lower numbers even than Timid imp fish. These aspects suggest that social isolation affects aggressive behavior independent of personality type, but does not cause them to become the opposite of their personality, and in combination with personality it can be said that even the boldest of isolated fish is less courageous than shy fish together.

Despite literature data that zebrafish would prefer the dark environment, in the present study all groups took significantly longer to enter the dark side compared to the light side. Moreover, consistent with the observations made in the other trials that ketamine has anxiolytic effects, all study groups given ketamine (either single exposure or combined with other substances) entered the light arm more quickly and later in the dark arm compared to the other groups. In terms of time spent in each of the two arms, the methionine groups spent less time in the dark zone and more time in the light zone compared to the other groups. In addition, all groups spent significantly more time in the light area compared to the dark area.

In terms of personality, the shy fish in most study groups entered the dark compartment later and the light compartment faster compared to the groups treated with microplastics and/or pharmaceuticals. Similar results were obtained for the time spent in each of the arms, where in most groups the shy fish spent significantly more time in the light compartment and less time in the dark compartment. Regarding the distance moved, significant personality- and treatment-dependent differences were identified where the administered substances significantly increased the exploratory tendency of shy fish.

Compared to studies in the literature that illustrated that zebrafish prefer the color red over yellow (94,95), In the present study, our groups chose y.ellow as their preferred color (86) and red as the least favorite

Based on the fact that zebrafish have cognitive flexibility and the ability to make choices (e.g. choose a color, especially when motivated by rewards)(96), we wanted to make a considerable contribution to the information available and already existing in the specialized literature regarding their learning ability by positively conditioning them (reward in the form of food) to choose the less preferred color over the preferred one. This experiment idea was based on the hypothesis of dopamine dysregulation in schizophrenia since it analyzes reward-based behavior (87) which influences cognitive functions, especially memory and decision-making (51). Surprisingly, the group with the most fish choosing the color red first was the methionine group, while the group with the fewest fish to choose red was the ketamine group.

There is a possible correlation between the level of anxiety observed in the novel tank test and the cognitive performance in the memory test described in the study published by our group (86).

Ketamine is a dissociative anesthetic drug that has become of interest in the scientific world because of its unique effects on the conscious mind. When administered in subanesthetic doses, ketamine can induce a dissociative state characterized by profound alterations in perception, cognition, and self (97). Ketamine can distort sensory perception leading to changes in the way individuals perceive the environment, sounds and other sensory stimuli. Individuals may experience visual distortions such as changes in depth perception, color perception or visual hallucinations (98).

In the case of tracks and heatmaps, the changes in the exploration behavior are evident between pre- and post-treatment with the increase in the level of exploration and the decrease in the tendency to stay near the walls, respectively at the bottom of the tank. Moreover, the fish entered the upper half faster, spent more time in the upper half of the tank and less in the lower half, entered the upper half and the lower half less often. Regarding the time spent touching the walls and the number of touches, the post-treatment values were lower. Based on the number of clockwise and counterclockwise rotations, post-treatment anxiety levels were lower (more clockwise rotations and fewer counterclockwise rotations). So it could be concluded that ketamine can change the personality traits of zebrafish post-chronic treatment (5 days) and could be used in modeling behavioral symptomatology and in the context of personality disorders, besides psychotic ones.

In the groups that received ketamine, *PCNA*-positive cells were observed in the two areas, periventricular (ZPV) and cerebellar valve, but a lower number of migrating cells was also noted. It is possible that ketamine not only alters the expression pattern of *PCNA* but also the migration/differentiation timing of cerebellar cell types in adults, contributing to an overall altered state of CNS cell proliferation.

The presence of *TNFαI8* expression in the nervous system of fish from the experimental groups denotes the presence of an inflammatory process generated by MP, K, M and their combination.

COX4-1 expression is linked to hypoxia-inducible factor (*HIF-1α*), and *COX4-1*-deficient cells have been shown to have increased levels of *HIF-1α* located in their nuclei (99). The presence of *COX41-i* overexpression in some groups denotes the increase in the energy requirement to restore the cellular metabolism of nerve cells, as a result of oxidative stress and neurotoxicity produced by various exposures.

BDNF could act as an antioxidant factor, as it is known to increase the activity level of some antioxidant enzymes (100). The presence of an intense expression in the experimental groups, demonstrates the ability to activate the system of antioxidant enzymes to restore the damage produced by MP, M, K and their combination alike.

The influencing character on oxidative stress was highlighted both in the case of schizophrenia models and for single and co-administered microplastics. Moreover, MDA increases were also identified in the same situations (101). In a study aimed at evaluating transgenerational effects in the case of methionine exposure, it was highlighted that the level of MDA shows increases compared to the control group, similarly in the case of SOD (102). Moreover, a significant decrease in SOD was shown after withdrawal of methionine exposure (103). Regarding the administration of ketamine, the specialized literature indicates that at different concentrations the levels of SOD and MDA increase, even after the cessation of administration (104).

Studies on the level of alpha-amylase in zebrafish are non-existent at the moment, but nevertheless there are a number of studies that highlight the possibility of its use as a biomarker for physiological stress. The main conclusions highlighted in the literature are that alpha-amylase is more sensitive to physiological stress than cortisol, in the sense that it can be affected without affecting cortisol levels, and alpha-amylase levels either do not change according to the normal (diurnal) pattern or be hypo- or hyper-secretion (105–107). Even though in the present study no significant differences were identified between the study groups, the fact that it obtained different levels of alpha-amylase activity suggests its usefulness for assessing the level of physiological stress. Moreover, the fact that we identified weak and moderate correlations between this parameter and the behavioral ones suggests that also in zebrafish there is a relationship between physiological stress and behavior.

Conclusions

The current study brought to light and highlighted different aspects associated with neurotoxicity determined by different pharmacological substances and/or plastic materials in order to highlight possible alternatives for modeling some disorders of a neuro-psychiatric nature with a significant reduction in the suffering of the individuals used. The main and interesting conclusions that can be drawn from the present study are:

1. Social isolation on its own could faithfully mimic the negative symptoms of schizophrenia through the prism of the values obtained in each parameter of the conducted behavioral tests.
2. Combined administration of ketamine and methionine could yield a more robust model of schizophrenia that explores both negative and positive symptoms, novel approach at least at the national level.
3. We highlighted a correlation between anxiety level and cognitive performance, an aspect that is also observable in humans and is relevant for future studies.
4. The preference of fish for a particular color is relative, which emphasizes that for any color-based study and not only, it is important to test the study lots before starting the experiment because there is a possibility that the available animal lots will not react/ not behave the same as the data identified in the literature. Animals are sensitive and strongly influenced by environmental, genetic and social factors.
5. The methodology used for the color-based memory test and positive conditioning is new nationally and internationally.

6. The hypotheses of differentiating the personality of zebrafish based on behavioral parameters were mostly confirmed and highlight the existence of personality in these fish.
7. We highlighted and demonstrated for the first time nationally and internationally that zebrafish have personality and even complex traits that cannot have clear boundaries and cannot be strictly delimited in the context of behavioral tests considered standard for them.
8. Each of the personality results illustrates that not all shy fish are the same, and neither are all bold fish. Within each category there are subcategories and it is important to note that even if a shy fish might have a slightly higher value in a parameter compared to shy conspecifics, it does not mean that it is misclassified.
9. In the case of the present study, the dissociative effect of ketamine was shown through the change of multiple parameters and swimming patterns from shy to bold fish categorization.
10. Immunohistology results illustrate that all substances used affect the fish up to the tissue level and up to the stage of necrosis.
11. Paradoxically, microplastics significantly increase the level of oxidative stress.
12. Obtaining different values of alpha-amylase activity highlights its possible utility for the assessment of

physiological stress, while the obtained correlations can serve as behavioral predictive factors.

The results and observations obtained from this study open multiple opportunities for future studies in which fish are socially isolated for a longer period of time to more faithfully model the negative symptoms of schizophrenia, the learning period is extended and tested, and short-term memory (not only the long-term one as in the present study), to repeat the light-dark test without other influences or other types of training done simultaneously, to repeat the aggression test according to the new methods of its assessment, to study the dissociative effects of ketamine in more depth and on a larger batch of fish.

References

1. Jablensky A. The diagnostic concept of schizophrenia: its history, evolution, and future prospects. Dialogues Clin Neurosci. 2010 Sep 30;12(3):271–87.
2. Arango C, Dragioti E, Solmi M, Cortese S, Domschke K, Murray RM, et al. Risk and protective factors for mental disorders beyond genetics: an evidence-based atlas. World Psychiatry. 2021 Oct 1;20(3):417–36.
3. Petrova NN, Khvostikova DA. Prevalence, Structure, and Risk Factors for Mental Disorders in Older People. Advances in Gerontology. 2021 Oct 1;11(4):409.
4. McKee J, Brahm N. Medical mimics: Differential diagnostic considerations for psychiatric symptoms. Ment Health Clin. 2016 Nov 1;6(6):289.
5. Seng J, Miller J, Sperlich M, van de Ven CJM, Brown S, Carter CS, et al. Exploring dissociation and oxytocin as pathways between trauma exposure and trauma-related hyperemesis gravidarum: a test-of-concept pilot. J Trauma Dissociation. 2013 Jan;14(1):40.
6. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Vol. 94, Physiological Reviews. American Physiological Society; 2014. p. 909–50.
7. Ortiz GG, Pacheco Moisés FP, Mireles-Ramírez M, Flores-Alvarado LJ, González-Usigli H, Sánchez-González VJ, et al. Oxidative Stress: Love and Hate History in Central Nervous System. In: Advances in protein chemistry and structural biology [Internet]. 2017 [cited 2019 Apr 1]. p. 1–31. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1876162317300032
8. Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of the zebrafish, Danio rerio. Biological Reviews. 2008 Feb;83(1):13–34.
9. Ashwin C, Chapm E, Howells J, Rhydderch D, Walker I, Baron-Cohen S. Enhanced olfactory sensitivity in autism spectrum conditions. Mol autism. 2014;5(53).

10. Pervin LA. Personality. In: APA: Encyclopedia of Psychology. 2000.
11. Forkosh O. Animal behavior and animal personality from a non-human perspective: Getting help from the machine. Patterns. 2021 Mar 12;2(3):100194.
12. Mumtaz F, Khan MI, Zubair M, Dehpour AR. Neurobiology and consequences of social isolation stress in animal model-A comprehensive review. Biomed Pharmacother. 2018 Sep 1;105:1205–22.
13. Stefan M, Travis M, Murray RM. An atlas of schizophrenia. The Parthenon Publishing Group; 2002.
14. Howes OD, McCutcheon R, Owen MJ, Murray RM. The role of genes, stress, and dopamine in the development of schizophrenia. Biol Psychiatry. 2017;81(1):9–20.
15. Kesby JP, Eyles DW, McGrath JJ, Scott JG. Dopamine, psychosis and schizophrenia: the widening gap between basic and clinical neuroscience. Transl Psychiatry. 2018;8(1):30.
16. Grace AA, Gomes F V. The circuitry of dopamine system regulation and its disruption in schizophrenia: insights into treatment and prevention. Schizophr Bull. 2019;45(1):148–57.
17. Patel KR, Cherian J, Gohil K. Schizophrenia: Overview and Treatment Options. P T. 2014;39(9):638–45.
18. Egerton A, Grace AA, Stone J, Bossong MG, Sand M, McGuire P. Glutamate in schizophrenia: Neurodevelopmental perspectives and drug development. Schizophr Res. 2020;223:59–70.
19. Uno Y, Coyle JT. Glutamate hypothesis in schizophrenia. Psychiatry Clin Neurosci. 2019;73(5):204–15.
20. Baxter PS, Bell KFS, Hasel P, Kaindl AM, Fricker M, Thomson D, et al. Synaptic NMDA receptor activity is coupled to the transcriptional control of the glutathione system. Nat Commun. 2015 Apr 10;6.
21. Hardingham GE, Do KQ. Linking early-life NMDAR hypofunction and oxidative stress in

schizophrenia pathogenesis. Nature Publishing Group. 2016 Feb 14;17(2):1–9.
22. Becquet P, Vazquez-Anon M, Mercier Y, Wedekind K, Mahmood T, Batonon-Alavo DI, et al. A systematic review of metabolism of methionine sources in animals: One parameter does not convey a comprehensive story. Animal Nutrition. 2023 Jun;13:31–49.
23. Dash PK, Hergenroeder GW, Jeter CB, Choi HA, Kobori N, Moore AN. Traumatic Brain Injury Alters Methionine Metabolism: Implications for Pathophysiology. Front Syst Neurosci. 2016 Apr 29;10.
24. Jin X, Liu L, Liu D, Wu J, Wang C, Wang S, et al. Unveiling the methionine cycle: a key metabolic signature and NR4A2 as a methionine-responsive oncogene in esophageal squamous cell carcinoma. Cell Death Differ. 2024 May 3;31(5):558–73.
25. Tsao D, Diatchenko L, Dokholyan N V. Structural Mechanism of S-Adenosyl Methionine Binding to Catechol O-Methyltransferase. PLoS One. 2011 Aug 31;6(8):e24287.
26. Tang KL, Antshel KM, Fremont WP, Kates WR. Behavioral and Psychiatric Phenotypes in 22q11.2 Deletion Syndrome. Journal of Developmental & Behavioral Pediatrics. 2015 Oct;36(8):639–50.
27. Green T, Steingart L, Frisch A, Zarchi O, Weizman A, Gothelf D. The feasibility and safety of S-adenosyl-l-methionine (SAMe) for the treatment of neuropsychiatric symptoms in 22q11.2 deletion syndrome: a double-blind placebo-controlled trial. J Neural Transm. 2012 Nov 8;119(11):1417–23.
28. Hasan Anik A, Hossain S, Alam M, Binte Sultan M, Hasnine MDT, Rahman MdM. Microplastics pollution: A comprehensive review on the sources, fates, effects, and potential remediation. Environ Nanotechnol Monit Manag. 2021 Dec;16:100530.
29. Sun A, Wang WX. Human Exposure to Microplastics and Its Associated Health Risks. Environment & Health. 2023 Sep 15;1(3):139–49.

30. Lee Y, Cho J, Sohn J, Kim C. Health Effects of Microplastic Exposures: Current Issues and Perspectives in South Korea. Yonsei Med J. 2023;64(5):301.
31. Polderman TJC, Benyamin B, de Leeuw CA, Sullivan PF, van Bochoven A, Visscher PM, et al. Meta-analysis of the heritability of human traits based on fifty years of twin studies. Nat Genet. 2015 Jul 18;47(7):702–9.
32. Savuca A, Curpan A, Nicoara M, Ciobica AS. POSSIBLE RELATIONSHIP BETWEEN ENVIRONMENTAL POLLUTANTS/MICROPLASTICS AND SCHIZOPHRENIA ETIOLOGY AND PROGRESSION. In: Proceedings of the Romanian Academy - Series A: Mathematics, Physics, Technical sciences, information Science. Romanian Academy; 2023.
33. Curpan AS, Savuca A, Hritcu LD, Solcan C, Nicoara MN, Luca AC, et al. A new approach to explore the correlation between declarative memory and anxiety in animal models of schizophrenia and microplastic pollution. Behavioural Brain Research. 2024 Feb;458:114742.
34. Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcoraci V, et al. Oxidative Stress: Harms and Benefits for Human Health. Vol. 2017, Oxidative Medicine and Cellular Longevity. Hindawi Limited; 2017.
35. Liu C, Gao X, Yuan J, Zhang R. Advances in the development of fluorescence probes for cell plasma membrane imaging. Vol. 133, TrAC - Trends in Analytical Chemistry. Elsevier B.V.; 2020. p. 116092.
36. Younus H. Therapeutic potentials of superoxide dismutase. Int J Health Sci (Qassim) [Internet]. 2018 [cited 2024 May 18];12(3):88. Available from: /pmc/articles/PMC5969776/
37. Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine. 2018 Dec 1;54(4):287–93.

38. Flohé L, Toppo S, Orian L. The glutathione peroxidase family: Discoveries and mechanism. Free Radic Biol Med. 2022 Jul;187:113–22.
39. Rhee SG, Cho CS. Blot-Based Detection of Dehydroalanine-Containing Glutathione Peroxidase with the Use of Biotin-Conjugated Cysteamine. In 2010. p. 23–34.
40. Ansarin K, Khoubnasabjafari M, Jouyban A. Reliability of malondialdehyde as a biomarker of oxidative stress in psychological disorders. BioImpacts. 2017 Aug 23;5(3):123–7.
41. Dharmajaya R, Sari DK. Malondialdehyde value as radical oxidative marker and endogenous antioxidant value analysis in brain tumor. Annals of Medicine & Surgery. 2022 May;77.
42. Ermakov EA, Dmitrieva EM, Parshukova DA, Kazantseva D V., Vasilieva AR, Smirnova LP. Oxidative Stress-Related Mechanisms in Schizophrenia Pathogenesis and New Treatment Perspectives. Vol. 2021, Oxidative Medicine and Cellular Longevity. Hindawi Limited; 2021.
43. Fraguas D, Díaz-Caneja CM, Ayora M, Hernández-Álvarez F, Rodríguez-Quiroga A, Recio S, et al. Oxidative Stress and Inflammation in First-Episode Psychosis: A Systematic Review and Meta-analysis. Schizophr Bull. 2019 Jun 18;45(4):742–51.
44. Gonzalez-Liencres C, Tas C, Brown EC, Erdin S, Onur E, Cubukcoglu Z, et al. Oxidative stress in schizophrenia: A case-control study on the effects on social cognition and neurocognition. BMC Psychiatry. 2014 Sep 24;14(1):1–9.
45. Guidara W, Messedi M, Naifar M, Maalej M, Grayaa S, Omri S, et al. Predictive value of oxidative stress biomarkers in drug-free patients with schizophrenia and schizo-affective disorder. Psychiatry Res. 2020 Nov 1;293:113467.
46. Khan F, Sultana S, Mullick M, Akhter N. Oxidative Stress and Antioxidant Status in Schizophrenia Patients. JAFMC Bangladesh. 2016;12(2):40–3.

47. Nucifora LG, Tanaka T, Hayes LN, Kim M, Lee BJ, Matsuda T, et al. Reduction of plasma glutathione in psychosis associated with schizophrenia and bipolar disorder in translational psychiatry. Transl Psychiatry. 2017 Aug 22;7(8):e1215.
48. Bosch JA, Carroll D. Mucosal Secretory Immunity, Stress and. In: Encyclopedia of Stress. Academic Press; 2007. p. 768–74.
49. Damodaran T V. Peripheral nervous system toxicity biomarkers. In: Biomarkers in Toxicology. Academic Press; 2014. p. 169–98.
50. Brierley-Bowers P, Sexton S, Brown D. Measures of autonomic nervous system regulation. Arlington, VA: Defense centers of excellence for psychological health and traumatic brain injury; 2011. 1–24 p.
51. Ieda M, Miyaoka T, Wake R, Liaury K, Tsuchie K, Fukushima M, et al. Evaluation of autonomic nervous system by salivary alpha-amylase level and heart rate variability in patients with schizophrenia. European Archives of Psychiatry and Clinical Neuroscience 2013 264:1. 2013 May 5;264(1):83–7.
52. Stogios N, Gdanski A, Gerrentsen P, Chintoh AF, Graff-Guerrero A, Rajji TK, et al. Autonomic nervous system dysfunction in schizophrenia: impact on cognitive and metabolic health. NPJ Schizophr. 2021;22.
53. Inagaki T, Miyaoka T, Okazaki S, Yasuda H, Kawamukai T, Utani E, et al. High salivary alpha-amylase levels in patients with schizophrenia: A pilot study. Prog Neuropsychopharmacol Biol Psychiatry. 2010 May;34(4):688–91.
54. Kanayama M, Miyaoka T, Araki T, Hayashida M, Hashioka S, Horiguchi J. Salivary Alpha-Amylase Activity Levels in Catatonic Schizophrenia Decrease after Electroconvulsive Therapy. Case Rep Psychiatry. 2018;2018.
55. Meshalkina DA, Kizlyk MN, Kysil E V, Collier AD, Echevarria DJ, Abreu MS, et al. Zebra fish models of autism spectrum disorder. Exp Neurol. 2018;207–16.

56. Kalueff A V., Echevarria DJ, Stewart AM. Gaining translational momentum: more zebrafish models for neuroscience research. Prog Neuropsychopharmacol Biol Psychiatry. 2014 Dec 3;55:1–6.
57. Vaz R, Hofmeister W, Lindstrand A. Zebrafish models of neurodevelopmental disorders: Limitations and benefits of current tools and techniques. Int J Mol Sci. 2019 Mar 2;20(6).
58. Kalueff A V., Gebhardt M, Stewart AM, Cachat JM, Brimmer M, Chawla JS, et al. Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond. Zebrafish. 2013 Mar 1;10(1):70.
59. López-Schier H. Neuroplasticity in the acoustic startle reflex in larval zebrafish. Curr Opin Neurobiol. 2019 Feb;54:134–9.
60. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Developmental Dynamics. 1995 Jul;203(3):253–310.
61. Sökmen TÖ, Sulukan E, Türkoğlu M, Baran A, Özkaraca M, Ceyhun SB. Polystyrene nanoplastics (20 nm) are able to bioaccumulate and cause oxidative DNA damages in the brain tissue of zebrafish embryo (Danio rerio). Neurotoxicology. 2020 Mar 1;77:51–9.
62. Goldsmith P. Zebrafish as a pharmacological tool: the how, why and when. Curr Opin Pharmacol. 2004 Oct;4(5):504–12.
63. Meshalkina DA, Kysil E V, Warnick JE, Demin KA, Kalueff A V. Adult zebrafish in CNS disease modeling: a tank that's half-full, not half-empty, and still filling. Lab Anim (NY). 2017 Oct 1;46(10):378–87.
64. Curpăn A Ștefania, Alin C. Do zebrafish have personality? Possible associations between behavioural patterns and personality traits in Danio rerio in the context of schizophrenia. Bulletin of Integrative Psychiatry. 2023 Dec 15;99(4):23–33.
65. Yuan M, Chen Y, Huang Y, Lu W. Behavioral and metabolic phenotype indicate personality in zebrafish (Danio rerio). Front Physiol. 2018 May 30;9(MAY):653.

66. Chen Y, Li W, Xiang L, Mi X, Duan M, Wu C. Fish personality affects their exposure to microplastics. Ecotoxicol Environ Saf. 2022 Mar 15;233:113301.
67. Daniel DK, Bhat A. Bolder and Brighter? Exploring Correlations Between Personality and Cognitive Abilities Among Individuals Within a Population of Wild Zebrafish, Danio rerio. Front Behav Neurosci. 2020 Aug 12;14:138.
68. Amato-Lourenço LF, Carvalho-Oliveira R, Júnior GR, dos Santos Galvão L, Ando RA, Mauad T. Presence of airborne microplastics in human lung tissue. J Hazard Mater. 2021 Aug;416:126124.
69. Avdesh A, Chen M, Martin-Iverson MT, Mondal A, Ong D, Rainey-Smith S, et al. Regular care and maintenance of a zebrafish (Danio rerio) laboratory: an introduction. J Vis Exp [Internet]. 2012 [cited 2023 Jun 23];(69). Available from: https://pubmed.ncbi.nlm.nih.gov/23183629/
70. Forkosh O. Animal behavior and animal personality from a non-human perspective: Getting help from the machine. Patterns. 2021 Mar 12;2(3):100194.
71. White JR, Meekan MG, McCormick MI, Ferrari MCO. A Comparison of Measures of Boldness and Their Relationships to Survival in Young Fish. PLoS One [Internet]. 2013 Jul 16 [cited 2023 Jul 19];8(7):68900. Available from: /pmc/articles/PMC3712919/
72. Goold C, Newberry RC. Aggressiveness as a latent personality trait of domestic dogs: Testing local independence and measurement invariance. PLoS One [Internet]. 2017 Aug 1 [cited 2023 Jul 19];12(8). Available from: https://pubmed.ncbi.nlm.nih.gov/28854267/
73. Gartland LA, Firth JA, Laskowski KL, Jeanson R, Ioannou CC. Sociability as a personality trait in animals: methods, causes and consequences. Biol Rev Camb Philos Soc [Internet]. 2022 Apr 1 [cited 2023 Jul 19];97(2):802–16. Available from: https://pubmed.ncbi.nlm.nih.gov/34894041/
74. MacKinlay RD, Shaw RC. A systematic review of animal personality in conservation science.

Conservation Biology [Internet]. 2023 Feb 1 [cited 2023 Jul 19];37(1):37. Available from: /pmc/articles/PMC10084254/
75. Costa JHC, Neave HW, Weary DM, von Keyserlingk MAG. Use of a food neophobia test to characterize personality traits of dairy calves. Sci Rep [Internet]. 2020 Dec 1 [cited 2023 Jul 19];10(1). Available from: /pmc/articles/PMC7188825/
76. Steimer T. Animal models of anxiety disorders in rats and mice: some conceptual issues. Dialogues Clin Neurosci [Internet]. 2011 [cited 2023 Jul 19];13(4):495. Available from: /pmc/articles/PMC3263396/
77. Daniel DK, Bhat A. Bolder and Brighter? Exploring Correlations Between Personality and Cognitive Abilities Among Individuals Within a Population of Wild Zebrafish, Danio rerio. Front Behav Neurosci. 2020 Aug 12;14:138.
78. Amin B, Slabbekoorn H, Schaaf M, Tudorache C. "Early birds" take it easy: diurnal timing is correlated with overall level in activity of zebrafish larvae. Behaviour [Internet]. 2016 Jan 1 [cited 2022 Sep 11];153(13–14):1745–62. Available from: https://brill.com/view/journals/beh/153/13-14/article-p1745_10.xml
79. Martins EP, Bhat A. Population-level personalities in zebrafish: aggresion-boldness across but not within populations. Behavioral Ecology. 2014;25(2):368–73.
80. Curpăn A Ștefania, Ciobică A. Do zebrafish have personality? Possible associations between behavioural patterns and personality traits in Danio rerio in the context of schizophrenia. Bulletin of Integrative Psychiatry. 2023;4(99).
81. Shams S, Seguin D, Facciol A, Chatterjee D, Gerlai R. Effect of Social Isolation on Anxiety-Related Behaviors, Cortisol, and Monoamines in Adult Zebrafish. Behavioral Neuroscience. 2017;131(6):492–504.
82. Wang L, Jiang W, Lin Q, Zhang Y, Zhao C. DNA methylation regulates gabrb2 mRNA expression:

developmental variantions and disruptions in l-methionine-induced zebrafish with schizophrenia-like symptoms. Genes Brain Behav. 2016;15(8):702–10.
83. Bruzzone M, Gatto E, Xiccato TL, Valle LD, Fontana CM, Meneghetti G, et al. Measuring recognition memory in zebrafish larvae: Issues and limitations. PeerJ [Internet]. 2020 [cited 2023 Sep 13];2020(4). Available from: /pmc/articles/PMC7192156/
84. Estaphan S, Curpan A, Khalifa D, Rashed L, Ciobica A, Cantemir A, et al. Combined Low Dose of Ketamine and Social Isolation: A Possible Model of Induced Chronic Schizophrenia-Like Symptoms in Male Albino Rats. Brain Sciences 2021, Vol 11, Page 917 [Internet]. 2021 Jul 11 [cited 2023 Jun 2];11(7):917. Available from: https://www.mdpi.com/2076-3425/11/7/917/htm
85. Zanandrea R, Wiprich MT, Altenhofen S, Rubensam G, dos Santos TM, Wyse ATS, et al. Withdrawal Effects Following Methionine Exposure in Adult Zebrafish. Mol Neurobiol [Internet]. 2020 Aug 1 [cited 2024 Mar 5];57(8):3485–97. Available from: https://link.springer.com/article/10.1007/s12035-020-01970-x
86. Curpan AS, Savuca A, Hritcu LD, Solcan C, Nicoara MN, Luca AC, et al. A new approach to explore the correlation between declarative memory and anxiety in animal models of schizophrenia and microplastic pollution. Behavioural Brain Research. 2024 Feb 26;458:114742.
87. Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, et al. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behavioural Brain Research. 2009 Dec 14;205(1):38–44.
88. Campanari ML, Bourefis AR, Buee-Scherrer V, Kabashi E. Freezing activity brief data from a new FUS mutant zebrafish line. Data Brief [Internet]. 2020 Aug 1 [cited 2023 Sep 14];31. Available from: /pmc/articles/PMC7352050/

89. Riehl R, Kyzar E, Allain A, Green J, Hook M, Monnig L, et al. Behavioral and physiological effects of acute ketamine exposure in adult zebrafish. Neurotoxicol Teratol. 2011 Nov 1;33(6):658–67.
90. Wang L, Alachkar A, Sanathara N, Belluzzi JD, Wang Z, Civelli O. A Methionine-Induced Animal Model of Schizophrenia: Face and Predictive Validity. International Journal of Neuropsychopharmacology [Internet]. 2015 Nov 1 [cited 2023 Jun 23];18(12):1–11. Available from: /pmc/articles/PMC4675974/
91. Benvenutti R, Gallas-Lopes M, Marcon M, Reschke CR, Hermann AP, Piato A. Glutamate NMDA Receptor Antagonists with Relevance to Schizophrenia: A Review of Zebrafish Behavioral Studies. Curr Neuropharmacol. 2022;20(3):494–509.
92. Tunbak H, Vazquez-Prada M, Ryan TM, Kampff AR, Dreosti E. Whole-brain mapping of socially isolated zebrafish reveals that lonely fish are not loners. Elife. 2020 May 5;9.
93. Dreosti E, Lopes G, Kampff AR, Wilson SW. Development of social behavior in young zebrafish. Front Neural Circuits [Internet]. 2015 [cited 2019 Nov 4];9:39. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26347614
94. Park JS, Ryu JH, Choi TI, Bae YK, Lee S, Kang HJ, et al. Innate Color Preference of Zebrafish and Its Use in Behavioral Analyses. Mol Cells [Internet]. 2016 Oct 10 [cited 2023 Jun 20];39(10):750. Available from: /pmc/articles/PMC5104883/
95. Roy T, Suriyampola PS, Flores J, López M, Hickey C, Bhat A, et al. Color preferences affect learning in zebrafish, Danio rerio. Sci Rep [Internet]. 2019 Dec 1 [cited 2023 Sep 13];9(1). Available from: /pmc/articles/PMC6787237/
96. Zabegalov KN, Khatsko SL, Lakstygal AM, Demin KA, Cleal M, Fontana BD, et al. Abnormal repetitive behaviors in zebrafish and their relevance to human brain disorders. Behavioural brain research [Internet]. 2019 Jul 23 [cited 2023 Jun 23];367:101–10.

Available from: https://pubmed.ncbi.nlm.nih.gov/30926483/
97. Gitlin J, Chamadia S, Locascio JJ, Ethridge BR, Pedemonte JC, Hahm EY, et al. Dissociative and Analgesic Properties of Ketamine Are Independent. Perioperative medicine. 2020;133:1021–8.
98. Ballard ED, Zarate CA. The role of dissociation in ketamine's antidepressant effects. Nature Communications 2020 11:1 [Internet]. 2020 Dec 22 [cited 2024 Mar 5];11(1):1–7. Available from: https://www.nature.com/articles/s41467-020-20190-4
99. Fukuda R, Zhang H, Kim J whan, Shimoda L, Dang C V., Semenza GL. HIF-1 Regulates Cytochrome Oxidase Subunits to Optimize Efficiency of Respiration in Hypoxic Cells. Cell. 2007 Apr;129(1):111–22.
100. Tapia-Arancibia L, Aliaga E, Silhol M, Arancibia S. New insights into brain BDNF function in normal aging and Alzheimer disease. Brain Res Rev. 2008 Nov;59(1):201–20.
101. Sheng C, Zhang S, Zhang Y. The influence of different polymer types of microplastics on adsorption, accumulation, and toxicity of triclosan in zebrafish. J Hazard Mater [Internet]. 2021 Jan;402:123733. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0304389420317192
102. Zanandrea R, Wiprich MT, Altenhofen S, Rubensam G, dos Santos TM, Wyse ATS, et al. Paternal exposure to excessive methionine altered behavior and neurochemical activities in zebrafish offspring. Amino Acids [Internet]. 2021 Jul 22;53(7):1153–67. Available from: https://link.springer.com/10.1007/s00726-021-03019-2
103. Zanandrea R, Wiprich MT, Altenhofen S, Rubensam G, dos Santos TM, Wyse ATS, et al. Withdrawal Effects Following Methionine Exposure in Adult Zebrafish. Mol Neurobiol [Internet]. 2020 Aug 12;57(8):3485–97. Available from: https://link.springer.com/10.1007/s12035-020-01970-x

104. Liao PH, Yang WK, Yang CH, Lin CH, Hwang CC, Chen PJ. Illicit drug ketamine induces adverse effects from behavioral alterations and oxidative stress to p53-regulated apoptosis in medaka fish under environmentally relevant exposures. Environmental Pollution [Internet]. 2018 Jun;237:1062–71. Available from: https://linkinghub.elsevier.com/retrieve/pii/S026974911732345X
105. Nater UM, Rohleder N, Gaab J, Berger S, Jud A, Kirschbaum C, et al. Human salivary alpha-amylase reactivity in a psychosocial stress paradigm. International Journal of Psychophysiology. 2005 Mar;55(3):333–42.
106. Fischer S, Nater UM, Strahler J, Skoluda N, Dieterich L, Oezcan O, et al. Psychobiological impact of ethnic discrimination in Turkish immigrants living in Germany. Stress. 2017 Mar 4;20(2):167–74.
107. Ali N, Nater UM. Salivary Alpha-Amylase as a Biomarker of Stress in Behavioral Medicine. Int J Behav Med [Internet]. 2020 Jun 1 [cited 2023 Jul 11];27(3):337–42. Available from: https://link.springer.com/article/10.1007/s12529-019-09843-x

List of published articles

ISI articles:

1. **Curpăn** A.S., Luca A.-C., Ciobica A. Potential novel therapies for neurodevelopmental diseases targeting oxidative stress, Oxidative medicine and cellular longevity, 2022; https://doi.org/10.1155/2021/6640206 (IF = 6,761 la momentul publicării)
2. **Curpăn** AS., Balmus I-M, Dobrin RP, Ciobica A, Chele GE, Gorgan DL, Boloș A. 2022. A Mini-Review Regarding the Modalities to Study Neurodevelopmental Disorders-Like Impairments in Zebrafish—Focussing on Neurobehavioural and Psychological Responses, Brain Sciences 2022; 12(9):1147. https://doi.org/10.3390/brainsci12091147 (IF= 3.3 la momentul publicării)
3. **Curpan** AS, Savuca A, Hritcu LD, Solcan C, Nicoara MN, Luca AC, et al. A new approach to explore the correlation between declarative memory and anxiety in animal models of schizophrenia and microplastic pollution. Behavioural Brain Research. 2024 Feb 26;458:114742. (IF=3.352 la momentul publicării)

IDB articles:

1. **Curpăn** AS, Alin C. Do zebrafish have personality? Possible associations between behavioural patterns and personality traits in *Danio rerio* in the context of schizophrenia. Bulletin of Integrative Psychiatry. 2023 Dec 15;99(4):23–33. (indexată BDI)

Conferences papers published *in extenso:*

1. Savuca A, **Curpan** A, Hritcu L, Ciobîcă A, Plavan G, Nicoar M. Preliminary study on the behavioral response of zebrafish to the presence of methionine and polypropylene residues in water, 2022;45:89-96. 10.35219/ann-ugal-math-phys-mec.2022.2.08. – publicarea *in extenso* a lucrării prezentate la conferință

2. Savuca A, **Curpan** A, Nicoara M, Ciobica AS. POSSIBLE RELATIONSHIP BETWEEN ENVIRONMENTAL POLLUTANTS/MICROPLASTICS AND SCHIZOPHRENIA ETIOLOGY AND PROGRESSION. In: Proceedings of the Romanian Academy - Series A: Mathematics, Physics, Technical sciences, information Science. Romanian Academy; 2023. – publicarea *in extenso* a lucrării prezentate la conferință

3. **Curpan** A, Savuca A, Ciobîcă A. Behavioural analysis of potential new approach in modelling schizophrenia using zebrafish. Current trends in natural sciences, 2023; 12. 06-16.

10.47068/ctns.2023.v12i24.001. – publicarea în extenso a lucrării prezentate la conferință

List of attended conferences

National conferences:

1. Workshop "Abordări moderne ale feedbackului între procese de mediu și schimbările climatice" - Universitatea Dunarea de Jos din Galati – A. Săvucă, **A.Ş. Curpăn**, L.D. Hrițcu, A.S. Ciobîcă, G. Plăvan, M. N. Nicoară- „*Preliminary study on the behavioural response of zebrafish to the presence of methionine and polypropylene residues in water*" - 6-9 iulie 2022, https://www.rexdan.ugal.ro/index.php/ro/anunturi-si-evenimente/conferinte/workshop-abordari-moderne-ale-feedbackului-intre-procese-de-mediu-si-schimbarile-climatice/acasa-5 - conferință națională cu publicare
2. CONFERINŢA ŞTIINŢIFICĂ DE PRIMĂVARĂ a AOSR, 2023, BUCURESTI, Romania –- **A.-S. Curpăn**, A. Săvucă, A.S. Ciobîca – " *Au peștii zebră personalitate ? Posibile asocieri între tiparele comportamentale și trăsăturile de personalitate la Danio rerio*" 19-20 Mai 2023, https://www.aosr.ro/conferinta-nationala-stiintifica-editia-de-primavara-transformarea-digitala-in-stiinte/ - conferință națională cu publicare

International conferences:

International Scientific Symposium ”CURRENT TRENDS IN NATURAL SCIENCES”, Pitesti, Romania – **A.-S. Curpan**, A. Savuca, A.S. Ciobica -”*Behavioral analysis of potential new approach in modelling schizophrenia using zebrafish*” - 18 - 20 Mai 2023, https://www.natsci.upit.ro/international-symposium/ - conferință internațională cu publicare