Exploring the impact of salinity tolerance on gill function and antioxidant enzyme activities on Melon barb, *Haludaria fasciata* (Jerdon, 1849): A Comprehensive study.

A Dissertation for

Course code and Course Title :ZOO-651 Dissertation

Credits: 16

Submitted in partial fulfilment of Masters Degree in Zoology

by

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Under the Supervision of

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Exploring the impact of salinity tolerance on gill function and antioxidant enzyme activities on Melon barb, *Haludaria fasciata* (Jerdon, 1849): A Comprehensive study"** is based on the results of investigations carried out by me in the Zoology at School of Biological Sciences and Biotechnology, Goa University under the Supervision of **Ms. Gandhita V. Kundaikar** and the same has not been submitted elsewhere for any award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of experimental or other findings given in the dissertation.

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Seat Number: 22P0440030

Date:

Place: Goa University.

COMPLETION CERTIFICATE

This is to certify that the dissertation report entitled "**Exploring the impact of salinity tolerance on gill function and antioxidant enzyme activities on Melon barb, *Haludaria fasciata* (Jerdon, 1849): A Comprehensive study**" is a bonafide work carried out by Miss. Sania Bepari under my supervision in partial fulfilment of the requirements for the award of the degree of Masters of Science in the Discipline Zoology at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

The thesis is submitted in partial fulfilment of the requirement for the degree of Master's in Zoology and comprises research work carried out by me under the guidance of Ms. Gandhita V. Kundaikar Assistant Professor of Zoology, Goa University from 2023-2024.

Freshwater ecosystems worldwide face escalating threats from human activities and environmental changes, including salinity intrusion. This intrusion, driven by factors like climate change, rising sea levels, reduced rainfall, and changes in water flow patterns, poses a serious risk to the delicate balance of aquatic ecosystems. Among the inhabitants most affected are indigenous ornamental fish species like *Haludaria fasciata*, whose survival hinges on the stability of their freshwater environments. This study emerged from a deep concern for the well-being of these freshwater species in the face of growing environmental challenges. We realized the urgent need to understand how changes in salinity levels affect the health and physiology of *Haludaria fasciata*, a species crucial to the health of freshwater ecosystems. Our study takes a comprehensive approach to expand our knowledge of the impacts of salinity intrusion. We aim to not only evaluate the salt tolerance of *Haludaria fasciata* but also analyze its physiological, biochemical, and histological responses to varying salinity concentrations. By revealing the complex relationship between salt stress and fish well-being, we hope to identify ways to protect these valuable freshwater species and their habitats through potential mitigation strategies and conservation efforts.

The journey began with the survival rate measurement in fish in response to different salinity levels, which lays foundation to our investigation into the effects of salinity stress on *Haludaria fasciata*. Through careful research and dedication, we aim to discover insights that can guide conservation efforts and ensure the future health of freshwater ecosystems and their inhabitants.

The thesis comprises four main chapters. The first Chapter introduces the study, covering its background, goals, objectives, hypotheses, and scope. The second chapter delves into the literature review. In the third chapter, a comprehensive methodology for conducting various estimations is outlined. Finally, the fourth chapter presents the analysis and conclusions, encompassing results related to the survival rate, physiology, and microanatomy of *Haludaria fasciata*, along with discussions and conclusions.

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TABLES

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ABBREVIATIONS USED

Entity	Abbreviation
Alkaline Phosphatase	ALP
Acid Phosphatase	ACP
Alanine aminotransferase	ALT
Thiobarbituric acid reactive substances assay	TBARS
Catalase	CAT
Superoxide dismutase	SOD
Reduced Glutathione	GSH
Malondialdehyde	MDA
Secondary Lamellar Fusion	SLF
Disintegration of primary lamellae	DPL
Extracellular cartilaginous matrix	ECM
Chondrocytes	CH
Cartilago	CR
Lacuna, capillary lumen	L
Epithelial lifting	EL
Interlamellar fusion	ILF
Shortening and thickening of secondary lamellae	ST
Lymphocytic infiltration	LI
Interlamellar space	ILS
Aneurysm	A
Degeneration of erythrocytes in stromal region of primary lamellae	DE
Epithelial hyperplasia	H
Curling of the top of secondary lamellae	Cr
Blood congestion	BC
Venous sinus	VS
Detachment of secondary lamellae	DSL
Diffuse epithelial hyperplasia	DEH
Proliferation of lymphocyte	PL
Mucous cell Proliferation	MCP
Chloride cells	C
Pavement cells	PVCs
Pillar cells	P
Mucus cells	M
Primary lamellae	Pl
Secondary lamellae	SL
Cubbing of secondary lamellae	Cu
Lamellar fusion	LF

ABSTRACT

Salinity intrusion, attributed to factors such as sea level rise, decreased precipitation, reduced runoff, and increased temperature, poses a significant threat to freshwater ecosystems, particularly impacting indigenous ornamental fish species inhabiting rivers, streams, and lakes in India. Among these species, *Haludaria fasciata*, commonly known as melon barb and belonging to the Family Cyprinidae, is prevalent in the Indian trade and endemic to the rivers of the southwestern ghat region of India. This study aimed to investigate the salinity tolerance of *Haludaria fasciata* and its effects on gill function and antioxidant enzyme activities.

The experimental protocol involved a 21-day acclimatization period of the fish in laboratory conditions, Subsequently, a 45-day survival rate experiment was conducted, exposing the fish to varying salinity levels while monitoring water quality parameters and mortality daily. Following this, a 21-day experimental setup exposed the fish to salinities ranging from 2 to 6 ppt, along with a control group. On days 7, 14, and 21, fish from each salinity group were euthanized using the ice chilling method for biochemical, antioxidant enzyme activity, and histological evaluations.

Results indicated a decrease in survival rate with increasing salinity levels. Biochemical tests revealed a decrease in protein and carbohydrate levels with increasing salinity, while significant increases in enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and non-enzymatic antioxidant thiobarbituric acid reactive substances (TBARS), as well as liver enzyme alanine transaminase (ALT), were observed within the experimental group across

treatment days, indicating oxidative stress. Enzymatic antioxidants including reduced glutathione (GSH), alkaline phosphatase (ALP), and acid phosphatase (ACP) showed insignificant variabilities within experimental groups across days. Ion transporting enzymes demonstrated an insignificant increase in magnesium adenosine triphosphatase (Mg-ATPase) and significant increase in sodium/potassium adenosine triphosphatase (Na/K+ ATPase) within the experimental group across treatment days, suggesting increased energy requirements for maintaining osmoregulation and ion balance.

Histological evaluation of gill tissue revealed a dose-dependent response to salinity, characterized by hyperplasia, aneurysm, detachment of secondary epithelium, rupture of pillar cells, hyperplasia of mucous cells, hypertrophy of chloride cells, and severe disintegration of secondary lamellae with increasing salinity levels. The study reveals that *Haludaria fasciata* can withstand salt levels up to 6 ppt despite reduced survival rates, showing heightened antioxidant activity and significant gill tissue changes. Optimal survival occurs at 2 ppt, highlighting the resilience of the species. Hence, urgent monitoring and action are imperative to safeguard freshwater ecosystems from the detrimental effects of salt intrusion.

Keywords: Salinity intrusion*,* *Haludaria fasciata*, salt tolerance, gill function, antioxidant enzyme activity, survival rate, indigenous freshwater fish species, ion transporting enzymes.

CHAPTER 1: INTRODUCTION

1.1 Background

Ornamental fishes, characterized by their appealing colours and docile behavior, are well-suited for being kept as pets in enclosed environments like aquariums or garden ponds, providing an avenue for the enjoyment of their aesthetic appeal (Mukherjee et al., 2000). Ornamental fish keeping is turning into an easy and stress relieving hobby (Ghosh et al. 2003). They are referred to as “living jewels” because of their colour, shape and behaviour, aquarium keeping is the second largest hobby, next to photography (Das et al. 2005).

Tlusty et al. (2008) suggest that properly managing the habitats and populations of native ornamental fish could offer economic benefits to local communities. However, differing perspectives, such as those outlined by Moreau and Coomes (2007) and Rowley et al. (2008), argue that unregulated harvesting from natural habitats may compromise the sustainability of the trade, leading to declines in important species populations.

India exports mostly wild-caught ornamental fish, with approximately 85% sourced from rivers in the North-East and South-East regions (Gupta et al., 2019). The North-East alone has 155 ornamental species out of 195 recorded species. The Western Ghats, recognized as a biodiversity hotspot, harbors 40 ornamental freshwater fish species, with 37 being endemic (Gupta et al., 2019). While 90% of India's ornamental fish trade involves freshwater species, only 2% are wild-caught, with the rest cultivated. Marine ornamental fish account for 10% of the trade, with 98% captured and 2% cultivated (Gupta et al., 2019).

Most ornamental fish breeders in India focus on exotic species, neglecting indigenous, marine, and brackish water varieties. Maintaining optimal water quality is crucial for the health and well-being of aquatic organisms, which ultimately determines the success of ornamental fish cultivation (Lipton, 2006).

Water quality is vital for aquatic environments, directly affecting the well-being of fish and other organisms. Studies emphasize the importance of physico-chemical and biological factors, as pollutants can disrupt optimal conditions. Organisms thrive within specific ranges, and deviations can impair physiological functions (Davenport, 1993; Kiran, 2010). Good water quality is essential for fish survival and growth, influenced by factors like temperature, clarity, pH, and nutrient levels (Davenport, 1993; Kiran, 2010).

Salinity, the concentration of dissolved ionized salts in water, profoundly affects the density, behavior, and development of aquatic organisms, especially fish (Jamabo, 2008). It influences various thermodynamic aspects of water, such as density, heat capacity, and solvation capacity, shaping habitat characteristics for aquatic life. Salinity also modulates biochemical processes both intra- and extracellularly, predominantly through dissolved inorganic ions like sodium chloride (NaCl) (Jamabo, 2008).

Rising salinity levels in freshwater ecosystems pose a threat to indigenous ornamental fish populations, driven by human activities such as irrigation and mining (Feistel et al., 2015). This salinization, accompanied by pollutants, presents a complex challenge with unknown

toxicological effects on fish species (Feistel et al., 2015). It's a significant global water quality issue, marking the Anthropocene era (Stets et al., 2018).

Salinity stress disrupts physiological balance, leading to increased reactive oxygen species generation and gill microarchitecture modifications in fish species like *Dicentrarchus labrax*, *Thymallus arcticus*, and *Oncorhynchus mykiss.* Human-induced salinization threatens indigenous freshwater ornamental fish populations, creating a complex mixture of pollutants and marking a significant global water quality challenge in the Anthropocene era (Feistel et al., 2015).

Barbs comprise a group of cyprinid fish characterized by small one or two pairs of barbels surrounding their mouth. Globally, approximately 400 species of barbs exist, out of which only 150 species possess remarkable ornamental value. *Haludaria fasciata*, commonly known as the Melon Barb, stands out as one of the prominent species traded in India, alongside other popular barbs such as *Puntigrus tetrazona* (Tiger Barb), *Pethia conchonius* (Rosy Barb), *Puntius titteye* (Cherry Barb), *Barbodes everetti* (Clown Barb), *Balantiocheilos melanopterus* (Silver Shark), *Desmopuntius johorensis* (Striped Barb). *Haludaria fasciata*, (formerly known as *Puntius fasciatus*), commonly referred to as the Melon Barb, is assessed as Least Concern on The IUCN Red List of Threatened Species (Abraham, 2015) is an indigenous freshwater fish species found in rivers throughout the Western Ghats region of South India, inhabit various water bodies across Tamil Nadu, Kerala, Karnataka, Goa, and Andhra Pradesh. Their natural habitats are ecologically diverse and comprise wetlands, rivers both permanent and seasonal, canals, lakes, ditches, and ponds, as well as hill streams and foothill areas. They thrive in a tropical climate with specific water parameters: a pH range of 5.5-7.5, General soft to moderate

water hardness ranging from 50-150 ppm, and a temperature between 22-26°C and prefer soft, acidic, moderately fast-flowing waters teeming with aquatic life. They exhibit an egg-scattering reproductive strategy, preferring open water and substrate for spawning. Adults display no parental care towards the eggs. Typically, melon barbs reach a maximum length of 6 centimeters.

The increasing effects of global warming, such as sea level rise and changes in precipitation patterns, have led to salinity intrusion in estuarine rivers, posing a significant threat to indigenous freshwater fish species like *Haludaria fasciata*, commonly known as the Melon Barb. Salinity intrusion disrupts osmoregulation and behaviors of freshwater fish, ultimately leading to population declines and threatening their survival and reproduction. Particularly in coastal areas like Goa, freshwater sources are vulnerable to contamination by saltwater intrusion from the Arabian Sea, exacerbating salinity levels in freshwater bodies.

Freshwater ornamental fish species, adapted to specific freshwater conditions, are highly sensitive to salinity fluctuations. Salinity intrusion not only affects fish species but also disrupts the balance of aquatic flora and fauna, leading to biodiversity loss and imbalances in the freshwater ecosystem's food chain. Moreover, it degrades water quality, decreasing oxygen levels and increasing pollution, further harming freshwater fish populations.

Given these challenges, conducting scientific research on indigenous freshwater ornamental fish, particularly focusing on their salinity tolerance, is crucial for their sustainable management and conservation. Such research provides a foundation for developing targeted interventions to protect the cultural, ecological, and economic value of freshwater ornamental fish populations amidst escalating environmental threats.

The present study aims to investigate the impact of salinity tolerance on the indigenous freshwater ornamental fish *Haludaria fasciata*, assessing parameters like antioxidant enzyme activities and histological characteristics. By addressing this research gap, we can gain insights into the adaptation mechanisms of freshwater fish to salinity stress and inform conservation efforts to mitigate the impacts of salinity intrusion on freshwater ecosystems.

1.2 Scope of the study

The research investigates how salinity tolerance affects gill function and antioxidant enzyme activities in *Haludaria fasciata* (Melon Barb), a freshwater ornamental fish. It aims to determine the species response to varying salinity levels and identify thresholds causing gill histological alterations. By focusing on *Haludaria fasciata's* responses to salinity stress, the study contributes to the knowledge of aquatic biology and environmental physiology. It also sheds light on the species' ecological adaptability, conservation significance, aquaculture optimization, physiological stress responses, and potential biomedical implications. The research informs conservation strategies, aquaculture practices, and welfare standards for both captive and wild populations while providing insights into oxidative stress mechanisms relevant to biomedical sciences.

1.3 Objectives of the study

The freshwater indigenous ornamental species *Haludaria fasciata* lacks information on its tolerance to salinity. Despite extensive studies on various factors like temperature effects and dietary effects, there's a notable gap in understanding its salinity thresholds and how it affects

antioxidant defenses and histological characteristics. Considering the above scenario present study was undertaken to achieve the following objectives:

1. To assess the tolerance of *Haludaria fasciata* exposed to varying salinity levels.

2. To evaluate the impact of salinity fluctuations on the biochemical and antioxidant enzyme activities in *Haludaria fasciata*.

3. To investigate the salinity thresholds causing histological alterations in the gills of *Haludaria fasciata*.

1.4 Hypothesis of the research work

The study hypothesizes that elevated salinity levels within the aquatic habitats could potentially elicit a dose-dependent impact on gill structure, physiological function and antioxidant enzyme activities of *Haludaria fasciata.*

CHAPTER 2: LITERATURE REVIEW

Leite et al. (2022) conducted a study examining the sub-lethal effects of salinization on the behaviour of the Iberian barbel (*Luciobarbus bocagei*), a widespread native cyprinid species. They observed significant alterations in swimming activity and shoaling behaviour as salinity levels increased in the flume channels. Specifically, there was a marked reduction in swimming activity and shoal cohesion, accompanied by an increase in boldness, characterized by a higher frequency of attempts to escape the altered environment. Thus, suggesting that salinization can induce behavioural shifts in freshwater fish, potentially reflecting changes in their ecological context.

Ain et al. (2021) examined how salinity affected the development of hybrid Malaysian mahseer fry (*Tor tambroides* ♂ × *Barbonymus gonionotus* ♀) by subjecting the fish to varying salinity concentrations. The water salinity of 3 ppt showed the highest growth and survival rates, while 0 ppt showed the lowest growth and survival rates. Significant histological changes were observed in conjunction with the largest body gains and lengths at 9 ppt water salinity with water salinity of 3 ppt being the ideal condition for the fries of Malaysian mahseer hybrids.

The effects of four distinct salinities (SW, 50% SW, 100% SW, and 150 percent SW) on the growth of *Carassius auratus* and *Carassius carassius* were investigated by Küçük (2013). The SW and 50% SW had greater specific growth rates, while the 150 percent SW had the lowest. In summary, both species can be introduced to brackish water with a salinity of no more than 8 parts per thousand. Crucian carp and goldfish had maximum salinity tolerances of 20 ppt.

Sharma et al. (2020) studied the effect of salinity on growth, survival and biochemical alterations in the freshwater fish *Labeo rohita* (Hamilton 1822) and found that increased salinity exposure decreased the growth and survival rates and tissue ascorbic acid values in *Labeo rohita*, with maximum mortality and oxygen consumption occurring at 4.5 ppt and 2.5% respectively. The maximum weight gain was observed at 0%, followed by 2.5%, 3.5%.

Murmu et al. (2020) examined the salinity tolerance of genetically improved rohu (*Labeo rohita)*, with a finding that Jayanti rohu fingerlings could survive up to 8 ppt salinity. Survival declined beyond this level, with hematological changes observed above 6 ppt. Histological analysis revealed mild gill lesions at 8 ppt. Their findings suggest potential for cultivating Jayanti rohu in low-salinity environments, offering insights for aquaculture practices and further research.

Oguz (2023) in their study investigated histological and immunohistochemical changes in *sailfin velifera* gills exposed to different salinities (20, 30, 40, and 50 ppt) in lab conditions. Analysis revealed increased cell numbers, particularly hyperplasia in primary lamellae with higher salinity. Groups at 20 and 30 ppt showed rapid increases in mucus cells (MCs) and heat shock protein (HSP70+) cell numbers. Proliferating cell nuclear antigen (PCNA+) cells were present in all salinity groups. Additionally, there was an increase in mitochondria-rich cells (MRC) with rising salinity, suggesting the role of gill cell's in regulating ion and salt balance in *sailfin velifera* under varying salinity.

The study carried out by Nofal et al. (2019) investigated the effects of increasing salinity levels on the structural integrity of *Coptodon zillii,* a highly salt-tolerant tilapia species, focusing on Qaroun Lake in Egypt. Utilizing histological, histochemical, immunohistochemical, and genetic analyses, severe histological alterations in gill and liver tissues were observed, including degeneration, necrosis, hemorrhage, and changes in carbohydrate distribution and collagen content. Elevated caspase-3 expression indicated cellular stress, while genetic analysis revealed the prolactin gene's sensitivity to salinity fluctuations. These findings underscore the significant impact of salinity rise on both fish production and internal tissue morphology in *C. zillii.*

In a study conducted by Moghadam et al. (2013), gill histopathological changes were investigated in Golden Grey Mullet (*Liza aurata*) fry exposed to acute fluctuations in salinity levels, employing light and scanning electron microscopy techniques. Fry acclimated to 12 ppt salinity were exposed to salinity levels ranging from 40 to 70 ppt. Mortality rates peaked between 45 to 60 ppt, reaching 100% beyond 60 ppt. Histopathological examination revealed edema, breakdown of pillar cells, hypertrophy of epithelial and chloride cells, and hyperplasia. Scanning electron microscopy identified two chloride cell types, with Type II exhibiting an increase at higher salinities. LC50 values ranged from 49.91 to 50.11 ppt. The findings highlight the gill as the primary site of salinity-induced effects, suggesting that alterations in chloride cells and gill structure contribute to mortality in Golden Grey Mullet fry under acute salinity stress.

Study conducted by Romano (2017), examined the impact of varying salinity levels (0, 3, 6, and 9 ppt) on the survival, growth, and histological features of five-week-old silver barb fry

over 17 days. Results indicated decreased growth and increased condition factor with higher salinities. Survival rates were similar up to 6 ppt but significantly dropped at 9 ppt. Histological analysis revealed depleted energy stores and reduced mucous cell count in gills at 9 ppt. The findings suggest that while silver barb fry are sensitive to high salinity due to their age, short-term exposure to 3–6 ppt can mitigate freshwater diseases during early nursery culture.

The study carried out by Ramee et al. (2020), explores environmental stressors influencing sexual differentiation in Rosy Barb and Dwarf Gourami fish. Stressors like stocking density, salinity, and temperature were applied to elevate cortisol levels in larvae, potentially inducing masculinization. While some experiments showed differences in growth, survival, and cortisol levels, only temperature manipulation had a potential influence on sex ratios, particularly in Dwarf Gouramis. Limited sexual plasticity was observed, likely due to strong genetic sex determination systems. These findings offer insights into larval stress physiology in ornamental aquaculture.

Jiang et al. (2022) carried out a 56-day study investigating the effects of freshwater (FW) and brackish water (BW) on juvenile silver carp. Results showed increased antioxidant activity in BW-exposed gills, while kidney tissue exhibited varied responses. Significant changes in Na^+/K^+ ATPase levels were observed. Histological analysis revealed structural damage in gills under BW conditions. Transcriptome analysis identified genes associated with salt tolerance mechanisms. These findings offer insights into silver carp osmoregulation and brackish water aquaculture optimization.

Ahmed et al. (2023) in their study examined how common carp respond to salinity stress at both molecular and biochemical levels. Hypersalinity exposure led to significant changes in serum markers, such as decreased protein levels and increased cortisol and sodium. Molecular analysis revealed upregulation of genes, particularly in the liver, indicating a robust response to salinity stress. Immune-related genes showed modulation in the kidney and spleen, suggesting involvement of multiple physiological systems in coping with salinity stress. These findings deepen our understanding of fish adaptation to environmental stressors, crucial for effective aquaculture management.

Chacko and Sekharan (2022) examined the impact of temperature on the embryonic development of *Haludaria fasciata*, utilizing varying incubation temperatures ranging from 10 to 35°C. It was discovered that eggs incubated at 35°C developed and hatched sooner than eggs incubated at 20°C, which exhibited the slowest development and late hatching. A higher hatching and survival rate was observed in eggs incubated at 26°C.

Chacko and Sekharan (2024) investigated how six distinct diets affected the *Haludaria fasciata's* larval development and juvenile output. Live *Artemia nauplii*, LAP, and decapsulated Artemia eggs all had greater average specific growth rates. It has been noted that the larval feeding program of *H. fasciata* does not require live feeds and can be replaced by Micro diets like EL and Prince Wean without significantly affecting the larvae's ability to grow and survive.

Eagderi (2018) investigated how the early developmental stage of the sword tail (*Xiphophurus helleri*) was influenced by the isosmotic point of salinity on its body shape. The body shape of

the two treatments differed significantly between them, with the group exposed to higher salinity exhibiting a longer dorsal fin, anteriorly positioned gills, lower head and body depth, shorter snout, head, and caudal peduncle.

2.1 Lacunae

The scientific literature on the endemic freshwater ornamental species *Haludaria fasciata,* reveals a conspicuous gap in research concerning its salinity tolerance. While extensive studies have explored factors such as temperature effects on embryonic development, dietary influences, sexual dimorphism, disinfectant efficacy, and parasite dynamics, investigations into the salinity thresholds of this species and its effect on antioxidant defense mechanisms and histological characteristics remains notably lacking. Despite the extensive studies conducted on other similar parameters in exotic freshwater fishes, the investigation into these specific aspects in *H. fasciata* remain limited. Addressing this gap would provide valuable insights into *Haludaria fasciata's* ability to physiologically adapt to varying environmental conditions, thereby contributing to its more effective conservation and management strategies in both wild and captive environments.

CHAPTER 3: METHODOLOGY

3.1. Model Organism

Classification:

Kingdom- Animalia

Phylum- Chordata

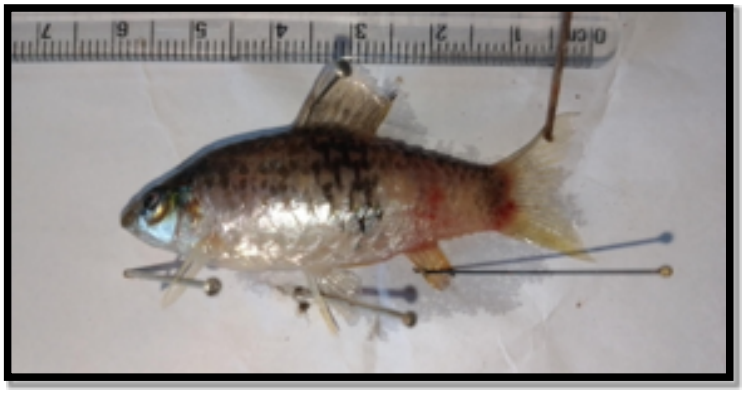
Class- Actinopterygii

Order- Cypriniformes

Family- Cyprinidae

Genus- *Haludaria*

Species- *H. fasciata*



Fish comprise the most extensive array of vertebrates, offering valuable alternatives for research compared to warm-blooded animals. This study focuses on utilizing *Haludaria fasciata* as a model organism for research purposes. The Melon barb, scientifically known as *Haludaria fasciata*, is a cyprinid fish endemic to rivers within the Western Ghats of South India, specifically found in Goa, Karnataka, Kerala, and Tamil Nadu. This species thrives in a tropical climate with specific water parameters, including a pH range of 6.0-6.5, water hardness around 50-150 ppm, and a temperature range of 22-26°C. Melon barbs are commonly sought after in the aquarium trade. They exhibit an egg-scattering reproductive strategy, preferring open water and substrate for spawning and reach a maximum length of 6 centimeters with its adults displaying no parental care towards the eggs.

Haludaria fasciata has gained recognition as a research model organism due to its physiological and ecological relevance. Its small size, short generation time, and ease of maintenance make it conducive to laboratory studies. Furthermore, its phylogenetic proximity to other fish species facilitates comparative analyses. Researchers utilize *H. fasciata* to investigate various aspects of biology, including but not limited to ecology, behavior, physiology, and genetics. Its adaptability to diverse environmental conditions renders it valuable for exploring responses to environmental stressors and evolutionary dynamics.

3.2 Fish Material and Acclimatization

In this study, 150 commercially obtained *Haludaria fasciata*, averaging 4.3 ± 0.73 cm in length and weighing 1.47 ±0.65 g, were acclimated in aquarium tanks of varying capacities (30L, 60L, and 100L) equipped with aerators. During the subsequent 25-day acclimatization period, the fish were initially fed freeze-dried tubifex worms with a minimum crude protein content of 60%, transitioning to flake feed with a crude protein content of 54%. Daily maintenance included siphoning off faeces and uneaten feed from tank bottom and a 25% water exchange to avoid deposition of nitrogenous waste.

3.3 Experimental design

Following acclimatization period, Survival rate experiment was conducted for a period of 45 days according to Murmu et al. (2020). Firstly, brine solution was prepared in one of the aquarium tanks (60L Capacity) by adding the Sodium Chloride (NaCl) to freshwater until the desired level of salinity was obtained due to its capacity to provide controlled conditions,

reproducibility and ethical considerations. The salinity level was measured using a handheld refractometer. Different concentrations of saline solutions (0, 2, 4, 6, 8, and 10 parts per thousand) were prepared by mixing brine solution with freshwater. The sum of 16 acclimatized fishes in each were subjected to salinity regimes of 0, 2, 4, 6, 8 and 10 ppt in aquarium tanks respectively (60L capacity) with 50% of water replaced weekly with the corresponding salinity solution. Uneaten food and faecal matter was siphoned off daily from the tank bottom to reduce the nitrogenous load. Every day, mortality if any was recorded for each salinity treatment group. On 45th day of experiment, the survival rate of fishes (%) was calculated. Following which 21 day experimental setup was conducted by eliminating the groups which showed 100% mortality before the desired survival period. During this period, the fish were fed with freeze-dried tubifex worms and flake feed having 54-60% protein content twice a day. Six fishes from each salinity treatment were sampled on day 7, 14 and 21. Fishes sampled were euthanized by ice chilling method, following which the liver and gills were excised from the fish, washed immediately with phosphate-buffered saline, weighed and utilized for carrying out biochemical tests and antioxidant enzyme activities. Gill tissue was fixed immediately in 10% Neutral buffered formalin for histological preparations.

3.4 Mainteinance of water quality parameters

Water quality parameters such as pH, temperature, conductivity, salinity and TDS (Table 3.1) were monitored weekly in accordance with standard methods (APHA, 1988) using PCS Testr 35 Multi-Parameter. Dissolved oxygen in water was performed using Winkler's method, according to (Welsh and Smith, 1960).

Fig. 3.1 showing 21 day acclimatization period under laboratory conditions.

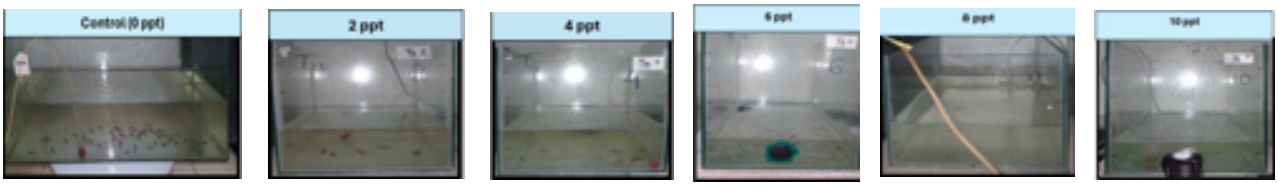


Fig 3.2 depicting experimental setup for Survival rate experiment for a period of 45 days.

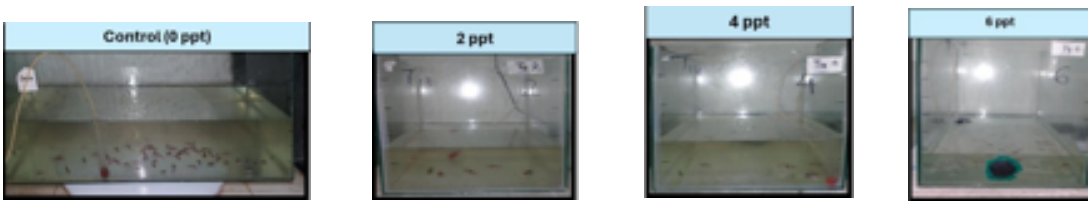


Fig 3.3 demonstration 21 day experimental set-up.

Fig 3.4 A. depicting freeze-dried tubifex worms and B. flake feed.

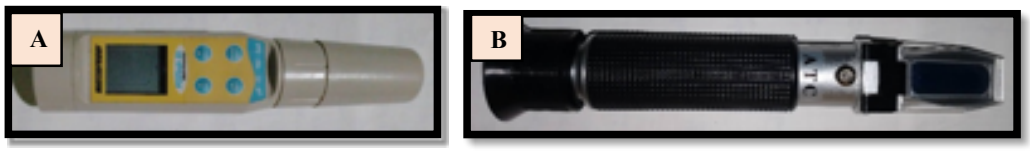


Fig 3.5 A. depicting PCS testr 35 and B. hand held refractometer used for maintaining water quality parameters and salinity in ppt respectively.

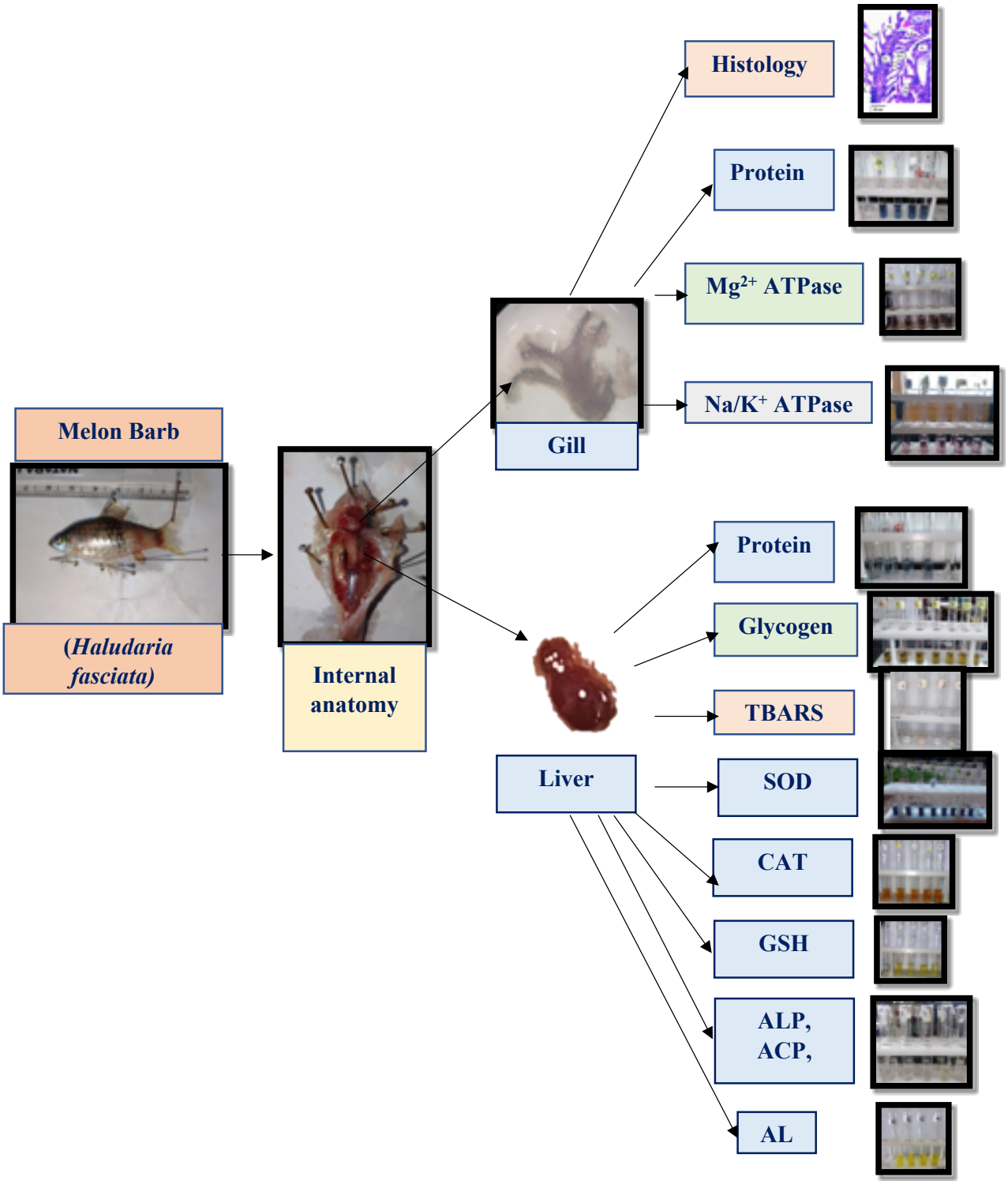


Fig 3.6 Brief overview of Methodologies carried out.

3.5 Biochemical estimations

3. 5.A Protein estimation (Lowry et al. 1951)

The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue coloured complex. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdotungstate components of the Folin-Cio-Calteau reagent to give bluish products which contribute towards enhancing the sensitivity of this method.

Reagents:

- **Lowry's reagent :**

To 50 ml of 2% sodium carbonate prepared in 0.1 N sodium hydroxide add 1ml of 0.5% copper sulphate prepared freshly in 1% potassium sodium tartarate.

Extraction:

Liver tissue was homogenised in 1ml of Potassium Phosphate Buffer pH 7.4. The solution was centrifuged and the supernatant was utilised as the sample source.

Procedure:

0.2 ml of the tissue extract for protein was diluted up to 1 ml with distilled water. To this, 5ml of Lowry's reagent was added and incubated for 10 minutes at room temperature. 0.5 ml of Folin Cio-Calteau (1:1 N NaOH) reagent was then added and the mixture was further incubated in dark for a period of 30 minutes at room temperature. The intensity of the blue coloured complex was measured against a suitable blank at 660nm (Lowry et al. 1951). Quantification of the protein content of the sample was done with the help of a standard curve of Bovine Serum Albumin (200ug/ml).

3.5.B. Carbohydrate Estimation (Carroll, 1956)

Carbohydrates are dehydrated by conc. H_2SO_4 to form furfural. Active form of the reagent is anthranol, the enol tautomer of anthrone, which reacts by condensing with the carbohydrate furfural derivative to give a green colour in dilute and a blue colour in concentrated solutions, which is determined colorimetrically. The blue-green solution shows absorption maximum at 620 nm.

Reagents:

- **Anthrone reagent:**

0.2g of Anthrone was dissolved in 100 ml of concentrated sulphuric acid.

Extraction:

Tissue samples are initially homogenized in a 5M KOH solution and then subjected to boiling in a water bath for 30 minutes. After cooling, 10% PCA is added to the mixture, followed by centrifugation at 3000 rpm for 10 minutes to separate components. The resulting precipitate is dissolved in distilled water. Subsequently, a mixture of 30% ice-cold ethanol and diethyl ether in a 1:1 ratio is added, and the solution is centrifuged again at 3000 rpm for 10 minutes to induce further precipitation. Finally, the precipitate formed is dissolved in distilled water for subsequent analysis or processing.

Procedure:

To this 0.1 ml of deproteinized aliquot, 5ml of anthrone reagent was added and incubated for 10 minutes in a boiling water bath. The intensity of the colour developed was measured at 620 nm against a suitable blank (Carroll, 1956). Quantification of the total carbohydrate content was done with the help of standard curve of total carbohydrate (100ug glycogen /ml).

3.6 Antioxidant enzyme activities

3.6.A. Catalase (CAT) (Aebi 1974)

The reaction of catalase is the decomposition of hydrogen peroxide into water and molecular oxygen.

The remaining H_2O_2 in the samples, which was not decomposed by the enzyme reacts with dichromate to give a blue precipitate of perchromic acid. This unstable precipitate is then decomposed by heating to give a green coloured stable compound. The intensity of the green colour can be measured at 620 nm.

Reagent:

- **Dichromate acetic acid reagent :**

5% Potassium dichromate and glacial acetic acid were taken in a 1:3 ratio to prepare this reagent.

Extraction:

Liver tissue was homogenized using 0.0M Potassium Phosphate Buffer, pH 7.0 and centrifuged. The supernatant was utilized as a sample source and the precipitate was discarded.

Procedure:

To 0.9 mL of phosphate buffer (0.01 M, pH-7.0) 0.1 mL of tissue homogenate and 0.5ml of H_2O_2 (0.2 M) were added. After 60 sec, 2ml of dichromate acetic acid reagent (5%) was added. The tubes were kept in a boiling water bath for 10 min and the colour developed was read at

600 nm. Standard H_2O_2 in the range of 0.2-1.0 ml was taken with blank containing reagent alone. CAT activity was measured proportionately to the rate of H_2O_2 reduction.

Dichromate in acetic acid was converted to perchromic acid then chromic acetate, when heated in the presence of H_2O_2 Chromic acetate formed was measured at 620 nm. Absorbance values were compared with a standard curve generated from a known catalase and the activities were expressed as mol/hr/mg protein. The amount of protein /mg of tissue was determined by the standard method (Lowry et al. 1951).

3.6.B Superoxide dismutase (SOD) (Beauchamp and Fridovich, 1971)

Superoxide dismutase catalyzes the dismutation of the superoxide (0_2^-) into hydrogen peroxide and molecular oxygen.

$$2O_2^{\circ-} + 2H^+ \xrightarrow{\textbf{Superoxide dismutase}} H_2O_2 + O_2$$

Illumination of riboflavin in the presence of oxygen and electron donors like methionine or EDTA generates a flux of superoxide anions and produces a blue formazan compound, the intensity of which can be measured at 560 nm.

Reagents:

- **SOD Substrate**

To 25 ml of 0.2M potassium phosphate buffer (ph7.0), 14mg of methionine, 4.93 ml of nitroblue tetrazolium chloride (1mg/ml in 50mm potassium phosphate buffer, ph;7.0) and 0.63 ml of riboflavin (1mg/ml in 0.05M potassium phosphate buffer) were added and the volume was made up to 100ml with double distilled water.

Extraction:

Tissue was homogenized using 0.01M of ice cold Potassium Phosphate Buffer, pH 7 and it was brought down to 2.5% with 0.05 M Potassium Phosphate Buffer, pH 7.0 and then centrifuged. The clear supernatant was used as a sample source and the residue was discarded.

Procedure:

To 2.9 ml of freshly prepared SOD substrate, 0.1 ml liver tissue homogenate enzyme blank was prepared by adding 0.1ml of 0.05M potassium phosphate buffer instead of sample. Illumination was carried out in the aluminium foil lined box fitted with 15 V florescent lamp for exactly 10 minutes. The reaction was stopped by switching off the source of illumination. The intensity of the colour was read at 560 nm (Beauchamp and Fridovich, 1971). Enzyme activity was quantified with the help of a standard curve of SOD (400ug/ml). Protein content in the enzyme was estimated as described as earlier. SOD activity was expressed as IU/mg protein. One unit of the enzyme can be defined as the amount of SOD required to inhibit 50% of NBT reduction at standard condition.

Reduced Glutathione (GSH) (Moron et. al., 1979)

GSH reacts with 5,5'-dithiobis,2-nitrobenzoic acid to produce a yellow-coloured compound. The intensity of the colour can be measured spectrophotometrically at 412 nm.

Reagents

- **5,5'-dithiobis 2- nitrobenzoic acid (DTNB) reagent**

19.8 mg of 5,5'-dithiobis 2-nitobenzoic acid was dissolved in 100 ml of 0.1% Sodium nitrate to prepare this reagent.

Extraction:

Tissue was homogenized in 1ml 5% TCA and cold centrifuged at 500 xg for 5 minutes to obtain the supernatant which serves as the sample source and the precipitate was discarded.

Procedure:

To 1.0 ml of diluted tissue extract or serum, 2 ml of 5,5'-dithiobis , 2-nitrobenzoic acid (DTNB) reagent was added to make the final volume 3.0 ml. (Moron et. al., 1979). Absorbance was read at 412 nm against a suitable blank. Reduced Glutathione content of the samples was quantified with the help of a standard curve of Reduced glutathione (0.2umol/ml in 5% TCA).

3.7. Non enzymatic antioxidant

Thiobarbituric acid reactive substances (TBARS) (Niehaus and Samuelsson, 1968)

Malondialdehyde forms 1:2 adduct with thiobarbituric acid to produce a coloured complex. The intensity of the colour can be measured spectrophotometrically at 535 nm. Other lipoproteins are precipitated out by trichloroacetic acid and avoided from interfering in the reaction. Only water-soluble malondialdehyde reacts with thiobarbituric acid and produces a coloured complex.

Reagents:

- **TBA-TCA-HCL Reagent**: 0.37% Thiobarbituric acid, 15% Trichloroacetic acid and 0.25 N Hydrochloric acid were mixed in 1:1:1 ratio to prepare this reagent.

Extraction:

The liver tissue was homogenized by using Tris HCl buffer, pH 7 to prepare a tissue homogenate. The supernatant served the purpose of sample source and the residue was discarded.

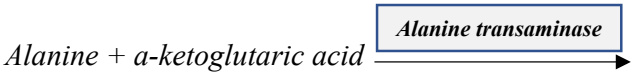
Procedure:

0.1 ml of tissue homogenate was treated with 2 ml of TBA-TCA-HCL reagent and placed in boiling water bath for 15 minutes, cooled and centrifuged at room temperature for 10 min at 500 xg. The absorbance of clear supernatant was measured against a suitable blank at 535 nm (Niehaus and Samuelsson, 1968). Thiobarbituric acid reactive substance concentration was estimated with the help of a standard curve of malondialdehyde (10 nmole/ml).

3.8 Liver enzymes

3.8.A. Alanine aminotransferase (ALT) (Reitman and Frankel, 1957)

a-ketoglutaric acid reacts with alanine and undergoes a transamination reaction. Alanine donates it's amino group to a-ketoglutaric acid. As a result, alanine becomes pyruvate and a-ketoglutaric acid becomes glutamic acid. Pyruvate reduces 2,4-dinitrophenyl hydrazine to dinitrophenyl hydrazone. In an alkaline medium, hydrazone produces a coloured complex. The intensity of the colour can be measured at 540 nm.



Reagents:

- **ALT Substrate:** 0.532g alanine and 6.0mg a-ketoglutaric acid were added to 0.1 ml of 1N NaOH prepared in phosphate buffer (0.01M, pH 7.5). The final quantity was adjusted to 20 ml with phosphate buffer (pH 7.5).

Extraction:

Liver tissue was homogenised using 0.0M Potassium Phosphate Buffer, pH 7.0 and centrifuged. The supernatant was utilised as a sample source and the precipitate was discarded.

Procedure:

0.5 ml of ALT substrate prepared in phosphate buffer (0.01 M, pH-7) was incubated at 37ºc for 5 minutes. To this 0.1 ml of liver tissue homogenate was added to assay the enzyme activity. A suitable enzyme blank was prepared by taking 0.5 ml of the substrate and 0.1 ml of distilled water. They were incubated further for another 15 min at 37ºc. The reaction was stopped by the addition of 0.5 ml dinitrophenyl hydrazine reagent (DNPH). They were mixed thoroughly and kept at room temperature for about 15 min. 5 ml of 0.4 N NaOH was added to develop the colour, intensity of which was read against the enzyme blank at 540 nm (Reitman and Frankel, 1957). The product formed (pyruvate) during this reaction was quantified with the help of a standard curve of pyruvate (220ug/ml). The enzyme activity was expressed as ug of pyruvate formed/ min/ reaction/mg of enzyme protein. Protein content in the enzyme was estimated as described earlier.

3.8.B. Alkaline phosphatase (ALP) (King and Armstrong, 1934).

Paranitrophenyl phosphate is colourless. The enzyme splits off the phosphate group from it to form p-nitrophenol, which in the acid medium is also colourless. Under alkaline conditions, this is converted to p-nitrophenoxide ions, which exhibit yellow colour. The intensity of the yellow colour is directly proportional to the enzyme present in the specimen and can be measured at 405 nm.

Reagents:

- **ALP substrate:**

680mg of p-nitrophenyl phosphate was added to 8.0mg of $MgCl_2$ solution which was prepared by adding 30 mg of $MgCl_2$ to 10 ml glycine buffer.

Extraction:

Liver tissue was homogenized using 0.2M glycine buffer, pH 7. Clear supernatant was used as a sample source and the precipitate was discarded.

Procedure:

To 2.7 ml of glycine buffer, 0.2 ml of ALP substrate (freshly prepared) was added and incubated at 37°c for 5 minutes. To this 0.1 ml of liver tissue homogenate was added to assay the enzyme activity. Simultaneously an enzyme blank was prepared by mixing 2.7 ml of buffer, 0.2 ml substrate and 0.1 ml of distilled water. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was stopped by adding 5 ml of 0.25 N NaOH. The intensity of the products of this reaction was (p-nitro phenol) was measured at 405 nm (King and Armstrong, 1934). The amount of p-nitrophenol released by the action of Alkaline phosphatase was quantified by p-nitro phenol standard curve (250ug/ml). Protein content in the enzyme was estimated as described earlier. The enzyme activity was expressed as (IU/mg protein). Unit can be defined as the quantity of Alkaline phosphatase that liberates 1 mg of phosphate ion from glycerol-2-phosphate in one hour under standard conditions.

3.8.C Acid Phosphatase (Leinhardt and Walter, 1965)

The enzyme acid phosphatase catalyses the conversion of the substrate, p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate (Pi). The enzyme activity is measured by recording the absorbance due to the formation of p-nitrophenol, spectrophotometrically at 410 nm. The concentration of p-nitrophenol is calculated from the standard curve.

- **Substrate buffer solution:**

Dissolve 165mg P-nitro phenyl phosphate in 100ml citrate buffer (0.1M, PH 4.8) prepared by adding 410mg citric acid and 1.125g sodium citrate in distilled water.

Procedure:

1.0 ml of substrate buffer (citrate) was added to each of the tube and the tubes were equilibrated at 37°C for 10 min. To the sample tube 0.2 ml of the respective homogenate was added. All the tubes were incubated at 37.5°C for 30 min. The activity was arrested by addition of 4.0 ml 0.1N NaOH in all the tubes. After arresting the enzyme activity, 0.2 ml of homogenate was added to blank tube, if necessary the tubes are centrifuged. p-nitrophenyl liberated in the assay was measured as optical density at 405 nm with spectronic- 20 adjusting blank at zero.

Unit of enzyme activity was defined as the amount of p-nitrophenyl phosphate required to release l M of p-nitrophenol per minute under standard assay conditions.

3.9 Estimation of ion-transporting enzymes (ATPases):

The activities of ATPases are directly linked with the hydrolysis of ATP. The released inorganic phosphate, upon hydrolysis of ATP, is measured with ammonium molybdate reagent to form phosphomolybdate acid which was reduced by ascorbic acid to give blue colour.

Reagents:

- **Ammonium Molybdate Reagent:**

0.42% of Ammonium Molybdate solution prepared in 1N Sulphuric acid and 10% aqueous Ascorbic acid solution were taken in the ratio of 6:1 to prepare this reagent.

Reagent A: Copper acetate buffer (pH-4)

Dissolve 1.25g of Copper sulphate and 23g of Sodium acetate in 500ml of 2mol/L acetic acid.

Reagent B: 5% Ammonium molybdate solution

Reagent C: 2% Metol prepared in 5% NaCl

Reagent mixture: Dissolve 2.364g Tris HCl, 0.014g $MgCl_2$, 0.0074g of KCl and 0.292g of NaCl in 500 ml of distilled water.

Extraction:

The tissue was homogenized in 1ml of 0.32M sucrose solution. The homogenate was cold centrifuged at 5000 rpm for 15 minutes. The residue was discarded and the supernatant was used as a sample source for the estimation of Na^+/K^+ ATPases, Mg^{2+} ATPases.

3.9.A. Protocol for estimation of Na^+/K^+ ATPases:

0.1 ml of the homogenate was taken and to this 0.2 ml of reagent mixture was added and incubated for 10 minutes at 100℃ in a boiling water bath. After cooling the contents of the test tubes 0.2ml of 0.5mM ATP was added and incubated at 37℃ for 10 minutes. To this 0.2 ml 10% TCA was added and centrifuged at 3000 RPM for 5 minutes. After centrifugation, to the above reaction mixture, 3 ml of Reagent A, 0.5 ml of Reagent B and 0.5 ml of Reagent C- was added and incubated at room temperature for 7 minutes. The blue colour developed was measured against a suitable blank at 820 nm.

3.9.B Protocol for estimation of Mg^{2+} ATPases:

In a test tube, 0.1ml of tissue homogenate was taken and to this 0.2ml of reagent mixture, 0.1ml of 0.1mM Oubain was added and incubated at 100℃ in a boiling water bath for 10 minutes. To this 0.2ml 10% TCA was added and centrifuged at 3000 rpm for 5 minutes. After

centrifugation, to the above reaction mixture, 3 ml of Reagent A, 0.5 ml of Reagent B and 0.5 ml of Reagent C was added and incubated at room temperature for 7 minutes. The blue colour developed was measured against a suitable blank at 820 nm.

The released inorganic phosphate was estimated following the modified methods of Fiske and Subbarow, (1925). Standard phosphorus in the range 0.2-1.0 ml, was taken with blank containing reagent alone and 3.5 ml of ammonium molybdate reagent and 0.2 ml of ascorbic acid and incubate the mixture at 60℃ for 30 minutes. After 30 minutes, the intensity of the colour developed was measured at 820 nm. The quantification of released phosphate was done with the help of standard curve of phosphate (0.1mg/ml).

3.10 Histology

Histological analyses were conducted in accordance with the Histopathology guide for freshwater fish (Martins et al., 2018). Following exposure to salinity treatments, fish from each group were euthanized on days 7, 14, and 21 using the ice chilling method for 45 minutes. Subsequently, gills were excised, washed with phosphate-buffered saline, and fixed in 10% neutral buffered formalin in labelled vials for 24-48 hours before being transferred to 70% alcohol. Histological processing was of the gill samples was done at Ashwini Pathology Laboratory, Panjim, where tissue samples were dehydrated, embedded in paraffin, and sectioned into 5µm thick cross and sagittal sections. These sections were then stained with Haematoxylin and Eosin (HE) stain. The resulting histology slides were analyzed for anomalies under a compound microscope at 40x magnification.

3.11 Data analysis

The survival percentage was recorded as; (No of fishes survived after 45 days/Initial No of fishes stocked) X 100, and the scatter graph displayed in Fig 4.1 was plotted using the data obtained. The data were analyzed by using the two-way ANOVA method, with Salinity concentration and days being the two independent variables tested on a sole response variable i.e. the enzyme activity. Data were expressed as Mean ± Standard Deviation of the means. Pearson's correlation test was performed for different enzymatic tests carried out for different time periods and the corresponding r value is given in the table (Table 4.1- 4.6).

CHAPTER 4: ANALYSIS AND CONCLUSION

4.1 Results

4.1.1 Survival Rate

In this investigation, the salinity tolerance of Melon barb (*Haludaria fasciata*) was examined by exposing them to various salinity levels (control (0), 2, 4, 6, 8, and 10 ppt) over a period of 45 days. Complete mortality occurred at 10 ppt by day 4 and at 8 ppt by day 8 (Fig 4.1). Subsequent experimentation involved exposing the fish to salinity levels of 0, 2, 4, and 6 ppt for 21 days to evaluate enzyme activities and histology. Survival rates of Melon barb (*H. fasciata*) were as follows: 100%, 100%, 95.4%, and 72.7% at control (0), 2, 4, and 6 ppt, respectively (Table 4.1).

4.1.2.A. Biochemical estimations

Liver and gill tissues were sampled at 7, 14, and 21 days for biochemical and histological Analyses. Protein estimation in liver tissue (Fig 4.2) displayed varied concentrations across groups and time points, with significant main effects of both time ($f = 11.75$, $p = 0.0011$) and salt concentration ($f = 5.135$, $p = 0.0284$). In gill tissue, (Fig. 4.3) protein concentration exhibited similar trends, with a significant main effect of time ($f = 20$, $p < 0.0001$) but no significant interaction between time and salt concentration ($f = 0.2645$, $p = 0.95$).

Carbohydrate estimation (Fig 4.4) revealed substantial variability among experimental groups over time, with the highest concentrations observed in the control group. Both time ($f = 13.98$, $p = 0.0011$) and salt concentration ($f = 61.76$, $p < 0.0001$) had significant main effects on

carbohydrate content, while no significant interaction between these factors was observed ($f = 1.94$, $p = 0.1362$).

4.1.2.B. Enzyme activities

The enzyme activity profiles of Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Acid phosphatase (ACP), Reduced glutathione (GSH), Superoxide dismutase (SOD), Catalase (CAT), Malondialdehyde (MDA), Magnesium ATPase (Mg^{2+} ATPase), and sodium/potassium ATPase (NA/K^{+} ATPase) exhibit distinct patterns across varied salinity levels and exposure durations in Melon barb (*Haludaria fasciata*).

ALT activity demonstrated a consistent elevation in group C (6 ppt) throughout the study (Fig 4.8), with significant influences from both time ($f = 33.3$, $p = 0.0002$) and salinity concentration ($f = 41$, $p < 0.0001$), as well as their interaction ($f = 6.8$, $p = 0.0010$).

ALP activity displayed slight fluctuations, yet the control group showed notable peaks on days 7 and 21 (Fig 4.7). While salt concentration ($f = 6$, $p = 0.0188$) and the interaction ($f = 6.5$, $p = 0.0013$) significantly impacted ALP activity, the effect of time was insignificant ($f = 0.07$, $p = 0.93$).

ACP activity peaked in group C (6 ppt) on days 7 and 21 (Fig 4.6), with a dose-dependent rise by day 21. Time significantly influenced ACP activity ($f = 6$, $p = 0.0368$), while the main effect of concentration was not significant ($f = 2.5$, $p = 0.13$).

Reduced glutathione (GSH) levels varied across groups, with distinct trends observed over different exposure periods (Fig 4.9). Significant impacts from salinity concentration ($f = 11.7$, $p = 0.002$) and its interaction with time ($f = 9.1$, $p = 0.0002$) were evident, whereas the effect of time alone was non-significant ($f = 0.72$, $p = 0.49$).

SOD activity showed a general increase, notably in group C (6 ppt) on days 7 and 21 (Fig 4.10). Both time ($f = 133.8$, $p < 0.0001$) and salinity concentration ($f = 31$, $p < 0.0001$), along with their interaction ($f = 22$, $p < 0.0001$), significantly influenced SOD activity.

Catalase levels rose progressively from control to group C (6 ppt) by days 7 and 21 (Fig 4.11), with minor fluctuations on day 14. Time ($f = 6.4$, $p = 0.0176$), concentration ($f = 31.4$, $p < 0.0001$), and their interaction ($f = 8.1$, $p = 0.0004$) significantly impacted catalase activity.

MDA concentrations were notably higher in group C (6 ppt) across all days (Fig 4.5), influenced significantly by both time ($f = 36.8$, $p = 0.0001$) and salinity concentration ($f = 48.2$, $p < 0.0001$), along with their interaction ($f = 22.6$, $p < 0.0001$).

4.1.2.C. Ion transporting enzymes

Mg^{2+} ATPase activity exhibited fluctuations over time (Fig 4.12), with a significant interaction between time and salinity concentration ($f = 3.6$, $p = 0.0202$), while Na/K^{+} ATPase activity (Fig 4.13) showed an upward trend on days 7 and 14, with significant effects from time ($f =$

27.1, $p < 0.0001$) and salinity concentration ($f = 16.3$, $p = 0.0009$), along with their interaction ($f = 6.5$, $p = 0.0013$).

These results underscore the intricate responses of Melon barb (*Haludaria fasciata*) to varying salinity levels over time, emphasizing the necessity of considering both factors in comprehending enzyme dynamics in fish under environmental stress.

Correlation analysis

According to Pearson correlation analysis, in liver tissue on day 7 (Table 4.2), Superoxide dismutase (SOD) exhibited a significant negative correlation with Alkaline phosphatase (ALP) ($p = 0.003$, $r = -0.776$) and glycogen ($p = 0.03$, $r = -0.63$). Acid phosphatase (ACP) showed a significant positive correlation with catalase ($p = 0.036$, $r = 0.61$), while SOD was positively correlated with Malondialdehyde (MDA) ($p = 0.021$, $r = 0.65$).

On day 14 (Table 4.3) alanine aminotransferase (ALT) displayed a significant positive correlation with MDA ($p = 0.055$, $r = 0.57$), and was positively correlated with reduced glutathione ($p = 0.014$, $r = 0.68$).

By day 21, ALT exhibited significant positive correlations with catalase ($p = 0.0037$, $r = 0.77$) and sod ($p = 0.0073$, $r = 0.73$), (Table.4.4) whereas a significant negative correlation was observed between SOD and ALT ($p = 0.054$, $r = -0.57$).

On day 7, no significant correlations were found between protein, Na/K^+ ATPase and Mg^{2+} ATPase. However, on day 14, gill protein showed significant negative correlations with Mg^{2+}ATPase ($p = 0.00003$, $r = -0.91$) and Na/K^+ ATPase ($p = 0.012$, $r = -0.59$), and a significant positive correlation was observed between Mg^{2+} ATPase and Na/K^+ ATPase ($p = 0.12$, $r = 0.61$) (Table 4.5).

On day 21, gill protein exhibited significant negative correlations with Mg^{2+}ATPase ($p = 0.0031$, $r = -0.26$) and Na/K^+ ATPase ($p = 0.000005$, $r = -0.3$) (Fig 4.6). Additionally, a significant positive correlation was observed between Na/K^+ ATPase and Mg^{2+}ATPase ($p = 0.0000007$, $r = 0.58$).

4.1.3 Histology

Histological analysis of fish gill tissues exposed to varying salinity levels (0, 2, 4, and 6 ppt) over 7, 14, and 21 days revealed significant alterations. At day 7, 4 ppt (Fig 4.14.C,E &F) and 6 ppt (Fig.4.14, D,G &H) exposures led to anomalies like aneurysms, hyperplasia, and blood congestion, resulting in lamellar fusion. Gill filaments of control group (0 ppt salinity) retained normal architecture with intact primary and secondary lamellae having abundant erythrocytes with the presence of normal cells of lamellar epithelium including Pavement cells (PVCs), Pillar cells (PCs), Mucous cells (MCs) and Chloride cells (CC), while salinity-related alterations included loosening of gill filament architecture and decreased erythrocyte count at 6 ppt, accompanied by increased mucous cell numbers.

By day 14, treated groups exhibited pronounced fusion of secondary lamellae, (Fig 4.12.J) venous sinus congestion (2 ppt), and vasodilation (4 ppt), alongside enhanced mucous cell proliferation (Fig 4.14.K). Exposure to 6 ppt (4.14.L) resulted in noticeable lymphocytic infiltrates. By day 21, treated group gill filaments (Fig 4.14.Q-V) displayed severe degeneration, epithelial lifting, pillar cell clustering, and complete lamellar fusion with cubbing. Chloride cell hypertrophy, mucous cell hyperplasia, decreased erythrocytes, and increased chondrocyte count within primary lamellae were also observed, correlated with rising salinity levels. Thus, escalating salinity levels induced progressive structural and cellular alterations in fish gills, indicating disruption of ion exchange mechanisms and potential physiological stress.

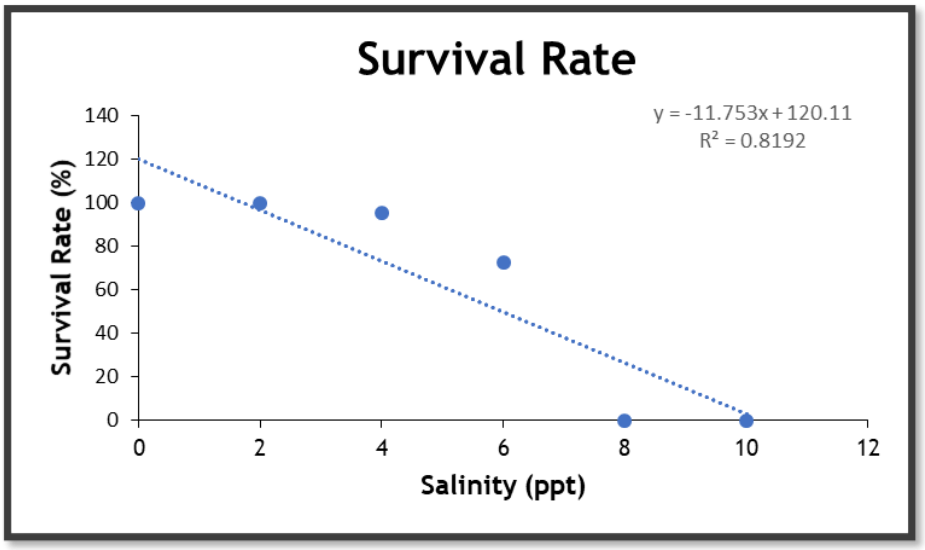


Fig 4.1 Graph showing Survival rate (%) of *H. fasciata* along with increasing salinity levels (ppt).

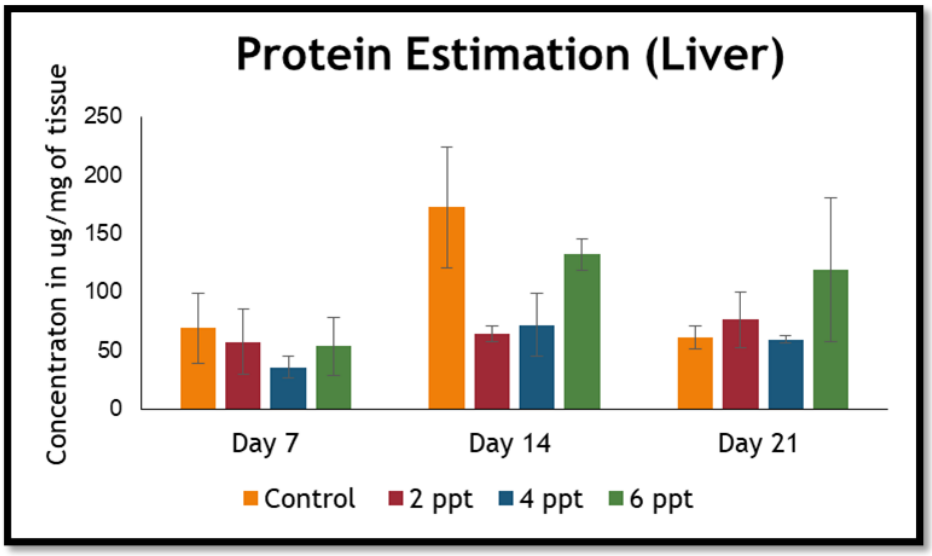


Fig 4.2 Estimation of Total Protein content in liver tissue of *H. fasciata.*

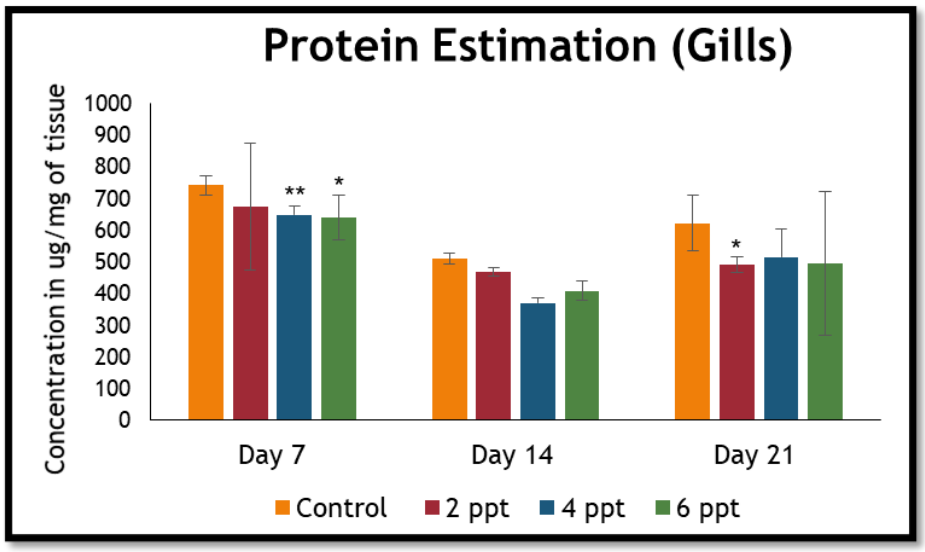


Fig 4.3 Estimation of Total Protein content in the gill tissue of *H. fasciata.*

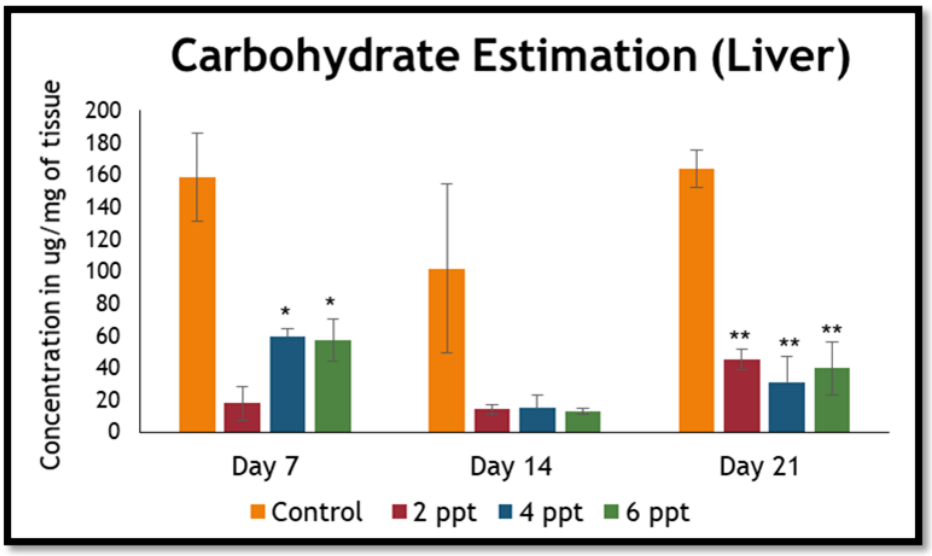


Fig 4.4 Estimation of Total Carbohydrate content in the liver tissue of *H. fasciata.*

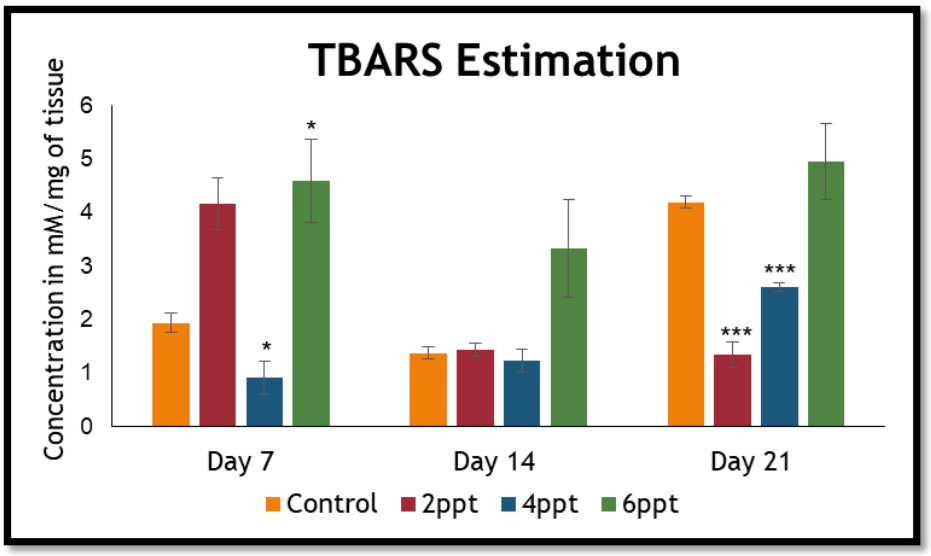


Fig 4.5 Estimation of Thiobarbituric acid reactive substance assay (TBARS) in the liver tissue of *H. fasciata.*

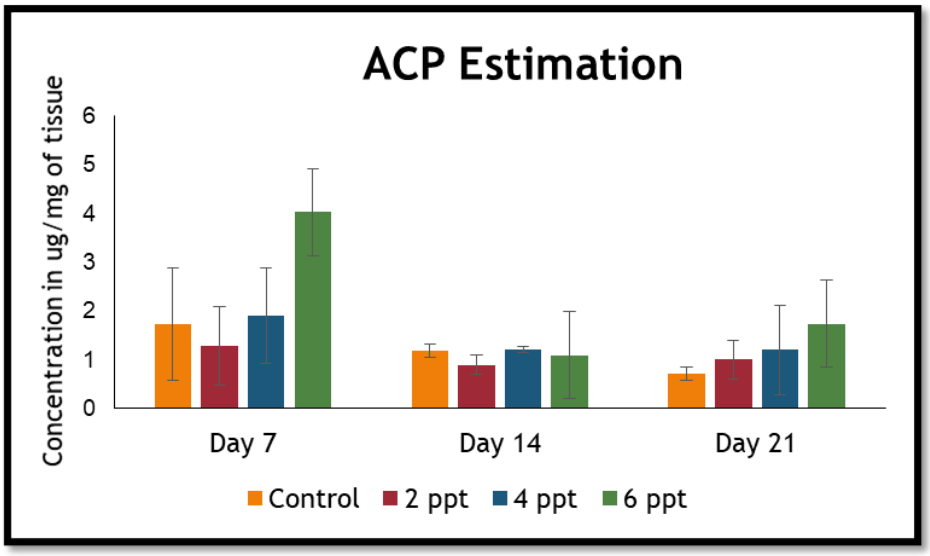


Fig 4.6 Estimation of Acid phosphatase (ACP) activity in the liver tissue of *H. fasciata.*

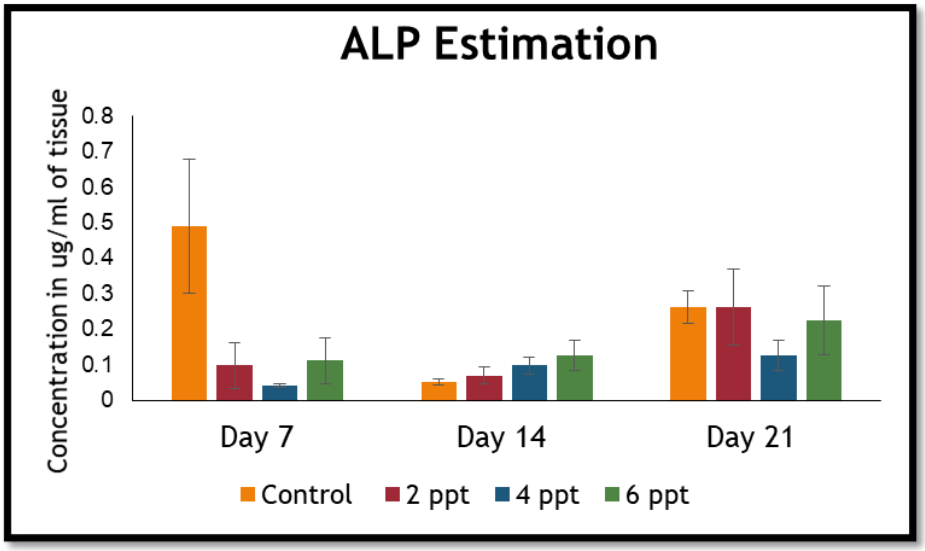


Fig 4.7 Estimation of Alkaline phosphatase (ALP) activity in the liver tissue of *H. fasciata.*

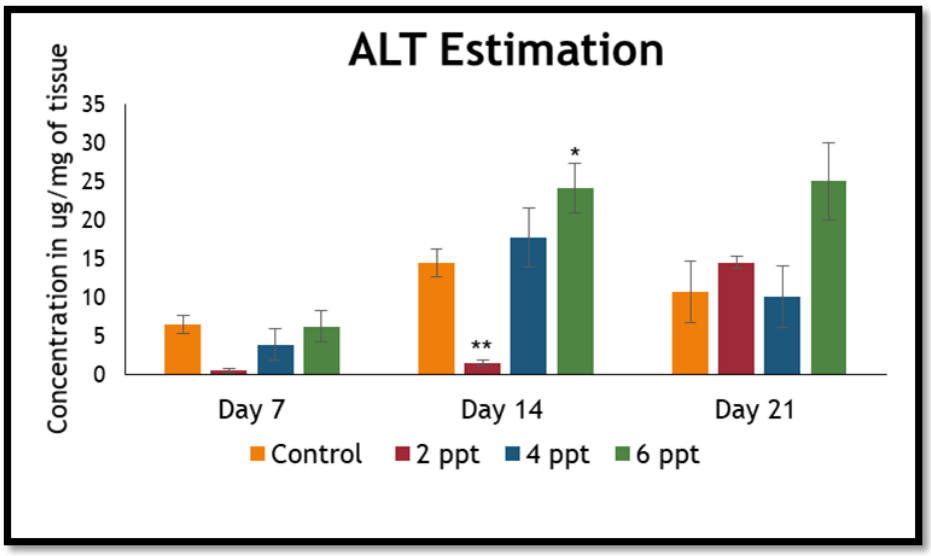


Fig 4.8 Estimation of Alanine aminotransferase (ALT) activity in the liver tissue of *H. fasciata.*

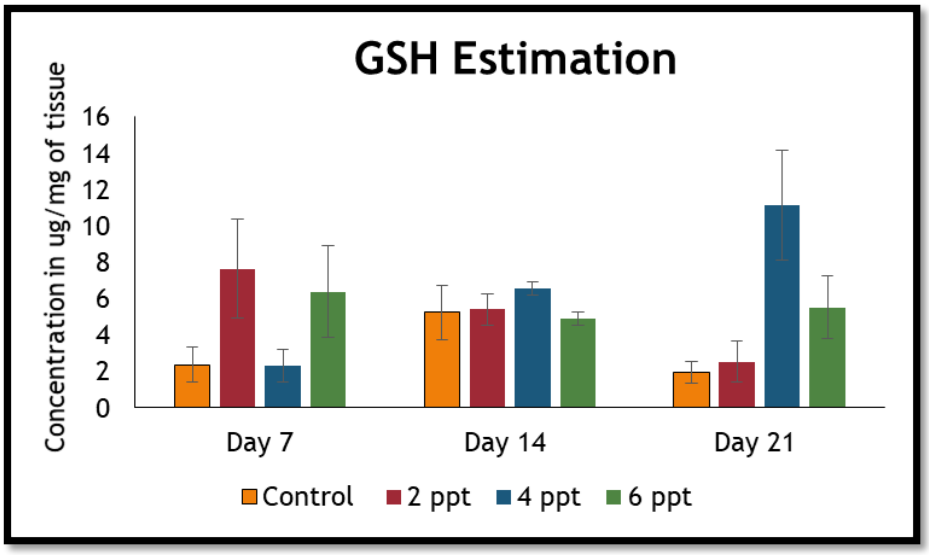


Fig 4.9 Estimation of Reduced Glutathione activity in the liver tissue of *H. fasciata.*

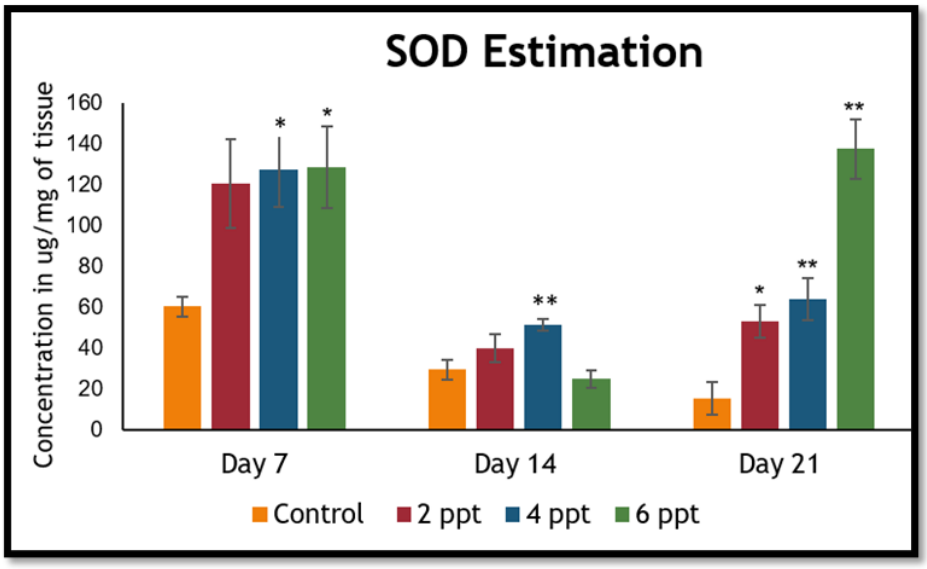


Fig 4.10 Estimation of Superoxide dismutase (SOD) activity in the liver tissue of *H. fasciata.*

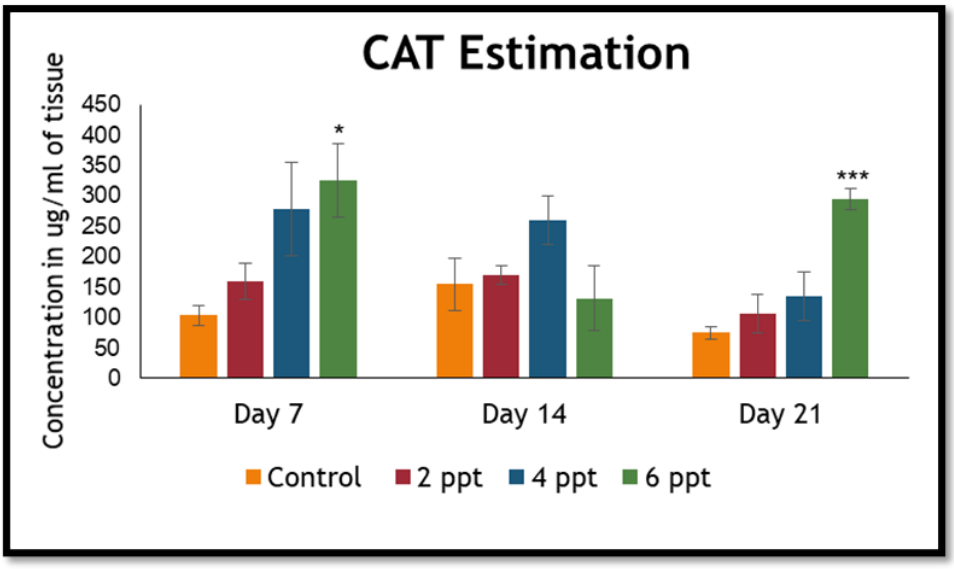


Fig 4.11 Estimation of Catalase activity in the liver tissue of *H. fasciata.*

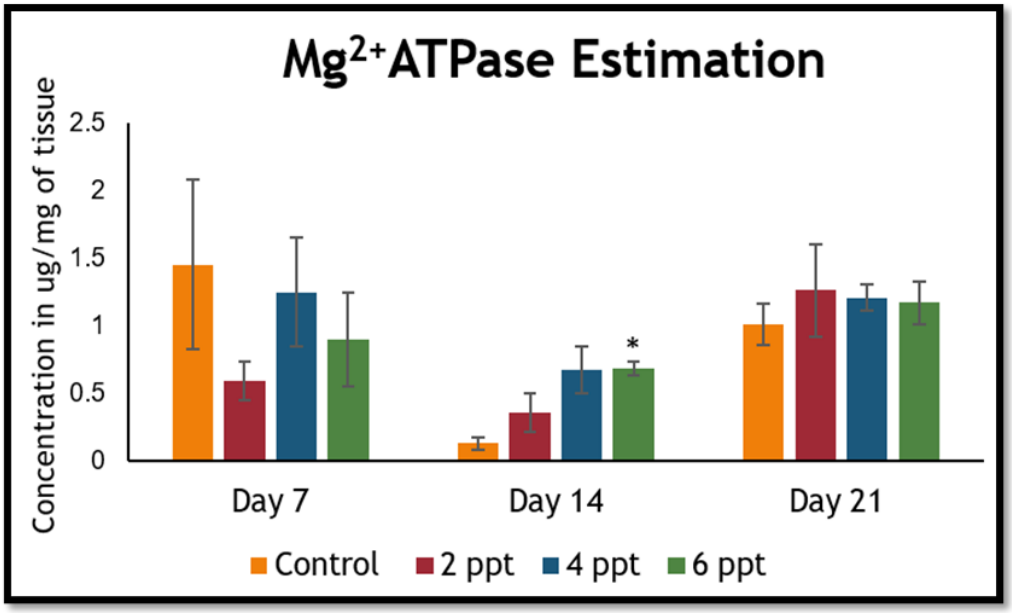


Fig 4.12 Estimation of Mg^{2+}ATPase in the gill tissue of *H. fasciata.*

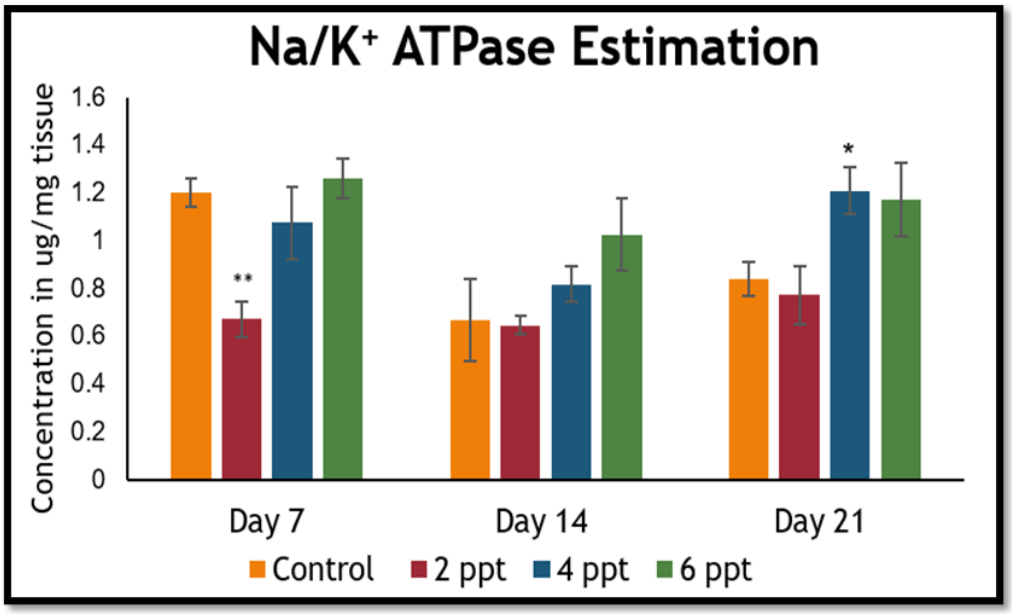


Fig 4.13 Estimation of Na/K^{+}ATPase in the gill tissue of *H. fasciata.*

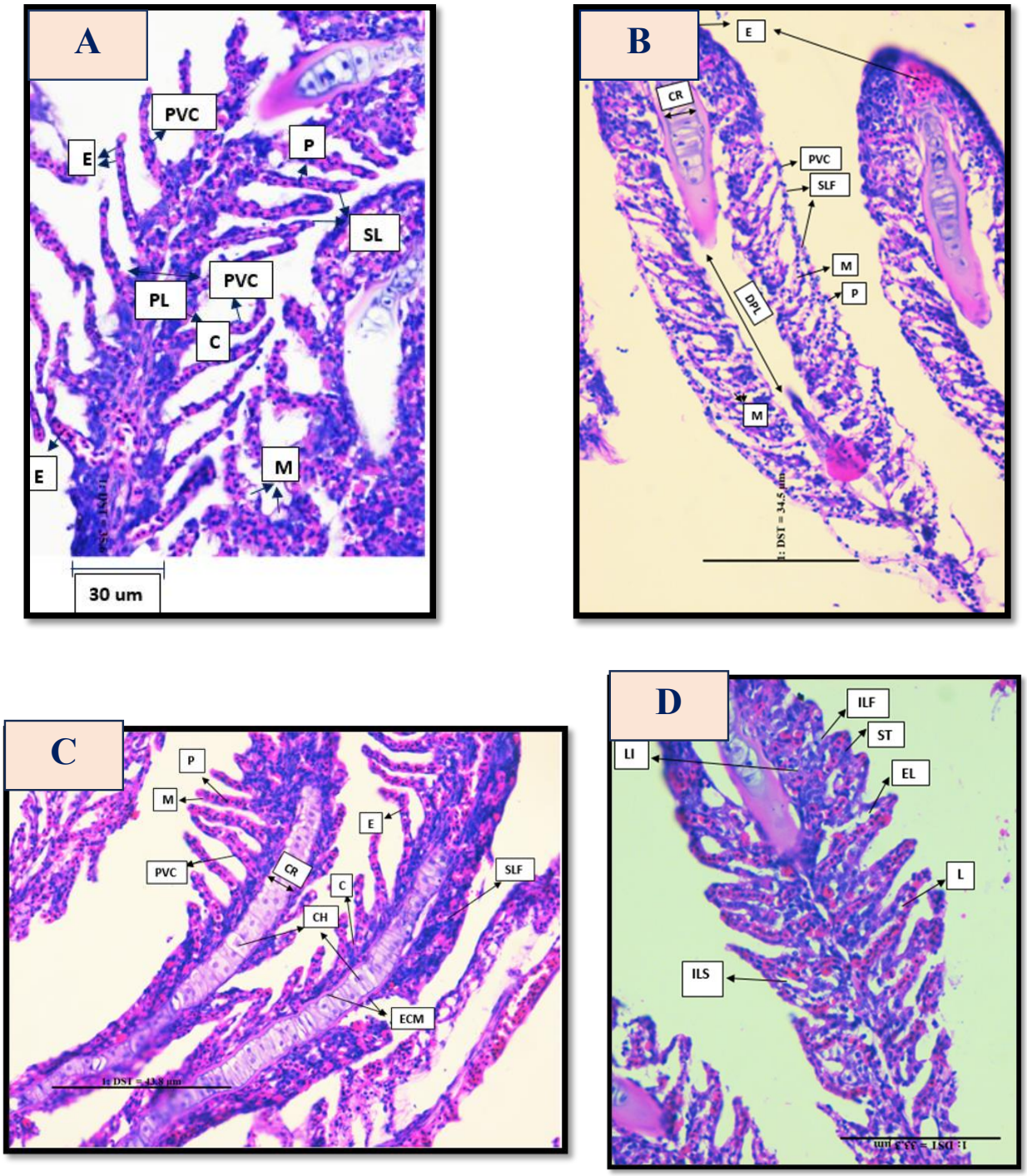
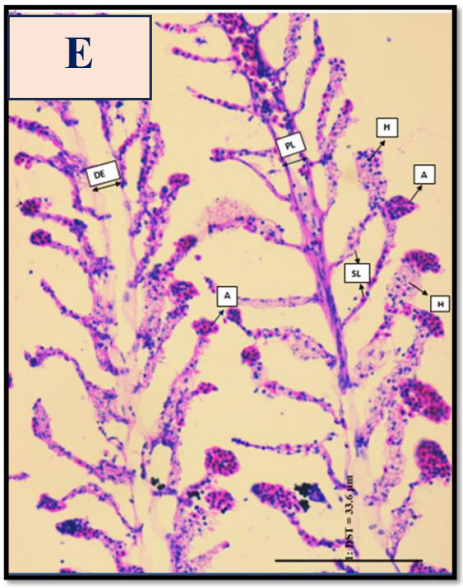
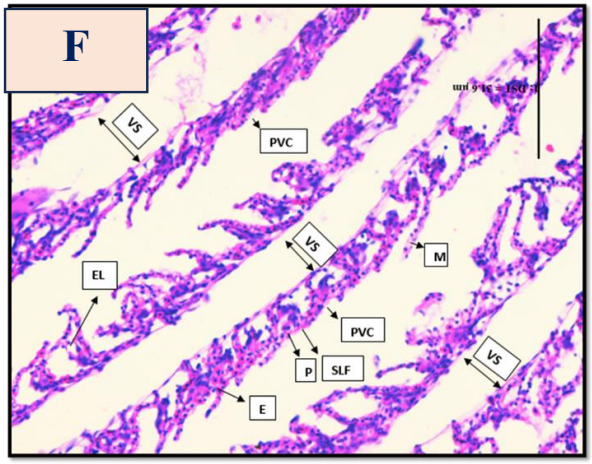


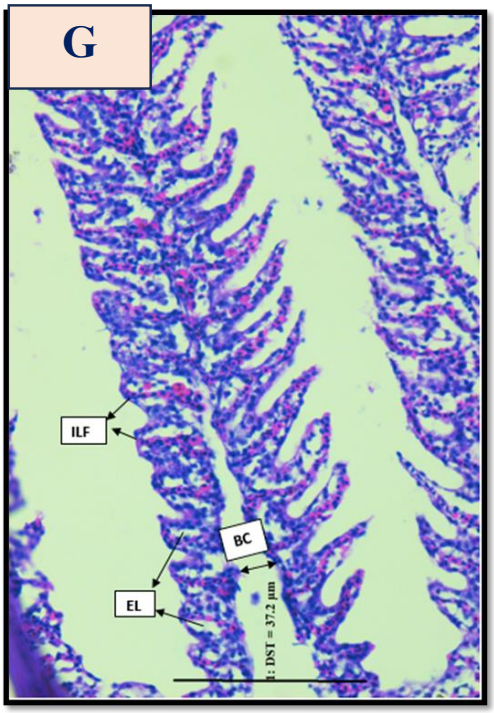
Fig 4.14 A-V: Histological evaluation of gills exposed to varying salinity for different time periods.

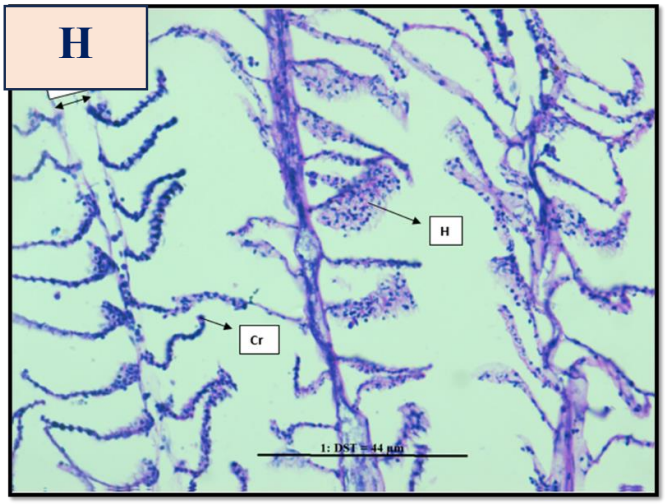
Saggital section depicting histological alterations in *H. fasciata* gills exposed to different salinity at 7 days.

A:Control-PL(Primary lamellae)**, SL** (Secondary lamellae), **PVC** (Pavement cells), **E** (Erythrocytes)**, M** (Mucous cells), **and C** (Chloride cell)**; Fig B:2 ppt-E**(clump of erythrocyte in proximal portion of gill fiament), **SLF**(Secondary lamellar fusion)**, DPL**(Disintegration of primary lamellae)**; C:4 ppt-ECM** (Extracellular cartilaginous matrix)**, CH** (Chondrocytes)**, CR** (Cartilago constituting primary lamellae)**; D:6 ppt-L** (Lacuna; capillary lumen), **EL** (Epithelial lifting), **ILF** (Interlamellar fusion), **ST** (Shortening and thickening of secondary lamellae), **LI** (Lymphocytic infiltration), **ILS** (Interlamellar space).



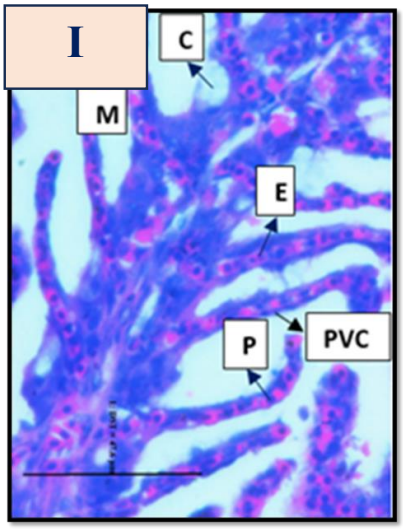


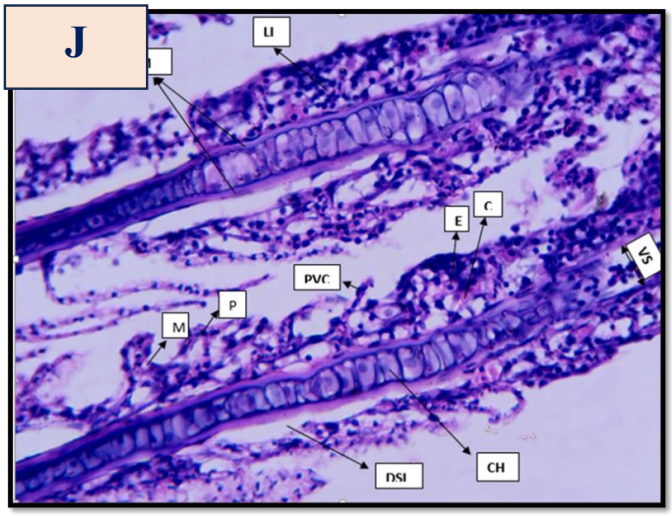


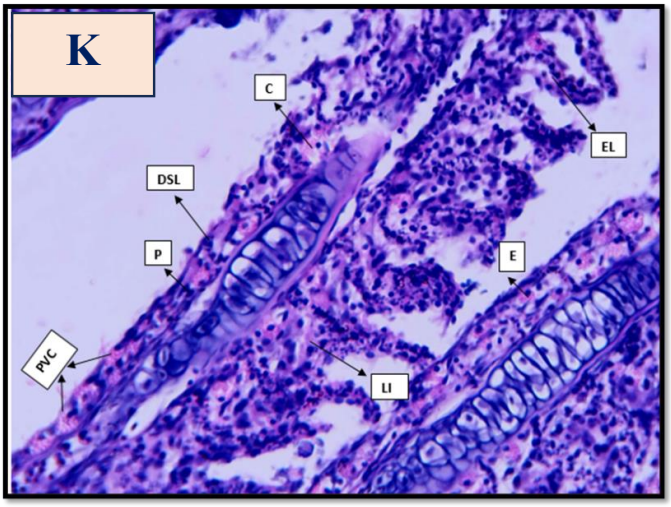


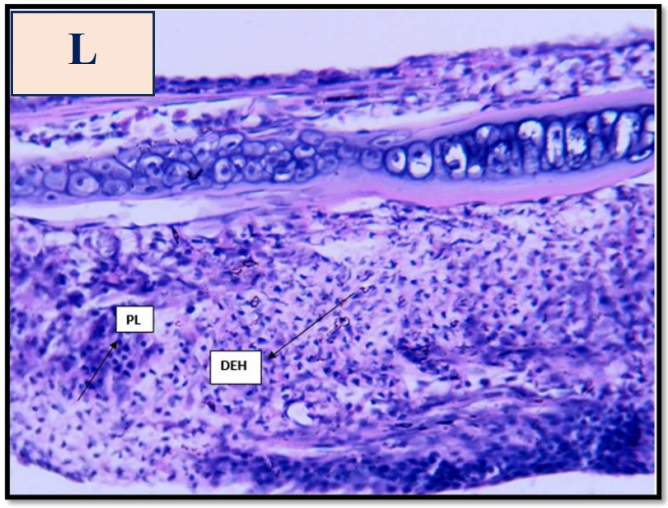
Saggital section depicting histological alterations in *H. fasciata* gills exposed to different salinity at 7 days.

E and F: 4 ppt-A (Aneurysm), **DE** (Degeneration of erythrocytes in stromal region of primary lamellae), **H** (Epithelial hyperplasia in secondary lamellae), **SLF** (Secondary lamellar fusion), **VS** (Venous sinus, showing complete detachment of primary lamillary epithelium and complete lack of erythrocytes), **EL** (Epithelial lifting)**; G and H: 6 ppt- H** (Focal Hyperplasia of epithelium), **Cr** (Curling of the tip of the secondary lamellae), **BC** (Blood congestion), **ILF** (Incomplete lamellar fusion).



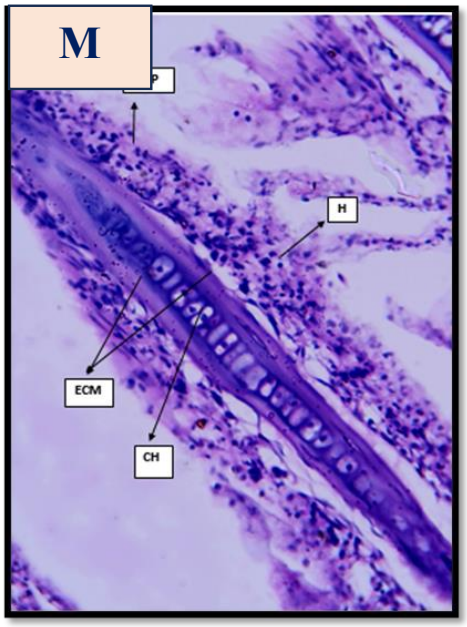


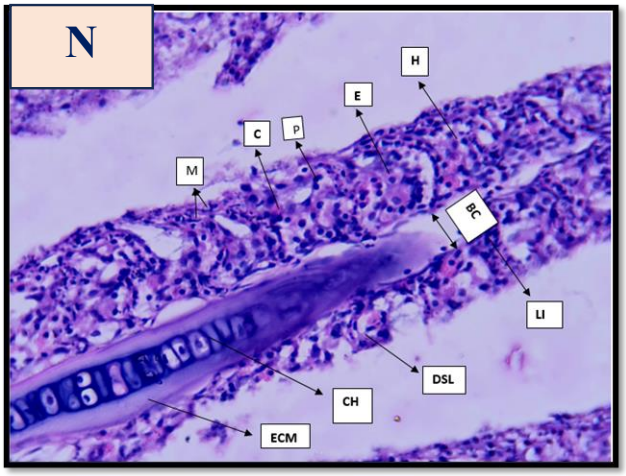


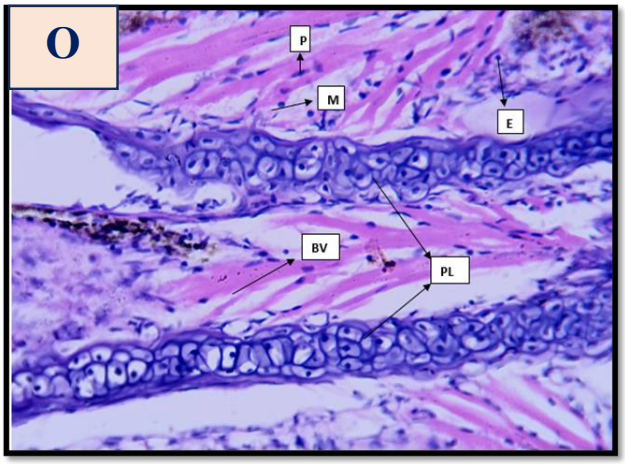


Saggital section depicting histological alterations in *H. fasciata* gills exposed to different salinity at 14 days.

I: Control, J:2 ppt-VS (Venous sinus), **DSL** (Detachment of Secondary lamellae), **K:4 ppt-LI** (Lymphocytic infiltrate), **EL** (Epithelial lifting)**; L:6 ppt-DEF** (Diffuse epithelial hyperplasia and fusion of secondary lamellae, **PL** (Proliferation of lymphocyte).

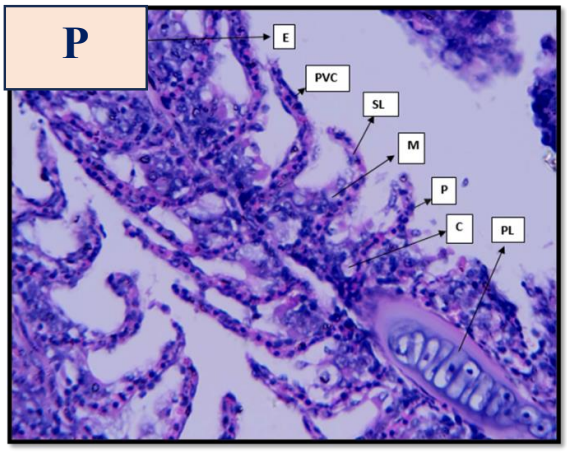


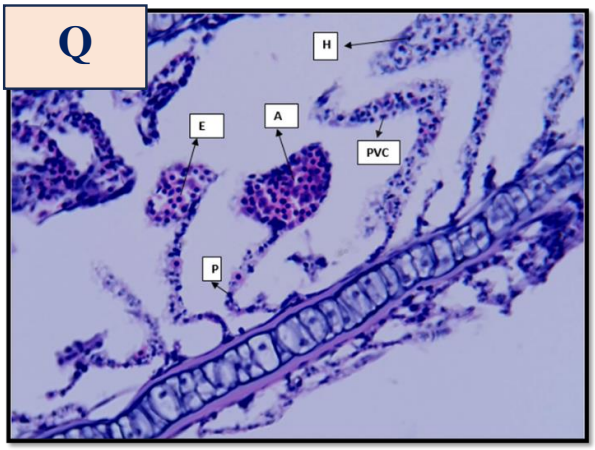


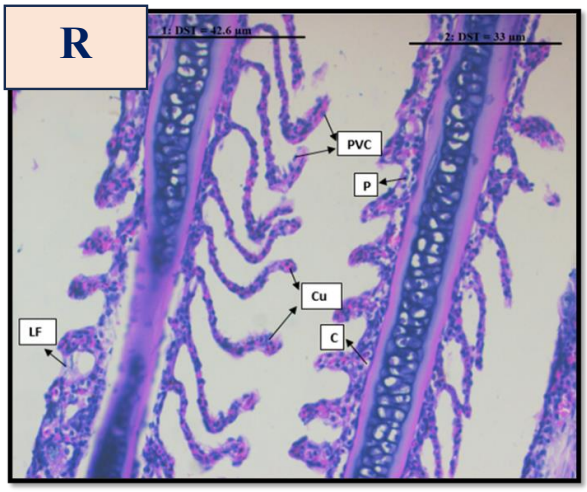


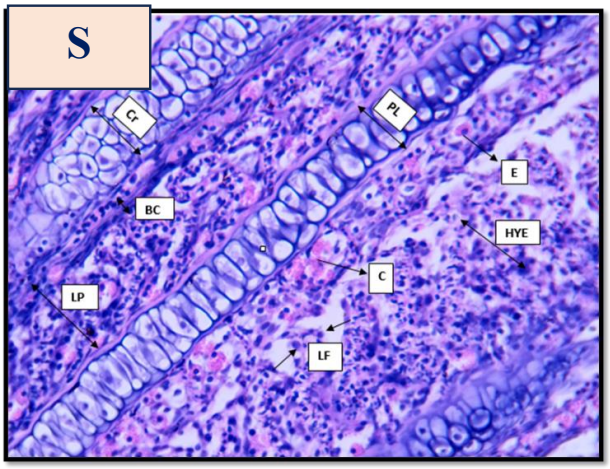
Saggital section depicting histological alterations in *H. fasciata* gills exposed to different salinity at 14 days.

M:2 ppt-MCP (Mucus cell proliferation)**; N:4 ppt-DSL** (Degeneration of secondary lamellae)**, LI** (Lymphocyte infiltration)**, BC** (Blood congestion)**; O:6 ppt-BV** (Blood vessels), **PL** (Primary lamellae), **P** (Pillar cell), **M** (Mucus cell), **E** (Erythrocyte).



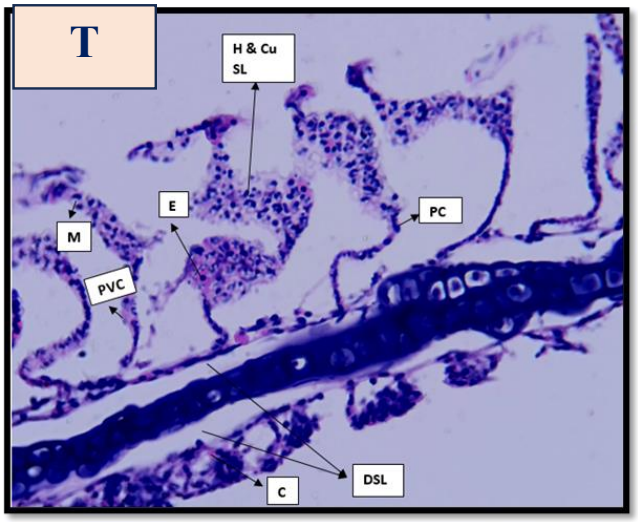


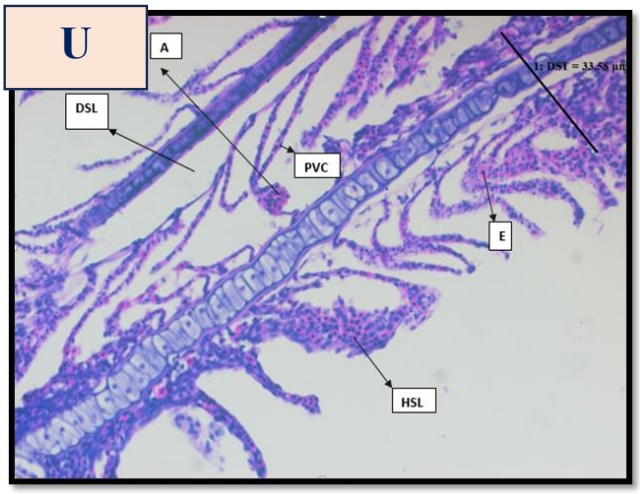


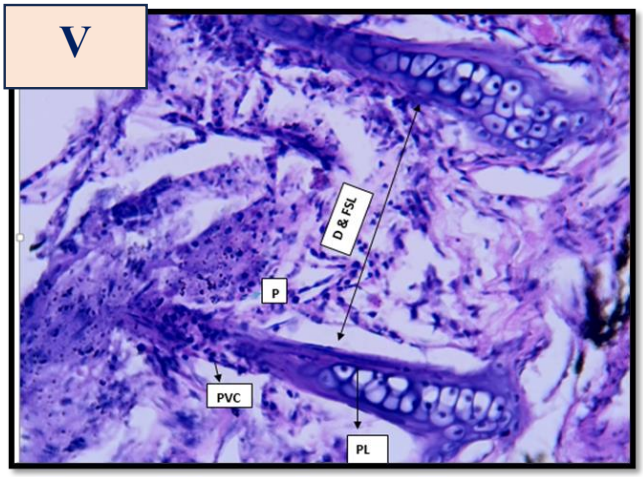


Saggital section depicting histological alterations in *H. fasciata* gills exposed to different salinity at 21 days.

P:Control-PVC (Pavement cells), **C** (Chloride cell), **P** (Pillar cell), **M** (Mucus cell), **PL** (Primary lamella), **SL** (Secondary lamella)**; Q: 2 ppt-H** (Hyperplasia of secondary lamellar epithelial cells)**; R:4 ppt-Cu** (Cubbing of secondary lamellae), **LF** (Lamellar fusion)**; S:6 ppt-C-**Cartilago), **HYE** (Hyperplasia of epithelium), **LP** (Lymphocyte proliferation), **BC** (Blood congestion).







Saggital section depicting histological alterations in *H. fasciata* gills exposed to different salinity at 21 days.

T:2 ppt: DSL (Detachment of Secondary lamellae)**, H&Cu SL** (Hyperplasia and cubbing of secondary lamellae)**; U:4 ppt- A (Aneurysm), HSL** (Hyperplasia of secondary lamellae)**; V: 6 ppt-D&FSL** (Disintegration and fusion of secondary lamellae).

Table 3.1. Average water quality parameters in aquarium tanks of *H. fasciata* (Melon barb) exposed to different salinities.

Parameter	**Salinity concentration (ppt)**			
	Control (0 ppt)	2 ppt	4 ppt	6 ppt
Temperature (ºC)	26.1 ± 0.23	26.27 ± 0.21	26.19 ± 0.42	26.22 ± 0.39
Conductivity (m S)	0.38 ± 0.01	8.23 ± 0.54	10.43 ± 1.0	13.22 ± 0.65
DO (mg/L)	0.58 ± 0.31	0.89 ± 0.05	0.69 ± 0.05	0.67 ± 0.17
TDS (ppt)	0.27 ± 0.01	5.61 ± 0.37	6.95 ± 0.55	9.22 ± 0.55
pH	8.43 ± 0.09	7.86 ± 0.15	7.99 ± 0.13	7.96 ± 0.14

Table 4.1: Survival rate (in percentage) of *Haludaria fasciata* (Melon barb) exposed to different salinities.

Treatments	**Survival % of *H.fasciata***
Control (0 ppt)	100
2 ppt	100
4 ppt	95.5
6 ppt	72.7
8 ppt	0
10 ppt	0

Table 4.2: Pearson correlation coefficient (r values) for Pearson correlation for different enzymatic tests at day 7

	PROTEIN	CARBOHYDRATE	MDA	ALT	CAT	ALP	ACP	GSH
CARBOHYDRATE	0.25							
MDA	0.11	-0.29						
ALT	0.14	0.49	-0.16					
CAT	-0.23	-0.40	0.16	0.12				
ALP	0.20	0.36	-0.32	0.27	-0.40			
ACP	-0.02	-0.17	0.42	0.52	0.61	-0.12		
GSH	0.31	-0.38	0.65	-0.30	0.06	-0.54	0.20	
SOD	-0.30	-0.62	0.33	-0.35	0.54	-0.78	0.04	0.36

Table 4.3: Pearson correlation coefficient (r values) for Pearson correlation for different enzymatic tests test at day 14.

	PROTEIN	CARBOHYDRATE	MDA	ALT	CAT	ALP	ACP	GSH
CARBOHYDRATE	0.47							
MDA	0.20	-0.27						
ALT	0.40	-0.04	0.57					
CAT	-0.52	-0.10	-0.55	-0.01				
ALP	0.09	-0.38	0.98	0.63	-0.40			
ACP	0.20	0.40	-0.08	0.46	0.39	-0.05		
GSH	-0.59	0.12	-0.41	-0.10	0.51	-0.32	0.26	
SOD	-0.72	-0.31	-0.50	-0.27	0.70	-0.36	0.07	0.68

Table 4.4: Pearson correlation coefficient (r values) for Pearson correlation for different enzymatic tests test at day 21.

	PROTEIN	CARBOHYDRATE	MDA	ALT	CAT	ALP	ACP	GSH
CARBOHYDRATE	-0.16							
MDA	0.44	-0.36						
ALT	0.50	-0.09	0.46					
CAT	0.54	-0.31	0.53	0.77				
ALP	-0.12	-0.02	0.22	-0.57	-0.30			
ACP	0.64	-0.15	0.14	0.42	0.53	-0.23		
GSH	0.00	-0.31	-0.05	-0.05	0.30	-0.30	-0.05	
SOD	0.59	-0.42	0.41	0.73	0.93	-0.29	0.47	0.32

Table 4.5: Pearson correlation coefficient (r values) for Pearson correlation test for ion transporting enzymes at day 14.

	GILL PROTEIN	Mg^{2+}ATPase
Mg^{2+}ATPase	0.12	
Na/K^{+} ATPase	0.01	0.51

Table 4.6: Pearson correlation coefficient (r values) for Pearson correlation test for ion transporting enzymes at day 21.

	GILL PROTEIN	Mg^{2+}ATPase
Mg^{2+}ATPase	-0.26	
Na/K^{+} ATPase	-0.39	0.48

4.2. Discussion

The present study revealed that the enzyme activities, survival, and microscopic gill anatomy of indigenous freshwater ornamental fish *Haludaria fasciata* (Melon Barb) is greatly affected by salinity. This finding is similar to the earlier reports, by Jiang et al. (2022), Murmu et al. (2020), and Sarma et al. (2013).

4.2.1. Survival Rate

In the present study, the survival rate of *H. fasciata* was affected by higher salinities and duration of exposure (Fig 4.1). 100% mortality was achieved in fishes exposed to salinity concentration of 10 ppt for a duration of 4 days and 8 ppt salinity for a period of 8 days. No mortality was recorded for the first 3 hours, even at 10 ppt. Low survival rates of *H. fasciata* at higher salinity were comparable to those reported in *Labeo rohita* (Rohu) by Murmu et al. (2020), *Cyprinus carpio* by Mubarik et al. (2015) and *Clarias batrachus* by Sarma et al. (2013).

De-Boeck et al. (2000) reported that salt exposure reduced food intake by 70% in *C. carpio* which had adverse effects on the growth and survival in fishes. In the present study, the poor survival rate of *H. fasciata* at higher salinity could be attributed to the combined effect of confinement stress (Kangs Ombe & Brown et al. 2008) increasing osmotic maintenance requirements at higher salinities (Kilambi & Zdinak, 1980) and reduced food intake in addition to increased salinity stress.

4.2.2. Biochemical estimations and antioxidant enzyme activities

In our present study, insignificant variability in protein concentration was seen in liver tissue with the control group showing the highest protein concentration on days 7 and 14 while on 21 days it was taken over by group C i.e. 6 ppt. A notable decrease in protein concentration within the gill tissue was observed across the experimental groups during the treatment period. The reduction in total proteins during salinity elevation can be attributed to the heightened energy demand for osmoregulation. Numerous researchers have documented a significant decline in protein levels with increasing salinity, similar finding was reported by Kelly and Woo (1999). Additionally, reduced appetite in fish species at higher salinity levels, may also contribute to this reduction. Similar findings have been reported in prior studies by Usher et al. (1991); Plaut (1998). The highest concentration of protein in group C at day 21 may be due to the intensified osmoregulatory demands, prompting enhanced protein metabolism to support the energy requirements for maintaining internal salt concentrations amidst elevated salinity levels. Thus, the observed shift in protein concentration corresponds with the varying osmoregulatory demands imposed by different salinity levels.

In our investigation, we noted a significant decline in liver glycogen levels on days 7 and 21. This temporal pattern suggests a responsive reaction to acute exposure to high salinity which is in agreement to Bollen et al. (1998). The marked decrease in liver glycogen levels indicates a heightened rate of catabolism, likely driven by the increased energy demands in the highly salinized environment. Similar studies by De-Boeck et al. (2000) and Begum (2004) have demonstrated similar depletions in liver glycogen in species exposed to environmental stressors such as pesticides and salt stress. The observed decrease in liver glycogen may also be linked to stress-induced hyperglycemia, a common response in fishes experiencing stress or exhaustive

exercise, as indicated previously by Barton and Iwama (1991), Karsi and Yildiz (2005), and Hrubec et al. (1997). Consequently, liver glycogen serves as the primary carbohydrate reserve, supporting ion-secretion mechanisms in the gills which might have led to its reduction in liver. Similar findings have been reported by Soengas et al. (1995), Sangiao-Alvarellos et al. (2005). The insignificant decrease in glycogen content on day 14 could be attributed to regulatory mechanisms, partial adaptation to the high-salinity environment, compensatory processes, utilization of alternate energy sources, or sampling variability.

Alanine aminotransferase (ALT) is a vital enzyme facilitating the transfer of L-amino acids, thus playing a key role in gluconeogenesis and linking carbohydrate and protein metabolism. This study observed a notable increase in ALT levels on days 7 and 14, with a less significant change on day 21. Similarly, AL Khshali and Alshawi (2013) reported elevated levels of ALT and AST in goldfish subjected to gradually increasing salinity levels (4, 8, and 12 g/l) compared to a control treatment (0.1 g/l). Conversely, Vijayan et al. (1996) observed a significant increase in liver enzyme activity in Tilapia transferred from freshwater to seawater for two weeks, attributed to heightened protein breakdown in saltwater environments. Hegazi (2011) suggested that elevated enzyme levels during fish stress may trigger the secretion of ketonic acids in the tricarboxylic acid cycle. Additionally, Fazio et al. (2013) noted an enhancement in AST and ALT levels in gray mullet liver with increasing water salinity, indicating increased permeability of hepatocytes and cellular leakage which may also be attributed to our present findings.

Alkaline phosphatase is a glycoprotein enzyme that is mediated by the cytoplasmic membrane and is thought to be a useful biomarker for evaluating the integrity of the endoplasmic reticulum and

plasma membrane (Wagner & Congleton, 2004). ACP and ALP enzymes are essential for breaking down foreign materials in aquatic organisms (Liu et al., 2004). While ALP is involved in immune functions and sensitive to environmental changes, ACP helps immune responses by breaking down pathogens (Xing et al., 2002). However in the present study, negligible fluctuation in ALP activity except for group B (4 ppt) on day 21 and a negligible rise in Acid Phosphatase (ACP) was observed across the treatment days within the experimental groups which demonstrates how flexible they are in response to changes in salinity. Similar research by Liu et al. (2004); and Xing et al. (2002) revealed that despite of increase in salinity levels, blood parrotfish ACP and ALP activities in blood parrotfish did not change. Additionally, ALP is essential for aquatic species' bone mineralization according to Lan et al. (1995), Zikic et al. (2001) and the negligible levels in the present study indicates that there was no issue of bone mineralization.

Glutathione Reductase is an NADPH-dependent oxidoreductase that catalyzes the conversion of GSSG to GSH (Sirikanth et al., 2013). The level of Reduced Glutathione (GSH) exhibited a non-significant decline on days 7, 14, and 21 in group A (2 ppt), while it did not show significant differences in group B (4 ppt) and group C (6 ppt) across the various treatment durations. This pattern suggests that the reduction in GR may lead to a depletion of Glutathione (GSH) and the inability to maintain its redox status due to peroxidative effects as reported by Atli and Canli (2010). Additionally, a decrease in Reduced Glutathione activity occurs when salinity surpasses the body's tolerance limit (Yin et al., 2011). The consistent GSH activity in the present study indicates that the salinity levels likely did not exceed the tolerance limit of *H. fasciata* which is in line with BaySoy et al. (2012). This observation suggests that GRd plays a role in the detoxification process by eliminating xenobiotic substances from the tissue (Moniruzzaman et al., 2016).

Hepatic enzymatic antioxidants such as Catalase (CAT) and Superoxide Dismutase (SOD) exhibited significant elevation in the experimental groups across various treatment durations as salinity levels increased. These endogenous enzymatic antioxidant systems, play a crucial role in alleviating the adverse effects of oxidative stress in exposed organisms. Elevation in SOD and CAT activity serves as an indicator of excess reactive oxygen species (ROS). In the present study however, higher levels of antioxidants may be related to more vigorous metabolic activity in experimental groups. The rise in SOD activity shows an increase in the production of superoxide radicals, prompting a boost in the antioxidant system to repair the resulting damage as reported by Oruc (2012) and also CAT activity shows an increase in the production of free radicals to alleviate the excessive production of reactive oxygen species which is in agreement to the fidings of Carocho and Ferreira (2013).

Malondialdehyde (MDA) serves as the final product of lipid peroxidation. In the present study, hepatic Malondialdehyde (MDA), a nonenzymatic antioxidant showed a dose-dependent increase with escalating salinity exposure, reaching its peak in group C (6 ppt). Elevated malondialdehyde (MDA) levels, indicative of heightened lipid peroxidation under salinity stress, align with prior studies in aquatic organisms (Martinez-Alvarez et al., 2002). Elevated MDA levels impair cellular function and trigger the induction of antioxidative enzymes such as SOD and CAT to mitigate the ROS.

The fluctuations in malondialdehyde (MDA) levels, with significant increases at 7 and 21 days, may result from the dynamic stress response in aquatic organisms. Initial acclimation at 7 days could trigger elevated MDA levels, followed by potential adaptation at 14 days, and then renewed

stress at 21 days. The MDA increase corresponds to heightened cell toxicity as reported by (Abdel-Tawwab and Monier, 2018) which was also significant in the present study through way of histological evaluation of gills.

The Na^+/K^+ATPase, a vital membrane protein, plays a pivotal role in regulating ion balance and facilitating overall osmoregulation in organisms. In this study, fish exposed to different salinity levels showed a significant increase in Na+/K+-ATPase activity, consistent with previous findings by Shivkamat and Roy (2005) and Nolan et al. (1999). Additionally, findings by Kammerer et al. (2009) indicated that tilapia subjected to salinity stress for a duration of three days exhibited heightened Na^+-K^+-ATPase (NKA) activity in the gills. The rise in Na^+/K^+ ATPase activity across experimental groups is likely due to its predominant presence in tissues responsible for salt absorption and its essential role in regulating osmotic balance as this enzyme is particularly abundant in the gills as reported by Kulac et al. (2012) which is a salt-transporting organ. According to Benli and Yildiz's (2004) findings, these results could be explained by increased levels and activity of branchial Na+-K+- ATPase, which is primarily found in mitochondrial-rich cells, as well as the effects of rising salinity on renal tissue.

Mg^{2+}ATPase activity, however, did not significantly differ from controls except for group C (6 ppt) at day 14. These results suggest dynamic responses of ATPase activities to salinity variations in fish osmoregulatory tissues. However insignificant variabilities in Mg^{2+} ATPase could be due to the changes in ion levels and the adverse effects of salt exposure.

4.2.3. Histological evaluation

Control gill filament showed normal gill architecture with intact primary and secondary lamellae with an abundant number of erythrocytes along with all the normal epithelial cells of the gill lamellae, such as Pavement Cells (PVCs), Pillar Cells (PC) which are modified endothelial cells, Mucous cells (MC) and Chloride cells (CC) which showed variations in number with increasing salinity levels.

Microscopic findings such as epithelial hypertrophy, cell detachment, and inflammatory infiltration, hyperplasia and microaneurysm formation reflect the formation of chronic lesions. Vascular congestion was observed in all the gill filaments which were exposed to salinity levels.

There was a substantial increase in the number of mucous, epithelial, and chloride cells (ionocytes) present in the gills in fish at 21 days suggesting that it is sensitive to chronic salinity exposure. According to Mallat (1985), Hinton and Lauren (1990), these changes occur initially as an adaptation to new stressors for protecting against excessive penetration of toxins from water to the blood vessels in the gills and thus to the blood.

Elevated gill epithelium commonly referred to as epithelial lifting was observed at day 7 in fish gill exposed to 6 ppt, which was then common across days, according to Singhadach et al. (2009) it arises from the acute inflammation.

Common symptom associated with salinity exposed fish gills were hyperplasia of varying degrees, partial at salinities 2 and 4 ppt whereas complete hyperplasia was observed at 6 ppt at 14 and 21 days. Aneurysms developed at the tips of secondary lamellae at 7 days in 4 ppt and at 21 days in 2 ppt which is due to congestion of blood vessels in a fish gill exposed to 4 ppt which indicates the rupture of supporting pillar cells, whose initial phase depicted by dilation of marginal channels, leading to telangiectasia which later turns into terminal aneurysms, similar process of aneurysm formation at the tips of secondary lamellae due to salt stress is reported by Camargo and Martinez (2007). Similar observations were also reported by Ghorashi et al. (2013).

At 6 ppt swelling and fusion of secondary lamellae was observed at 14 and 21 days which according to Okomoda et al. (2019), is responsible for providing a layer of protection by reducing exchanges with the external environment. According to Fernandes and Mazon (2003), this layer interferes with the fishes gaseous and ionic exchanges with the environment. At 6 ppt it was observed that chloride cells responsible for excretion of Cl^- ions, increased in number similar observation was reported by Hibiya et al. (1982), Mc Donald et al. (1991) suggesting that it is an adaptive process that responds to changes in ion balance. With increase in salinity levels, mucous cell production increased in the gill lamellae serving as an adaptive mechanism. Similar findings were reported by Paulino et al. (2014) for providing more effective protection to epithelial cells against environmental agents. Epithelial detachment was most common after hyperplasia which serve as osmoregulaory defense mechanisms according to Mohavedinia et al. (2012).

4.3 Conclusion

The findings of this study emphasize the critical impact of salinity intrusion on freshwater ecosystems, particularly highlighting its effects on indigenous ornamental fish species like *Haludaria fasciata*. Through careful experimentation, it was revealed that increasing salinity levels led to a decrease in survival rates and triggered significant alterations in biochemical parameters, antioxidant enzyme activities, and histological changes in gill tissues.

Notably, *Haludaria fasciata* demonstrated a remarkable degree of resilience, tolerating salinity levels up to 6 ppt, albeit with reduced survival rates. However, optimal survival occurred at 2 ppt, indicating the species' capacity to adapt to moderate salinity levels while maintaining feed uptake and moderate antioxidant enzyme activities, with slight alterations in 4 ppt. Conversely, exposure to 6 ppt salinity induced severe histological alterations, heightened antioxidant enzyme activities, and greatly reduced feed uptake, underlining the critical threshold beyond which osmotic stress becomes detrimental.

These findings have profound implications for the management of *Haludaria fasciata* populations and the conservation of freshwater ecosystems. By elucidating the species' salinity tolerance and physiological responses, this research provides valuable insights for guiding melon barb culture, breeding in captivity, and informing fisheries management practices. Moreover, it emphasizes the urgent need for comprehensive monitoring and proactive measures to mitigate the adverse effects of salinity intrusion on aquatic habitats and fish populations.

Looking ahead, future studies may delve into the precise molecular mechanisms governing osmoregulation and oxidative stress responses in melon barb, potentially leading to the development of targeted therapies to alleviate the negative impacts of salinization. Additionally, exploring hematological and genotoxicity aspects could further enhance our understanding of the physiological adaptations of *Haludaria fasciata* to salinity stress.

In essence, this investigation into Melon barb (*Haludaria fasciata*) represents a significant contribution to the fields of aquatic biology and environmental physiological adaptation. By identifying critical thresholds and elucidating adaptive responses, this research not only advances scientific knowledge but also provides practical guidance for the sustainable management, conservation, and preservation of native fish populations and freshwater ecosystems.

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