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From Green to Serene: Investigating the Effects of Algal and Light Conditions on Stress Levels and Welfare in Fish

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Biotechnology

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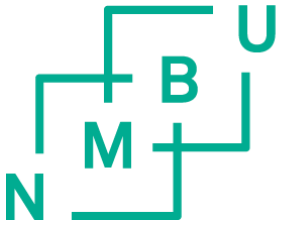

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ABSTRACT

The increasing use of fish in laboratory research and aquaculture threatens fish welfare. Thus, environmental enrichment strategies have gained increasing attention to promote the well-being of captive fish. This study investigated the preferences and stress responses of Japanese medaka (*Oryzias latipes*) to different environmental enrichments and conditions. In the behavioral experiments for specific light colors and intensities, male medaka exhibited a preference for colored LED lights, especially in purple over white lights when assessed individually and in groups. Females did not show clear color preferences but tended to prefer colors over white when tested in groups, suggesting social influences. The medaka favored a lower light intensity in purple lights but tolerated a wider intensity range in blue, green and white lights. Additionally, separate experiments found that medaka preferred environments with reflective tanks walls and algae-free conditions over excessive algal growth present. Medaka may have potentially perceived mirror reflections in the tank walls as conspecifics. The open field test revealed a higher exploratory, lower anxiety-like behaviors in medaka from the clean tanks compared to medaka from algae tanks. It was however, unexpectedly found higher physiological stress in medaka from the same clean tanks compared to algae tanks when measuring cortisol levels. The findings highlight the medaka's responsiveness and preference to environmental enrichments and factors like lighting, habitat complexity/cleanliness, social settings and reflective surfaces. By implementing enrichment tailored to their preferences it could significantly improve their welfare for this research model.

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INTRODUCTION

1.1 Laboratory Fish and Aquaculture

Fish are known for their high levels of biodiversity and have become increasingly significant in various fields of research, continuing to expand across different disciplines (Volff, 2005). Fish in laboratory studies are used to research fields like nutrition, physiology, genetics, disease and more. To make this possible, fish are maintained under controlled conditions to conduct experiments, sampling and analysis (Isyagi, 2005). Fish species such as zebrafish (*Danio rerio)* and medaka (*Oryzias latipes)* are commonly used as model organisms in laboratories, due to their transparent embryos and rapid development. This is valuable in the studies of genetics, development processes and disease mechanisms (Porazinski et al., 2011). There is an increased use of fish as animal models in scientific research, but little is still known about the optimum conditions to increase the welfare of the fish kept in captivity. This is crucial not only for ensuring ethical treatment, but also for maintaining the integrity and accuracy of research findings (Toni et al., 2019).

At the same time there has been a surge in global aquaculture production, beginning at slightly over 1 million tons food in 1950 and peaking at 80 million tons by 2017. Aquaculture is a massive industry that produces high-quality food, but also helps in restocking fish populations for commercial and conservation purposes (Brown & Day, 2002). To optimize production in aquaculture, research focuses on parameters such as growth, feed utilization, water quality and disease management. This involves testing different feed formulations, rearing conditions, husbandry practices and monitoring fish performance and their health (Aljehani et al., 2023). Fish like Atlantic salmon, oysters, tilapia, sea bass and many more are used in aquaculture (Azra et al., 2022). With projections indicating continued growth, a growing concern for the welfare of teleost fish has emerged (Hvas et al., 2021).

1.2 Fish Welfare

Seeing as fish in captivity are used in many aspects, they are also affected by many common factors such as handling stress, lack of environmental complexity, experimental testing, suboptimal water quality, high stocking density and disease susceptibility (Segner et al., 2019). A review of European

research papers discovered that there was a general lack of information related to optimal environmental conditions and husbandry practices for ensuring fish welfare (Toni et al., 2019).

Animal welfare is not only about physical pain, but also the mental state of the animal. Fish welfare is therefore about understanding the state of the fish in relation to its ability to cope with its environment and their associated mental experience (Segner et al., 2019). This means that to understand fish welfare, it is also needed to consider the emotional or affective states that fish may experience. Feelings such as pain, fear and psychological stress are essential to understand fish welfare (Dara et al., 2023).

There have been efforts to ensure fish welfare by creating protocols used in laboratories and aquaculture, however information that is provided about fish is sometimes too generic and brief to be used as a tool for ensuring fish health (Toni et al., 2019). For example, welfare protocols provided by Directive 2010/63/EU lack consideration of species-specific features, guidance on how to measure chemical and physical parameters and does not provide specific instructions for each species nor reference documentation that laboratories can rely on (Toni et al., 2019).

Therefore, it is necessary to improve protocols with adequate knowledge in the biology of the housed fish species and provide the appropriate equipment to create a more suitable environment. There are after all more than 25 000 teleost fish species and more research is needed of fish species and the adoption of welfare protocols that could potentially reduce overall stress levels in fish (Toni et al., 2019). Reducing stress levels in fish could remove unwanted behavior and physiological alterations that could potentially bias experimental results. In the long run, improving fish welfare can also positively impact growth performance, flesh quality, fish health and reproductive success in farmed fish populations, ultimately increasing profits and productivity in aquaculture (Toni et al., 2019).

1.3 Fish Welfare Indicators

To this day, welfare indicators have been identified to ensure the welfare of fish and animals in captivity. Potential welfare indicators for fish should be minimally invasive, reliable, and recognizable (Toni et al., 2019). As fish are sentient beings capable of experiencing pain, stress and suffering, several indicators must be determined and brought to light (Lambert et al., 2022).

There are various factors related to behavioral and physiological performance that should be considered when selecting welfare indicators. When it comes to behavioral indicators such as reduced feed intake, abnormal swimming behavior and abnormal breathing rates, it can signalize the fish's poor welfare (Martins et al., 2012). There are some disadvantages to these behavioral indicators because they can be inconsistent over time and difficult to quantify, thus the caretakers must have sufficient skills to observe the fish's body language. Physiological indicators for example can be cortisol/corticosterone levels, glucose, and others. Other indicators include water quality, stocking density and environmental conditions. (Farming, 2023). To attain a more robust assessment of fish welfare, more combined aspects need to be investigated to ensure the fish's well-being (Martins et al., 2012).

Stress is the most important physiological indicator that is often used to assess poor welfare. This is because stress can represent a long-term response that may eventually cause detrimental consequences (Toni et al., 2019). This means that promoting good health and minimizing stress that fish can perceive may improve fish welfare. Short-term stress can have both advantageous and detrimental effects. The fish gain benefit of being mildly challenged which stimulates neural plasticity and potentially improves stress coping mechanisms. However, acute stress may impede neural plasticity instead and compromise the fish welfare (Dara et al., 2023; Palamarchuk & Vaillancourt, 2021). It is therefore important to regulate stress levels to avoid excessively stressful situations and long-term stress (Sørensen et al., 2013), as it is not only stress that threatens an animal's well-being, but also eustress and distress. Eustress is everyday experiences that benefits survival, such as avoiding predators or searching and competing for food, which often does not have a negative impact on the animal unless it becomes chronic. In contrast, distress is consciously experienced as an unpleasant or aversive state, potentially resulting from factors such as water quality issues, lack of enrichment, handling stress, social isolation, or overcrowded conditions and many more (Barreto et al., 2022).

In fish there are three phases of stress, primary, secondary and tertiary. In the primary phase, there are series of neuroendocrine responses, which includes the release of glucocorticoids and catecholamines. The secondary phase involves physiological reactions, including increased heart rate and utilization of metabolic energy stores. Lastly, the tertiary phase triggered by chronic

stressors that affect overall bodily performance. To understand the these responses, it is further needed to look at mechanisms behind stress (Barreto et al., 2022).

1.4 Stress Markers: Glucocorticoids Hormones in Fish

As stress is such a key factor in fish welfare, it is necessary to study cortisol which is known as the primary stress hormone in fish and many mammals by playing a crucial role in physiological responses. The production of cortisol happens within the inter-renal cells located in the head kidney and is released into the blood (Sadoul & Geffroy, 2019). Unbound cortisol in the plasma is its physiologically active form, where most cortisol is bound to cortisol-binding globulin (CBG) and albumin (le Roux et al., 2003). While corticosterone also serves as a stress indicator, it plays a more significant role in reptiles and birds. Improving our understanding of these hormonal dynamics contributes to a thorough evaluation of stress and its effects on the well-being of fish (Sadoul & Geffroy, 2019).

The roles of cortisol are important in fish to maintain hydromineral balance by stimulating ion transport mechanisms in the gills, intestine and kidney to regulate salt and water balance (Vijayan et al., 2010). Cortisol also affects the immune system, behavior, and reproduction of the fish. Elevated levels of cortisol can have immunosuppressive effects by suppressing inflammatory responses and immune functions like antibody production. Cortisol can also alter aggression and reproduction in fish in negative ways. However, very high cortisol levels can suppress aggression due to loss of energy (Best & Vijayan, 2018; Martinez-Porchas et al., 2009; Vijayan et al., 2010).

While cortisol is often associated with stress, it serves other catabolic functions under normal conditions such as energy mobilization. Cortisol can for example increase availability of all fuel substrates for muscles by executing lipolysis (Thau et al., 2024). Lipolysis is a catabolic process that releases glycerol and free fatty acids from the adipose tissue. The catabolic effects of cortisol in other words, are related to increasing blood glucose levels through gluconeogenesis (Thau et al., 2024). When fish experience stress, cortisol levels in the plasma dramatically increase preparing them for a “fight or flight” response (Mommsen et al., 1999). There are other factors that may affect the intensity of cortisol response in fish. Intrinsic factors such as heritability, age,

sexual maturity, and social status and also extrinsic factors like environmental color, temperature, nutritional status, and previous response to stressors (Martinez-Porchas et al., 2009).

Cortisone is an inactive metabolite from the active glucocorticoid cortisol that has been metabolized by enzymes in the scales and skin of fish (Martinez-Porchas et al., 2009). Since this allows for local inactivation of cortisol, when cortisol from the circulation reaches the skin or scales, a portion of it can be converted into cortisone which has a lower biological activity compared to cortisol. The presence of cortisone can therefore underestimate the actual levels of active cortisol (Martinez-Porchas et al., 2009; Mazzi et al., 2023; Sadoul & Geffroy, 2019). As the detrimental consequences of stress have been brought forth, it needs to be mentioned that environmental enrichments can be implemented to alleviate these negative effects from prolonged stress (Brown et al., 2003), as will be discussed the following chapter.

1.5 Environmental Enrichments

In most fish laboratories and aquaculture, the typical housing condition is considered "sterile" characterized by plain environments. This setup is designed for ease of cleaning, space efficiency, and cost-effectiveness, aiming to standardize behavior across various experimental groups while also promoting good physical health. However, environments as such may drastically limit the fish's natural behavioral, potentially compromising their welfare if the fish has a desire to engage in specific behaviors (Brydges & Braithwaite, 2009).

For example, environmental enrichment for laboratory rodents is now widely accepted for ensuring good welfare and reliable research. Studies have shown that enrichment reduces anxiety, aggression, injury, disease, improves cognition and allows expression of natural behaviors (Näslund & Johnsson, 2016; Stevens et al., 2021). Based on the positive effects observed in rodents, principles such as appropriate stimulation and opportunities for natural behaviors should be applicable to other species including fish (Stevens et al., 2021) (Stevens et al., 2021).

There are many ways to enrich the target environment such as providing hiding places, light adjustments, changing the tank environment or providing social interactions, although this does not mean that every enrichment results in positive effects. Thus, conducting experiments to find the preference of fish species is needed (Näslund & Johnsson, 2016). Research conducted with

commercial fish species has demonstrated that enriching the complexity of their rearing environment can influence their behavior (Brydges & Braithwaite, 2009). For instance, providing structural enrichment and feeding live prey has been shown to enhance foraging performance on novel live prey in Atlantic salmon (*Salmo salar*) (Brown et al., 2003). Similarly, environmental enrichment in captive-bred North Atlantic cod (*Gadus morhua*) has been found to increase exploratory behaviors and reduce the time needed to recover from exposure to stressors (Brydges & Braithwaite, 2009). Laboratory fish such as zebrafish increased their exploratory behavior, reduced aggression and improved spatial learning when their environment was enriched with plastic plants, rocks and gravel substrate (Favero Neto & Giaquinto, 2020).

Introducing environmental enrichment is therefore often considered advantageous for the welfare of aquaculture and laboratory fish, as these environments can alter behavior and physiology (Brydges & Braithwaite, 2009).

1.5.1 Color Light Enrichment

When it comes to enrichment, colors and lighting are effortless ways to enrich fish environments in laboratory and aquaculture settings to provide a more natural and stimulating environment. Species such as zebrafish show preference for certain colors like blue and green over red and yellow (Noureldin et al., 2021). Even tank colors such as blue and black are preferred over white and transparent tanks and can reduce anxiety and cortisol levels (Stevens et al., 2021). The light enrichment and color schemes within the tank may influence the preferences and hormonal shifts associated with pigmentation changes in fish species. These environmental factors could have a direct impact on behavioral factors such as mating (Juntti & Fernald, 2016; Sköld et al., 2016). Another study demonstrated that rearing goldfish under blue monochromatic LED light improved their growth, behavior and immune-physiological responses to stress (Noureldin et al., 2021). Most studies regarding light however have focused more on the effects of light duration, rather than the different light colors and intensity on fish welfare (Kleiber et al., 2023). Although there are fewer studies about light colors and how they affect fish, studies on Nile Tilapia have shown some promising results. Blue light was found to be effective in inhibiting stress-induced cortisol response, while green light had a much lower impact. Another study done on Nile Tilapia showed

that red light stimulates feeding motivation, but not growth. The light intensity in these studies were all the same, suggesting that the results were color-based (Volpato & Barreto, 2001).

Light preference studies are often conducted in controlled laboratory settings, however in an experiment conducted by Sakamoto et al. (2017) in a natural coastal environment, it was found that fish were most attracted to blue LED lights and less attracted as the wavelengths increased at night. As sunrise arrived and the underwater background increased in intensity, the attracted fish number decreased. This demonstrates that light colors, intensity and underwater brightness were important factors, with light color being the most influential (Sakamoto et al., 2017). By selecting the right lighting spectrum, intensity and color combinations, a suitable tank environment can be created. This leads us further to what effects tank colors can have.

1.5.2 Tank colors

The color of the tank itself can also have significant impacts on fish welfare (McLean, 2021). These tanks are designed to optimize efficient waste collection and increased recycling capabilities. While tanks have a benefit of being produced in a wide range of colors, most commercial facilities limit the production of these tanks to three shades, black, blue and green (Sköld et al., 2016).

Studies have shown that some species have a better growth and feed conversion when maintained in alternative-colored tanks (Sköld et al., 2016). For example, a study by Duray et al. (1996) examined the effect of background color on rotifer intake, growth, and survival of grouper larvae, highlighting the importance of tank color in influencing fish behavior and performance. Similarly, Papoutsoglou et al. (2000) investigated the effects of background color on the growth and physiological responses of scaled carp reared in a closed circulation system, emphasizing the significance of colors in aquaculture settings.

A study for salmon was conducted to see growth and feed conversion by using red, blue and aqua-green cloths to surround the tanks (Browman & Marcotte, 1987). By feeding different colored-feed in different background colors, the results seemed to show that prey and background color gave a significantly higher feed-rate conversion where aqua-green background color had

the worst feed-rate conversion. This suggests that the visual environment in aquaculture tanks plays a role in salmon feeding and growth. Factors like tank color and light likely impact the visibility and contrast of prey items for the salmon (Browman & Marcotte, 1987). For medaka a study showed that a black or white background for 10 days induced an increase or decrease of the coloration and pigments, indicating morphological color changes in medaka (Ohshima et al., 2013). Additionally, the transparent strain of medaka has lower fitness and generally lower energy levels, activity and robustness compared to a pigmented WT medaka (Ohshima et al., 2013). This may suggest that background color could impact factors beyond appearance, potentially affecting the fitness and growth of medaka as well. The different colorations and patterns play an important role in intra-play and inter-play, especially when it comes to mating behaviors (Sköld et al., 2016). Fish perceive and interpret visual cues, such as colors and lighting intensities to choose their mate, which eventually contributes to speciation (Juntti & Fernald, 2016).

These findings show the importance of considering colors as one of many factors, that may impact growth rates, stress responses and feed conversion efficiency in teleost fish, ultimately improving fish welfare, research findings and farming (Duray et al., 1996; Papoutsoglou et al., 2000).

1.5.3 The role of algae in fish tanks

While light color enrichments are important, the presence of algae in the tank can also impact fish welfare and behavior. Algae growth is common in fish, aquaculture ponds and rearing systems, which can have beneficial and detrimental effects on fish in captivity. Algae are naturally rich nutrient sources and the primary food producer in the aquatic food chain, having antioxidant, immunostimulant and antimicrobial properties (Roy & Pal, 2015). Excess nutrients like nitrogen and phosphorus can be removed by algae as they act as biofilters improving water quality, thus algae help to reduce the harmful impact of nutrient pollution on water ecosystems (Ramli et al., 2020). Algae also produce more oxygen which may benefit the fish, but high levels of algae can reduce dissolved oxygen at night when respiring, potentially stressing the fish (Roy & Pal, 2015).

However, algal growth indicates high nutrient levels and possibly the presence of biofilms, as such they can be used as an indicator of poor water quality. Excessive algae growth can also hinder the visual inspection of fish, making it difficult to assess the health and behavior of the fish. These opportunistic pathogens hiding in the algal growth can potentially cause a disease outbreak in the fish tank and compromise the fish welfare (Smith, 2023). The filtration systems can be clogged by algae, reducing their efficiency causing the accumulation of waste products and worsened water quality. In some cases, excessive algae growth can disrupt natural behaviors of fish, such as breeding, feeding or social interactions, by altering the visual cues or environmental conditions they rely on (Islam et al., 2024)

1.6 Photoreception in teleost fish

The visual system is important when it comes to mate selection, foraging, avoiding predators, and communication (Lu et al., 2023). Teleost fish have retinas that are multilayered tissues housing photoreceptor cells. These photoreceptor cells express opsin genes that make the molecular foundation of vision. This means that the opsins convert light signals into neural signals, which the brain can interpret as visual information. The retina is not the only photoreceptor organ that teleost fish have, but also the dominant extraretinal organ, the pineal gland (Lu et al., 2023).

When it comes to researching visual development and function, the medaka and zebrafish are remarkable model organisms since they have excellent vision and a diverse set of opsin genes. The medaka has eight opsin genes, while humans only have three cone opsins (Wang & Takeuchi, 2017). The only missing opsin is the middle to long wavelength-sensitive (red-green). Fish seem to frequently express various opsin subtypes, which probably reflect their evolutionary adaptation to different aquatic light environments (Matsumoto et al., 2006). Medaka has a unique eye placement as their big eyes are found at the upper part of the head, between the thin conjunctivae and the optic cups (Carson et al., 2012). The medaka's eyes like evolved to enhance visual perception above the water for the detection of predators and capturing of prey (Wang & Takeuchi, 2017)

The number and types of visual pigments in teleost fish usually correlate with their ecological habitats, which can vary from clear oceans to murky lakes, deep seas and turbulent waters (Carleton et al., 2020). There have been studies showing that teleost fish, including species like goldfish

(*Carassius auratus*) and tench (*Tinca tinca*) can distinguish different colors and perceive them consistently and accurately (Rosa Salva et al., 2014). Observations have shown that fish species such as *Schizothorax waltoni* exhibit preferences for specific light colors, favoring green and blue shades over red and yellow. This behavior indicates that their color preferences may be influenced by environmental cues (Wheeler, 1982).

1.7 Medaka (*Oryzias latipes*) as a Model Fish

The wild medaka is originally from Asia and primarily resides in Japan but can also be found in parts of eastern China and Korea. The name "Medaka" originates from Japan and translates to "small fish with big eyes". The medaka is also called “rice fish” and this reflects on their natural habitat in rice fields (Wittbrodt et al., 2002). Medaka has been used in research due to their genetics, physiology and embryology and has an important role in various scientific disciplines such as biomedicine, toxicology, cancer research, genetics, and genomics (Larsen, 2015; Lin et al., 2016; Padilla et al., 2009; Schartl & Walter, 2016).

The Japanese medaka is highly advantageous for laboratory research due to its small size (2,5 – 3 cm), making them easy to manage and maintain in laboratories. Additionally, these fish exhibit remarkable robustness, resilience to a variety of common diseases, and the ability to thrive across a wide temperature range, from 0 °C to 40 °C (Larsen, 2015). Their spawning time is strongly influenced by light cycles such as a 14/10h light/dark cycle (Murakami & Kinoshita, 2018; Wittbrodt et al., 2002). Each day, they lay between 30 to 50 eggs, with up to 3,000 eggs produced during the mating season. However, it is not a general practice to simulate seasonal changes in the laboratory environment, which means that the medaka can breed year-round and produce eggs continuously, if proper temperature, right lighting and nutrition are provided (Larsen, 2015; Wittbrodt et al., 2002). The eggs are attached to filaments that are connected to the female's body, which makes identification and breeding processes easier. Generation time is 6-8 weeks under laboratory conditions for medaka (Wittbrodt et al., 2002). Following the spawning of eggs, they can be fertilized for 8 hours in vitro. In studies including transplantation and microinjection, the medaka embryos can be maintained at low temperatures (4 °C) to decelerate development for up to 3 months (Wittbrodt et al., 2002).

Embryos can be cultivated up to a density of 200 eggs per 9-cm petri dish and within 7 days, they can progress into feeding larvae (Wittbrodt et al., 2002). After fertilization it is possible to determine sex within their transparent eggs by analyzing the number of germ cells present within 2 days. Medaka have a short generation time which makes them an asset in analyzing complex mechanisms of neurodegeneration more rapidly compared to other vertebrate animal models, as sex can influence the development and progression of neurodegenerative condition (Wang & Cao, 2021). The medaka is therefore a valuable candidate as they have shorter generation time and can be studied more rapidly. (Larsen, 2015). Medaka also contributes to the study of human diseases and offers insight into complex pathways involved in cancer development and metastasis processes (Larsen, 2015).

Social behaviors such as shoaling medaka makes a good model fish for studying responses to environmental enrichment as well (Gatto et al., 2022). By conducting research on medaka to understand the impacts from environmental enrichments and light on aquaculture species, the rearing conditions and fish welfare (Ryu & Gong, 2020). Short generation time of medaka allows researchers to study the effects of enrichment over generations (Li et al., 2020), while their transparency makes for studying the effects of different light colors and intensities on early life stages and development (Merino et al., 2020; Ogino et al., 2023).

1.8 Cortisol and Enzyme-linked Immunosorbent Assay (ELISA)

EIAs, also known as enzyme immunoassays, use catalytic properties of enzymes to measure and identify immunologic responses (Alhajj et al., 2023). Due to their specific interactions between antigens and antibodies, immunoassays have been used globally in areas such as diagnosis, quality control of commercial products and pharmacokinetic studies through drug monitoring (Sakamoto et al., 2018). ELISA kits are extensively used to quantify cortisol levels, serving as a valuable method for assessing stress in laboratory animals (Kinn Rød et al., 2017).

As there are some different ELISA formats, competitive ELISA is the format used in this study, where the sample cortisol (antigen) competes with a fixed amount of cortisol-enzyme (enzyme horseradish peroxidase) conjugate for binding to a limited number of antibody sites (Sesay et al., 2013). The intensity of fluorescence/color is inversely proportional to the cortisol amount in the

sample. That means a higher cortisol level in the sample results in a lower intensity of fluorescence/color. A standard curve of known cortisol concentrations is necessary to determine the unknown concentrations as the curve acts as reference concentrations. The advantage of this technique is that it is suitable for detecting small molecules such ‘as cortisol which cannot be easily immobilized on a plate. Due to the competitive binding mechanism, there is also high sensitivity and specificity as well (Sesay et al., 2013). The ELISA do not rely on radioactive substances and only involves minimal amounts of organic solvents as well, ensuring safety and environmental friendliness (Leila Soledade Lemos et al., 2023).

By taking blood samples, cortisol can be measured although this alone does not necessarily reflect a state of chronic stress. Cortisol concentrations may also be influenced by circadian rhythms, environmental cycles, sex, maturity, and reproductive stages. Adaptive behaviors or eustress can result in positive responses, such as foraging and breeding, and may change the concentration of the stress-related hormones. By taking these factors into account, cortisol levels, stress responses and adaptive behaviors, decisions regarding fish welfare may be improved. Therefore, it is also important to establish a normative range within a healthy population for concentrations associated with stress-related indicators (L. S. Lemos et al., 2023).

Water samples is a well-known method as well, when it comes to measuring cortisol levels in fish. The free form of cortisol can diffuse through the gill membranes into the surrounding water in the tank. This means that cortisol can be sampled without the need to handle the fish directly, avoiding stressful situations for the fish. If cortisol molecules have gone through metabolism and inactivation, it can also be excreted through urine and feces that contribute to cortisol levels in the water (L. S. Lemos et al., 2023).

Directly sampling the fish can pose challenges, as the initial sampling may cause other fish to stress, potentially biasing the remaining medaka’s cortisol levels. Only within a few minutes of experiencing stress, fish can release cortisol into the plasma which can lead to difficulties in accurately measuring cortisol levels. Water samples are therefore a valuable approach in addition (Sadoul & Geffroy, 2019).

Aim of thesis

The aim of this study was to contribute to the understanding of fish welfare and best husbandry practices in laboratories, by investigating what fish prefer and dislike in their environment. Exploring different light colors, intensities and tank environments, I aim to improve enrichment in laboratory settings, ultimately improving the fish welfare.

In this project, medaka (*Oryzias latipes*) was used as a model organism for all the experiments. LED lights of assorted colors and intensities were used in order to determine medaka preferences. A tank wall reflection test was incorporated to aid in identifying potential biases. Algae-covered tanks were used to assess both preference and stress levels as algae growth is common in laboratories, aquaculture and hobbyist aquariums. The open field test was used to study the fish' exploratory behavior as this can be related to stress. Finally, by performing ELISA for cortisol concentration, HPLC for monoamines levels and if relevant, RT-qPCR for gene expression analysis, stress and preferences can be evaluated.

2.MATERIALS AND METHODS

Different experiments in this study were conducted to assess the preference and stress response of medaka fish when exposed to different environmental conditions, including light color and intensity, and algae covered or clean tank. The experiments consisted of the following components:

1. Behavioral analysis: detailed observations and video analysis of the behavioral patterns of medaka fish in the experiments.

2. Neurochemical analysis: brain and pituitary samples were collected for high-performance liquid chromatography (HPLC) analysis and reverse transcription-quantitative PCR (RT-qPCR) to investigate neurochemical and gene expression changes if relevant.

3. Physiological stress assessment: blood and water were sampled to measure cortisol levels using enzyme-linked immunosorbent assay (ELISA) to assess the physiological stress response.

2.1 Preference Test

2.1.1 Group Light Intensity Preference Test

This experiment investigated the medaka's preference in light intensities. A cross-maze tank was placed in a dark room, preheated, with no personnel passing to minimize disturbances. Two RGB LED lights (Namron, Norway) of the same color but with different intensities were placed on the two opposite arms while the two other arms were closed off with transparent walls (Figure 1). Light intensities (Table 1) were measured in both lux (light intensity) and PAR (photosynthetically active radiation) using a miniature spectrometer (Ocean Insight, Flame Miniature Spectrometer, USA).

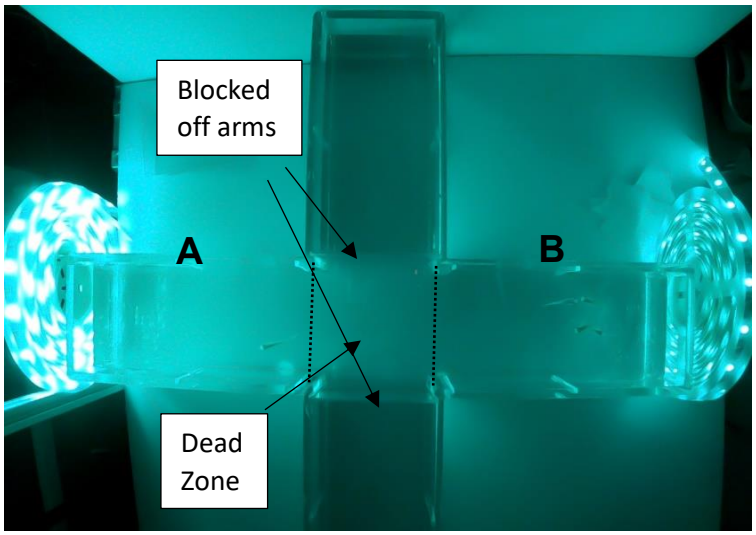

Figure 1. This picture shows a cross-maze placed in a dark room. *The cross maze shows that only arm A and B were employed with two RGB LED light strips on each side, while the remaining arms were closed off using transparent walls. The two black dotted lines represent a "dead zone" within the maze, indicating an area where no preference was assigned.*

Several trials were conducted with different colors. For each trial, a group of five randomly selected adult medaka were introduced into the maze tank. Prior to the experiment, all fish were housed in the same tank consisting of twelve medaka fish (8 liters). All fish were allowed a 10-minute acclimation time to adjust to the new environment before the recording began with a GoPro Hero 7 camera (GoPro, USA) located above the tank for 15 minutes each trial.

To determine fish preference, the number of fish present in each arm of the maze was counted by analyzing snapshot of the video every 30 seconds throughout the 15-minute trial duration. Each color preference test was performed in triplicates with a new group of five fish each time.

Table 1 Colors, light intensity, PAR are presented in the table.

Colors	White	Blue	Red	Purple	Green
Light intensity (lm/m^2)	715 vs 115	220 vs 40	340 vs 85	200 vs 60	480 vs 95
Light intensity difference	600	180	255	140	385
PAR ($\mu mol/m^2/s$)	16 vs 2.25	18.5 vs 2.9	10 vs 1.9	4.6 vs 0.9	10 vs 3.6
PAR difference	13.75	15.6	8.1	3.7	6.4

2.1.2 Individual and Group Color Preference Test

The experiment was designed to explore two research goals: the color preferences of individual male and female medaka fish, as well as their color preferences when tested as a group.

The cross-maze tank was placed in a dark and preheated room, with no personnel passing. Similar to above, two LED lights (Namron) of different colors (Table 2) were positioned at the extremities of the two open arms (Figure 2). Transparent walls block off the remaining unlit arms in the middle. Light intensity was kept similar between both arms by focusing on PAR measurement (Table 2), as it is not possible to get both PAR and LUX equal at the same time as they measure slightly different things. To measure light a miniature spectrometer was used (Ocean Insight, Flame Miniature Spectrometer, USA).

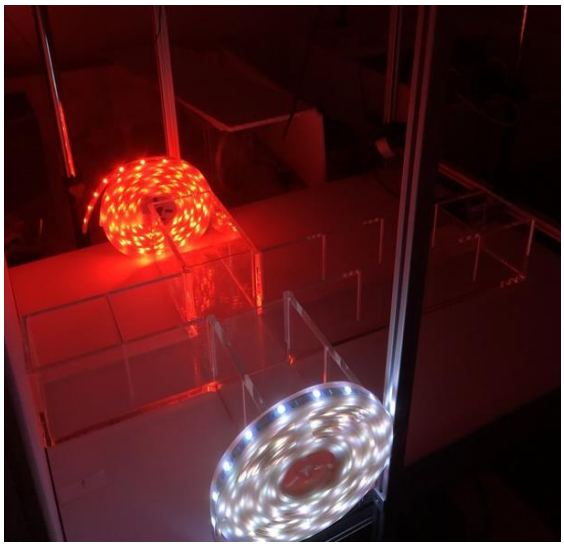

Figure 2. Set up; a cross-maze employed with two LED lights. *One arm is employed with white light and the other arm with colored lights. The arms without LED lights are closed off with transparent walls, where the middle as shown in Figure 1, is the dead zone where no preference was assigned.*

A different group of medaka fish was used in this individual and group behavior test. This group consisted of eight adult medaka (4 males and 4 females) that were housed together in an 8-liter tank. Prior to this experimental tank, they were housed in 8-liter tanks consisting of twelve fish each.

For each trial, a GoPro Hero 7 camera (GoPro, USA) was used to record the fish from above for 10 minutes. An acclimation time of 10 minutes was given to each fish after its introduction into the

maze before recording started. As described above, fish preference was determined by counting the number of fish present in each arm of the maze. This was done by analyzing snapshots of the video every 30 seconds throughout the 10-minute trial duration, as this time was shown to be sufficient in the previous behavior test.

First, trials were conducted for each of the eight fish individually (males first and then females). Second, group trials were conducted using the same fish from the individual experiment, four fish of the same sex (male group first followed by female group). The trials were conducted in duplicates.

Table 2. Color combinations, PAR and lux are presented from the experiment.

Color	Green vs White	Red vs White	Blue vs White	Purple vs White
PAR (μmol/m²/s)	6.8	6.8	6.8	6.8
Lux (lm/m²)	600 vs 350	250 vs 310	125 vs 300	125 vs 300
Lux difference	250	60	175	175

2.1.3 Reflection Preference Test

As fish can be attracted to its mirror reflection, behavioral experiments may not accurately represent natural behaviors. To test this hypothesis, an experiment was designed to test fish attraction to their own reflection. An 8-L tank was prepared by using sandpaper to scratch the tank walls on only the left side (Figure 3). This tank was in the recirculating water system, providing a constant flow of water throughout the experiment.

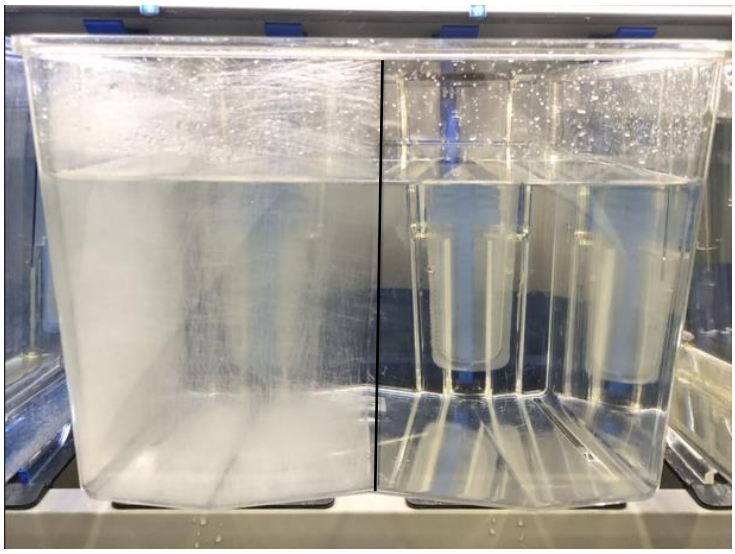

Figure 3. An 8-litre transparent tank is depicted, where half is eliminated of mirror reflections (left side), while the right side was kept transparent and reflective. *The middle line separated the reflective and non-reflective environments to determine if they are attracted to their own mirror reflection.*

Twelve medaka were housed in an 8-liter tank prior to the experiment. For each trial, individuals or groups of four fish (2 males and 2 females) were placed in the designated tank. First, eight randomly selected fish went through individual trials in the designed tank, resulting in a total of eight individual trials. Second, two males and two females were grouped for the group trials. Eight group trials were conducted, where a new group of medaka was introduced every trial.

Their behavior was recorded for minutes using a GoPro Hero 7 camera (GoPro, USA) placed in the tank's front. An acclimation time of 10 minutes was given each time to the fish to acclimate before recording started.

The fish preference was determined by counting the number of fish present in each side of the tank, analyzing snapshots extracted from the video every 30 seconds throughout the 10-minute trial duration.

2.1.4 Algae/Algae-Free Preference Test

Since algae growth is common in laboratory, aquaculture and hobby tanks, an experiment was conducted to assess whether medaka fish exhibit a preference for algae or algae-free environments. Algae were left to naturally grow for 2-3 weeks in a tank empty of fish kept within the recirculating

system. Prior to the experiment half of the tank was cleaned to remove the algae, creating algae/algae-free conditions (Figure 4). The tank was kept in the recirculating water system throughout the experiment.

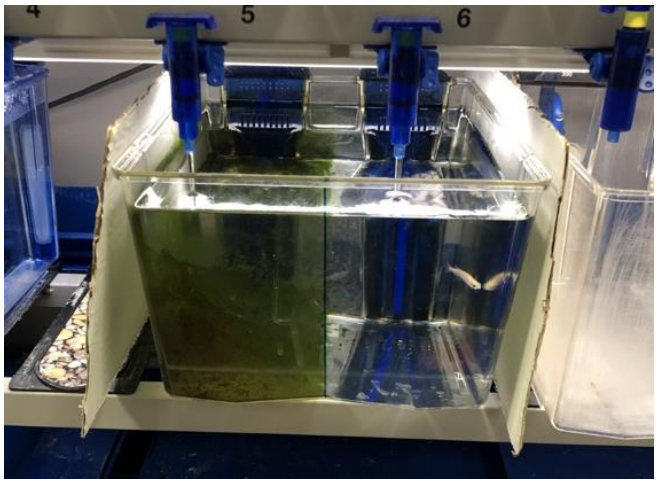

Figure 4. A transparent 8-litre fish tank was used where half of the tank's surfaces were covered in algae. *A dividing line in the middle separates the two conditions to aid in video analysis. The card boxes on each side of the tank blocks the fish view to other tanks/fish.*

Medaka fish were randomly chosen from their common 8-liter tank, each new recording trial. For this experiment, both individuals and groups of four fish (2 males and 2 females). Eight trials were conducted firstly for individuals and then the groups of four medaka. A new group of fish were introduced each new trial, resulting in sixteen trials.

Fish behavior was recorded with the GoPro Hero 7 camera (GoPro, USA) located at the tank's front for a duration of 10 minutes. An acclimation time of 10 minutes was given to the fish before to record their behavior.

The number of fish in each side of the tank were counted by analyzing every 30 second snapshot throughout the 10-minute trials to determine the preference for algae-free or excessive algae.

2.2 Stress Responses of Medaka in Algae vs. Algae-Free Environments

In this experiment, the effects of algae and algae-free tanks were tested on medaka stress levels by looking at different timepoints (Figure 6) in blood and water cortisol levels, monoamine chemistry and potentially stress related gene expression levels in the brain. In addition, fish behavior in an open field test was investigated as an additional stress indicator.

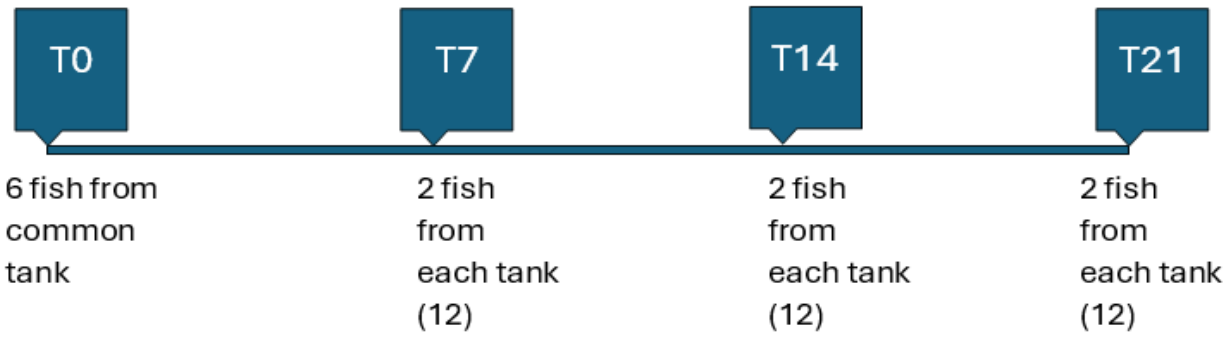

Figure 6. A timeline of the dedicated time points for fish samplings. T0 indicates the start of the experiment, day 0. *All time points including T0, T7, T14, and T21 were designated for sampling blood, pituitary, and brain parts from the medaka fish. At T0 however, the control group from common tank were the only tank sampled.*

In preparation for the experiment, algae were left to grow in three tanks (3.5 L) over 2-3 weeks (algae tanks, Figure 5C). Three other tanks (3.5 L) were maintained free of algae (algae-free tank group, Figure 5B), meaning that whenever algae grew in these tanks, the fish were relocated to new clean tanks. All tanks were kept in the recirculating water system. Twelve (6 males and 6 females) WT adult medaka at around 4-month-old were used per tank in this experiment.

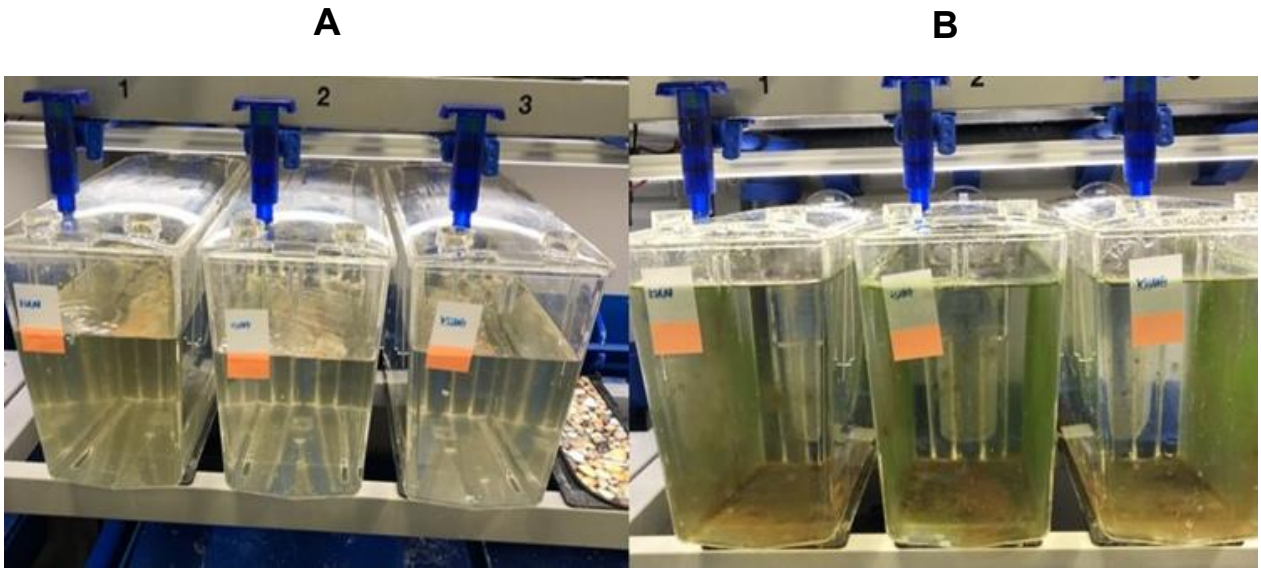

Figure 5. Pictures are showing the two tank environments, algae-free and algae. *Picture A depicts the clean tanks free of algae. Picture B shows the algae tanks covered in green and brown algae. The front wall of all tanks had to be free of algae to monitor fish health and behavior.*

2.2.1 Water sampling

Cortisol is a hormone that is continuously released by fish into the water. First, the water circulation in the tank was stopped for one hour so that the cortisol can build up in the water and reach a more detectable stable concentration. This is a common technique used to improve the reliability of water-borne hormone measurements in aquaculture studies (Midttun et al., 2022).

To measure cortisol levels in the water, water samples were then collected from each of the clean and algae-covered tanks at timepoints T0 (before distributing the fish in algae and algae-free tanks), 7, 14 and 21 days after the fish were split in the two conditions (Figure 6).

At each time point 100 mL water samples were collected from each tank without disturbing the fish, using a pipette controller and disposable pipettes. Since 2 out of 12 fish were taken out for sampling from each tank at every timepoint, T7-T21 (except the control tank), a corresponding water volume had to be drained with a hose before just after stopping the flow to ensure the same concentration of cortisol in water compared to the number of fish. This means that 0.58 L (1:6) of water was removed from the tanks since the tanks were of 3.5 L.

The water samples were stored frozen at -20°C for later cortisol extraction and analysis. However, due to the lack of a water filter required for cortisol extraction, this experiment could not be completed.

2.2.3 Blood, brain and pituitary sampling

2.2.3.1 Fish euthanasia

The euthanasia of fish is a critical aspect of humane and ethical research practices. This procedure follows the guidelines of Norwegian University of Life Sciences, ensuring the welfare and care of research animals involved. MS-222 (Tricaine methane sulfonate) is a common agent used in this experiment for blood sampling (Closs et al., 2022) . A lethal dose of MS-222 was prepared using one tube of 0.6% stock solution diluted with 10 mL aquarium water. The fish were euthanized by immersing them in a beaker containing the lethal solution for 2-3 min. To ensure that the fish was euthanized properly, breathing was checked and the tail was pinched to look for responses. Afterwards, the fish were measured of length and weight. By using an electronical caliper to determine the length of each fish, the tip of the snout to the caudal peduncle, which is the narrow part of the body where the caudal fin (tail) begins, were measured. The fish were then weighed in a weighing chamber. This enclosed chamber provided a controlled environment, minimizing external factors that could influence the weight readings.

2.2.3.2 Blood sampling

Following euthanasia, blood was collected as previously described (Royan et al., 2020). Needles were prepared by pulling a glass capillary in a needle puller. The needle tips were gently broken off with forceps, creating an opening. To prevent the blood samples from clotting in the needle, sodium heparin solution was suctioned throughout the whole needle using an aspirator tube and allowed to dry before use.

The blood was sampled by introducing the needle in the caudal peduncular vein located along the body axis and posterior to the anus, in the region of the dorsal aorta. To gain easier access to the peduncular vein, the scales covering the fish were removed with forceps prior needle introduction. Using the mouthpiece of an aspirator tube to create a negative pressure, the blood was suctioned in the needle. The needle was then centrifuged to collect the blood into heparin coated (1.5 mL) tubes. The tubes were then stored at -80 °C awaiting testing.

2.2.3.3 Brain and Pituitary sampling

Internal standard solution was prepared with 10 mL of a solution containing 94.2 ng/mL of 3,4-dihydroxybenzyl amine hydrobromide (DHBA). As for the sodium acetate buffer, it was added 1.075 mL glacial acetic acid, 0.75 g sodium acetate and 4 sodium hydroxide pellets in 250 mL

Milli-Q water. Finally, pH was adjusted to a value of 5 through titration, using concentrated phosphoric acid (H3PO4). The solutions were then tested in the HPLC machine and stored at 4 °C (Vindas et al., 2014). The preparation of this procedure was required for the quantitative analysis of monoamine neurotransmitters levels in the brain of medaka fish using HPLC.

The pituitary and the following brain parts were separated and collected: hypothalamus, telencephalon, optic tectum, cerebellum, and brain stem. After blood sampling, the fish were placed in an ice water bath to harden the tissue to make it easier for brain and pituitary sampling. The medaka was then quickly dried on paper towels. Under a stereomicroscope, the skull bones were removed with fine forceps, lifting it off carefully exposing the whole brain. First the spinal cord was severed allowing the hindbrain to be lifted. Pituitaries were then taken from the central point of the inferior lobe and stored in 2 mL tubes containing 300 μL Trizol reagent and 4 zirconium oxide beads (NaOH). The sampled tubes were quickly stored in dry ice during the procedure to later be stored in a –80 °C freezer until later use for gene expression analysis of stress-related genes (RT-qPCR). Next, the brain was carefully dissected and collected, and stored in RNase free 1,5 mL pre-weighted tubes containing 100 μL sodium acetate buffer including tested internal standard. The tubes were then frozen and kept in a –80 °C freezer until later use for HPLC analysis. Unfortunately, because of HPLC machine failure and time constraints, the brain parts could not be analyzed further.

2.2.3.4 Cortisol Extraction from Blood and Quantification

Cortisol is often referred to as the primary stress hormone and plays an important role in physiological responses in many organisms, including fish. In this case, blood samples were collected from medaka fish to assess total cortisol concentration by using the DetectX Cortisol Enzyme Immunoassay Kit (Arbor Assays).

Extraction

The initial step in the process involved the extraction of cortisol from blood using a dissociation reagent, diethyl ether. This extraction was necessary to isolate the free, unbound cortisol from the sample, as the ELISA assay is specifically designed to detect this form of cortisol. If the cortisol remained bound to carrier proteins in blood such as corticosteroid-binding globulin and albumin, it would not be properly recognized and quantified by the ELISA antibodies. The dissociation reagent therefore helped to release the cortisol from these binding proteins, thus allowing for

accurate measurements of the free cortisol levels in the sample. Additionally, the volume of blood collected from the medaka were limited, making it necessary to perform this dissociation step to extract the maximum amount of cortisol from the available sample.

To extract cortisol from the blood, 49 µL of PBS (phosphate-buffered saline) was first added to 1 µL of blood. In the subsequent step while working under a fume hood, 200 µL of diethyl ether was added, followed by vortexing for 20 seconds to ensure thorough washing. By letting the solution rest for 3 minutes, it allowed for the heterogenization of the solution. The solution was then quickly frozen on dry ice, allowing the transfer of the organic layer to be more easily accessible either by pipetting or pouring it into a clean tube. The bottom separation freezes more quickly hence making it easier to transfer the upper liquid phase. After the solution thawed, the same procedure was repeated 4 times. The tubes were then placed in a heat block at 45 degrees Celsius, allowing evaporation of the liquid by keeping the lid open. The tubes were then frozen at -20 degrees and stored for later utilization with the ELISA kit.

ELISA

The ELISA was performed according to supplier's instructions (Arbor Assays). A series of dilutions were made to prepare standards, concentrations of cortisol: 3,200, 1,600, 800, 400, 200, 100, and 50 pg/mL.

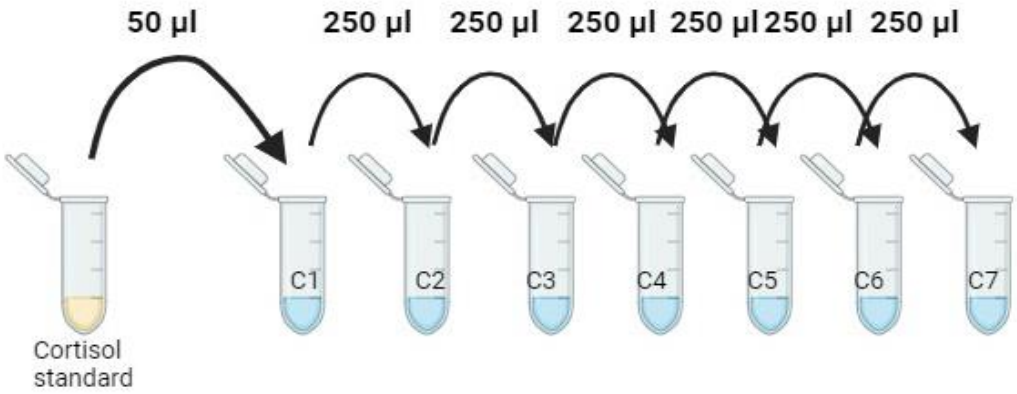

Figure 7. Illustration showing the dilution-series of cortisol stock. Made by author using Biorender.

While the standards were run in duplicate, because of the low concentration of cortisol measured in our samples, the samples could not be run in duplicates. Positive and negative controls were set.

A non-specific binding (NSB) well does not get the antibodies that binds to cortisol, providing information on background binding of other substances. NSB are used to subtract from the total binding data in the other wells. B0, known as the zero standard or maximum binding well, represents the maximum binding of the enzyme-linked analyte to the antibody in the absence of any free analyte (the molecule being measured). The reaction was initiated by adding 100 µL of the TMB Substrate solution to each well. After 30 minutes of incubation, the colorless solution turned blue. The reaction was then stopped by adding 50 µL of stop solution to each well (total volume in which the concentration is measured: 150µl). The stop solution caused the blue solution to turn yellow. The ELISA plate was then analazyed within 15 minutes, because the color produced by the oxidized TMB substrate can start to fade after the reaction has stopped, leading to inaccurate absorbance measurements.

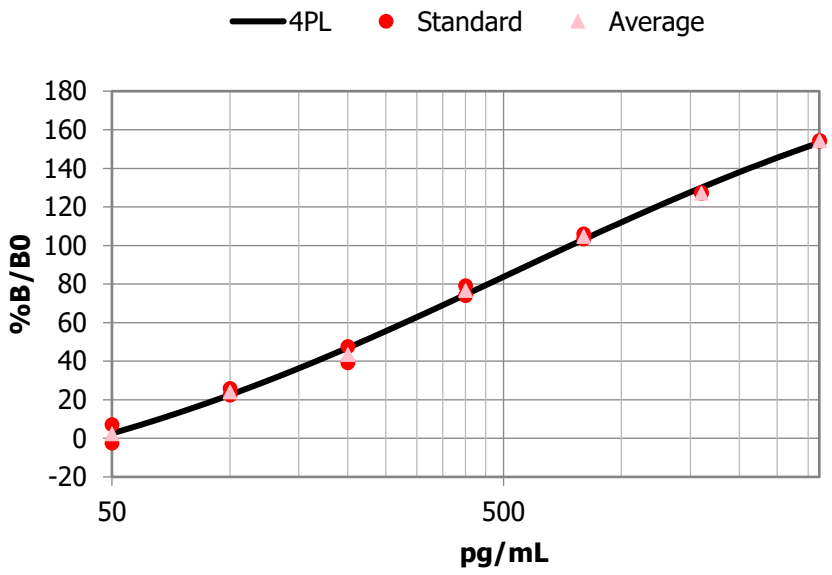

Figure 8. This line diagram shows the standard curve for known cortisol concentrations. *It was used to read cortisol in unknown blood samples from medaka.* Retrieved from MyAssays.

By using a plate reader, the optical density at 450 nm was measured in each well. Finally, by using the plate reader's built-in 4PLC software and MyAssays tool, cortisol concentration was calculated

for each sample. A recalculation was done to get initial concentrations of the actual amount of cortisol in 1 μL blood.

2.2.4 Open Field Test

An open field test was conducted to assess the exploratory behavior of two groups of adult medaka fish. This was done to gain insights into the impact of their previous housing conditions on their stress levels and adaptability to a new environment (Figure 5).

The fish were housed in the two tested environments (algae and algae-free tanks) with 5 fish per 3.5L-tank (3 tanks per condition) for 1 month before the behavioral test was performed, totaling 15 fish.

For each trial, one fish was placed at the periphery of an open field arena, a square maze with black walls and a see-through bottom measuring approximately 30x30 cm. The trials were recorded with the acA1300-60gm Basler camera. Their activity was recorded for 10 minutes, for every trial. Water (4L) was changed each time a new fish was introduced to maintain proper water temperature (26-28 ℃) and avoid any excretes from previous fish that may affect fish behavior.

Videos were automatically analyzed using the Ethovision XT 13 software and the following characters were investigated:

1. The amount of time spent in the center zone.
2. How often the fish entered the center zone.
3. How early the fish would start swimming in the center zone.

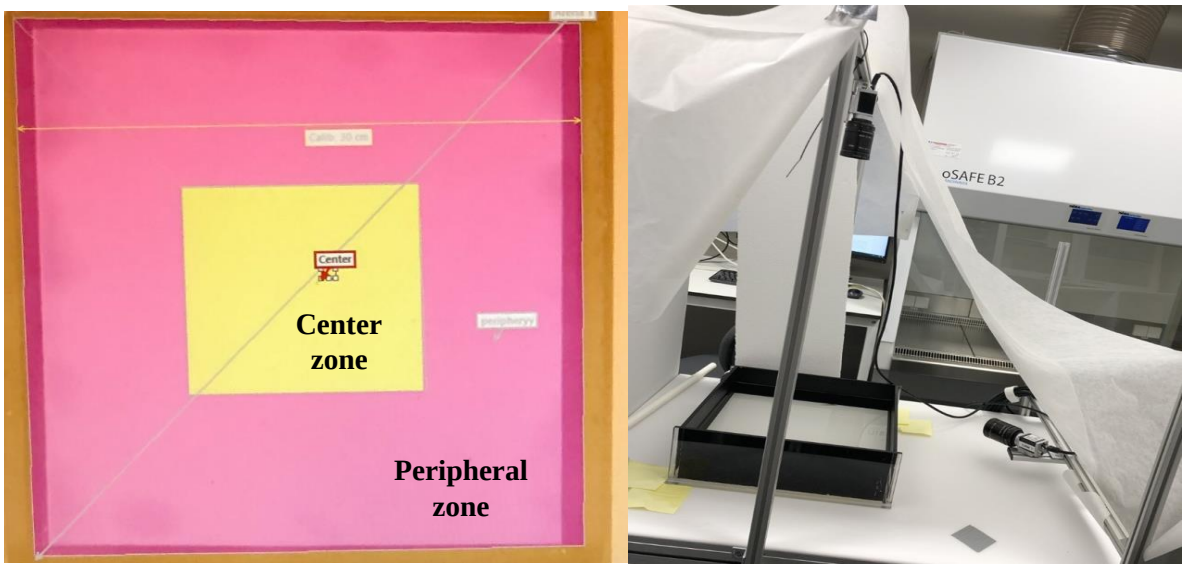

Figure 9. Ethovision XT 13 was used to create an arena for open field analysis. *The arena was shielded with baking paper sheets to minimize reflections and ensure even lighting environment throughout the experiment.*

2.3 Statistical analysis

All data collected in this study are presented as scatter plots with bars showing the respective means of each variable/condition on the x-axis. Standard error of mean (± SEM) is used for all collected data. Unpaired t-tests were performed for the for collected data, but in some experiments, one-way ANOVA and nonparametric test such as Mann-Whitney test were also used. In all tests where $p < 0.05$ (95%), significant differences were observed.

Before performing the t-tests, Shapiro-Wilk normality test was performed to determine if the data was normally distributed (Gaussian). This is important, as violations to normal distribution can impact the validity and interpretation of parametric statistical analyses. Sample groups that were normally distributed, could be tested with a parametric test assuming equal variance. Data that were not normally distributed were log-transformed, transforming the skewed datasets to pass normal-distribution test. Sample groups that could not be normalized underwent nonparametric tests, Mann-Whitney test (for unpaired data).

Unpaired One-way ANOVA assuming equal variance were used for experiments with three groups. Data were log-transformed to achieve normal distribution as well. When data could not be log-transformed, Kruskal-Wallis (non-parametric) test was performed.

3.RESULTS

3.1 Light Intensity Preference Across Colors

In this experiment, the group preference of five medaka were explored for light intensities across a range of colors. Figure 10 summarizes medaka fish's preference to high or light intensity levels.

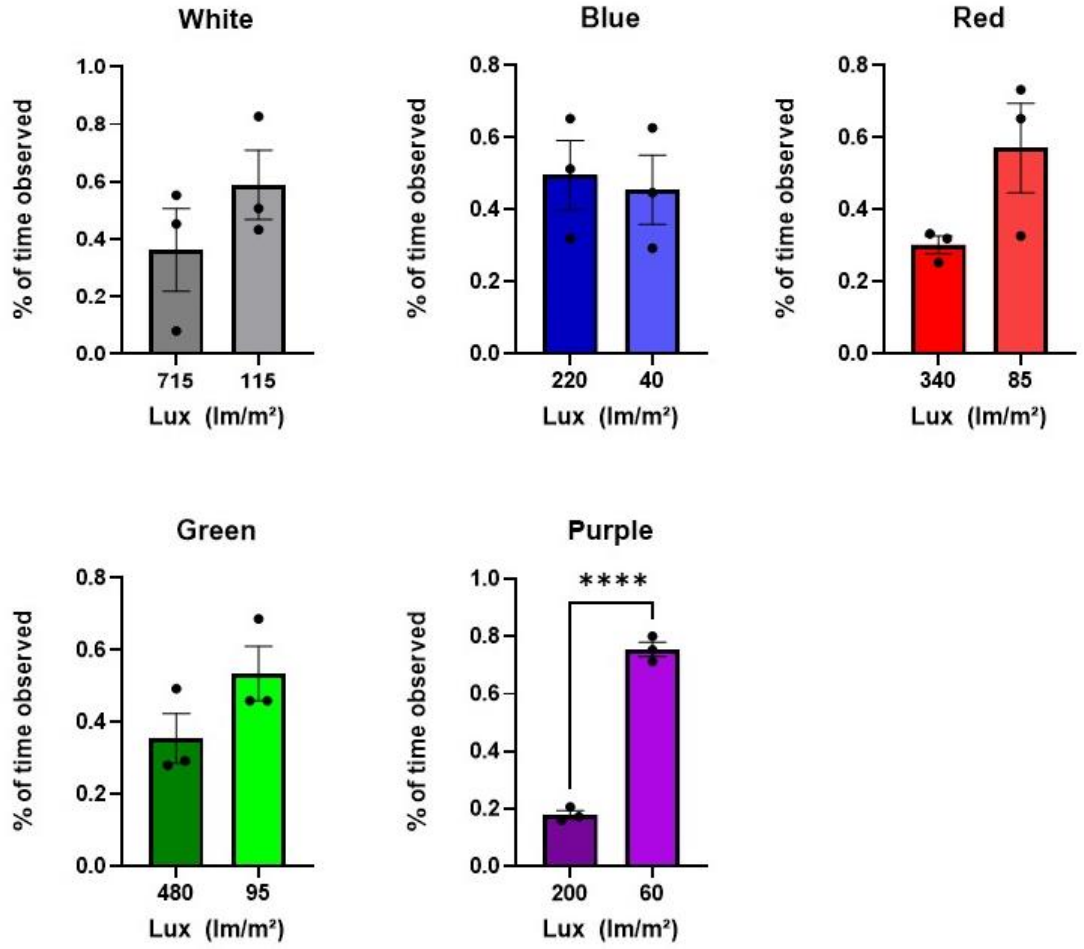

Figure 10. The graphs present mean time medaka spent in areas with different illumination levels. *Both high and lower light intensity levels were evaluated within the same color treatment. **** indicates p-value < 0.0001 as shown in Table 3.*

Table 3. Statistical analyses present p-values and F-values (Figure 10).

Graphs	Test	P-value ($p<0.05$)	F value (DFn, DFd)
White	T-test	0.2943	1.423 (2, 2)
Blue	T-test	0.7840	1.006 (2, 2)
Red	T-test	0.1013	25.27 (2, 2)
Green	T-test	0.1534	1.199 (2, 2)
Purple	T-test	<0.0001****	3.256 (2, 2)

In Figure 10, the graphs show tendencies in medaka preference for lower light intensities across the colors, except blue. In contrast, medaka in blue lights showed a slightly stronger preference for higher light intensity levels, although the preference was similar between the two conditions. However, only the purple light shows a statistically significant difference (Table 3). This indicates there are significant differences in the time medaka spent between the two light intensity levels for purple light.

It is important to note that both bars in a graph do not add up to 100 %, as medaka could be in the "dead zone" of the maze, showing no clear preference, as described in Figure 1. The medaka spent 3-10 % in the dead zone across the light colors.

3.2 Individual & Group Preferences in Male and Female Medaka

The results from exploring medaka light color preferences are presented in Figures 11 and 12. The aim of these experiments was to discover if there were any sex, individual or group differences in medaka fish preference for light colors when PAR amount was similar.

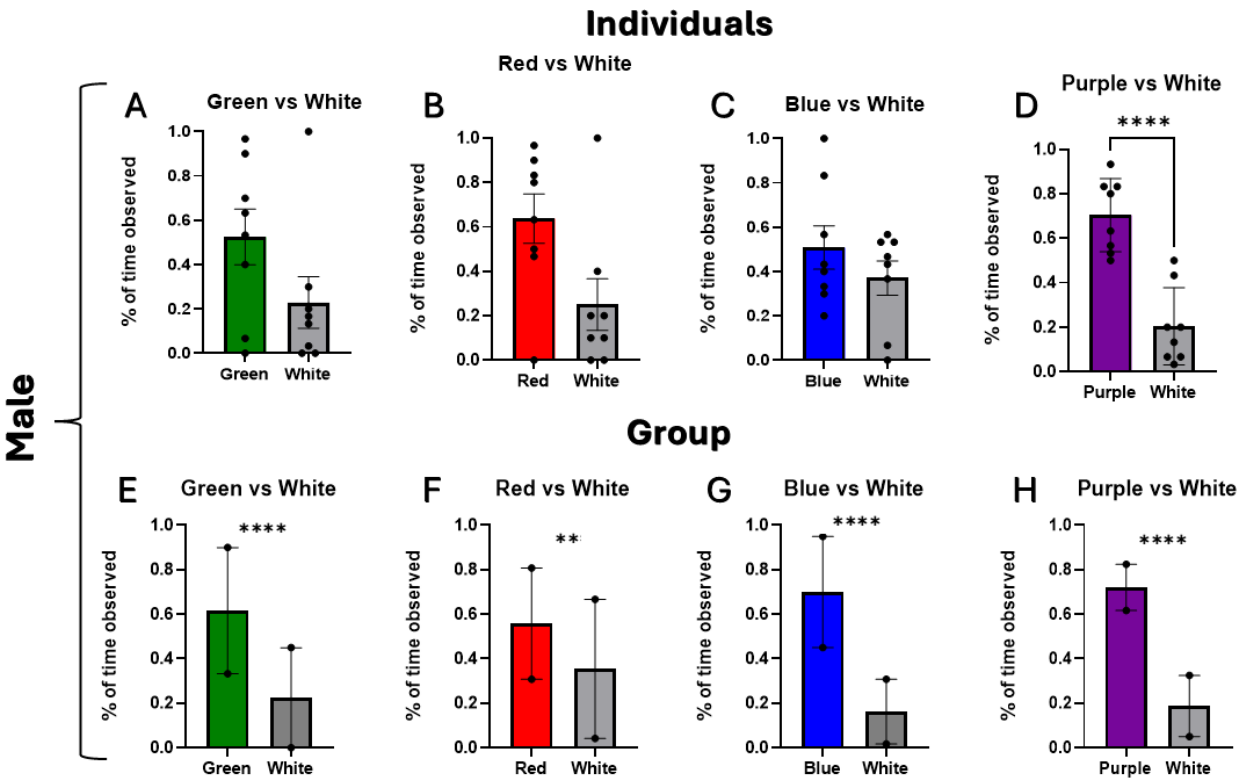

Figure 11. The graphs present the mean time that male medaka spent in each light color. *Graph A-D present individual male medaka, while graph E-H present the same individuals as a group of 4 medaka.* ******** *indicates p-value < 0.0001.*

Table 4. Statistical analyses are presented; tests, p-value and (r). *Graph A, B, E and H has detected outliers for white lights (see appendix). Tests done for group of 4 medaka's contained the raw data values instead of means.*

Graphs	Test	P-value	F value (DFn, DFd)
A	T-test	0.1588	1.162 (7, 7)
B	Mann-Whitney test	0.0600	1.091 (7, 7)
C	Mann-Whitney test	0.7012	1.607 (7, 7)
D	T-test	<0.0001****	1.117 (7, 7)
E	Mann-Whitney test	<0.0001****	1.420 (59, 59)
F	Mann-Whitney test	0.0032**	1.127 (59, 59)
G	Mann-Whitney test	<0.0001****	1.966* (59, 59)
H	Mann-Whitney test	<0.0001****	1.290 (59, 59)

As seen in Figure 11, the male medaka fish prefer the colored light over the white light both as individuals and groups. The male's preferences are very consistent between the individual trials and group trials. However, one notable exception are the blue light colors (Graph C & G), where the group of males spend approximately 20 % more time under blue lights compared to the individuals.

In all the trials for male medaka, there were 10-20 % where no clear preference was observed, especially for green light shown in graph A and E. The scatter plots for green and blue (Graph A and C) exhibits a higher degree of spread, whereas the remaining colors are more clustered.

Individual male medaka, preferred purple light significantly over white, while the male groups preferred all colors over white lights.

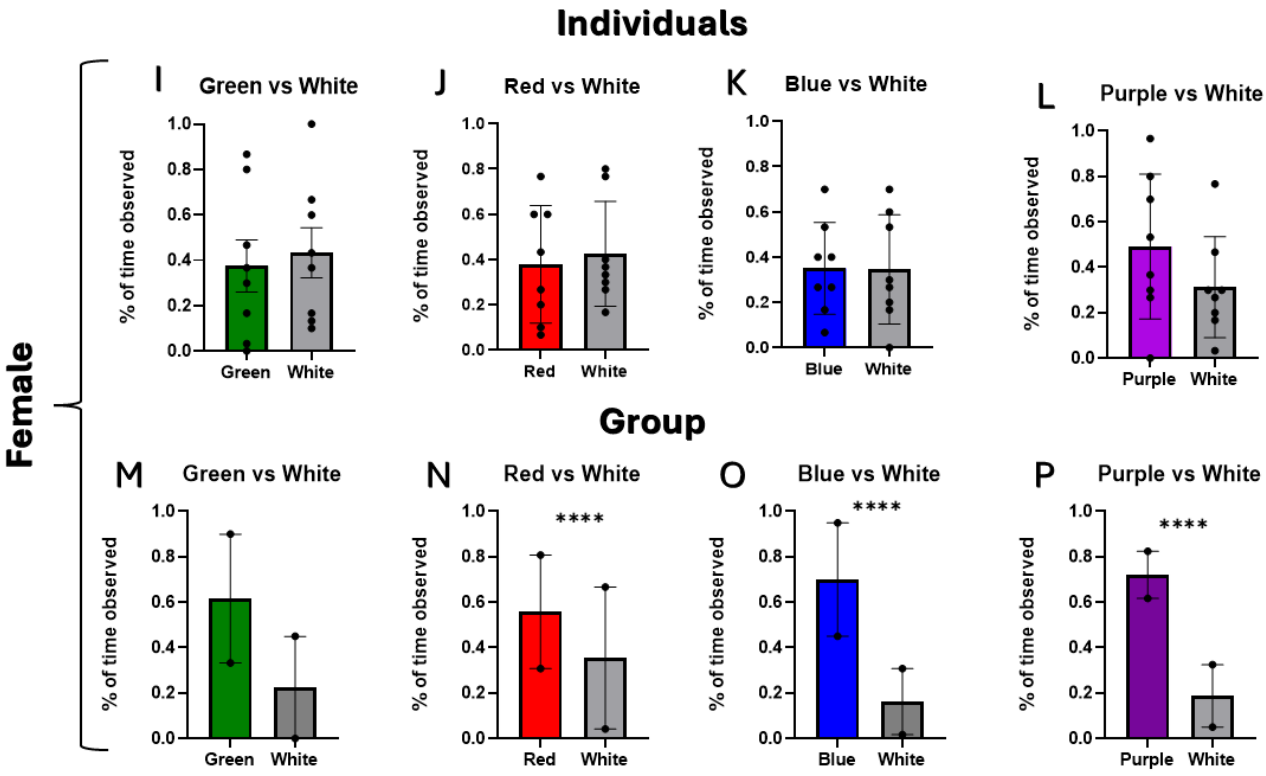

Figure 12. The graphs show the mean time of female medaka spent in each light color. *Graph I-P show 4 individual female medaka, while graph E-H present the same individuals as a group of 4 medaka. **** indicates p < 0.0001*

Table 5. Statistical analyses present tests, p-value and F-values for Figure 12. *There are found many outliers in graph I and M for green light. Outliers are also found in white lights for graphs, M-P (See appendix). Tests done for group of 4 medaka's contained the raw data values instead of means.*

Graphs	Test	P-value ($p < 0.05$)	F value (DFn, DFd)
I	T-test	0.7193	1.084 (7, 7)
J	T-test	0.7152	1.249 (7, 7)
K	T-test	0.9707	1.407 (7, 7)
L	T-test	0.2128	2.064 (7, 7)
M	Mann-Whitney test	0.0211	1.546 (59, 59)
N	Mann Whitney test	<0.0001****	3.180**** (59, 59)
O	Mann Whitney test	<0.0001****	2.105** (59, 59)
P	Mann Whitney test	<0.0001****	1.128 (59, 59)

In females (Figure 12), there is no clear preference towards color light over white light when tested as individuals, except purple light where a tendency for a preference towards the purple light over the white light seems to occur. However, in the group trials, the female medaka suddenly seem to prefer all colored light, except for green over white light (Graph M-P). Yet, the time female medaka spent in the dead zone increases in group compared to in individual tests. In the individual female trials, they spend 20-30 % of the time in the dead zone (no preference), while in the group experiment, females spent up to 70 % of the time (in the green light test, Graph M).

Statistical analysis in Table 5, shows that all the female group trials had p-values that were significant (Graph M-P). Thus, the group of female medaka had a significant preference for colors over white lights as similarly observed in male medaka, whereas individual females showed no clear preferences between the colors.

3.4 Fish Interest in Tank Mirror Reflections

To investigate the interest of medaka fish in tank mirror reflections, a transparent tank with a reflective and a non-reflective side was prepared, as shown in Figure 5.

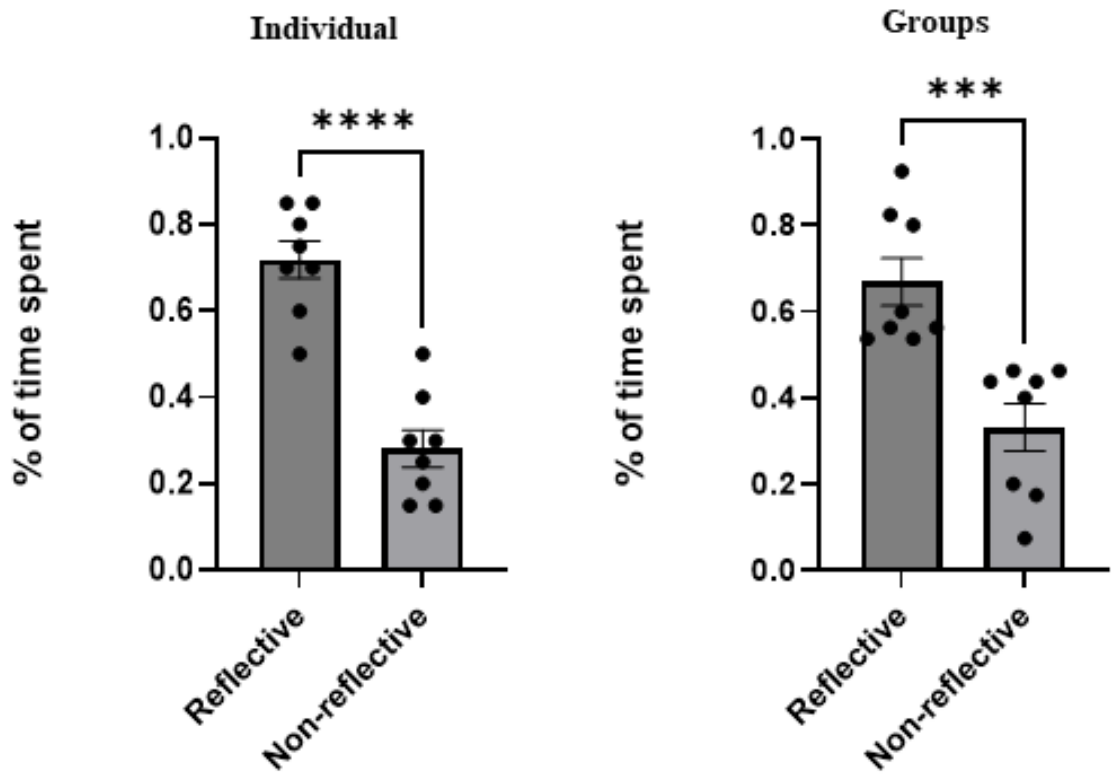

Figure 13. Graphs present the mean time spent in the reflective and non-reflective conditions for both individuals and groups. *The scatter plot shows the mean of 8 trials. Individuals are random solitary medaka fish, while groups consist of 2 males and 2 females.* ******** *indicates p < 0.0001.*

In Figure 13 it is shown that individuals and groups of medaka (2 males and 2 females) spent most of their time in the reflective side compared to the non-reflective side. The means of the two experiments show no significant differences, with individuals spending slightly more time in the reflective side than groups. An unpaired t-test revealed a clear preference by medaka for the reflective side in both experiments. For the individual fish experiment, the unpaired t-test yielded a p-value< 0.0001****, indicating a highly significant difference in time spent in the reflective versus non-reflective sides. Similarly, the group experiment also showed a significant difference between the two conditions, with a p-value of 0.0007***.

3.3 Evaluating Medaka Preference for Algae-Free Environments

This chapter describes the preference of medaka in algae and algae-free (clean) sides in the same tank (Figure 4).

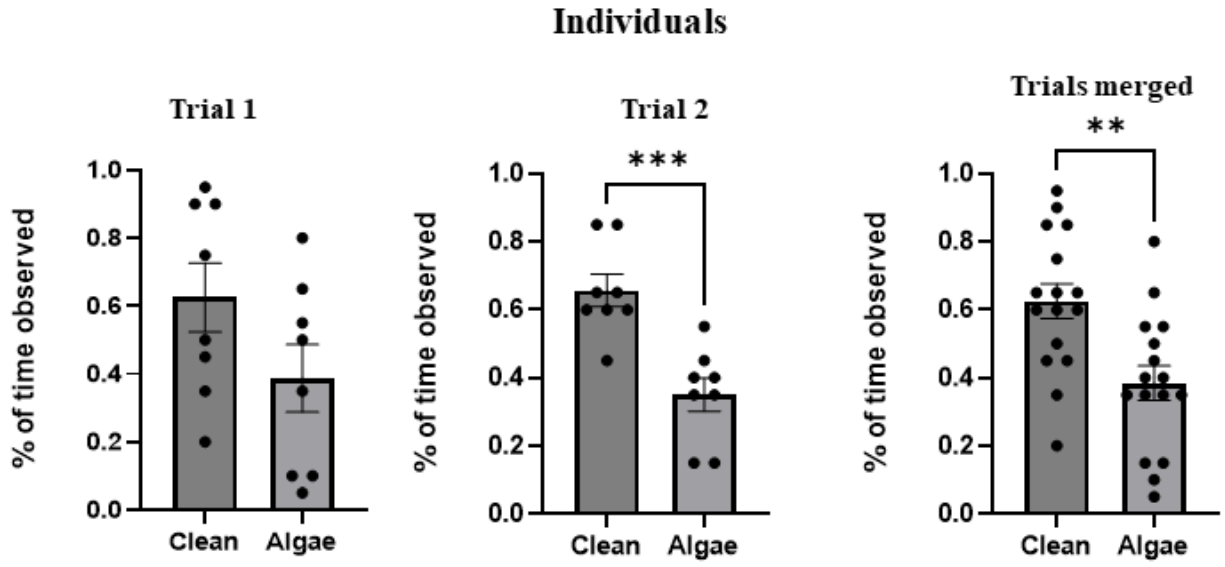

Figure 14. The graphs illustrate the mean time spent by individual medaka fish in clean and algae-covered environments. **** indicates p<0.001.*

Table 6. Statistical analyses are presented for individual medaka in algae/algae-free environments, Figure 14. *Tests present p-values and F-values*

Graphs	Test	P-value (p<0.05)	F-value (DFn, DFd)
Trial 1	T-test	0.1173	F=1.029 (7, 7)
Trial 2	T-test	0.0005***	F=1.061 (7, 7)
Merged	T-test	0.0024**	F=1.018 (15, 15)

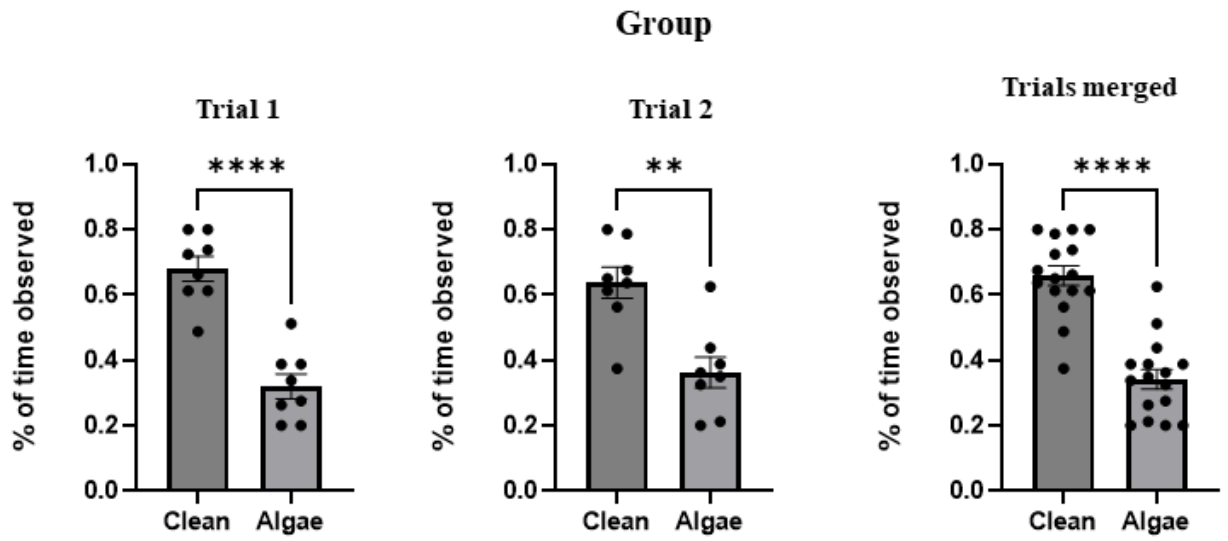

Figure 15. The graphs illustrate the time spent by medaka fish in algae and algae-free environments. *Groups consist of 4 medaka (2 males and 2 females). **** indicates p < 0.0001.*

Table 7. Statistical analyses for medaka groups in algae/algae-free environments (Figure 15). *Tests present p-values and F-values for the group trials.*

Graphs	Test	P-value (p<0.05)	F-value (DFn, DFd)
Trial 1	T-test	<0.0001****	1.000 (7, 7)
Trial 2	T-test	0.0011**	1.000 (7, 7)
Merged	T-test	<0.0001****	1.000 (15, 15)

Figures 14 and 15 demonstrate that medaka individuals and groups spent significantly more time in clean environments compared to the algae environments in all trials, except for trial 1 as individuals. On average, the medaka spent over 60% of their time in all clean condition and less than 40% in all algae condition. There are only minor differences between medaka individuals and the groups for the environments.

The scatter plot of the merged data shows that all data points for the clean condition reside above the mean for the algae condition. This consistent preference for the clean tank environment is across the experiments.

An unpaired t-test showed significant differences between the clean and algae conditions for all trials except trial one in the individual experiment, where the p-value was 0.2756 (table 6).

3.5 Open Field Test

The open field test was conducted to examine stress-related behavior by analyzing fish activity in the open field maze. The designated center zone is shown in Figure 9. Three key parameters are of interest to determine: Time observed in center zone, frequency of entries into center zone, and time of first entry into the center zone (Figure 16).

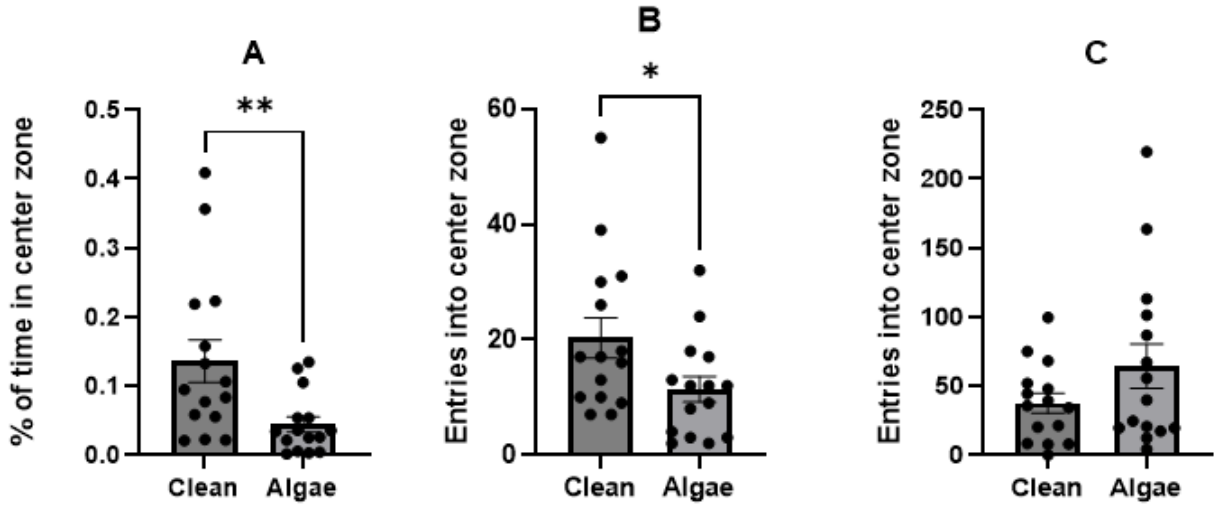

Figure 16. Graphs present individual medaka in the open field test. *Graph A present mean time of medaka spent in the center zone. The number of entries into the center zone (B), and the latency (in seconds) for each individual fish to first enter the center zone (C). Each trial was analyzed for a total duration of 600 seconds (10 minutes), with the remaining time were spent in the periphery excluded from the analysis.*

Table 8. Statistical analyses of open field test, p-values and F-values are presented for Figure 16. *** present p-value <0.01.*

Graphs	Test	P-value (p<0.05)	F-value (DFn, DFd)
A	T-test	0.0035**	F=1.995 (14, 14)
B	T-test	0.0177*	F=2.041 (14, 14)
C	Mann-Whitney test	0.3669	F=4.910** (14, 14)

Graph A in figure 16 demonstrates that medaka fish raised in a clean tank environment spent significantly more time in the center zone compared to those raised in an algae environment. Note that he data for male and female medaka were merged. The medaka spent approximately three times longer in the center zone than medaka from the algae environment. Medaka in the clean tank spent an average of 13.6% of the total time (82 seconds) in the center zone, while medaka in the algae tank spent only 4.4% of the time (27 seconds) in the center. The remaining time was spent in the periphery zone outside the center, which was 86.4% (518 seconds) for clean tank and 95.6% (573 seconds) for algae tank. The scatter plot indicates a more dispersed distribution for the clean group with fish spending over 40 % of their time in the center zone. The algae group appears more clustered with fish spending maximum 15 % of their time in the center zone. Table 8 presents a p-value that shows significant differences between the clean and algae group.

Graph B presents approximately a mean of 20 entries into the center zone by the medaka from clean tanks and 11 entries for medaka from algae tanks. In the clean condition, one individual fish had nearly 60 entries, despite the overall mean being around 20. In the algae tank, some fish had close to 40 entries although the mean was 11. There were observed significant differences of entries into the center zone (Table 8).

Graph C in figure 16 shows that medaka from the algae-covered tank had a higher mean latency to first enter the center zone compared to the clean tank group. The mean latency was 41 seconds for the clean group and 64 seconds for the algae group. Interestingly, medaka from algae tanks used as long as 220 seconds before entering the center zone, whereas a from clean tanks the medaka only used up to 100 seconds. However, it was revealed no significant difference between the two groups (table 8).

3.6 Cortisol Concentration in Medaka

This chapter presents the results of cortisol levels on blood samples collected from medaka fish in the clean, algae, and “control” group (environment prior to the experiment) tank conditions. The cortisol concentrations, fish weight, and size are analyzed and compared between the experimental groups.

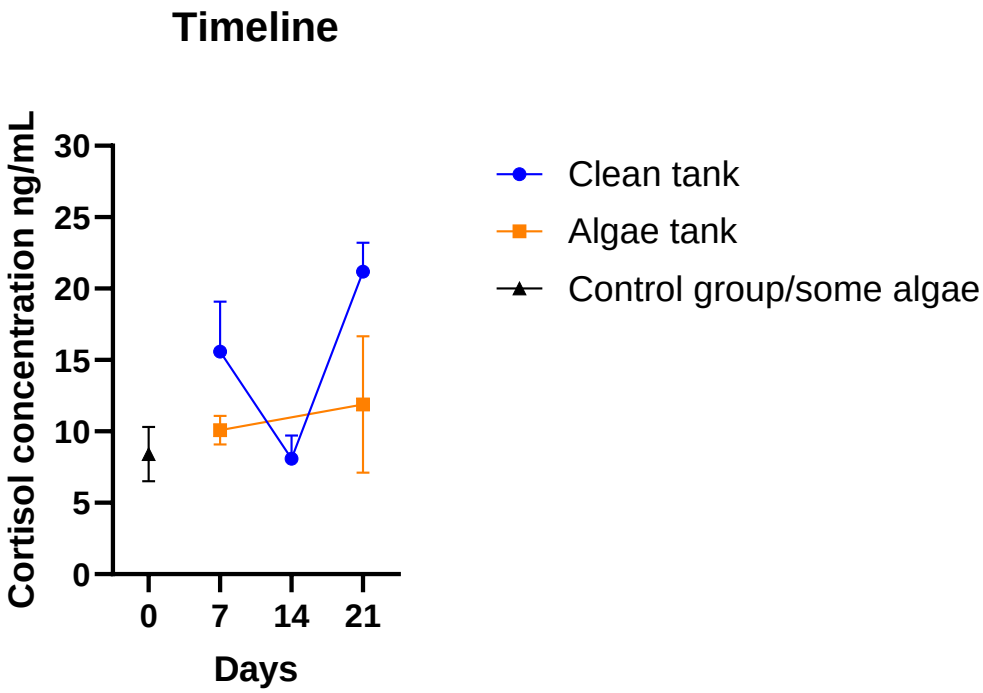

Figure 17. Line plot presents the mean (+/-SEM) cortisol concentration at timepoint; day 0, 7, 14 and 21. *Algae group missing all data for day 14, whereas "control" group are only sampled at day 0.*

Since the cortisol samples were collected from medaka fish in different time points, Figure 17 and 18 show the mean and scatter of the cortisol concentration. The "control" group yielded the lowest mean concentration of cortisol along with day 14 of the clean group. The clean group also shows the highest concentrations of all medaka in day 7 and 21. Day 7 is high, day 14 is decreased, and then day 21 at the highest concentration for clean group.

The algae group miss cortisol measurements from day 14, though day 7-21, shows a slight increase in cortisol level (see methodological considerations)

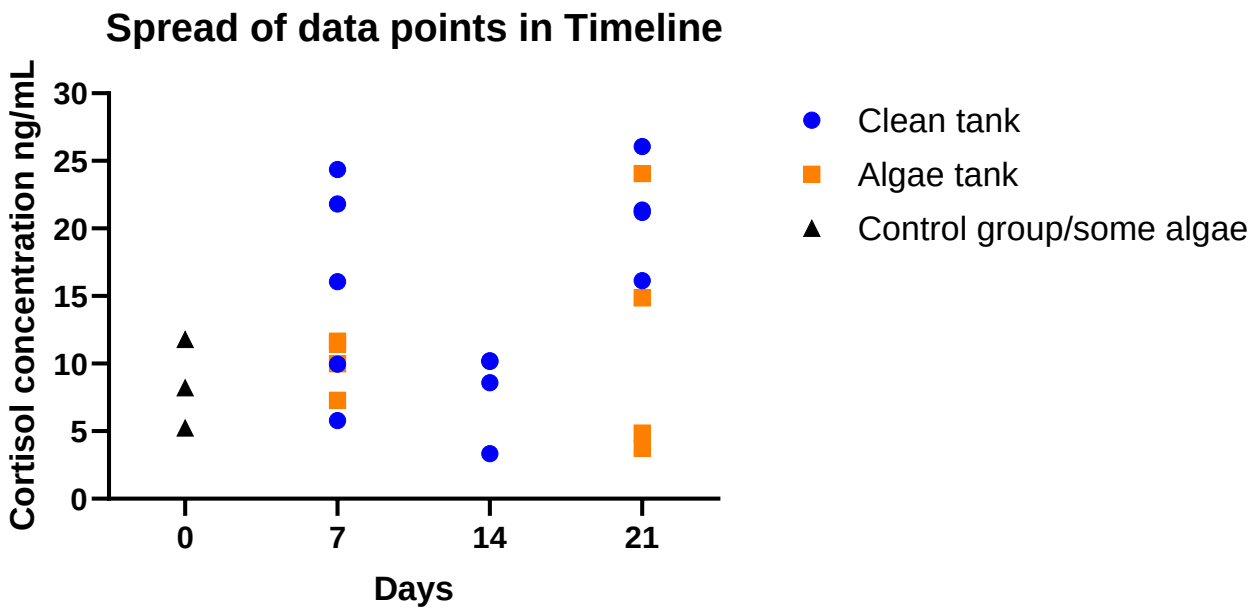

Figure 18. Scatter plot presents the spread of individual cortisol concentrations within medaka in Figure 17. *For the clean tank on day 14, there are two data points clustered as one at around 10 ng/mL. Meaning there are 4 data points on day 14.*

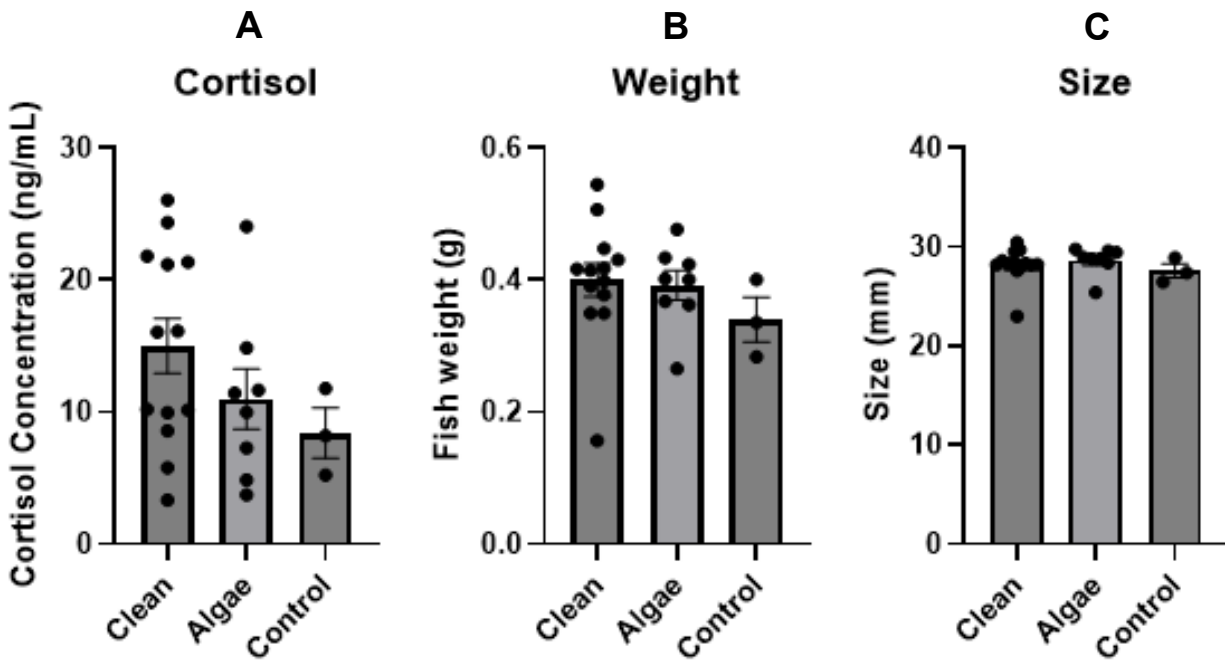

Figure 19. The cortisol concentrations of medaka from clean, algae, and control tanks are shown in graph (A), with individual data points ||overlaid as a scatter plot. *The clean tank contained 13 fish (6 males, 7 females), the algae tank had 8 fish (4 males, 4 females), and the control tank had 3 fish (2 males, 1 female). Graph B presents the weight and C presents the size of the fish.*

Table 9. Statistical analyses present p-values, and F-values of figure 19.

Graphs	Tests	P-value ($p < 0.05$)	F value (DFn, DFd)
A	One-way ANOVA	0.3279	1.176 (2, 21)
B	One-way ANOVA	0.5147	0.685 (2, 21)
C	Kruskal Wallis	0.2075	0.026 (2, 21)

Cortisol concentration in Figure 19, graph A, the clean group (13 fish) has a higher mean cortisol concentration compared to all the other groups. Notably, this group also contains both the highest and lowest individual cortisol concentration values observed across all conditions. The clean group has a mean cortisol concentration at 15.6 ng/mL while the algae group (8 fish) has 11.8 ng/mL and finally the control group (3 fish) with the least cortisol concentration at around 8.4 ng/mL. Table 9, bar plot A shows a p-value of 0.3279 for the cortisol data, failing to reject the null hypothesis, meaning that there are no significant differences between the groups.

Graph B presents the mean weights for each group, where the clean group has the highest mean weight overall at 0.400 g. Like the cortisol data, this group also contains the highest and lowest individual weights measured across the groups. The algae group only has a slightly lower mean weight at 0.392 g compared to the clean group, but a higher mean than the control group. The clean group has the lowest mean weight of all groups at 0.340 g. The weight differences are not significant between groups.

Finally, graph C presents the mean fish sizes for each group. The bar plots appear very similar across the groups at around 28 mm. The algae group has the highest mean size, while the control group has the lowest mean size, although the means are almost identical across the groups. Table 9 shows no significant differences between groups for the size data in bar plot C.

4.DISCUSSION

4.1 Light Color and Intensity Preferences

The preference for light colors and illumination levels were investigated for WT medaka. The results from the light experiments show that medaka fish significantly prefer a lower light intensity of purple LED lights over high light intensity. For other colors like white, red and green, there were tendencies towards lower light intensity observed, but these were not significant. This may be due to medaka not disliking any of the light intensities, which may suggest that medaka have a more tolerant threshold for the light intensities.

The tendency towards lower light intensities might align with previous research on medaka as they in their natural habitat prefers plant-rich and shallow habitats where light intensities usually are lower (Hilgers & Schwarzer, 2019). This may seem contradictory since shallow waters would have higher light intensities than deep water, but the presence of plants and shoreline vegetation are common and can create pockets of low light intensities that medaka may prefer. As the medaka prefer light intensities around 100-150 lux for a 14-hour day period (Niwa, 2024), this may suggest that their natural habitats have similar light intensities as well.

Many fish species avoid light environments as a refuge-seeking behavior when they are stressed or feel anxious, although this does not fully explain the medaka's behavior. This may be cause of medaka's body coloration/transparency that reduces their visibility and helps them blend in with the background, even in brighter environments (Lucon-Xiccato et al., 2022). Although this may be, fish will in general, including medaka, seek safety and cover when they are stressed or frightened. The medaka's body coloration may just reduce their need to hide as much (Ruzzante & Doyle, 1991).

Within the cross maze, a problem was encountered with the medaka sometimes spending a lot of time in the dead zone (middle of the maze), showing no clear preference for light colors and intensities. Reasons for this may be caused by stress or their shoaling behavior. The medaka were allowed a 10-minute acclimation time, which should be enough time for them to be more comfortable and exploratory, rather than stressed. Despite this, there are studies that suggest a longer acclimation time (Miyanishi et al., 2016). It may be that housing only 4 medaka fish together may not provide enough social interaction and group dynamics to fully support their shoaling

behavior, compared to keeping them in larger groups as recommended (Padilla et al., 2009). Since the walls in the dead zone are transparent, and the light experiments consisted of solitary and small groups of fish, they might have searched for conspecifics in their own mirror reflection in these transparent walls. If we look at the results for fish interest in tank reflections (Figure 13), this experiment demonstrated that the medaka in our lab did indeed prefer the reflective side of the tank, especially in the experiment with individual fish. Our study also aligns with another study where fish considered mirror reflections as other individuals (Desjardins & Fernald, 2010). As medaka are social fish and thrive best in groups, this means that it is natural for them to seek other medaka fish. This may result in medaka chasing their own reflection on the walls or trying to reach the unlit arms if they were stressed or simply prefer the darker parts.

However, the medaka spent only a short time in the dead zone for light intensity preferences. That may suggest the medaka were not stressed and did not dislike the light colors and intensities themselves, as the dead zone and the remaining blocked arms would have been less illuminated for refuge seeking.

When it comes to light intensity thresholds, a study with fish species *S. waltoni*, showed that they had a low phototaxis threshold for blue light, but even lower for red lights (Xu et al., 2022). Phototaxis refers to the movement of an organism in response to light source. When the lights were under the threshold, blue light was preferred, while the fish avoided red. But when the light intensities increased, the fish were more attracted to the blue lights and avoidance of red decreased as well. These results along with our findings may suggest that medaka have a certain intensity threshold for different light colors. When it comes to light intensities for blue light, the medaka fish showed a slightly stronger tendency for higher light intensity. However, it is important to note in Table 1, the lux difference between the two blue-lit maze arms were the lowest across the colors. Since the time spent between the two blue light intensities were similar, this observation might suggest that the light intensity threshold for blue lights was within a comfortable range for the medaka. This may also mean that the light intensity for purple were too high, causing the medaka to prefer the lower intensity. For green, red and white lights, the medaka may have a more tolerant threshold, especially for white lights with the highest lux difference, however more data is needed to further explore thresholds in diverse colors.

Moving on to color preference, where purple lights were highly preferred by male medaka as individuals and groups. Females did not display any clear preference in the individual trials. However, when assessed in a group, the females suddenly showed tendencies towards colors over white lights, suggesting that social dynamics in a group may influence their environmental preference. But these results need to be interpreted carefully because of outliers found in many of these trials, especially for groups.

Outliers may have arisen, because medaka individuals may have spent more time searching for other conspecifics in their own mirror reflection, rather than showing preference for any of the lights. Their mirror reflection may be perceived in the whole transparent maze somehow causing the medaka to spend their entire time in one arm or in the dead zone. If the medaka first entered an arm and saw their own reflection, then it would be understandable if they remained in that arm for the entire trial. Since they are social animals and a group of 4 medaka may be too small for the fish to not chase their own reflections after a conspecific. This again may lead to a cascade reaction where other fellow medaka chase this same outlier, causing the medaka to explore less and favor a specific light color or intensity.

Females spent significantly more time in the dead zone of the maze compared to males. The significant preference for colors over white lights in female groups may be biased, since a great amount of time were spent in the dead zone. Although, the difference in arms were significant, the dead zone itself were almost more preferred. The reasons for this are difficult to determine, but as mentioned earlier, they might have preferred the dead zone in the middle due to mirror reflection or wrong light intensity threshold in the lights. As the lights had high intensity, the female medaka may be more sensitive and prefer lower light intensities. Further experiments need to be conducted to find these sex specific light thresholds. Additionally, the female medaka showed a substantial difference between individual and group trials, highlighting a more exploratory behavior in solitary females compared to the female group. This could mean that preference for colors, intensities and other enrichments may be easier to determine if the female medaka is solitary. Thus, addressing the need to further explore this suggestion.

Nonetheless, when comparing the male fish to females in light color preference, male medaka tend to show preference for colored lights over white. However, female medaka showed some

tendencies in preference to white lights as individuals. If further explored, then the individual preferences in males and females could be revealed as significantly different.

It is also important to note, these experiments happened in a dark room, where the only light sources were the LED lights. The light colors were therefore high in contrast compared to the background darkness. It has been shown by a Japanese study that a higher background lighting intensity from the sunrise, reduced the number of light-attracted fish at night (Sakamoto et al., 2017). The medaka placed in a dark room might be more influenced by the colors and intensity of the LED lights more than they would have been in a naturally lit environment. The obvious contrast between the colored lights and the surrounding darkness may have amplified the medaka's preferences towards the specific light colors, thus exaggerating their actual preferences in an otherwise naturally lit environment. This may suggest the need to also perform these experiments in lit environments to best understand the effects of color enrichments.

4.2 Medaka in Tank Reflections and Algae Free Environments

These experiments explored the preference of individuals and groups of medaka for transparent tank wall reflections and algae-free environments as shown in Figure 3 and 4.

Medaka showed a preference for the reflective side (Figure 13), both as individuals and in groups. The individual medaka had a slightly stronger preference for the reflective side and as previously mentioned, medaka are social fish exhibiting shoaling behavior. Therefore, we could expect them to be more drawn to the reflective side, and even more so as individuals, since fish may perceive mirror reflections as other conspecifics. As there were minor differences between individual medaka and groups, this may be explained with a group of 4 medaka that may not provide enough social interaction.

Although the medaka spent more time in the reflective side, this does not necessarily correlate to their interest in mirror reflections. Studies have shown that medaka may perceive their mirror reflections, however, they did not show a significant preference for mirror stimulus with the modified shoaling test (Lucon-Xiccato et al., 2022). In the octagonal mirror test, the medaka were observed swimming near the mirror, showing behavior that could not be described as aggressive, whereas many other species would show aggression (Lucon-Xiccato et al., 2022). This suggests

that while medaka can perceive mirror reflections, they do not necessarily respond to them strongly as a social stimulus or a rival. In the previous light experiments, they exhibited behavior as if trying to break through the transparent walls, as if trying to reach their own mirror reflection. While the medaka do possess shoaling behavior, their response to social cues may be weaker than other highly social fish species like zebrafish.

The results for medaka preference in algae-free environments shown in Figure 14, may shed further light on the medaka's preference for reflective tank walls. Although algae are a quite common occurrence in tanks in laboratories, aquaculture and hobbyist aquariums, it is unclear whether fish actively dislike the algae. It is shown that individuals and groups of medaka preferred the clean sides of the tank compared to algae. This may indicate that medaka dislikes the algae or they just prefer their own mirror reflections in the clean side. There are slight differences in the individual and group trials but in trial 1 for individual medaka, there were some fish spending almost all their time on the clean side. The medaka may have chased their own reflection in the walls, barely exploring the tank. In this sense, the fish might be more attentive of finding other conspecifics in their reflection, than exploring the environment. Yet, all results did show a preference for the algae-free side. However, replicates of these experiments need to be further conducted, as one trial was not significant, but the second one was. Merging these together resulted in significant differences.

4.3 Open Field Test

Further a due, the open field test was conducted as explained in Figure 9, to determine stress responses of medaka in algae versus clean environments.

Firstly, when examining the time medaka spent in the center zone of the maze, there was a significant difference observed between the algae and clean tanks. Medaka raised in the clean tanks exhibited less anxiety-like behavior by exploring more into the center zone, compared to medaka raised in the algae environment which stayed mostly in the periphery, avoiding the center (Myklatun et al., 2018).

An observation we have seen earlier in our experiments, is that the medaka preferred the algae-free environments. This aligns with what is observed in this experiment. Although this preference seems

likely, there are a few medaka individuals from the clean tanks spending a big amount of time of time in the center zone.

How often medaka entered the center zone gives greater insight into the time spent in the center zone. Time spent in the center zone is considered a stronger sign of exploration and lower wall-hugging anxiety (Lucon-Xiccato et al., 2020). Frequency of entries into the center zone may reflect the medaka's activity levels or impulsivity. The higher frequency of entries may indicate that the medaka is not stressed, as they tend to freeze or have reduced activity when stressed (Lucon-Xiccato et al., 2020; Otsuka et al., 2021). This aligns with the finding that clean tank medaka spent more total time in the center and had higher frequency of entries compared to the algae tank medaka. However, there are a study that suggests that frequent transitions between the center zone and the periphery may indicate anxiety and hesitation to fully explore the center zone (Myklatun et al., 2018). The higher frequency of entries is, more likely to indicate increased exploratory activity in our case. This interpretation is also supported by previous findings, where medaka from enriched environments display more exploratory behaviors (Matsunaga & Watanabe, 2010).

Lastly, the latency of the first entry into the center zone will give more insight into the stress-related behavior of medaka from the conditions (Ratuski et al., 2021). Medaka from algae tanks use longer time to first enter the center zone, though differences between the two conditions is not significant. Longer latencies to first enter the center zone are associated with higher levels of anxiety and stress responses. If the experiment was conducted several times for more medaka fish, the difference between the conditions might have been significant. However, this tendency still aligns with our findings of medaka avoiding algae-free environments more.

Since the control group is not included in this experiment, the results and discussion reflect only on medaka fish from clean and algae tanks. Although the control group itself would not necessarily be the ideal tank condition, it would have been valuable to include to further gain more insight to the different potential environments preferred.

4.4 Cortisol Evaluation as a Stress Indicator

This chapter will discuss the cortisol levels found in medaka from the clean and algae environments, to reflect on their preferences or dislikes, based on the previous chapters as well.

Previous experiments showed results suggesting that medaka do not prefer the algae tanks. However, medaka from the clean tanks had higher cortisol levels compared to those in algae and control tanks. As cortisol is a primary stress hormone, the elevated levels observed in medaka from the clean tanks contradicts our previous findings, which suggested that medaka preferred clean tank environments.

The control group had the lowest cortisol levels indicating that the medaka were least stressed in their "normal" tank environments and most stressed in clean environments. These findings might suggest that medaka preferred a dirtier tank with algae, than a clean sterile one, though not significantly. However, as there are missing cortisol data and the control group has very few readings compared to other conditions, it may be biased to assume that the "normal" tank environment with some algae, was the least stressful environment. Whether the cortisol measurements are the most important indicators for stress or their behavior for cleaner conditions, will be further discussed.

First, we need to understand that while lower stress could make an environment more preferrable for the medaka, it does not necessarily determine that reduced stress corresponds to stronger environmental preference in medaka (Lucon-Xiccato et al., 2020; Lucon-Xiccato et al., 2022). It may mean the medaka only dislike other conditions less, rather than preferring them. This is why the control group is not as important since it is not known as the ideal environment.

If we look at the weight and size, there seems to be a trend in less weight and size, the lower the cortisol levels. A study that shows that weight, size and sex matter in steroid levels in medaka and since cortisol is involved in regulating metabolic and physiological processes, the sex specific steroid profiles could influence cortisol levels as well (Kawajiri et al., 2015). While size and weight were not associated with cortisol, it is possible that these factors could influence cortisol levels if it reflects differences in metabolic demands. However, since our study misses a lot of samples and the sex ratio is skewed, medaka's sex are not included in the cortisol analysis.

Since there were insignificant differences between the conditions in weight and size, and many more studies have found little correlation between these factors and cortisol, it may be no reason to interpret these results any further, as these results are insignificant.

Monoamines such as serotonin, dopamine and their metabolites can be affected by environmental enrichment, which could give a clearer picture of medaka preference. However, the monoamine change does not directly correlate to the medaka's environmental preference, it only shows how the clean and algae tanks changed the monoamine neurochemistry (Höglund et al., 2005). Since the collection of these monoamines failed, it is harder to interpret the results.

The timeline presented in Figure 17 and 18, shows us the variation of cortisol concentrations within 21 days. The control and clean group show the lowest mean cortisol concentrations during this timeline. However, the clean group shows a tendency of a remarkably high concentration first, then decreasing and then spiking extremely. This tendency is hard to interpret as there were done no behavioral tests at each time point. Since the control and algae group also miss many data points, it is difficult to make a fair comparison between the conditions. However, the tendencies seen in cortisol levels and in the timeline, shows no logical pattern.

When it comes to medaka preference, behavior may be a more direct measure of it since cortisol is an indirect physiological measure that do not always align with preference. Behavioral responses like spatial positioning, exploration and activity levels do directly reflect the fish's motivation, exploration and preference for the environments (Mathuru, 2016; Zekoll et al., 2021). Though both behavioral and physiological data will often be better than only one or the other.

4.5 Methodological considerations

4.5.1 Light Color Preference in Medaka

The experiments for light color preference had many outliers detected as seen in Table 4 and 5. The statistics were done with these outliers which may have resulted in biased results. The outliers might have been favoring colored arms, rarely exploring, creating a biased result for low preference for white light. The exact reasons are difficult to determine for these outliers, but a few reasonable explanations can be caused by the mirror reflections in the walls. Medaka's shoaling behavior as

individuals and even groups might be enough reason for to create outliers. Some individuals may also be too stressed if acclimation time was not enough causing them to be inactive/freeze.

Group data used for statistical tests of color preference contained raw data, due to complications of too few means. Using raw data allowed the statistical analysis to function, while individual data consisted of means.

4.5.2 Open Field Test

While the Ethovision XT 13 software provided a useful tool for tracking the medaka fish behavior, there were several challenges encountered during the video analysis process. During the video recordings, the camera would occasionally become unfocused, resulting in blurry footage. This made it more difficult for the software to accurately detect and track the fish movements. To mitigate this issue, the recordings and camera had to be constantly monitored and manually refocused back. The software had difficulty differentiating the fish from the background or other elements in the arena, occasionally leading to inconsistent or failed detection of the fish. The data collected for the fish's activity and time spent in the peripheral zone and center zone was not adding up to the 10-minute trials. Due to the unreliable nature of the peripheral zone data, it was decided not to include the periphery data in the analysis. Analysis and interpretation will primarily be on the fish's behavior and activity in the center zone of the open field arena. The analysis did not include the movement of medaka due to uncertainty about the threshold settings. Initially, the movement threshold was set too high, starting at 1.5 cm/s and stopping movement detection at 1.25 cm/s.

Despite the efforts to minimize disruptions during the open field test, I had to manually refocus the camera during the trials which may have caused disturbances to the fish because of the camera malfunction. The fish may have been able to perceive the camera adjustments, which could have influenced their natural exploratory behavior. The camera might have slightly moved as well, making errors in periphery and center zone not adding up to the actual trial time, 10 min = 600 sec. It was chosen to use the center zone data and then base the periphery data from that to add up the total to 600 seconds, although the center zone data was of main interest.

The “control group”, is the environment they were housed in prior to the experiment and is not an actual control group. This group was not included as there were time constraints, complications and many blood samples missing. With little to compare with, the control group was discontinued.

4.5.3 Cortisol Level in Medaka in Algae vs Clean environments

Unfortunately, a significant portion of medaka blood samples were lost due to unknown reasons. The absence of cortisol concentration measurements might have negatively influenced the results, since the clean group had more data, while algae and control had less.

Medaka are small fish that makes it hard to retrieve blood from and thus requires practice and good technique. During blood sampling and storage there were errors, resulting in only 24 blood samples out of 42 from the experiment. During the blood sampling process, there were some medaka fish we failed to retrieve blood from.

For the sampling process, heparin was used to coat needles and tubes. However, we had problems with the blood coagulating after thawing the samples. The heparin coating procedure is an easy method; thus, the fault may have been in the heparin itself. The heparin might have lost its anticoagulative properties if antithrombin III was depleted. Without enough antithrombin III, heparin loses its blood thinning effects (Beurskens et al., 2020). Since the heparin was not newly made, these antithrombin III may have been depleted and caused the coagulation in many of our blood samples. It was also chosen not to compare genders as the data collected were too small and not normally distributed between the different conditions.

It is also recommended to run duplicates of the samples on two separate ELISA plates, since it allows assessment of variability between the samples. Potential errors including pipetting inconsistency, contamination and insufficient washing may be discovered. In this study, this was not possible to do because of lack of blood samples from the experiment.

5.CONCLUSION

The findings in this study established medaka's preference for clean tanks, certain light colors and intensity. Factors such as light intensity thresholds, social dynamics, and sex-based tendencies are important factors to consider for implementing effective light enrichment. In husbandry it is important to maintain an enriched and clean tank, as excessive algae growth is not preferrable for medaka and may in some case, compromise their well-being.

Nonetheless, thresholds for various colors need further exploration to fine-tune color preference accuracy. By replicating the experiments on tank environments, color preference and further exploring light intensity thresholds for medaka, husbandry practices and their well-being may be improved.

The insights gained can serve as a steppingstone and guide further development of more humane and enriched housing conditions, which may have further implications in aquaculture and aquarium settings as well.

6.APPENDIX

6.1 Supplementary Tables of Outliers

Individual & Group Color Preferences in Male and Female Medaka

Tabell 10. ROUT (Q = 1%). Outlier numbers are presented for individual and group experiment for male medaka color preference. *Group data consists of raw data due to not enough mean numbers to do statistical tests.*

MALE		
Color Combination	**Individual Outlier Number**	**Group Outlier Number (raw data)**
Green vs White	0 - 1	0 - 8
Red vs White	0 - 1	0 - 0
Blue vs White	0 - 0	0 - 0
Purple vs White	0 - 0	0 - 4

Tabell 11. ROUT (Q = 1%). Outlier numbers are presented for individual and group experiment for female medaka color preference. *Group data consists of raw data due to not enough mean numbers to do statistical tests.*

FEMALE		
Color Combination	**Individual Outlier Number**	**Group Outlier Number (raw data)**
Green vs White	0 - 0	4 - 16
Red vs White	0 - 0	0 - 1
Blue vs White	0 - 0	0 - 8
Purple vs White	0 - 0	0 - 5

6. 2 Supplementary Graphs

Cortisol Concentration in Medaka

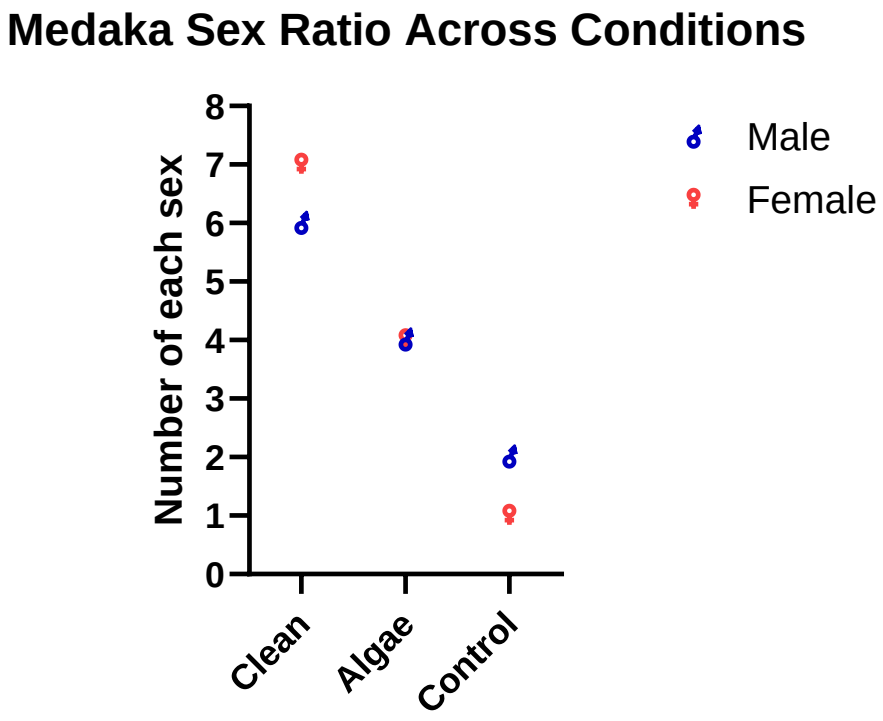

Figure 20. This scatter plot presents how many medaka there are of each sex for cortisol concentration comparison.

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