DIETARY AND ENVIRONMENTAL MODULATION OF GUT MICROBIOTA AND BEHAVIOURAL RESPONSES IN ZEBRAFISH

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the Faculty of Science
University of Calicut

By

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JANUARY 2026

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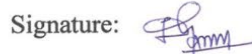
I, **Dhanusha S**, hereby declare that the work embodied in this thesis, **"DIETARY AND ENVIRONMENTAL MODULATION OF GUT MICROBIOTA AND BEHAVIOURAL RESPONSES IN ZEBRAFISH"** submitted to the University of Calicut in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Zoology is a bona fide record of the research work carried out by me under the supervision of Dr. Binu Ramachandran, Assistant Professor, Department of Zoology, University of Calicut. No part of the thesis has formed the basis for the award of any degree, diploma or similar titles of any university. The contents of the thesis have undergone a plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index was found to be with in the permissible limit. I also declare that this thesis is free from AI-generated content.

Signature: 

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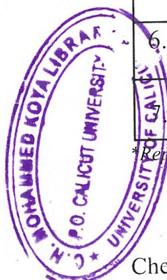

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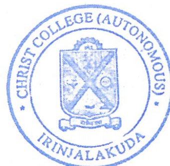

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Dhanusha S.

Dedication

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Table 4.4	*Alpha diversity values*

സംഗ്രഹം

ആന്ത്ര-മസ്തിഷ്ക അച്ചുതണ്ട് എന്ന് വിളിക്കുന്നത്, ആന്ത്രത്തിലെ സൂക്ഷ്മജീവികളെയും കേന്ദ്ര നാഡീവ്യൂഹത്തെയും ബന്ധിപ്പിക്കുന്ന ഉഭയദിശ ആശയവിനിമയ ശൃംഖലയാണ്. ഇത് നാഡീ–രാസ വിനിമയത്തെയും പെരുമാറ്റത്തെയും സ്വാധീനിക്കുന്നു. ഈ അച്ചുതണ്ടിലെ തകരാറുകൾ മസ്തിഷ്ക പ്രവർത്തനത്തെ ബാധിക്കുകയും നാഡീ-പെരുമാറ്റ സംബന്ധമായ വൈകല്യങ്ങൾക്ക് കാരണമാകുകയും ചെയ്യുന്നു. മനുഷ്യരുമായി ജനിതക, ശാരീരിക, നാഡീ–രാസ സാദൃശ്യമുള്ള സീബ്ര മത്സ്യങ്ങൾ സൂക്ഷ്മജീവസമൂഹവും പെരുമാറ്റവും സംബന്ധിച്ച ഗവേഷണങ്ങൾക്ക് വിലപ്പെട്ട രോഗചികിത്സാപരമായ മാതൃകയായി പരിഗണിക്കപ്പെടുന്നു. ഈ പ്രബന്ധത്തിൽ, ഭക്ഷണക്രമം, ജൈവാണു നാശിനികളായ രാസവസ്തുക്കളുമായുള്ള സമ്പർക്കം, പരിസ്ഥിതി സമ്പുഷ്ടീകരണം, ചാക്രിക ഇരുട്ട്–വെളിച്ച സമ്പർക്കം (പ്രകാശദൈർഘ്യം) എന്നിവ സീബ്ര മത്സ്യങ്ങളുടെ ആന്ത്രത്തിലെ സൂക്ഷ്മജീവികളുടെ ഘടനയിലും പെരുമാറ്റ ലക്ഷണങ്ങളിലും ഉണ്ടാക്കുന്ന സ്വാധീനങ്ങൾ പഠനവിധേയമാക്കി. ഗവേഷണ നിരീക്ഷണങ്ങൾ അഞ്ചു അധ്യായങ്ങളിലായി അവതരിപ്പിച്ചിട്ടുണ്ട്. 16S റൈബോസോമുകളിലെ റൈബോ–ന്യൂക്ലിക് അമ്ലങ്ങളെ അടിസ്ഥാനമാക്കി, സൂക്ഷ്മജീവികളെ അവരുടെ സ്വാഭാവിക ജീവിതപരിസരത്തിലെ എല്ലാ ജനിതക വസ്തുക്കളെയും ക്രമീകരിച്ച് സൂക്ഷ്മജീവികളുടെ സമൂഹഘടന മനസ്സിലാക്കി. മത്സ്യങ്ങളുടെ പെരുമാറ്റ പരിശോധനകൾ വഴി ചലനശേഷി, ആകുലത, ആക്രമണം, സാമൂഹിക ഇടപെടൽ, ഭയത്തെ അടിസ്ഥാനമാക്കിയുള്ള ഒഴിവാകൽ എന്നിവ വിലയിരുത്തി. കൂടാതെ, ജൈവാണു നാശിനികളായ രാസവസ്തുക്കൾ ആന്ത്രത്തിലെ വില്ലികളിലും മസ്തിഷ്ക കോശങ്ങളിലുമുണ്ടാക്കുന്ന സ്വാധീനം കലകളുടെ ഘടനാരീതി അവലംബിച്ച് പഠിച്ചു. ദിനദൈർഘ്യം ജീവികളിൽ ചെലുത്തുന്ന സ്വാധീനം മസ്തിഷ്ക ജനിതക പകർപ്പുകളുടെ പഠനത്തിലൂടെ മനസ്സിലാക്കി. ഫലങ്ങൾ സൂചിപ്പിക്കുന്നത്, ഭക്ഷണത്തിലുള്ള മാറ്റങ്ങളും പ്രോബയോട്ടിക് ബാക്ടീരിയയുടെ സാന്നിധ്യവും സൂക്ഷ്മജീവികളുടെ വൈവിധ്യവും പെരുമാറ്റസ്ഥിരതയും വർധിപ്പിച്ചു. അതേസമയം,

ഉയർന്ന തോതിലുള്ള ജൈവാണു നാശിനികളുമായുള്ള ചെറുകാല സമ്പർക്കം ആകുലതയും ക്ഷണികാഘാത സാധ്യതയും വർധിപ്പിച്ചു. കുറഞ്ഞ തോതിലുള്ള ദീർഘകാല സമ്പർക്കം ആകുലത കുറച്ചെങ്കിലും ആക്രമണ സ്വഭാവം വർധിപ്പിച്ചു. കൂടാതെ, ഭയം മൂലമുള്ള ഒഴിവാകൽ പഠനം എന്നിവയിൽ കുറവ്, രോഗാണുക്കളുടെ കോളനീകരണം, സൂക്ഷ്മജീവികളുടെ അസന്തുലിതാവസ്ഥ, ആന്ത്രവില്ലികളുടെയും മസ്തിഷ്ക കോശങ്ങളുടെയും കേടുപാടുകൾ എന്നിവ കണ്ടെത്തി. പരിസ്ഥിതി സമ്പുഷ്ടീകരണം പുതുമയോടുള്ള ഭയം, ആകുലത, കുറഞ്ഞ സാമൂഹിക ഇടപെടൽ എന്നിവ സൃഷ്ടിച്ചു, എന്നാൽ ആക്രമണത്തിലും പഠനശേഷിയിലും മാറ്റമുണ്ടായില്ല. സൂക്ഷ്മജീവികളുടെ വൈവിധ്യം കുറഞ്ഞെങ്കിലും ഗുണകരമായ ബാക്ടീരിയയുടെ സാന്നിധ്യം ആന്ത്രവും മസ്തിഷ്ക കോശങ്ങളെയും സംരക്ഷിച്ചു. ദിനദൈർഘ്യത്തിൽ വരുത്തിയ മാറ്റങ്ങൾ ജൈവഘടികാരവുമായി ബന്ധമുള്ള സൂക്ഷ്മജീവികളുടെ സ്വഭാവത്തിലും, മസ്തിഷ്ക ജനിതക പകർപ്പുകളിലും, പെരുമാറ്റ രീതികളിലും വ്യതിയാനം സൃഷ്ടിച്ചു. ദീർഘകാല പ്രകാശാവസ്ഥ ആക്രമണ സ്വഭാവം വർധിപ്പിക്കുകയും ദീർഘകാല ഇരുട്ട് ആകുലത കുറയ്ക്കുകയും ചെയ്തു. മൊത്തത്തിൽ, ഈ ഗവേഷണം ആന്ത്ര–മസ്തിഷ്ക അച്ചുതണ്ട് പ്രവർത്തനരീതി വ്യക്തമാക്കുന്നതിൽ സീബ്ര മത്സ്യങ്ങൾ ഒരു ശക്തമായ മാതൃകയാണെന്ന് തെളിയിക്കുന്നു. കൂടാതെ, സൂക്ഷ്മജീവികളുടെ സന്തുലിതാവസ്ഥ നിലനിർത്തുന്നതിനുള്ള ലക്ഷ്യമിട്ട ഇടപെടലുകൾ നാഡീ–പെരുമാറ്റ വൈകല്യങ്ങളുടെ ചികിത്സയ്ക്ക് സാധ്യതയുള്ളതാണെന്നും ഈ പഠനം വിരൽ ചൂണ്ടുന്നു.

ABSTRACT

The gut-brain axis is a bidirectional communication network connecting the intestinal microbiota with the central nervous system, influencing neurochemical signalling and behaviour. Disruptions in this axis can alter brain function and contribute to neurobehavioral disorders. Zebrafish (*Danio rerio*), with their genetic, physiological, and neurochemical similarities to humans, offer a valuable translational model for microbiome and behaviour research. This thesis examines the effects of diet, antibiotic exposure, environmental enrichment, and photoperiod on gut microbiota composition and behavioural phenotypes in zebrafish, and the objectives are explained in five different chapters. 16S rRNA amplicon-based metagenomic sequencing was used to profile microbial communities, and behavioural assays assessed locomotion, anxiety, aggression, social interactions, and avoidance learning. Complementary histological analyses were conducted to explore the potential effects of antibiotics on intestinal villi and brain cells. Brain transcriptomics was employed to assess the effect of photoperiod. The results demonstrated that dietary modifications and the presence of probiotic strains enhanced microbial diversity and behavioural stability, whereas acute high-dose antibiotic exposure induced heightened anxiety-like behaviour and increased seizure susceptibility. Chronic low-dose antibiotics reduced anxiety but increased aggression. Moreover, impaired fear-induced avoidance learning, along with pathogen colonization and dysbiosis, damaged villi morphology and brain cells. Environmental enrichment induced neophobic, anxiety-like, and less social behaviours, whereas aggression and learning were not affected. Lowered microbial diversity with beneficial bacteria protected intestinal and brain cells, whereas altered photoperiods disrupted circadian-associated microbial profiles, brain transcriptomics, and behavioural patterns, with extended light periods increasing aggression, while extended dark periods reduced anxiety. Overall, this work reinforces zebrafish as a robust model for untangling gut-brain axis mechanisms and supports the translational potential of microbiome-targeted interventions for neurobehavioral disorders.

Key words: Zebrafish, Behaviour, Gut microbiota, Diet, Environment, Photoperiod

"Every emotion starting in the brain will be reflected in the gut, and anything that happens in gut will be reflected in some way at the brain level. It's simple, echoing, like a whisper that crosses the hollow of the body unified..."

- Emeran Mayer

GENERAL INTRODUCTION

Ⅰ. Zebrafish - Biogeographical distribution and biological features

Zebrafish (*Danio rerio*), an Ostariophysian cyprinid fish, first described by Francis Hamilton in 1822, is a tiny (2.5-5 cm in length) tropical freshwater fish, and it can easily be recognised by its distinctive horizontal blue and silver stripes (Hamilton, 1822; Lawrence, 2007; Spence et al., 2008). Zebrafish are native to South Asia, specifically in India, Nepal, Bhutan, Bangladesh and Myanmar. They are frequently spotted in shallow, slow-moving fresh water ponds, streams, rice paddies and seasonal pools with dense vegetation. Their natural environment mainly consists of the Ganges and Brahmaputra River basins, particularly in the Indian states of Odisha, West Bengal, and Assam (Spence et al., 2006a; Engeszer et al., 2007; Spence et al., 2008; Whiteley et al., 2011; Arunachalam et al., 2013). Also reported from the river basins of Gandak, Karnal, Rapti, Kali, Ramganga and Yamuna (Menon, 1962). Their spreads in Nadhave and Kalauma rivers in the Kumaon Himalayas and Rajmahal were also recorded (Hora, 1937; Hora, 1938). Two distributional records from the Thunga River in Karnataka and the Kabini River in Wayanad districts of Kerala have been found in the Western Ghats (Arunachalam et al., 2003).

The Bengali name "dhani", which means "of the rice field," is where the name *Danio* originates (Talwar & Jhingran, 1991). The danios are included in the subfamily Rasborinae (Howes, 1991). Male zebrafish are usually slender compared to females. They are more golden in colour, while females are larger, lighter in colour, and characterised by the presence of a C-shaped belly (Avdesh et al., 2012). Also marked by the presence of a "danionin notch" in the dentary's ventromedial

margin (Spence et al.,2008). The body is laterally compressed and fusiform, with an upward-facing terminal oblique mouth. Whereas the eyes are central and hidden view from

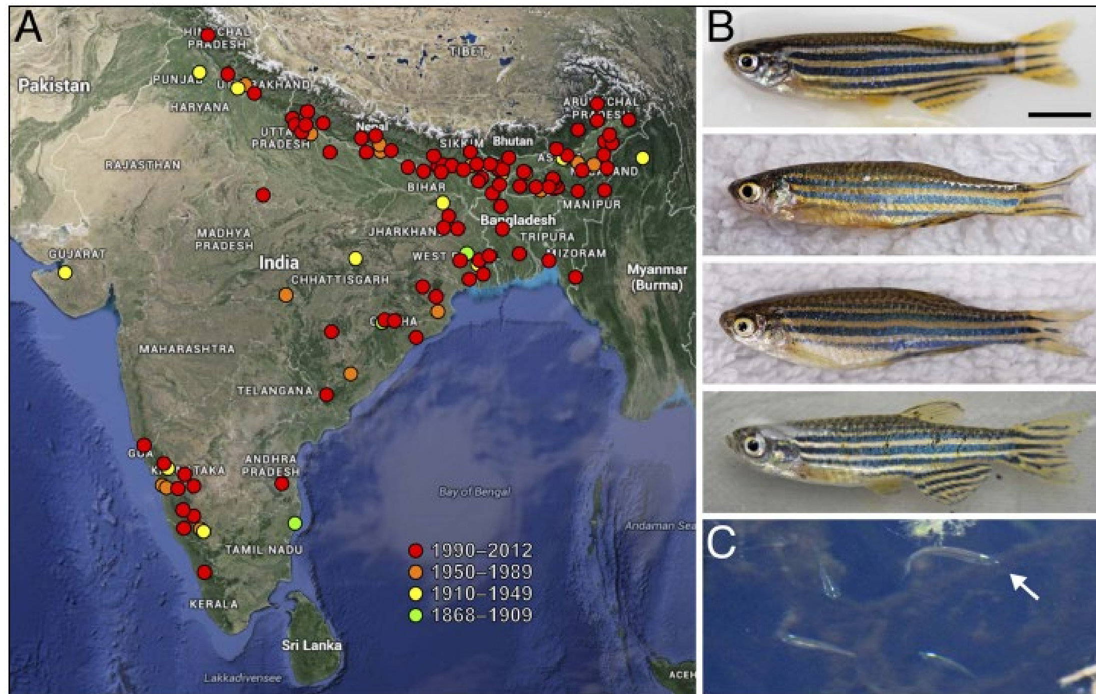

Figure 1: Zebrafish and their Geographic Distribution: *(A) Zebrafish documented across various locations in India, Nepal, Bangladesh, and possibly parts of Myanmar (Spence et al., 2006; Engeszer et al., 2007; Spence et al., 2008; Whiteley et al., 2011; Arunachalam et al., 2013). (B) Specimens collected from different populations in northeastern India (Engeszer et al., 2007). The top two individuals shown are males, distinguished by a subtle yellowish hue on their ventral side, while the bottom two are females. (C) A school of zebrafish observed in a shallow stream pool located in Meghalaya, India, north of the Bangladesh border. One zebrafish is marked with an arrow for identification. Scale bar for panel B: 5 mm (*Image: Parichy, 2015).

above, the lower jaw extends more than the upper. Features of the diagnosis for the species include two pairs of barbels, five to seven dark blue longitudinal lines from below the operculum to the caudal fin, and an incomplete lateral line that extends to the pelvic fin base (Barman, 1991; Spence et al., 2008).

Two lines form centrally at the beginning of development, and then more lines appear above and below in succession (McClure, 1999). The dorsal fin has a dark blue upper edge with white trimmings, and the anal fin is striped similarly. Males often have bigger anal fins with yellow colouration, and females have genital papilla in front of the anal fin (Laale, 1977; Schilling, 2002). Three different pigment cell types make up the colour pattern: iridescent iridophores, gold xanthophores, and dark blue melanophores (Parichy, 2006). Like many teleosts, melanophores' ability to concentrate or disperse in response to stimuli appears to serve two purposes: signalling, as fish tend to darken during aggressive displays (Gerlai, 2003; Larson, O'Malley & Melloni, 2006), and camouflage, as melanophores gather or spread in response to light intensity (Guo, 2004). Zebrafish take a wide array of foods due to their omnivorous and opportunistic feeding habits. Their diet is mainly composed of aquatic insects and zooplankton, suggesting that they prefer planktonic foraging in the water column. However, the consumption of arachnids and terrestrial insects indicates surface feeding behaviour, and the gut contents' detritus, sand, and mud also suggest sporadic bottom-feeding, reflecting their adaptive feeding strategies in a variety of environmental niches (Dutta, 1993; McClure et al., 2006; Spence et al., 2007). In laboratory settings, zebrafish are usually kept in transparent tanks with minimal or no environmental enrichment that mimics their natural habitat (Lavery & Manson, 2023). The laboratory-reared zebrafish exhibit slightly larger body size compared to wild fish (Wright et al., 2006). Although spawning is more seasonal, domesticated zebrafish strains breed year-round (Spence et al., 2006).

II. Zebrafish - a model organism in Biomedical research

Zebrafish is a well-established model organism in research areas of vertebrate neuro-anatomy, developmental biology, and genetics (Moorman, 2001; Kato et al.,

2004; Risner et al., 2006; Wright et al., 2006). George Streisinger was initially introduced zebrafish into the field of research in the 1980s (Streisinger et al., 1981). Now it has become an established and still growing model organism in various fields of biology, including behavioural neuroscience (Moorman, 2001; Kato et al., 2004; Risner et al.,

Figure 2: *Zebrafish housed in laboratory environment.*

2006). Zebrafish consist of numerous advantages compared to rat and mouse models, including ease of maintenance, transparent embryos, high fecundity, rapid reproductive cycle, and the potential for genetic manipulation (Shin & Fishman, 2002; Lieschke & Currie, 2007).

The generation time is usually 3-4 months. In comparison to other teleosts, the egg size is notably larger and transparent. Therefore, developmental stages can be easily monitored using a microscope and thus emerged as one of the premier models for studying developmental processes (Kimmel et al., 1995). Rapid embryonic development occurs, with all major organ precursors developing within 36 h of fertilisation. Hatching occurs after 72 hours, 2-3 days after hatching, they start

exhibiting food-seeking as well as active avoidance behaviours (Kimmel et al., 1995). About the human genomic references, it suggests that close to 70% of human genes hold a minimum of one obvious zebrafish orthologue, of which over 80% are linked to human diseases (Howe et al., 2013). Due to their high genetic similarity, especially in genes associated with disease, zebrafish are observed to be a leading model for the research of human diseases and creating possible therapeutic strategies (Howe et al., 2013; Callaway, 2013). With the use of cutting-edge techniques like CRISPR/Cas9, transgenesis, and morpholino knockdowns, this genomic similarity allows precise genetic modifications for gene function research and disease modelling (Hwang et al., 2013; Patton & Zon, 2001).

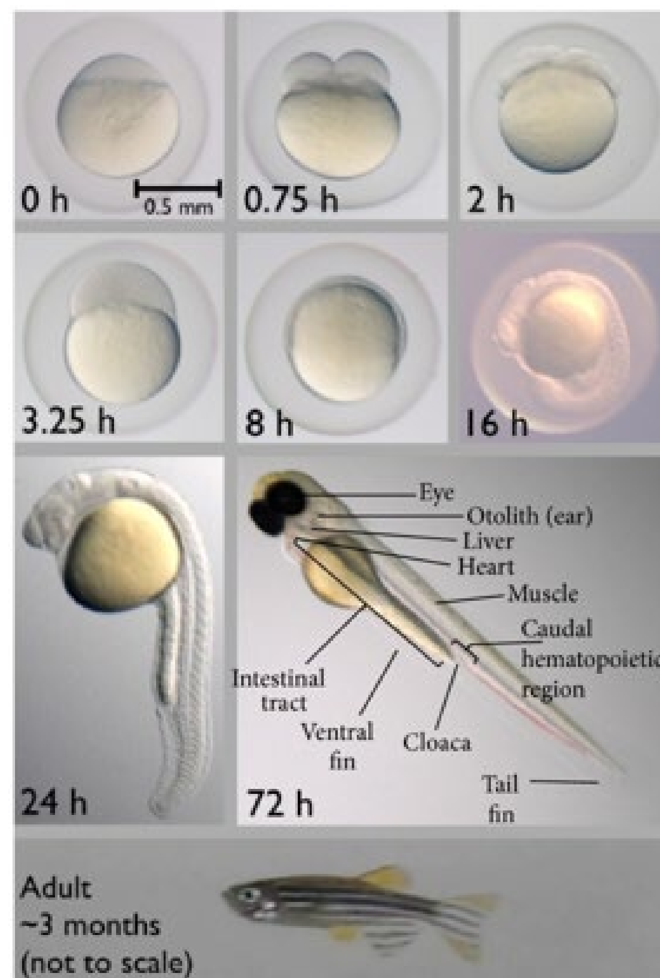

Figure 3*: Zebrafish developmental stages (Tabassum et al., 2015).*

The utilisation of zebrafish in the research of human diseases, such as heart disease, metabolic syndromes, cancer and neurodevelopmental disorders, is growing (Lieschke & Currie, 2007). Therefore, zebrafish is suitable for high-throughput drug screening, which permits the quick evaluation of toxicity and efficacy by treating hundreds of embryos with different compounds in multi-well plates (MacRae & Peterson, 2015). An important advantage over conventional mammalian models is the direct observation of drug-induced phenotypes made possible by the external development of embryos. As a vertebrate, zebrafish share organs and cell types with mammals. Since their biology is similar to that of mammals, zebrafish are referred to as "the canonical vertebrate" (Rubinstein, 2003). Many ethical guidelines do not classify their early developmental stages as protected animals, which permits researchers to conduct extensive experiments within ethical and legal boundaries (Lawrence, 2007).

III. Zebrafish brain- neuroanatomical structure

The brain organisation shows substantial conservation between zebrafish and humans as they exhibit similar neuroanatomic and biochemical pathways, making it an ideal model for studying neurological processes (Wullimann et al., 1996; Saleem & Kannan, 2018; Kalueff et al., 2014). The zebrafish brain possesses three main sections, the forebrain, midbrain, and hindbrain (diencephalon, telencephalon, and cerebellum). Each section has specific subregions that regulate behaviour, motor control, neuroendocrine activity, and sensory processing (Mueller & Wullimann, 2015). The forebrain region, involving the telencephalon, similar to the mammalian cortex, regulates cognitive behaviour, social behaviour, memory and emotional control. The dorsal telencephalon, known as pallium, acts like the mammalian hippocampus and amygdala, whereas the ventral telencephalon, or subpallium, is

engaged in a reward and motivation loop (Rink & Wullimann, 2002). Another area in the forebrain, including hypothalamus and thalamus are found in the diencephalon (Mueller et al., 2011).

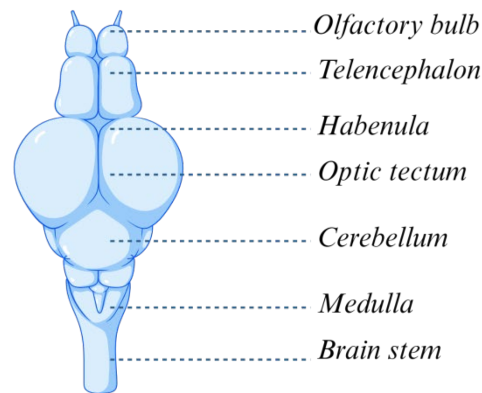

***Figure 4**: Schematic representation of adult zebrafish brain (Created in https://BioRender.com).*

Optic tectum, an area where visual sensory stimulus is processed, is part of the midbrain. The superior colliculus structure in mammals is similar to that of optic tectum in zebrafish, it regulates behaviour driven by visual stimulus. The mid brain also consists of dopaminergic neurons, specifically in the tegmentum (Kaslin & Panula, 2001). The cerebellum and medulla oblongata, important for coordination, posture, autonomic control and involuntary functions. However, in zebrafish, the cerebellum is very simple in structure and involved in learning and other functions similar to the mammalian counterpart. Coordination of sensory information as well as regulation of reflex actions, including fish swimming and their posture, are under the control of medulla oblongata (Wullimann et al., 1996; Bae et al., 2009). Zebrafish have distinctly conserved neurochemical network in the brain, with the

expression of neurotransmitters such as dopamine, serotonin, glutamate, GABA, epinephrine, and nor-epinephrine.

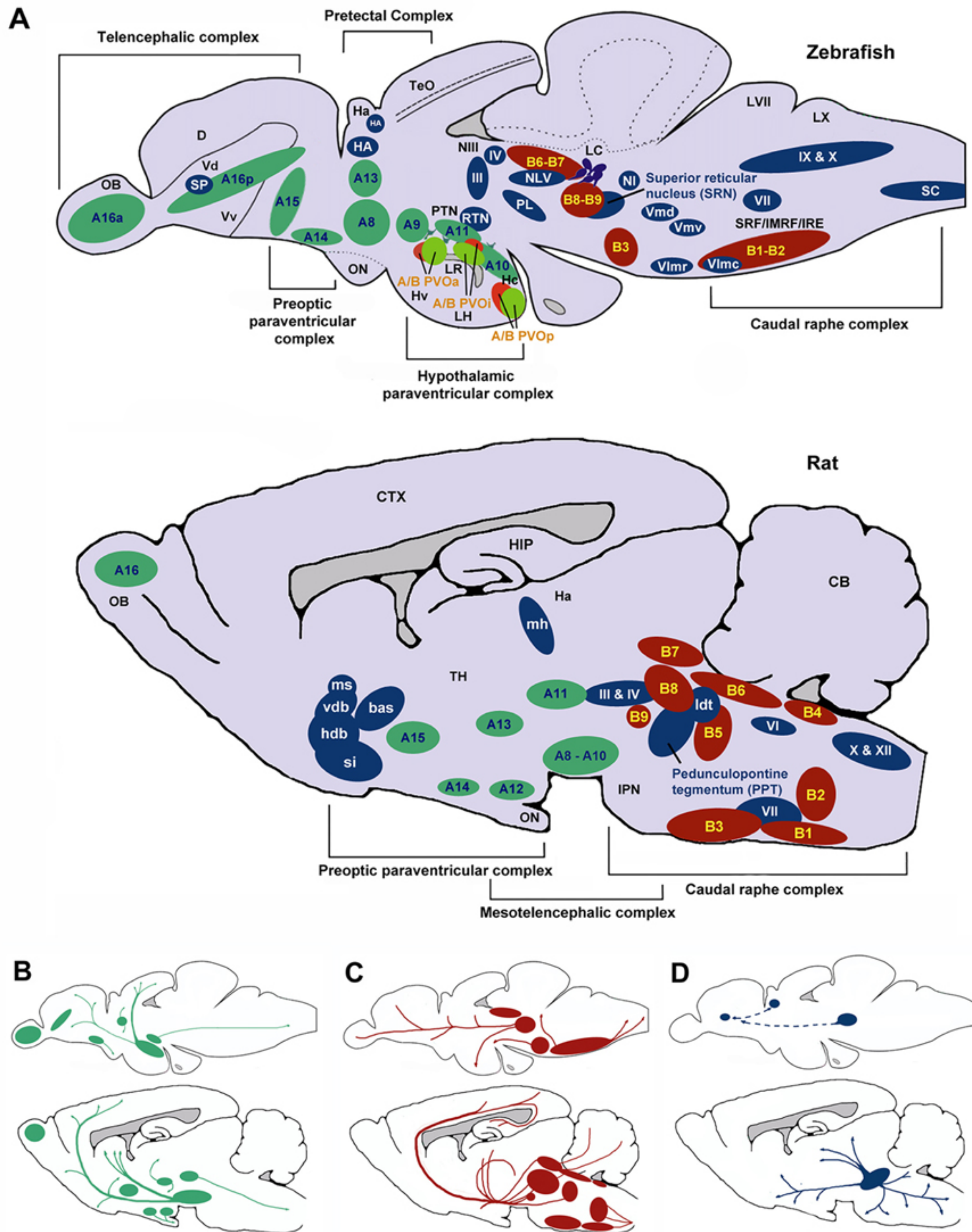

***Figure 5**: Comparing the dopaminergic (green), serotonergic (red), and cholinergic (blue) neuronal populations in the brains of zebrafish (upper) and rats (lower), sagittal view (Parker et al., 2013).*

Zebrafish behaviours are regulated by these neurotransmitter systems (Panula et al., 2010). There are various methods we can utilise to detect the real time activity of neurons, like confocal microscopy, in vivo calcium imaging, and two-photon microscopy (Deng et al., 2024). In transgenic zebrafish (GAL4/UAS system-based), we can specifically target or label certain neurons. Moreover, other techniques such as immunochemistry and in situ-hybridisation can provide data regarding neurochemical distributions, and the gene expression data can make available using brain atlases such as Z-Brain and MapZebrain (Knust et al., 2019; Randlett et al., 2015; Shainer et al., 2023). Thus, by the close examination of structural and functional combination of zebrafish brain we can connect it to the behavioural outcomes. For example, the optic tectum is involved in visual tracking, the hypothalamus in feeding and hormonal control, and the telencephalon is connected to social preference and fear responses. The cerebellum and hindbrain regulate motor activities such as locomotion, avoidance, and exploration (Stewart et al., 2014; Firdous et al., 2024). We can use these associations to simulate behavioural tests related to human neuropsychiatric, neurodevelopmental, and neurodegenerative disorders. Thus, zebrafish is a good model to study anxiety, depression, aggression, and social preference. Also, with the available genetic tools and high-throughput screening, zebrafish provide an excellent system for drug screening and translational neuroscience (Mohamed & Ekker, 2023; Chia et al., 2022).

IV. Zebrafish in behavioural neuroscience

Behavioural neuroscience is the study of relationships between brain function and behaviour, the zebrafish has now emerged as a powerful model organism in this field. Due to the preserved neuroanatomy, genetic tractability, and standardisation of various behaviours in zebrafish, they become highly valuable in neurobehavioural

research (Kalueff et al., 2014). Since zebrafish behaviours are well studied and constantly improving, we can make use of those behavioural patterns to study neurobehavioural alterations and psychiatric disorders (Kalueff et al., 2013; Stewart et al., 2014). From early developmental stages, zebrafish display a wide range of behaviours (Gerlai, 2010). Because these behaviours can be measured and replicated, trustworthy experimental paradigms are made possible. Additionally, they facilitate in vivo imaging and high-throughput behavioural screening, which are challenging to accomplish in conventional mammalian models (Stewart et al., 2014). Having similar neural circuitry, including the majority of important neurochemical signal transduction pathways, the zebrafish and human brains are similar in both morphology and function (Blader & Strahle, 2000). The neurotransmitter system of zebrafish is conserved from the very beginning of their development. It generates neurotransmitters such as dopamine, glutamate, histamine, acetylcholine, serotonin, and GABA, and it has many traits in common with mammalian systems, such as dopaminergic cell clusters in the olfactory bulb and hypothalamus (Horzmann & Freeman, 2016). Whereas, like in mammals, serotonergic systems are dispersed throughout the spinal cord and hindbrain (Panula et al., 2010). These similarities are useful in modelling neurological disorders and creates more translational relevance.

In order to study stress and anxiety, sequences of behavioural tests are available for zebrafish, which includes open field tests, light/dark box tests, and novel tank tests. In open-field tests, experimenters assess how fish behave when they are opened to a new place that make them anxious, by utilising a square or rectangular shaped tank with less height (Champagne et al., 2010). Whereas, the novel tank test evaluates the zebrafish vertical movement and exploration, in which time spent in the upper or lower halves determine their anxiety (Cachat et al., 2010). In case of light/dark box

test, zebrafish's scototaxis behaviour is measuring, the time spent in dark compartment is an indicator of anxiety. The anxiogenic agents can increase anxiety while it can be decreased by anxiolytic drugs (Maximino et al., 2011). Therefore, the light/dark test in zebrafish is suitable for screening various psychoactive substances (Basenet et al., 2019; Irons et al., 2010; Maeda et al., 2021). The behavioural alterations in autism spectrum disorders can be studied using social preference and shoaling tests, they assess social cohesiveness and preference for conspecific individuals (Rea & Van, 2020). Different types of maze tasks including P, Y, and Plus tests, the place preference test, novel object recognition test, and avoidance tests are performed to measure the cognitive behaviour including learning and memory in zebrafish (Tan et al., 2022; Andersson & Olsson, 2015; Levin & Cerutti, 2011; Gerlai, 2016; Cho et al., 2025).

The human neurological disorders including autism, ADHD, epilepsy, Parkinson's and Alzheimer's diseases are modelled and studied using zebrafish (Stewart et al., 2014). For example, Shank3b gene mutation in zebrafish is related to autism, exhibit disrupted neuronal signalling and disturbed social behaviour (Liu et al., 2018). In this study we used pentylenetetrazol (PTZ)-induced seizure zebrafish model, showed epileptic behaviours (Sivarajan & Ramachandran, 2023). Canzian et al. (2021) verified stress is one the potential factor that increase susceptibility towards seizure behaviour in zebrafish. Therefore, chronic environmental stressors including variations in circadian rhythm and alteration in gut microbiota can cause neurobehavioural variations in zebrafish (Eachus et al., 2021; Demin et al., 2021; Silva et al., 2022; Mohanta et al., 2020), it will provide information regarding the influence of environmental conditions on mental health.

Behavior Endpoints (units)	Definition	Applicable to	Data Interpretation
Average speed (cm·s^{-1})	Total distance traveled divided by total time duration	Novel tank test, mirror-biting test, predator test, social interaction test, shoaling test	Reflects general motor/neurological phenotypes
Maximum speed (cm·s^{-1})	Maximum speed fish capable to reach		
Minimum speed (cm·s^{-1})	Minimum speed fish capable to reach		Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test
Distance traveled (cm)	Total distance the zebrafish traveled within the novel tank		
Freezing time percentage (%)	Total percentage of time when speed less than 1 cm·s^{-1}		Indicates increased anxiety and freezing time is generally higher in stressed zebrafish
Swimming time percentage (%)	Total percentage of time when speed 1–10 cm·s^{-1}		
Rapid time movement percentage (%)	Total percentage of time when speed more than 10 cm·s^{-1}		
Time spent in top percentage (%)	Total time spent in the top portion of the novel tank	Novel tank test, predator test, shoaling test	Increasing value indicates lower anxiety levels
Time spent in top/bottom ratio	Total time spent in the top and bottom portion of the novel tank ratio		
Distance traveled in the top (cm)	Total distance traveled in the top portion of the novel tank		
Distance traveled top/bottom ratio	Total distance traveled in the top and bottom portion of the novel tank ratio		
Number of entries to the top	Total times zebrafish swims to the upper half of the tank	Novel tank test	
Average entry duration (s)	Time spent in top divided by the number of entries to the top		
Latency to enter top (s)	The amount of time it takes the fish to cross into the upper half of the tank		Increasing value indicates higher anxiety levels
Mirror biting time percentage (%)	Total percentage of time when zebrafish bit the mirror	Mirror-biting test	Increasing value indicates higher aggression levels
Longest duration in mirror side percentage (%)	Total percentage of zebrafish longest duration stayed in front of mirror		
Predator approaching time percentage (%)	Total percentage of time when zebrafish approached the predator	Predator test	Decreasing value indicates higher anxiety levels
Average distance to predator separator (cm)	Average distance of zebrafish to separator for predator		Increasing value indicates higher anxiety levels
Zebrafish interaction time percentage (%)	Total percentage of time when zebrafish interacted with another zebrafish	Social interaction test	Increasing value indicates higher sociability levels
Longest duration in separator side percentage (%)	Total percentage of zebrafish longest duration stayed in front of separator		
Average distance to zebrafish separator (cm)	Average distance of zebrafish to separator for another zebrafish		Decreasing value indicates higher sociability levels
Average inter-fish distance (cm)	Average distance between the body center of every member of the shoal	Shoaling test	Increasing value indicates lower anxiety levels
Average shoal area (cm^2)	Average size of the shoal		
Thigmotaxis (cm)	The average distance of the group from the center of the tank		Decreasing value indicates lower anxiety levels
Average nearest neighbor distance (cm)	Distance for the body center of each fish to the closest neighboring fish		Increasing value indicates lower anxiety levels
Average farthest neighbor distance (cm)	Distance for the body center of each fish to the farthest neighboring fish		

***Table 1**: Summary of behaviour endpoints frequently utilised in quantifying anxiety, aggression, and social preference (Audira et al., 2018).*

V. Gut microbiota- a dynamic and functional organ system

Our intestinal tract is inhabited with lot of microorganisms including bacteria, archaea, fungi, and viruses, they are collectively called the gut microbiota (MoSZak et al., 2020). Around 100 trillion microorganisms are found to be inhabited in gastrointestinal tract (Ley et al., 2006). Research suggests that it should be considered as a functional organ, since it regulates various functions in our body including metabolism, immune response, colonisation of microbes, pathogen resistance, intestinal epithelium maintenance, and even behaviour through the gut-brain axis (Saxena & Sharma, 2016; Shreiner et al., 2015). Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia are the major bacterial phyla involved in the human gut microbiota. Among these, Firmicutes and Bacteroidetes are the most common phyla (Laterza et al., 2016). A healthy and well-functioning gut microbial system is marked by the presence of stable core microbiota with high taxonomic diversity and richness (Fan & Pedersen, 2021). The primary colonisation process is crucial, as it determines the growth and development of gastrointestinal tract and immunity of newborns (Caicedo, 2005; Houghteling & Walker, 2015). Mode of delivery also greatly contributing to the initial microbial colonisation process (Rutayisire et al., 2016). Other environmental factors such as diet, maternal weight, antibiotics, and stress further determine the colonisation process (Collado et al., 2010; Zijlmans et al., 2015; Scott et al., 2013; Zimmermann & Curtis, 2019). Diet is the principal factor significantly influence the microbiota composition, high fat or protein diet contributes to the enterotype with Bacteroides species, whereas diets with high carbohydrates have supported enterotype with *Prevotella* species (Wu & Hui, 2011).

The age of an individual significantly affect gut microbiota transformation, since childhood is marked by the presence of higher abundance of Bifidobacterium, essential for immune development. While young adult stage consists of more balanced microbiota with diverse genera (Yang et al., 2020). Whereas, in older stages, studies suggest that the increased microbial diversity may persist but with a reduction in beneficial bacteria like *Lactobacillus.* The reduction in the abundance of beneficial bacteria may cause potential health issues, revealing that increased inflammation and decline in immunity in old age groups are due to the imbalances in gut microbiota (Gamez-Marcias et al., 2024).

Since the gut microbiota is involved in appetite regulation and insulin responsiveness, it functions like an endocrine organ. They also produce neurotransmitters such as GABA, dopamine, and serotonin, thereby affecting mood and behaviour. Therefore, this virtual organ is important in regulating systemic functions other than gastrointestinal health (Pires et al., 2024). The short-chain fatty acids (SCFAs) are secondary metabolites having ability to influence neuronal activities. They are the byproduct of gut microbiota fermentation of resistant starch and dietary fibers, including butyrate, propionate, and acetate (Silva et al., 2020). The gut microbiota also synthesises some vitamins, particularly vitamin K, and B group vitamins including biotin, cobalamin, folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine (Rowland et al., 2018). These bacterial derived products help in maintaining the intestinal barrier integrity and permeability. The faecal microbiota transplantation (FMT) from a healthy donor to diseased persons seemed to improve intestinal barrier function and reduce intestinal permeability via microbial modulation (Gupta et al., 2021; Di Vincenzo et al., 2024). Therefore, the gut microbiota is more dynamic and it exhibits microbiome plasticity by reacting to

stress, food, disease, and drugs (David et al., 2014). Also contributing to the formation of the gut-brain axis, thereby influencing behaviour (Carabotti et al., 2015; Martin & Mayer, 2017).

VI. Gut-brain axis - Mechanism of microbial neural crosstalk

The gut-brain axis (GBA) is the to-and-fro interaction between the central neural network and the enteric system through hormonal, neural and immunological pathways, and any dysfunction in this axis causes pathophysiological consequences (Cryan & Dinan, 2012). Apart from digestive function and satiation, GBA is important in physiological functions (Aziz & Thompson, 1998; Tache et al., 1980; Konturek et al., 2004). The primary function of GBA is to merge intestinal functions and connect emotional as well as intellectual centres of the brain with pathways involved in intestinal permeability, immune activation, enteric reflex, and entero-endocrine signalling (Carabotti et al., 2015). The alteration of the GBA mechanism is connected with variations in the stress response and behaviour (Rhee et al., 2009). The interactions of microbiota with the brain probably start at early foetal development. Bacteria, as well as their metabolites, get transferred between mother and developing baby via amniotic fluid, and the actual colonisation begins after birth upon exposure to environmental microbes (Toh & Allen-Vercoe, 2015). Interaction via direct and indirect pathways between the beneficial gut microbiota and the central nervous system (CNS) signals positive effects on neurological health. For instance, certain strains of *Lactobacillus* contribute positive effect on stress and anxiety-related behaviours (Borrelli et al., 2016). The vagus nerve provides pathway for bidirectional communication between gut and brain (Forsythe et al., 2014). Other pathways influence GBA are immune system modulation, hypothalamic-pituitary-

adrenal (HPA) axis, and tryptophan metabolism (Erny et al., 2015; Sudo et al., 2004; O'Mahony et al., 2015).

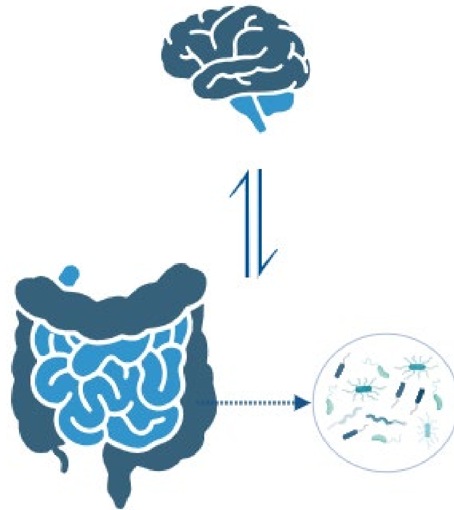

Figure 6: *Gut-Brain axis illustration (Created in https://BioRender.com)*

The gut bacteria can also influence GBA via production of neurotransmitters and SCFAs, which are considered as the important brain modulators (Calvani et al., 2018; Dalile et al., 2019; Stilling et al., 2016). Acetate, propionate, and butyrate, are the fermentation products of dietary fibers and complex starchs in the colon by gut bacteria (Pascale et al., 2018). The main transporters of SCFAs are monocarboxylate transporters (MCTs), they transfer SCFAs directly into the colonocytes. The MCTs are also present in other tissues but their expression is different in different tissues (Vijay & Morris, 2014; Schönfeld & Wojtczak, 2016). Greater expression of MCTs found in the endothelial cells, they help in transferring SCFAs through the blood-brain barrier (BBB) and enter into the brain tissue (Oldendorf, 1973; Kekuda et al., 2013). The supplementation of *Clostridium butyricum* enhanced the concentration of butyrate in the brain of diabetic mice and exhibited neuroprotective effects (Sun et al., 2016; Liu et al., 2015). Acetate is involved in the appetite regulation, it increased the expression of anorexigenic neuropeptide in mice, which in turn modified the

levels of the neurotransmitters such as glutamate, glutamine, and GABA in the hypothalamus (Frost et al., 2014). Butyrate and propionate influence cell signalling through maintaining intracellular potassium levels (Oleskin & Shenderov, 2016). These SCFAs together affect brain neurochemistry by regulating the expression of two enzymes tyrosine hydroxylase and tryptophan 5-hydroxylase. The tyrosine hydroxylase is involved in the production of dopamine, norepinephrine, and adrenaline, whereas tryptophan 5-hydroxylase is involved in the serotonin production (Nankova et al., 2014; Clarke et al., 2014; Reigstad et al., 2015; Yano et al., 2015; Dalile et al., 2019; Silva et al., 2020).

In rodents, sodium butyrate facilitates long-term memory consolidation, BDNF (Brain-derived neurotrophic factor) expression, and neurogenesis (Levenson et al., 2004; Wei et al., 2015; Yoo et al., 2011; Kim et al., 2009; Silva et al., 2020). In humans, SCFAs help neural progenitor cells to increase their growth rate and regulate circadian rhythm, appetite, and sleep (Yang et al., 2019; Torres-Fuentes et al., 2019; SZentirmai et al., 2019; Silva et al., 2020). Through the activation of GPCR and inhibiotion of HDACs, SCFAs perform their functions (Nankova et al., 2014; Patnala et al., 2017; Silva et al., 2020; Wang et al., 2025). The probiotic genus, *Lactobacillus* can synthesis neurotransmitters, such as serotonin, glutamate, GABA, dopamine, histamine, and acetylcholine (Tanous et al., 2005; Zareian et al., 2012; Kim & Shim, 2023). Whereas, *Lactococcus* produce histamine, glutamate, GABA, dopamine, and serotonin. *Escherichia coli* is involved in the synthesis of GABA, dopamine, norepinephrine, and serotonin (Roshchina, 2010; Shishov et al., 2009; Kim & Shim, 2023). Bacillus species aid in the production of acetylcholine, dopamine, and norepinephrine (Özogul, 2011; Shishov et al., 2009; Kim & Shim, 2023).

Other genera, such as *Streptococcus* and *Enterococcus*, release histamine and serotonin; *Streptococcus* also synthesizes dopamine. Furthermore, Bifidobacterium and *Pseudomonas* have been linked to the production of GABA, and *Corynebacterium glutamycum* is a prominent producer of glutamate (Kim & Shim, 2023). This evidence highlights the varied neurochemical repertoire of gut microbiota and its likely function in modifying host behaviour through the gut-brain axis. Autism spectrum disorder (ASD) involves a spectrum of behavioural aberrations, including repetitive behaviours, communication impairments, and sensitivity to environmental changes. Also, in ASD individuals, compositional differences in gut microbiota have been observed (Downs et al., 2014; Taniya et al., 2022). The microbial composition in autistic patients is marked by an increase in Proteobacteria, Lactobacillus, Bacteroides, Clostridium, and Faecalibacterium, while Bifidobacterium, Blautia, and Prevotella were found to be lower (Mehra et al., 2022). Because of the altered gut microbiota, autistic patients show disrupted intestinal permeability or leaky gut. It results in the release of endotoxin known as lipopolysaccharide (LPS), which can alter the CNS through increased activities in the amygdala, a brain centre that regulates emotions and behaviour (Haba et al., 2012; Mehra et al., 2022). Microglial cells act as primary immune cells in the brain and are involved in various activities, including immune surveillance, neuroprotection, synaptic patterning, and cellular phagocytosis. The gut microbiota also influences the development of microglial cells. For instance, mice exposed to antibiotics or germfree conditions resulted in altered microglial ratios and immature phenotypes (Erny et al., 2015; Kim & Shim, 2023). So, any perturbations in the gut microbiota can significantly affect behavioural profiles via modulations in brain neurochemistry.

VII. Zebrafish intestinal framework

The intestine is the principal organ where the breakdown and absorption of nutrients occur, as well as the waste removal. The development, structural composition, and biological roles of the intestines of zebrafish and mammals are remarkably similar. Also, the distribution of leukocytes and antimicrobial gene expression along the intestine is moderately preserved between zebrafish and mammals (Oehlers et al., 2011; Wang et al., 2010; Flores et al., 2020). Zebrafish intestine consists of three segments with unique morphological features: S1-S5, S6, and S7. S1 to S4 are functionally similar to the mammalian small intestine and are marked by the absorption of proteins and lipids. S5 constitutes an intermediate region. Whereas, S6 and S7 are analogous to the mammalian large intestine as they exhibit increased expression of molecular markers, examples include aquaporin3 (aq3p) and cofilin1 (cfl1), which are independently involved in water absorption and actin filament stabilisation (Wang et al., 2010; Xia et al., 2022). However, the architectural differences in the digestive system are apparent between mammals and zebrafish. In mammals, the oesophagus, stomach, small intestine (duodenum, jejunum, and ileum), and colon are the four separate compartments that make up the gastrointestinal tract, which is a long and twisted tube. Adult zebrafish, on the other hand, have no stomach; instead, their intestine is a straightforward tapered tube that is divided into three sections: the anterior, middle, and posterior intestines. The anterior intestinal bulb in zebrafish possesses a larger lumen than its posterior; it may serve as a reservoir similar to the stomach of mammals (Flores et al., 2020; Carten & Farber, 2009; Ramos-Morales, 2012; Xia et al., 2022).

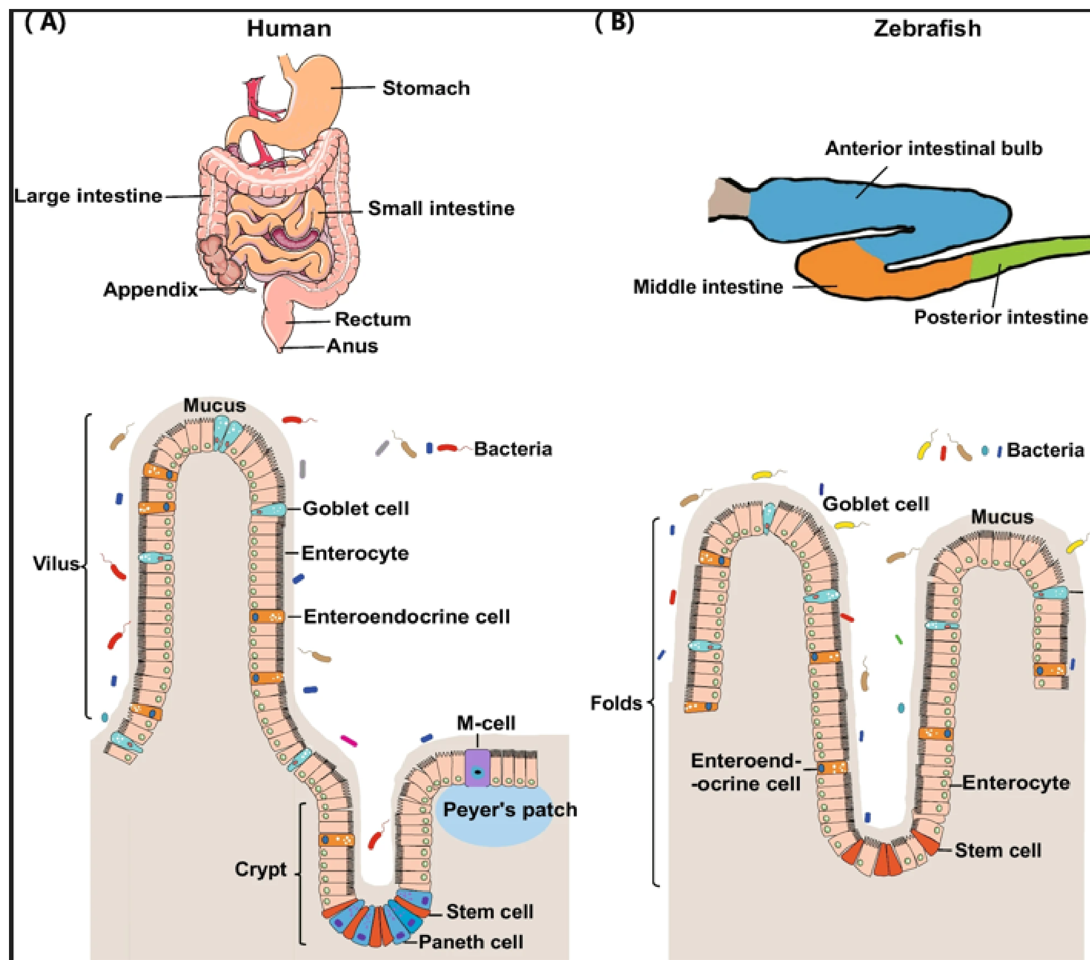

Figure 7: Comparison of Human and Zebrafish Intestinal Anatomy: *The structure of the intestines in humans (A) and adult zebrafish (B) shows some similarities and differences. In both, epithelial cells become either secretory cells or nutrient-absorbing enterocytes as they move upwards-along finger-like villi in humans or folds in zebrafish. The zebrafish intestine has three main sections: the front part called the anterior intestinal bulb (blue), the middle section (orange), and the back part or posterior intestine (green). In zebrafish, the mature intestinal lining contains enterocytes, enteroendocrine cells, and mucus-producing goblet cells. Unlike humans, zebrafish do not have intestinal crypts, Paneth cells, or M-cells (Xia et al., 2022).*

The tissue organisation in the zebrafish gut is also simpler than that of mammals, as they possess only mucosa, muscularis externa, and serosa layers. The lamina propria, which contains muscle fibers, lymphatic vessels, and blood capillaries, lies

beneath the mucosa and epithelium. The lamina propria is covered by muscularis externa, consists of circular and longitudinal enteric nerve cells and smooth muscle fibers (Wallace & Pack, 2003). The mucosa lining is folded into several large intestinal folds similar to mammalian villi and intestinal crypts (Pack et al., 1996; Wallace et al., 2005; Wang et al., 2010; Flores et al., 2020). The goblet cells in zebrafish are distributed throughout the mucosa, instead of being restricted to crypts (Wang et al., 2010). Zebrafish lack the muscularis mucosa, the submucosa layer, Paneth cells and Peyer's patches. Hence, they possess goblet cells, enterocytes, and enteroendocrine cells with conserved roles in mucus secretion, hormone synthesis, and nutrient absorption (Flores et al., 2020; Xia et al., 2022). Moreover, the enteric neurons and glial cells make up the enteric nervous system (ENS), a functionally significant part of the GI system in both zebrafish and mammals (Flores et al., 2020). Therefore, the bacterial metabolites can act upon the nervous system, for example, the tryptophan catabolites produced by gut microbiota can trigger 5-HT secretion and Trpa1 channels in enteroendocrine cells, which in turn activate zebrafish's enteric and vagal neurons (Ye et al., 2021).

VIII. Zebrafish gut microbiome: A translational model for host

Zebrafish have gained increased attention as a significant model organism for gut microbiota studies. By virtue of their affordability, remarkable genetic stability and developmental fidelity, ability to generate germ-free (GF) larvae, efficient drug screening, and suitability for real-time imaging, they are widely used in studying gut microbiome and host-microbiota interactions (Roeselers et al., 2011; Xia et al., 2022; Zhong et al., 2022). The external fertilisation in zebrafish and less microbial exposure at hatching make them suitable for defined strain colonisation in GF or single-strain colonisation conditions (Pham et al., 2008). When a group of zebrafish

is housed in the same aquatic environment, it facilitates a higher magnitude of biological replication and knowledge about microbial populations in the particular environment (Stephens et al., 2016). Though zebrafish gut shows a simpler structure and microbial composition compared to mammals, they exhibit functional homology in many areas, making them more appealing for microbiome-related functional analysis (Abu-Siniyeh et al., 2025; Xia et al., 2022).

A study regarding the core gut-microbiome of zebrafish by Roeselers et al. (2011 revealed that γ-Proteobacteria and Fusobacteria classes are ordinary members of the gut microbiota in zebrafish, and the majority of Fusobacteria sequences isolated from the zebrafish intestine were closely related to *Cetobacterium somerae* (Roeselers et al., 2011), followed by Firmicutes, Bacteroidetes and Actinobacteria (Stephens et al., 2016). Microbial analysis revealed that mammals and zebrafish share most of the gut microbial population, and they show similar functions in the digestive tract (Rawls et al., 2006; Xia et al., 2022). Firmicutes and Bacteroidetes phyla dominate in case of mammals, while zebrafish microbiota predominates with Proteobacteria and Fusobacteria phyla (Roeselers et al., 2011; Garibay-Valdez et al., 2024).

The gut microbiota of larval zebrafish is less diverse with major phyla include Proteobacteria, while adult zebrafish exhibit greater taxonomic diversity (Burns et al., 2017; Stephens et al., 2016). The major genera found in the larval stage are composed of *Vibrio, Aeromonas, Plesiomonas, Pseudomonas, Shewanella*, and *Acinetobacter*. Whereas, the juvenile stage is characterised by the presence of Proteobacteria along with Fusobacteria and Bacteroidota as dominant phyla. *Cetobacterium, Plesiomonas, Aeromonas, Vibrio,* and *Flavobacterium* are the major genera observed in juvenile stage. However, a balanced microbiota is seen in adult

stages with the presence of Proteobacteria and Fusobacteria as major phyla. *Cetobacterium, Aeromonas, Acinetobacter, Plesiomonas, Vibrio,* and *Firmicutes* are the major genera in adult stages (Garibay-Valdez et al., 2024).

This microbial composition is very dynamic in zebrafish and is influenced by host genotype, developmental stages, diet and environmental exposures (Wong et al., 2015). Particularly, microbial diversity and richness seem to rise with age, the same patterns observed in both human and mouse models (Stagaman et al., 2020). Therefore, zebrafish can be used to study how dietary or environmental changes affect the gut microbial community. This gut microbial colonisation influence Gut morphology, including the development of enterocytes, goblet cells, and enteroendocrine cells (Walker, 2014). Also stimulates innate immunity by the activation of toll-like receptors (TLRs), nuclear factor-kappa B (NF-κB), and cytokines including IL-1β, TNF-α, and IL-10, to resist pathogenic invasion and maintain intestinal homeostasis (Kumar, 2020). Thereby stabilising the bidirectional communication between the gut and the brain. The omics-based approaches, including 16S rRNA gene sequencing, metagenomics, metabolomics, proteomics and transcriptomics, are extensively utilised in zebrafish microbiome research to derive knowledge about host–microbe interactions at the molecular level (Yang et al., 2025). The gnotobiotic procedures, microbiota transplantation techniques, and imaging-based microbial localisation further strengthen the experimental flexibility of zebrafish microbiome research (Pham et al., 2008; Rawls et al., 2006; Høgset et al., 2020). Moreover, CRISPR/Cas9 gene editing techniques facilitate targeted alterations of host genes associated with microbial detection and signalling pathways (Li et al., 2016). Research has shown that numerous factors, including dietary conditions, environment, antibiotic exposure, prebiotics, probiotics, microplastics

and heavy metals, significantly shift microbial communities (Sieler et al., 2023; Sharpton et al., 2023; Almeida et al., 2019; Ehsannia et al., 2022; López Nadal et al., 2020; Ye et al., 2019; Yu et al., 2023). Furthermore, zebrafish are also used in investigating microbiome-targeted treatments involving synbiotics and psychobiotics; their capability is proven preclinically (Zhong et al., 2022; Cruz et al., 2020; Allen et al., 2016). Considering the limitations, zebrafish has now become a robust model in translational research.

IX. Significance of the Study

Gut-brain axis refers to a two-way system that has an essential function in controlling neurological, metabolic and immune processes. The recent findings indicate that gut microbiota composition may be influenced by internal and external environmental factors. These microbial communities also have an impact on behaviour, mood, cognition and even neurodevelopment. Though rodent models have extensively utilised in gut microbiome research, zebrafish are presently being acknowledged as a strong translational model organism, owing to their genetic tractability, behavioural traits, transparency, and high through put screening. However, the environmental modulation of the zebrafish gut microbiome and how these alterations influence behaviour is not comprehensively assessed, especially in relation to several real-world variables like diet, antibiotics, environmental enrichment, and photoperiod. Thus, we carried out this research to address the influence of environmental factors on gut and behaviour which in turn form gut-brain-behaviour axis.

X. Objectives of the Study

- To establish and maintain zebrafish under controlled experimental conditions, including: (a) varied dietary regimes, (b) antibiotic treatments and enriched environmental settings, and (c) distinct photoperiod schedules.
- To examine how different environmental conditions influence the composition and dynamics of gut microbial communities in zebrafish.
- To explore how environmental modulation influence behavioural phenomic patterns in zebrafish. [All these objectives are discussed in following chapters]

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CHAPTER 1

Influence of dietary regimes on behaviour, morphometry, and gut-microbiota in zebrafish

Rationale: Diet is a key determinant of an organism's overall health and well-being. Modifications in dietary regimes can lead to significant changes in growth, metabolism, and other physiological functions. In zebrafish, studies linking diet to behavioural patterns and gut microbiota dynamics remain scarce. Elucidating this relationship could provide valuable insights into how dietary factors modulate the gut-brain-behaviour axis

1.1 Graphical Abstract

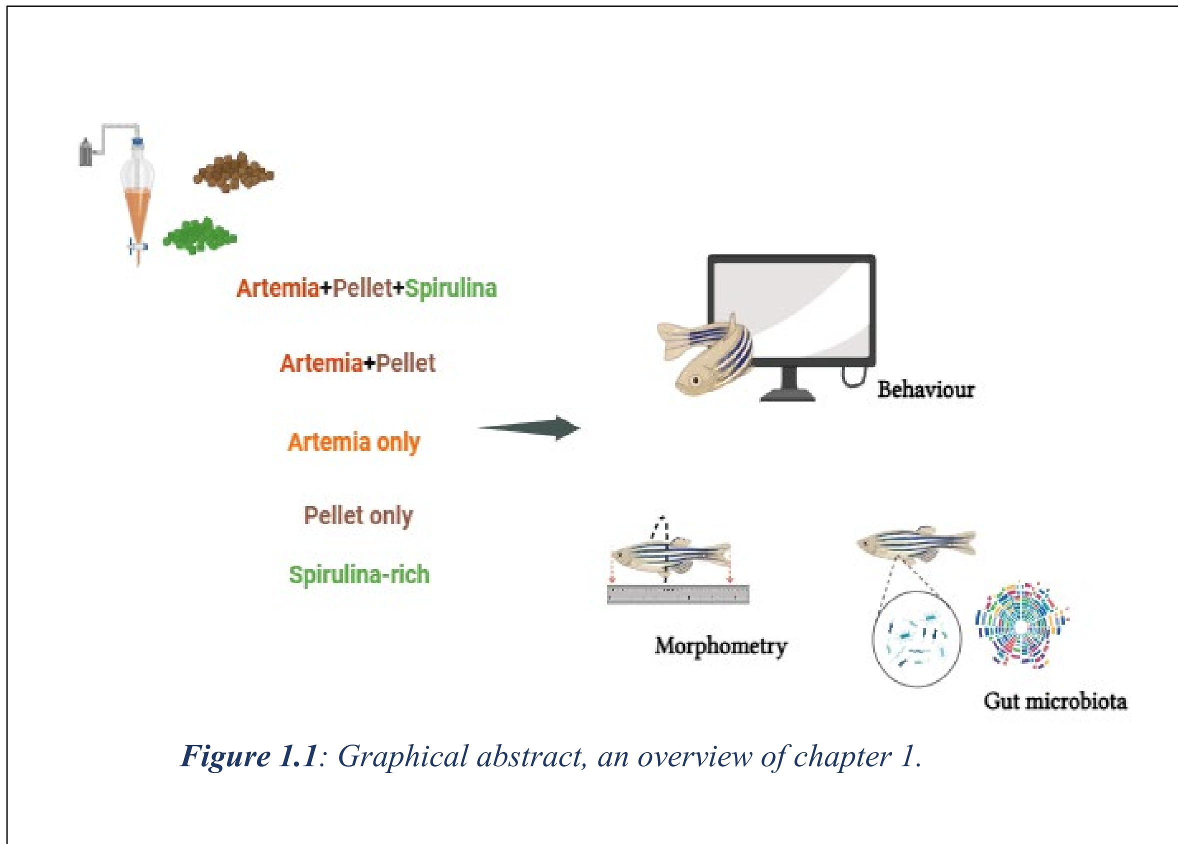


***Figure 1.1**: Graphical abstract, an overview of chapter 1.*

1.2. Introduction

The interplay between genetic and environmental factors is fundamental in shaping animal behaviour (Breed & Sanchez, 2010). While genetic factors provide the blueprint and are modulated by intrinsic mechanisms, the external environment acts as a governing factor influencing the expression of these genes (Virolainen et al., 2023). Diet as an environmental factor interacts with genetic predispositions to modulate the physical and mental states of an organism. Nutritional imbalances at any stage of life can influence behaviour (Han & Dingemanse, 2015; Heianza & Qi, 2017). Although diet is a primary determinant of gut microbiota composition (Song & Chan, 2018; Nova et al., 2022; Liu et al., 2024), its modulation can have far-reaching effects on various metabolic, physiological, and neurological processes (Fujisaka et al., 2023; Andoh, 2016; Ullah et al., 2023), potentially triggering neurobehavioural changes, namely anxiety and depression (Butler et al., 2023; Kumar et al., 2023; Xiong et al., 2023). For instance, research on Mediterranean fruit flies has conveyed that early nutritional conditions can predict distinct behaviours expressed in adulthood (Romanyukha et al., 2004). Similarly, in fish, the quality of diet appears to affect welfare significantly by influencing both physical condition and behaviour (O'Brine et al., 2015). Zebrafish (*Danio rerio*) have come to be regarded as a noteworthy model organism across diverse research domains, including nutrition, behavioural neuroscience, and gut microbiota studies (Witten et al., 2017; Baranasic et al., 2022; Nittoli et al., 2021; Kalueff et al., 2014; Fowler et al., 2019; Demin et al., 2020; Chia et al., 2019; Borrelli et al., 2016). There are numerous established behavioural tests in zebrafish to quantify locomotion, exploration, anxiety, and aggression (Cachat et al., 2011; Maximino, 2018; Midttun et al., 2020). Also, research on the gut microbiota is increasing rapidly in this model

species (Tan et al., 2019; Wang et al., 2021; Lin et al., 2022; Castro et al., 2023; Kiran et al., 2024). Therefore, we utilised zebrafish to study gut microbiota and behaviour.

Diet is regarded as a key factor, capable of influencing one's behaviour. In insects, the physical activity is greatly influenced by diet qualities, studies indicate that insects raised with high-quality diets exhibit greater physical activity compared to those with low-quality diets (Tremmel & Müller, 2013). Whereas, in zebrafish, short time high-fat diets induced neurobehavioural alterations, including increased aggression and anxiety (Picolo et al., 2021). Zebrafish growth and morphology are greatly influenced by their diets, zebrafish reared with *Artemia*-only diet resulted smaller and less sexually dimorphic individuals compared to those reared with mixed diets (Craig et al., 2012; Gonzales, 2012). Obesity and cardiovascular strain are found to be increased in zebrafish fed with high-fat diets (Meguro et al., 2019; Vargas & Vasquez, 2017). In contrast, diet rich in docosahexaenoic acid (DHA), have been reported to enhance the gamete quality and larval health (Nowosad et al., 2017). The microalgae, spirulina is considered as an effective component in zebrafish diet, because of their high-nutritional value. It improves growth, digestive health, immune responses, and fertility in zebrafish (Roohani et al., 2019; Coli et al., 2024; Ahmadifar et al., 2023; Calabro et al., 2021). Live and commercial pellet feeds are usually used in laboratories to rear and maintain zebrafish cultures. Paramecium, rotifers, tetrahymena, and *Artemia* nauplii are the common live feeds, in which *Artemia* (brine shrim) is the most widely used (El-Magsodi et al., 2014; Leger et al., 1986; MacRae, 2003; Persoone & Sorgeloos, 1980; Triantaphyllids et al., 1998; Avdesh et al., 2012; Lahnsteiner et al., 2023).

The physiological process such as feeding, digestion, metabolism, immune response, and energy equilibrium are greatly influenced by the gut microbiota (Burokas et al., 2015; Goncalves & Gallardo-Escarate, 2017; Johnson & Foster, 2018; Mayer et al., 2015). Dietary choices shape gut microbial ecosystems, which, in turn, influence host health and disease sensitivity. High-Fiber diets impart positive influence on gut microbiota, it supports beneficial microbes that generate short-chain fatty acids (SCFAs) and are critical for host health (Dapa & Xavier, 2024). In contrast, diets rich in carbohydrates result dysbiosis and cause reduced microbial diversity and increased pathogenic microbes (Zhu, 2024). In zebrafish, dietary composition has been observed to shape gut microbiota significantly. For instance, bacterial protein-based diets support distinct microbial communities and metabolic pathways compared to fish protein hydrolysate diets (George et al., 2024). Deviations in dietary formulations influence microbial diversity and composition. Also affecting the gut microbiome's role in growth, development, and immune responses (Sieler et al., 2023).

The gut microbiome has a central role in the gut-brain axis, facilitating bidirectional communication between the gastrointestinal and central nervous systems. Through its influence on gut and brain, microbiome contributes to maintaining host physiological equilibrium (Cryan & O'Mahony, 2011; Sherwin et al., 2016; Vigneri, 2014; Bienenstock et al., 2015). Despite the recognised importance of diet and gut microbiota on behaviour, studies exploring how dietary regimes influence gut microbial composition and behaviours such as anxiety, exploration, and aggression in zebrafish remain limited. To address this gap, the present study investigates the impact of different dietary regimes, comprising *Artemia* (*OSI Red Ring Artemia Cysts*), pellet feed (*The Tiqlid nursery floating pellet, 0.8 mm*, widely used in

ornamental fish culture), and a spirulina-rich pellet diet (*Maalavya Spirulina-rich fish food*)-on zebrafish exploratory, anxiety, and aggression behaviours, as well as morphology. Additionally, we evaluate the influence of these diets on gut microbial populations, aiming to elucidate diet-driven microbial dynamics that contribute to behavioural outcomes.

1.3. Materials and methods

1.3.1. Ethical statement

The use of zebrafish in this study was permitted by the Institutional Animal Ethics Committee (IAEC) of Calicut University (permit number: 426/GO/Re/S/01/ CCSEA). All experimental procedures covering animals were conducted in strict compliance with the guidelines outlined by the Committee for the Control and Supervision of Experiments on Animals (CCSEA), 2021. Initiatives were undertaken to minimise the number of animals used and to ensure their welfare, with all efforts made to reduce potential distress and suffering during experimental procedures.

1.3.2. Dietary Groups and Experimental Setup

At 5 days post-fertilisation (dpf) zebrafish larvae were randomly segregated into five dietary groups: *Artemia*+Pellet+Spirulina mixed feeding group (APS), *Artemia*+Pellet group (AP), *Artemia*-only group (A), Pellet-only group (P), and Spirulina-rich group (S). Each group was maintained in triplicate within tanks measuring 36 cm × 26 cm × 19 cm, with a total volume of 16 L. Twenty larvae were housed in each tank under optimal water quality conditions, with water temperature maintained at 27 ± 1°C, pH between 6.5 and 7.0, and a 14:10 light/dark cycle at an intensity of 300-500 lux.

1.3.3. Feeding Regimens

All groups were fed twice daily. For the *Artemia*-only group (A), live *Artemia* (3 mL per 10 fish, as per Westerfield, 2007) was administered both in the morning and evening. The Pellet-only group (P) received Tiqld nursery pellets (30 mg/10 fish per feeding; pellet size: 0.8 mm) twice daily. For the APS group, an alternating feeding regimen was adopted to ensure dietary variety, consisting of *Artemia*, pellets, and spirulina-enriched pellets. The AP group was fed *Artemia* in the morning and pellets in the evening. The Spirulina-rich group (S) was exclusively provided with spirulina-enriched pellets. During the first month, larvae in the pellet-fed and spirulina-enriched groups were given powdered forms of their respective feeds to accommodate their smaller oral anatomy. From the second month onward, whole pellets were introduced as their oral capacity developed. Tanks were aerated beginning in the second month to maintain water quality, and one-third of the water volume was replaced daily to control ammonia levels, following established ZFIN protocols and CCSEA guidelines for zebrafish housing and maintenance. Following a 4-month feeding period, zebrafish were subjected to a series of behavioural assays over a subsequent 2-month period. During this time, each group continued on its designated dietary regimen to ensure consistency and minimise confounding factors. The components present in Pellet nursery feed, *Artemia* nauplii, and Spirulina-rich pellets;

Tiqlid nursery feed composition: Fish meal, corn starch, wheat gluten, whole egg powder, wheat flour, Ca-caseinate, spirulina, brewer’s yeast, fish oil, alfalfa, spinach, carrots, garlic, beta-glucanase, astaxanthin. [Crude fat min 4%, Crude fibre min 5%, Protein 52%]. *Artemia* nauplii: [Total protein min 37%, Total lipid min 15%, Total carbohydrate 11%, Ash 2%]. Spirulina-rich pellet: fish meal, spirulina,

wheat flour, soybean meal, corn meal, yeast, fish oil, rice flour, vitamins and minerals. [Crude protein 28%, Crude fibre 4%, Crude fat 3%, Crude ash 3%].

1.3.4. Novel Tank Test (NTT)

After completing the 120-day feeding regimen, zebrafish from all five dietary groups were subjected to a novel tank diving test to assess exploratory behaviour and anxiety-like responses (Fontana et al., 2022). Prior to testing, individuals were acclimatised for 5 minutes in an opaque beaker to minimise handling stress. Subsequently, each fish was gently transferred into an experimental tank (dimensions: 27.9 cm × 7 cm × 15 cm, with a bottom length of 22 cm) containing 1.35 L of system water. Behavioural activity was recorded for 6 minutes using a Logitech C270 HD 720p webcam positioned 35 cm from the front of the tank. To ensure uniform testing conditions, all behavioural trials were performed in between 10:00 AM and 4:30 PM in a controlled, closed-room environment, following the protocols described by Cachat et al. (2011). The experimental tank was virtually divided into two zones: the top and bottom, prior to video analysis. Video recordings were analysed using SMART 3.0 software (Panlab-Harvard Apparatus, USA) for automated tracking of zebrafish movement and time spent in each zone. This zonation approach enabled quantitative assessment of vertical exploratory behaviour and anxiety-like tendencies.

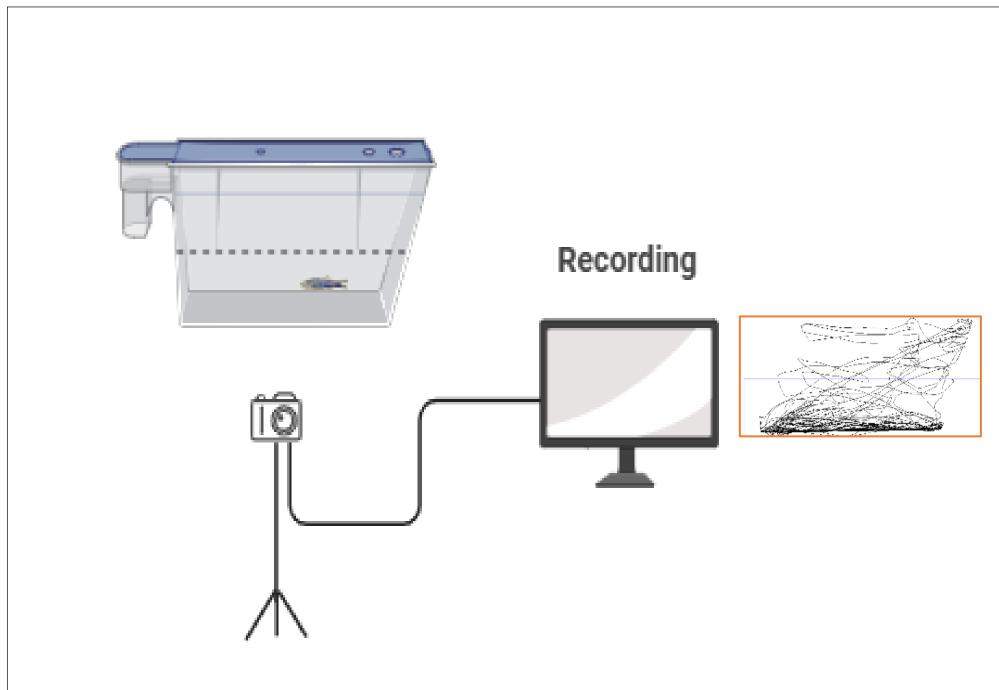


Figure 1.2: *Novel Tank Test, illustration representing behavioural recording.*

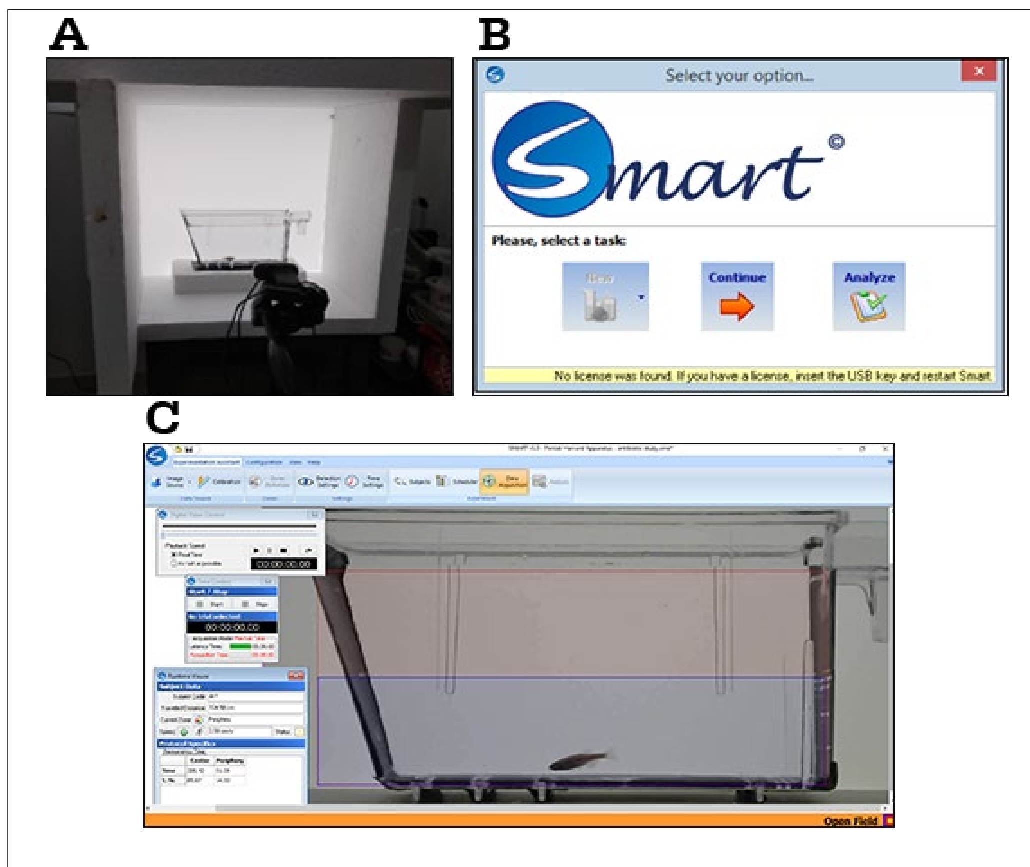


Figure 1.3: *Novel Tank Test, (A) Experimental set up (B) Smart software 3.0 (C) Video analysis interface of Smart 3.0.*

1.3.5. Light and Dark Test (LDT)

The light/dark preference test, a widely employed behavioural assay in zebrafish neuroscience, assesses anxiety-like behaviours based on the intrinsic preference of adult zebrafish for dark versus light environments. An acrylic tank (dimensions: 45 cm × 10 cm × 15 cm; L × W × H) was constructed, with the interior equally divided into black and white compartments. The water column height was maintained at 10 cm, corresponding to a total tank volume of 4.5 L. Central sliding doors, colour-matched to the respective compartments, created a central chamber (dimensions: 15 cm × 10 cm × 10 cm) used for acclimatisation. Individual zebrafish were transferred to the central chamber using a fine-mesh net and allowed to acclimatise for 3 minutes. Following acclimatisation, the sliding doors were gently removed, permitting the fish to explore the tank freely for a 10-minute testing period. Behavioural activity was recorded from a top-mounted camera to ensure good and proper video capture. The recorded videos were analysed by two experienced observers, they are not aware of the experimental conditions to minimise the bias. And ensured inter-rater reliability greater than 0.90. The above-mentioned methodology is previously standardised and published in research papers (Maximino, 2018). This light/dark test is a reliable method to measure anxiety-like behaviours in zebrafish, where anxiogenic effect is marked with reduced time spent in the white compartment and increased latency to enter this area.

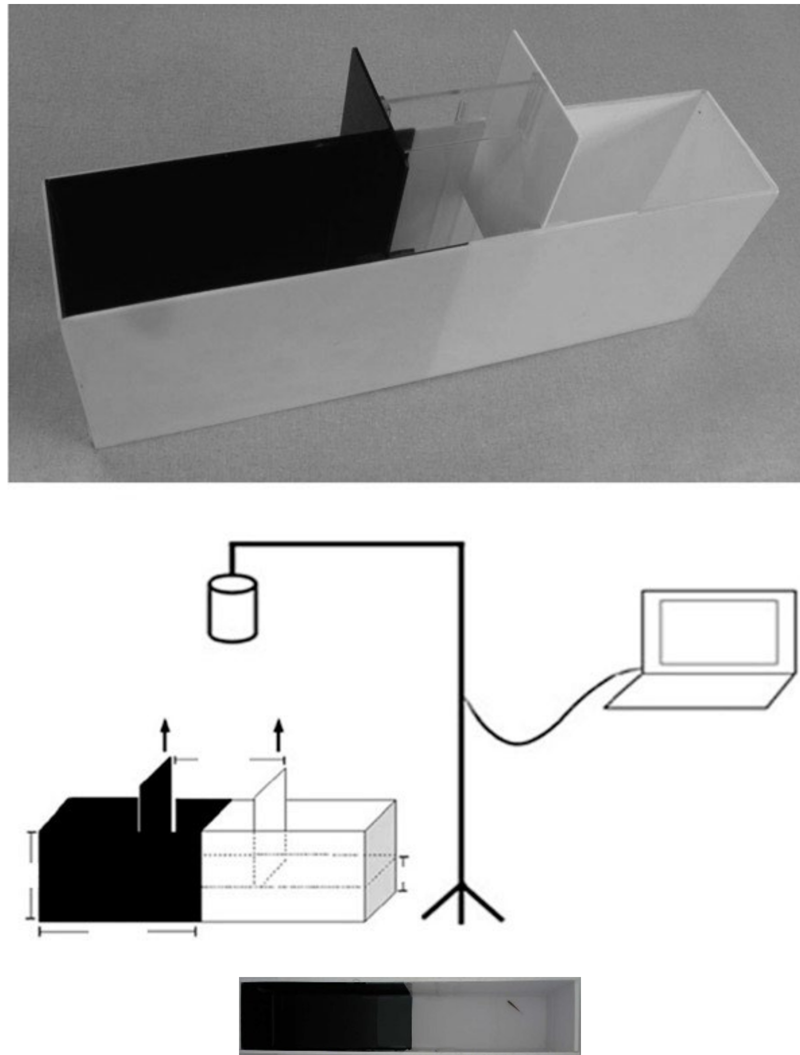


Figure 1.4: Light-Dart Test, the test tank having equally divide dark and white compartment and the experimental setup (Maximino, 2018).

1.3.6. Mirror Induced Aggression (MIA)

The mirror-biting test was performed to measure aggression by analysing various behavioural parameters, including the time spent in the mirror zone, latency to enter the mirror zone, the number of entries into the mirror zone, and the frequency of mirror bites. The test utilised a three-chambered apparatus with dimensions of 15 × 60 × 20 cm (W × L × H) was equally divided into three compartments and filled with 4 L of water. Individual fish were introduced into the central chamber and video-recorded for an initial period of 6 minutes. Following this acclimation period,

a mirror was placed on either the left or right wall of the apparatus, adjacent to the central chamber, in an alternating pattern to ensure unbiased placement. The fish, confined to the central chamber, were then allowed to interact with their mirror image, and behaviour was recorded for an additional 6 minutes, as described by Midttun et al. (2020). The central chamber was virtually divided into two zones: the mirror zone-MZ (comprising 1/3 of the chamber, also referred to as the approach zone) and the non-mirror zone-NMZ (comprising the remaining 2/3 of the chamber, also referred to as the avoidance zone). Behavioural recordings were analysed using Smart 3.0 software, while the frequency of mirror bites was manually quantified by two observers unaware of the experimental conditions (The inter-rater reliability was> 0.90), ensuring consistency and accuracy in data collection.

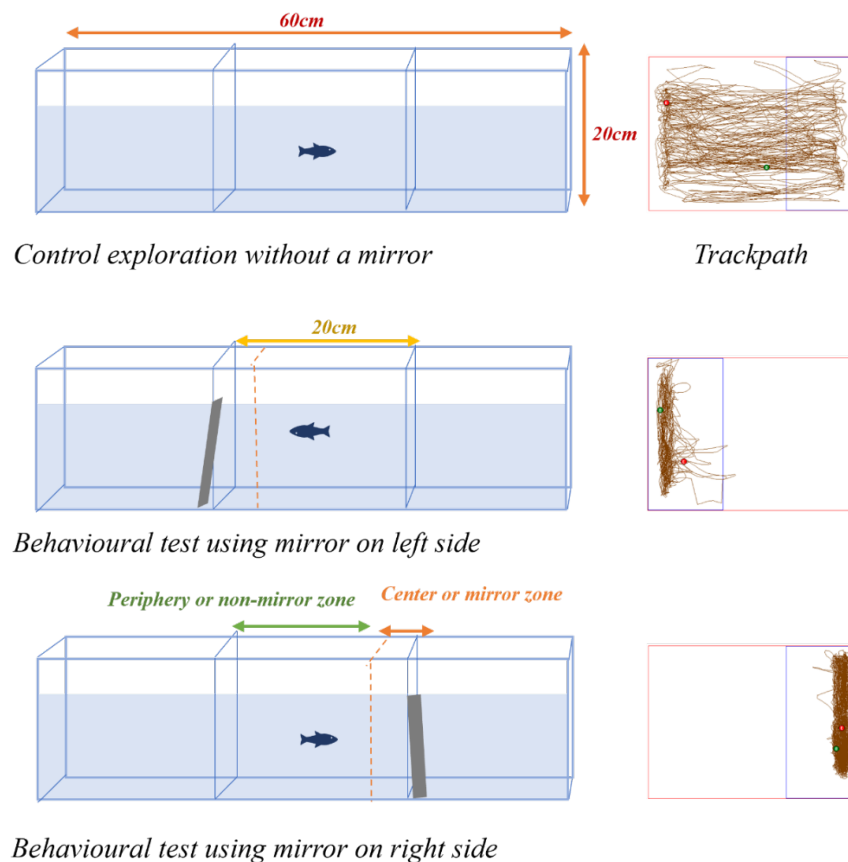


Figure 1.5: *Mirror-Biting Test, illustration showing zebrafish exploration in the presence and absence of mirror.*

1.3.7. Morphological measurements

After completing the behavioural experiments, morphometric measurements, including body weight, standard length, and girth, were recorded. Hypothermia was applied as an anaesthetic agent. The water temperature was gradually lowered to approximately 4-6°C using chilled water or ice, as mentioned by Collymore et al. (2014). Zebrafish were immobilised temporarily through cooling and placed on a scaled platform to measure standard length and girth. Body weight was determined using a high-precision electronic balance. To minimise stress and ensure the accuracy of measurements, all procedures were performed promptly during the brief window of immobility induced by hypothermia. Following the measurements, zebrafish were immediately transferred to a recovery tank with aerated water, where the temperature was gradually restored to normal conditions and ensured complete recovery before subsequent handling or observations.

1.3.8. Statistical analysis

Statistical analyses were conducted to evaluate data distribution and group differences. The Kolmogorov-Smirnov test, combined with the Dallal-Wilkinson-Lilliefors p-value test, was used to assess data normality, which is appropriate for moderate sample sizes and does not assume known population parameters. Bartlett's test was employed to examine the homogeneity of variances, which determine the suitabitity of parametric comparisons. For normally distributed data, group means were compared using One-Way ANOVA, with Tukey's post hoc test applied for multiple comparison corrections. Results were reported with 95% confidence intervals to provide an estimate of effect precision. For data with non-normal distribution, the Kruskal-Wallis test was performed, followed by Dunn's multiple comparison tests to evaluate differences between groups while adjusting for multiple

testing. Changes within groups were quantified using paired Cohen's *d* to estimate effect size, enabling interpretation of biological relevance beyond statistical significance. Correlations between variables were assessed using Spearman's rank correlation coefficient, chosen due to its robustness to non-normal distributions and suitability for monotonic relationships. All statistical analyses and visualisations were performed using GraphPad Prism (version 9.5) and Python v3.10. A significance threshold of $p < 0.05$ was applied throughout the analyses.

1.3.9. Gut dissection and DNA extraction

Dissection tools were autoclaved and thoroughly dried before use. Surface disinfection was performed using 70% ethanol. Six adult zebrafish were randomly selected, with two individuals (one male and one female) sampled from each tank in the triplicate experimental setup. Zebrafish were euthanised using hypothermia for 30 seconds and pinned on a dissecting mat. The skin and underlying musculature were carefully removed, and the gastrointestinal tract was dissected out from the body following the protocol described by Gupta *et al.* (2010).

The dissected whole gut samples were homogenised using a grinding pestle (separate new grinding pestle for each sample), and the resulting homogenate of each sample was transferred into clean DNA free microcentrifuge tubes. DNA was extracted from the homogenate using the ORIonX Genomic DNA Kit (Origin Lab, India). The extraction protocol involved adding 200 µL of Buffer GA and 20 µL of Proteinase K to the homogenate, followed by thorough mixing via vortexing. Samples were incubated at 56°C until complete tissue lysis was achieved. Subsequently, 200 µL of Buffer GB was added and vortexed thoroughly, and the samples were incubated at 70°C for 10 minutes to obtain a homogeneous solution. After a brief centrifugation, 200 µL of ethanol (96–100%) was added and vortexed

for 20 seconds. The mixture was then pipetted into a CB3 spin column (placed in a 2 mL collection tube) and centrifuged at 12,000 rpm for 45 seconds. The flow-through was discarded, and the spin column was reinserted into the collection tube. A total of 500 µL of Buffer GD was added to the spin column, followed by centrifugation at 12,000 rpm for 30 seconds. The flow-through was discarded, and the spin column was washed with 700 µL of Buffer PW, and centrifuged for 30 seconds, and the flow-through was discarded. The spin column was centrifuged again for 2 minutes to ensure the membrane was completely dry. The spin column was then transferred to a clean 1.5 mL microcentrifuge tube, and 50–200 µL of Buffer TE was added to elute the DNA. After incubating at room temperature for 2–5 minutes, the sample was centrifuged for 2 minutes. The eluted DNA was stored at -20°C for subsequent 16S rRNA amplicon-based metagenomic analyses

1.3.10. 16S rRNA gene amplicon sequencing

The DNA concentration in the filtrate was quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, USA) and confirmed to be at least ~30 ng/µL. PCR amplification targeted the V4 region of the 16S rRNA gene, and the resulting PCR products were visualised on a 2% agarose gel to confirm amplification. The PCR products were subsequently purified according to the Illumina HiSeq protocol for amplicon preparation. Library construction was performed using the NEB Next Ultra DNA Library Preparation Kit (New England Biolabs). The quality and quantity of the constructed libraries were assessed using the Agilent 2200 Tape Station system. Sequencing was conducted on the Illumina HiSeq 2500 platform, following the manufacturer's protocol, to generate high-quality reads for downstream metagenomic analysis (Sieler et al., 2023; Pothayi et al., 2024).

1.3.11. Bioinformatics analyses

Quality assessment of paired-end sequences generated from Illumina HiSeq sequencing was performed using FastQC-v0.11.9 (Andrews, 2010). Adapter removal and sequence trimming were conducted with FastP-v0.20.1 (Chen, 2023). Processed sequences were then analysed and taxonomically classified using Kraken 2, a K-mer-based sequence classification tool that utilises a pre-constructed PlusPFP database of k-mers derived from known genomes for rapid categorisation of input sequences (Lu & Salzberg, 2020). To refine and quantify taxonomic outputs, Bracken was employed to compute both absolute and relative abundance values based on Kraken 2 results (Lu et al., 2017). Alpha diversity metrics, including Observed Sequence Variants (OSVs), Chao1, ACE, Shannon, Simpson, and Fisher's diversity indices were calculated to evaluate within-group diversity. Beta diversity between groups was determined using the Bray-Curtis dissimilarity index. Visualisation of diversity metrics was achieved using specific R-packages to generate Principal Coordinates Analysis (PCoA) plots (vegan), alpha diversity plots (phyloseq, ggplot 2, dplyr, ggpubr), beta diversity plot (pheatmap, RColor Brewer, dplyr, ggplot2), and sankey plots (pavian). Bar plots depicting taxonomic compositions were created using Python.

1.4. Results

1.4.1. Effects of diet regimes on Novel Tank Test

Vertical exploratory and anxiety-like behaviours of zebrafish in response to a novel environment were assessed (Sabadin et al., 2022). behavioural endpoints were compared across dietary groups (APS, AP, A, P, and S), revealing significant differences. The representative heatmap of the area explored by different diet groups is shown in Figure 1.6. For the number of entries into the top zone, a statistically

significant effect of diet was observed ($F (4,120) = 34.77$, $p < 0.0001$). Post hoc analysis demonstrated that the APS diet group (52.44 ± 3.174) had significantly more entries compared to the AP (16.64 ± 2.286, $p < 0.0001$), A (13.24 ± 1.436, $p < 0.0001$), P (29.84 ± 3.422, $p < 0.0001$), and S (29.44 ± 2.248, $p < 0.0001$) groups. The AP and A groups showed significantly fewer entries compared to the P (AP vs. P: $p = 0.0046$; A vs. P: $p = 0.0002$) and S (AP vs. S: $p = 0.0065$; A vs. S: $p = 0.0002$) groups, with no significant difference between the AP and A ($p = 0.8886$) or between the P and S groups ($p > 0.9999$) (Fig. 1.7A). The highest number of entries was observed in the APS group, followed by the S and P groups, with the A and AP groups showing the lowest values. Significant differences were also observed for cumulative time spent in the top zone ($p < 0.0001$). Post hoc analysis revealed that the APS group (146.2 ± 10.65) spent more time in the top zone compared to AP (41.93 ± 6.826, $p < 0.0001$), A (33.83 ± 3.782, $p < 0.0001$), P (78.01 ± 8.741, $p = 0.0130$), and S (68.63 ± 6.720, $p = 0.0028$) groups. The A group spent less time in the top zone compared to the P ($p = 0.0037$) and S ($p = 0.0169$) groups, while the AP group spent less time in the top zone than the P group ($p = 0.0462$). No differences were detected between the AP and A, AP and S, or P and S groups ($p > 0.05$) (Fig. 1.7B). The higher time in the top zone for the APS group indicates reduced anxiety potentially due to a balanced nutritional composition. In contrast, the lower values for the A and AP groups suggest heightened anxiety might be due to nutrient deficiencies (Nabinger et al., 2021). The P and S diets partially met nutritional requirements. The diet also had a significant effect on mean swimming speed ($F (4,120) = 32.66$, $p < 0.0001$). Post hoc analysis revealed that the APS group (6.543 ± 0.2533) exhibited higher mean speed than the AP (4.522 ± 0.1687, $p < 0.0001$), A (3.720 ± 0.1634, $p < 0.0001$), and P (5.248 ±

0.2345, $p = 0.0001$) groups. The S group (6.123 ± 0.1694) also showed an increased speed compared to the P ($p = 0.0218$), A ($p < 0.0001$), and AP ($p < 0.0001$) groups. In contrast, the A diet demonstrated reduced mean speed compared to both the P ($p < 0.0001$) and the AP ($p = 0.0441$) groups. No statistically significant differences were observed between the APS and S groups or between the AP and P groups ($p > 0.05$) (Fig. 1.7C). These findings suggest that APS and S diets enhance activity levels. In contrast, the A and AP diets are associated with reduced mobility. Significant variations in total distance travelled were observed ($p < 0.0001$). Post hoc analysis showed a marked reduction in total distance travelled in the A group (1293 ± 56.76) compared to the APS (2310 ± 100.1, $p < 0.0001$), P (1829 ± 83.45, $p = 0.0007$), and S (1912 ± 78.95, $p < 0.0001$) groups. The APS group travelled significantly further than the AP ($p = 0.0003$) and P ($p = 0.0454$) groups. No statistically significant differences were observed between the APS and S, P and S, AP and P, or AP and A groups ($p > 0.05$) (Fig. 1.7D). These results further underscore the influence of dietary composition on zebrafish locomotion with the APS diet yielding the highest activity levels. Freezing time was also influenced by diet ($p < 0.0001$) with a post hoc examination finding sharp decrease in freezing time in the APS (0.5804 ± 0.3114) and S (0.3148 ± 0.0763) groups relative to AP (50.84 ± 7.006, $p < 0.0001$), A (85.65 ± 10.71, $p < 0.0001$), and P (63.40 ± 9.800, $p < 0.0001$) groups. No statistically significant variations were observed between APS and S, A and P, AP and P, or AP and A groups ($p > 0.05$) (Fig. 1.7E). Reduced freezing in the APS and S groups may be attributed to the presence of spirulina, which likely enhances zebrafish energy levels.

Stress level indices, calculated based on time spent in the top and bottom zones, also showed significant diet-related differences ($p < 0.0001$). The post hoc analyses

confirmed that the APS group showed the lowest stress levels (0.1878 ± 0.0591), significantly lower than those in the AP (0.7670 ± 0.03792, $p < 0.0001$), A (0.8121 ± 0.0210, $p < 0.0001$), P (0.5666 ± 0.0485, $p = 0.0130$), and S (0.6187 ± 0.0373, $p = 0.0028$) groups. The P group also expressed reduced stress compared to the AP ($p = 0.0462$) and A ($p = 0.0037$) groups. The stress level in A ($p = 0.0169$) was higher compared to the S group. No significant differences between AP and A, AP and S, or P and S ($p > 0.05$) (Fig. 1.8A).

The ratio of distance travelled in the top to the bottom zones provided further insights into anxiety and exploratory behaviour. A higher ratio indicates reduced anxiety and confident exploration of the exposed area of the novel tank. A lower ratio suggests increased anxiety or a preference for staying in the safer, sheltered bottom zone. Equal distance distribution can indicate normal behaviour, showing that the fish is neither overly anxious nor usually bold (Adam et al., 2017; Anwer et al., 2021). The changes in this ratio across diet groups reflected the influence of diet factors on zebrafish mood and behaviour ($p < 0.0001$). The post hoc analysis confirmed higher ratios were observed in the APS group with a higher median (M) and interquartile range (IQR) (M = 0.9267, IQR = 0.4309-1.181) compared to AP (M = 0.2525, IQR = 0.1405-0.6238, $p = 0.0294$), A (M = 0.1941, IQR = 0.0789 -0.3043, $p < 0.0001$), and P (M = 0.2583, IQR = 0.1455-0.3691, $p = 0.0035$) groups, with no variations between APS and S, AP and A, AP and P, AP and S, A and P, or P/S ($p > 0.05$) (Fig. 1.8B).

The pairwise relationship between variables across five dietary groups is depicted in a pair plot (Fig. 1.9A), and their correlation coefficient values are plotted in the Spearman correlation matrix plot. Inverse correlations were seen between parameters like freezing and mean speed in APS ($r = -0.04$, 95% CI [-0.4375 to

0.3725]), AP (r = -0.76, 95% CI [-0.8951 to -0.5126]), A (r = -0.60, 95% CI [-0.8099 to -0.2412]), and S (r = -0.09, 95% CI [-0.4798 to 0.3254]) groups, in the P group it was nearly zero (r =0.03, 95% CI [-0.3821 to 0.4284]). Freezing and distance in top zone also found to be negatively correlated in APS (r = -0.11, 95% CI [-0.4944 to 0.3083]), AP (r = -0.23, 95% CI [-0.5889 to 0.2020]), A (r = -0.34, 95% CI [-0.6581 to 0.0909]), and S (r = -0.35, 95% CI [-0.6589 to 0.06946]) groups, but it was contradictory in P group (r = 0.28, 95% CI [-0.1448 to 0.6135]). An inverse correlation was observed between freezing and entries to the top between all the diet groups APS (r = -0.44, 95% CI [-0.7183 to -0.04376]), AP (r = -0.13, 95% CI [-0.5134 to 0.3035]), A (r = -0.07, 95% CI [-0.4711 to 0.3533]), P (r = -0.11, 95% CI [-0.4928 to 0.3102]), and S (r = -0.32, 95% CI [-0.6411 to 0.1001]). A negative correlation also noticed between freezing and time in top zone in AP (r = -0.08, 95% CI [-0.4747 to 0.3492]), A (r = -0.27, 95% CI [-0.6152 to 0.1620]), P (r = -0.01, 95% CI [-0.4138 to 0.3971]), and S (r = -0.38, 95% CI [-0.6789 to 0.03335]) groups except APS (r = 0.13, 95% CI [-0.2904 to 0.5091]) group. Strong positive correlation witnessed between time in top and distance in top between all the diet groups APS (r = 0.79, 95% CI [0.5705 to 0.9067]), AP (r = 0.73, 95% CI [0.4537 to 0.8787]), A (r = 0.81, 95% CI [0.5921 to 0.9157]), P (r = 0.63, 95% CI [0.2981 to 0.8236]), and S (r = 0.87, 95% CI [0.7192 to 0.9432]) (Fig. 1.9B). The zebrafish that exhibit higher freezing time tend to have lower mean speed, distance in top, entries to top, and time in top and zebrafish that spend more time in top cover a greater distance in the top portion of the tank. These results suggest that diet-induced anxiety might be a limiting factor for exploratory behaviours in novel environments.

1.4.2. Diet-influenced morphometric changes

The morphometric analysis included body length, weight and girth (belly size) measurements across the dietary groups. No significant differences in body length were observed among the diet groups ($p = 0.0259$) (Fig. 1.10A). However, considerable variations in body weight were detected ($p < 0.0001$). Post hoc analysis revealed that the P group had the highest median body weight (M = 0.6060, IQR = 0.5478-0.7363) compared to all other groups: APS (M = 0.4615, IQR = 0.3710 - 0.5850, $p = 0.0177$), AP (M = 0.3930, IQR = 0.3650-0.4530, $p < 0.0001$), A (M = 0.3440, IQR = 0.2093-0.4188, $p < 0.0001$), and S (M = 0.4050, IQR = 0.3313-0.4380, $p < 0.0001$). Additionally, the APS group exhibited a significantly higher median body weight compared to the A ($p = 0.0074$) group. No significant variations were found between APS and AP, APS and S, AP and A, AP and S, or A and S ($P > 0.05$) (Fig. 1.10B).

Regarding girth, significant differences were also noted among the diet groups ($p < 0.0001$). Post hoc analysis indicated that the P group had the highest and most consistent median belly size (M = 2.5, IQR = 2.5-2.5) compared to the APS (M = 2.3, IQR = 2-2.5, $p = 0.0132$), AP (M = 2, IQR = 2-2.3, $p < 0.0001$), A (M = 2, IQR = 2-2.3, $p < 0.0001$), and S (M = 2, IQR = 2-2.3, $p < 0.0001$) groups. No variations were observed in girth among the APS, AP, A, and S groups ($p > 0.05$) (Fig. 1.10C). Overall, the morphometric characteristics were more prominent in the P group, with a moderate positive correlation between body weight and girth ($r = 0.47$) (Fig. 1.10D). These findings suggest that the P diet supports enhanced growth metrics, potentially attributable to its nutritional composition.

1.4.3. Light-Dark Test- a confirmation of anxiety

The Light-dark compartment test, a standard assay for assessing anxiety-like behaviour in zebrafish, corroborated the anxiety levels observed during the Novel tank test. Prominent fluctuations were observed in the number of entries to the light zone ($F (4, 120) = 21.01$, $p < 0.0001$). Post hoc evaluation unveiled that zebrafish in the AP (33.88 ± 2.887) and A (24.96 ± 2.379) groups exhibited significantly fewer entries into the light zone compared to the APS (52.56 ± 4.440, APS/AP $p = 0.0031$, APS/A $p < 0.0001$), P (55.92 ± 4.737, P/AP $p = 0.0003$, P/A $p < 0.0001$), and S (64.60 ± 2.807, S/AP $p < 0.0001$, S/A $p < 0.0001$) groups. No statistically significant differences were observed between APS and P, APS and S, P and S, or AP and A groups ($p > 0.05$) (Fig. 1.11A). A substantial change was also found in the latency to enter the light zone ($p < 0.0001$), and a follow-up analysis brought to light that zebrafish in the A (87.12 ± 17.99) group showed increased latency compared to all other groups: APS (20.24 ± 7.019, $p = 0.0010$), AP (31.84 ± 12.46, $p = 0.0190$), P (7.400 ± 2.978, $p < 0.0001$), and S (8.120 ± 4.242, $p < 0.0001$). No statistically significant variations were found among APS, AP, P, and S groups ($p > 0.05$) (Fig. 1.11B). Significant differences were detected in the time in the light zone ($F (4, 120) = 17.78$, $p < 0.0001$). The post hoc analysis confirmed that A group (74.88 ± 9.033) spent distinctively less time in light zone, indicating elevated anxiety levels, compared to the APS (199.8 ± 17.57, $p < 0.0001$), AP (172.2 ± 10.22, $p < 0.0001$), P (206.2 ± 18.42, $p < 0.0001$), and S (219.6 ± 11.08, $p < 0.0001$) groups. The mean differences were non-significant among APS, AP, P, and S ($p > 0.05$) (Fig. 1.11C). The anxiety index, calculated based on the duration spent in the dark zone, confirmed that zebrafish in the A group exhibited significantly higher anxiety levels (0.7496 ± 0.0299) compared to all other groups ($p < 0.0001$) (Fig. 1.12A).

Additionally, a strong negative correlation was seen between the anxiety index and the time spent in the light zone across all dietary groups (r = -0.9 to -1) (Fig. 1.12B). These findings highlight that the A diet is associated with elevated anxiety-like behaviour in zebrafish, whereas the APS, AP, P and S diets appear to mitigate anxiety more effectively.

1.4.4. Mirror Biting Test to quantify aggression

The Mirror Biting Test provided a comprehensive assessment of how different diet regimes influence zebrafish behaviour, particularly aggression. Significant deviations were observed in the number of entries to the mirror zone ($F (4, 95) = 11.20$, $p < 0.0001$). Post hoc analysis revealed increased entries in the APS (M = 35.50, IQR = 25.5-62) group relative to A (M = 20, IQR = 4.25-27, p = 0.0042) and P (M = 10, IQR = 2.25-26, $p < 0.0001$) groups. Similarly, the S (M = 33.50, IQR = 28-39.75) group showed more entries relative to P ($p < 0.0001$), A (p = 0.0014), and AP (M = 22, IQR = 14.25-33.75, p = 0.0370) groups. No significant differences were found between APS and AP, APS and S, AP and A, AP and P, or A and P groups ($p > 0.05$) (Fig. 1.13A). Latency to enter the mirror zone also displayed significant variation ($p < 0.0001$). Subsequent evaluation indicated that the A group (M = 5.86, IQR = 0-15.43) had increased latency towards the mirror compared to AP (M = 0, IQR = 0, $p < 0.0001$), P (M = 0, IQR = 0, $p < 0.0001$), and S (M = 0, IQR = 0, p = 0.0003) groups. The APS group (M = 0, IQR = 0-5.81) showed slightly higher latency compared to the P (p = 0.0457) group, but no significant differences among AP, P, and S ($p > 0.05$) (Fig. 1.13B).

A great shift was observed in the time in the mirror zone ($p < 0.0001$). Follow-up analysis revealed that time spent in mirror zone was heightened in P group (M = 317.5, IQR = 290.2-357.6) compared to other groups: APS (M = 200.1,

IQR = 158.5-240.2, $p < 0.0001$), AP (M = 229.2, IQR = 155-315.2, $p = 0.0041$), A (M = 245.5, IQR = 171.4-317.1, $p = 0.0211$), and S (M = 224.3, IQR = 200.9-298.8, $p = 0.0046$). There were no statistically significant variations among APS, AP, A, and S ($p > 0.05$) (Fig. 1.13C). Diet regimes also influenced distance travelled ($p < 0.0001$) and mean speed ($p < 0.0001$) in the mirror zone. Post hoc analysis revealed that zebrafish in the A group (M = 492.6, IQR = 328.3-651.3) travelled significantly less distance compared to APS (M = 858.4, IQR = 546.0-968.1, $p = 0.0063$), AP (M = 679.8, IQR = 548.5-863.6, $p = 0.0314$), P (M = 1175, IQR = 955.4-1412, $p < 0.0001$), and S (M = 852.4, IQR = 687.3-1172, $p = 0.0002$) groups. When comparing APS ($p = 0.0098$) and AP ($p = 0.0017$) with P, the P group showed a greater distance travelled in front of the mirror. No significant variations were observed between APS and AP, APS and S, AP and S, or P and S ($p > 0.05$) (Fig. 1.13D). The mean speed in the mirror zone was reduced in A (M = 2.255, IQR = 1.9-2.6) group compared to other groups: APS (M = 4.095, IQR = 3.48-4.68, $p < 0.0001$), AP (M = 2.85, IQR = 2.40-3.42, $p = 0.0052$), P (M = 3.95, IQR = 3.34 - 4.25, $p < 0.0001$), and S (M = 3.89, IQR = 3.32-4.19, $p < 0.0001$). The AP group also showed reduced mean speed compared to the APS ($p = 0.0001$), P ($p = 0.0020$), and S ($p = 0.0019$) groups. No significant differences were found between the APS, P, and S groups ($p > 0.05$) (Fig. 1.13E). Mirror bites, which indicate aggression levels, demonstrated fluctuation ($p < 0.0001$). Post hoc analysis explicitly stated mirror bites were prominent in the P group (M = 671, IQR = 483.5-926.8) compared to other groups: APS (M = 100.5, IQR = 65.75-190.5, $p < 0.0001$), AP (M = 72, IQR = 37-137.3, $p < 0.0001$), A (M= 55.5, IQR = 0-107, $p < 0.0001$), and S (M = 133.5, IQR = 80.25-210.3, $p < 0.0001$). No significant differences were observed between APS, AP, A, and S groups ($p > 0.05$) (Fig. 1.13F).

These findings suggest that the P diet strongly promotes aggression in zebrafish, as indicated by lower latency, increased time in the mirror zone, greater distance travelled, higher mean speed, and more mirror bites. The APS, AP, and S diets promoted increased entries and decreased latency, with moderate time, distance, and greater speed. Still, fewer mirror bites suggest that these diets may foster boldness while mitigating aggression. The A-only diet, on the other hand, exhibited increased latency, decreased distance and mean speed, and fewer mirror bites, indicating reduced boldness and aggression. The parallel index, which quantifies the straightness of zebrafish movement, decreased in the mirror zone across all dietary groups. This index ranges from -1 to 1, where values closer to 1 indicate more straight-line movement and values closer to -1 reflect more frequent directional changes. (Smart user manual). The parallel index was lower in the mirror zone compared to the non-mirror zone for all groups, with Cohen's d values as follows: APS (Cohen's d -1.41, [95.0%CI -1.97, -1.02]), AP (Cohen's d -2.59, [95.0%CI -3.57, -1.7]), A (Cohen's d -1.01 [95.0%CI -1.89, -0.311]), P (Cohen's d -1.35 [95.0%CI -2.53, -0.557]), and S (Cohen's d -2.62 [95.0%CI -3.7, -1.67]) (Fig. 1.14A). When considering the groups, statistically significant differences were observed in the P group, where the parallel index was significantly reduced compared to APS ($p = 0.0005$), A ($p = 0.0216$), and S ($p = 0.0199$) groups (Fig. 1.14B).

The turning tendency is measured by the rotation of the zebrafish movement. Positive values indicate rightward rotation and negative values indicate leftward rotation (Cruz & Oliveira, 2015; Koyama et al., 2016) was assessed in the mirror zone across dietary conditions. The polar scatter dot plot of turn angles in the mirror zone under the five dietary conditions (APS, AP, A, P, and S) revealed that most of

the data points group around 180°, suggesting that zebrafish primarily exhibited straight-line swimming or reversals in the mirror zone, irrespective of diet. The APS group showed a more concentrated distribution near 180°, indicating a more consistent turning behaviour. The AP and A groups demonstrated slightly broader angular distributions, suggesting moderate variability in turning behaviour. The P and S groups exhibited more dispersed patterns, implying greater variability and potentially less stereotypic responses in the mirror zone. A few data points in A group deviated significantly, suggesting occasional sharp turns or changes in swimming direction when interacting with the mirror. The APS diet appeared to encourage straight swimming or more uniform turning responses in the mirror zone. Conversely, the P and S diet showed a higher spread of turn angles, which may indicate less inhibition or a more exploratory approach towards the mirror (Fig. 1.14C). Though not statistically significant, the vector length reflecting the strength and consistency of directional movement, showed diet-dependent differences. The APS diet resulted in more consistent directional movement, whereas the P and S diets were associated with greater variability in movement patterns, suggesting less consistency in behavioural responses (Fig. 1.14D).

Finally, the valence index, a measure of aggression, is calculated based on the balance between approach and avoidance behaviours. The approach behaviour involves time spent, number of entries and bites in the mirror zone. The avoidance behaviour comprises freezing and latency in the mirror zone and time spent in the non-mirror zone. Significant mean variation was found between groups ($p < 0.0001$). Post hoc analysis assured that P group (0.8908 ± 0.02293) exhibited a higher valence index than all other groups: APS (0.3264 ± 0.04718, $p < 0.0001$), AP (0.3984 ± 0.08878, $p < 0.0001$), A (0.2609 ± 0.1056, $p < 0.0001$), and S ($0.5163 \pm$

0.05067, $p = 0.0011$). No significant differences were observed between APS, AP, A, and S groups ($p > 0.05$) (Fig. 1.14E).

1.4.5. The gut microbial shift

The metagenomic analysis revealed significant shifts in the gut microbiota composition across the different dietary groups. A total of 34, 33, 32, 29, and 34 OTUs were present in APS, AP, A, P, and S, respectively. From that, the top five common OTUs were considered here. The phylum Pseudomonadota was predominantly found in the A diet group (69%), with a noticeable reduction in the APS (27.3%) and P diet groups (12.8%). Its abundance was significantly lower in the AP (4.9%) and S (3.2%) diet groups. In contrast, the phylum Bacillota showed higher abundance in the AP (89.7%), P (83.9%) and S (93.8%) diet groups, while its prevalence was notably reduced in APS (32.1%) and A (16.3%) diet groups. The phylum Bacteroidota was observed in A (9.1%) and APS (2.9%) groups but was negligible in AP (0.8%), P (0.7%), and S (0.2%) groups. The Actinomycetota (19.3%) and Fusobacteriota (11.5%) phyla were most prominent in the APS diet group (Fig. 1.15A). At the genus level, the A diet group showed high diversity with 582 OTUs, in which *Pseudomonas* (30.4%) and *Escherichia* (9.24%) were dominant, accompanied by moderate levels of *Sodalis* (5.44%), *Streptococcus* (4.63%) and *Salmonella* (3.82%). In the APS diet group, out of 478 OTUs, *Streptococcus* (20.96%) and *Cetobacterium* (11.30%) were predominant, followed by *Streptomyces* (8.49%), *Bacillus* (6.50%), *Mycobacterium* (3.60%) and *Ralstonia* (3.52%). The AP diet group with 419 OTUs, predominantly supported *Bacillus* (38.99%), *Paenibacillus* (22.64%), and *Staphylococcus* (14.04%), with smaller contributions from Brevibacillus (2.05%). The P diet group with the lowest OTUs (335), had a high abundance of *Paenibacillus* (55.47%), *Brevibacillus* (26.41%) and

Acinetobacter (9.21%) while *Cetobacterium* was reduced to 1.46%. In the S diet group (383 OTUs), the dominant genera included *Bacillus* (31.71%), *Brevibacillus* (28.63%), *Staphylococcus* (13.81%) and *Paenibacillus* (8.12%) (Fig. 1.15B).

At the species scale, out of 917 OTUs, the A diet group was dominated by *Escherichia coli* (9.49%) and *Pseudomonas aeruginosa* (6.80%). Additionally, *Sodalis ligni* (5.29%), *Pseudomonas sp*. (4.05%) and *Salmonella enterica* (3.77%) were also present in moderate levels. In the APS diet group, having 609 OTUs, *Streptococcus thermophilus* (19.91%) and *Cetobacterium somerae* (11.61%) were found to be dominant with smaller contributions from *Bacillus subtilis* (6.17%) and *Streptomyces sp.* (4.63%). In the AP diet group with 695 OTUs, *Paenibacillus polymyxa* (18.13%), *Staphylococcus aureus* (12.30%) and *Bacillus thuringiensis* (10.09%) were prevalent, alongside *Bacillus velezensis* (7.48%) and *Bacillus cereus* (7.04%). The P diet group with the lowest OTUs (497) was enriched with *Paenibacillus polymyxa* (32.61%), *Brevibacillus choshinensis* (20.60%) and *Paenibacillus lautus* (10.29%), with *Acinetobacter sp.* contributing 5.81%. Similarly, the S diet group with 566 OTUs harboured *Brevibacillus choshinensis* (20.22%), *Staphylococcus aureus* (11.74%), *Bacillus thuringiensis* (8.84%), *Bacillus cereus* (7.22%), and *Bacillus velezensis* (5.08%) (Fig. 1.15C).

In Beta Diversity analysis, Bray-Curtis dissimilarity metrics revealed variations in species composition across dietary groups. The value ranges from 0 to 1, where 0 indicates identical composition and 1 indicates no shared species. A few shared species were observed among AP/P (0.71), S/P (0.63), and AP/S (0.37). Groups APS and A exhibited higher dissimilarity compared to others (close to 1) (Fig. 1.15D). The differences were visualised in a Principal Coordinates Analysis (PCoA) plot, where the X-axis (PCoA1) explained 48.86% of the variance and the Y-axis

(PCoA2) explained 29.91%, accounting for 78.77% of the total variation. The position of each sample reflects its similarity or difference in composition. Samples closer to each other are more similar, while those farther apart are more dissimilar. Diet group A clustered distinctly at the lower right edge, while APS was located in the upper-right corner, showing a distinct composition of the microbiota. In contrast, the AP, P, and S groups formed a tighter cluster, reflecting greater similarity in their microbiota (Fig. 1.15E).

The Alpha Diversity measures species richness and evenness within a single group. The observed value indicates the number of observed Operational Taxonomic Units (OTUs). The species richness was highest in the A diet group (917 OTUs), indicating the most diverse microbial community, while the P diet had the lowest richness (497 OTUs). The APS (609), AP (695), and S (566) groups exhibited moderate diversity. Chao1 and ACE indices estimate the total species richness, including unobserved species. The AP diet group had the highest estimated richness (Chao1: 929.53, ACE: 894.33), implying a greater presence of rare species. In contrast, the P diet group had the lowest estimated richness (Chao1: 670.88, ACE: 673.64), confirmed presence of fewer species and some rare species remained undetected. Moderate richness with rare species well represented in APS (Chao1: 757, ACE: 771), with observed richness close to estimated richness, suggesting most species had been captured. High richness with fewer rare species in A (Chao1: 917, ACE: 917) indicates no significant hidden species. Moderate richness with better representation of rare species observed in S (Chao1: 777, ACE: 755).

The Shannon index measures both richness and evenness. A higher value in the A diet group (4.74) indicated a more even distribution and a diverse community. The P diet group had the lowest Shannon index (2.68), reflecting dominance by a few

species. A moderate evenness was found among APS (3.87), AP (3.62), and S (3.42). The Simpson and Inverse Simpson indices confirmed these trends, with the A diet group achieving the highest values (0.973, 37.71), while the P diet group had the lowest (0.832, 5.97). APS (0.93,15.11), AP (0.92, 13.47), and S (0.92, 12.81) showed intermediate diversity. Fisher's alpha index quantifies species richness and abundance, and these findings are also supported, with the A diet group exhibiting the highest diversity (139.41) and the P diet group the lowest (77.86). Moderate diversity was observed in AP (114.19), APS (105.35), and S (88.03) diet groups (Fig. 1.15F). The top bacterial species present in each group are depicted in Figure 1.15G. The major taxa, including phylum, family, genus, and species, are displayed in a sankey plot (Fig. 1.15H).

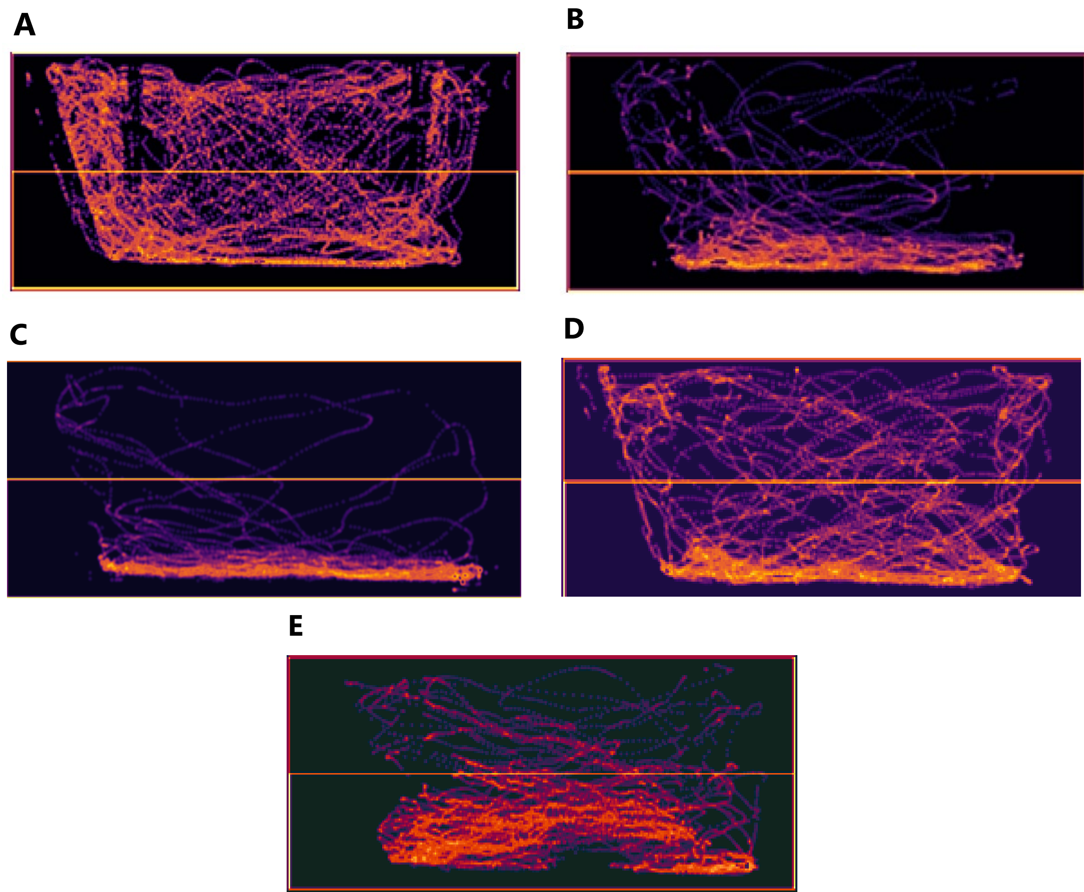


Figure 1.6*: Novel Tank Test, representative heatmap of zebrafish exploration obtained from Smart 3.0 software (A) APS (Artemia+Pellet+Sprulina), (B) AP (Artemia+Pellet), (C) A (Artemia only) (D) P (Pellet only), and (E) S (Spirulina- rich).*

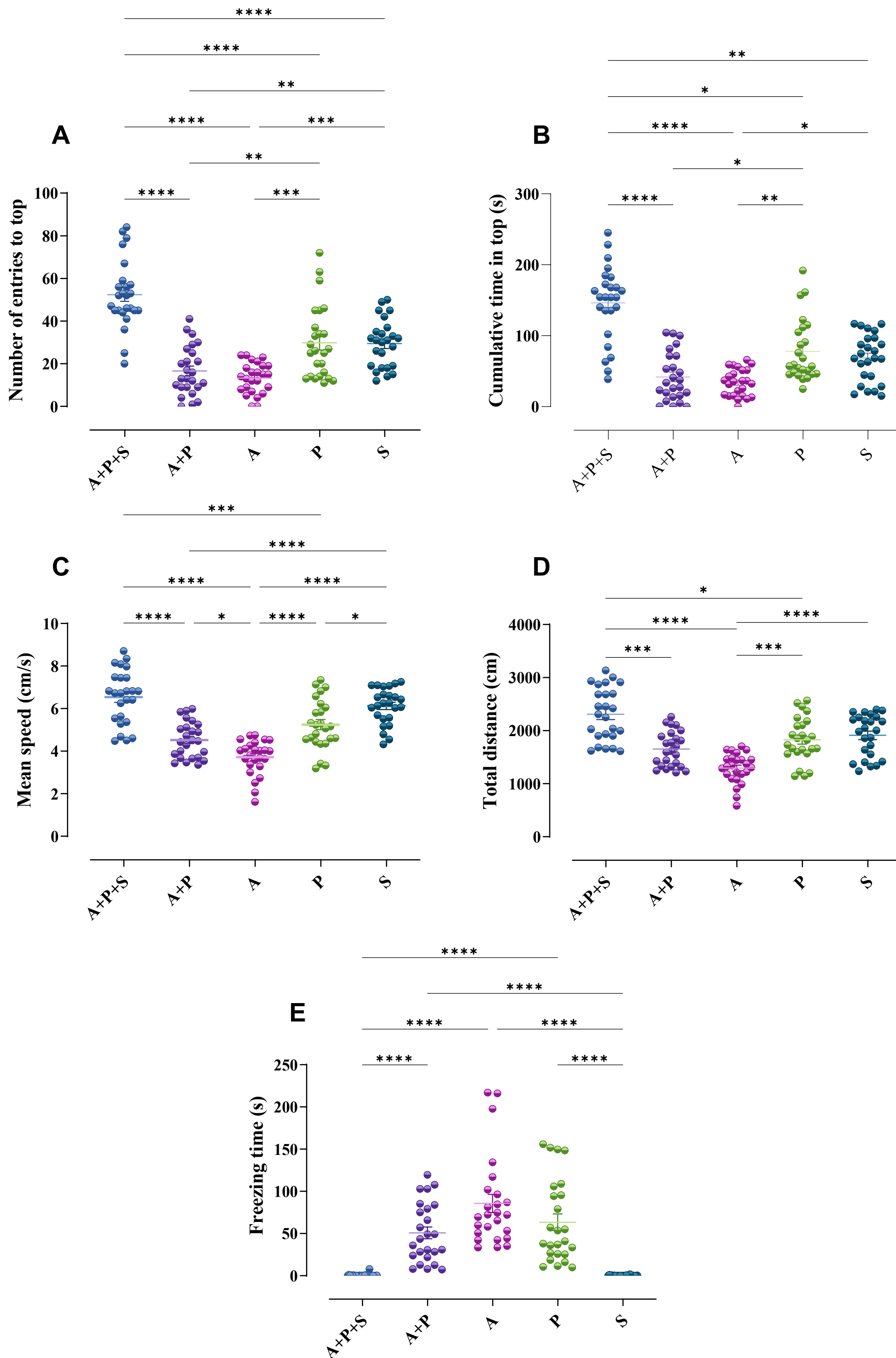


Figure 1.7: *Novel Tank Test, the Scattered dot plot represents behavioural endpoints derived from recorded videos upon analysed by Smart 3.0 tracking software (A) Number of entries to top zone. The number of times each fish crossed the virtual mid-line and made entries into the upper half of the novel tank. APS diet promotes higher entries to the top*

*while A and AP diet affects the entrance behaviour significantly. (B) Cumulative time in the top zone. The time spent in the upper half of the novel tank measures anxiety levels. APS diet subsides anxiety levels in zebrafish while the A diet elevates anxiety. (C) Mean speed. The average speed exhibited by each group. APS diet facilitates increased locomotion and A diet weakens locomotory abilities. (D) Total distance travelled. Indicates exploratory behaviour of each group. APS diet positively affects exploratory behaviour whereas A diet is insufficient to support greater exploration. (E) Freezing time. The total immobility (movement<1cm/s) time, APS and S diet groups show sharply minimised immobility time. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001) (n=25).*

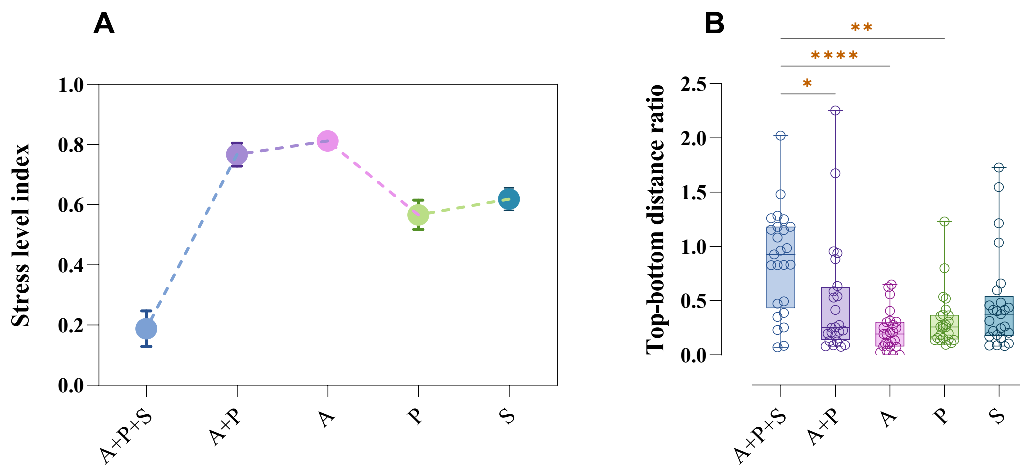
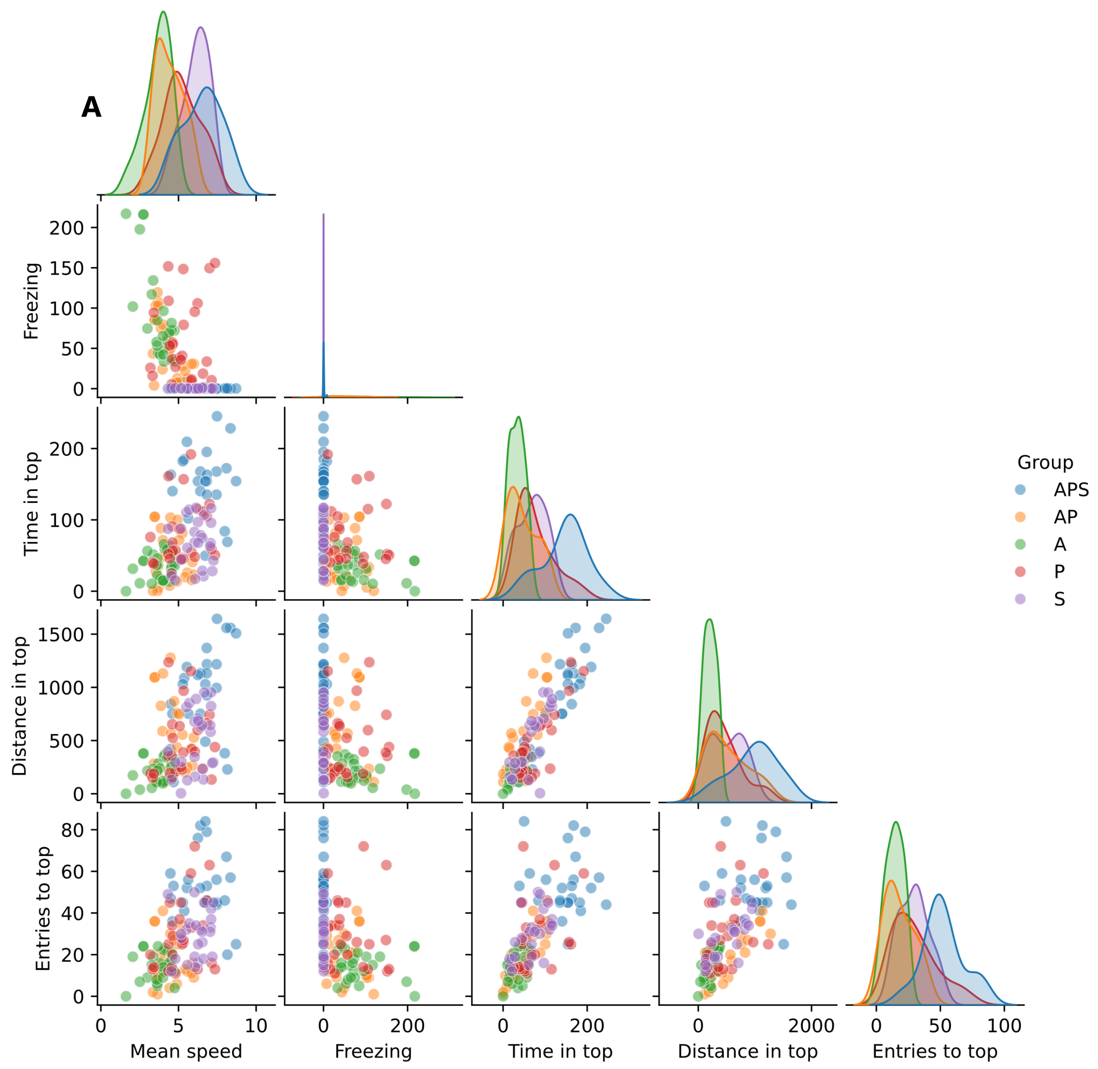


Figure 1.8**: Novel Tank Test, the dotted line plot displays (A) Stress level index. The control diet AP and A diet surged the novelty-induced stress level in zebrafish the APS diet considerably deteriorated novel-induced stress and the P and S diet induced moderate stress levels in zebrafish. The data were represented as mean ± SEM. The box and whisker plot with individual data points represents (B) Top-bottom distance ratio. The median and interquartile range were higher in APS and were more consistent in the A and P diet groups exhibiting lower ratios than in APS. *(P<0.05), **(P<0.01), and *(p<0.0001) (n=25).*


A
Freezing
Time in top
Distance in top
Entries to top
Mean speed
Group
APS
AP
A
P
S

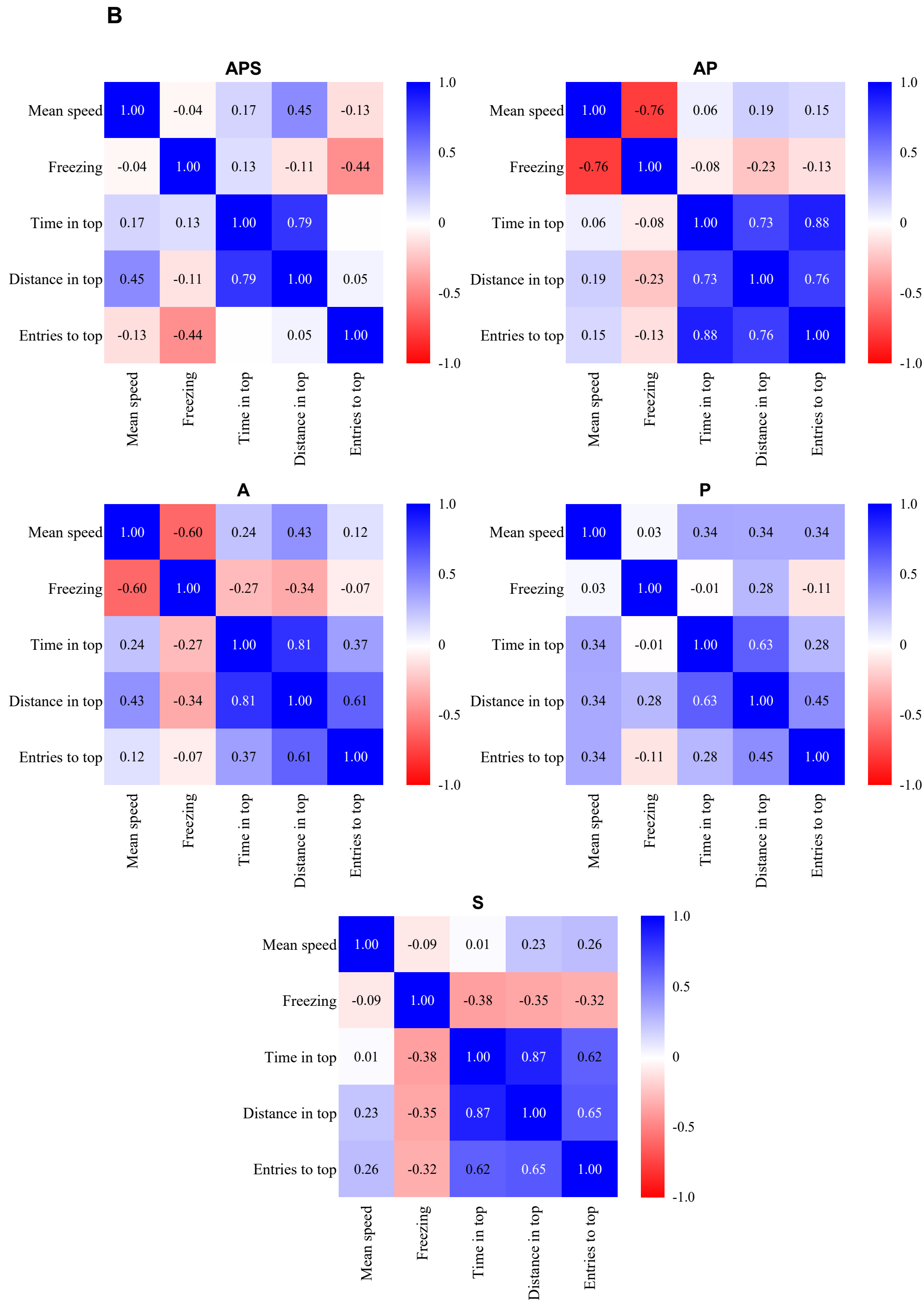


Figure 1.9: *Relation between behavioural endpoints (A) A pair plot illustrating the behavioural metrics across different diet groups, showing the relationships between groups for each behaviour through scatter plots combined with KDE (Kernel Density Estimate) plots. (B) A correlation matrix presents how each behaviour correlates with others within each group and the corresponding correlation coefficient values*

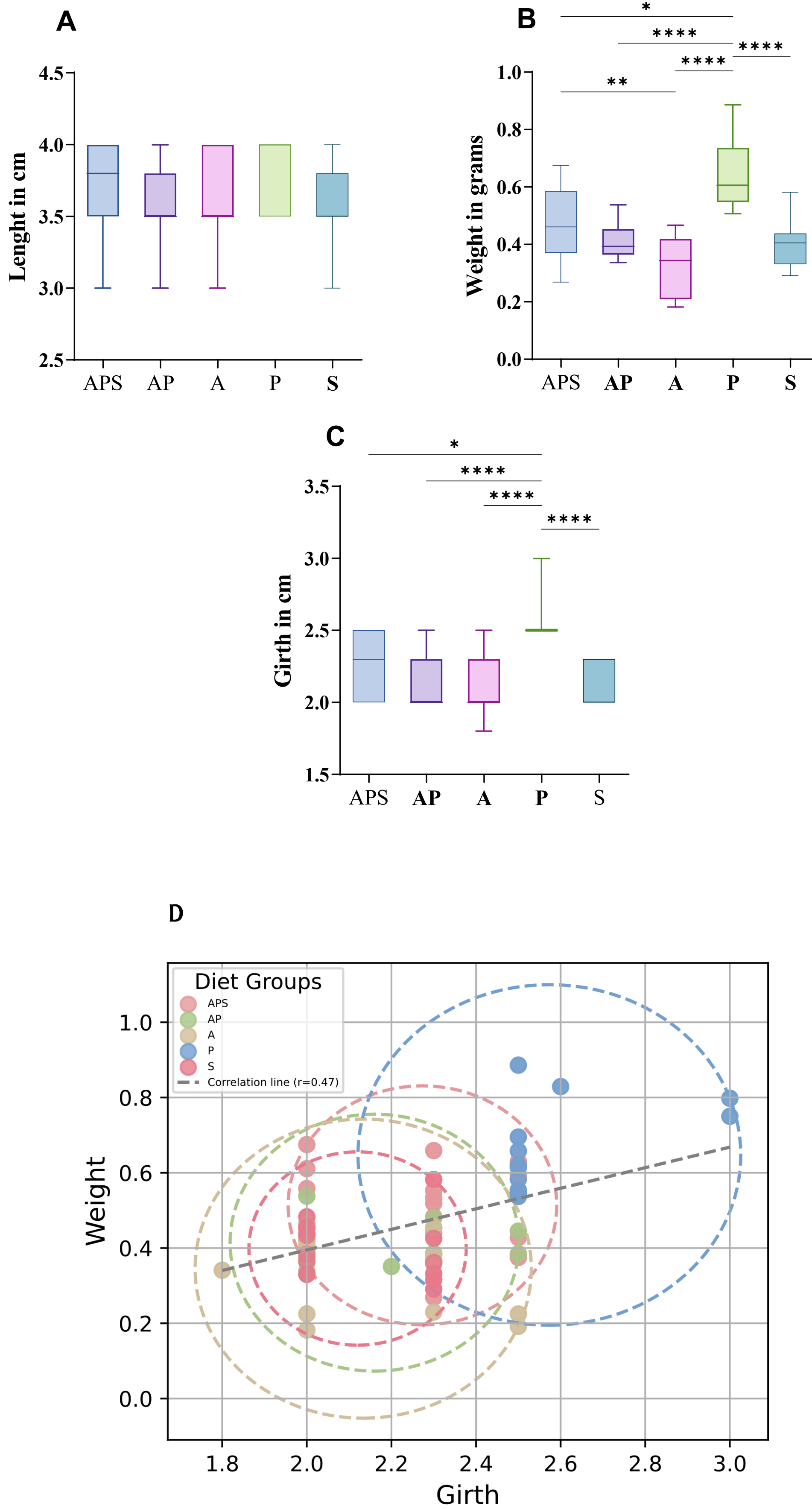

A
Lenght in cm
4.5
4.0
3.5
3.0
2.5
APS
AP
A
P
S
B
Weight in grams
1.0
0.8
0.6
0.4
0.2
0.0
*
****
****
****
**
C
Girth in cm
3.5
3.0
2.5
2.0
1.5
D
Diet Groups
APS
AP
A
P
S
Correlation line (r=0.47)
Weight
Girth
1.8
2.0
2.2
2.4
2.6
2.8
3.0

Figure 1.10**: Morphometrics (A) Length in cm. The box and whiskers plot shows the length of zebrafish across different diet groups. No significant differences were observed between groups. (B) Weight in grams. The median and IQR are higher in the P diet group and lower in the A diet group. (C) Girth in cm. The IQR of the P diet group is higher and narrow suggesting greater girth compared to other groups. The data is represented as Median IQR. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), and *(p<0.0001) (n = 15-20). (D) The scatter plot depicts the relationship between girth (x-axis) and weight (y-axis) across five zebrafish diet groups: APS, AP, A, P, and S. Each data point represents an individual observation, and the coloured dashed ellipses highlight the distribution and variance within each diet group. A black dashed line indicates the overall correlation between girth and weight, with a correlation coefficient of r=0.47 suggesting a moderate positive relationship.*

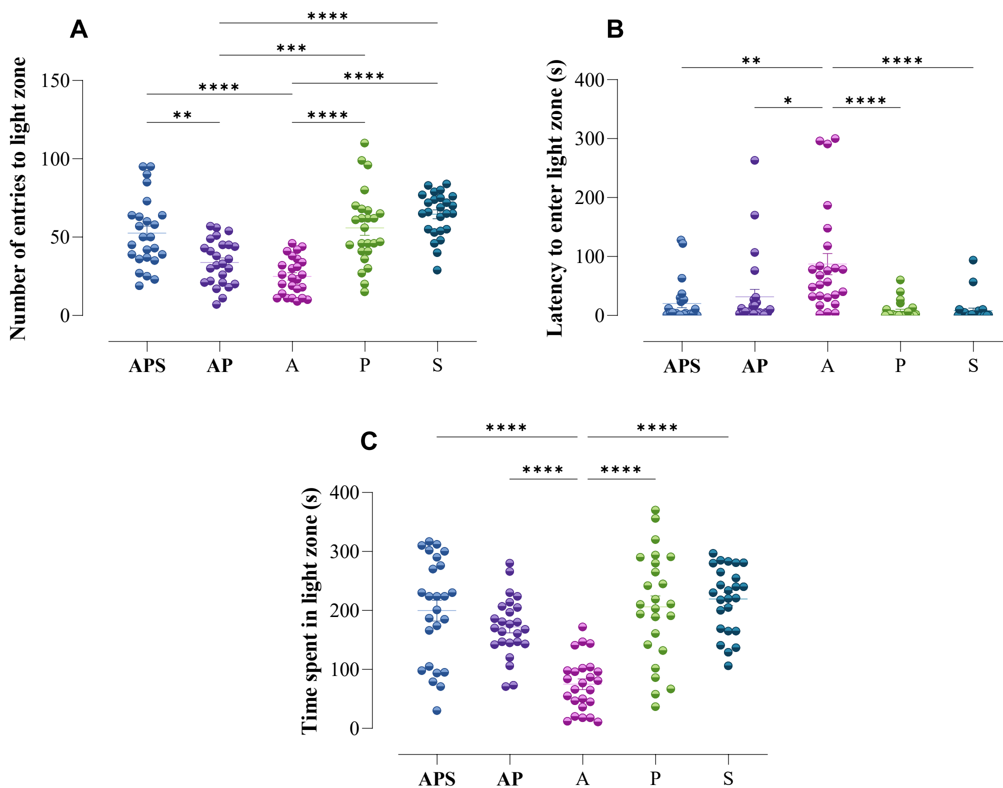


***Figure 1.11**: Light and Dark Test, Scatter dot plot displaying (A) Number of entries to light zone. APS, P, and S diet groups showed an increased number of entries while AP and A diet groups made fewer entries. (B) Latency to enter the light zone. The time taken to enter into the light zone is higher in A diet group indicating*

*greater anxiety. (C) Time spent in the light zone. The amount of time spent in the light zone is severely restricted in A diet group. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001) (n = 25).*

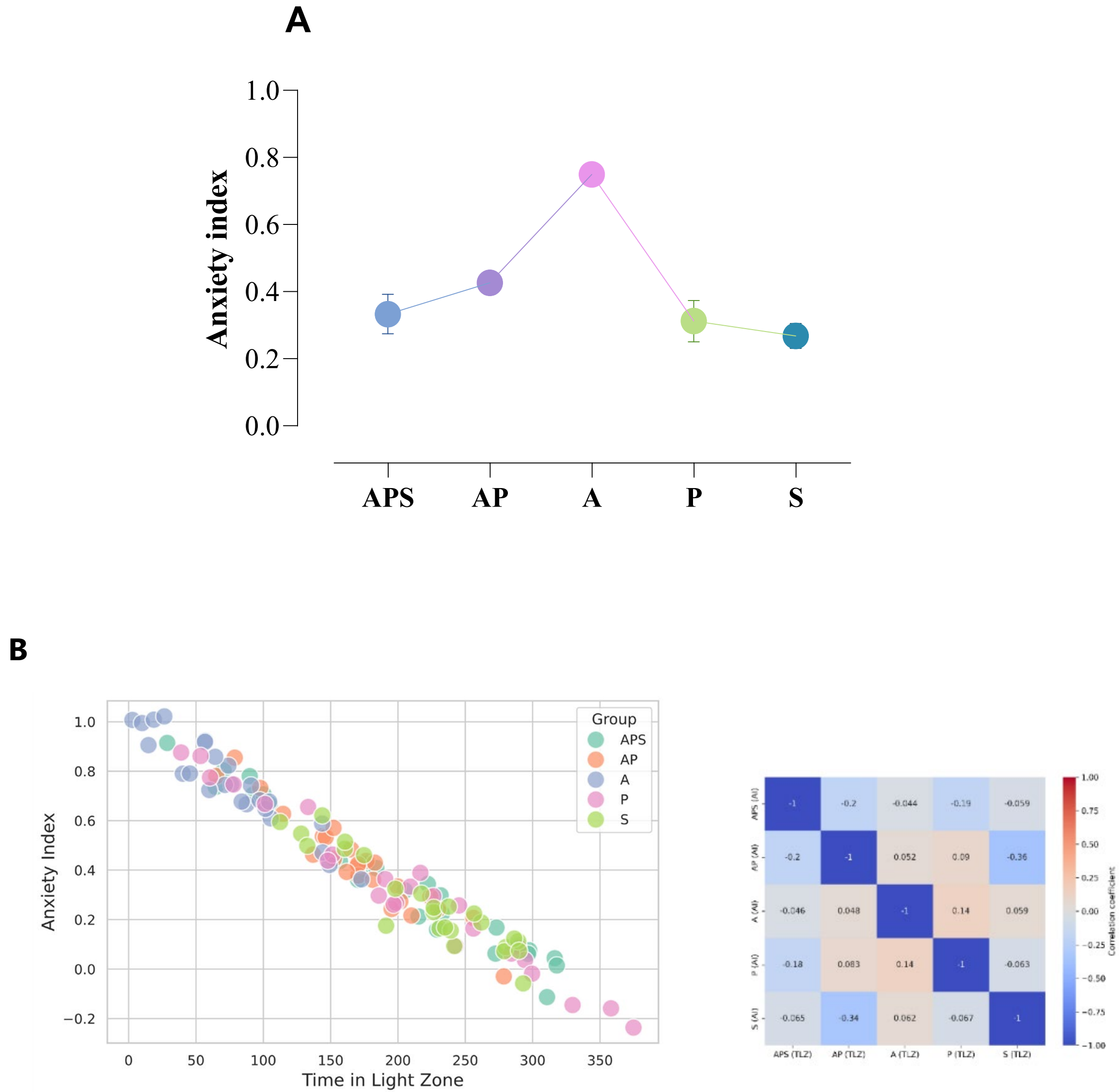


***Figure 1.12**: (A) Dotted line plot illustrating anxiety index values across groups. A diet group reflects its higher anxiety index. (B) The scatter plot depicts the relationship between the Time in Light Zone (x-axis) and the Anxiety Index (y-axis) across different diet groups in zebrafish. There exists a negative correlation between anxiety index and time spent in light zone.*

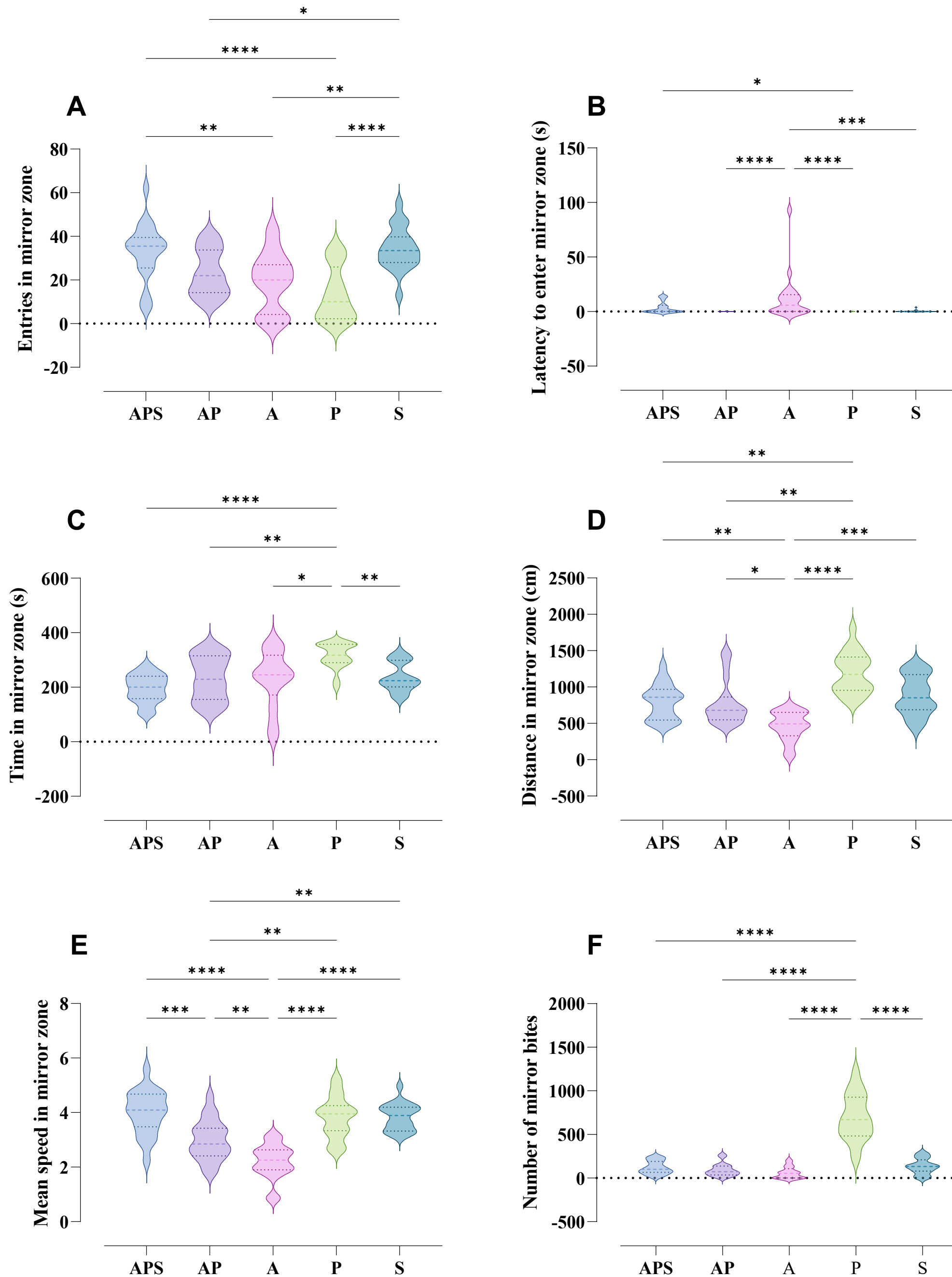
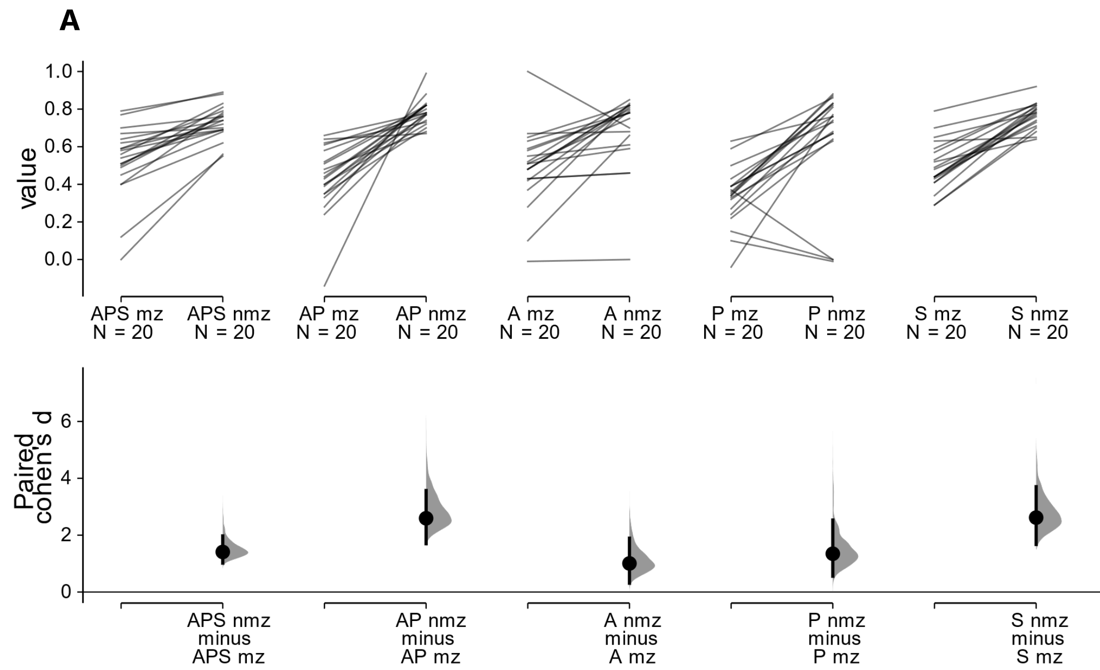
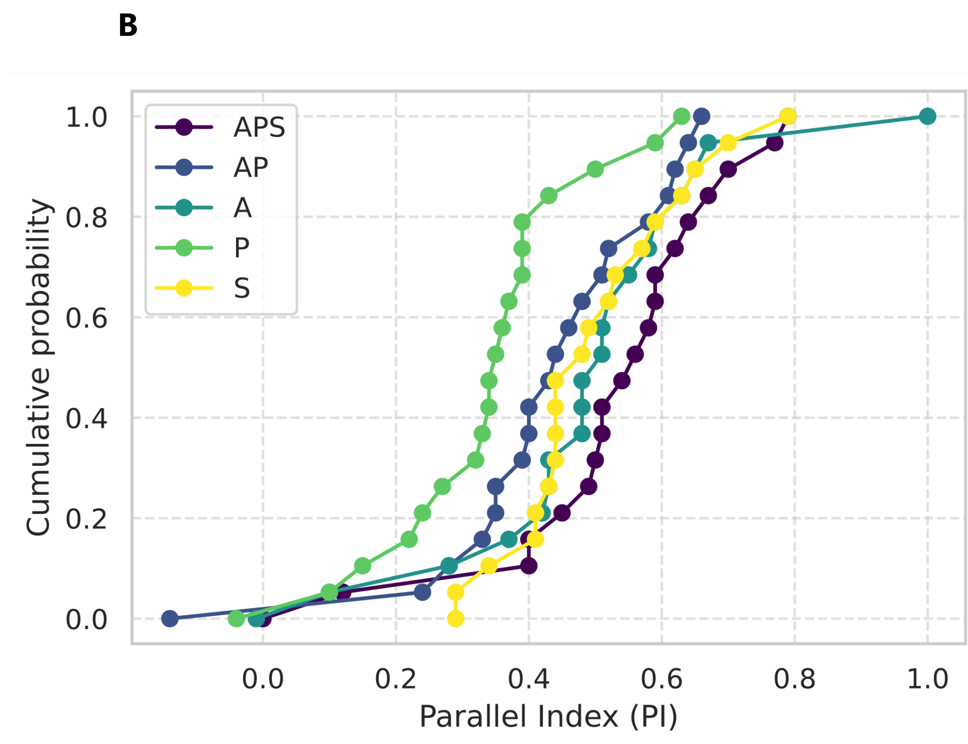


***Figure 1.13**: Mirror Biting test, Violin plot represents behavioural endpoints derived from the activities in a three-chambered tank with a mirror. (A) Entries to mirror zone. The number of entries was higher in APS and S diet groups, and lower in AP, A, and P. (B) Latency to enter mirror zone. A diet increased the time taken to enter the mirror zone. (C) Time spent in mirror zone. P diet promoted the amount of time in front of the mirror. (D) Distance in mirror zone. P diet stimulates locomotory activities while A diet restrains movement in the presence of a mirror.*

*(E) Mean speed in mirror zone. A diet lowered the average speed in the mirror zone. (F) Number of mirror bites. P diet enhanced the mirror bites pointing to the exacerbated boldness and aggressive nature. The data is represented as Median IQR. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001) (n = 20).*





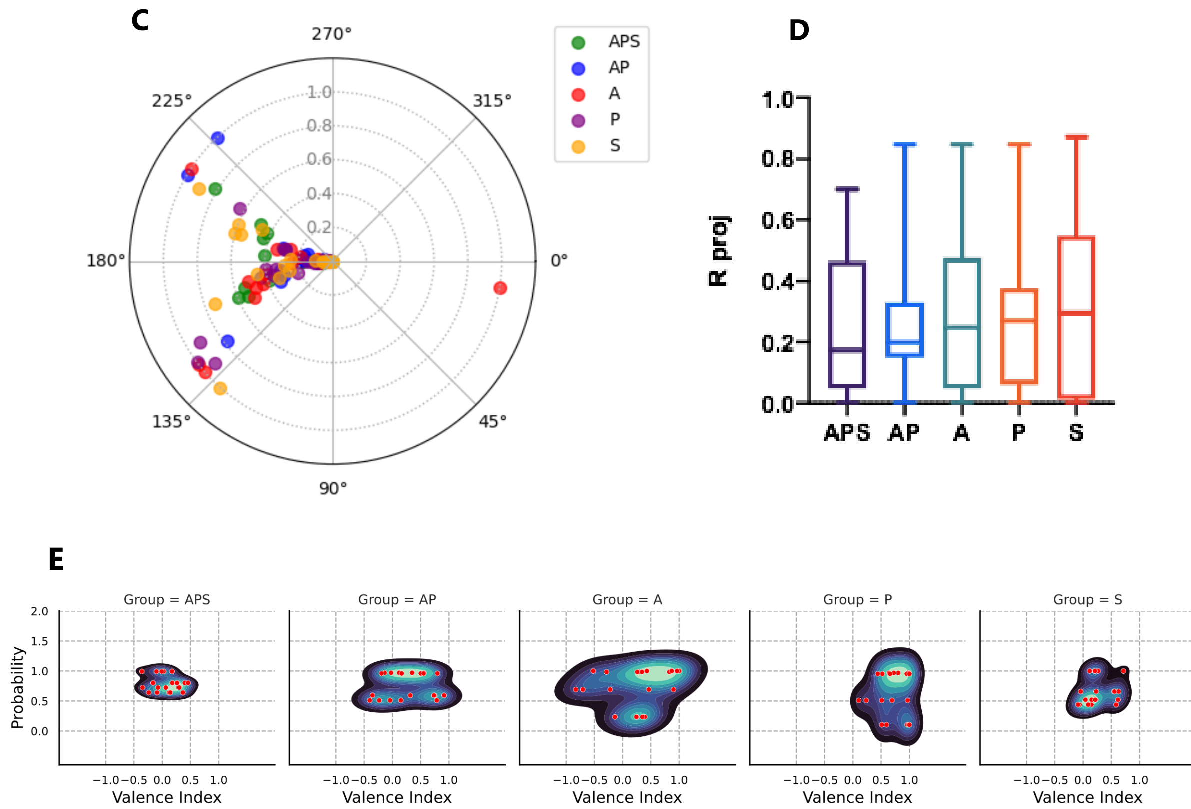


Figure 1.14*: Mirror Biting Test, (A) Parallel Index in the non-mirror and mirror zones within groups. The Cumming estimation plot illustrates paired Cohen's d comparisons across different diet groups. Lines connect paired observations, and the paired mean difference is represented as a bootstrap sampling distribution. Mean differences are depicted as dots; the ends of the vertical error bars indicate 95% confidence intervals. Decreased parallel orientation is observed in the mirror zone. (B) Parallel index between groups. Lower in the P diet group. (C) Turning tendency. Polar scatter plot of the five different diet groups' individual mean resultant vector's angles α (0˚ to 360˚) combined with corresponding vector lengths R (0 to 1), for each group. (D) R-projection. Box and whiskers plot of the diet groups resultant vector's lengths R projected (R proj) onto the mirror direction (180˚) and corresponding group mean value Rg proj (median), for each group. Positive values indicate directional focus towards the mirror, zero indicates no directionality, and negative values indicate directional focus opposite to the mirror. (E) Valence index. The valence index measures the tendency of animals to either approach or avoid the stimulus. When the index is positive, zebrafish is more aggressive, showing engagement with its reflection. Negative valence indicates more avoidant, possibly perceiving the mirror image as threatening. Neutral or zero indicates an equal balance of approach and avoidance behaviour.*

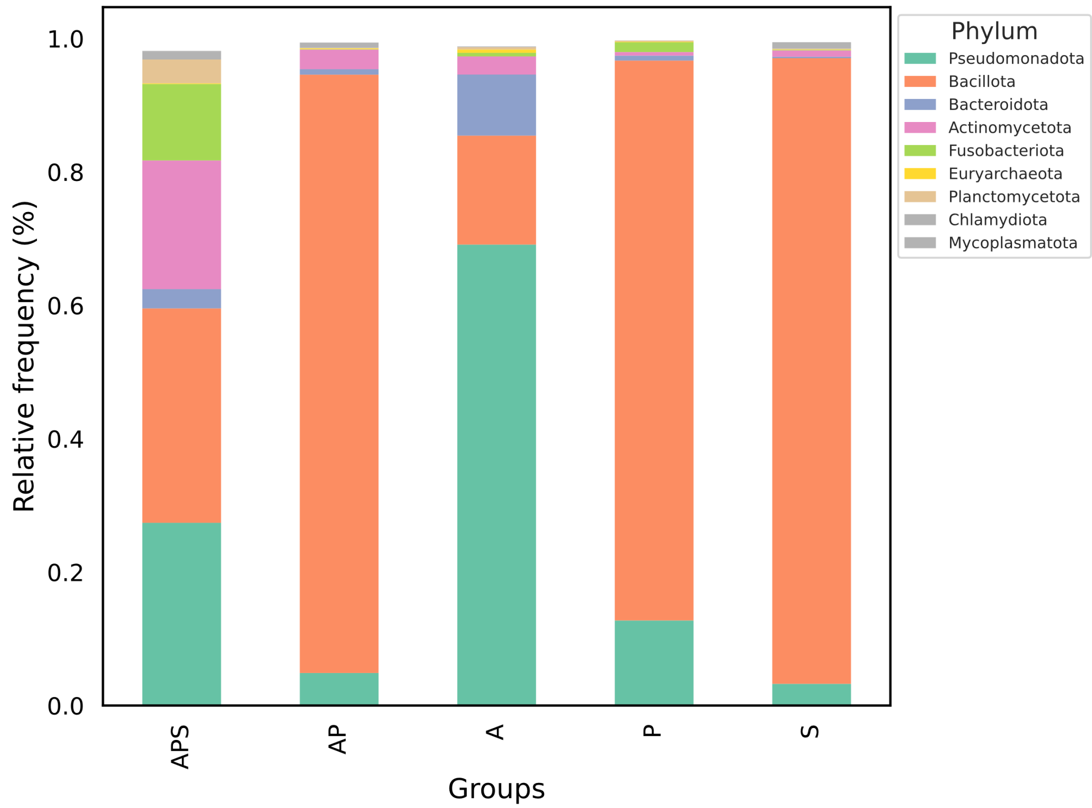


Figure 1.15A*: Gut microbiome analysis, the stacked bar plot displays the relative frequency of microbial abundance of five different diet groups at Phylum level.*

Name	APS	AP	A	P	S
Pseudomonadota	27.40%	4.88%	69.11%	12.75%	3.25%
Bacillota	32.15%	89.71%	16.34%	83.95%	93.81%
Bacteroidota	2.88%	0.82%	9.15%	0.73%	0.22%
Actinomycetota	19.30%	2.95%	2.72%	0.54%	0.95%
Fusobacteriota	11.46%	0.06%	0.54%	1.47%	0.09%

Table 1.1*: Relative abundance of top five microbial phyla present in different dietary groups*

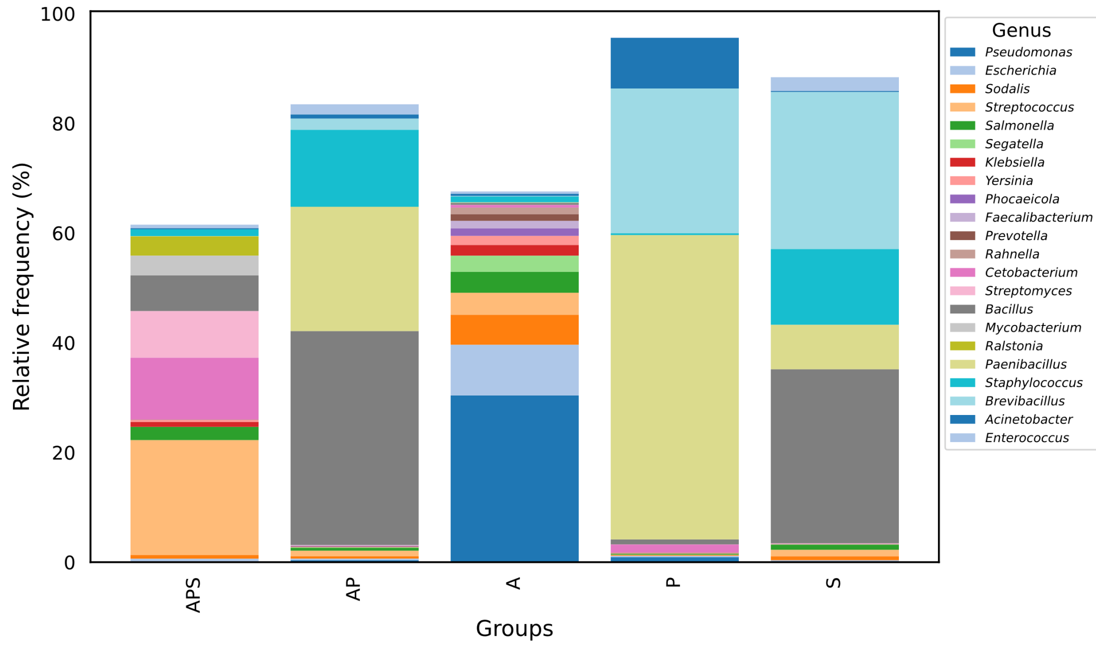


Figure 1.15B: *Gut microbiome analysis, the stacked bar plot displays the relative frequency of microbial abundance of five different diet groups at Genus level.*

Name	APS	Name	AP	Name	A
Streptococcus	20.96%	*Bacillus*	38.99%	*Pseudomonas*	30.40%
Cetobacterium	11.30%	*Paenibacillus*	22.64%	*Escherichia*	9.24%
Streptomyces	8.49%	*Staphylococcus*	14.04%	*Sodalis*	5.44%
Bacillus	6.50%	*Brevibacillus*	2.05%	*Streptococcus*	4.03%
Mycobacterium	3.60%	*Enterococcus*	1.84%	*Salmonella*	3.82%
Ralstonia	3.52%	*Priestia*	1.24%	*Segatella*	2.95%
Name	P	Name	S		
Paenibacillus	55.47%	*Bacillus*	31.71%		
Brevibacillus	26.41%	*Brevibacillus*	28.63%		
Acinetobacter	9.21%	*Staphylococcus*	13.81%		
Cetobacterium	1.46%	*Paenibacillus*	8.12%		
Pseudomonas	0.91%	*Enterococcus*	2.49%		
Bacillus	0.90%	*Streptococcus*	1.20%		

Table 1.2: *Relative abundance of top six genus present in different dietary groups.*

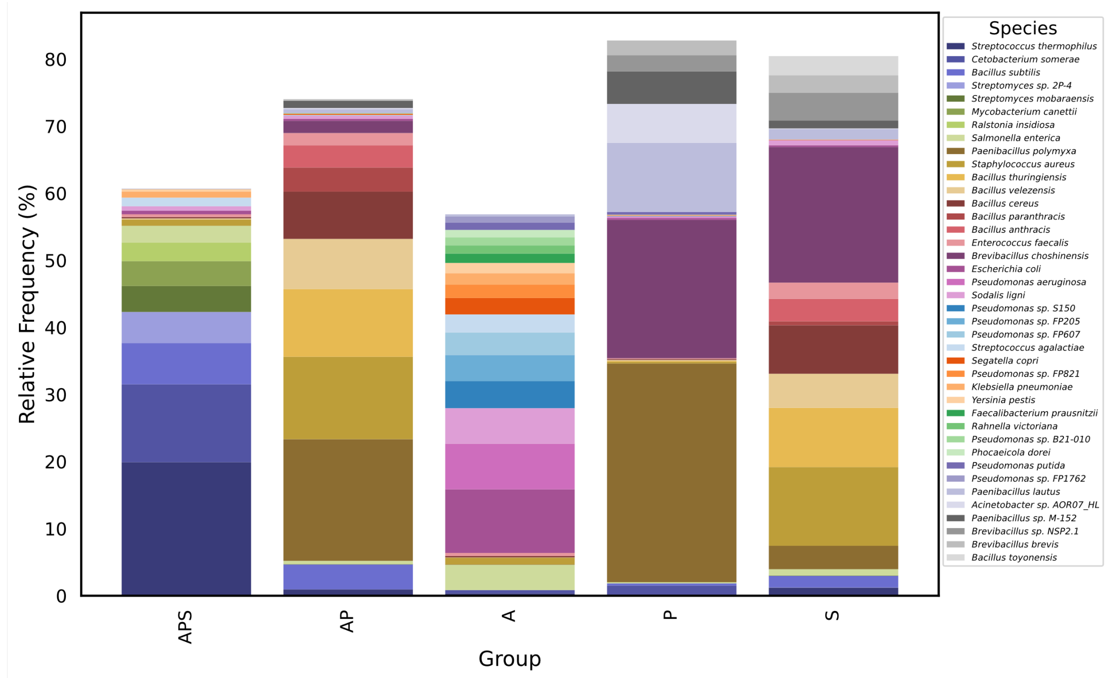


***Figure 1.15C**: Gut microbiome analysis, the stacked bar plot displays the relative frequency of microbial abundance of five different diet groups at Species level.*

Name	APS	Name	AP
Streptococcus thermophilus	19.91%	*Paenibacillus polymyxa*	18.13%
Cetobacterium somerae	11.61%	*Staphylococcus aureus*	12.30%
Bacillus subtilis	6.17%	*Bacillus thuringiensis*	10.09%
Streptomyces sp. 2P-4	4.63%	*Bacillus velezensis*	7.48%
Streptomyces mobaraensis	3.87%	*Bacillus cereus*	7.04%
Name	A	Name	P
Escherichia coli	9.49%	*Paenibacillus polymyxa*	32.61%
Pseudomonas aeruginosa	6.80%	*Brevibacillus choshinensis*	20.60%
Sodalis ligni	5.29%	*Paenibacillus lautus*	10.29%
Pseudomonas sp. S150	4.05%	*Acinetobacter sp. AOR07_HL*	5.81%
Pseudomonas sp. FP205	3.88%	*Paenibacillus sp. M-152*	4.86%
Name	S		
Brevibacillus choshinensis	20.22%		
Staphylococcus aureus	11.74%		
Bacillus thuringiensis	8.84%		
Bacillus cereus	7.22%		
Bacillus velezensis	5.08%		

***Table 1.3**: Relative abundance of top five species present in different dietary groups.*

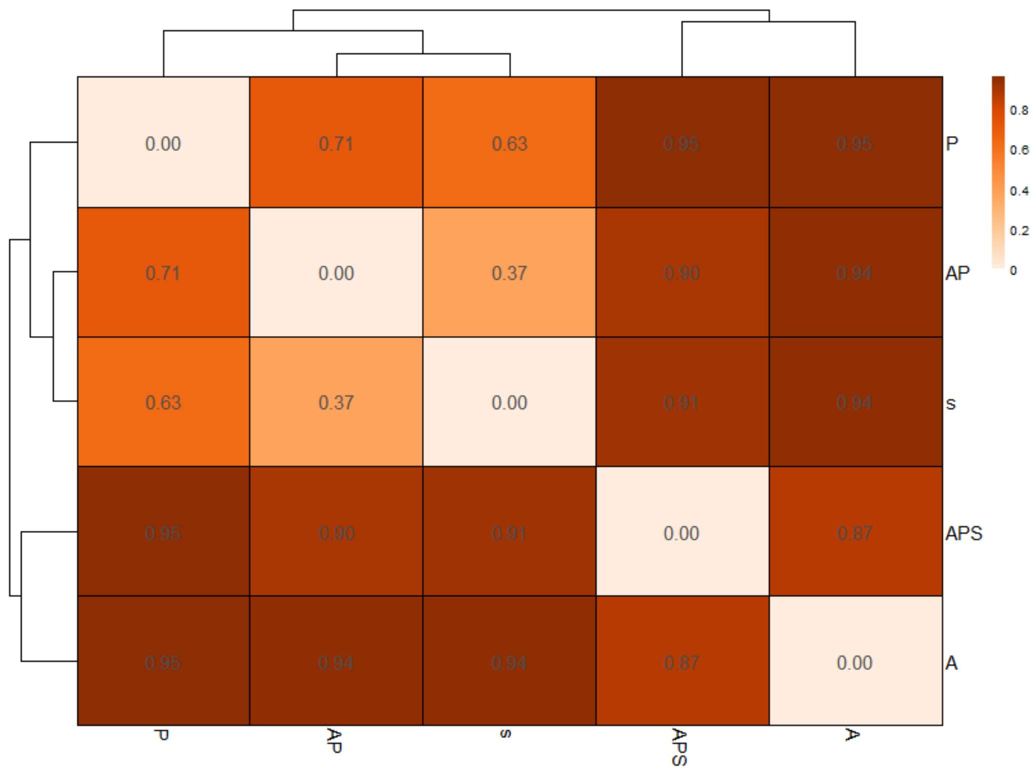


***Figure 1.15D**: Gut microbiome analysis, Beta diversity. Bray Curtis Dissimilarity Matrix between species present in each sample.*

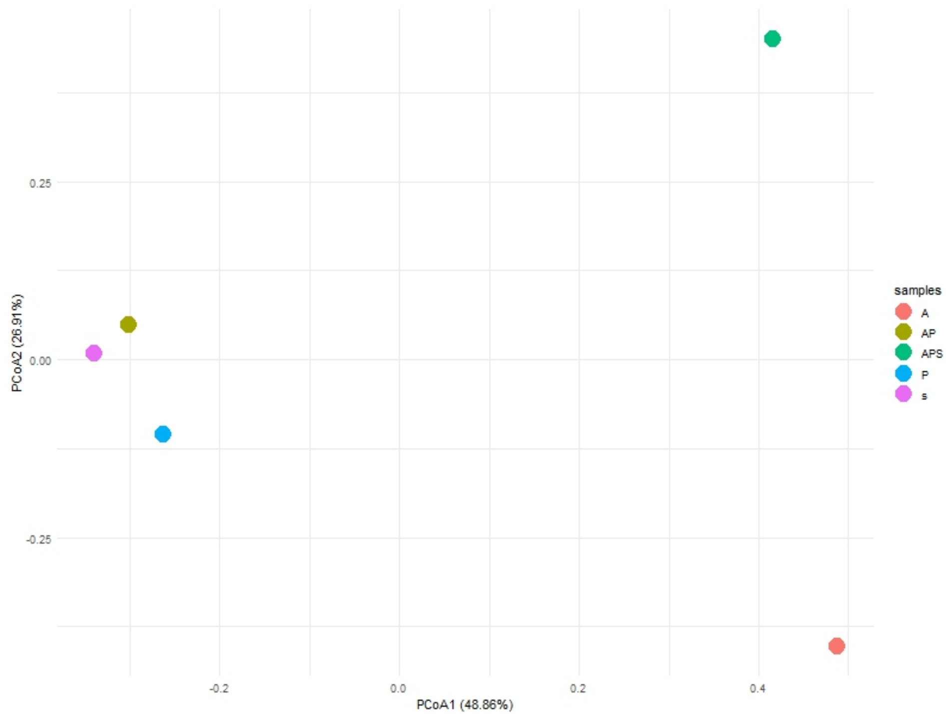


***Figure 1.15E**: Gut microbiome analysis, Principal Coordinates Analysis (PCoA) plot illustrates the beta diversity or dissimilarities among samples based on their microbiota composition*

Group	Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
APS	609	757.3483	30.59782	771.2107	14.06147	3.874403	0.93382	15.11038	105.3504
AP	695	929.5316	45.47432	894.3387	15.34142	3.627904	0.92581	13.47881	114.1967
A	917	917	0	917	15.00861	4.74417	0.973484	37.71311	139.4073
P	497	670.88	36.37004	673.6385	13.60476	2.684412	0.832598	5.973628	77.85765
S	566	777.9859	43.30146	755.6962	14.19264	3.422174	0.921971	12.8157	88.03281

***Table 1.4**: Alpha Diversity indices of different dietary groups.*

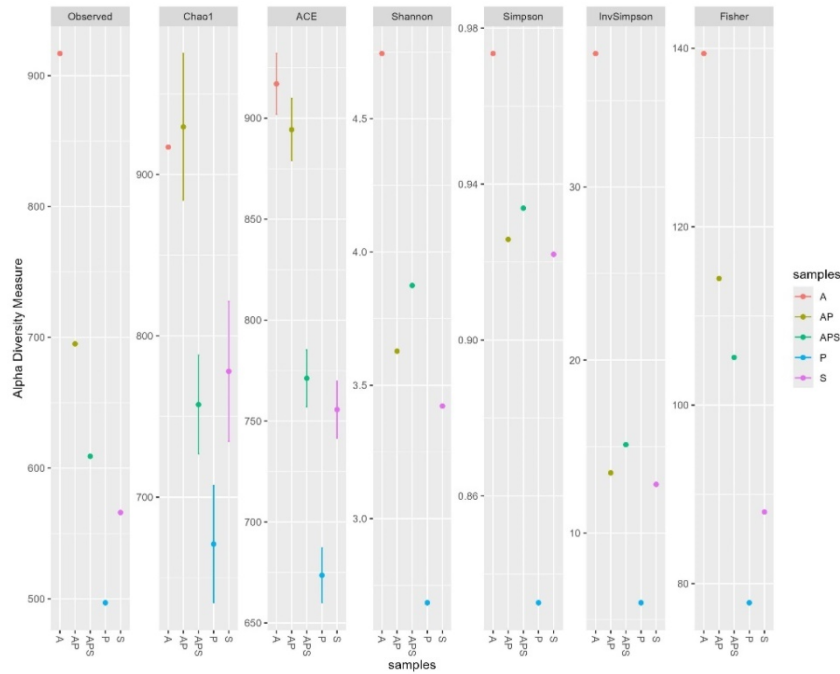


***Figure 1.15F**: Gut microbiome analysis, Alpha Diversity indices.*

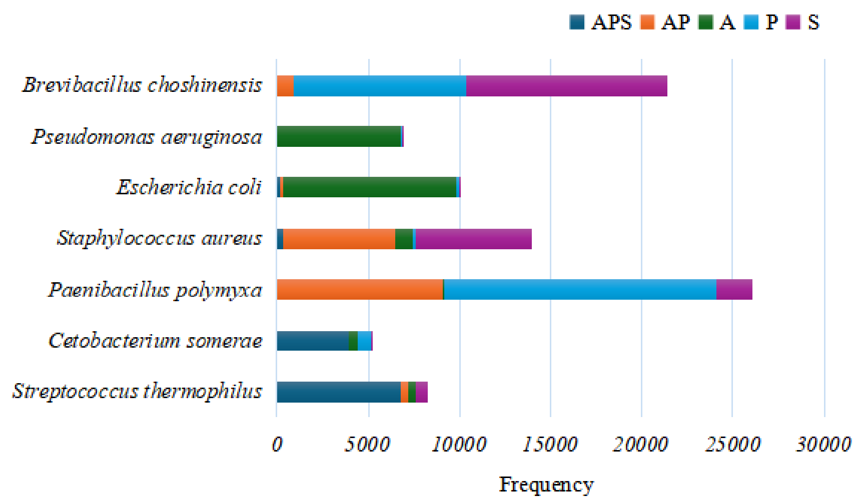
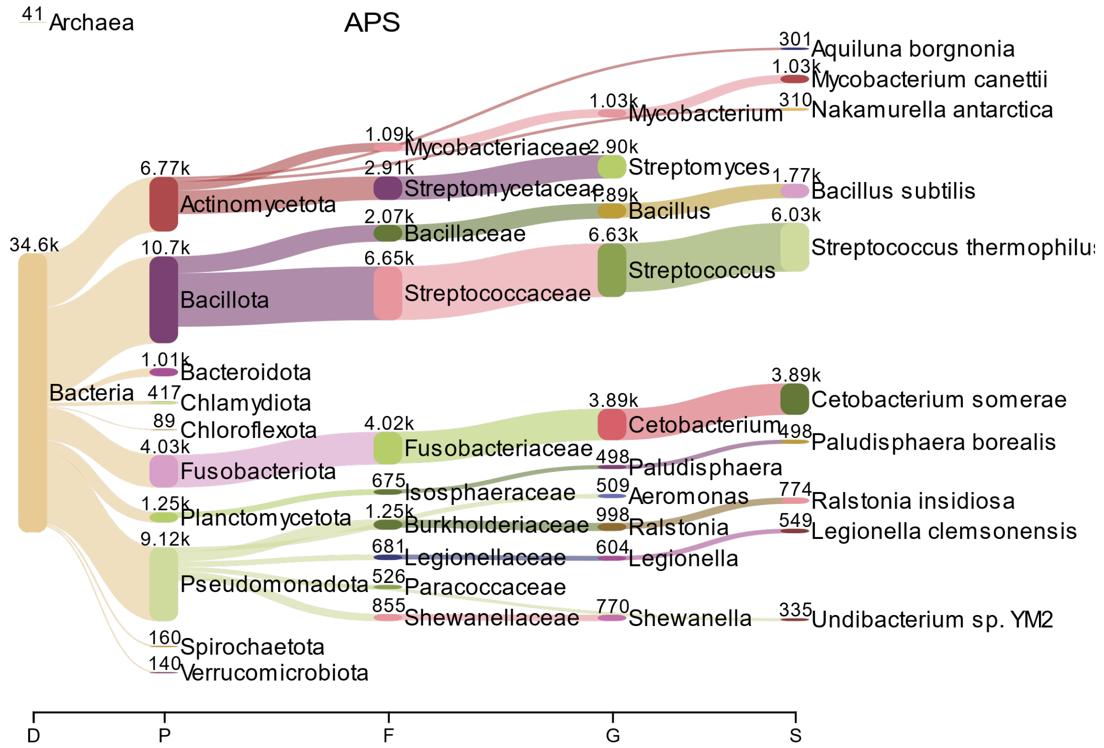
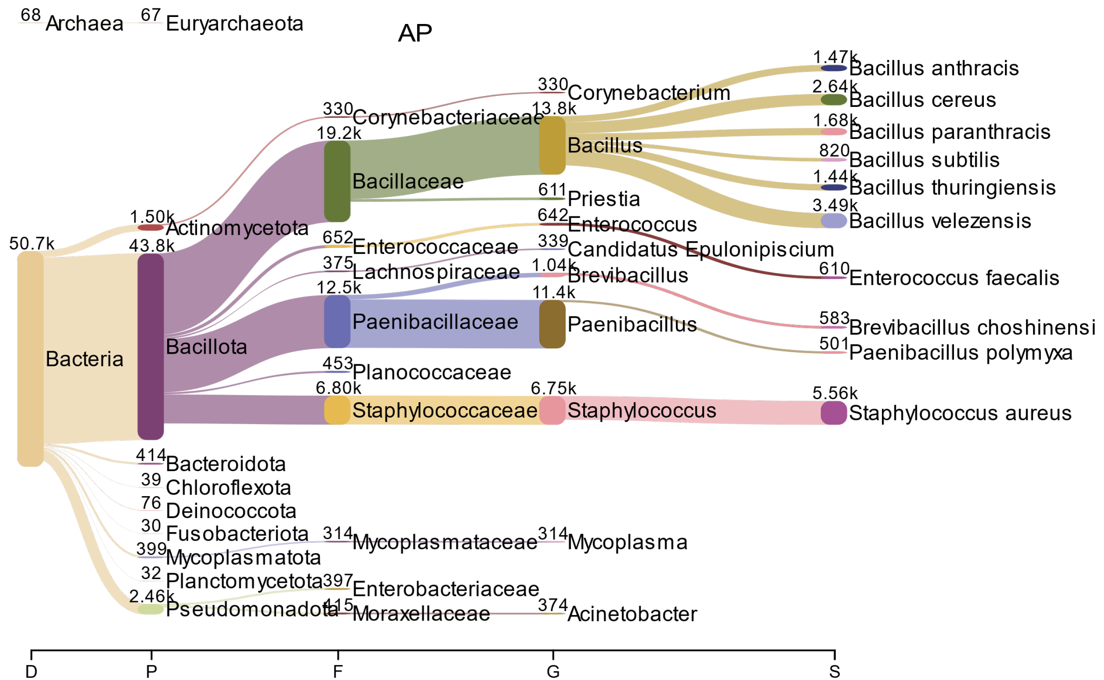
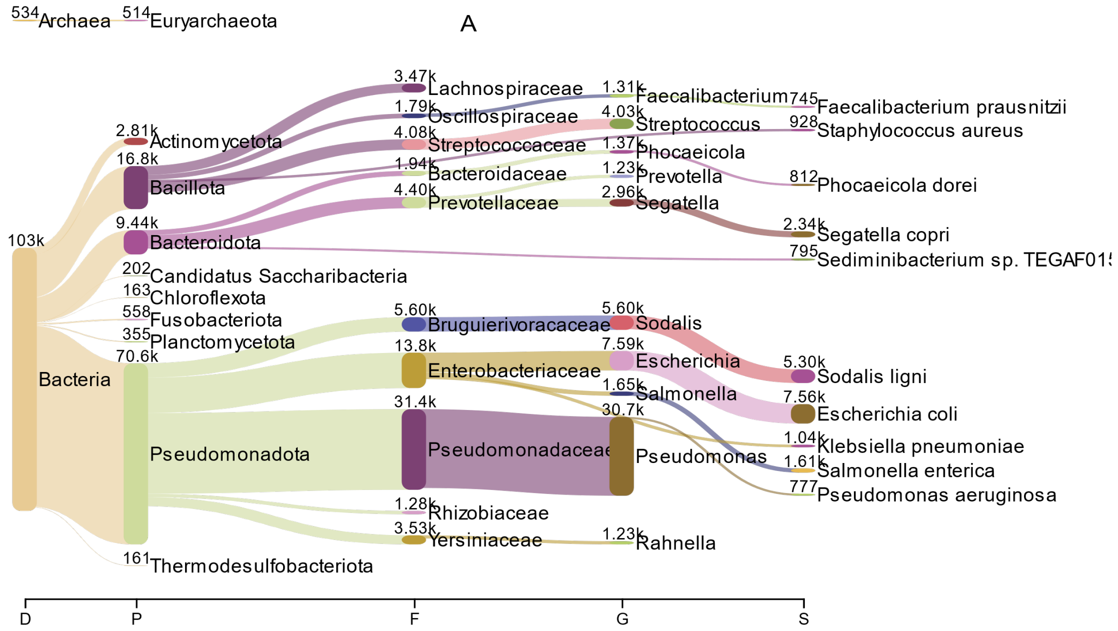
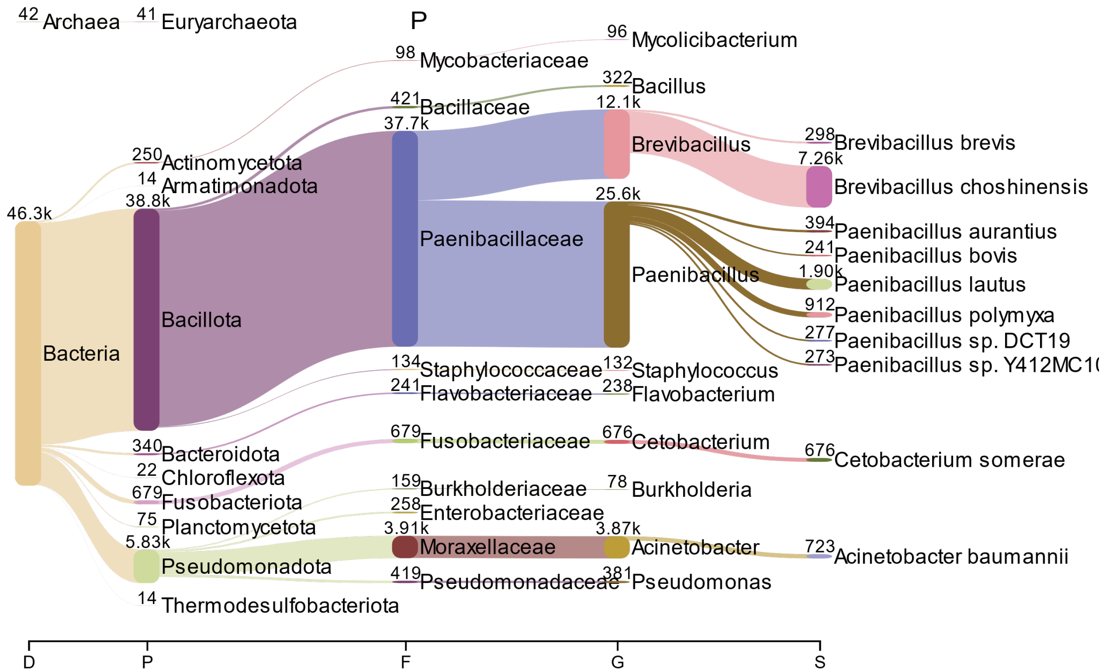


***Figure 1.15G**: Gut microbiome analysis, A horizontally stacked bar plot depicts the top two-level bacterial species across groups.*


APS
41 Archaea
34.6k Bacteria
6.77k Actinomycetota
10.7k Bacillota
1.01k Bacteroidota
417 Chlamydiota
89 Chloroflexota
4.03k Fusobacteriota
1.25k Planctomycetota
9.12k Pseudomonadota
160 Spirochaetota
140 Verrucomicrobiota
1.09k Mycobacteriaceae
2.91k Streptomycetaceae
2.07k Bacillaceae
6.65k Streptococcaceae
4.02k Fusobacteriaceae
675 Isosphaeraceae
1.25k Burkholderiaceae
681 Legionellaceae
526 Paracoccaceae
855 Shewanellaceae
1.03k Mycobacterium
2.90k Streptomyces
1.89k Bacillus
6.63k Streptococcus
3.89k Cetobacterium
498 Paludisphaera
509 Aeromonas
998 Ralstonia
604 Legionella
770 Shewanella
301 Aquiluna borgnonia
1.03k Mycobacterium canettii
310 Nakamurella antarctica
1.77k Bacillus subtilis
6.03k Streptococcus thermophilus
3.89k Cetobacterium somerae
498 Paludisphaera borealis
774 Ralstonia insidiosa
549 Legionella clemsonensis
335 Undibacterium sp. YM2
D
P
F
G
S


AP
68 Archaea
67 Euryarchaeota
50.7k Bacteria
1.50k Actinomycetota
43.8k Bacillota
414 Bacteroidota
39 Chloroflexota
76 Deinococcota
30 Fusobacteriota
399 Mycoplasmatota
32 Planctomycetota
2.46k Pseudomonadota
330 Corynebacteriaceae
19.2k Bacillaceae
652 Enterococcaceae
375 Lachnospiraceae
12.5k Paenibacillaceae
453 Planococcaceae
6.80k Staphylococcaceae
314 Mycoplasmataceae
397 Enterobacteriaceae
415 Moraxellaceae
330 Corynebacterium
13.8k Bacillus
611 Priestia
642 Enterococcus
339 Candidatus Epulonipiscium
1.04k Brevibacillus
11.4k Paenibacillus
6.75k Staphylococcus
314 Mycoplasma
374 Acinetobacter
1.47k Bacillus anthracis
2.64k Bacillus cereus
1.68k Bacillus paranthracis
820 Bacillus subtilis
1.44k Bacillus thuringiensis
3.49k Bacillus velezensis
610 Enterococcus faecalis
583 Brevibacillus choshinensis
501 Paenibacillus polymyxa
5.56k Staphylococcus aureus
D
P
F
G
S


A
534 Archaea
514 Euryarchaeota
103k Bacteria
2.81k Actinomycetota
16.8k Bacillota
9.44k Bacteroidota
202 Candidatus Saccharibacteria
163 Chloroflexota
558 Fusobacteriota
355 Planctomycetota
70.6k Pseudomonadota
161 Thermodesulfobacteriota
3.47k Lachnospiraceae
1.79k Oscillospiraceae
4.08k Streptococcaceae
1.94k Bacteroidaceae
4.40k Prevotellaceae
5.60k Bruguierivoracaceae
13.8k Enterobacteriaceae
31.4k Pseudomonadaceae
1.28k Rhizobiaceae
3.53k Yersiniaceae
1.31k Faecalibacterium
4.03k Streptococcus
1.37k Phocaeicola
1.23k Prevotella
2.96k Segatella
5.60k Sodalis
7.59k Escherichia
1.65k Salmonella
30.7k Pseudomonas
1.23k Rahnella
745 Faecalibacterium prausnitzii
928 Staphylococcus aureus
812 Phocaeicola dorei
2.34k Segatella copri
795 Sediminibacterium sp. TEGAF01
5.30k Sodalis ligni
7.56k Escherichia coli
1.04k Klebsiella pneumoniae
1.61k Salmonella enterica
777 Pseudomonas aeruginosa
D
P
F
G
S


P
42 Archaea
41 Euryarchaeota
46.3k Bacteria
250 Actinomycetota
14 Armatimonadota
38.8k Bacillota
340 Bacteroidota
22 Chloroflexota
679 Fusobacteriota
75 Planctomycetota
5.83k Pseudomonadota
14 Thermodesulfobacteriota
98 Mycobacteriaceae
421 Bacillaceae
37.7k Paenibacillaceae
134 Staphylococcaceae
241 Flavobacteriaceae
679 Fusobacteriaceae
159 Burkholderiaceae
258 Enterobacteriaceae
3.91k Moraxellaceae
419 Pseudomonadaceae
96 Mycolicibacterium
322 Bacillus
12.1k Brevibacillus
25.6k Paenibacillus
132 Staphylococcus
238 Flavobacterium
676 Cetobacterium
78 Burkholderia
3.87k Acinetobacter
381 Pseudomonas
298 Brevibacillus brevis
7.26k Brevibacillus choshinensis
394 Paenibacillus aurantius
241 Paenibacillus bovis
1.90k Paenibacillus lautus
912 Paenibacillus polymyxa
277 Paenibacillus sp. DCT19
273 Paenibacillus sp. Y412MC1
676 Cetobacterium somerae
723 Acinetobacter baumannii
D
P
F
G
S

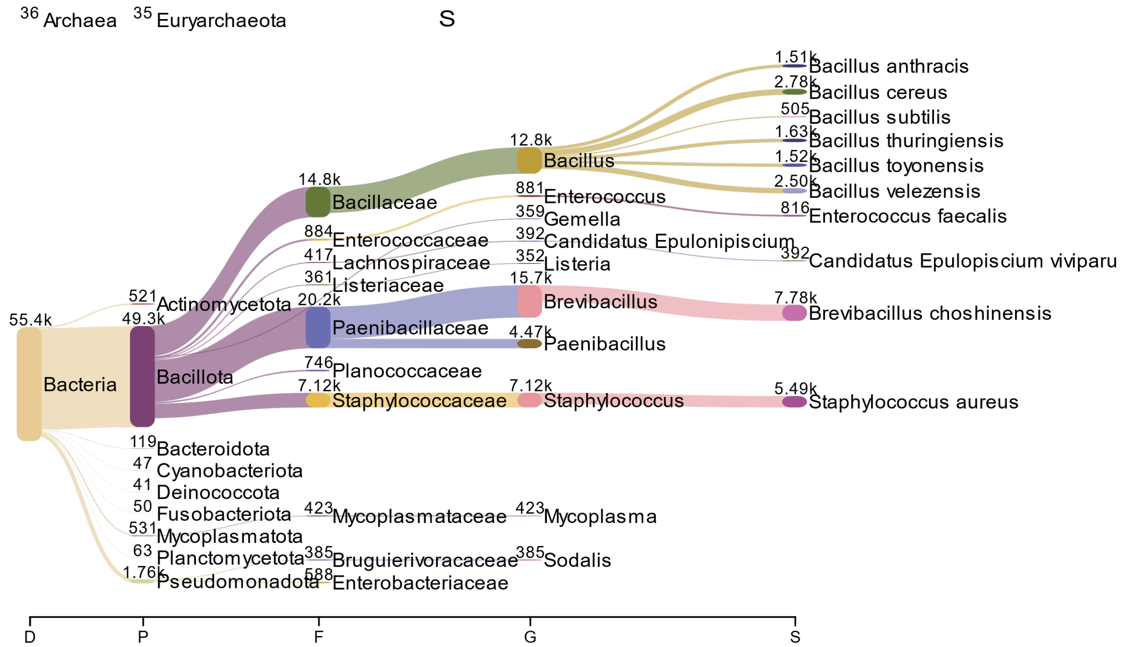


***Figure 1.15H**: Gut microbiome analysis, Sankey plots representing major taxa of APS, AP, A, P, and S. (Data expressed in relative percentage wise from three different samples)*

1.5. Discussion

Although zebrafish are prominently used in behavioural studies (Barreiros et al.,2021; Petersen et al., 2022; Selvaraj et al., 2022), the impact of gut microbial fluctuations, driven by changes in dietary regimes, on behavioural transitions has not been extensively explored. This study investigated the effects of five distinct dietary regimes-*Artemia*+Pellet+Spirulina (APS), *Artemia*+Pellet (AP), *Artemia* only (A), Pellet only (P), and Spirulina-rich diet (S) on zebrafish gut microbiota and behaviour. The findings provide light into the influence of dietary combinations on gut microbial dynamics and behavioural endpoints. This study demonstrated that behavioural outcomes, such as exploration, locomotion, anxiety, and aggression, are significantly modulated by diet.

The Novel Tank Test (NTT), a widely established behavioural protocol leveraging zebrafish's innate responses to novel environments and the test was employed to assess anxiolytic and anxiogenic effects (Bencan et al., 2009; Egan et al., 2009;

Kalueff et al., 2013). The behavioural metrics including the number of entries into the top zone, mean speed, distance travelled, and top-bottom distance ratio were markedly higher in the APS group. This indicates the enhanced locomotor and exploratory capabilities of mixed diet. Whereas these metrics were substantially reduced in the A group. Additionally, the APS group exhibited increased time spent in the top zone and reduced freezing time, suggestive of anxiolytic effects. Whereas the opposite pattern in the A group indicated an anxiogenic response. These findings are attributed to the balanced nutritional composition of the APS diet. Such balance in the nutritional profile can positively influence the gut-brain axis. While the A diet likely lacks critical nutrients essential for normal behaviour. Previous studies have demonstrated that zebrafish fed chloroplast-rich diets and diverse protein sources exhibited improved growth and nutritional profiles (Gedi et al., 2019; Williams et al., 2023). Though *Artemia* offers immunomodulatory benefits, enhanced pigmentation, and sensory stimulation (Wee et al., 2021), its nutritional inadequacies, notably the absence of essential polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and arachidonic acid (ARA), which are crucial for zebrafish development and growth, might be contributed to the heightened anxiety-like behaviours in zebrafish (Lawrence et al., 2012; Cueto-Escobedo et al., 2022; Figueiredo et al., 2009). Enrichment of *Artemia* cultures can partially address these deficiencies but is insufficient to compensate for the nutritional gaps in newly hatched *Artemia* nauplii (Navarro et al., 2014; Wan-Loy, 2004).

Zebrafish fed P and S diets exhibited moderate exploratory and anxiety-related behaviours, suggesting partial fulfilment of nutritional requirements. Variations in the nutritional composition of commercial diets could impact growth, development, and reproductive competence in adult stages. Although *Artemia* provides initial

growth advantages during early developmental stages, its exclusive use as an adult diet results in insufficient nutrient provision (Fowler et al., 2019). In contrast, the AP diet, commonly used in laboratories (Del Vecchio et al., 2021) supported normal exploration in zebrafish. However, the zebrafish fed AP diet exhibited increased freezing time and reduced top-zone activity compared to the APS group, indicating the advantages of additional supplementation of spirulina in diet. Ranking stress levels among the groups revealed the following order: APS < P < S < AP < A. These results emphasize that a varied, nutrient-dense diet, such as APS, promotes greater exploration and lower anxiety, consistent with the neurobehavioural benefits of nutrients like those found in spirulina.

The increased frequency of entries into the light zone observed in APS, P, and S groups again supports their anxiolytic nature. Even though the AP diet group showed a lower number of entries to light zone, their decreased latency and higher time in light zone similar APS, P, and S suggests that diets containing spirulina and pellets may contribute to decreased anxiety levels and an inclination towards exploring brighter, more exposed areas. Prior research on the spirulina-based diets suggests that their capacity to boost serotonin production and improve gut-brain connectivity contributes to stress-relieving effects (Ma et al., 2022). Conversely, the limited amino acid profile of *Artemia* nauplii, including deficiencies in free amino acids critical for neurotransmitter synthesis (Fyhn et al., 1993; Helland, 1995; Verma et al., 2021), may underlie the heightened anxiety observed in the A group. Nutritional deficits in diet, especially vitamin D, dramatically reduced the vertical position and swimming activity of fish (Oliveri et al., 2020). The changes in behaviour and development have also been linked to iron deficiency as it impacts neurotransmitters, including serotonin, noradrenaline, and dopamine (Shah et al.,

2021). Moreover, insufficient levels of vitamin B, minerals like calcium, magnesium, zinc, and omega-3 fatty acids, documented to affect stress and anxiety in humans (Durrani et al., 2022; Du et al., 2022; Cuciureanu & Vink, 2011; Hagmeyer et al., 2015; Antao et al., 2023). This is probably that *Artemia*-only diet exacerbated the anxiogenic effects in zebrafish (Kandathil Radhakrishnan et al., 2019).

When two zebrafish are placed in the same tank, they often display aggression and form a hierarchy; the dominant fish shows more aggression and occupies more tank space (Teles et al., 2016; Larson et al., 2006; Paull et al., 2010). In zebrafish, aggression can be induced using mirror stimulation to avoid direct confrontations (Gerlai et al., 2000; Norton et al., 2011; Barbosa et al., 2019; Reichmann et al., 2022). Zebrafish on a P diet displayed significantly elevated aggression. It was evident in higher mirror-bite frequencies, while APS, AP, and S diets elicited increased entries with fewer bites, indicating moderated aggression. The A group exhibited diminished aggression and boldness, characterised by increased latency and reduced mirror-bite frequency, speed, and distance travelled. Studies referred to the fact that aggressive behaviour in zebrafish exhibits a moderate heritability index of 0.36 (Ariyomo et al., 2013), indicating that this is influenced by both genetic and environmental factors, including diet (Reichmann et al., 2022). Diets rich in protein, such as the nursery pellet diet, are associated with increased aggression in zebrafish. This result is consistent with findings in other species, such as southern field crickets, which revealed that young crickets fed high-protein diets exhibited increased weight, heightened aggression, greater activity during mating, and less consistent aggressive behaviour patterns (Han & Dingemanse, 2017). Conversely, diets similar to the natural intake of zebrafish with moderate protein and fiber

optimise gut function and support behavioural stability (Leigh et al., 2018). Excessive protein or fat intake, however, can lead to obesity-associated neurobehavioural alterations, including heightened aggression (Picolo et al., 2021). As evidenced by studies, the altered estrogen production has been shown to cause increased aggression in zebrafish (Liu et al., 2020). The abnormal monoamine neurotransmitter activity and irregular neuroendocrine reactions have been observed to cause aggressive behaviours in humans and non-human vertebrates (Zhang-James & Faraone, 2017; Freudenberg et al., 2016). For instance, the low serotonin levels, impaired dopamine signalling, reduced GABA signalling, increased adrenaline, noradrenaline, histamine, steroid deficit, and stress-related hormones are the associated underlying mechanisms that lead to the generation of aggressive behaviours (Umukoro et al., 2013; Zabegalov et al., 2018).

The reduction in parallel orientation is an indicative of exploratory behaviour, in the mirror zone all groups showed reduced parallel orientation. However, the P diet group exhibited the most pronounced decrease. The parallel index has been proposed to reflect the overall tendency to turn and reflect subtle changes in the pattern of locomotor activity, which explains characteristics of the exploration of an unfamiliar environment compared to the locomotor movement in frequently visited areas (Horstick et al., 2020). And it was found to decrease with the familiarity of the area being explored. Turn angle analysis revealed changes in zebrafish angular movement in response to a stimulus, particularly their own mirror image. Consistent turning behaviour was observed in the APS group, whereas the P and S groups exhibited greater variability, reflecting differences in locomotor responses. Spinal projection neurons (SPNs) mediate these turning and modulating movement based on stimuli and turning angles (Huang et al., 2013). Considering morphometry,

compared to other groups, the P group exhibited higher mean weight and girth suggesting a propensity for diet-induced obesity. It can potentially disrupt forebrain neurogenesis and brain homeostasis (Ghaddar et al., 2020), further contributing to the behavioural variations. The genetics, nutritional status, medications, and physiological processes influence gut microbiota and its structure and function (Matsuzaki et al., 2023). The intestinal microbiome plays a vital role in mediating numerous metabolic processes in the host, such as preserving homeostasis, facilitating immune function, maintaining mucosal barrier integrity, providing metabolic assistance, and assisting brain development (Kubelkova et al., 2016). In fish, as in many other organisms, this microbial community forms a symbiotic relationship with its host, performing essential functions in pathogen defences, as well as nutritional, hormonal, neurological, and physiological processes (Vargas-Albores et al., 2023; Garibay-Valdez et al., 2024). Research using gnotobiotic or germ-free models has provided convincing evidence for the influence of microbiota on brain communication. Key findings highlight the role of microbiota in modulating normal stress responses, anxiety-related behaviours, and other central nervous system (CNS) functions (Luczynski et al., 2016). The present study demonstrated that dietary changes lead to significant alterations in gut microbiota composition, which may underlie observed behavioural abnormalities.

Among the various dietary groups in this study, six principal bacterial phyla were identified: Pseudomonadota, Bacillota, Bacteroidota, Actinomycetota, Fusobacteriota, and Planctomycetota. Notably, Pseudomonadota dominated the A group, whereas Bacillota was predominant in all other groups. Significant decrease in the abundance of Pseudomonadota was observed in both AP and S diet groups, while the APS diet group exhibited higher proportions of Actinomycetota,

Fusobacteria, and Planctomycetota. The microbial phyla identified in this study are consistent with findings from a comprehensive meta-analysis of 16S rRNA sequences from zebrafish intestinal microbiomes, identified a core microbiota comprising Proteobacteria (Pseudomonadota), Fusobacteriota, Planctomycetota, Firmicutes (Bacillota), Actinobacteriota (Actinomycetota), and Bacteroidota (Garibay-Valdez et al., 2024). The APS diet group exhibited a more balanced microbiota composition at the phylum level.

The A diet group favoured genera such as *Pseudomonas*, *Escherichia*, *Sodalis*, *Streptococcus*, and *Salmonella*, encompassing pathogenic species including *Pseudomonas aeruginosa*, *Escherichia coli*, *Sodalis ligni*, *Salmonella enterica*, and *Streptococcus agalactiae*. Pathogenicity studies indicate that *P. aeruginosa* infects zebrafish through virulence factors disrupting host cellular functions and evading immune responses (Clatworthy et al., 2009; Pont & Blanc-Potard, 2021; Bergeron et al., 2017). Additionally, *Pseudomonas* has been shown to influence appetite-related genes, suggesting a potential role in CNS-mediated feeding (Song et al., 2022). In the zebrafish intestine, the increment of *E. coli* strains can affect epithelial barrier integrity, inflammatory responses (Flores et al., 2023), nutrient-processing gene function, innate immune functions, and cellular proliferation. Colonisation by specific *E. coli* strains, such as DH5α and MG1655, has been associated with decreased hyperactivity and changes in lipid metabolism-related genes in zebrafish (Tan et al., 2019; Nag et al., 2018). Similarly, *S. enterica* and *S. agalactiae* (Group B *Streptococcus*, GBS) have been implicated in inflammatory responses. The pathogenicity may further lead to endotoxemia and trigger neuroinflammation, anxiety, and cognitive impairments in zebrafish (Howlader et al., 2016; Kim et al., 2015; Hayley Patterson et al., 2012; Buttini et al., 1996; Goel et al., 2018). The

presence of these pathogenic microbes in the A diet group may account for reduced exploratory behaviour, heightened anxiety, and decreased aggression.

In contrast, the APS diet promoted the growth of beneficial genera such as *Streptococcus*, *Cetobacterium*, *Streptomyces*, and *Bacillus*. The APS diet primarily supported species such as *Streptococcus thermophilus*, *Cetobacterium somerae*, *Bacillus subtilis*, and various *Streptomyces* species. While studies directly linking *S. thermophilus* to zebrafish gut health and its influence on behaviour are limited, the existing research suggests that *S. thermophilus* is found to exhibit a symbiotic relationship with *Lactobacillus rhamnosus* (Falcinelli et al., 2015; Gioacchini et al., 2012; Gioacchini et al., 2014), a potential psychobiotic in mice and humans (Bravo et al., 2011; Partty et al., 2015). The probiotic supplementation with *L. rhamnosus* increased the level of *Streptococcus* in the zebrafish gut with a decreasing percentage of Pseudomonadota, and it has been associated with improved serotonergic gene expression and exploratory behaviour in zebrafish (Borrelli et al., 2016). These studies supported our result that zebrafish fed the APS diet displayed higher exploratory and lower anxious behaviour with higher abundance of Bacillota and reduced Pseudomonadota. *C. somerae*, a predominant bacterium in the zebrafish gut (Yu et al., 2020) belonging to the phylum Fusobacteria, plays a crucial role in maintaining gut health and immune responses. It contributes to the synthesis of vitamin B_{12}, which in turn enhances the interactions within the gut microbiota and thereby improving the host's resistance against infections (Qi et al., 2023). Additionally, *C. somerae* produces short-chain fatty acids (SCFA), particularly acetate, which positively influences glucose homeostasis in zebrafish. This effect is mediated through parasympathetic activation, highlighting the gut-brain axis in regulating metabolic functions (Wang et al., 2021). Their role in maintaining gut

health and modulating metabolic functions suggests potential indirect effects on behaviour. Additionally, *B. subtilis* serve as a probiotic in zebrafish and has been shown to alter the intestinal microbiota profile of zebrafish. Immersion of zebrafish in *B. subtilis* KM0 for 48 hours reduced potentially pathogenic bacteria such as *Flavobacterium*, *Plesiomonas*, and *Pseudomonas*, thereby enhancing gut health (Castro et al., 2023). In laying hens, *B. subtilis* supplementation reduced stress-induced injurious behaviour by modulating the microbiota-gut-brain axis (Jiang et al., 2021). *Streptomyces* species are known for their bioactive compounds, including antibiotics and enzymes (Quinn et al., 2020). In zebrafish, *Streptomyces* has gathered attention for its potential probiotic properties, which may contribute to host health and behaviour (Tan et al., 2016; Butt et al., 2024). *Streptomyces sp.* RL8 alone or in combination with *Bacillus* influenced the composition of gut microbiota, thereby improving the host's digestive efficiency and immune response in white shrimp (Mazon-Suastegui et al., 2020). These findings align with the observed behavioural improvements and microbial composition in the APS diet group.

The AP diet predominantly supported genera such as *Bacillus*, *Paenibacillus*, *Staphylococcus*, and *Brevibacillus*, with enriched species including *Paenibacillus polymyxa*, *Staphylococcus aureus*, *Bacillus thuringiensis*, *Bacillus velezensis*, and *Bacillus cereus*. While direct studies on *P. polymyxa* in zebrafish are limited, research on related *Paenibacillus* species, such as *P. ehimensis* administration, resulted in potential benefits to gut health and overall metabolism in zebrafish (Lin et al., 2022). *B. velezensis* and *B. cereus* have demonstrated probiotic properties in aquatic species, including zebrafish, and their supplementation improved the beneficial bacterial taxa (Chen et al., 2023). In Pengze crucian carp (*Carassius auratus var. Pengze*), dietary supplementation with *B. cereus* reshaped the intestinal

bacterial community altered by a high plant protein diet, notably reducing opportunistic pathogens (Li et al., 2022). The presence of *S. aureus*, generally not regarded as a natural pathogen of fish, has been documented to induce illness in zebrafish embryos. Notably, utilizing neutrophils to circumvent destruction by the host immune system may explain the anxiety-like behaviours observed in this group (Prajsnar et al., 2012; Fries et al., 2023).

The P diet, characterised by increased protein and fat content, favoured genera such as *Paenibacillus*, *Brevibacillus*, *Acinetobacter*, and *Cetobacterium*. The predominant species associated with this diet include *Paenibacillus polymyxa*, *Brevibacillus choshinensis*, *Paenibacillus lautus*, and *Acinetobacter* species. *B. choshinensis* is widely utilised in biotechnology for the production of recombinant proteins. Its low extracellular proteolytic activity and high secretion efficiency make it an ideal candidate for producing various enzymes and therapeutic proteins (Horne et al., 2004; Mizukami et al., 2018), while *B. choshinensis* itself has not been extensively studied as a probiotic; other species within the *Brevibacillus* genus have shown probiotic potential (Cao et al., 2024). Although specific studies on *P. lautus* in zebrafish are missing, members of the *Paenibacillus* genus are known for their diverse metabolic capabilities, including the production of antimicrobial compounds and enzymes involved in nutrient cycling (Grady et al., 2016). In zebrafish, *Acinetobacter* can become part of the gut microbiota, especially under certain dietary conditions. For instance, zebrafish fed a high-fat diet exhibited an increased abundance of *Acinetobacter* species in their intestines (Ye et al., 2019), which might promote obesity and elevated aggression. Studies also support that overweight or obese individuals are more physically aggressive (Tso et al., 2017), and it is

associated with higher body mass index and fat (Derks et al., 2019), backing the findings in this group.

The S diet promoted bacterial genera similar to the AP diet, including *Bacillus*, *Brevibacillus*, *Staphylococcus*, and *Paenibacillus*, but with a higher enrichment of *Brevibacillus*. The key bacterial species associated with the Spirulina diet include *Brevibacillus choshinensis*, *Staphylococcus aureus*, *Bacillus thuringiensis*, *Bacillus cereus*, and *Bacillus velezensis*. Studies reported that spirulina supplementation can increase the relative abundance of beneficial bacteria (Yu et al., 2020) and also showed that a reduced ratio of Firmicutes to Bacteroidetes (Bacillota to Bacteroidota) (Yu et al., 2020; Kumar et al., 2024). In contrast, our study witnessed an increased ratio of Bacillota to Bacteroidota. Despite the beneficial effects of spirulina supplementation, the observed anxiety-like behaviours might be linked to diet-induced microbial shifts (Kumar et al., 2024). Research reveals that having a wide variety of gut bacteria and certain types of bacteria is linked to how fish explore and recognize novel objects. This behaviour is often used to study fear and anxiety, also suggesting the link between gut bacteria and fish behaviour. These factors can change the gut bacteria, and thereby influence the gut-brain-behaviour axis (Anka et al., 2025), possibly through the production of secondary metabolites, including SCFAs. The metabolites generated by gut microbiota can influence the secretory activities of enterocytes, which in turn affect the production of gut peptides that regulate gut motility (Borre et al., 2014; Cani & Knauf, 2016). These microbial substances can impact behaviour either by directly entering the bloodstream and crossing the blood-brain barrier (BBB) or by indirectly stimulating the vagal neurons (Breton et al., 2016; Wostmann et al., 1983; Cussotto et al., 2018; Kim & Serre, 2018). The bidirectional interaction between the gut and brain can

impact brain biochemistry, including GABA, serotonin, and tryptophan metabolites, which are crucial for the regulation of the central nervous system (Haque et al., 2022).

In nutshell, the present study suggests that among the five different feeding regimes tested, the mixed diet consisting of *Artemia*, pellet, and spirulina (APS) is particularly effective in modulating the gut-brain axis. This diet positively influenced exploratory, anxiety-related, and aggressive behaviours, also promoted beneficial composition of gut microbiota. Thereby highlighting the significance of a balanced diet. Although the standard mixed diet with *Artemia* and pellet (AP) supported zebrafish microbiome and behaviour, it induced higher levels of anxiety in the novel environment compared to the APS, P, and S diets. Pellet-based diets, including both nursery pellet and spirulina-enriched pellets, are generally considered complete meals, however the high protein content in the nursery pellet diet appeared to elevate mirror-induced aggression in zebrafish, potentially due to increased body weight and girth. The spirulina-rich diet showed behavioural effects similar to APS but induced slightly higher anxiety in novel environment. In contrast, the *Artemia*-only diet was insufficient to support a healthy gut microbiota, as it harboured many pathogenic species and negatively influenced exploratory behaviour, anxiety, and boldness. These findings highlight that the monotypic diet is suboptimal, and combining diverse dietary components can substantially enhance both gut microbiota composition and behaviour in zebrafish. The possible implication of this study is that differential nutrient requirements can influence the behaviour and gut microbiome of adult zebrafish. The results also suggest that the laboratory-reared zebrafish need live as well as commercial feeds to balance all the nutrient requirements which in turn get manifested in their gut-brain axis. Overall, the results

emphasise critical influence of dietary composition on zebrafish behaviour, highlighting the role of nutrient density and diversity in modulating exploratory, anxiety-related, and aggressive behaviours.

1.6. References

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CHAPTER 2

Acute antibiotics treatment influences both the incidence and onset of PTZ-induced epileptic seizures in zebrafish

Rationale: In addition to diet, the surrounding environment plays a critical role in determining the health and well-being of an organism. Antibiotics, while essential for treating bacterial infections, must be used with caution under appropriate guidance and regulation. Overuse or misuse can lead to detrimental effects on both individual health and the environment. Considering that certain antibiotics exhibit neurotoxic properties, investigating the impact of short-term, high-dose antibiotic exposure on anxiety-like and seizure-related behaviours in zebrafish may provide valuable insights and raise awareness regarding epilepsy.

2.1. Graphical Abstract

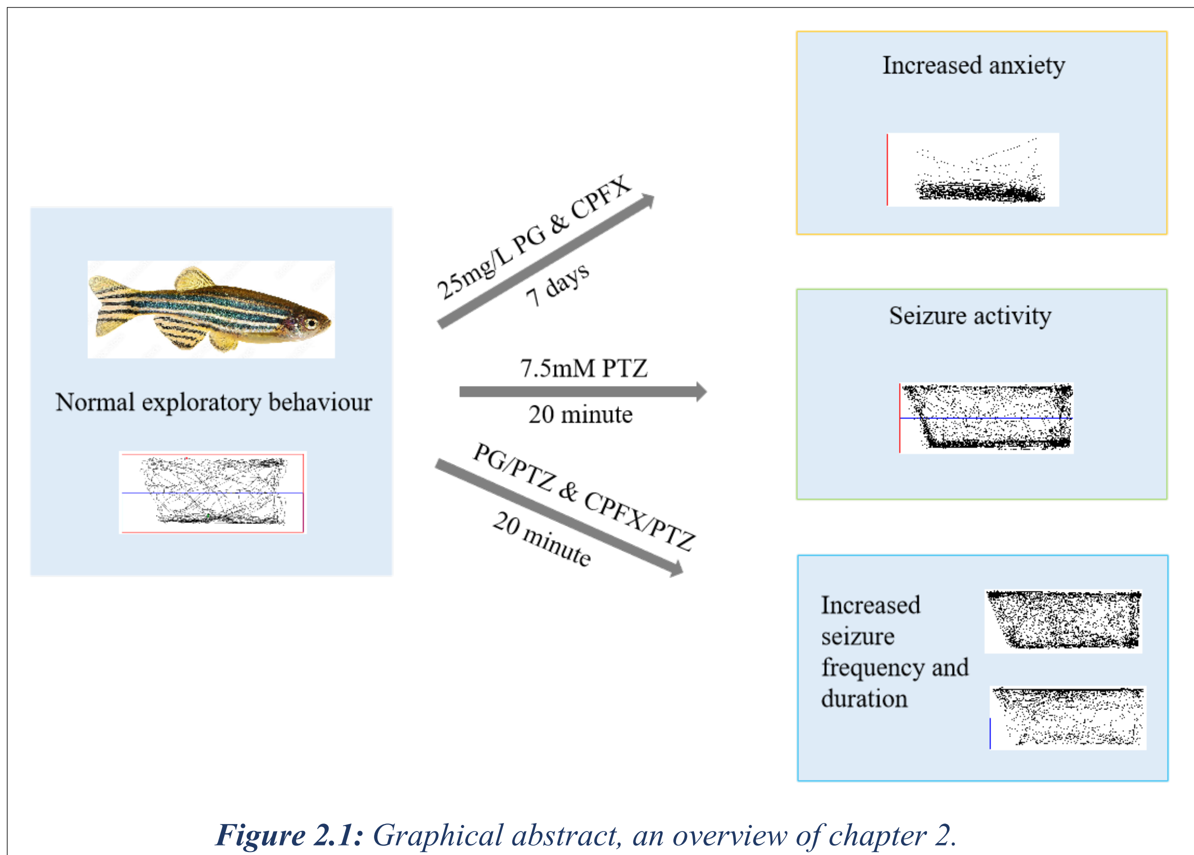

Figure 2.1: *Graphical abstract, an overview of chapter 2.*

2.2. Introduction

Antibiotics are widely used as chemotherapeutic means for controlling infectious diseases in human beings, plants and animals. Besides their beneficial effects, possible consequences of them also should have taken into consideration. The inclusion of antibiotics in various non-therapeutic purposes as growth promoters in animal agriculture is massive scale, (Chattopadhyay, 2014) they appear to elevate the development of antibiotic-resistant strains (Yap, 2013). Antibiotics used in agriculture, livestock and humans can escalate the antibiotic load in the environment, which is a major concern in the evolution of antibiotic-resistant bacteria and consequential health problems (Koch et al., 2021). Moreover, it was reported that antibiotics cause allergies, nephritis, gastrointestinal issues, haematological problems, electrolyte imbalance and nervous system dysfunction in patients undergoing periodontal treatments (Heta & Robo, 2018). The neurotoxic side effect is of major concern. Among the different classes of antibiotics, β-lactams and fluoroquinolones are frequently linked to the central nervous system (CNS) dysfunction (Wanleenuwat et al., 2020; Zhang et al., 2013), including pro-convulsive or epileptogenic effects (Grondahl & Langmoen, 1993). The unopposed mechanisms underlying the situation are (Gamma-aminobutyric acid A) $GABA_A$ receptor antagonism and (N-methyl-D-aspartate) NMDA receptor agonism (Sugimoto et al., 2003; Wanleenuwat et al., 2020). Other side effects of β-lactam and fluoroquinolones are the production of reactive oxygen species (ROS) and mitochondrial dysfunction, which induce DNA, protein and lipid damage in mammalian cells and contribute to neurotoxicity (Kalghatgi et al., 2013; Salimiaghdam et al., 2022). Here, we chose penicillin G (PG) and ciprofloxacin (CPFX) from the β-lactam and fluoroquinolone groups respectively. PG prevents

penicillin-binding proteins (PBP) from assisting in the formation of bacterial cell walls and disengages the machinery responsible for peptidoglycan biosynthesis (Cho et al., 2014). CPFX, a fluorinated quinolone, is typically employed as a broad-spectrum anti-microbial drug against both gram-positive and gram-negative bacteria. By impeding DNA topoisomerase II and IV, it prevents DNA replication (Plhalova et al., 2014).

Epilepsy is a neurological condition characterised by aberrant brain function. It is a neuropsychiatric illness marked with convulsive symptoms that influence social and economic well-being (Birbeck et al., 2007). Epilepsy is a disorder with complex symptoms, not a disorder with single manifestation and aetiology. There are numerous risk factors in epilepsy with significant hereditary tendency that impacts more than 70 million people globally (Thijs et al., 2019). Increased intracranial pressure, metabolic abnormalities, electrolyte disturbances, drug intoxication, stress, depression, psychosis, and sleep deprivation are responsible for the generation of epilepsy (Alldredge et al., 1989; Canzian et al., 2021; Castilla-Guerra et al., 2006; Kim et al., 2003; Trinka et al., 2012; Vespa et al., 2007). In order to understand about the pathophysiological underpinnings of epilepsy and how seizure-related behaviours change in response to environmental and pharmaceutical influence, we should make use of kindling models.

A kindling model is an animal model with epilepsy induced by chemical or electrical stimulus. The kindling study was first carried out by Goddard in 1967. Pentylenetetrazole (PTZ), a $GABA_A$ receptor antagonist, is a pharmacological stimulant widely used to induce epilepsy (Dhir, 2012). Anticonvulsant properties of several drugs can be screened in animal models with chronic epilepsy induced by the PTZ-kindling method (Gall et al., 2022). The zebrafish (*Danio rerio*), a tiny

tropical freshwater fish, has been validated as a model organism in various research domains globally (Trigueiro et al., 2020) particularly in neuro-behavioural and CNS disorders, including epilepsy (Kalueff et al., 2014; Sakai et al., 2018; Stewart et al., 2014). In translational neuroscience, zebrafish are advantageous due to their amenability to genetic manipulation, physiological resemblance to mammals, cost-effectiveness, robust behaviour, and capacity for high-throughput screening. Approximately 190 behavioural phenotypes in zebrafish have been documented (Kalueff et al., 2013). Using multichannel electroencephalogram (EEG) recordings from single or multiple adult zebrafish brains, researchers were able to analyse the mode of seizure propagation (Cho et al., 2017; Lee et al., 2020). The detailed behavioural profile of adult zebrafish under PTZ exposure was previously systematised and designated as scores. The intensity of seizure and fluctuations in latency to each score varied in response to changes in PTZ concentration (Mussulini et al., 2013).

A few studies have confirmed the epileptogenic properties of antibiotics (de Oliveira Vilaça et al., 2018; Grondahl & Langmoen, 1993; Sugimoto et al., 2003). However, it is unclear if antibiotics affect the frequency and duration of seizures in the zebrafish model. To comprehend different kinds of epilepsies and related seizures rodents have contributed to a great extent. Currently, zebrafish has had emerging as an animal model for investigating seizures and associated comorbidities (Yaksi et al., 2021). This study emphasises the zebrafish as an appropriate model organism for exploring the neuro-behavioural screening of various drugs and their convulsive properties. Here we attempt to follow up on whether antibiotics such as PG and CPFX have any oscillatory effect on PTZ-challenged zebrafish. Furthermore, we attempted to corroborate the fact that the β-

lactam and fluoroquinolone antibiotic families have a pro-convulsive effect or epileptogenic property.

2.3. Materials and Methods

2.3.1. Ethical statement

All research involving animals was undertaken by following the Ethics committee of the University of Calicut and as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulation 2021. All efforts were made to minimise the number of animals used and their suffering. All experimental protocols followed the ARRIVE guidelines (Kilkenny et al., 2010)

2.3.2. Animals

Zebrafish were obtained from a commercial supplier in their native place, West Bengal, Kolkata. The fish were quarantined and acclimatized in lab conditions for one month in separate tanks (16L) filled with RO water (reverse osmosis) and pH adjusted to 6.5-7. The temperature was set at 28 degrees Celsius. Ammonia levels were controlled by waste removal and one-third of water was changed daily. After that, they were transferred to a continuously circulating three-rack standalone zebrafish housing facility with a mechanical, biological, and UV filtration unit. They were fed twice daily with live *Artemia* in the morning and Tiqld nursery feed in the evening and provided a 14/10 light-dark cycle. Experimentally naive four to six-month-old adult fish having an average body weight of 0.45g and an average length of 3.1cm were selected for experiments. The behavioural tests were carried out between 10 a.m. and 4: 30 p.m.

2.3.3. Antibiotic Treatment

A total of 30 individuals were separated from the housing facility into separate rectangular tanks with dimensions of 36cm × 26cm × 19cm (L×W×H). Animals

were exposed to 25mg/L concentrations of CPFX and PG (one of the maxima-reported concentrations in water bodies) (Petersen et al., 2021). Individuals were divided into three groups; the control group, PG group and the CPFX group. PG was dissolved in tank water and CPFX was dissolved in 0.1 N HCL and then added to the tank water. The treatment period was extended to 7 days (to mimic the antibiotic course duration in human adults) and the treatment water was renewed every other day (24-hour interval). The control group was maintained in the tank water.

2.3.4. Pentylenetetrazole Exposure

After antibiotic treatment, fishes were individually exposed to a 7.5mM PTZ solution for 20 minutes. The concentration was selected according to previous studies, which ensures a lower mortality rate and generation of consistent seizures (Canzian et al., 2021; Mussulini et al., 2013). The zebrafish were transferred to the experimental tank having dimensions of 29.9cm × 7cm × 15cm (L×W×H) and recorded for a single session of 20 minutes. Seizure-associated behavioural scores were observed by individuals unaware of experimental conditions. The seizure-like behavioural phenomics is represented as scores; "score 0 – normal swimming in the bottom of the tank; score 1 – enhanced swimming activity; score 2 – abrupt changes in swimming directions; score 3 – circular movements in the top of the tank; score 4 – clonic-like seizure behaviour (i.e., fish display a characteristic corkscrew swimming); score 5 – loss of posture" (Canzian et al., 2021).

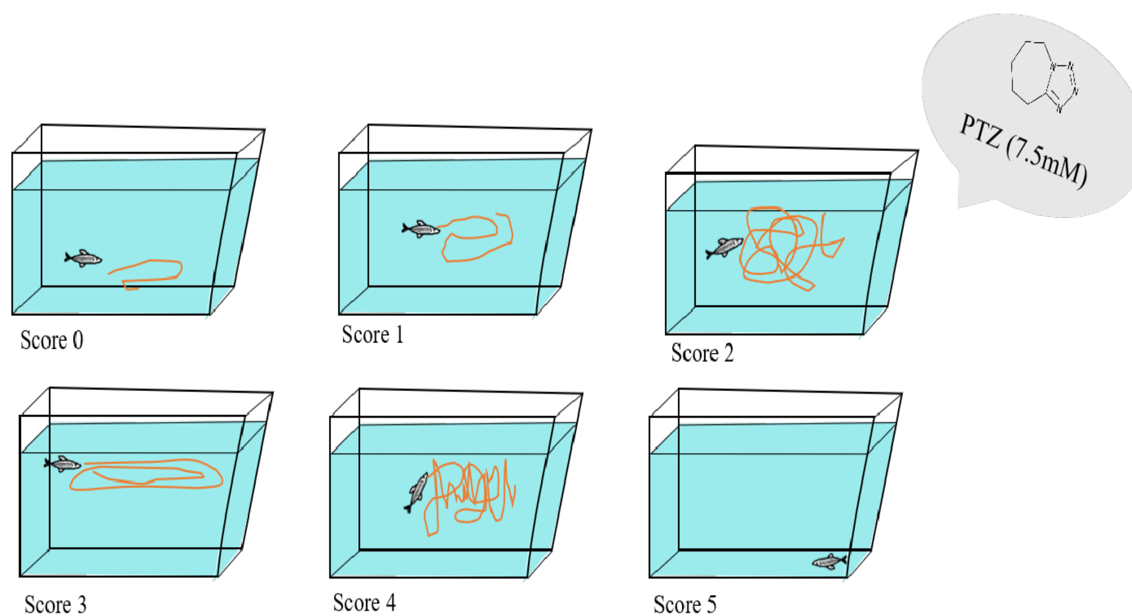

Figure 2.2: *Illustration represents seizure-like behavioural scores*

2.3.5. Novel tank behavioural test

Antibiotic-treated zebrafish were subjected to a novel tank diving test as per a previously described standard protocol to measure the anxiety and exploratory behavioural endpoints (Chin et al., 2019). A trapezoidal experiment tank having dimensions of 27.9cm in length, 15cm in height, 7cm in width, and 22cm bottom, filled with 1.350L of system water was used for experiments. The video was recorded for 6 minutes using a webcam (Logitech C270 HD 720p). The tank was virtually divided into two zones: top and bottom. Behavioural endpoints were measured using Smart 3.0 software. All behavioural tests were carried out in a closed room (Cachat et al., 2011).

2.3.6. Statistical analysis

The average values of the data points were analysed using the non-parametric Mann-Whitney-U-test when data were compared between groups. $P < 0.05$ was considered statistically significant. All the statistical analyses were performed using

Graph Pad Prism software (Graph Pad Software, Inc., San Diego, CA, USA) and Microsoft Excel.

2.4. Results

2.4.1. Exploratory behaviour and antibiotic treatment

The exploratory behaviour was evaluated after a 7- day treatment with PG and CPFX (25mg/L). Analysis of novel tank test parameters showed distinct variations in behavioural endpoints. The track path of the control group resembled a uniform pattern and covered a significant portion of the novel tank. They spent nearly the same amount of time in the arena's bottom and top zones. In contrast, the track path for PG and CPFX groups is limited to the bottom portion of the tank (Figure 2.3). The time spent at the top is significantly less in PG ($p = 0.0029$) and CPFX ($p = 0.0016$) groups when compared to the control group; however, no statistically significant differences were observed in the numbers of entries to the top. Also, the distance traveled was greater in PG ($p = 0.0011$) and CPFX ($p < 0.0001$) groups. Statistically significant drop-down in resting time (PG, $p = 0.0440$ & CPFX, $p < 0.0001$), lift in fast time (> 15 cm/s) (PG, $p < 0.0001$ & CPFX, $p < 0.0001$) and mean speed (PG, $p = 0.0018$ & CPFX, $p < 0.0001$) all these indicates restlessness in antibiotic-treated groups and represent hyperactivity. It could be due to antibiotic-induced anxiety. Among antibiotic groups, there were no statistically significant differences in exploratory behavioural endpoints (Figure 2.4).

2.4.2. PTZ exposure and Seizure-like behaviour

The antibiotic treatment later followed by PTZ exposure (20-minute, 7.5mM) testified that PG/PTZ and CPFX/PTZ groups exhibit intense seizure activity. The track path of PTZ-challenged zebrafish exhibits abrupt turns and erratic swimming patterns, depicting seizure-like behaviour (Figure 2.5a). Seizure severity was

calculated by measuring the frequency (PG/PTZ, $p = 0.0001$ and CPFX/PTZ $p = 0.0004$) and duration (PG/PTZ, $p < 0.0001$ and CPFX/PTZ $p = 0.0001$) of score 4 events which were far exceeded in antibiotic-treated groups than WATER/PTZ group. No statistically significant differences were obtained among antibiotic-treated groups. These results conclusively demonstrate that PG and CPFX have a profound effect on seizure severity (Figure 2.5b).

The seizure scores across time are illustrated in Figure 2.6 and the behavioural scores were measured at 30 seconds intervals. The early generation of seizure scores in antibiotic-treated groups was apparent in the graph showing the percentage of animals attaining each score across time (Figure 2.7). The prior generation of each score was more obvious in the individual score graphs. In the WATER/PTZ group, score 1 began at 60th s and all animals in the group attained the particular score at 120th s, whereas in PG/PTZ and CPFX/PTZ groups it was 30th - 60th s. However, score 2 in the WATER/PTZ and PG/PTZ groups was initiated at 150th s and all animals in that group displayed the specific score at 270th s and in CPFX/PTZ group it was 90th s - 240th s. The score 3 (WATER/PTZ: 420th s - 570th s, PG/PTZ: 180th s - 570th s and CPFX/PTZ: 270th s - 270th s) and score 4 (WATER/PTZ: 360th s - 570th s, PG/PTZ: 210th s - 390th s and CPFX/PTZ: 270th s - 480th s) (Figure 2.8). Clonic seizure-like behaviour persisted in antibiotic groups till the end. These results better demonstrate the prior development of seizure-like behavioural phenomics.

Reduced latency to score 1 was exhibited by the PG/PTZ ($p = 0.0030$) and CPFX/PTZ ($p = 0.0010$) group when compared to the WATER/PTZ group, as the majority of participants bypass score 0 and entered into score 1 directly. There were no discernible variations observed in latency to score 2. In comparison with

the WATER/PTZ group, lowered latency to score 3 was shown by PG/PTZ ($p = 0.0029$) and CPFX/PTZ ($p = 0.0182$) groups. Statistically notable differences in latency to score 4 were obtained in PG/PTZ ($P = 0.0265$) when compared to the WATER/PTZ group but, not in CPFX/PTZ ($p = 0.1311$) group (Figure 2.9). Nonetheless, the evidence substantiates that PG and CPFX have a potent impact on seizure frequency, duration, and early generation.

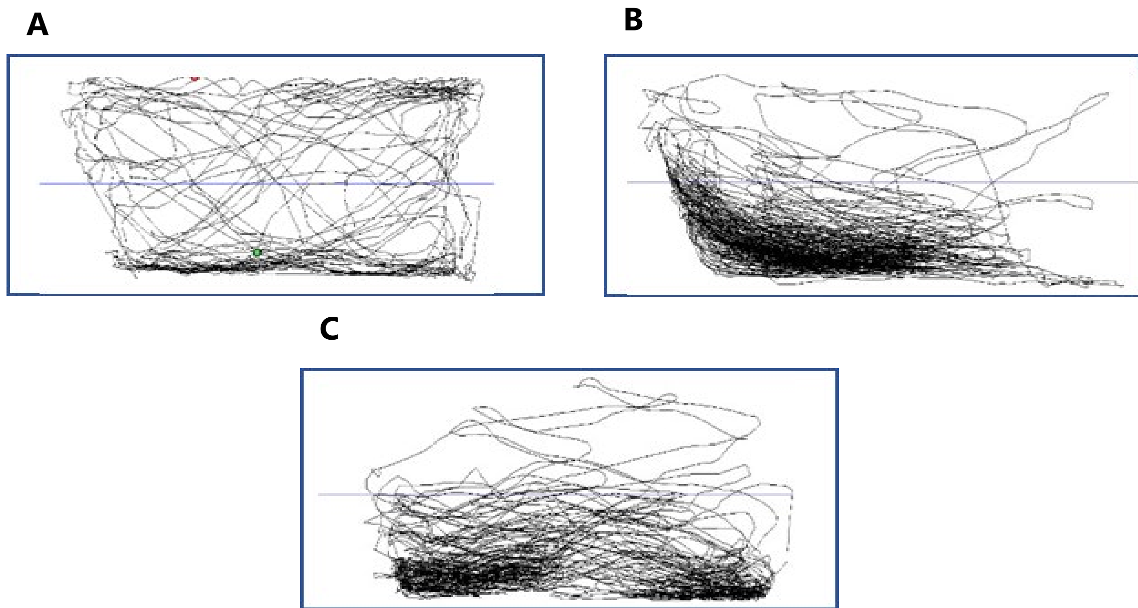

Figure 2.3: *Antibiotic treatment (25mg/L for 7 days), the behavioural analysis reflects anxiety in zebrafish. Representative track-path of zebrafish exploration in the novel tank (6-minute) extracted from Smart 3.0 software (A) Control group (B) PG treatment group (C) CPFX treatment group.*

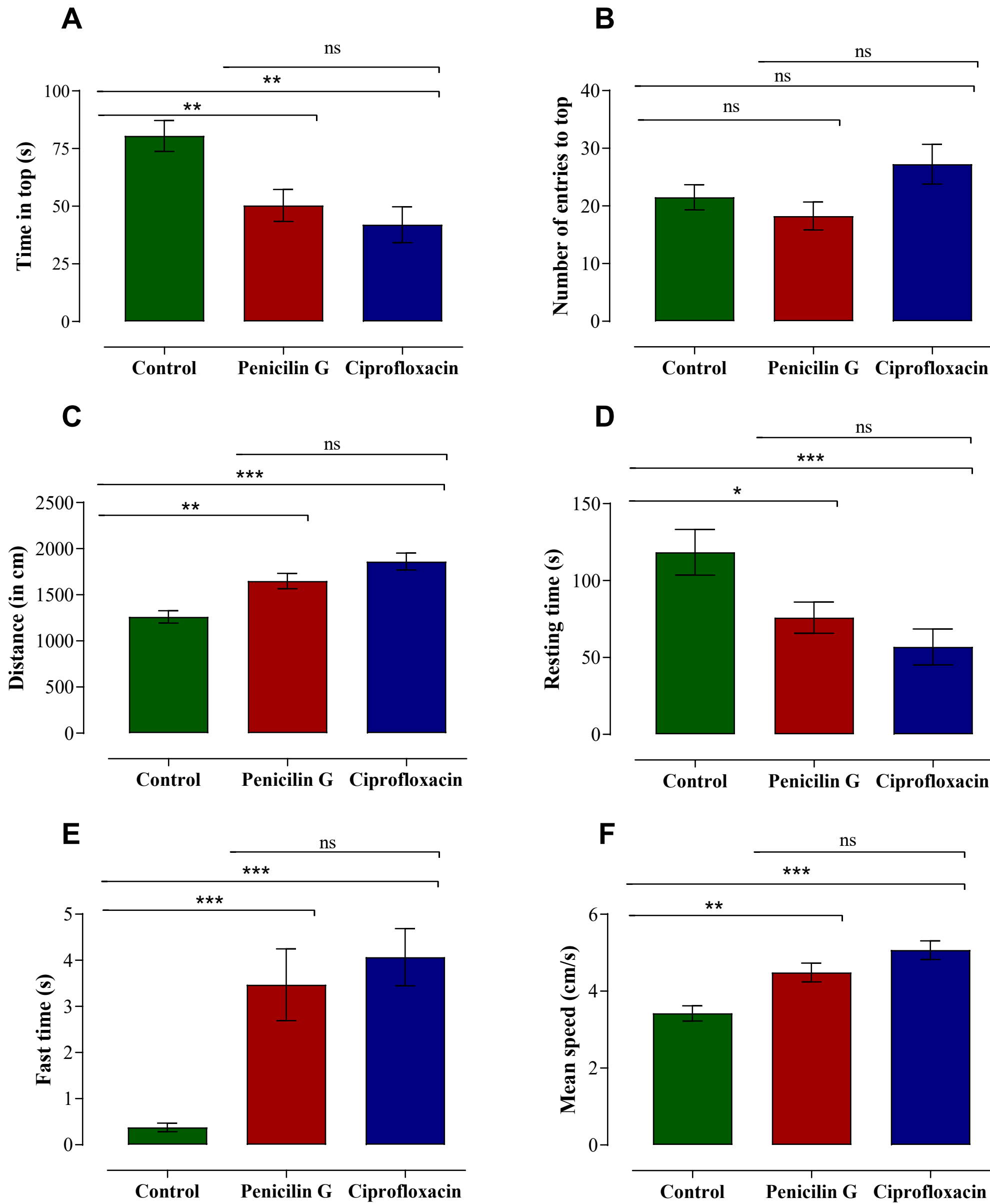

Figure 2.4: *Antibiotic treatment (25mg/L for 7 days), behavioural end-points represented as bar diagrams. (A) Time spent in top zone of the tank (B) Number of entries to top zone (C) Total distance travelled (D) Resting time (E) Fast time (>15cm/s) (F) Mean speed, data represented as mean ± SEM (*p<0.05; **p<0.01; ***p<0.001, 'ns' refers to statistical non-significance, n = 20-30 per group).*

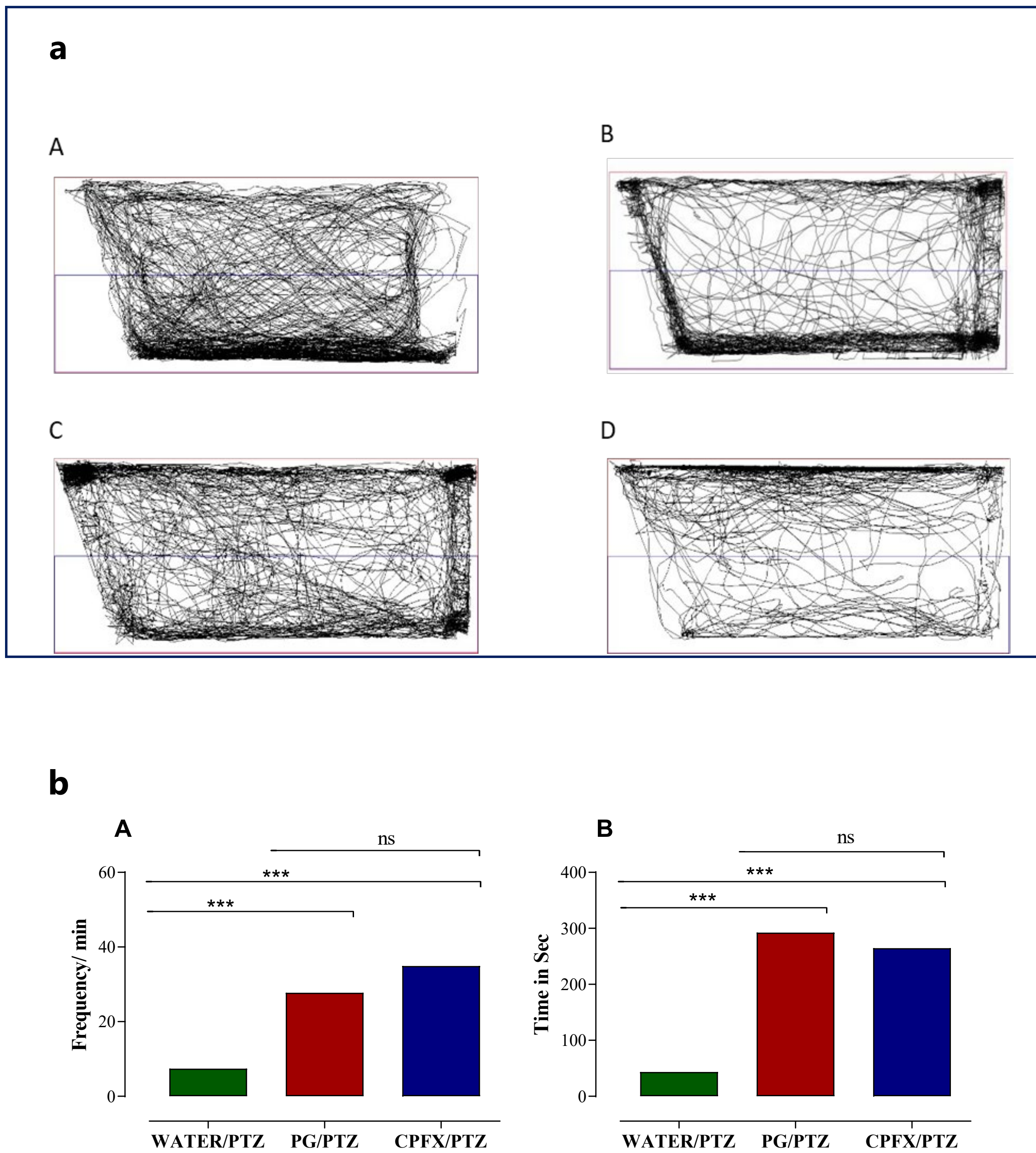

Figure 2.5: (2.5a) *Representative track path of PTZ-challenged zebrafish (7.5mM, 20 minutes) analysed using smart3.0 software (A) Negative control (only system water) (B) WATER/PTZ (C) PG/PTZ (D) CPFX/PTZ. (**2.5b**) The bar diagram indicates seizure (A) Frequency and (B) duration. Data represented as mean ± SEM (***p≤0.0001, n = 14 per group).*

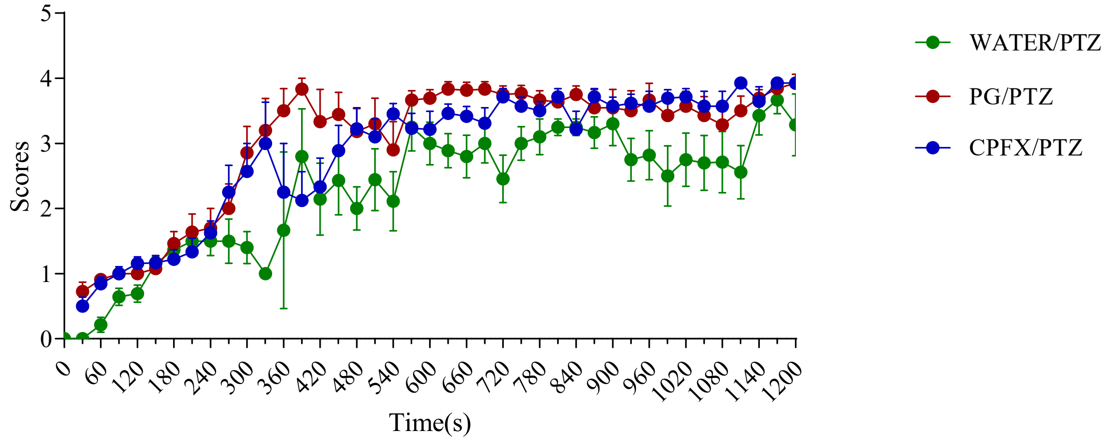

Figure 2.6: Time course of seizure-like behavioural scores obtained during PTZ-exposure (7.5mM, 20 minutes) formerly treated with PG and CPFX. Data represented as mean ± SEM (n=14 per group).

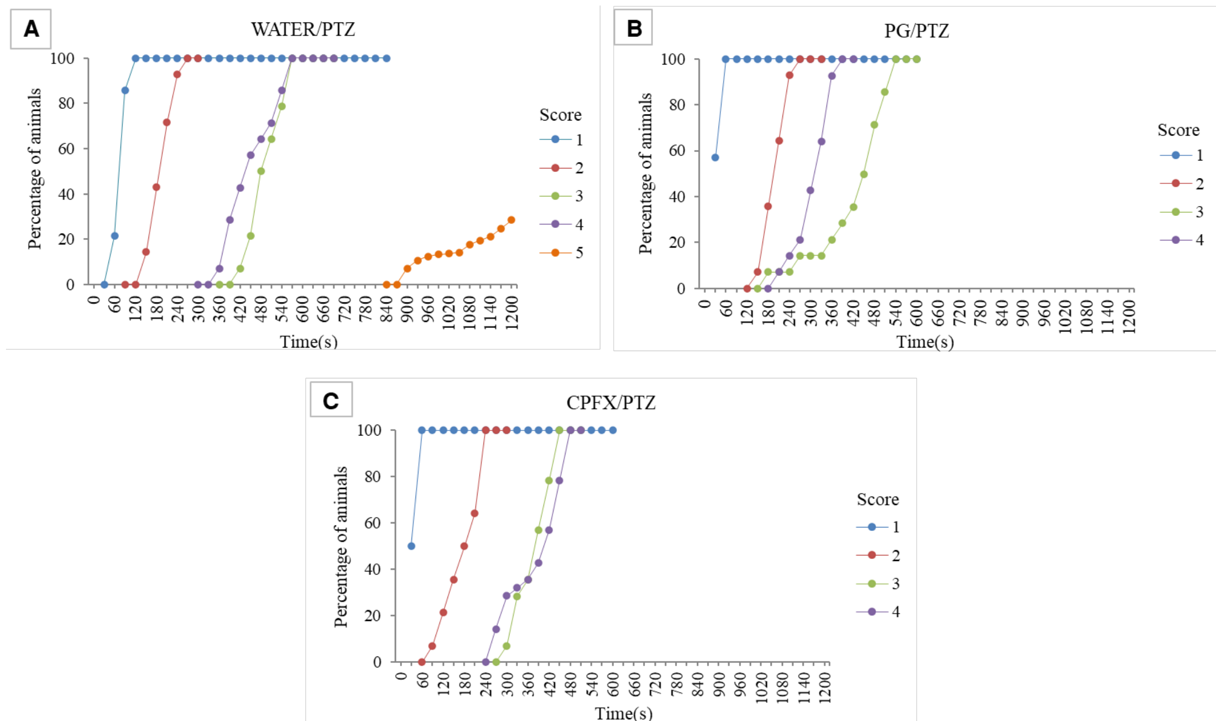

Figure 2.7: *Behavioural profile after PTZ- exposure (7.5mM, 20 minutes) earlier treated with PG and CPFX (25mg/L) and control group maintained at system water. Graph showing percentage of animals attained each score (score1-4) across time in (A) WATER/PTZ group (B) PG/PTZ and (C) CPFX/PTZ group (n=14 per group).*

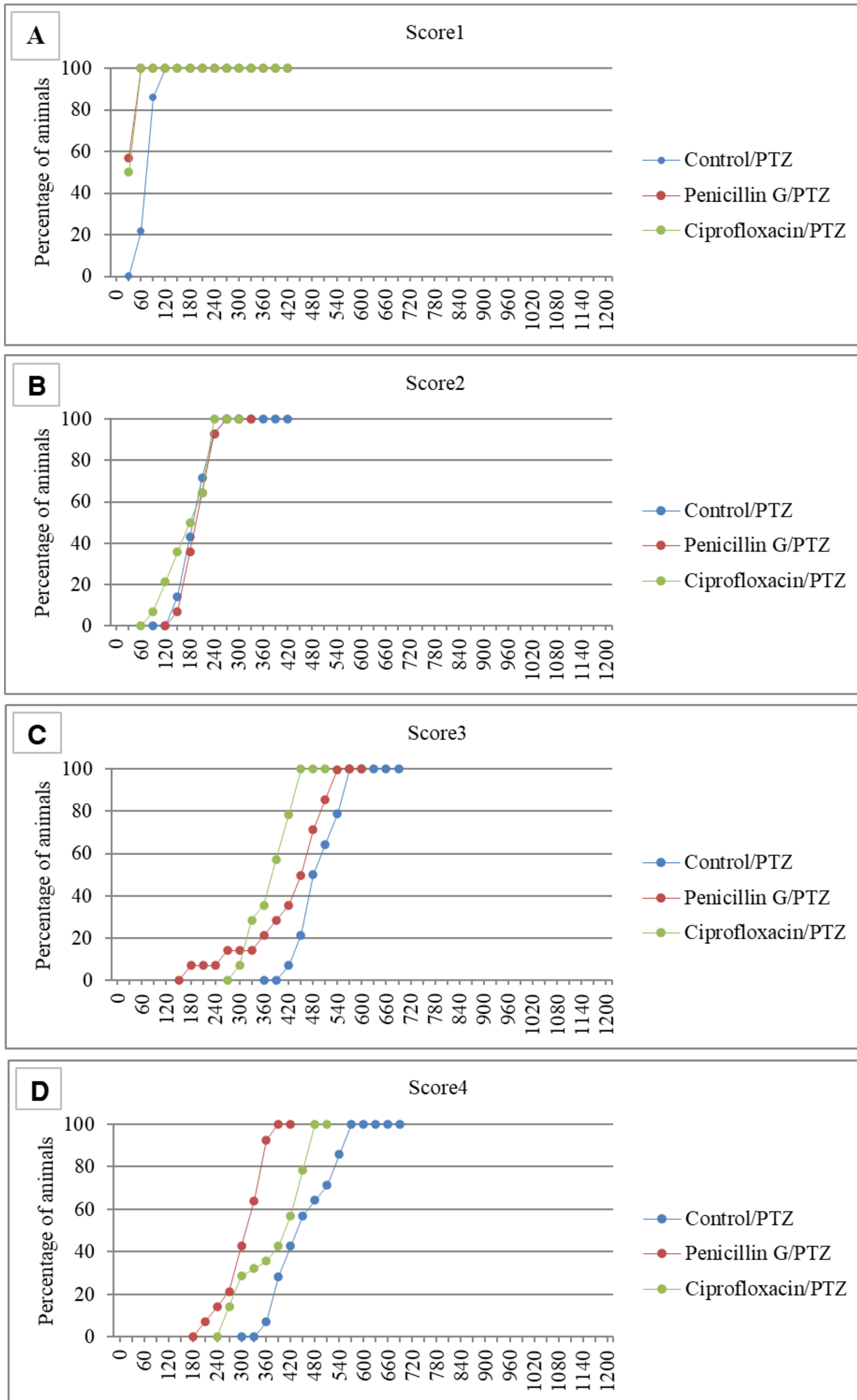

Figure 2.8: *Graph displaying the percentage of animals that reached individual scores across time (A) score 1, (B) score 2, (C) score 3, (D) score 4) (n= 14 per group).*

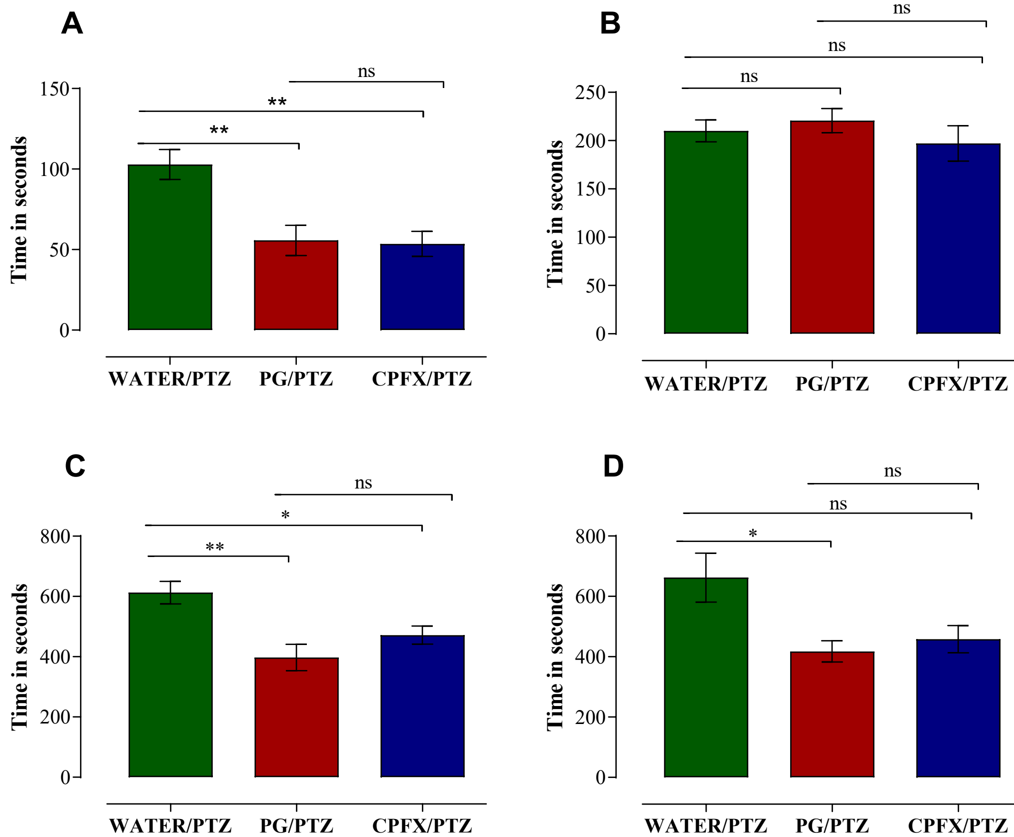

Figure 2.9: *The bar diagram shows the latency to reach each score and the data expressed as mean ± SEM (A. score 1, B. score 2, C. score 3, D. score 4) (*p<0.05, **p<0.01).*

2.5. Discussion

The present study examined whether an antibiotic from the β-lactam and fluoroquinolone family such as PG & CPFX have any effect on seizure severity in adult zebrafish. PG and CPFX are found to be positive modulators of seizure, as shown by the findings that antibiotic therapy increases seizure duration and frequency, and decreases the latency to various scores. The epileptogenic potential of several antibiotics has been extensively discussed, but experimental evidence is limited. This study, for the first time, investigated the adverse effect of antibiotics as a seizure modulator using the zebrafish model.

As demonstrated by previous research, antibiotics exposure alters the behaviour of zebrafish in a variety of ways. Some antibiotics influence exploratory parameters in a short time of exposure, but others do not have the same capacity. Therefore, it depends on the time of exposure (Petersen et al., 2021). Our results reflect that PG & CPFX affect the normal exploratory behaviour of zebrafish. The PG and CPFX groups spent less time in the top zone of the tank, which correlates with previous research that evaluated the effect of the β-diketone antibiotic group. The earlier report suggests that exposure to 25mg/L concentration of CPFX on zebrafish for three-month decreased the time spent in the upper portion of the test tank and also reduced social cohesion, while the concentration of 6.25mg/L exhibited anxiolytic effects (Wang et al., 2016). Even though the average speed and distance traveled have increased, the majority of exploration is limited to the bottom and corners, indicating a form of avoidance behaviour. It reflects the anxiety caused by antibiotics.

Lower resting and higher fast time are congruent with a slightly agitated nature, which corresponds to the emergence of depressive symptoms. An investigation in humans using the biomonitoring method revealed that an increased risk of depression in older adults was found when exposed to some antibiotics such as azithromycin, sulfaclozine, tetracycline and veterinary antibiotics (Liu et al., 2021). The severity of symptoms in adults clinically diagnosed with attention-deficit/hyperactivity disorder (ADHD) was associated with depressive and avoidance behaviour (Knouse et al., 2013). Similarly, the long-term effect of oxytetracycline (OTC) on zebrafish behaviour results in hyperactivity and freezing during light periods (Almeida et al., 2019). Presumably, antibiotic-induced microbiome perturbation causes depressive-like behaviour that could be normalised

by chronic administration of probiotics (Guida et al., 2018). Additionally, stress increases seizure susceptibility. Fish with elevated anxiety exhibited heightened sensitivity to PTZ-induced convulsions (Canzian et al., 2021). Studies proposed that gut microbiota can mediate antiseizure effects. It was manifested in research based on the ketogenic diet can alter the composition of the microbiome in mouse models of epilepsy, preventing the animals from developing seizures (Mu et al., 2022; Olson et al., 2018). The irregular pattern in the track path of PTZ-challenged fish better demonstrates their abrupt movement during clonic seizures. The surprising factor is, in the antibiotic-treated group seizure frequency and duration were increased, which underscores the fact that PG and CPFX have adequate potency to lift seizure intensity by raising frequency and duration. In PG and CPFX treatment groups, seizures remained throughout, and the latency to different scores was also found to be reduced. The repetitive seizure condition may be due to the elevated excitability and the pro-convulsive effect of PG and CPFX.

The neurotoxicity of various antibiotics, including cephalosporins, penicillin, quinolones, and carbapenems, has been investigated in a few studies. In dialysis patients, third-generation cephalosporins are associated with a high risk of neurotoxicity and the neurotoxicity involves interactions with the GABA receptors (Zhang et al., 2013b). A medicinal dose of 50 mg/kg CPFX received by mice exhibited a reduction in the levels of GABA and serotonin in their brain (Ilgin et al., 2015). It has been suggested that the pro-convulsive effect of β-lactam antibiotics is through competitive inhibition of the benzodiazepine-binding site or down-regulation of the expression of $GABA_A$ receptors. Similarly, fluoroquinolones when entering to CNS act as $GABA_A$ receptor inhibitors (Flaus et al., 2019, Cannizzaro et al., 2021). Comparably, treatment on hippocampal slices

with PG (1.2 g/L) and CPFX (50 mg/L) demonstrates the epileptogenic potential of these antibiotics as well (Grondahl & Langmoen, 1993). Fluoroquinolones, one of the most regularly prescribed antibiotics, were reported to pose a considerable health risk. In some individuals, it might be brought about side effects ranging from psychological and sensory disturbances to muscle, tendon, and nerve problems. These results suggest that antibiotics are not only deleterious to microbes but also harmful to host cells. The incorporation of fluorine atoms into quinolones made it easier to infiltrate tissues all over the body, including the central nervous system and boosted their efficiency to counteract a broad spectrum of infections (Kabbani et al., 2018).

Various animal models have been employed by researchers to investigate the physiological implications and behavioural abnormalities of epilepsy (Loscher, 2017). Electrode-mediated kindling and pharmacological seizure induction are used to develop animal models of epilepsy. PTZ-based pharmacological induction is among the most prevalent of these techniques (Kandratavicius et al., 2014; Shimada & Yamagata, 2018). As a $GABA_A$ receptor antagonist, PTZ inhibits the activity of inhibitory synapses, thereby increasing excitability and leading to seizures (Squiresl et al., 1984; Tourov et al., 1996). PTZ also cause oxidative stress in the hippocampal tissue. Assays reported an increased lipid peroxidation, nitric oxide (NO) synthesis and decreased level of antioxidants. The neuroinflammation and neuronal apoptosis followed by seizures were also recorded in a research paper aimed to evaluate the protective effect of proanthocyanidins against epilepsy (Alyami et al., 2022). Rat hippocampus slices, cultures of cortical neurons, and intact hippocampi with commissural connections constitute an in vitro model of epilepsy. Studies using these models unravelled the signalling process behind the

onset of seizures. Epileptogenesis is characterised by the activation of excitatory neurotransmission in the central nervous system via glutamate receptors such as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). NMDA receptor activation increases the $Ca2^{+}$ influx. A higher intracellular $Ca2^{+}$ concentration activates $Ca2^{+}$/calmodulin-dependent protein kinase (CaMKII) and calcineurin. CaMKII assists in the insertion of AMPA receptors on the synaptic site, which confers long-term potentiation (LTP) of excitatory synapses. Calcineurin binds to $GABA_A$ receptors and promotes their endocytosis, thereby inhibiting GABA-mediated inhibition and initiating epileptogenesis (McNamara et al., 2006). We considered adult zebrafish since they are more suitable for studying neurological disorders furthermore, physiologically stable than larval zebrafish. The partially formed nervous system in larvae may restrict the translation of certain neurological conditions. The complete development of brain structures and their functions including the blood-brain barrier and adult neurogenesis are important elements in epilepsy studies (Cho et al., 2020).

Here, we chose an experimental protocol with an acceptable lower concentration of PTZ, it ensures minimal mortality and can induce consistent seizure-like behaviour (Canzian et al., 2021; Mussulini et al., 2013). Since only two antibiotics, PG and CPFX, were chosen for this study, we cannot generalise that all antibiotics affect seizures. However, a comprehensive examination of the modulatory effects of all current antibiotics in the pharmacological field is required. This study validates that zebrafish develop heightened seizure susceptibility following antibiotic treatment, which consequently impairs their normal exploratory behaviour. The antibiotic exposure arouses anxious behaviour in zebrafish, thereby proliferating the seizure

intensity. The seizure proliferative effect of antibiotics in zebrafish suggests that further investigation of the neuro-modulatory effects of antibiotics and several other drugs can be easily tested using this zebrafish model. Future research will be required to elucidate the molecular pathway of long-term antibiotic therapy. This study demonstrates once again the significance of zebrafish as a model for investigating epilepsy.

2.6. References

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CHAPTER 3

Chronic exposure to antibiotics and natural enrichment on behaviour, gut-brain cells, and microbiota in zebrafish

Rationale: Antibiotic contamination in the environment can disrupt gut microbiota dynamics. Beyond their role in modulating seizure activity, antibiotics may also alter the normal behavioural repertoire of an organism. Environments characterized by antibiotic pollution and those enriched with natural stimuli represent two contrasting ecological conditions. Investigating the effects of chronic, low-dose antibiotic exposure and natural environmental enrichment on gut microbiota composition and behaviour could provide valuable insights into the influence of environmental factors on organismal health and function.

3.1. Graphical Abstract

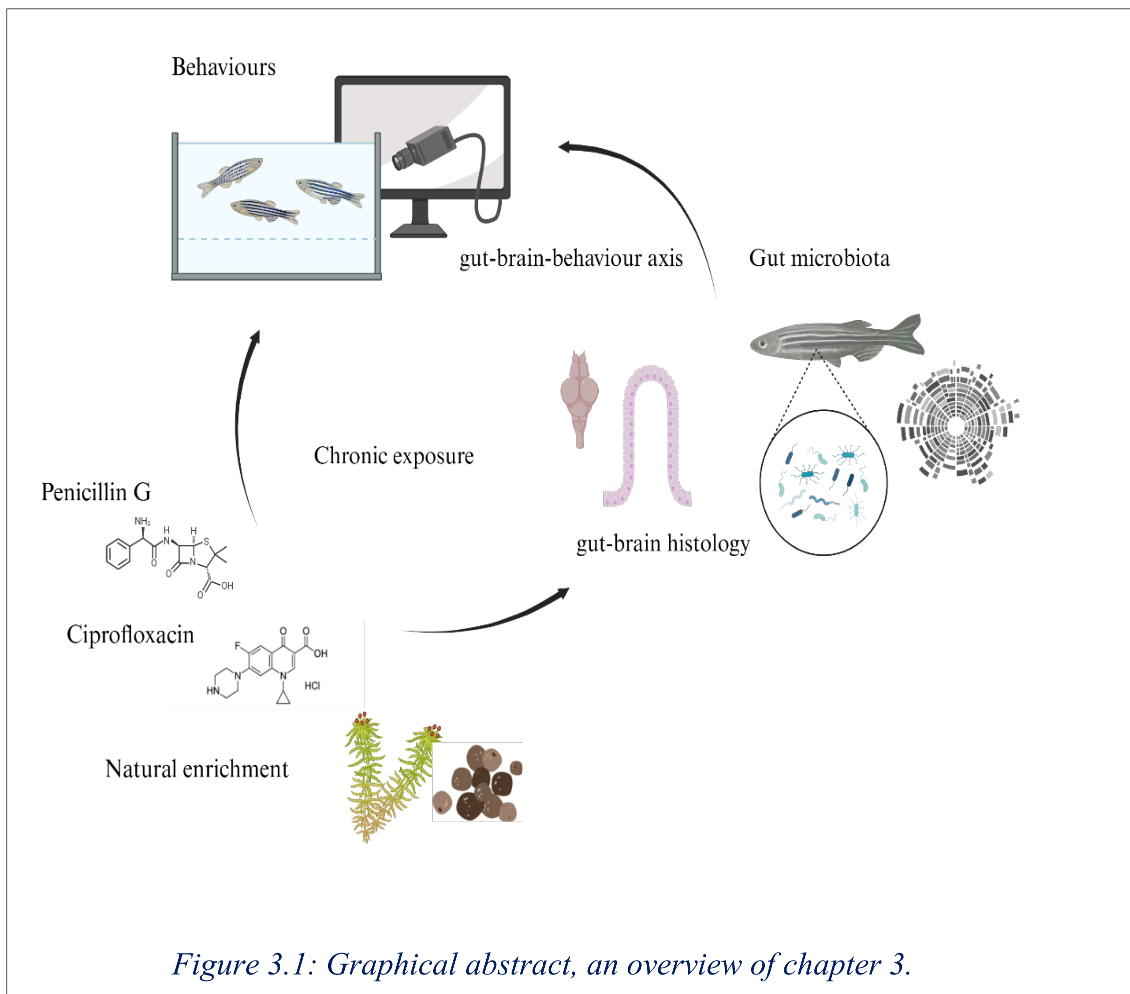


Figure 3.1: Graphical abstract, an overview of chapter 3.

3.2. Introduction

Environmental conditions play a crucial role in shaping the health, physiology, and overall stability of organisms (Chatterjee, 2022; National Research Council (US), 2013). Zebrafish are highly sensitive to environmental manipulations, which significantly influence their physiology and behaviour (Gatto et al., 2024). This small tropical freshwater fish is native to South Asia, particularly India, Bangladesh, and Nepal. They prefer shallow, slow-moving aquatic ecosystems, with water-filled channels of rice cultivation areas and still pools that form beside streams during monsoon intervals (Parichy, 2015; Spence et al., 2008). Their natural environments consist of silt or gravel at the bottom with aquatic plants (EngeSZer et al., 2007). Conversely, in laboratory settings zebrafish are housed in specialised aquatic tanks with optimal water quality conditions and husbandry practices (Kohale, 2021). However, these laboratory environments often not support environmental complexity, so fish remain in a barren environment with minimal stimulation (Williams et al., 2009). Studies regarding environmental enrichment suggests that introducing structural complexity, such as PVC pipes, plastic plants, marbles, and visual stimuli, can increase zebrafish well-being and reduce behavioural, biochemical, molecular, and reproductive abnormalities (Gallas-Lopes et al., 2023; Gazzano et al., 2025; Byrd et al., 2024; Krueger et al., 2020; Millington et al., 2024). Because of the non-standardisation of enrichment protocols, the variation in protocols result varied responses across studies. Still there are studies consistently report beneficial effects of enriched environments.

The change in environmental factors, including temperature fluctuations, pH variations, salinity changes, and the presence of pollutants or toxins, act as significant stressors in aquatic environments (Carrier-Belleau et al., 2021; Madesh et

al., 2024). Among these, we are particularly concerned about the presence of antibiotic residues in water bodies, as they contribute to environmental pollution and affect health and well-being of organisms (Bilal et al., 2020). Studies have shown that antibiotics alter zebrafish behaviour, microbiome composition, and overall toxicity responses (Petersen et al., 2021; Fröhlich et al., 2016; Desbonnet et al., 2015; Almeida et al., 2019; Zhang et al., 2023; Zhou et al., 2024; Suryanto et al., 2022). Our previous chapter revealed that exposure to Penicillin G (PG) and Ciprofloxacin (CPFX) increased the seizure frequency and reduced the latency of seizure generation in zebrafish (Sivarajan & Ramachandran, 2023). The PG and CPFX are belonging to the β-lactam and fluoroquinolone antibiotic families, and are often related with central nervous system dysfunctions (Wanleenuwat et al., 2020; Zhang et al., 2013). The environmental changes with enrichment resembling natural habitat and with antibiotic pollution represents two contrary environmental conditions. Studies compare these extremes are limited. In this chapter, we aim to research on the behavioural, gut microbiota, and histological changes in zebrafish reared in an environment with natural laterite stones, live aquatic plants (*Egeria najas*), and algal growth (natural enrichment) with that of zebrafish exposed to Penicillin G and Ciprofloxacin. This study contributes awareness into how natural environmental enrichment and antibiotic-induced stress modulate zebrafish physiology and behaviour.

3.3. Materials and methods

3.3.1. Animals

For this experiment, 400 zebrafish larvae of AB strain were collected from a licensed aquaculture facility (KL/04/OH/264/2021) located in Alappuzha, Kerala, India. The larvae were maintained at the University of Calicut, according to the

standard husbandry protocols and institutional animal welfare rules. Each tank contained 20 larvae, maintained under optimal conditions: water temperature at 27 ± 1°C, pH ranging from 6.5 to 7.0, and a light/dark cycle of 14:10 hours with 300-500 lux intensity. All experimental protocols followed the ARRIVE guidelines (Kilkenny et al., 2010) and were approved by the Committee for Control and Supervision of Experiments on Animals (CCSEA) under the 2021 regulations (Approval No. 426/GO/Re/S/01/CCSEA), conforming to the ethical standards established by the University of Calicut.

3.3.2. Antibiotic exposure

One-month-old zebrafish were categorised into two groups one was the Penicillin G (PG) exposed group and the other was the Ciprofloxacin (CPFX) exposed group. For each group, triplicate tanks were maintained. About 15 zebrafish were kept in a single tank having dimensions of about 36cm length×26cm width×19cm height (volume of 16 L). 10 mg/L concentration of Penicillin G and Ciprofloxacin were given to respective groups. The treatment water was completely renewed every 3 days and the treatment continued for 4 months. They were fed twice a day with Artemia in the morning and Tiqld nursery pellet feed in the evening.

3.3.3. Enriched environment

One-month-old zebrafish were transferred to an aquarium consisting of natural laterite pebbles and an aquatic plant called *Egeria najas,* the tank was kept in a place where natural light hit so that algae could be developed, and mild aeration was also given. Waste removal and 2/3rd of the water change was done every 3 days. They were fed twice a day with *Artemia* in the morning and Tiqld nursery pellet feed in the evening. The control group was maintained alongside without antibiotic and enrichment.

3.3.4. Behavioural Assays

After four months of continuous exposure in an environment with antibiotics and natural enrichment, they were subjected to various behavioural assays. The zebrafish were taken out from their environmental conditions during experimental time, and after completion of the experiment, they were duly returned to the respective environment. Experiments were carried out in a room with a controlled temperature set at 28°C. All trials were performed between 10:00 AM and 4:30 PM in a closed-room setting to maintain a consistent testing condition (Cachat et al., 2011). A Logitech 720p C270 webcam, operating at 30 frames per second, was utilised to capture the behavioural responses of the zebrafish subjects. The camera-position was adjusted based on the specific requirements of each behavioural test. To minimize external interference during the experiments, the setup was surrounded by a black curtain. The analysis of behavioural data was conducted using Smart 3.0 video tracking software (Panlab-Harvard Apparatus, USA).

3.3.5. Novel Tank Test (NTT)

Following the 4-months of different environmental conditions, zebrafish from Control, PG, CPFX, and EE groups underwent a novel tank diving test to evaluate their exploratory behaviour and anxiety-like responses (Fontana et al., 2022). To minimize handling stress, each fish was acclimated for 5 minutes in an opaque beaker before testing. The fish were then carefully transferred into an experimental tank (27.9 cm × 7 cm × 15 cm, with a 22 cm bottom length) containing 1.35 L of system water. Recorded the behavioural activity for 6 minutes. Prior to video analysis using software, the experimental tank was virtually segmented into top and bottom zones to track the activities in different zones.

3.3.6. Light-Dark Test (LDT)

The anxiety-like behaviour of adult zebrafish exposed to antibiotics and enrichment is evaluated using the light/dark preference test, which exploits their innate inclination towards either dark or light environments. An acrylic tank (45 cm long, 10 cm wide, 15 cm high) was divided equally into black and white sections. The tank contained 4.5 L of water, filling it to a depth of 10 cm. A central area (15 cm × 10 cm × 10 cm) was created using colour-matched sliding doors for initial fish acclimation. Each zebrafish was first introduced in the middle chamber using a fish net and they were given 3-minutes for acclimatisation. After acclimatisation, the sliding door was slowly detached from the tank, permitting the fish to freely explore the light and dark compartments of the tank for 10 minutes. The behaviour was recorded with top-mounted camera. Two individuals, unaware of the experimental conditions, analysed the recorded footage to reduce bias. The inter-rater reliability score exceeded 0.90 (Maximino et al., 2018).

3.3.7. Mirror Biting Test (MBT)

The experiment performed in a three-compartment tank measuring 15 × 60 × 20 cm (W × L × H), filled with 4 L of water. Each fish was introduced in the middle or focal chamber and recorded for 6 minutes. After initial 6-minutes, a mirror was positioned on either the left or right wall facing the central chamber, alternating to reduce bias. The fish was then allowed to interact with its reflection for another 6 minutes. The protocol described by Midttun et al. (2020). The central chamber was conceptually split into two areas: the mirror zone (MZ) (1/3 of the chamber, also called the approach zone) and the non-mirror zone (NMZ) (remaining 2/3, also known as the avoidance zone). Smart 3.0 software was used to analyse behavioural recordings, while two independent observers, unaware of the experimental

conditions, manually counted mirror bites (inter-rater reliability > 0.90) to ensure data collection accuracy and consistency.

3.3.8. Social Preference Task (SPT)

The three-chambered tank used for MBT was also adopted for SPT. The first chamber was kept empty, the middle chamber served as the experimental area, and the third chamber contained the conspecific stimuli. Six individuals of the conspecific group were alternately placed in the left and right chambers, with their positions balanced between tests. Initially, two black panels were positioned between the middle chamber to block the focal fish's view of the empty and conspecific chambers. A single zebrafish was introduced into the middle chamber and given 3 minutes to acclimate. The panels were then gently removed, allowing the fish to swim freely for 6 minutes and 30 seconds while being recorded by a webcam placed in front of the tank. The first 30 seconds were designated as the latency period. The experimental chamber was virtually split into two unequal zones: the Social Zone (SZ), comprising the 1/3 portion adjacent to the conspecific chamber where the fish was expected to prefer visual interaction with conspecifics, and the Non-Social Zone (NSZ), consisting of the 2/3 portion facing the empty chamber. The analysed parameters included the number of entries to SZ, cumulative time in SZ, distance traveled in SZ, time immobile in SZ, mean speed in SZ, parallel orientation, and turning tendency in SZ (Angiulli et al., 2020).

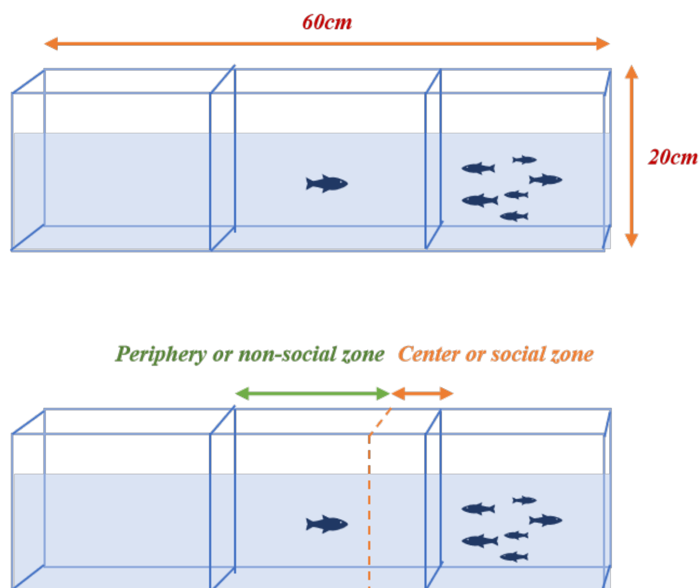


Figure 3.2: *Illustration representing social preference test experimental setup*

3.3.9. Statistical Analyses

To examine data distribution and group disparities, various statistical methods were employed. Data normality was evaluated using the Kolmogorov-Smirnov test in conjunction with the Dallal-Wilkinson-Lilliefors p-value test, while variance homogeneity was assessed through Bartlett's test. For data exhibiting normal distribution, One-Way ANOVA was utilised to compare group means, followed by Tukey's post hoc test for multiple comparison adjustments. Findings were presented with 95% confidence intervals. In cases of non-normally distributed data, the Kruskal-Wallis test was applied, succeeded by Dunn's multiple comparison tests to identify inter-group differences. Intra-group changes were measured using Wilcoxon-matched pairs signed rank test. Spearman's rank correlation coefficient was employed to analyse relationships between variables. GraphPad Prism (version 9.5), Python (version 3.10), and Microsoft Excel were used for all statistical analyses and data visualisation. Histology images were quantified using Image J. Throughout the study, a significance level of $p < 0.05$ was maintained.

3.3.10. Metagenomic Analyses

The gut tissue was dissected out from zebrafish, six samples from each condition and the DNA was extracted using the ORIonX Genomic DNA kit (Origin Lab, India). The extracted DNA was stored in -20-degree Celsius for further analyses. Using a Nanodrop spectrophotometer (Nanodrop Technologies, USA), the DNA concentration in the filtrate was measured and verified to exceed 30 ng/µL. The V4 region of the 16S rRNA gene was targeted for PCR amplification, and the resulting products were examined on a 2% agarose gel to verify successful amplification. Following the Illumina HiSeq protocol for amplicon preparation, the PCR products underwent purification. For the library construction, NEB Next Ultra DNA Library Preparation Kit (New England Biolabs) was used. The Agilent 2200 Tape Station system was used to analyse the quality and quantity of the constructed libraries. The sequencing was performed on the Illumina HiSeq 2500 platform to generate high-quality reads for subsequent metagenomic analysis, in accordance with the manufacturer's guidelines (Sieler et al.,2023; Pothay et al.,2024).

3.3.11. Bioinformatics

Quality assessment of paired-end sequences generated from Illumina HiSeq sequencing was performed using FastQC-v0.11.9 (Andrews, 2010). Adapter removal and sequence trimming were conducted with FastP-v0.20.1 (Chen, 2023). Processed sequences were then analysed and taxonomically classified using Kraken 2, a K-mer-based sequence classification tool that utilises a pre-constructed PlusPFP database of k-mers derived from known genomes for rapid categorisation of input sequences (Lu & Salzberg, 2020). To refine and quantify taxonomic outputs, Bracken was employed to compute both absolute and relative abundance values based on Kraken 2 results (Lu et al., 2017). Alpha diversity metrics, including

Observed Sequence Variants (OSVs), Chao1, ACE, Shannon, Simpson, and Fisher's diversity indices were calculated to evaluate within-group diversity. Beta diversity between groups was determined using the Bray-Curtis dissimilarity index. Visualisation of diversity metrics was achieved using specific R-packages to generate Principal Coordinates Analysis (PCoA) plots (vegan), alpha diversity plots (phyloseq, ggplot 2, dplyr, ggpubr), beta diversity plot (pheatmap, RColor Brewer, dplyr, ggplot2), and sankey plots (pavian). Bar plots depicting taxonomic compositions were created using Python.

3.4. Results

3.4.1. Chronic exposure to low-dose antibiotics elicited subtle anxiolytic effects, while prolonged environmental enrichment induced mild neophobia-related behaviours.

The results from NTT revealed that chronic exposure to PG (10 mg/L), CPFX (10 mg/L), and EE altered vertical exploration in zebrafish. Figure 3.2 illustrated the track path of zebrafish in a novel environment. Fig. 3.3A represents the C group exhibited a normal exploratory pattern, while PG (Fig. 3.3B) and CPFX (Fig. 3.3C) expressed an inclined track path more concentrated in a particular area. However, EE (Fig. 3.3D) restricted their exploration towards the bottom zone.

Regarding the endpoints, the number of entries to the top zone was significantly higher in the antibiotic groups, PG (37.97 ± 3.84) and CPFX (41.83 ± 3.24), compared to C (22.13 ± 2.74, PG/C $p = 0.0354$; CPFX/C $p = 0.0006$) and EE (13.37 ± 3.07, PG/EE $p < 0.0001$; CPFX/EE $p < 0.0001$). No significant differences in the number of entries to the top were observed between PG/CPFX and C/EE ($p > 0.05$) (Fig. 3.4A). CPFX exhibited a lower latency to enter the top (9.75 ± 2.91) than C (76.45 ± 15.05, $p = 0.0043$) and EE (180 ± 29.38, $p < 0.0001$), while PG (24.20 ± 6.89) showed lower latency than EE ($p = 0.0102$). No significant differences in

latency were observed among PG/C, PG/CPFX, and C/EE ($p > 0.05$) (Fig. 3.4B). The time spent in the top zone was significantly higher in CPFX (140.8 ± 8.82) compared to C (38.61 ± 4.89, $p < 0.0001$), PG (77.36 ± 6.30, $p = 0.0022$), and EE (56.66 ± 12.49, $p < 0.0001$). Compared with C, PG exhibited a higher time in the top zone ($p = 0.0202$). No differences were found between the C/EE and PG/EE groups ($P > 0.05$) (Fig. 3.4C). The total distance travelled in the novel environment, including the top and bottom zones, did not vary significantly among C (1821 ± 76.16), PG (1733 ± 88.26), and CPFX (1965 ± 73.84), but was reduced in EE (1154 ± 93.83) compared to all groups ($p < 0.0001$) (Fig. 3.4D). However, the distance covered in the top zone varied between groups, with CPFX (918.9 ± 64.41) exhibiting increased distance compared to C (289.4 ± 33.81, $p < 0.0001$), PG (536.6 ± 55.81, $p = 0.0048$), and EE (261.6 ± 58.63, $p < 0.0001$). PG covered a greater distance in the top zone than EE ($p = 0.0071$). The differences between the C/PG and C/EE groups were not significant ($p > 0.05$) (Fig. 3.4E). The resting time or immobility was significantly higher in the EE (73.36 ± 17.04) than in all other groups: C (1.46 ± 0.43, $p < 0.0001$), PG (2.31 ± 0.60, $p = 0.0030$), and CPFX (1.25 ± 0.38, $p < 0.0001$). The immobility time was similar for C, PG, and CPFX ($p > 0.05$) (Fig. 3.4F). The mean speed exhibited by EE (3.20 ± 0.26) was significantly lower than that of all other groups: C (5.05 ± 0.211, $p < 0.0001$), PG (4.81 ± 0.24, $p < 0.0001$), and CPFX (5.46 ± 0.20, $p < 0.0001$). The mean speed was also consistent among the C, PG, and CPFX groups ($p > 0.05$) (Fig. 3.4G).

The increased number of entries to the top, time spent in the top, distance travelled in the top, and reduced latency to enter the top indicate greater activity levels of PG and CPFX groups in the top portion of the novel tank upon exposure to chronic low concentration, with this effect being more pronounced in CPFX. However, their

immobility time, total distance travelled, and mean speed were closer to the C group. The EE group exhibited similar trends to C in entries to the top, latency to enter the top, time in the top, and distance in the top. In contrast, they displayed reduced overall distance, mean speed, and increased immobility, suggesting that their stress level is more similar with C. However, they possess novel-induced fear slightly greater than C, with reduced locomotion. When considering the antibiotic and EE groups, all the novel tank al parameters showed statistically significant variations, indicating hyperactivity or restlessness in the antibiotic groups.

Based on the time spent in the top and bottom, the novelty-induced stress level was calculated, and it was lower in CPFX (0.2178 ± 0.0490) compared to C (0.7855 ± 0.0272, $p < 0.0001$), PG (0.5703 ± 0.0350, $p = 0.0022$), and EE (0.6853 ± 0.0694, $p < 0.0001$). In PG, a slightly reduced stress level compared to C was observed ($p = 0.0205$). However, EE and C groups demonstrated similar stress levels in novel-induced environments ($p > 0.05$) (Fig. 3.5A). Similarly, the top-bottom ratio was higher in CPFX (M = 0.7261, IOR = 0.5850-1.221) compared to other groups: C (M = 0.1960, IQR = 0.1056-0.3108, $p < 0.0001$), PG (M = 0.3956, IQR = 0.2701-0.6536, $p = 0.0023$), and EE (M = 0.06311, IQR = 0-0.4475, $p < 0.0001$). In PG, the distance ratio was slightly higher compared to C ($p = 0.0238$), whereas the top-bottom distance ratio in the novel tank was similar in C and EE ($p > 0.05$) (Fig. 3.5B). The relationship between all endpoints derived from the novel tank test is collectively represented in a pair plot (Fig. 3.5C), and their Spearman correlation coefficient values are presented in a matrix plot. A positive correlation was observed between entries to the top, distance in the top, and time in the top in all groups: C ($r = 0.90, 0.82, 0.96$), PG ($r = 0.91, 0.84, 0.90$), CPFX ($r = 0.54, 0.46, 0.94$), and EE ($r = 0.98, 0.95, 0.97$). However, resting time was negatively correlated with entries to

the top (C r = -0.36, PG r = -0.65, CPFX r = -0.68, EE r = -0.81), time in the top (C r = -0.12, PG r = -0.56, CPFX r = -0.29, EE r = -0.74), distance in the top (C r = -0.18, PG r = -0.59, CPFX r = -0.38, EE r = -0.80), and mean speed (C r = -0.56, PG r = -0.65, CPFX r = -0.65, EE r = -0.91). In contrast to PG, CPFX, and EE, mean speed in C was negatively related to entries to the top (r = 0.12), time in the top (r = -0.30), distance in the top (r = -0.15), and resting time (r = -0.56) (Fig. 3.5D).

3.4.2. The EE group was more susceptible to light-dark induced anxiety, and the PG group was less affected compared to CPFX.

The number of entries to the light zone was significantly higher in EE (49.70 ± 3.328) compared to C (35.85 ± 3.083, p = 0.0116), PG (37.55 ± 3.288, p = 0.0339), and CPFX (34.85 ± 2.610, p = 0.0059). No significant differences were observed between C, PG, and CPFX (p > 0.05) (Fig. 3.6A). The latency to enter the light zone was also significantly higher in EE (30.55 ± 5.280) compared to CPFX (8.250 ± 1.894, p = 0.0006) and PG (5.250 ± 1.322, p = 0.0234). C (29 ± 8.076) also exhibited increased latency to the light zone compared to PG (p = 0.0256), whereas C and EE took 30 seconds to enter the light zone. The variation between C and CPFX was found to be statistically non-significant (p > 0.05) (Fig. 3.6B). Regarding time spent in the light zone, EE (67.02 ± 6.015) spent significantly less time compared to C (161.6 ± 11.44, p < 0.0001), PG (209.3 ± 19.44, p < 0.0001), and CPFX (129.1 ± 16.86, p = 0.0164). Between PG and CPFX, PG demonstrated an increased time in the light zone (p = 0.0010). When PG and CPFX were compared to C, the difference was statistically non-significant (p > 0.05) (Fig. 3.6C).

The light-induced anxiety was significantly higher in EE (0.776 ± 0.0200) compared to C (0.4612 ± 0.0381, p < 0.0001), PG (0.3023 ± 0.0648, p < 0.0001), and CPFX (0.5697 ± 0.0561, p = 0.0297). Compared to CPFX, the anxiety index was lower in PG (p = 0.0151) (Fig. 3.7A). An inverse relationship between anxiety index and

time spent in the light zone was apparent among groups (Fig. 3.7B). Although the number of entries to the light zone was higher in EE, subjects quickly escaped from the zone and spent the majority of their time in the dark area, indicating their fear or anxiety towards light. In contrast, only PG showed lower latency to the light zone than C and higher time spent in light than CPFX; otherwise, PG and CPFX were not significantly influenced by the presence of light.

3.4.3. The aggression was exacerbated in PG and CPFX but parallel in C and EE

The number of entries to the mirror zone (MZ) was not varied between groups (Fig. 3.8A), but the latency to enter into MZ was reduced to zero in PG and CPFX compared to C (M = 0, IQR = 0-1.29, $p = 0.0077$) and EE (M = 1.11, IQR = 0-3.675, $p < 0.0001$). No significant difference was found between C and EE ($p > 0.05$) (Fig. 3.8B). The time spent in MZ was also identical among groups whereas, CPFX (M = 274.6, IQR = 237.3-300) spent significantly more time in MZ compared to PG (M = 196.3, IQR = 168.5-231.7, $p = 0.0097$) (Fig. 3.8C). The CPFX (M = 946.8, IQR = 824.6-1121) demonstrated a higher distance travelled in MZ compared to C (M = 614.8, IQR = 531.8-718.3, $p < 0.0001$), PG (M = 578.7, IQR = 510.5-679, $p < 0.0001$), and EE (M = 707.2, IQR = 518.9-865, $p < 0.0001$). Statistically non-significant differences were found between C, PG, and EE ($p > 0.05$) (Fig. 3.8D). Similarly, CPFX (M = 3.79, IQR = 3.315-4.570) exhibited greater mean speed compared to others; C (M = 2.710, IQR = 2.225-3.565, $p < 0.0001$), PG (M = 3.180, IQR = 2.845-3.385, $p = 0.0164$), and EE (M = 2.970, IQR = 2.665-3.340, $p = 0.0005$). The differences were statistically non-significant among C, PG, and EE ($p > 0.05$) (Fig. 3.8E). Moreover, the number of mirror bites was significantly higher in CPFX (M = 180, IQR = 144-251) and PG (M = 165, IQR = 139-193) compared to C (M = 84, IQR = 64-132, $p < 0.0001$) and EE (M = 95, IQR = 58-121, $p < 0.0001$).

No significant changes were observed between C and EE ($p > 0.05$) (Fig. 3.8F). The EE group did not display any variations from the C group in the aggression test, whereas the CPFX group tended to remain in MZ and displayed a greater number of mirror bites. Although the PG group spent less time in MZ than CPFX, and their distance and speed in MZ were congruent with C and EE, their mirror biting tendency was greater compared to C and EE.

Therefore, the rate of aggression was also significantly higher in PG (0.8331 ± 0.0094) and CPFX (0.7066 ± 0.0222) compared to C (0.3941 ± 0.01495, $p < 0.0001$) and EE (0.3766 ± 0.02460, $p < 0.0001$) (Fig. 3.9A). The resting time or immobility time (movement <1cm/s) was significantly lower in CPFX (2.052 ± 0.3197) compared to C (17.47 ± 3.537, $p < 0.0001$), PG (5.674 ± 0.6564, $p = 0.0149$), and EE (12.12 ± 1.938, $p < 0.0001$). Slow time or normal swimming time (1-10 cm/s speed) was slightly reduced in PG (171.7 ± 7.304) compared to C (218.4 ± 12.60, $p = 0.0280$), CPFX (226.6 ± 10.07, $p = 0.0065$), and EE (215.8 ± 15.04, $p = 0.0419$). Fast time or high-speed swimming (>15cm/s) was significantly greater in PG (0.5116 ± 0.0949) and CPFX (0.9148 ± 0.2381) compared to C (0.1316 ± 0.0402, C/PG $p = 0.0035$, C/CPFX $p < 0.0001$) and EE (0.1529 ± 0.0412, PG/EE $p = 0.0076$, CPFX/EE $p < 0.0001$) (Fig. 3.9B). The lower resting time in MZ by CPFX, lower slow swimming time in MZ by PG, and higher fast time in PG and CPFX suggest that these groups exhibit increased restlessness or engagement in the presence of their mirror image.

The parallel index, which measures the linearity in zebrafish movement, was reduced in the MZ compared to NMZ across all conditions ($p < 0.0001$) (Fig. 3.9C). When comparing between groups, the parallel index was found to be slightly higher in PG (0.5212 ± 0.0264) compared to CPFX (0.4320 ± 0.0242, $p = 0.0362$) and EE

(0.4188 ± 0.0264, $p = 0.0268$) (Fig. 3.9D). Even though statistically not significant ($p > 0.05$), the turning tendency in MZ revealed that C and PG had a tendency to turn rightward and EE leftward but CPFX displayed balance between right and leftward turns (Fig. 3.9E).

3.4.4. The social preference was differently expressed in the EE group and no variation in PG and CPFX.

The antibiotic treatments using PG and CPFX did not alter social preference; their entries in the social zone (SZ) were similar to that of C. However, the EE group exhibited alterations when compared to the antibiotic groups and C. Although the number of entries to SZ was higher in EE (23.10 ± 3.187) compared to PG (9.167 ± 1.952, $p = 0.0021$) and CPFX (9.700 ± 2.201, $p = 0.0031$) (Fig. 3.10A), their time spent in SZ was lower (282.5 ± 12.67) compared to PG (317.3 ± 12.90, $p = 0.0280$) and CPFX (324.8 ± 10.70, $p = 0.0125$) (Fig. 3.10B), indicating less sustained social engagements only compared to antibiotic groups. No variation seen between C and EE ($p > 0.05$). Conversely, the locomotory s in EE, such as distance travelled (1097 ± 36.81) (Fig. 3.10C) and mean speed (3.796 ± 0.1386) (Fig. 3.10D), were higher in SZ compared to C (distance: 904.8 ± 32.11, $p = 0.0007$; mean speed: 2.942 ± 0.0869, $p = 0.0001$), PG (distance: 953.8 ± 33.15, $p = 0.0204$; mean speed: 3.038 ± 0.1040, $p = 0.0005$), and CPFX (distance: 945.9 ± 35.41, $p = 0.0138$; mean speed: 2.928 ± 0.1146, $p < 0.0001$). The resting time did not vary between groups; however, the slow time in EE (272.8 ± 12.21) was lower compared to PG (325.3 ± 6.513, $p = 0.0055$) and CPFX (317.7 ± 8.161, $p = 0.0212$). Conversely, fast time in EE (1.039 ± 0.3301) was higher in SZ compared to PG (0.1273 ± 0.04576, $p = 0.0049$) and CPFX (0.1397 ± 0.0509, $p = 0.0156$). No differences between C and EE ($p > 0.05$) (Fig. 3.11A).

The social preference was differently expressed in EE as their time spent seemed to be lower in SZ, their entries were more frequent. They were highly exploratory and instinctive in their social interactions but did not engage in prolonged social behaviours. However, social preference behaviour in the EE group was not significantly varied from the control group, indicating EE does not impair social preference but is differently expressed as their general activity and movements within the SZ increased compared to others. The parallel orientation was reduced in SZ compared to NSZ across all groups: C ($p < 0.0001$), PG ($p = 0.00142$), CPFX ($p = 0.0002$), and EE ($p < 0.0001$). However, the reduction was more consistent in C and EE (Fig. 3.11B). When comparing between groups in SZ, the EE group demonstrated increased linearity compared to C ($p = 0.0138$) and CPFX ($p = 0.0129$) (Fig. 3.11C). The PG group shown tendency to turn slightly rightward whereas, C, CPFX, and EE shown more leftward turning tendencies but they were not statistically significant ($p > 0.05$) (Fig. 3.11D). The social preference index appeared similar in C, PG, and CPFX ($p > 0.05$), while it was reduced in EE compared to PG ($p = 0.0335$) and CPFX ($p = 0.0200$) but not with C ($p > 0.05$) (Fig. 3.11E).

The relationship between novelty stress, light-dark anxiety, mirror aggression, and social preference among C, PG, CPFX, and EE was represented in a pair plot. Most relationships were weak and not statistically significant among all groups, suggesting no strong predictive power among these variables in the different conditions we provided. However, a weak moderate relation was found between light-dark index and aggression rate in CPFX ($R^2 = 0.1762$, $p = 0.02$) and EE ($R^2 = 0.1738$, $p = 0.02$), EE ($R^2 = 0.1465$, $p = 0.04$) also exhibited a relation between novelty index and light-dark anxiety (Fig. 3.12).

3.4.5. The intestinal morphology was altered in PG and CPFX groups but preserved in EE.

The intestinal tissue section is shown in Figure. 3.13. In comparison to the control group, the antibiotic groups exhibited significant alterations in anterior intestinal villus morphology. Villus height (VH) decreased markedly in PG (37.24%, $p < 0.0001$) and CPFX (43.33%, $p < 0.0001$). EE showed no statistically significant difference from C ($p > 0.05$) (Fig. 3.13A). Conversely, villus width (VW) expanded in PG (41.31%, $p = 0.0001$) and CPFX (35.43%, $p = 0.0011$). EE demonstrated no change in villus width relative to C ($p > 0.05$) (Fig. 3.13B). The crypt depth (CD) increased substantially in EE (67.91%, $p < 0.0001$) compared to C. PG and CPFX showed slight CD increases of 29.51% and 15.62% respectively, but these were not statistically significant ($p > 0.05$) when compared to C (Fig. 3.13C). Muscular thickness increased in CPFX compared to C (34.03%, $p = 0.0220$) and EE ($p = 0.0038$). PG showed a 14.45% increase and EE a 7.40% decrease in muscular thickness compared to C, but these changes were statistically insignificant (Fig. 3.13D). CPFX induced the most severe intestinal stress, as evidenced by the significant reduction in villus height, increased villi width, and thickened muscle layer. PG also demonstrated negative effects, including the villus height reduction and increased villus width. EE exhibited a substantial increase in crypt depth, indicating a distinct mechanism of gut modulation. In C, epithelial cells were uniformly arranged with intact goblet cells, while in PG and CPFX, the arrangement was highly distorted with increased vacuolization and epithelial cell swelling. Besides these, rupturing or shedding of villi was also visible in CPFX.

3.4.6. A sign of neuro-inflammation in the brain sections of PG and CPFX

The brain sections depict the midbrain or mesencephalon. The control brain displayed normal structure with evenly distributed cells. PG-treated zebrafish brain

tissue exhibited structural changes and increased cell loss. CPFX treatment also caused disruptions in cell clustering and potential tissue degradation, suggesting neuroinflammation. In contrast, EE brain tissue appeared more similar to the control, with fewer signs of damage or inflammation, indicating potential neuroprotective effects (Fig. 3.14).

3.4.7. The gut microbial shift was evident in all the treatment groups.

The environmental conditions have altered gut microbial populations. The metagenomic data revealed that 34, 35, 32, and 31 OTUs were present in C, PG, CPFX, and EE, respectively, at the phylum level. In the C group, Bacillota (32.15%), Pseudomonadota (27.40%), Actinomycetota (19.30%), and Fusobacteriota (11.46%) were found to be the most abundant phyla, while the minor phyla included Planctomycetota (3.56%), Bacteroidota (2.88%), Chlamydiota (1.19%), and Cyanobacteriota (0.24%). In the PG group, the most prevalent phylum was Pseudomonadota (64.13%), followed by Bacillota (27.72%), whereas Actinomycetota (3.48%) and Fusobacteriota (1.76%) were found to be reduced, and Planctomycetota (0.37%), Bacteroidota (0.99%), Chlamydiota (0.24%), and Cyanobacteriota (0.37%) were identified in negligible amounts. Additionally, in the CPFX group, Pseudomonadota (76.11%) was the dominant phylum, with Fusobacteriota (13.69%) as the second most prevalent. The minor phyla included Actinomycetota (4.66%) and Chlamydiota (2.56%). Others, such as Cyanobacteriota (0.59%), Bacillota (0.52%), Bacteroidota (0.46%), and Planctomycetota (0.13%) were considered negligible. The only phylum found exclusively in the EE group was Bacillota (96.04%), with Actinomycetota at 2.46%. Others, such as Pseudomonadota (0.87%), Fusobacteriota (0.03%), Planctomycetota (0.06%), Bacteroidota (0.17%), and Cyanobacteriota (0.01%) were observed in insignificant amounts (Fig. 3.15A).

At the genus level, 478 OTUs were identified in the C group, among which 20.96% were *Streptococcus*, 11.30% *Cetobacterium*, 8.49% *Streptomyces*, and 6.50% *Bacillus*. Minor levels of *Mycobacterium* (3.60%), *Ralstonia* (3.52%), *Shewanella* (2.50%), and *Salmonella* (2.41%) were also present. In the PG group, 544 OTUs were identified, with *Salmonella* (18.38%) and *Bacillus* (17.67%) being dominant. *Pseudomonas* (6.61%), *Cupriavidus* (6.20%), *Escherichia* (5.85%), *Aeromonas* (5.41%), and *Priesta* (5.07%) were also identified in lower amounts. In the CPFX group, out of 395 OTUs, 64.91% *Burkholderia* was identified, and 13.23% *Cetobacterium*; these were predominant, whereas *Microbacterium* (2.60%), *Protochlamydia* (2.34%), and *Bosea* (2.10%) were found to be less abundant genera in this group. In the case of the EE group with 310 OTUs, *Brevibacillus* (45.24%), *Paenibacillus* (21.28%), and *Bacillus* (20.01%) were considered predominant. *Domibacillus* (2.14%) and *Metabacillus* (2.00%) were also present in lower percentages (Fig. 3.15B).

At the species level, 609 OTUs were identified in the C group, with *Streptococcus thermophilus* (19.91%) and *Cetobacterium somerae* (11.61%) being the dominant species. *Bacillus subtilis* (6.17%) and *Streptomyces sp.* (4.63%) were present at subordinate levels. In PG, a higher number of OTUs (812) was observed, among which *Salmonella enterica* (18.56%) appeared to be dominant. *Bacillus velezensis* (6.74%), *Cupriavidus sp.* (6.23%), *Escherichia coli* (5.96%), and *Bacillus thuringiensis* (5.08%) were present at subordinate levels. In the CPFX group, which comprised 471 OTUs, *Burkholderia dolosa* (31.82%), *Burkholderia cepacia* (17.51%), and *Cetobacterium somerae* (13.30%) were the predominant species. *Burkholderia thailandensis* (5.72%) and *Burkholderia mallei* (4.15%) were the second most prevalent species. In the EE group, 508 OTUs were present.

Brevibacillus choshinensis (35.69%) and *Paenibacillus polymyxa* (16.80%) were the dominant species, while *Brevibacillus sp*. (5.88%), *Bacillus subtilis* (5.30%), and *Bacillus velezensis* (5.25%) were fewer dominant species in the EE group (Fig. 3.15C). The top species in each group were depicted in fig. 3.15D.

In the beta diversity analysis, the Bray-Curtis dissimilarity metrics revealed substantial distances of shared species between groups (here PG is denoted as Ab1 and CPFX as Ab2): C/Ab1 (0.79), C/Ab2 (0.77), C/EE (0.92), Ab1/EE (0.86), Ab2/EE (0.99), Ab1/Ab2 (0.88) (Fig. 3.15E). The dissimilarities were also depicted in a Principal Coordinates Analysis (PCoA) plot, with the x-axis (PCoA1) accounting for 45.9% of the variation, representing nearly half of the total variation. The y-axis (PCoA2) explained 29.29% of the variance. Ab1 (PG) was positioned at the bottom, separated from others. Ab2 (CPFX) was in the upper left, the C group was near the centre-left, and EE was at the top-right, suggesting that each sample group contained distinct microbial compositions, with EE and Ab1 having highly different microbiomes. C and Ab2 were closer, indicating possible similarity in their microbial profiles. Each sample was well separated, suggesting that the microbiome compositions were not highly overlapping (Fig. 3.15F).

In the assessment of alpha diversity measures, Ab1 exhibited the highest richness (observed: 812, Chao1: 1017.3, ACE: 987.4), suggesting that the Ab1 Group consisted of the most diverse microbial community. The C group expressed intermediate richness (observed: 609, Chao1: 757.3). Ab2 (471) and EE (508) had lower richness, indicating reduced microbial diversity. C and Ab1 had the highest Shannon diversity (3.87 and 3.82), indicating that their microbiomes were not only rich but also well distributed. EE and Ab2 had the lowest Shannon diversity (2.75 and 2.69), indicating microbial loss or dominance of a few taxa. The Simpson index

confirmed that C and Ab1 (0.93, 0.94) possessed higher evenness, while the microbiomes in EE and Ab2 (0.83, 0.84) were less even (Fig. 3.15G). Although diversity in Ab1 appeared to be higher, it harboured more pathogenic species than beneficial microbes. Ab2 demonstrated a more substantial microbiome depletion effect, and the lowered diversity in EE might be attributed to the selection of specific beneficial bacteria. The major taxa, including phylum, family, genus, and species, are displayed in a sankey plot (Fig. 3.15H).

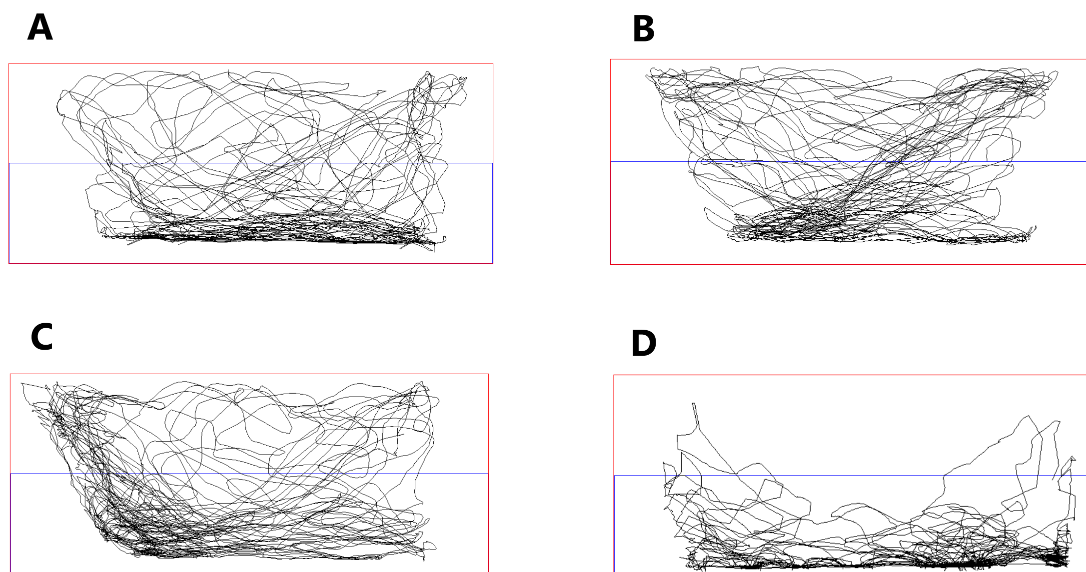


Figure 3.3*: The representative traces of zebrafish from novel tank behavioural experiment, raised under (A) Control, (B) Penicillin G, (C) Ciprofloxacin, and (D) Enriched Environment conditions, extracted from Smart 3.0 software.*

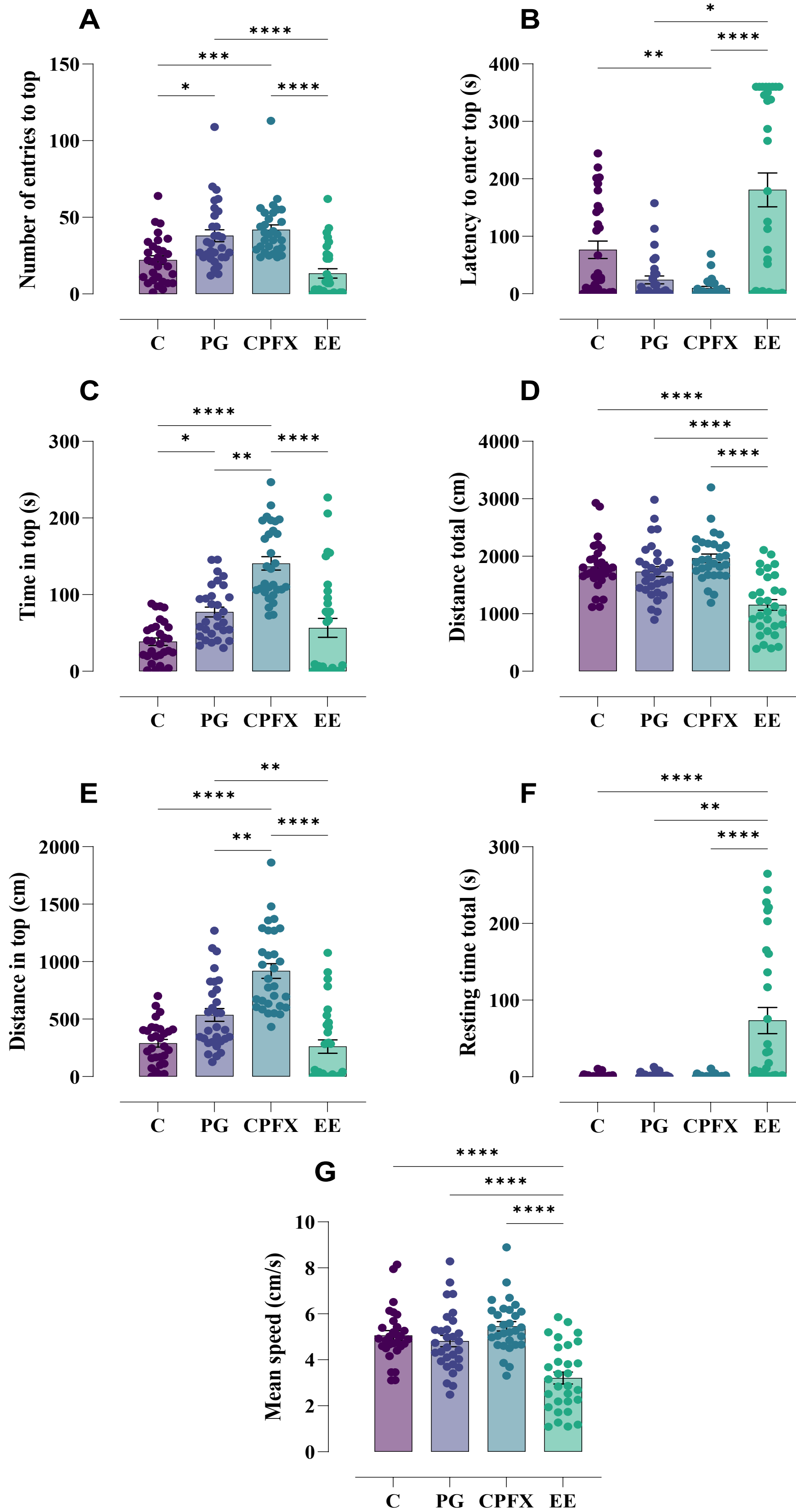
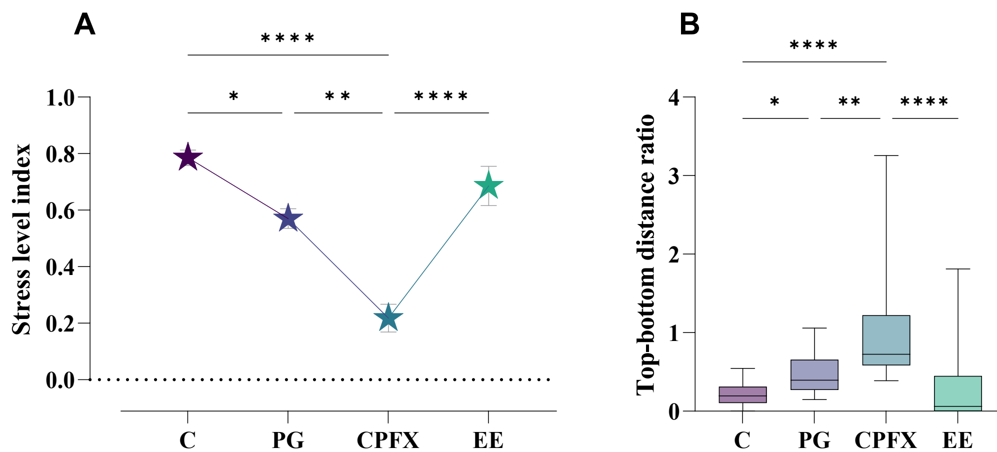
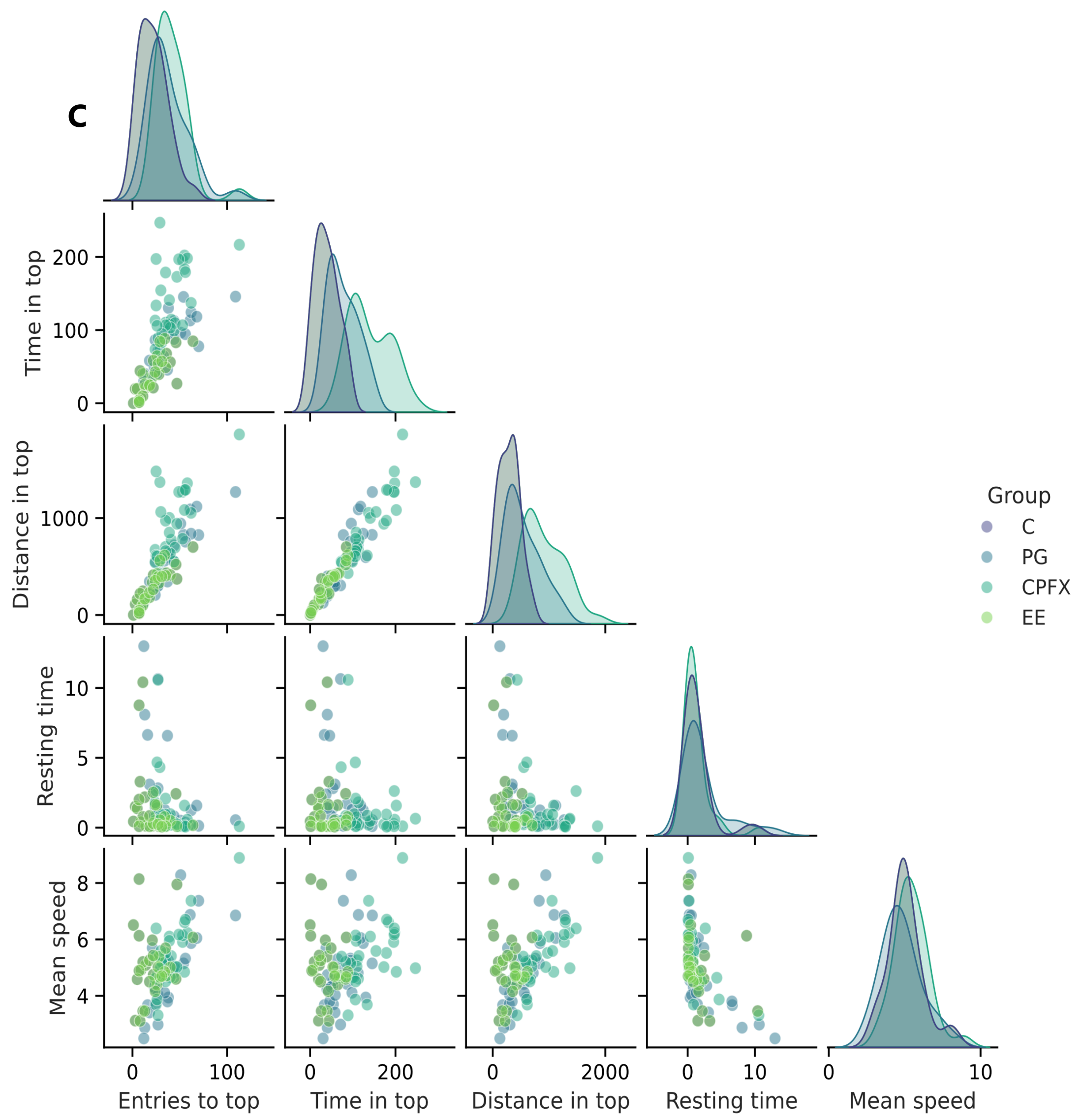


Figure 3.4: *Novel Tank Test, the bar plot with scattered points represents behavioural endpoints derived from recorded videos upon analysed by Smart 3.0*

*tracking software (A) Number of entries to top zone. The PG and CPFX group made significantly higher entries. (B) Latency to enter into the top zone. Lower latency by PG and CPFX. (C) Time in top. Higher in CPFX. (D) Total distance travelled. The novel tank exploration was reduced in EE. (E) Distance in top. The CPFX group tend to have greater exploration in top. (F) Resting time total. EE group have greater immobility. (G) Mean speed. Lower in EE. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001. n=30).*




C
Time in top
Distance in top
Resting time
Mean speed
Entries to top
Group
C
PG
CPFX
EE

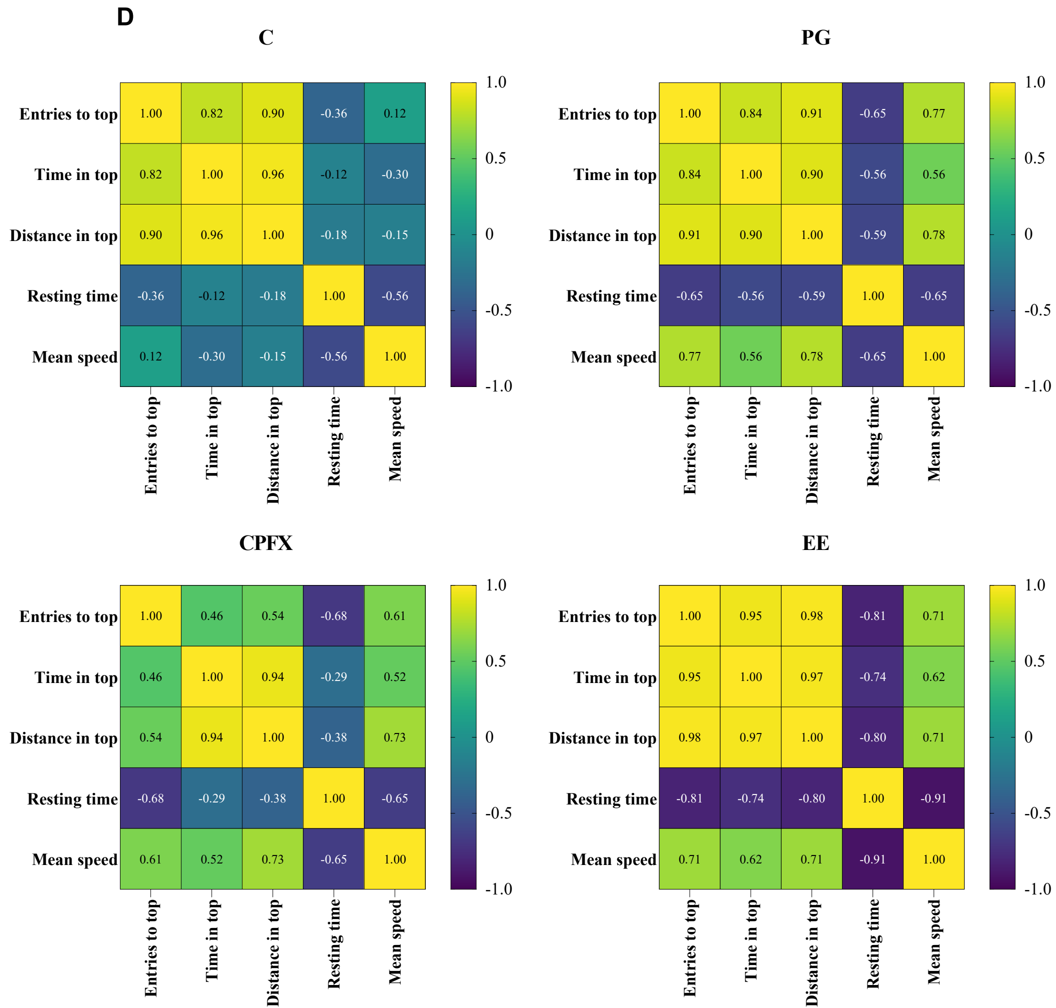


Figure 3.5: *The Novel Tank Test, (A) The dotted line plot representing Stress level index. Calculated on the basis of time spent in bottom versus top, appeared to be lower in CPFX. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), and ****(p<0.0001). (B) The box and whisker plot illustrating Top-bottom distance ratio. Higher ratio by CPFX and then PG. The data is represented as median interquartile range. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), and ****(p<0.0001). (C) Pair plot with kernel density estimation showing general relationship between novel tank behavioural parameters. (D) Correlation matrix plot, showing spearman correlation coefficient values of association between novel tank behavioural parameters.*

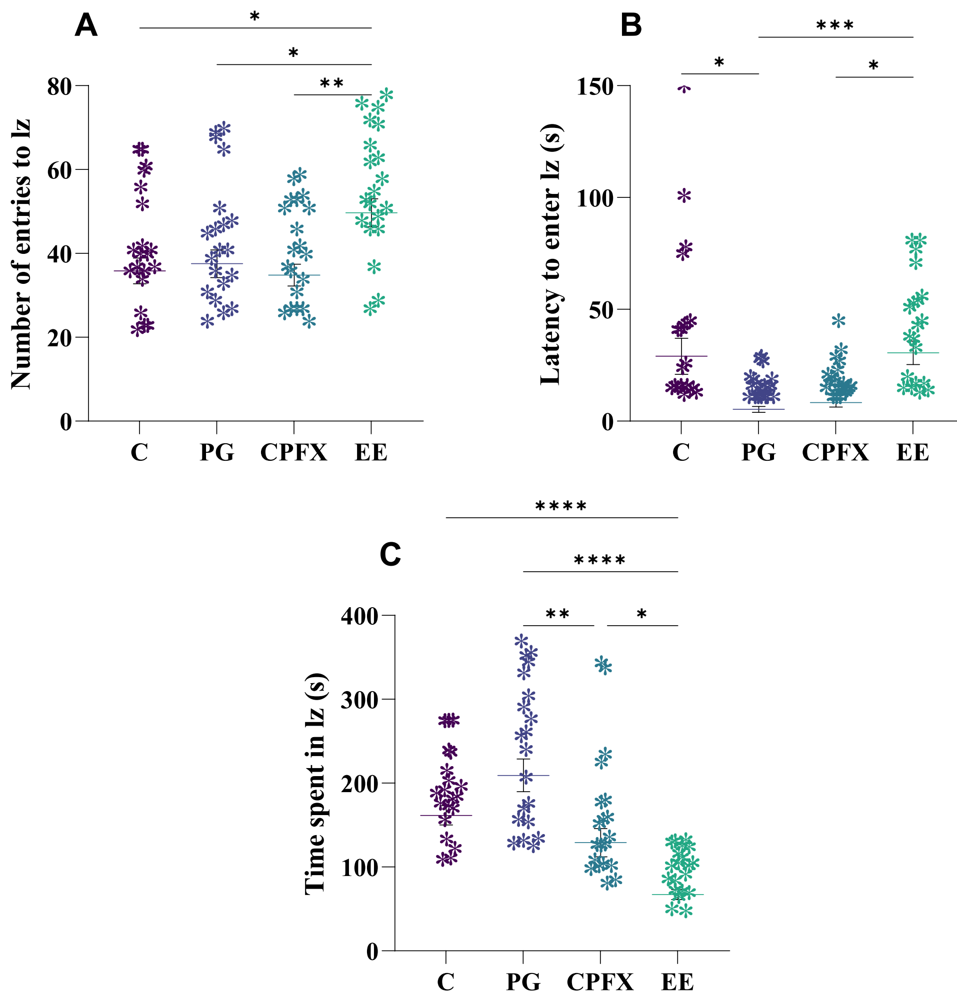


Figure 3.6: *Light and Dark Test, Scatter dot plot displaying (A) Number of entries to light zone. EE group displayed increased entries. (B) Latency to enter the light zone. The time taken to enter into the light zone is lower in PG and CPFX. (C) Time spent in the light zone. The amount of time spent in the light zone is severely restricted in EE group and increased in PG. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001. n=20).*

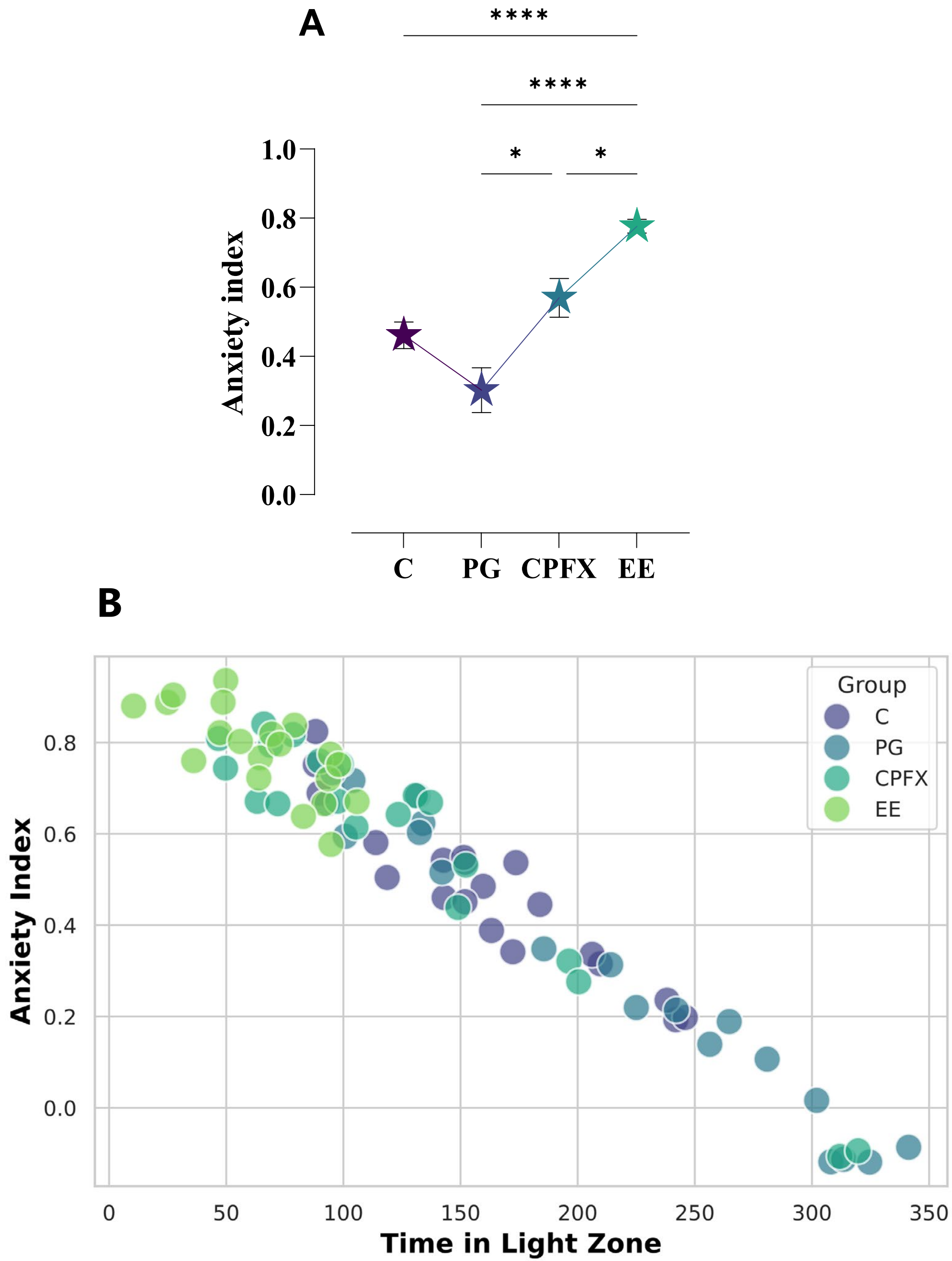


Figure 3.7. *Light-Dark Test (A) The dotted line plot representing Anxiety index. Calculated based on time spent in dark zone, lower in PG and higher in EE. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05) and ****(p<0.0001). (B) The Scattered dot plot displaying association between Anxiety index and Time in light zone. An inverse relationship found among all groups.*

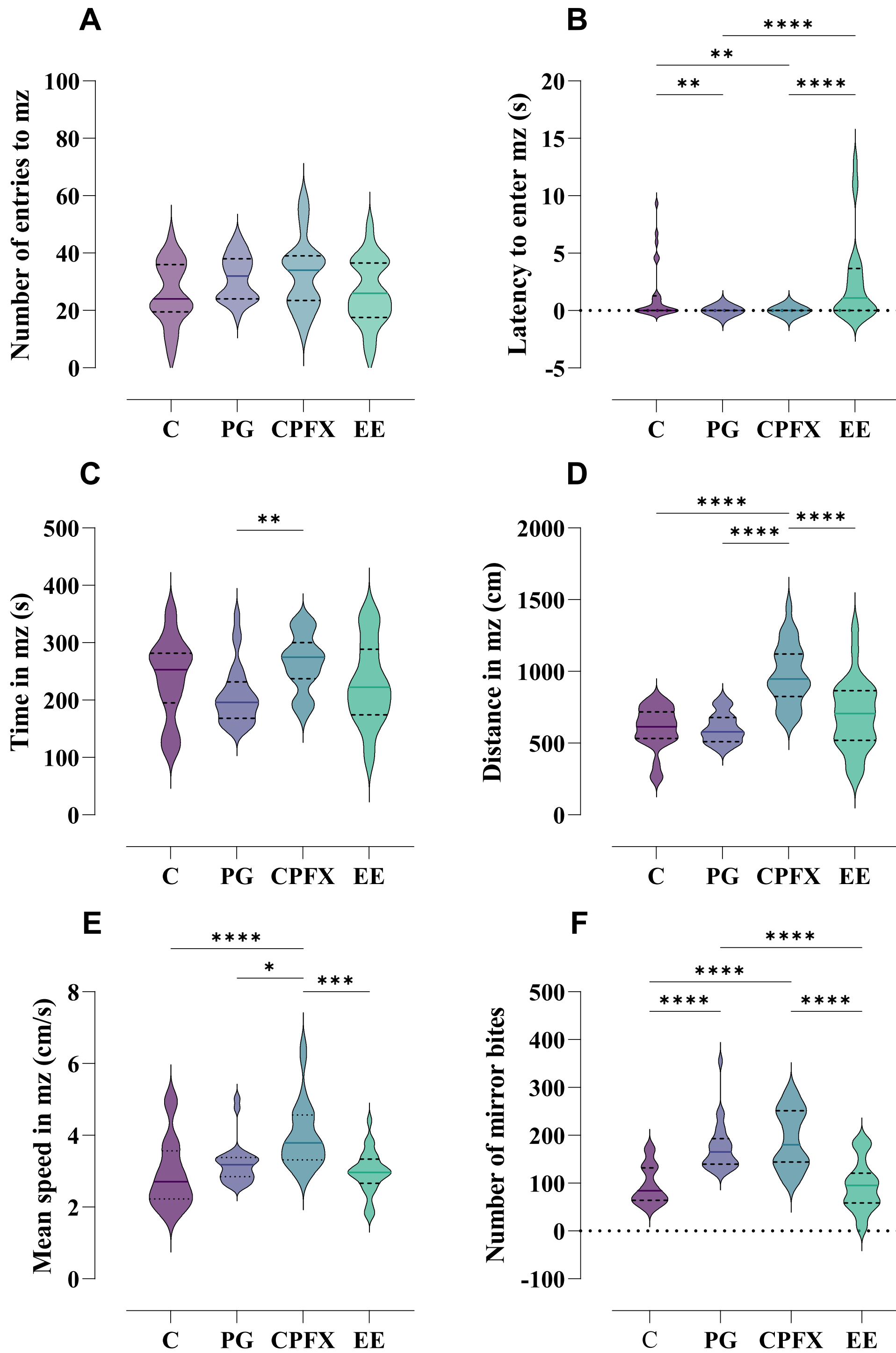
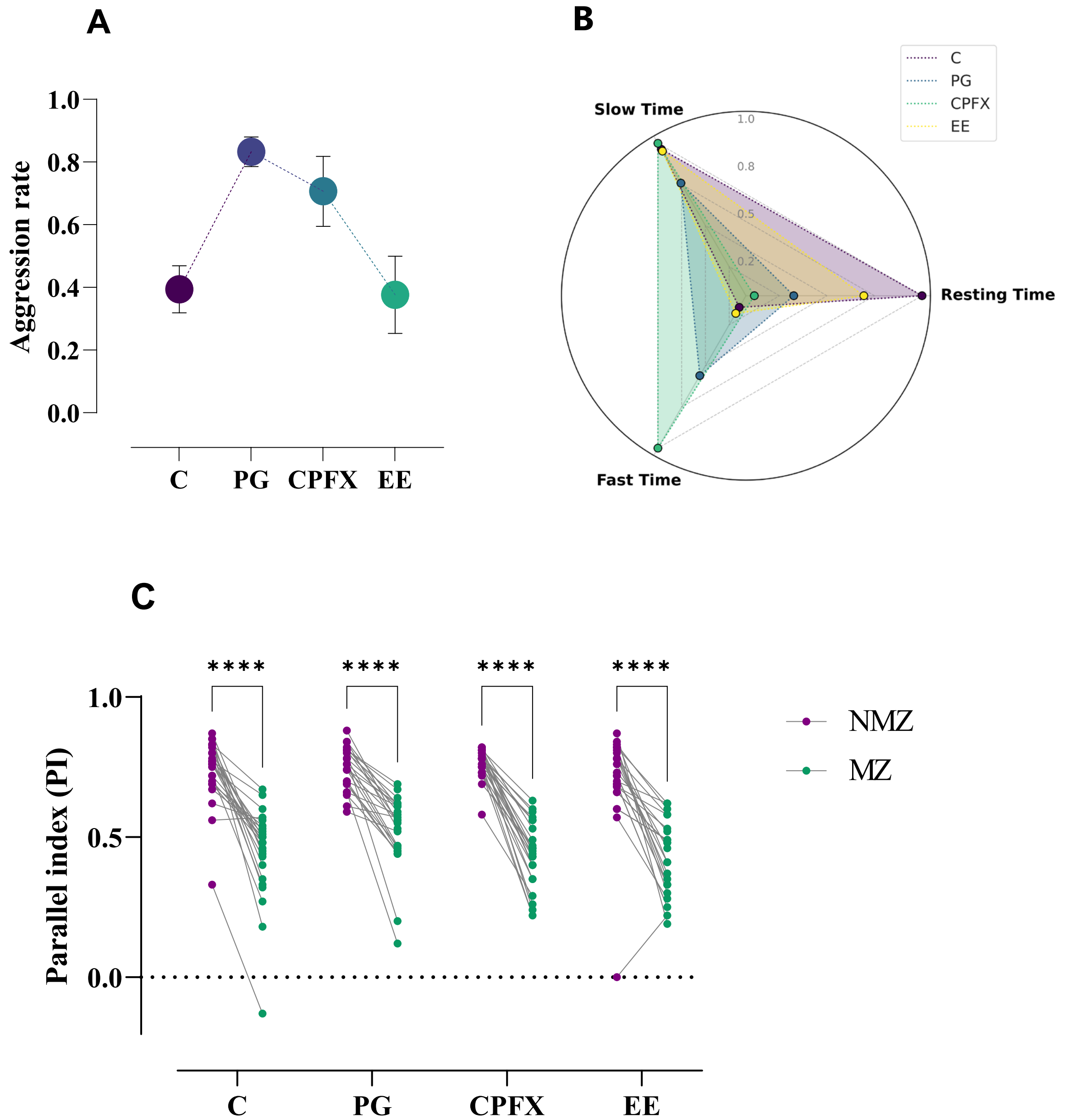


Figure 3.8: *Mirror Biting test, Violin plot represents behavioural endpoints derived from the activities in a three-chambered tank with a mirror. (A) Number of entries to mirror zone. (B) Latency to enter mirror zone. Reduced latency observed in PG and CPFX groups. (C) Time in mirror zone. Compared to PG, CPFX manifested increased time in MZ. (D) Distance in mirror zone. Increased distance exhibited by CPFX. (E) Mean speed in mirror zone. Higher in CPFX group. (F) Number of*

*mirror bites. PG and CPFX treatment enhanced the mirror bites pointing to the exacerbated aggressive nature. The data is represented as Median IQR. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001. n=25).*



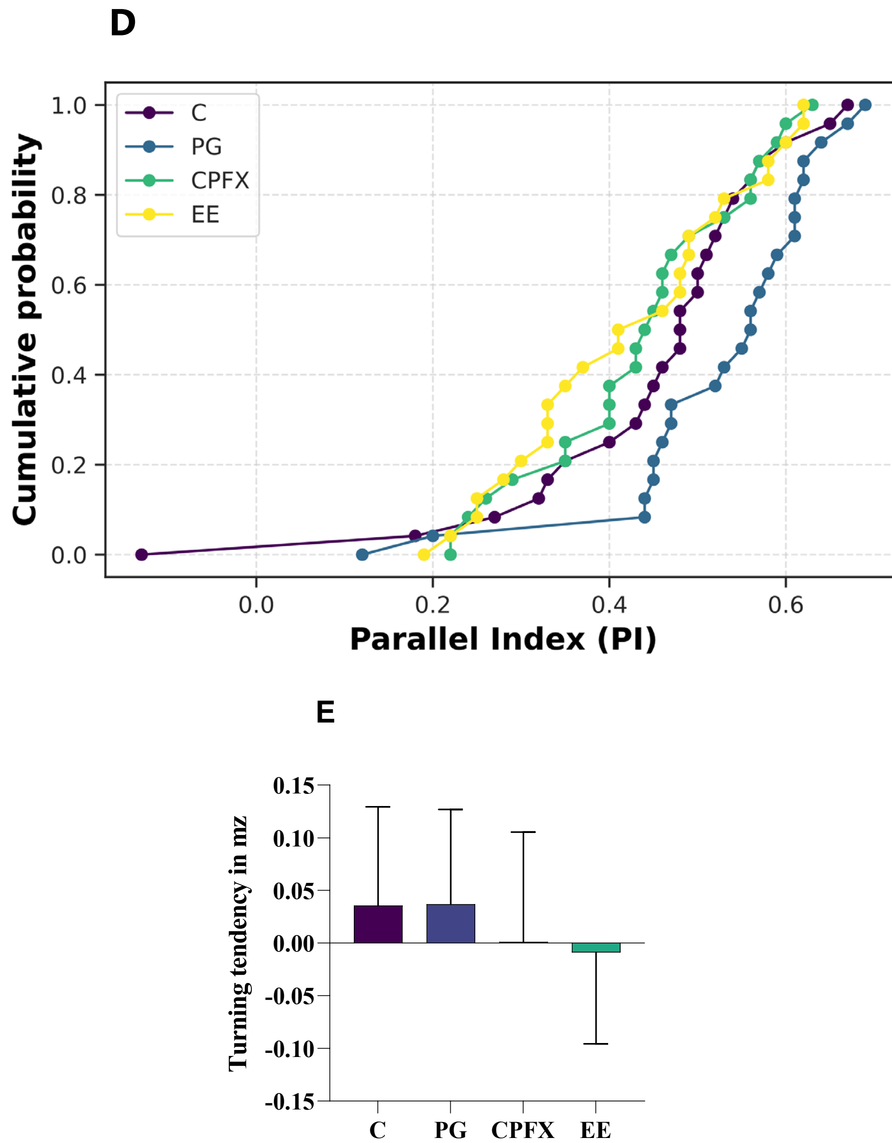


Figure 3.9: *Mirror Biting test, (A) The dotted line plot representing rate of aggression, it was higher in PG and CPFX. (B) The radar plot showing Resting, Slow, and Fast time. Lower resting time in CPFX, lower slow time in PG, and higher fast time in PG and CPFX. (C) The before-after plot displaying parallel orientation of zebrafish in the non-mirror zone (NMZ) and mirror-zone (MZ) with in group. The parallel orientation (parallel index) reduced irrespective of condition in MZ (D) The multiple line plot showing parallel orientation in MZ between groups. The parallel index was slightly higher in PG. (E) Turning tendency. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001).*

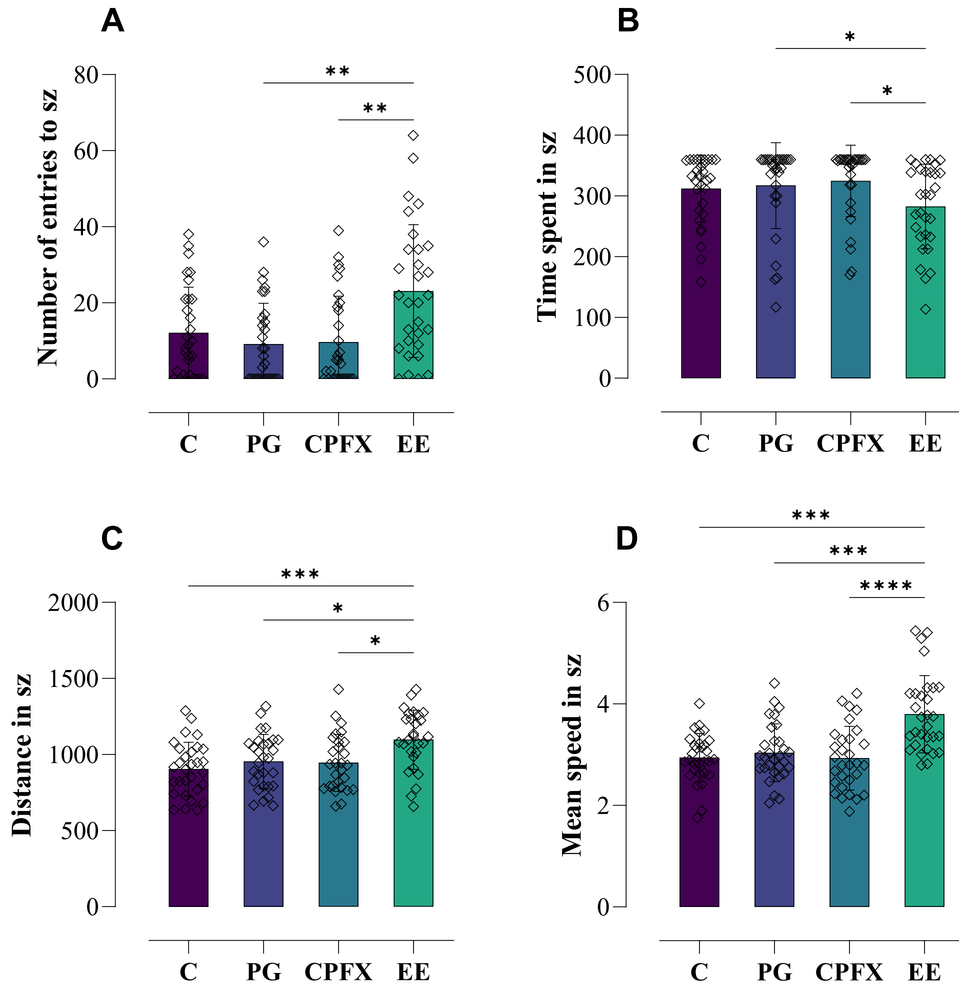
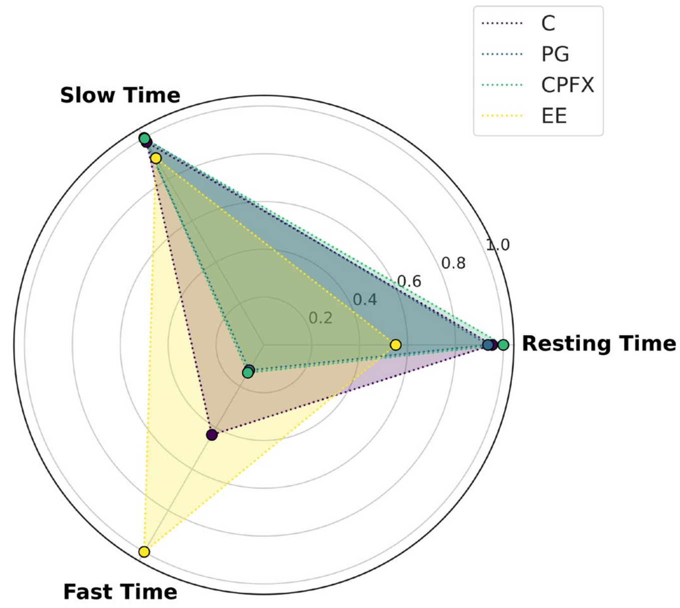
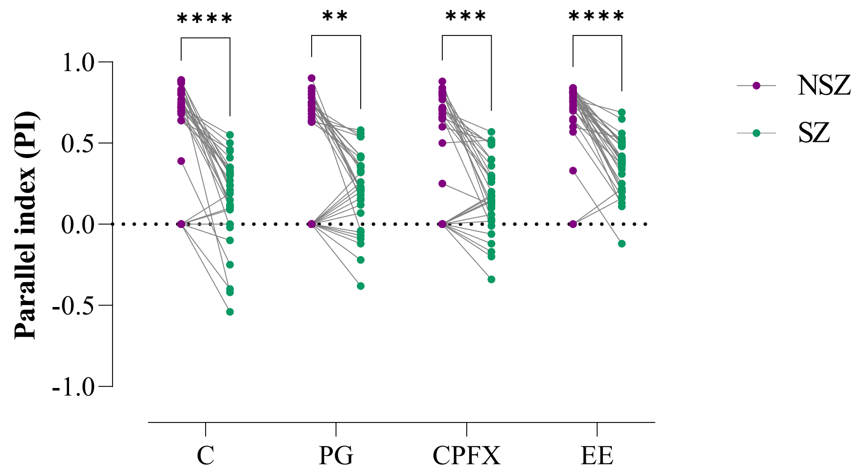
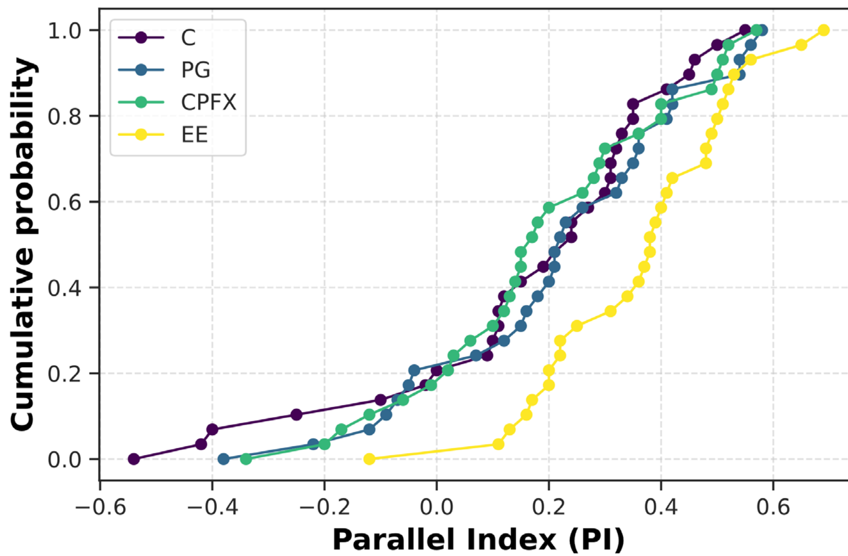


Figure 3.10: *The bar plot with scattered points represents behavioural endpoints from social preference test. (A) Number of entries to social zone, higher in EE compared to PG and CPFX. (B) Time spent in social zone, lower in EE compared to PG and CPFX. (C) Distance in social zone, higher in EE. (D) Mean speed in social zone, higher in EE. PG and CPFX treatment have no effect on social preference. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001. n=30).*


A
C
PG
CPFX
EE
Slow Time
Resting Time
Fast Time
0.2
0.4
0.6
0.8
1.0


B
****
**
***
****
Parallel index (PI)
1.0
0.5
0.0
-0.5
-1.0
NSZ
SZ
C
PG
CPFX
EE


C
C
PG
CPFX
EE
Cumulative probability
Parallel Index (PI)
0.0
0.2
0.4
0.6
0.8
1.0
−0.6
−0.4
−0.2
0.0
0.2
0.4
0.6

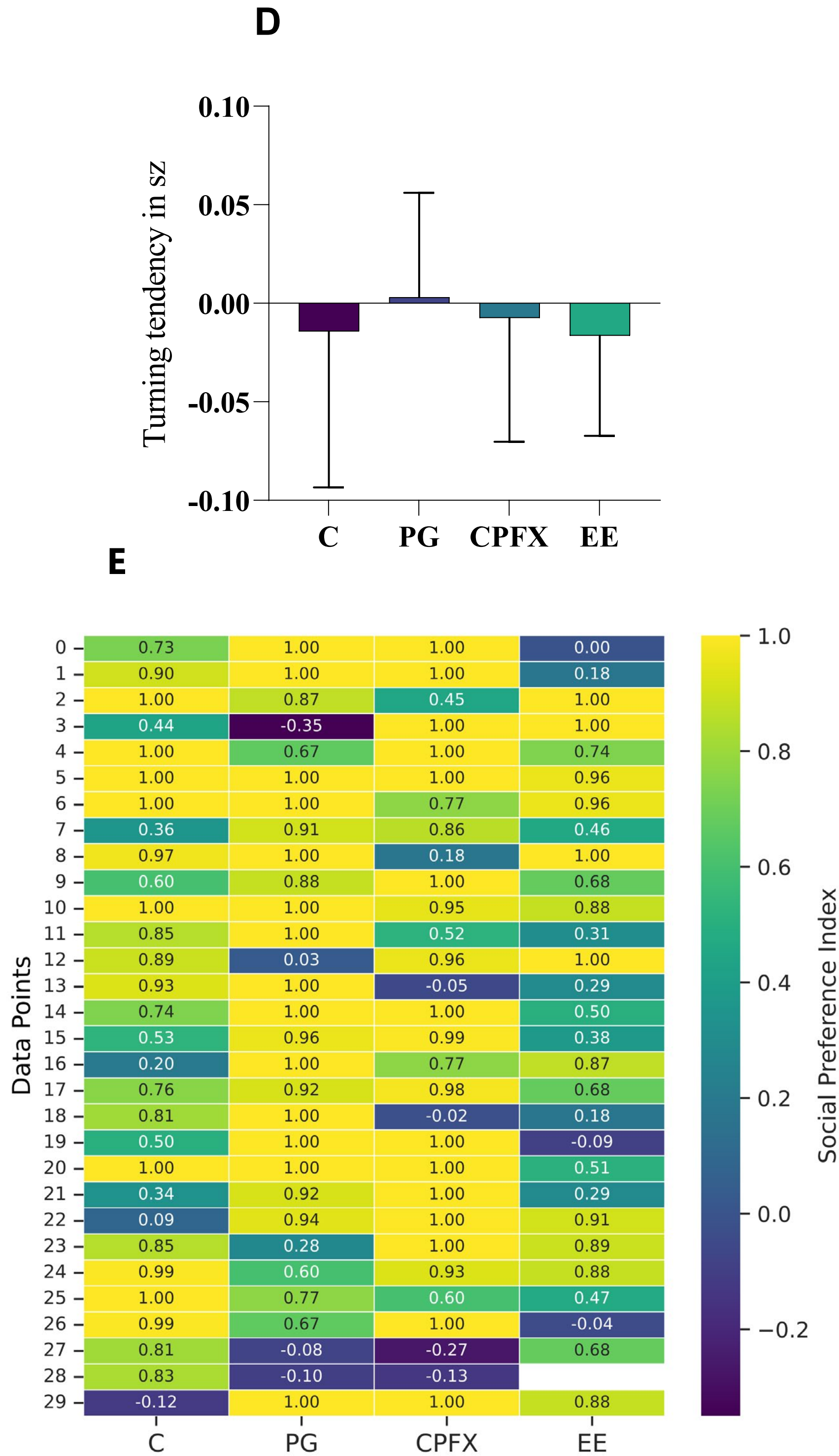


Figure 3.11: *Social preference test, (A) The radar plot showing Resting, Slow, and Fast time. Lower slow time and higher fast time in EE compared to PG and CPFX. No differences in resting time. (B) The before-after plot displaying parallel orientation of zebrafish in the non-social zone (NSZ) and social-zone (SZ) with in group. The parallel orientation (parallel index) reduced in all condition in SZ, but*

more consistent in C and EE. (C) The multiple line plot showing parallel orientation in SZ between groups. The parallel index was higher in EE. (D) Turning tendency (E) Heatmap representing social preference index (SPI), when comparing with C no changes in SPI was found in both antibiotics and enriched groups while comparison between antibiotics and EE the SPI was lower in EE.

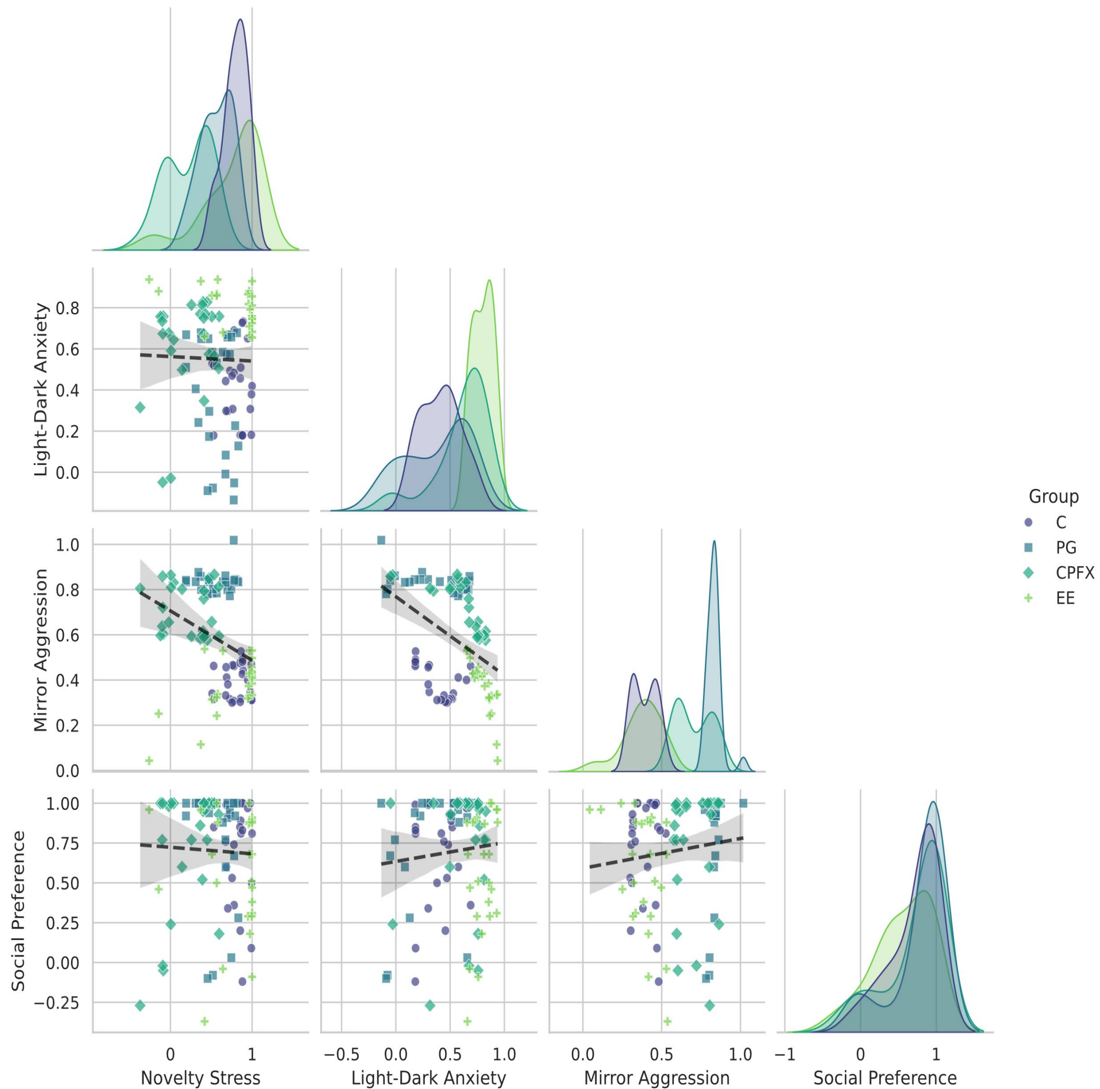


Figure 3.12: *The pair plot showing relationship between novelty stress, light-dark anxiety, mirror aggression and social preference. The diagonal elements displaying kernel density estimation (KDE) plots shows the distribution of individual variables. The off-diagonal elements contain scatterplots with regression lines showing relationship between pairs of variables. Different colours and markers represent different experimental groups.*

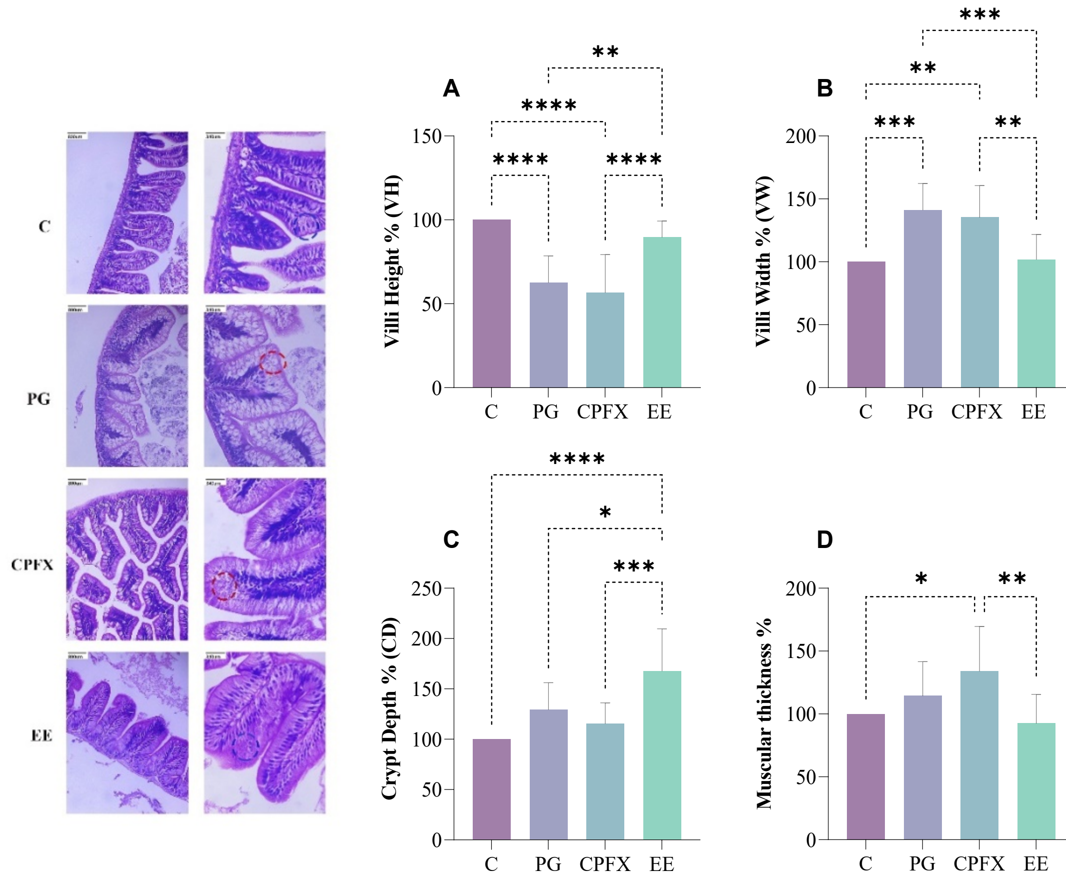


Figure 3.13: *Histology of intestinal tissue in C, PG, CPFX, and EE. Dotted circle (red) denotes vacuolisation and swelling of epithelial cells. The bar plot represents morphologic features of villi (A) Villi height, (B) Villi width, (C) Crypt depth, and (D) Muscular thickness. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001. n=10). The data plotted were derived from 10 randomly selected villi per group from three samples.*

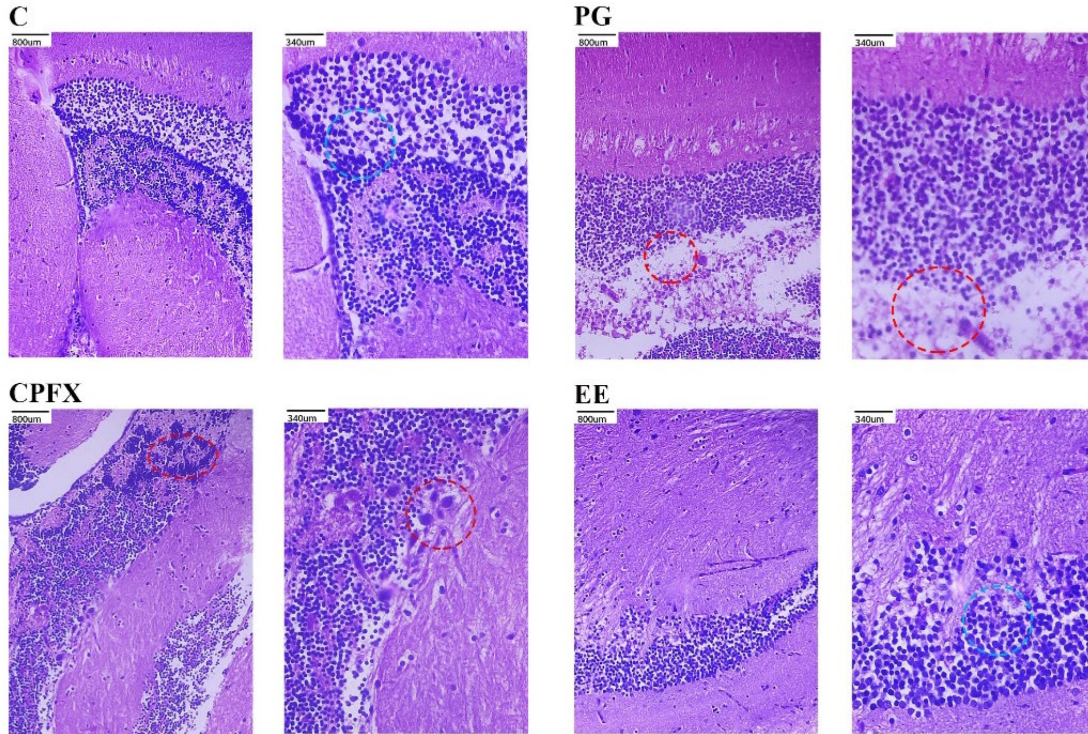


Figure 3.14*: Histology of brain tissue in C, PG, CPFX, and EE. Dotted circles (red) indicate inflammation in brain cells.*

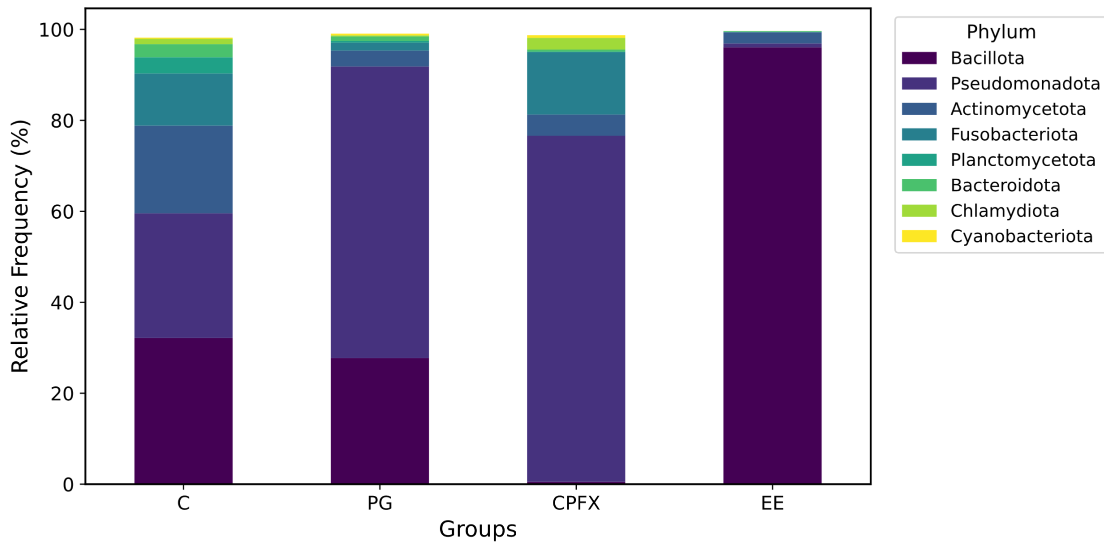


Figure 3.15A: *Gut bacterial analysis, the stacked bar plot displays the relative frequency of microbial abundance of four different conditions, Phylum -level.*

Name	C	PG	CPFX	EE
Bacillota	32.15%	27.72%	0.52%	96.04%
Pseudomonadota	27.40%	64.13%	76.11%	0.87%
Actinomycetota	19.30%	3.48%	4.66%	2.46%
Fusobacteriota	11.46%	1.76%	13.69%	0.03%
Planctomycetota	3.56%	0.37%	0.13%	0.06%
Bacteroidota	2.88%	0.99%	0.46%	0.17%

***Table 3.1**: Relative abundance of top six phyla present in antibiotic and enrichment groups.*

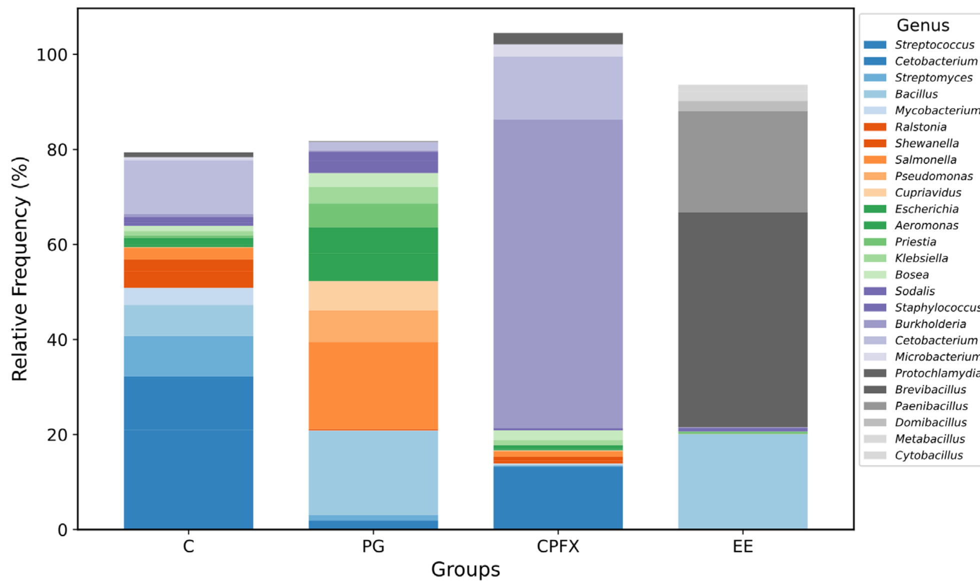


***Figure 3.15B**: Gut bacterial analysis, the stacked bar plot displays the relative frequency of microbial abundance of four different conditions, Genus-level.*

Name	C	Name	PG
Streptococcus	20.96%	*Salmonella*	18.38%
Cetobacterium	11.30%	*Bacillus*	17.67%
Cetobacterium	11.30%	*Pseudomonas*	6.61%
Streptomyces	8.49%	*Cupriavidus*	6.20%
Bacillus	6.50%	*Escherichia*	5.85%
Name	CPFX	Name	EE
Burkholderia	64.91%	*Brevibacillus*	45.24%
Cetobacterium	13.23%	*Paenibacillus*	21.28%
Cetobacterium	13.23%	*Bacillus*	20.01%
Microbacterium	2.60%	*Domibacillus*	2.14%
Protochlamydia	2.34%	*Metabacillus*	2.00%

***Table 3.2**: Relative abundance of top five genus present in antibiotic and enrichment groups.*

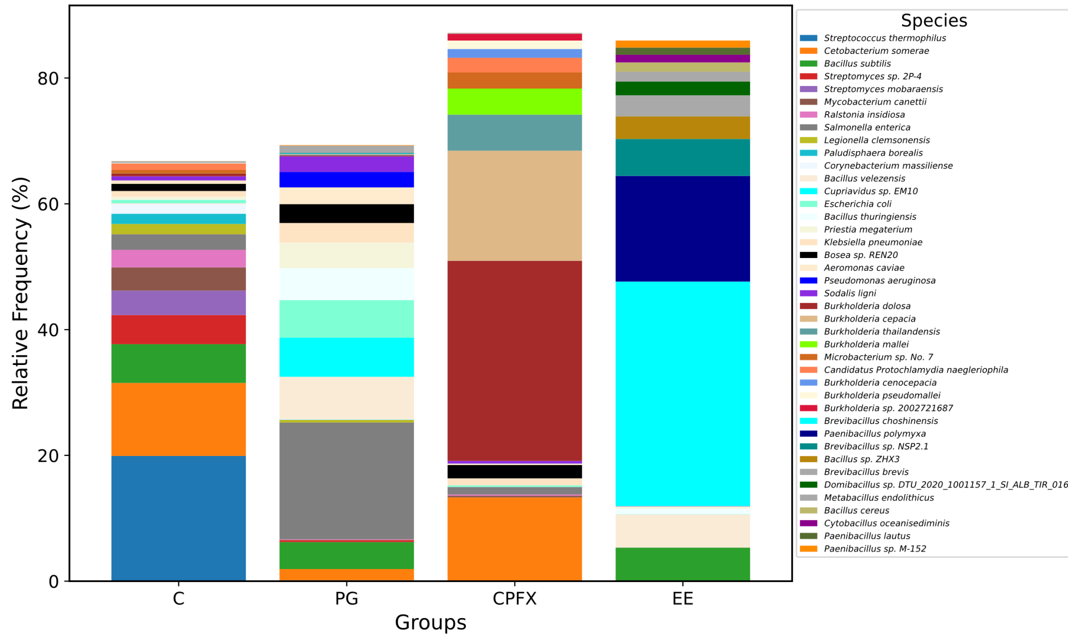


Figure 3.15C: *Gut bacterial analysis, the stacked bar plot displays the relative frequency of microbial abundance of four different conditions, Species-level.*

Name	C	Name	PG
Streptococcus thermophilus	19.91%	*Salmonella enterica*	18.56%
Cetobacterium somerae	11.61%	*Bacillus velezensis*	6.74%
Bacillus subtilis	6.17%	*Cupriavidus sp. EM10*	6.23%
Streptomyces sp. 2P-4	4.63%	*Escherichia coli*	5.96%
Streptomyces mobaraensis	3.87%	*Bacillus thuringiensis*	5.08%
		Bacillus subtilis	4.29%
		Priestia megaterium	4.06%
Name	CPFX	Name	EE
Burkholderia dolosa	31.82%	*Brevibacillus choshinensis*	35.69%
Burkholderia cepacia	17.51%	*Paenibacillus polymyxa*	16.80%
Cetobacterium somerae	13.30%	*Brevibacillus sp. NSP2.1*	5.88%
Burkholderia thailandensis	5.72%	*Bacillus subtilis*	5.30%
Burkholderia mallei	4.15%	*Bacillus velezensis*	5.25%
Microbacterium sp. No. 7	2.55%	*Bacillus sp. ZHX3*	3.59%

Table 3.3: *Relative abundance of top species present in antibiotic and enrichment groups.*

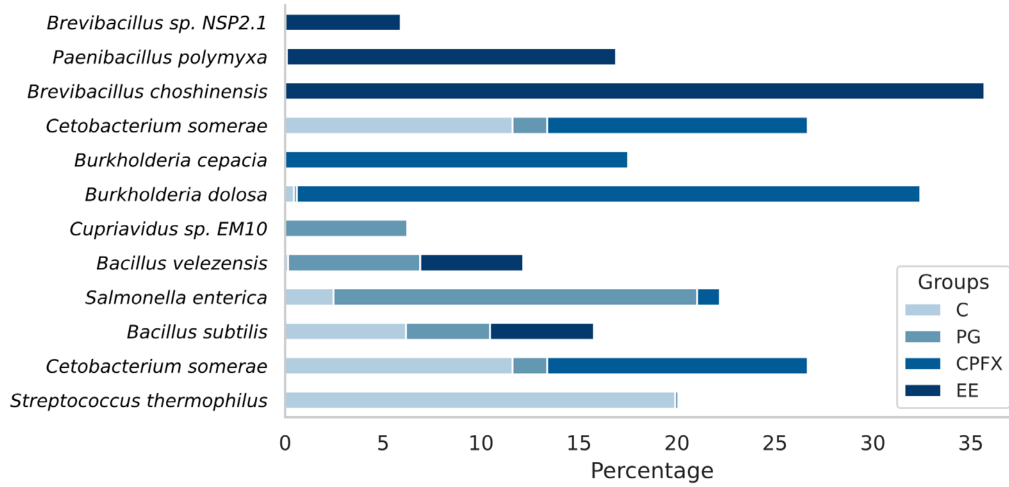


Figure 3.15D: *Gut bacterial analysis, horizontally stacked bar plot depicts the top two-level bacterial species across groups.*

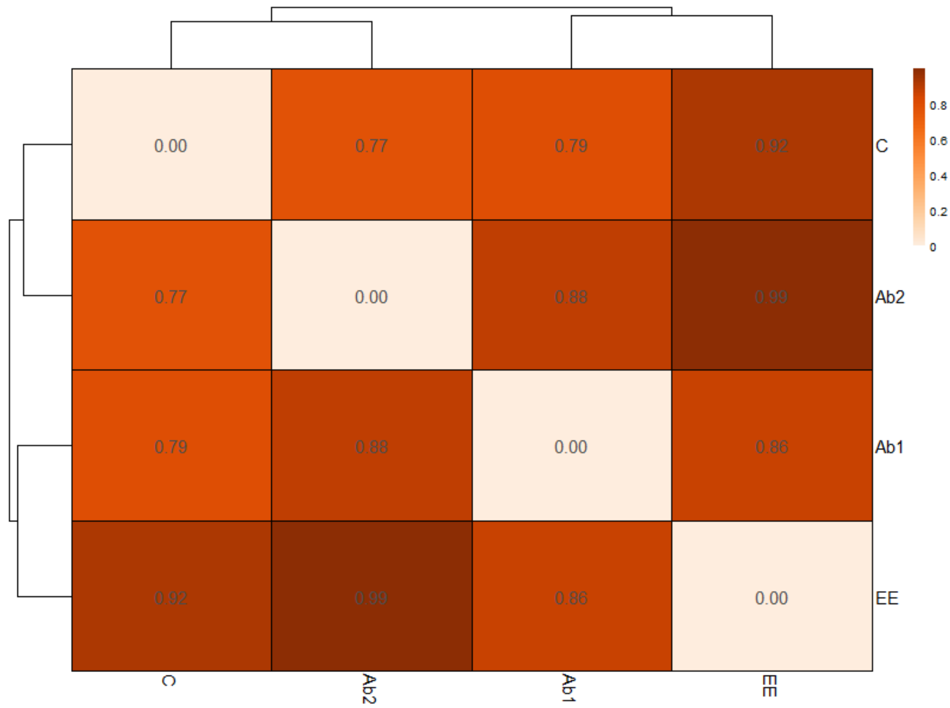


Figure 3.15E: *Gut bacterial analysis, Beta diversity. Bray Curtis Dissimilarity Matrix between species present in each sample.*

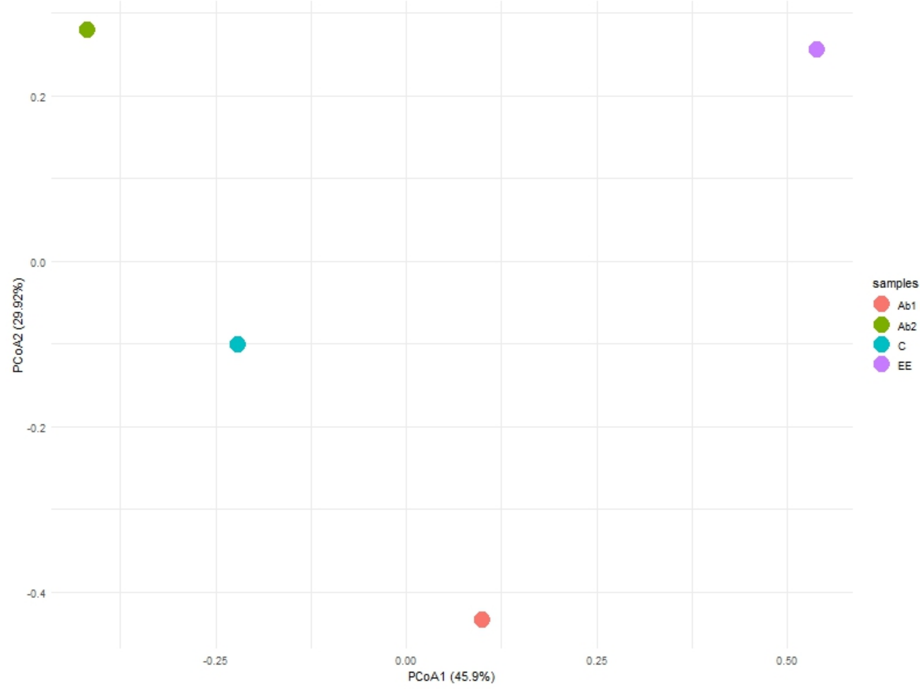


Figure 3.15F: *Gut bacterial analysis, Principal Coordinates Analysis (PCoA) plot illustrates the beta diversity or dissimilarities among samples based on their microbiota composition*

	Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
C	609	757.348	30.5978	771.211	14.0615	3.8744	0.93382	15.1104	105.35
Ab1	812	1017.3	38.7301	987.408	15.7596	3.82176	0.94153	17.1031	123.353
Ab2	471	643.667	38.0741	639.559	13.0941	2.69891	0.8426	6.35331	65.6473
EE	508	725.35	46.5322	685.149	13.7161	2.75148	0.83112	5.92146	74.5703

***Table 3.4**: Alpha Diversity indices of antibiotic and enrichment groups*

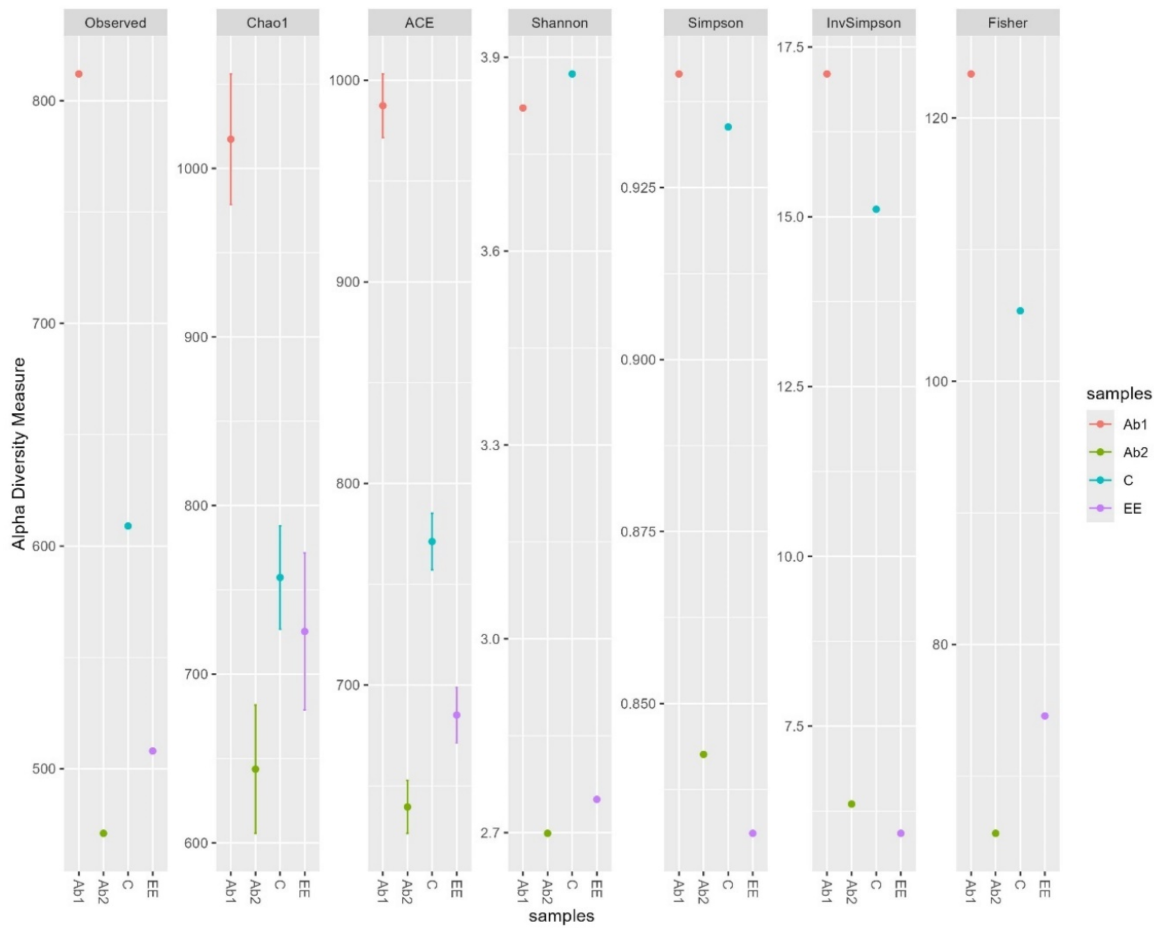
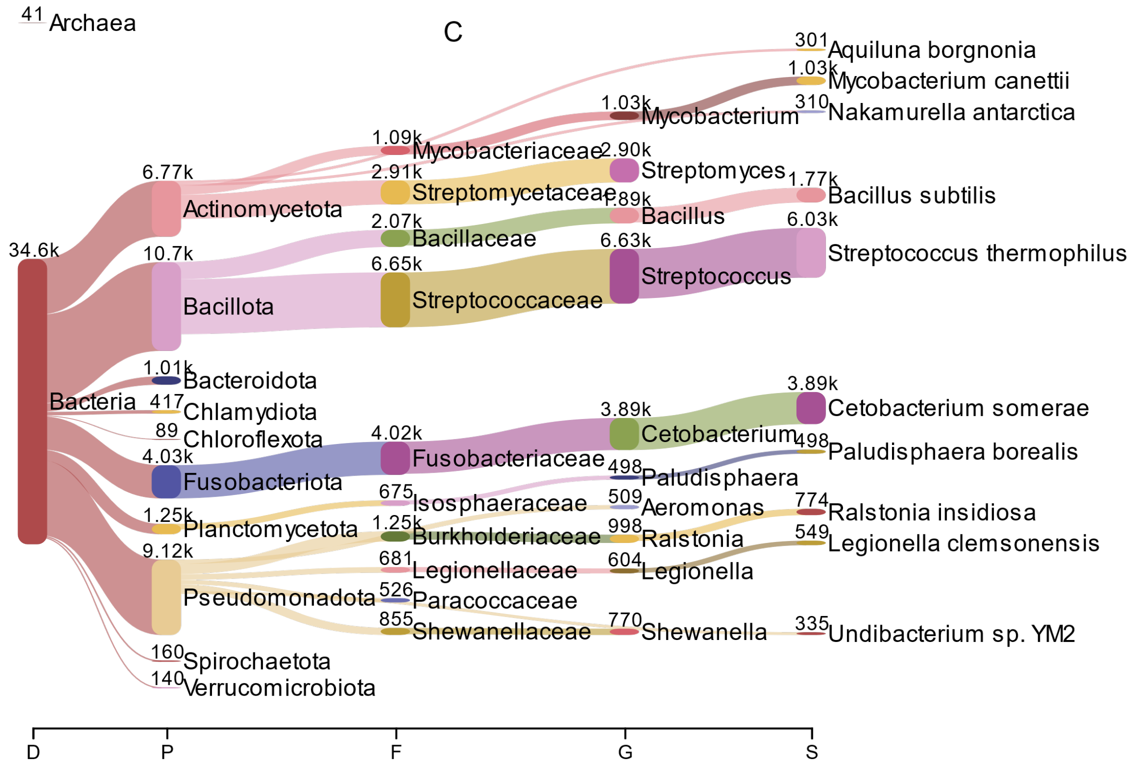
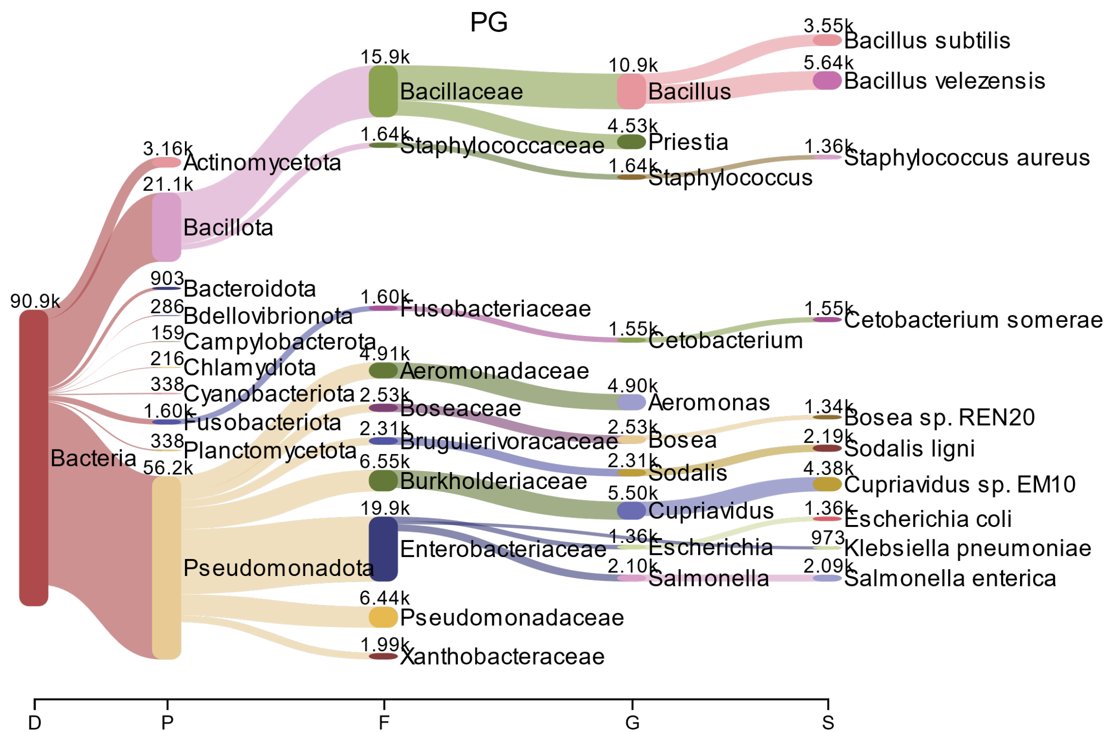


***Figure 3.15G:** Gut bacterial analysis, Alpha Diversity indices.*


C
41 Archaea
34.6k Bacteria
6.77k Actinomycetota
10.7k Bacillota
1.01k Bacteroidota
417 Chlamydiota
89 Chloroflexota
4.03k Fusobacteriota
1.25k Planctomycetota
9.12k Pseudomonadota
160 Spirochaetota
140 Verrucomicrobiota
1.09k Mycobacteriaceae
2.91k Streptomycetaceae
2.07k Bacillaceae
6.65k Streptococcaceae
4.02k Fusobacteriaceae
675 Isosphaeraceae
1.25k Burkholderiaceae
681 Legionellaceae
526 Paracoccaceae
855 Shewanellaceae
1.03k Mycobacterium
2.90k Streptomyces
1.89k Bacillus
6.63k Streptococcus
3.89k Cetobacterium
498 Paludisphaera
509 Aeromonas
998 Ralstonia
604 Legionella
770 Shewanella
301 Aquiluna borgnonia
1.03k Mycobacterium canettii
310 Nakamurella antarctica
1.77k Bacillus subtilis
6.03k Streptococcus thermophilus
3.89k Cetobacterium somerae
498 Paludisphaera borealis
774 Ralstonia insidiosa
549 Legionella clemsonensis
335 Undibacterium sp. YM2
D
P
F
G
S


PG
90.9k Bacteria
3.16k Actinomycetota
21.1k Bacillota
903 Bacteroidota
286 Bdellovibrionota
159 Campylobacterota
216 Chlamydiota
338 Cyanobacteriota
1.60k Fusobacteriota
338 Planctomycetota
56.2k Pseudomonadota
15.9k Bacillaceae
1.64k Staphylococcaceae
1.60k Fusobacteriaceae
4.91k Aeromonadaceae
2.53k Boseaceae
2.31k Bruguierivoracaceae
6.55k Burkholderiaceae
19.9k Enterobacteriaceae
6.44k Pseudomonadaceae
1.99k Xanthobacteraceae
10.9k Bacillus
4.53k Priestia
1.64k Staphylococcus
1.55k Cetobacterium
4.90k Aeromonas
2.53k Bosea
2.31k Sodalis
5.50k Cupriavidus
1.36k Escherichia
2.10k Salmonella
3.55k Bacillus subtilis
5.64k Bacillus velezensis
1.36k Staphylococcus aureus
1.55k Cetobacterium somerae
1.34k Bosea sp. REN20
2.19k Sodalis ligni
4.38k Cupriavidus sp. EM10
1.36k Escherichia coli
973 Klebsiella pneumoniae
2.09k Salmonella enterica
D
P
F
G
S

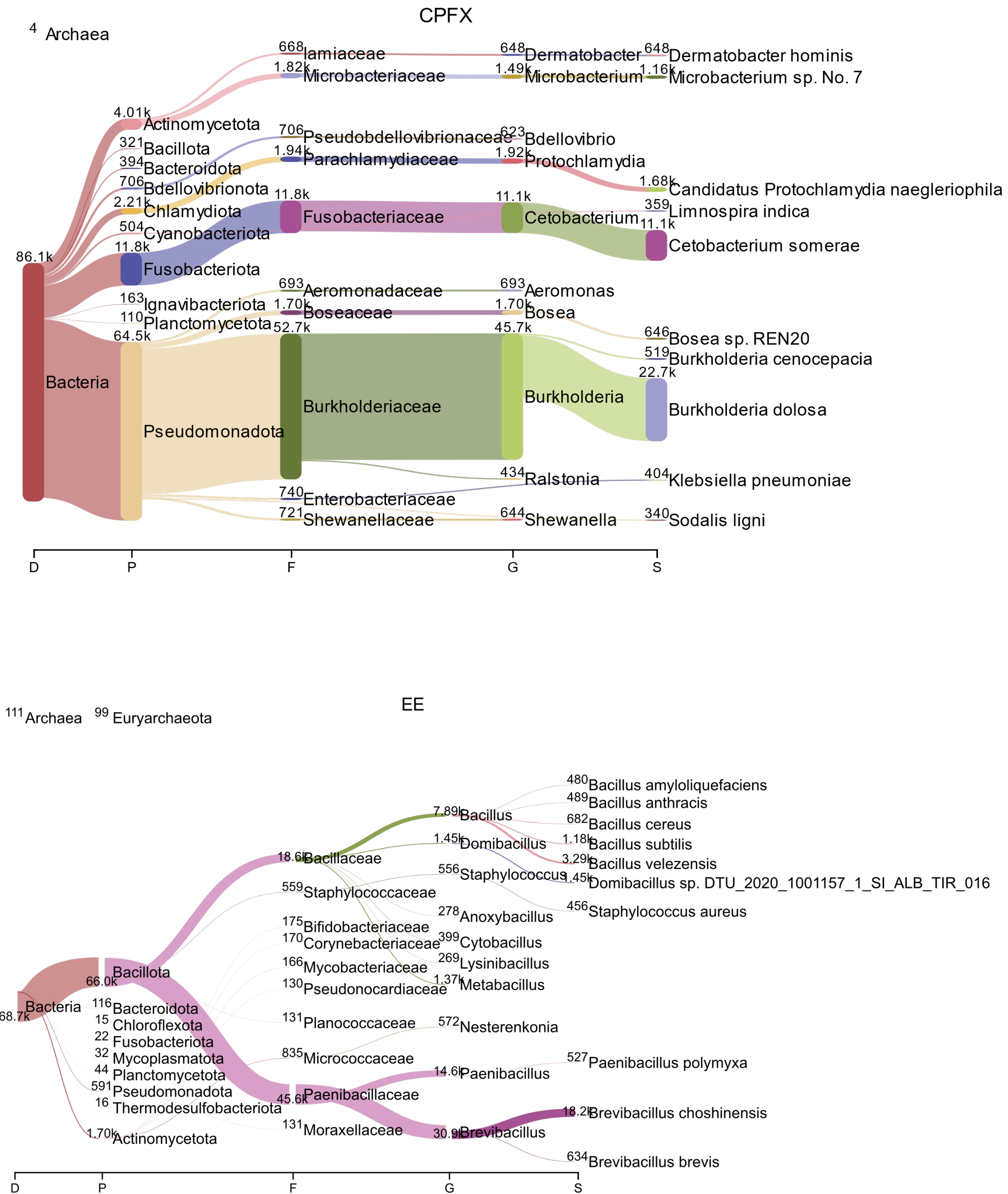


***Figure 3.15H:** Gut bacterial analysis, Sankey plots-C, PG, CPFX, and EE.*

3.5. Discussion

3.5.1 Environmental conditions can impact zebrafish behaviour

This study demonstrated that the environment inhabited by zebrafish significantly influences their behaviour. The investigation focused on the chronic exposure of zebrafish to PG (10 mg/L), CPFX (10 mg/L), and EE (with natural plants and

stones), examining how these contrasting environments affect zebrafish behavioural repertoires related to exploration, anxiety, aggression, and social preference. The PG and CPFX groups exhibited increased activity in the top zone of the novel tank; however, their overall distance travelled, speed, and resting time were not altered compared with the control group. Previous chapter involving a daily dose of 25 mg/L PG and CPFX for seven days indicated a decrease in time spent in the top zone and resting time, but an increase in distance traveled, mean speed, and time spent moving quickly in the novel tank test, suggesting signs of hyperactivity and restlessness (Sivarajan & Ramachandran, 2023). Another study found that treatment with antibiotics, including ciprofloxacin (6.25, 12.5, and 25 mg/L) for 96 hours, resulted in a greater distance traveled at the 25 mg/L dosage, with no influence on anxiety but an exacerbation of aggression, while sociability remained unaffected (Petersen et al., 2021). The results of the present study are consistent with these findings, as CPFX and PG exposure increased aggression rates without affecting social preferences. Conversely, another study reported that treatment with 100 mg/L amoxicillin decreased distance traveled and speed, increased freezing behaviour in the novel tank test, and reduced social interaction (Gonçalves et al., 2020). Although the CPFX group exhibited higher exploration and reduced anxiety in the novel environment than the PG group, they spent less time in the light zone, potentially due to the photosensitivity or phototoxic effects of CPFX (Tokura, 1998). A similar effect was observed in oxytetracycline-treated zebrafish as they showed increased exploration in a new environment but heightened stress responses, including hyperactivity and freezing during light periods (Almeida et al., 2019). In the context of environmental enrichment (EE), exploration and activity in the upper zone were unaffected; however, the observed reduction in overall distance traveled and speed,

coupled with increased resting or immobility, suggests neophobic-related behaviour. A previous study demonstrated that enrichment alleviates anxiety in zebrafish subjected to unpredictable chronic stress. Conversely, non-stressed fish exposed to enrichment exhibited anxiety-like behaviours in the novel tank test compared with non-stressed fish maintained under standard conditions (Marcon et al., 2018). Similarly, group-housed zebrafish displayed increased anxiety-like behaviours in the novel tank test compared with individually housed zebrafish (Parker et al., 2012; Shams et al., 2015).

The frequent tendency to approach the light zone, yet a reduced time spent there, suggests that zebrafish reared in an environment with natural plants and stones experience light-induced anxiety. Their natural environment, characterised by algal growth on tank walls, restricts some light, thereby creating a more comfortable shaded environment and leading them to prefer darker areas. The natural preference for dark compartments over light ones in zebrafish has been shown previously (Serra et al., 1999; Maximino et al., 2010; Facciol et al., 2017). Additionally, several factors, including tank design, test conditions, lighting, and water column depth, affect this preference (Facciol et al., 2017; Cordova et al., 2016). A naturally enriched environment for 15 days decreased cortisol release in zebrafish under stress, producing an effect similar to treatment with diazepam or fluoxetine, thereby reducing stress levels (Giacomini et al., 2016). Compared with synthetic enrichment materials, natural materials such as plants and stones lower stress levels, improve memory, and enhance exploratory behaviours in mice (Acklin & Gault, 2015). Moreover, natural enrichment facilitates species-typical behaviours and helps reduce abnormal behaviours, thereby promoting welfare and health (Würbel & Novak, 2024; Tsang & Gerlai, 2022). Research regarding rainbow trout has demonstrated

that aquatic environments with gravel substrate increased their well-being by reducing mortality rates as well as fungal infections (Reiser et al., 2019). These findings suggest that adding natural elements may provide a more suitable environment for animals. Generally, natural environments exert a beneficial effect on behaviour, whereas the neophobic response in the Novel Tank Test (NTT) and light avoidance behaviour in the Light/Dark Test (LDT) are presumably due to early exposure to natural enrichment or over-acclimatisation to such conditions. Physical enrichment elicits mixed responses concerning aggression. Some studies have reported increased aggression in zebrafish exposed to physical structures (Wilkes et al., 2012; Bhat et al., 2015; Woodward et al., 2019; Stevens et al., 2021; Gazzano et al., 2025), while Hamilton and Dill (2002) found that enrichment did not affect aggression levels. Other studies have also indicated lower aggression in the presence of structures (Basquill & Grant, 1998; Carfagnini et al., 2009; Keck et al., 2015).

Here, the environment with natural plants and stones, aggression levels were not elevated compared with the antibiotic-treated groups, although social preference was expressed differently. EE did not affect overall social preference compared with control groups but modified interaction styles, resulting in less sustained social engagements and more exploratory behaviour in social zone. In laboratory rats, EE enhanced social grooming, interaction, and prosocial behaviours, such as spending more time near conspecifics (Neal et al., 2018; Parra-Cruz et al., 2023). Similarly, EE increased the preference for social stimuli and sociability in BTBR mice (a model for autism) (Diamond, 2019), suggesting that EE can enhance the motivation to perform socially beneficial behaviours, particularly in animal models of disease or stressed individuals.

3.5.2. The chronic exposure to PG and CPFX reduced the gut and brain integrity while EE preserved it.

Chronic exposure to low doses of PG and CPFX caused changes in intestinal tissue. Vacuolization of epithelial cells was more evident in intestinal villi. Any deterioration in intestinal villi affects the protective function of the intestinal mucosa, reducing resistance and making fish more vulnerable to pathogens and other external contaminants. These further compromises their ability to withstand environmental changes (Wei, 2010; Mehinto et al., 2010). CPFX and PG exposure greatly affected villus morphology, causing the most significant reduction in villus height and an increase in villus width. Moreover, in CPFX-treated fish, the thickened muscle layer indicated intestinal stress. Similar study indicates that treatment with sulfamethoxazole affected villus morphology. Among the different concentrations tested (3 mg/L, 6 mg/L, 12 mg/L, and 24 mg/L), as the concentration increased, villus height decreased, and villus width increased. Muscular thickness also decreased at the highest concentration (24 mg/L) by 40% (Zhou et al., 2024).

In mouse models, the use of antibiotics resulted in variations in intestinal morphology. For example, neonatal mice with extensive antibiotic treatment resulted in a significant reduction in villus height, crypt depth, and a lower number of mucus-producing cells compared to controls (Chaaban et al., 2022). Chronic antibiotic treatment damaged the ileal villi in mice, and it was reversible after termination of antibiotic treatment (Romick-Rosendale et al., 2014). This indicates a direct consequence of antibiotics on villus structure. The use of antibiotics may compromise the structural integrity of villi, making them more susceptible to damage under challenging conditions. For instance, the gut microbial disruption upon antibiotic use can lead to ruptured intestinal villi by the subsequent exposure to allergens (Zhang et al., 2021). Antibiotics also affect the gut microbiota by reducing

bacterial diversity and disrupting the balance of microbial communities. This imbalance creates increased intestinal permeability and inflammation, potentially causing harm to the villi (Chen et al., 2021). Natural enrichment provides complex stimuli that mimic the natural habitat and positively influence physiology (Gallas-Lopes et al., 2023; Green & Swaney, 2023), including crypt depth. The increased crypt depth in EE might be due to increased epithelial turnover. This indicates an adaptive change to environmental conditions. Animals raised in enriched environments exhibit enhanced coping mechanisms reflecting their adaptation and resilience (Rojas-Carvajal et al., 2021). Regarding brain tissue, the mesencephalon displayed increased cell loss and cell clustering in PG and CPFX-treated fish, which might be a sign of neuroinflammation. Exposure to PG and CPFX causes gut microbial disruptions, resulting in dysbiosis. And in turn activates microglia in the brain. This can further increase the expression of pro-inflammatory markers, promoting neuroinflammation (Caliskan et al., 2022). Antibiotic-induced dysbiosis can also weaken the blood-brain barrier, thereby increasing its permeability and leading to the infiltration of immune cells into the brain, causing inflammation (Sun et al., 2021).

3.5.3. The population dynamics of gut-microbiota in PG, CPFX, and EE conditions

Environmental conditions significantly influence gut microbiota composition. The differential abundance of microbial taxa suggests that environmental stressors such as antibiotic exposure or environmental enrichment with natural plants and stones have a profound impact on gut microbial populations. The control group is marked with a balanced gut microbial phylum, with Bacillota and Pseudomonadota being dominant. At the same time, Actinomycetota and Fusobacteriota are also present in substantial proportions. These phyla are commonly linked to a healthy gut

microbiota, where Bacillota and Actinomycetota facilitate carbohydrate metabolism and short-chain fatty acid (SCFA) production, while Fusobacteria may be involved in gut immune modulation (Kanther & Rawls, 2010; Xia et al., 2022; Rinninella et al., 2019; Turnbaugh et al., 2006).

At the genus level, *Streptococcus* was dominant followed by *Cetobacterium, Streptomyces*, and *Bacillus*. Among these genera, *Streptococcus thermophilus* and *Cetobacterium somerae* were the most abundant species in control group. Studies have shown that *S. thermophilus* can adapt to the zebrafish gut environment. And enhance its immigration and inter-host transmission capabilities that are essential for establishing a stable gut microbiome (Robinson et al., 2018) and influencing host immune responses and metabolic activities (Willms et al., 2021; Uriot et al., 2021). As these bacteria aid in fermentation, they contribute to SCFA production (Cholan et al., 2020). *C. somerae* is associated with improved gut and liver health by reducing inflammation and hepatic triacylglycerol levels (Li et al., 2023; Xie et al., 2022) and enhancing antiviral responses (Liang et al., 2024). Administration of *C. somerae* in zebrafish has been found to improve glucose homeostasis and increase insulin expression, while also producing acetate, which mediates glucose regulation through parasympathetic activation, thereby highlighting its role in the gut-brain axis (Wang et al., 2021). These beneficial bacteria may influence behaviour by modulating neuroactive molecules (Borrelli et al., 2016). The moderate microbial diversity, richness, and higher evenness suggest a well-balanced microbiome.

Under PG and CPFX treatment, *Pseudomonadota* became the dominant phylum in both groups, with a decline in *Actinomycetota* and *Fusobacteriota* in PG, while *Actinomycetota* and *Bacillota* decreased in CPFX. This suggests that antibiotic exposure creates an environment favouring opportunistic bacterial groups in

response to dysbiosis (Clarke et al., 2014). Dysbiosis caused by antibiotics such as ciprofloxacin can alter vulnerability to pathogens and increase sensitivity to infection (Yang et al., 2023; Yang et al., 2024). Ciprofloxacin is known for eliminating beneficial bacteria while promoting the growth of resistant organisms (Dethlefsen et al., 2008). An increased level of phylum *Pseudomonadota* in zebrafish is associated with a reduced immune response, as indicated by reduced macrophage and neutrophil counts (Qiu et al., 2022). Additionally, treatment with amoxicillin greatly reduced gut microbiota diversity and allowed opportunistic bacteria such as *Pseudomonadota* to flourish (Deprey & Uno, 2016).

Salmonella and *Bacillus* were the most abundant genera in the PG group, while *Pseudomonas, Cupriavidus*, and *Escherichia* were also present in smaller proportions. The species *Salmonella enterica* was dominant. *S. enterica* is an enteropathogen, and its proliferation under PG exposure may indicate microbiota disruption, favoring opportunistic pathogens (Stecher & Hardt, 2011). Studies have shown that *S. enterica* can cause mortality in larval zebrafish when co-infected with human norovirus, leading to increased inflammatory responses and oxidative stress (Toh et al., 2024). The presence of *Escherichia coli* and *Cupriavidus* species, known to harbour antibiotic resistance genes, further suggests an antibiotic-mediated shift towards microbial communities dominated by resistant and potentially harmful species (Poirel et al., 2018; Zhang et al., 2018).

The CPFX group showed a prominent increase in the genus *Burkholderia*, with the major species being *Burkholderia dolosa* and *Burkholderia cepacia*, both of which are opportunistic pathogens known for their resistance to antibiotics and ability to thrive in disrupted microbiomes (Coenye & Vandamme, 2003). The presence of *B. thailandensis* and *B. mallei* indicates that CPFX treatment selects antibiotic-resistant

species, leading to a dysbiotic state (Dethlefsen et al., 2008). However, *Cetobacterium somerae* remained present in lower proportions, indicating some resilience under CPFX pressure. While PG exposure led to an increase in pathogenic species with higher microbial diversity and richness. Whereas the CPFX-treated group experienced greater dysbiosis, with a marked decline in both diversity and richness. This highlights the differential influence of β-lactam and fluoroquinolone antibiotics on gut microbiota. Studies also indicated that the use of both β-lactam and fluoroquinolone antibiotics can cause the proliferation of drug-resistant bacteria and disrupt gut microbiota balance (Gu et al., 2020). Such imbalances in the gut microbiota may also affect the gut-brain axis, thereby influencing neurological outcomes such as seizure susceptibility (Zou et al., 2024). Notably, the EE group displayed a higher abundance of *Bacillota*, whereas other phyla were present in smaller proportions. Studies support the idea that environmental enrichment facilitates the abundance of beneficial bacteria like *Bacillota*, which can outcompete pathogenic species (Karvonen et al., 2021) and contribute to improved immune responses (Wedekind et al., 2010). According to Sharpton et al. (2021), the presence of particular clades within *Bacillota* might be essential for maintaining fish gut health and overall well-being. Though enrichment stabilises beneficial phyla, zebrafish have a core microbiome that is highly sensitive to environmental changes (Sharpton et al., 2021).

Brevibacillus, Paenibacillus, and *Bacillus* were the predominant genera supported by EE, with the major species being *Brevibacillus choshinensis* and *Paenibacillus polymyxa*. Strains of *Brevibacillus* have probiotic properties that may help prevent intestinal inflammation by enhancing the gut barrier and regulating immune responses (Mengheri, 2008). The ability of *Brevibacillus choshinensis* to produce

secretory proteins also enhances its probiotic efficacy and making it a potential candidate for therapeutic interventions in intestinal health (Mizukami et al., 2010). Meanwhile, *P. polymyxa* is known to produce bioactive compounds that promote gut health (Grady et al., 2016). The lower microbial diversity and richness observed in the EE group suggest that environmental enrichment with natural materials can favour a limited bacterial phylum while providing beneficial effects.

The gut microbiota communicates with the brain via the vagus nerve and modulate neurotransmitter production and immune responses, thereby regulating mood and behaviour (Grzelak, 2024; Fang et al., 2025; Doenyas et al., 2025). Beneficial bacteria in control zebrafish likely maintain a healthy gut environment. They produce secondary metabolites (SCFAs) that mediate behavioural profiles (Vitetta et al., 2023). In contrast, chronic exposure to antibiotics disrupts the gut microbiome by promoting pathogenic species such as *Pseudomonas* or by depleting beneficial microbes. This leads to dysbiosis, which further affects gut and brain cells, alters neurotransmitter production, and ultimately influences behavioural phenotypes (Hayer et al., 2023; Thabet et al., 2024; Murugan, 2024). Conversely, prolonged environmental enrichment with natural plants and stones supports beneficial bacteria, protecting gut and brain tissues and enhancing overall well-being, including behaviour (Manzo et al., 2024; Sobko et al., 2020).

In conclusion, the environment we inhabit profoundly influences physiology and behaviour. This study investigated whether zebrafish inhabiting contrasting environments, antibiotic-treated versus naturally enriched, experience alterations in gut microbiota, intestinal morphology, and behaviour. Changes in the microbiome can manifest externally through behavioural shifts via the gut-brain-behaviour axis. Control zebrafish maintained a healthy gut and brain, exhibiting stable behavioural

patterns. However, elevated pathogen counts and dysbiosis in antibiotic-exposed fish led to gut and brain abnormalities, hyperactivity, and aggression. Compared to the PG group, zebrafish in the CPFX group displayed increased activity and reduced anxiety in a novel environment. However, they exhibited heightened anxiety in the light-dark test, increased aggression in the mirror-biting test, and no significant difference in social preference. Meanwhile, environmental enrichment with natural plants and stones promoted beneficial microbiota. Despite lower microbial diversity, enriched fish harboured a healthier microbiome, which protected intestinal integrity. However, prolonged exposure to a natural-like shaded environment may have induced mild neophobic tendencies and heightened light-induced anxiety in EE-exposed zebrafish. Importantly, these fish did not exhibit aggression but showed variations in social preference. This study emphasizes the role of the environment with antibiotic pollution and natural enrichment in behaviour and gut microbiome dynamics, which in turn modulates the gut-brain-behaviour axis. Further molecular and biochemical investigations regarding secondary metabolites and neurotransmitters are essential to elucidate the mechanisms by which gut microbes influence behaviour.

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CHAPTER 4

Influence of chronic photoperiod modulation on behaviour, gut-microbiota, and brain transcriptomics in zebrafish.

Rationale: In the previous chapters, we verified the influence of dietary and environmental modifications on behaviour and gut microbiota. In this chapter, we concentrate on photoperiod the primary zeitgeber regulating the circadian rhythm of an organism. Examining the effects of altered photoperiods on baseline behavioural patterns, gut microbiota composition, and brain transcriptomic profiles could offer deeper insights into how the sleep-wake cycle influence the gut-brain-behaviour axis.

4.1. Graphical Abstract

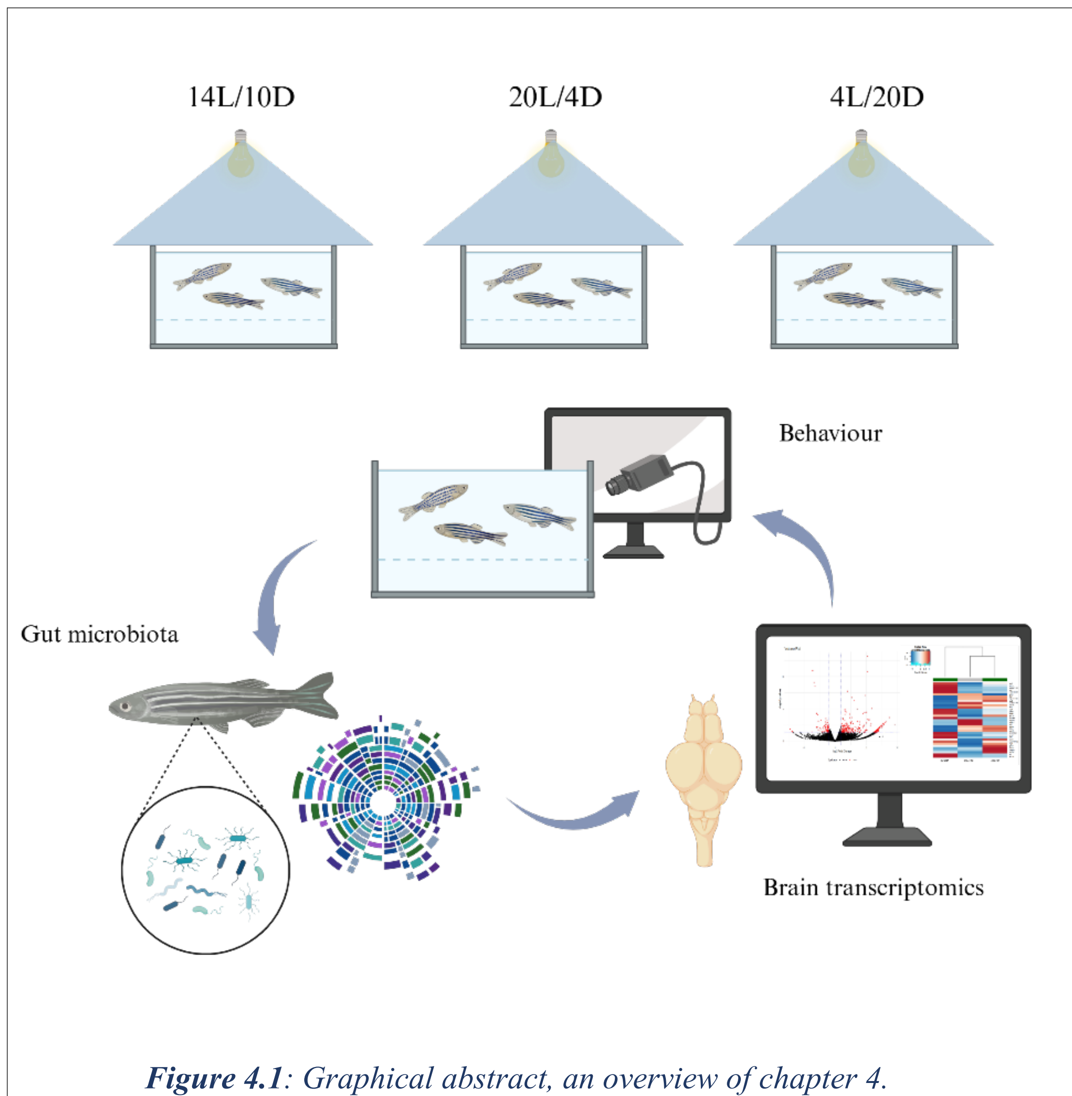


***Figure 4.1**: Graphical abstract, an overview of chapter 4.*

4.2. Introduction

Our biological clock follows 24-hour light-dark cycles and we are internalised that pattern as circadian rhythm. This circadian rhythm helps us to coordinate our biological and behavioural process with changing external environmental conditions (Dominoni et al., 2016; Navara & Nelson, 2007). We are connecting this internal clock to the external environment with the help of light stimulus, considered as the primary zeitgeber. This coordination is essential for keeping optimal physiology and homeostasis in animals (Lee & Kim, 2019; Touitou, 2017). Any deviations in this light-dark cycle results circadian disturbances, consequently poses increased health risks associated with sleep disorders, metabolic syndrome, and mood disorders (Figueiro, 2017; Adhikary et al., 2017; Maury et al., 2010; Vadnie & McClung, 2017).

Research suggests that late meals, shift work, and inconsistent sleep duration are responsible for increased risks of obesity, diabetes, and cardiovascular disease (Ansu Baidoo & Knutson, 2023; Peters et al., 2024). Circadian rhythm influences the working of enzymes, hormones, and transport systems in our body, thereby regulate energy homeostasis, metabolic activities, and behaviour in different species (Meiliana et al., 2015; Wafer Ameen et al., 2022; Depoy et al., 2024; Cloutier et al., 2022). Circadian disruption, especially during early life stages can cause long-lasting issues in the brain development and behavioural formation (Wafer Ameen et al., 2022). Any changes in brain can affect behaviour and the repetitive behavioural pattern in turn shape neural connections in the brain, stating the bidirectional relationship between brain and behaviour (Reite, 1987; Kelly & Garavan, 2005; Taylor, 1982). For example, an acute change in light-dark cycles in mice affected their locomotory activities, clock genes dysregulation, and changes in the expression

levels of neuroplasticity, motivation, and stress response genes (Berbegal-Saez et al., 2024). Disruptions in light-dark cycles also affect neural structures involving decreased dendritic length and complexity in prefrontal cortex neurons, which are important for cognitive control and emotional regulation (Karatsoreos et al., 2011). It also causes changes in brain regions responsible for mood regulation through direct neural circuits from intrinsically photosensitive retinal ganglion cells to the suprachiasmatic nucleus and other mood structures (Bedrosian & Nelson, 2017).

Research indicates that gut microbiota can be influenced by photobiomodulation through various light wave bands (Chen et al., 2024). Furthermore, the extended daylight has been shown to impact the diversity and functionality of gut microbiota across multiple species (Arreaza-Gil et al., 2022; Kissmann et al., 2023; Zhu et al., 2022; Shor et al., 2022; Zhang et al., 2023; Ma et al., 2024). In mice and Chinese mitten crab, light-dark cycle disruption has been found to alter the intestinal clock and gut microbiota (Ruple et al., 2025; Deaver et al., 2018; Zhang et al., 2022). Additionally, in Sprague Dawley rats, the prolonged light exposure increased sympathetic excitation and altered gut microbiota (Zhao et al., 2024).

Zebrafish is considered highly relevant model in translational neuroscience. Studies suggest that zebrafish larvae show variations in neuronal activity patterns during diurnal and nocturnal periods (de Vito et al., 2023). The suprachiasmatic nucleus (SCN) is a cluster of neurons situated in the ventral hypothalamus that serves as a crucial circadian rhythm regulator in mammals (Mistlberger, 2005). In zebrafish, the majority of the internal clock is regulated by the pineal system, a photosensory organ, and the retina, which contain circadian oscillators that control melatonin synthesis (Adatto et al., 2016). Dysregulation of locomotor activity patterns was observed in a genetically modified zebrafish strain expressing elevated levels of

agouti signalling protein 1, an antagonist of the melanocortin system, suggesting that locomotor activity is a significant output of the circadian rhythm (Godino-Gimeno et al., 2024). The diurnal rhythms of spawning, feeding, locomotor activity, shoaling, light-dark preference, and vertical position choice are considered circadian behaviours in zebrafish (Krylov et al., 2021; Dias et al., 2014; Nakagawa et al., 2022). However, in zebrafish, there is a paucity of studies addressing photoperiod and gut microbiota, including research documenting the effects of darkness on the microbial community in zebrafish (Feng et al., 2021; Basili et al., 2020). Therefore, this study aimed to evaluate how zebrafish behaviour, gut microbiome, and brain transcriptomics adapt to chronic photoperiod change, particularly extended light period (20L/4D) and extended dark period (4L/20D), in comparison to the control photoperiod (14L/10D).

4.3. Materials and methods

4.3.1. Zebrafish maintenance under different photoperiods

In this study, 400 AB strain zebrafish larvae were obtained from a certified aquaculture center (KL/04/OH/264/2021) located in Alappuzha, Kerala, India. The larvae were exposed to three distinct light-dark cycles: 14 hours of light and 10 hours of darkness (14L/10D), 20 hours of light and 4 hours of darkness (20L/4D), and 4 hours of light and 20 hours of darkness (4L/20D). The larvae were housed in food-grade high-density polyethylene tanks with 600 mm x 400 mm x 325 mm L x W x H, with a capacity of 64 liters. We provided an elevated roofing for these tanks with LED lighting, which can be controlled by a digital programmable timer to automatically switch on and off according to the photoperiod. 50 zebrafish larvae were introduced in each condition and were fed twice daily, *Artemia* in the morning and Tiqld nursery pellet feed in the evening. The zebrafish were maintained in these

conditions for a period of four months, with the temperature maintained at approximately 27±1°C and pH levels between 6.5 and 7. All experimental protocols followed the ARRIVE guidelines (Kilkenny et al., 2010) and were approved by the Committee for Control and Supervision of Experiments on Animals (CCSEA) under the 2021 regulations (Approval No. 426/GO/Re/S/01/CCSEA), conforming to the ethical standards established by the University of Calicut.

4.3.2 Behavioural Assays

Following exposure to different light-dark cycles for 4 months, the zebrafish were subjected to a series of behavioural assays. At the time of experiments, the fish were taken from their respective tanks and kept it in a holding tank. After experimental time they were returned to their original conditions. The experiments were conducted at a constant room temperature of 28°C. To ensure uniform testing conditions, all trials were performed between 10:00 AM and 4:30 PM in an enclosed room (Cachat et al., 2011). A Logitech 720p C270 webcam was used to record the zebrafish behaviour. The camera-position was adjusted according to the specific requirement of each behavioural test. To reduce external disturbances during the experiments, the setup was enclosed with a black curtain. behavioural data analysis was performed using Smart 3.0 video tracking software (Panlab-Harvard Apparatus, USA).

4.3.3. Novel Tank Test (NTT)

Zebrafish from the Control group (14L/10D) and the experimental groups (20L/4D and 4L/20D) were subjected to a novel tank diving test to examine their exploratory behaviour and anxiety-like responses (Fontana et al., 2022). To minimize handling stress, each fish was given a 5-minute acclimation period in an opaque beaker before the test commenced. Following this, the fish were gently introduced into an

experimental tank with dimensions of 27.9 cm × 7 cm × 15 cm and a bottom length of 22 cm, containing 1.35 L of system water. Their behaviour was observed and recorded for 6 minutes. Prior to video analysis with software, the tank was virtually divided into top and bottom zones to facilitate monitoring of activities within these areas.

4.3.4. Light-Dark Test (LDT)

The anxiety induced by change in photoperiod was evaluated using the light/dark preference test, which examine zebrafish's inclination towards either dark or light environments. An acrylic tank (45 cm long, 10 cm wide, 15 cm high) was divided equally into black and white sections. The tank contained 4.5 L of water, filling it to a depth of 10 cm. A central area (15 cm × 10 cm × 10 cm) was created using color-matched sliding doors for initial fish acclimation. Each zebrafish was first introduced in the middle chamber using a fish net and they were given 3-minutes for acclimatisation. After acclimatisation, the sliding door was slowly detached from the tank, permitting the fish to freely explore the light and dark compartments of the tank for 10 minutes. The behaviour was recorded with top-mounted camera. Two individuals, unaware of the experimental conditions, analysed the recorded footage to reduce bias. The inter-rater reliability score exceeded 0.90 (Maximino et al., 2018).

4.3.5. Mirror Biting Test (MBT)

The experiment used a three-compartment tank measuring 15 × 60 × 20 cm (W × L × H), filled with 4 L of water. Each fish was introduced in the middle or focal chamber and recorded for 6 minutes. After initial 6-minutes, a mirror was positioned on either the left or right wall facing the central chamber, alternating to reduce bias. The fish was then allowed to interact with its reflection for another 6 minutes. The

protocol described by Midttun et al. (2020). The central chamber was conceptually split into two areas: the mirror zone (1/3 of the chamber, also called the approach zone) and the non-mirror zone (remaining 2/3, also known as the avoidance zone). Smart 3.0 software was used to analyze behavioural recordings, while two independent observers, unaware of the experimental conditions, manually counted mirror bites (inter-rater reliability > 0.90) to ensure data collection accuracy and consistency.

4.3.6. Social Preference Task (SPT)

The three-chambered tank used for MBT was adopted for SPT. The first chamber was kept empty, the middle chamber served as the experimental area, and the third chamber contained the conspecific stimuli. Six individuals of the conspecific group were alternately placed in the left and right chambers, with their positions balanced between tests. Initially, two black panels were positioned between the middle chamber to block the focal fish's view of the empty and conspecific chambers. A single zebrafish was introduced into the middle chamber and given 3 minutes to acclimate. The panels were then gently removed, allowing the fish to swim freely for 6 minutes and 30 seconds while being recorded by a webcam placed in front of the tank. The first 30 seconds were designated as the latency period. The experimental chamber was virtually split into two unequal zones: the Social Zone (SZ), comprising the 1/3 portion adjacent to the conspecific chamber where the fish was expected to prefer visual interaction with conspecifics, and the Non-Social Zone (NSZ), consisting of the 2/3 portion facing the empty chamber. The analyzed parameters included the number of entries to SZ, cumulative time in SZ, distance traveled in SZ, time immobile in SZ, mean speed in SZ, parallel orientation, and turning tendency in SZ (Angiulli et al., 2020).

4.3.7. Statistical Analyses

To examine data distribution and group disparities, various statistical methods were employed. The outliers were removed based on ROUT method. Data normality was evaluated using the Kolmogorov-Smirnov test in conjunction with the Dallal-Wilkinson-Lilliefors p-value test, while variance homogeneity was assessed through Bartlett's test. For data exhibiting normal distribution, One-Way ANOVA was utilised to compare group means, followed by Tukey's post hoc test for multiple comparison adjustments. Findings were presented with 95% confidence intervals. In cases of non-normally distributed data, the Kruskal-Wallis test was applied, succeeded by Dunn's multiple comparison tests to identify inter-group differences. Intra-group changes were measured using Wilcoxon-matched pairs signed rank test. Spearman's rank correlation coefficient was employed to analyse relationships between variables. GraphPad Prism (version 9.5), Python (version 3.10), and Microsoft Excel were used for all statistical analyses and data visualization. Throughout the study, a significance level of $p < 0.05$ was maintained.

4.3.8. Metagenomic Analyses

The gut tissues were dissected out from zebrafish, six samples from each condition and the DNA were extracted using the ORIonX Genomic DNA kit (Origin Lab, India). The extracted DNA was stored in -20-degree Celsius for further analyses. Using a Nanodrop spectrophotometer (Nanodrop Technologies, USA), the DNA concentration in the filtrate was measured and verified to exceed 30 ng/µL. The V4 region of the 16S rRNA gene was targeted for PCR amplification, and the resulting products were examined on a 2% agarose gel to verify successful amplification. Following the Illumina HiSeq protocol for amplicon preparation, the PCR products underwent purification. For the library construction, NEB Next Ultra DNA Library

Preparation Kit (New England Biolabs) was used. The Agilent 2200 Tape Station system was used to analyse the quality and quantity of the constructed libraries. The sequencing was performed on the Illumina HiSeq 2500 platform to generate high-quality reads for subsequent metagenomic analysis, in accordance with the manufacturer's guidelines (Sieler et al.,2023; Pothay et al.,2024).

4.3.9. Bioinformatics

Quality assessment of paired-end sequences generated from Illumina HiSeq sequencing was performed using FastQC-v0.11.9 (Andrews, 2010). Adapter removal and sequence trimming were conducted with FastP-v0.20.1 (Chen, 2023). Processed sequences were then analysed and taxonomically classified using Kraken 2, a K-mer-based sequence classification tool that utilises a pre-constructed PlusPFP database of k-mers derived from known genomes for rapid categorisation of input sequences (Lu & Salzberg, 2020). To refine and quantify taxonomic outputs, Bracken was employed to compute both absolute and relative abundance values based on Kraken 2 results (Lu et al., 2017). Alpha diversity metrics, including Observed Sequence Variants (OSVs), Chao1, ACE, Shannon, Simpson, and Fisher's diversity indices were calculated to evaluate within-group diversity. Beta diversity between groups was determined using the Bray-Curtis dissimilarity index. Visualisation of diversity metrics was achieved using specific R-packages to generate Principal Coordinates Analysis (PCoA) plots (vegan), alpha diversity plots (phyloseq, ggplot 2, dplyr, ggpubr), beta diversity plot (pheatmap, RColor Brewer, dplyr, ggplot2), and sankey plots (pavian). Bar plots depicting taxonomic compositions were created using Python.

4.3.10. Transcriptomics

Zebrafish brain tissues were dissected and washed in PBS, about 40mg brain tissue (10-12 fish) from each group were collected and transferred in to a falcon tube and snap freezed the tissue using liquid nitrogen and transferred it into dry ice. The cells were collected by centrifugation (15 min, 4000g, 4˚C) and resuspended in TE buffer (100 mM Tris-HCl [pH 8], 100 mM EDTA [pH 8]). Total RNA extraction was performed using the TRIzol/chloroform method (Takara). RNA quality check was performed using Nanodrop (ThermoFisher) and RNA quantity was assessed using Qubit 4.0 (ThermoFisher). The RNA integrity was analysed using RNA Screentape in Agilent's 2100 Tapestation.

Transcriptome Library Construction: rRNA depletion using QIAseq FastSelect-5S/16S/23S Kit (Qiagen). Depleted RNA was fragmented into small pieces. First strand cDNA was synthesised using random primers and reverse transcription, second strand cDNA synthesis was performed and cDNA fragments were end-repaired and 3' adenylated. Adapters were ligated to the ends of the cDNA fragments. PCR amplification was performed using adapter-ligated cDNA. PCR products were purified using Ampure beads and dissloved in EB (RB solution). Library quality was analysed using Agilent 2100 Bioanalyzer. Library quantification performed using Qubit. Libraries were sequenced on the Nextseq 2000 sequencing system (Illumina). Paired-end (PE) sequencing with 150 nt read length was performed.

The sequence quality was assessed using Fast QC software and the pre-processing of Fastq sequence was done using Fastp. Genome alignment was based on *Danio rerio* reference genome obtained from NCBI. The alignment was performed using Hisat2 tool. Following alignment, the reads were subjected to feature counts for transcript

quantification. The raw counts were then normalised for library size and composition using DEseq2 package (Version 1.40.2). The normalised expression values were subsequently used for differential gene expression analysis.

The genes that were detected to be expressed above a cutoff in either of the condition, statistical testing for differential expression was carried out on a gene-by-gene basis. Genes were considered differentially expressed based on a combination of fold change ($\log_2(FC) > 1$ (upregulated) and < -1 (downregulated)) and adjusted p-value thresholds ($padj < 0.05$). Functional annotation and pathway enrichment analysis were performed using KOBAS (KEGG Orthology Based Annotation System) (Xie et al., 2011). Multiple testing correction was applied using the Benjamini–Hochberg procedure to control the false discovery rate (Benjamini and Hochberg, 1995).

4.4. Results

4.4.1. 4L/20D group showed reduction in novel-induced anxiety while 20L/4D exhibit mild stress-induced behavioural abnormalities.

The change in photoperiod influenced anxiety and locomotor behaviours in zebrafish. The result indicated that variation in number of entries to top, a post hoc analysis confirmed that 4L/20D group (39.57 ± 2.937) had greater tendency to make entries into the top zone compared to 20L/4D group (24.61 ± 2.293, $p = 0.0003$). Although no statistically significant difference was found in test groups ($p > 0.05$) with that of control 14L/10D (32.04 ± 2.568), the mean entries was lower in 20L/4D and higher in 4L/20D with that of control photoperiod (Fig. 4.2A). The variation was also apparent in latency to enter top, post hoc analysis confirmed that higher latency in 20L/4D (27.87 ± 5.905) compared to 4L/20D (11.90 ± 3.611, $p = 0.0188$). No differences in test groups with that of 14L/10D (13.92 ± 3.532) ($p > 0.05$) (Fig.

4.2B). The 4L/20D group exhibit increased time in top (154.0 ± 13.82) (Fig. 4.2C) and distance in top (943.8 ± 85.55) (Fig. 4.2E) compared to 14L/10D (time in top: 95.19 ± 8.959, $p = 0.0166$; distance in top: 644.6 ± 51.64, $p = 0.0142$) and 20L/4D (time in top: 82.61 ± 11.34, $p = 0.0006$; distance in top: 660.9 ± 79.17, $p = 0.0216$). There were no apparent variations among control and test groups in total distance travelled and mean speed in the novel environment ($p > 0.05$) (Fig. 4.2D and 4.2F). However, the resting time was varied among groups. The post hoc analysis revealed that 20L/4D group had greater resting or immobility time (7.004 ± 2.256) in a novel environment compared to 14L/10D (0.6564 ± 0.1093, $p < 0.0001$) and 4L/20D (0.2968 ± 0.0548, $p < 0.0001$). When compared to control, 4L/20D showed even lower resting time ($p = 0.0489$) (Fig. 4.2G). Variation was also found in slow swimming time; a post hoc analysis confirmed that lower slow swimming time in 20L/4D (350.1 ± 1941) compared to 14L/10D (355.7 ± 0.5702, $p = 0.0064$) and 4L/20D (357.3 ± 0.3230, $p < 0.0001$). No variation found between 14L/10D and 4L/20D ($p > 0.05$) (Fig. 4.2H). Besides, fast time or fast swimming time was also higher in 20L/4D (4.857 ± 0.9166) compared to 14L/10D (2.330 ± 0.3642, $p = 0.0196$). No statistically significant difference in 4L/20D (2.970 ± 0.3677) with that of 14L/10D and 20L/4D ($p > 0.05$) (Fig. 4.2I).

Based on the behavioural parameters in novel tank test the stress level index was calculated, revealed lower stress level index in 4L/20D (0.1447 ± 0.0767) compared to 20L/4D (0.5410 ± 0.0630, $p = 0.0007$) and 14L/10D (0.4894 ± 0.05137, $p = 0.0089$). There was a slight increase of stress level in 20L/4D with that of control, the variation was not statistically significant ($p > 0.05$) (Fig. 4.3A). Whereas, no statistically significant differences were found between 14L/10D (0.5599 ± 0.0626) and 20L/4D (0.6876 ± 0.1109), 4L/20D (1.099 ± 0.1707) groups in top-bottom

distance ratio ($p > 0.05$), still 4L/20D group had a tendency to exhibit increased top-bottom distance ratio (Fig. 4.3B). The general relationship between novel tank behavioural parameters such as entries to top, time in top, distance in top, resting time, and mean speed was depicted in a pair plot (Fig. 4.3C) and the relationships between parameters in control and test groups were statistically evaluated using the Spearman correlation method. In 14L/10D condition, the result revealed that there was a positive correlation between time in top vs. entries to top ($r = 0.84$), distance in top vs. entries to top ($r = 0.84$) and distance in top vs. time in top ($r = 0.86$). The resting time formed a negative correlation with entries to top ($r = -0.23$), time in top ($r = -0.06$), distance in top ($r = -0.18$) and mean speed ($r = -0.88$). The mean speed and time in top were also negatively correlated ($r = -0.12$), while the mean speed with entries to top and distance in top showed nearly zero correlation.

Regarding 20L/4D, a positive correlation was found among time in top vs. entries to top ($r = 0.86$), distance in top vs. entries to top ($r = 0.91$), time in top vs. distance in top (0.96), mean speed with entries to top ($r = 0.78$), time in top ($r = 0.67$), and distance in top ($r = 0.80$). Whereas, resting time had a negative correlation with entries to top ($r = -0.71$), time in top ($r = -0.49$), distance in top ($r = -0.55$), and mean speed ($r = -0.75$). A similar trend was observed in 4L/20D, entries to the top were positively correlated with time in the top ($r = 0.67$) and distance in the top ($r = 0.76$). And time in top was highly correlated with distance in top ($r = 0.96$). Mean speed also positively correlated with entries to top ($r = 0.71$), time in top ($r = 0.54$), and distance in top ($r = 0.72$). In case of resting time, it was negatively correlated with entries to top ($r = -0.58$), time in top ($r = -0.36$), distance in top ($r = -0.44$), and mean speed ($r = -0.58$) (Fig. 4.3D).

4.4.2. The light-induced anxiety was greatly reduced in 4L/20D.

Light-dark Test confirmed anxiety levels in zebrafish exposed to different photoperiods. Significant variation observed in the number of entries to the light zone. Post hoc test revealed that 4L/20D group (58.48 ± 3.164) made increased entries to light zone compared to 14L/10D (34.76 ± 3.074, $p = 0.0001$) and 20L/4D (29.84 ± 3.711, $p < 0.0001$). No differences were found between 14L/10D and 20L/4D ($p > 0.05$) (Fig. 4.4A). The latency towards the light zone was also altered in response to differences in photoperiod. Post hoc analysis declared that 4L/20D group (5.520 ± 2.172) exhibit lower latency compared to 14L/10D (90.24 ± 16.85, $p < 0.0001$) and 20L/4D (55.68 ± 12.14, $p = 0.0002$). No significant difference between 20L/4D and 14L/10D (Fig. 4.4B). Time in light zone which marked the anxiety level was significantly varied among groups, the post hoc analysis underlined that 4L/20D group (145.0 ± 14.44) exhibit reduced anxiety as their time in light zone was substantially increased compared to 14L/10D (42.79 ± 5.757, $p < 0.0001$) and 20L/4D (38.94 ± 8.849, $p < 0.0001$). The time in light zone was not significantly varied between 20L/4D and 14L/10D ($p > 0.05$) (Fig. 4.4C).

Therefore, the anxiety index from LDT was profoundly reduced in 4L/20D group (0.5168 ± 0.0481) compared to 14L/10D (0.8574 ± 0.0191, $p < 0.0001$) and 20L/4D (0.8702 ± 0.0295, $p < 0.0001$). The anxiety index was parallel in 14L/10D and 20L/4D ($p > 0.05$) (Fig. 4.5A). And there was a strong negative correlation ($r = -1$) of anxiety index vs time in the light zone (Fig. 4.5B).

4.4.3. The mirror-induced aggression was exacerbated in 20L/4D.

Although the number of entries (Fig. 4.6A), latency (Fig. 4.6B), time spent (Fig. 4.6C), and distance travelled (Fig. 4.6D) in mirror zone (MZ) were not showed any deviation between groups ($p > 0.05$), the mean speed (Fig. 4.6E) and number of

mirror bites (Fig. 4.6F) were altered in 20L/4D (mean speed: M = 3.690, IQR = 3.325 - 4.310; mirror bites: M = 212, IQR = 188 - 302) compared to 14L/10D (mean speed: M = 3.060, IQR = 2.460 - 3.745, $p = 0.0009$; mirror bites: M = 152, IQR = 103 - 233, $p = 0.0026$) and 4L/20D (mean speed: M = 3.120, IQR = 2.675 - 3.475, $p = 0.0012$; mirror bites: M = 158, IQR = 114 - 209, $p = 0.0028$). And the resting time was lower in 20L/4D (M = 3.050, IQR = 1.645 - 5.855) compared to 14L/10D (M = 7.220, IQR = 2.720 - 13.33, $P = 0.0168$). No statistically significant difference between 14L/10D vs. 4L/20D (M = 4.770, IQR = 2.500 - 9.115) and 20L/4D vs. 4L/20D ($P > 0.05$) (Fig. 4.6G). Whereas, the slow time was higher in 4L/20D (M = 272.5, IQR = 241.5 - 322.8) compared with 20L/4D (M = 235.2, IQR = 170.9 - 264.8, $P = 0.0137$). No difference between 14L/10D (M = 251.9, IQR = 221.6 - 322) and test groups ($p > 0.05$) (Fig. 4.6H). Fast time was considerably increased in 20L/4D (M = 1.680, IQR = 0.890 - 3.595) compared to 14L/10D (M = 0.600, IQR = 0.1509 - 1.055, $P = 0.0004$) and 4L/20D (M = 0.370, IQR = 0 - 0.720, $P < 0.0001$). No difference between 14L10D and 4L/20D ($p > 0.05$) (Fig. 4.6I).

The rate of aggression was seemed to be greater in 20L/4D (0.6486 ± 0.0393) compared to 14L/10D (0.4551 ± 0.0505, $p = 0.0037$) and 4L/20D (0.4562 ± 0.0291, $p = 0.0037$). No difference found between 14L/10D and 4L/20D ($p > 0.05$) (Fig. 4.7A). With in groups the parallel orientation of zebrafish was reduced in MZ compared to NMZ ($p < 0.0001$) (Fig. 4.7B). When parallel index between groups were considered, statistically significant variations were not found among 14L/10D (0.3776 ± 0.0300), 20L/4D (0.2884 ± 0.0383), and 4L/20D (0.3632 ± 0.0269). Still, the parallel index was appeared as lower in 20L/4D and higher in 4L/20D (Fig. 4.7C). The 14L/10D (0.0092 ± 0.0166) and 20L/4D (0.0040 ± 0.0091) groups

exhibited slightly rightward turning tendencies in MZ while 4L/20D (-0.0028 ± 0.0116) group showed a little leftward turning tendency (Fig. 4.7D).

4.4.4. Social preference was differently expressed in 20L/4D and 4L/20D.

The social preference behaviour was influenced by a change in photoperiod. The number of entries towards SZ was affected, post hoc analysis stated that 20L/4D group (M = 4, IQR = 0 - 17.25) exhibited reduction in number of entries towards SZ compared to 14L/10D (M = 18, IQR = 9.75-31, p = 0.0289) and 4L/20D (M = 30, IQR = 14.5 - 39.25, p = 0.0001). Even though 4L/20D group showed an increased entries but not statistically significant compared to 14L/10D (p > 0.05) (Fig. 4.8A). Considering time spent in SZ, post hoc analysis revealed no statistical variation between control 14L10D (M = 334.6, IQR = 308.8 - 355.5) and test groups, 20L/4D (M = 356.3, IQR = 325.3 - 360.0, p > 0.05), 4L/20D (M = 297.6, IQR = 253.9 - 343.6, p > 0.05). However, between test groups, the 4L/10D group exhibited lower time in SZ to that of 20L/4D (p = 0.0030) (Fig. 4.8B). Assessing distance traveled, post hoc analysis confirmed that 4L/20D group (M = 982.3, IQR = 893.7 - 1239) displayed lower distance in SZ compared to 14L/10D (M = 1199, IQR = 1053 - 1358, p = 0.0035). However no significant variation between 14L/10D and 20L/4D (M = 1109, IQR = 1006 - 1214), 20L/4D and 4L/20D (p > 0.05) (Fig. 4.8C). The mean speed (Fig. 4.8D), slow (Fig. 4.8F) and fast swimming time (Fig. 4.8G) were not altered between groups (p > 0.05) while the resting time was found to be higher in 20L/4D (M = 5.050, IQR = 3.305 - 9.025) compared to 14L/10D (M = 2.100, IQR = 1.063 - 4.560, p = 0.0021). No difference was found among 14L/10D and 4L/20D (M = 3.275, IQR = 2.003 - 5.680) (p > 0.05) (Fig. 4.8E).

The parallel orientation was reduced in SZ compared to NSZ across all the groups (p < 0.0001) (Fig. 4.9A) whereas, the parallel orientations were not much varied

between control 14L/10D and test groups 20L/4D and 4L/20D ($p > 0.05$) (Fig. 4.9B). The turn angles were shifted towards right direction in 14L/10D (0.0088 ± 0.008) and 4L/20D (0.0357 ± 0.0082) whereas, slightly leftward shift in 20L/4D (-0.0073 ± 0.0101). The variation was significant between 20L/4D and 4L/20D ($p = 0.0094$) (Fig. 4.9C).

4.4.5. Gut microbiota dynamics in response to photoperiod changes

The photoperiod or light-dark cycles contributing to the circadian rhythm had a profound influence on gut bacterial populations. The result demonstrated that there were 29 OTUs in the control condition 14L/10D, 33 OTUs in the extended light 20L/4D, and 28 OTUs in the extended dark group 4L/20D at the phylum level. Among them, the top six phyla were considered for analysis. In the 14L/10D group, Pseudomonadota (47.77%), Actinomycetota (21.60%), and Fusobacteriota (18.82%) were identified as the major phyla in this control group. Bacillota (5.42%), Planctomycetota (3.55%), and Bacteroidota (1.29%) were the minor phyla.

In the extended light group 20L/4D, phylum Actinomycetota (42.74%) and Pseudomonadota (41.97%) were dominant, followed by Planctomycetota (6.24%), Bacillota (3.66%), Fusobacteriota (2.74%), and Bacteroidota (1.28%) were considered as the minor phyla. Whereas, in the extended dark group 4L/20D, Bacillota (86.38%) was regarded as the dominant phylum followed by Pseudomonadota (8.63%), Actinomycetota (2.55%), Fusobacteriota (0.78%), Planctomycetota (0.28%) and Bacteroidota (0.20%) as subordinate (Fig. 4.10A).

Taking the genus level into consideration, the 14L/10D group consisted of 451 OTUs, in which *Actinobacter* (19.20%), *Cetobacterium* (18.62%), and *Streptomyces* (15.43%) were considered as prominent genera. Whereas, *Burkholderia* (6.04%), *Bosea* (4.00%), and *Aerococcus* (2.03%) were the subsequent genera found in

normal light-dark conditions. While the 20L/4D group contained 515 OTUs, where *Streptomyces* (24.52%) and *Burkholderia* (17.97%) were regarded as key genera and *Paludisphaera* (4.48%), *Bosea* (3.86%), *Corynebacterium* (3.11%), and *Nakamurella* (2.83%) were considered as subordinate genera. However, the 4L/20D group encompassed 369 OTUs in which genus *Bacillus* (68.5%) was the most distinguished genus. *Staphylococcus* (8.68%), *Sodalis* (3.79%), *Priestia* (3.36%), *Streptomyces* (1.22%) and *Salmonella* (1.00%) were the succeeding genera in the extended dark group (Fig. 4.10B). Regarding species, the 14L/10D group comprised 606 OTUs, in which *Cetobacterium somerae* (18.86%), *Streptomyces sp*. (12.99%), *and Acinetobacter baumanii* (9.25%) were considered as major species. Minor species, including *Acinetobacter sp*. (5.45%), *Bosea sp*. (4.05%), and *Burkholderia dolosa* (2.74%). The 20L/4D group with 708 OTUs supported *Streptomyces sp*. (21.16%) and *Burkholderia dolosa* (8.59%) as major species. *Paludisphaera borealis* (4.56%), *Burkholderia cepacia* (4.05%), *Bosea sp*. (3.93%), and *Corynebacterium massiliense* (3.05%) were the following minor species. Whereas the 4L/20D group consisted of 472 OTUs, among them *Bacillus velezensis* (28.52%) and *Bacillus thuringiensis* (22.71%) were found to be dominant. *Bacillus sp*. (8.53%), *Staphylococcus aureus* (7.63%), *Bacillus* cereus (5.20%), and *Sodalis ligni* (3.82%) were the minor group comprising the top six species (Fig. 4.10C).

In the analysis of beta diversity, the Bray-Curtis dissimilarity metrics indicated significant differences in shared species between 14L/10D and 4L/20D (0.91), as well as between 20L/4D and 4L/20D (0.91). In contrast, 14L/10D and 20L/4D exhibited a higher degree of shared species (0.64) (Fig. 4.10D). These dissimilarities were also illustrated in a Principal Coordinates Analysis (PCoA) plot, where the x-axis (PCoA1) accounted for 47.84% of the variation, representing nearly half of the

total variation. The y-axis (PCoA2) explained 25.17% of the variance. The 4L/20D group was located at the bottom left, distinct from the others, indicating greater dissimilarities. Meanwhile, 14L/10D and 20L/4D were positioned in the upper right, reflecting similarities between the control photoperiod and the extended light group (Fig. 4.10E).

In evaluating alpha diversity metrics, the 20L/4D group demonstrated the greatest richness, with observed values at 708, Chao1 at 993.34, and ACE at 936.56, indicating it had the most varied microbial community. The 14L/10D group showed moderate richness, with observed values of 606, Chao1 at 828.51, and ACE at 814.71. In contrast, the 4L/20D group exhibited a less diverse microbial community, with observed values of 472, Chao1 at 596.6, and ACE at 582.87. Both the 14L/10D and 20L/4D groups had the highest Shannon diversity scores (3.71 and 3.86), suggesting their microbiomes were not only rich but also well-balanced. The 4L/20D group had the lowest Shannon diversity score (2.75), indicating a predominance of a few taxa. The Simpson index further confirmed that the 14L/10D and 20L/4D groups (0.92, 0.93) had greater evenness, whereas the 4L/20D group (0.84) was less even. The Fisher index was also higher for the 14L/10D and 20L/4D groups (92.5, 108.4) compared to the 4L/20D group (64.74) (Fig. 4.10F). Although the diversity in the 14L/10D and 20L/4D groups appeared higher, they also contained some pathogenic microbes. The 4L/20D group might be associated with the selection of specific beneficial bacteria. The major taxa, including phylum, family, genus, and species, are displayed in a sankey plot (Fig. 4.10G).

4.4.6. Altered transcriptomic profile in response to varied photoperiod

We examined the transcriptome data of zebrafish brain tissue samples to obtain photoperiod-based gene expression and pathway enrichment patterns in 14L/10D,

20L/4D, and 4L/20D groups. A total of 37.003966 M, 30.622970 M, and 36.976640 M reads were obtained from 14L/10D, 20L/4D, and 4L/20D groups, respectively, with an average read length of 159 bp. After quality trimming and filtering, 97.38%, 96.98%, and 97.79% genes were retained in respective groups. The mapping rate to the *Danio rerio* reference genome obtained from NCBI was 70.2% in 14L/10D, 70.84% in 20L/4D, and 71.11% in 4L/20D. The uniquely mapped read pairs include 58.48% in 14L/10D, 58.32% in 20L/4D, and 57.43% in 4L/20D.

To examine global transcriptional differences across photoperiod conditions, the Principal Component Analysis (PCA) on log-transformed gene expression data was performed. The PC1: 67.19% and PC2: 32.81% explained the total variability in gene expression. PCA analysis revealed a clear difference among 14L/10D, 20L/4D, and 4L/20D conditions, indicating that gene expression patterns differed significantly based on photoperiod. The 14L/10D group clustered independently from both the 20L/4D and 4L/20D groups, suggesting a baseline expression profile. Whereas, 20L/4D and 4L/20D groups were positioned on opposite ends of PC1, indicating opposing transcriptional responses to prolonged light and dark exposure (Fig. 4.11A). There were 105 differentially expressed (DE) genes between 20L/4D compared to control 14L/10D, 64 DE genes between 4L/20D and 14L/10D, and 136 DE genes between 20L/4D and 4L/20D. Based on Z-score normalised expression values, the hierarchical clustering heatmap of upregulated genes was generated. Results revealed that an increased number of upregulated genes in 20L/4D compared to 14L/10D, while a lower number of upregulated genes in 4L/20D, where most of the genes remained closer to baseline. Compared to 14L/10D genes such as *gnat1*, *exorh*, *LOC100537* (*plcb4a*), *saga*, *zgc:112320* (*pde6ga*), *rcvrn3*, *asmt*, *rpl3a*, *knop1*, *eno1a*, *rpl9*, *nr4a1*, *six6a*, *h1fx*, *LOC100332*, *btg2*, *histh1l*, *fance*,

serpina10a, *uimc1*, *vtg1*, *gngt1*, and *socs3a* were seemed upregulated in 20L/4D and 4L/20D. Among them highly upregulated genes in 20L/4D included *gnat1*, *exorh*, *LOC100537* (*plcb4a*), *saga*, *zgc:112320* (*pde6ga*), *rcvrn3*, *asmt*, *rpl3a*, *knop1*, *eno1a*, *rpl9* with that of 14L/10D and 4L/20D. In 4L/20D group genes such as *nr4a1*, *six6a*, *h1fx*, *LOC100332*, *btg2*, *histh1l*, *fance*, *serpina10a*, and *uimc1* were highly upregulated compared to 14L/10D and 20L/4D (Fig. 4.11B).

The genes that highly expressed under normal light condition (14L/10D) become downregulated in both extended light (20L/4D) and extended dark (4L/20D) which includes *ndufb9*, *ndufa1*, *mt2*, *mrps26*, *coq9*, *glipr1b*, and *ba1l* (Fig. 4.11C). The expression pattern of upregulated genes across experimental conditions were identified using coexpression analysis. Gene expression levels varied significantly, with most genes showing the highest expression in 20L/4D (*LOC100537* (*plcb4a*), *saga*, *zgc:112320* (*pde6ga*), *rcvrn3*, *gnat1*). The genes like *nr4a1*, *h1fx*, *six6a*, *btg2*, and *histh1l* were highly expressed in 4L/20D (Fig. 4.11D). The coexpression analysis of down-regulated genes revealed transcriptional suppression in 20L/4D and 4L/20D conditions. Genes such as *coq9*, *mt2*, *ndufb9*, *ndufa1*, and *mrps26* demonstrated a sharp decline in both conditions, while *glipr1b* and *si:dkey-238o13.4* exhibited modest downregulation (Fig. 4.11E).

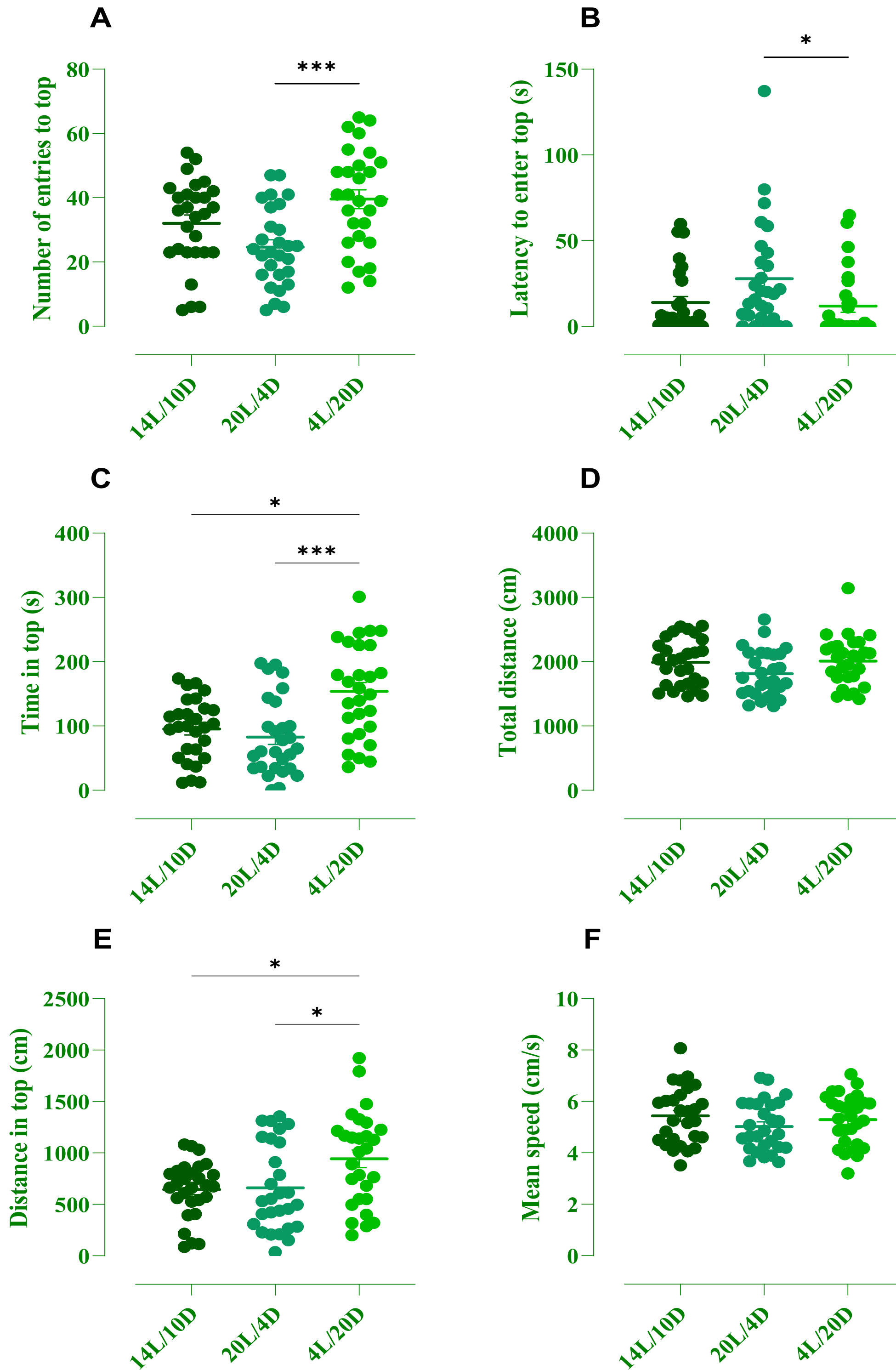
The KEGG pathway enrichment analysis was performed to identify differentially regulated biological pathways. When considering 20L/4D with that of 14L/10D, the most enriched pathways were related to lipid metabolism, with a strong enrichment ratio of fatty acid biosynthesis and fatty acid metabolism. Additionally, amino acid metabolism (valine, leucine, and isoleucine degradation) was enriched. Several immune and signalling pathways, such as apoptosis, toll-like receptor signalling, MAPK signalling, and Salmonella infection, were also highly enriched. Further,

intracellular signalling pathways (inositol phosphate metabolism, phosphatidylinositol signalling system) and cellular degradation processes (lysosome, phagosome) were also enriched. Finally, structural metabolic pathways, including carbohydrate metabolism (pentose and glucuronate interconversions), protein synthesis (ribosome), and vesicular transport mechanisms (SNARE interactions, cell adhesion molecules), were also upregulated (Fig. 4.11F).

Regarding 4L/20D, compared to 14L/10D, showed significant metabolic and physiological responses. Enriched metabolic pathways, such as nitrogen metabolism, glycolysis/gluconeogenesis, and amino acid biosynthesis, implied a metabolic response to the long darkness. Heightened immune activation, as evidenced by Toll-like receptor signalling and Salmonella infection pathways. Cellular homeostasis pathways such as mitophagy, protein export, and phagosome function were also strongly upregulated. Cardiac-related pathways (adrenergic signalling, cardiac muscle contraction) were also enriched. Lastly, homologous recombination, RNA decay, and spliceosome pathways indicate a transcriptional and translational adjustment (Fig. 4.11G).

20L/4D vs. 4L/20D demonstrated robust metabolic and physiological adjustments. Metabolic processes including nitrogen metabolism, glycolysis, and carbon metabolism were prominently enriched. Immune response-related processes such as Toll-like receptor signalling and AGE-RAGE signalling were also increased. This is reflecting an inflammatory reaction, possibly in response to oxidative stress due to prolonged light exposure. Hedgehog and GnRH signalling pathways were enriched, implying modulation of neuroendocrine function, potentially connecting photoperiod change to hormonal balance. Also, protein processing and cell maintenance-related pathways (phagosome, ER protein processing) were

upregulated. Lastly, oxidative stress response pathways, such as glutathione metabolism and p53 signalling, were enriched, implying prolonged light exposure causes oxidative stress and necessitates increased protection mechanisms (Fig. 4.11H).



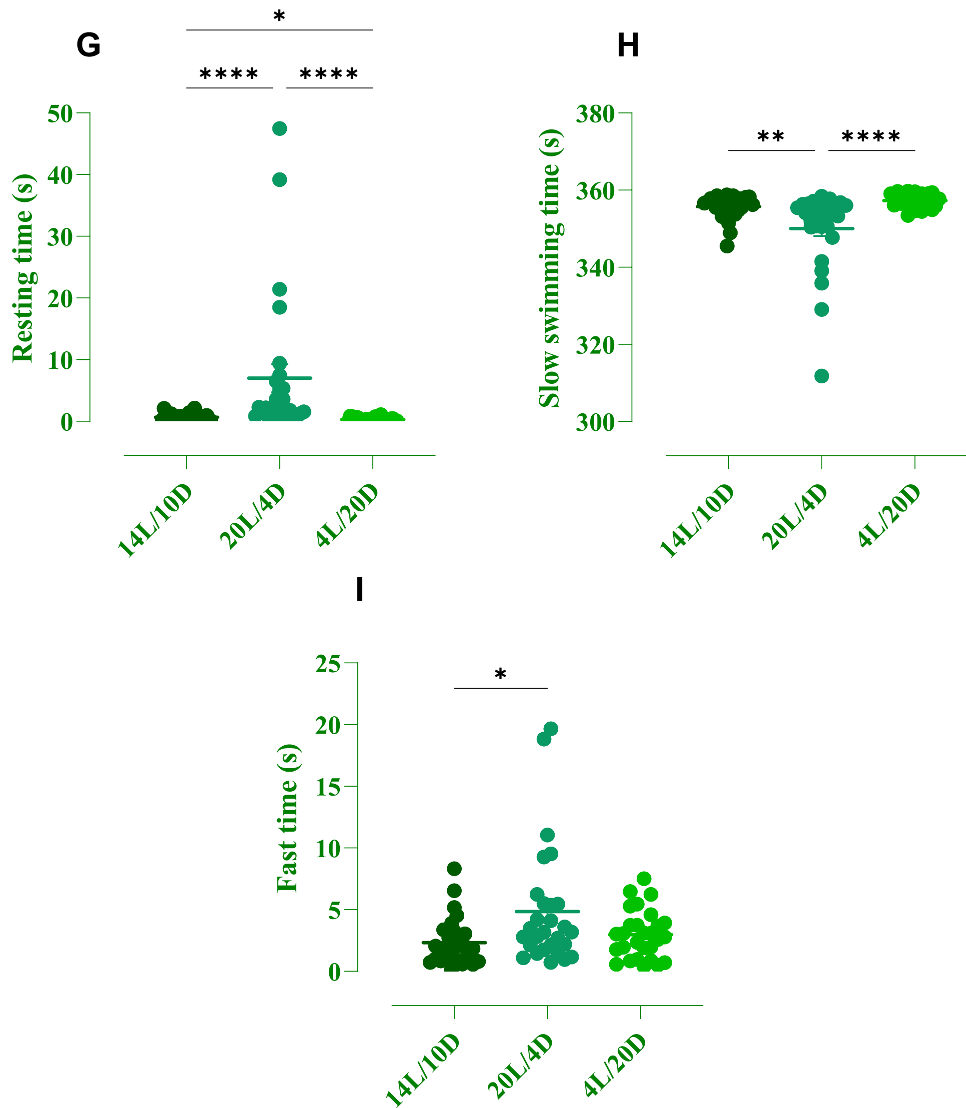
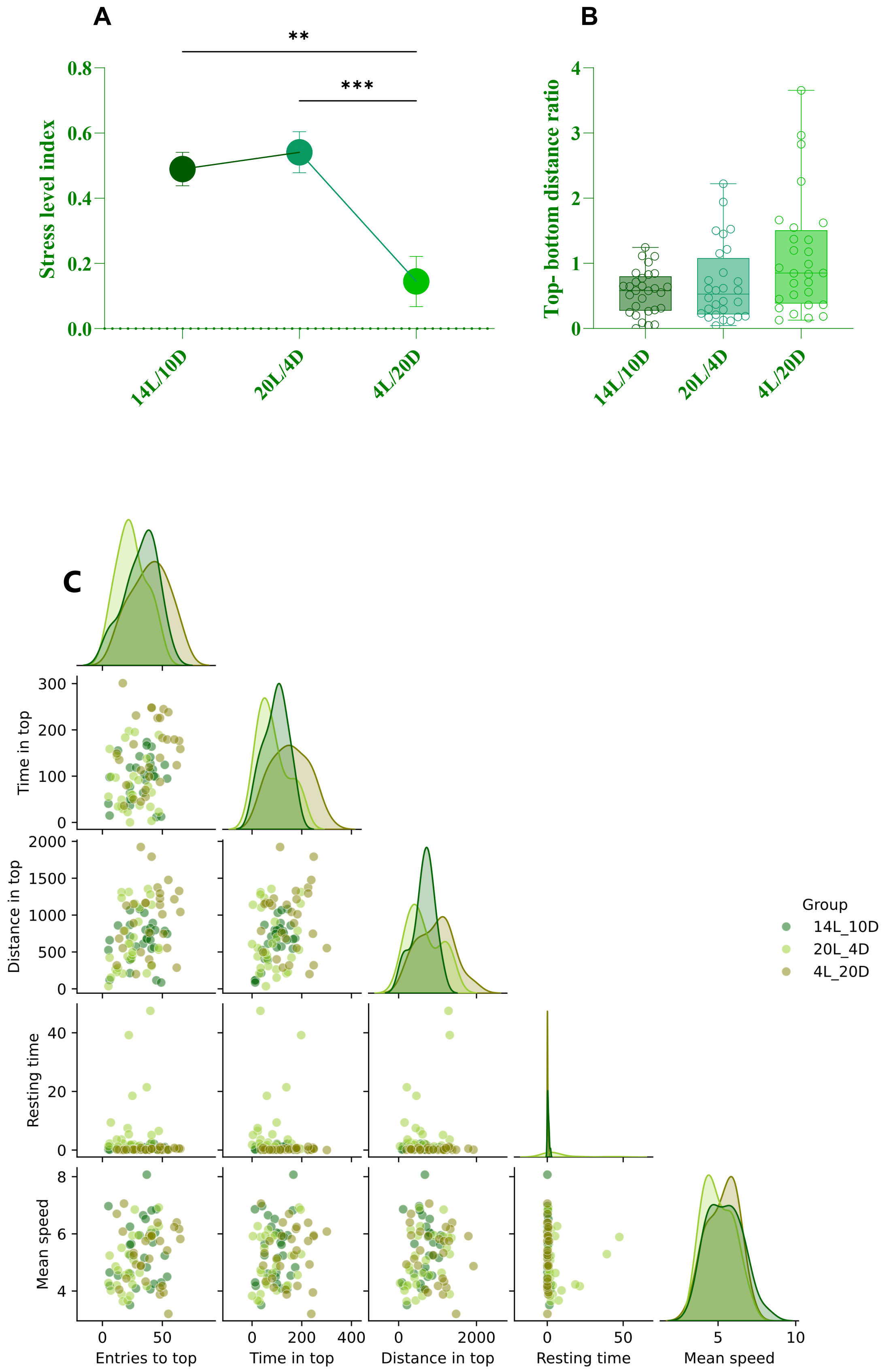


Figure 4.2: *Novel Tank Test, the scattered dot plot represents behavioural endpoints derived from recorded videos upon analysed by Smart 3.0 tracking software (A) Number of entries to top zone. The 4L/20D group made significantly higher entries. (B) Latency to enter into the top zone. Higher latency in 20L/4D with that of 4L/20D. (C) Time in top (D) Total distance travelled (E) Distance in top (F) Mean speed (G) Resting time. Higher in 20L/4D (H) Slow swimming time. Lower in 20L/4D (I) Fast time. Higher in 20L/4D. The data is represented as Mean ± SEM. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001. n=28)*


A
**
***
Stress level index
0.0
0.2
0.4
0.6
0.8
14L/10D
20L/4D
4L/20D
B
Top- bottom distance ratio
0
1
2
3
4
14L/10D
20L/4D
4L/20D
C
Time in top
Distance in top
Resting time
Mean speed
Entries to top
Group
14L_10D
20L_4D
4L_20D

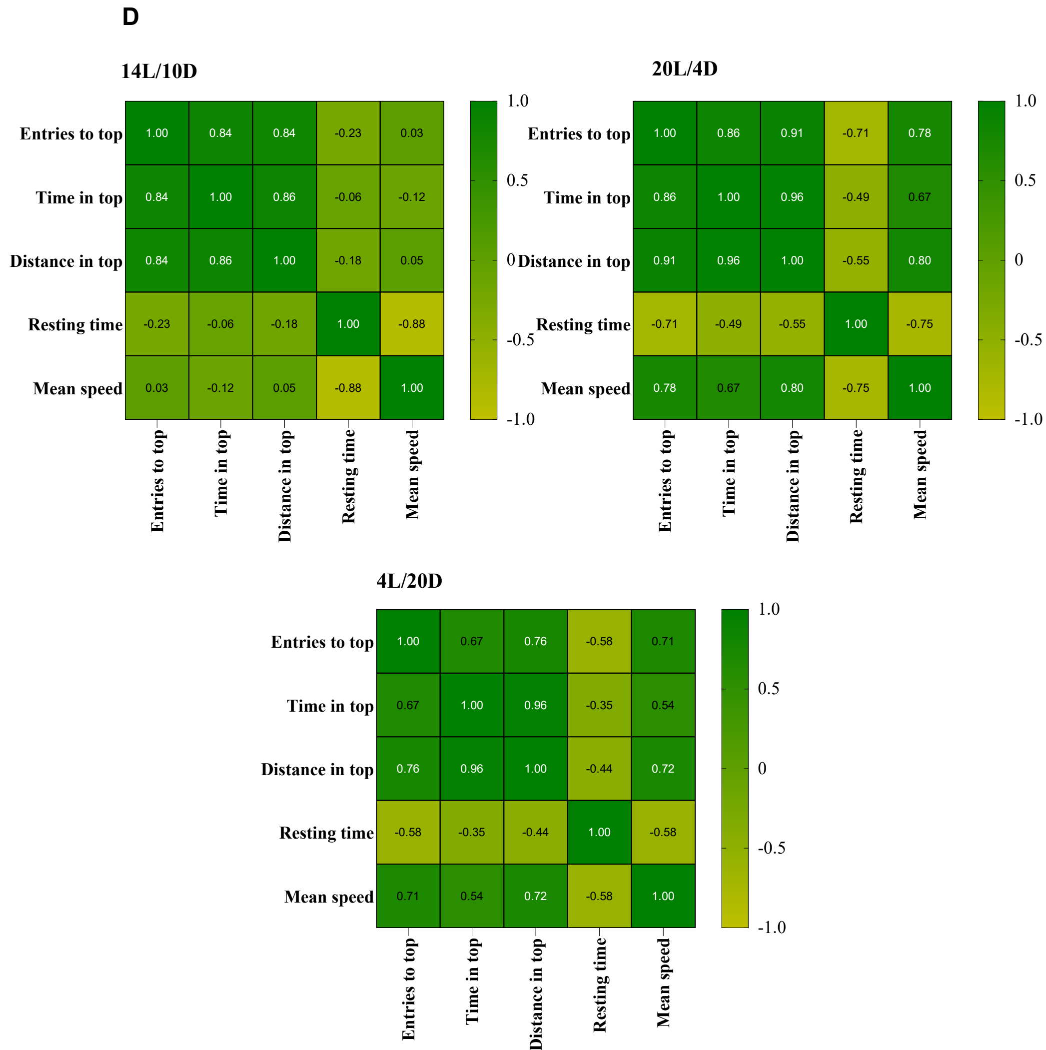


Figure 4.3: *The Novel Tank Test, (A) The dotted line plot representing Stress level index. Calculated on the basis of time spent in bottom versus top, appeared to be lower in 4L/20D. The data is represented as Mean ± SEM. The statistically significant differences are indicated by **(P<0.01) and ***(p<0.001). (B) The box and whisker plot with data points illustrating Top-bottom distance ratio. The data is represented as median interquartile range. (C) Pair plot with kernel density estimation showing general relationship between novel tank parameters. (D) Correlation matrix plot, showing spearman correlation coefficient values of association between novel tank parameters.*

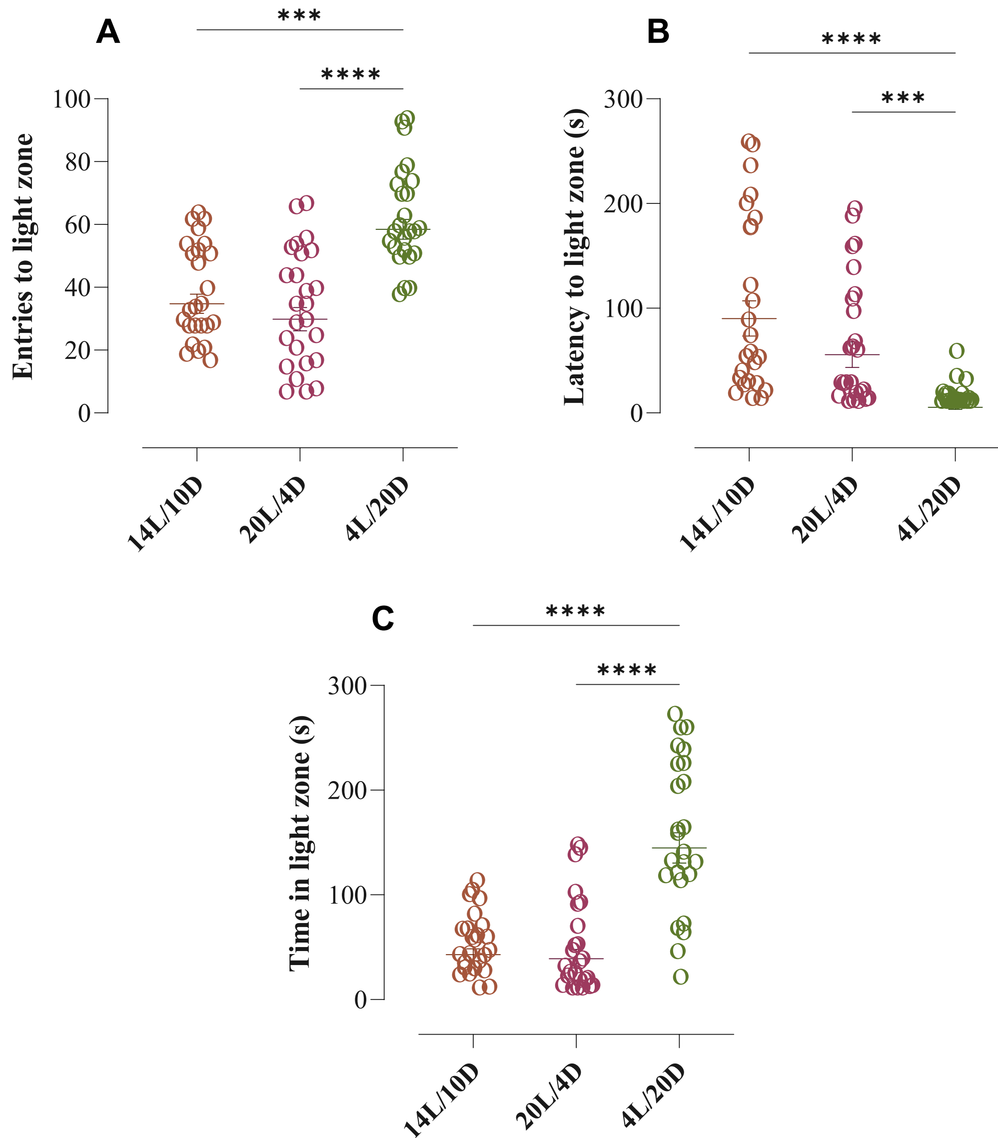


Figure 4.4: *Light and Dark Test, Scattered ring plot displaying (A) Number of entries to light zone. 4L/20D group displayed increased entries. (B) Latency to enter the light zone. The time taken to enter into the light zone is lower in 4L/20D. (C) Time spent in the light zone. The amount of time spent in the light zone is higher in 4L/20D. The data is represented as Mean ± SEM. The statistically significant differences are indicated by ***(P<0.001) and ****(p<0.0001. n=25).*

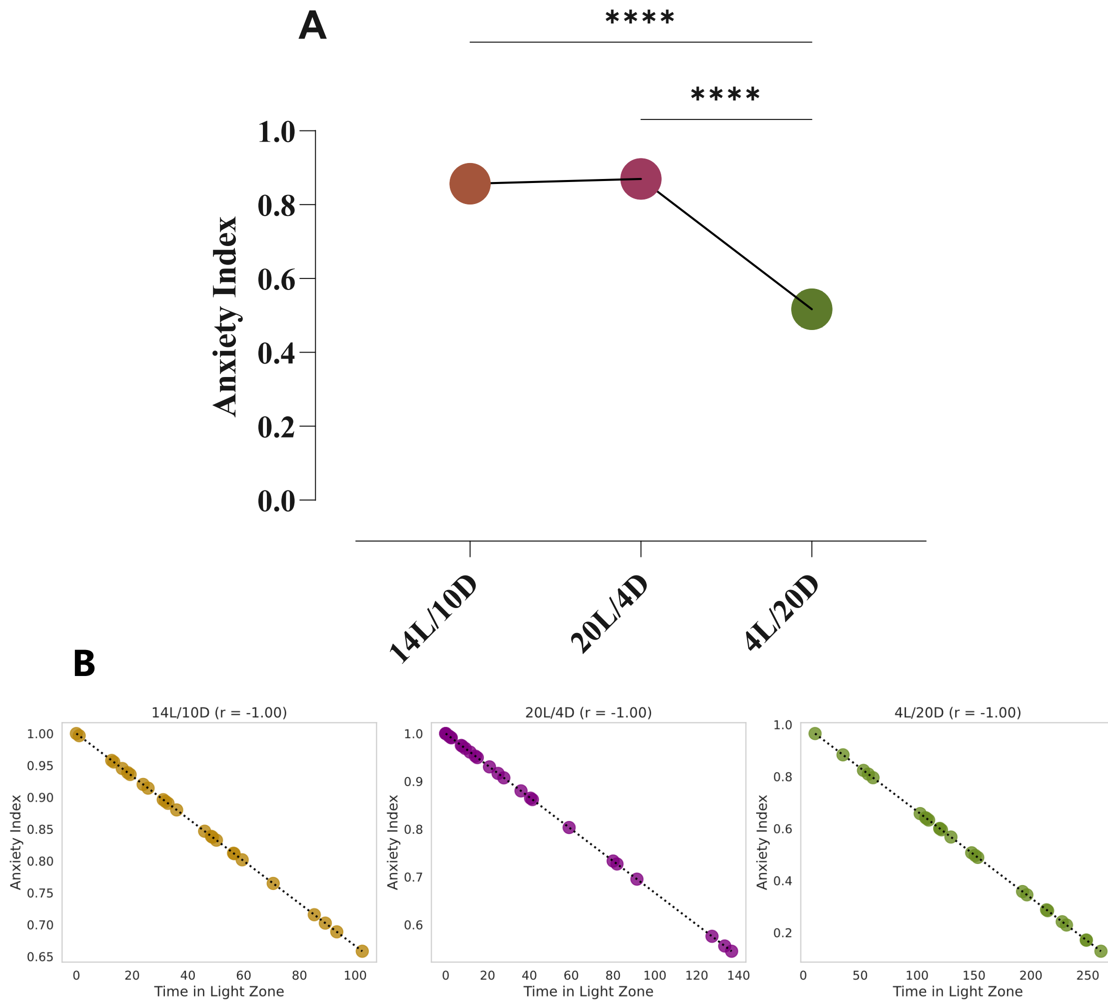
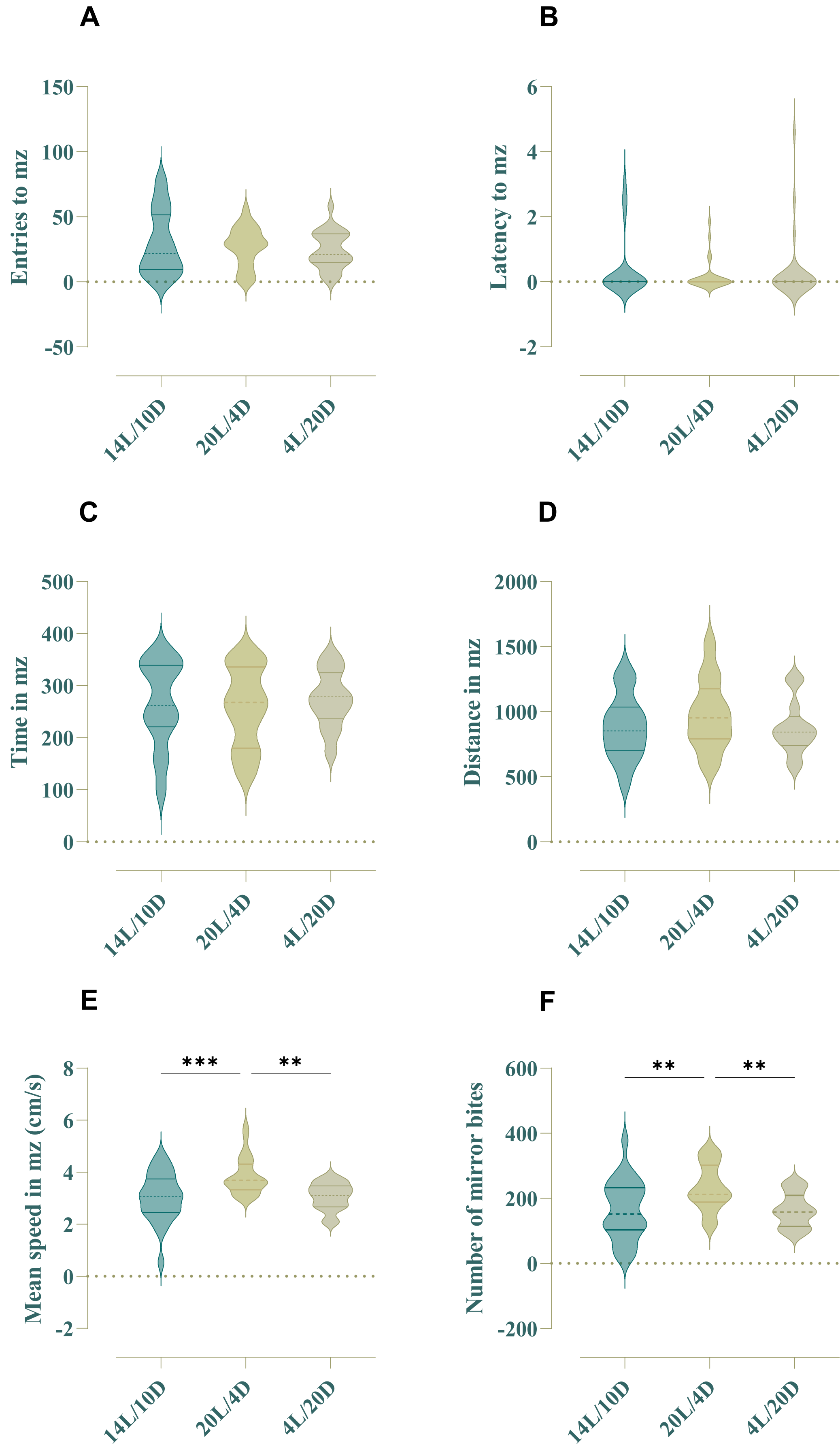


Figure 4.5: *Light-Dark Test (A) The dotted line plot representing Anxiety index. Calculated based on time spent in dark and light zone, lower in 4L/20D. The data is represented as Mean ± SEM. The statistically significant differences are indicated by ****(p<0.0001). (B) The Scattered dot plot displaying association between Anxiety index and Time in light zone. An inverse relationship found among all groups (r=-1).*


A
B
C
D
E
F
Entries to mz
Latency to mz
Time in mz
Distance in mz
Mean speed in mz (cm/s)
Number of mirror bites
14L/10D
20L/4D
4L/20D
***
**

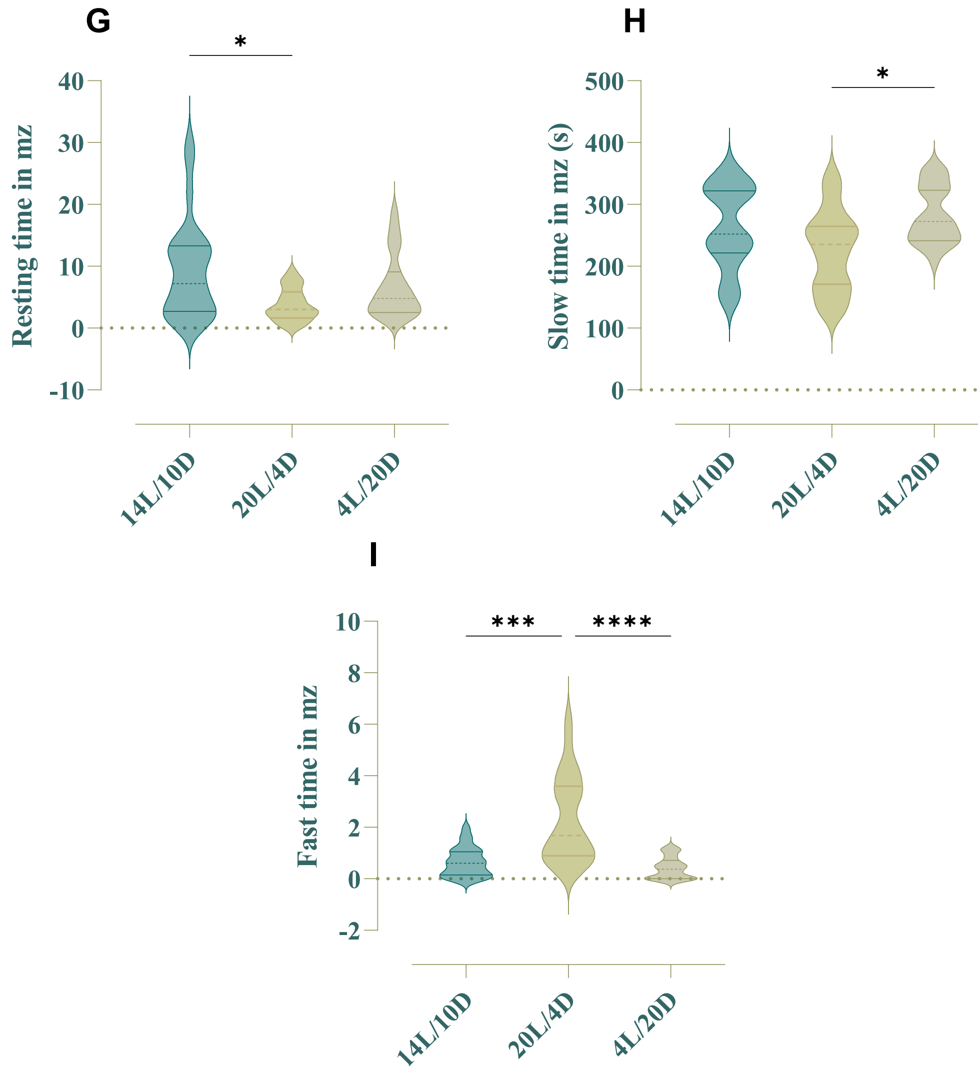
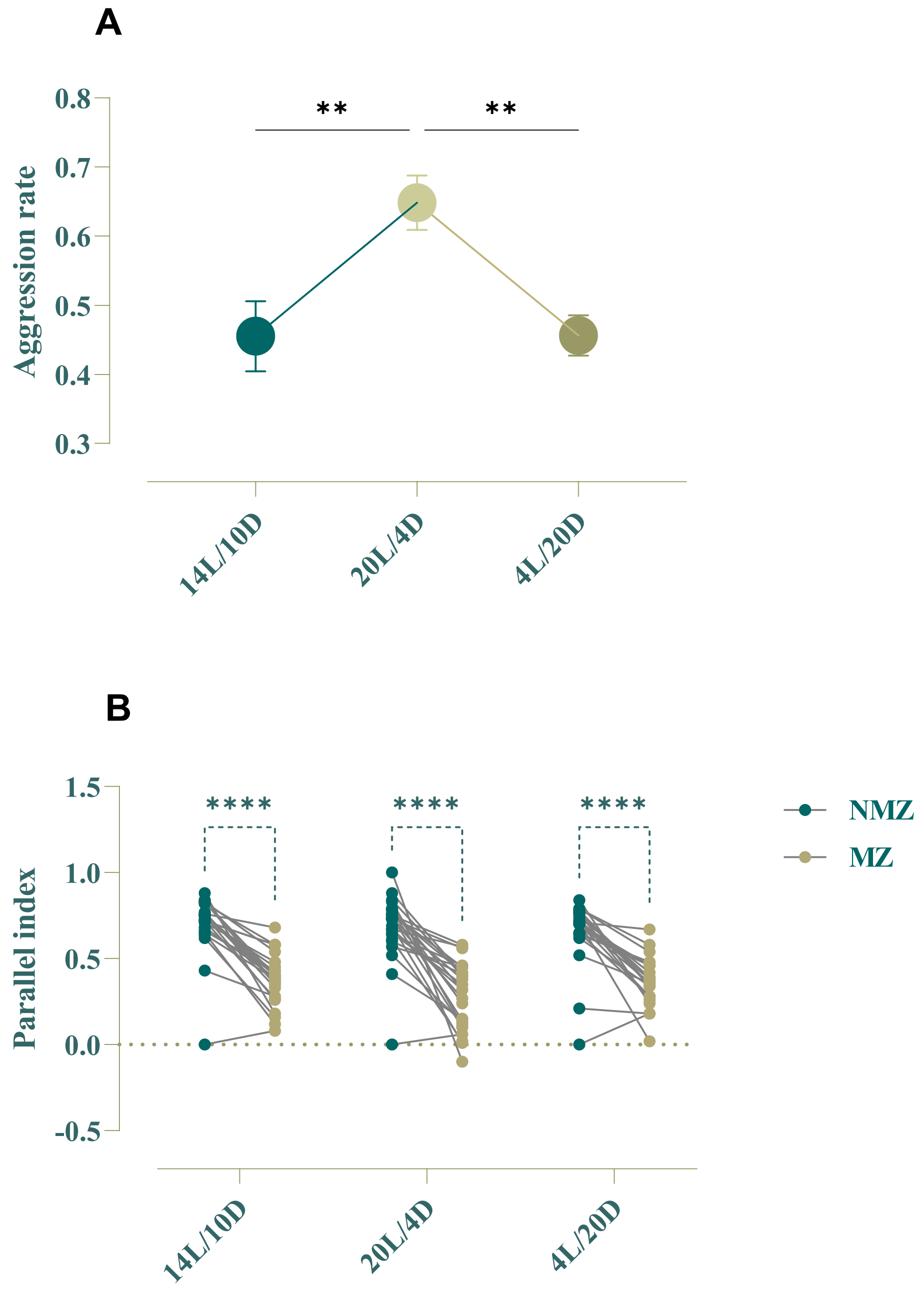


Figure 4.6: *Mirror Biting test, Violin plot represents behavioural endpoints derived from the activities in a three-chambered tank with a mirror. (A) Number of entries to mirror zone. (B) Latency to enter mirror zone (C) Time in mirror zone (D) Distance in mirror zone. (E) Mean speed in mirror zone. Higher in 20L/4D group. (F) Number of mirror bites. 20L/4D, the extended light condition enhanced the mirror bites pointing to the elevated aggressive nature. (G) Resting time in mirror zone. Lowered in 20L/4D. (H) Slow time in mirror zone. 4L/20D group exhibit increased slow time with that of 20L/4D. (I) Fast time. Increased fast time in mirror zone in 20L/4D. The data is represented as Median IQR. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001), (n=25).*


A
Aggression rate
0.8
0.7
0.6
0.5
0.4
0.3
**
**
14L/10D
20L/4D
4L/20D
B
Parallel index
1.5
1.0
0.5
0.0
-0.5
****
****
****
NMZ
MZ
14L/10D
20L/4D
4L/20D

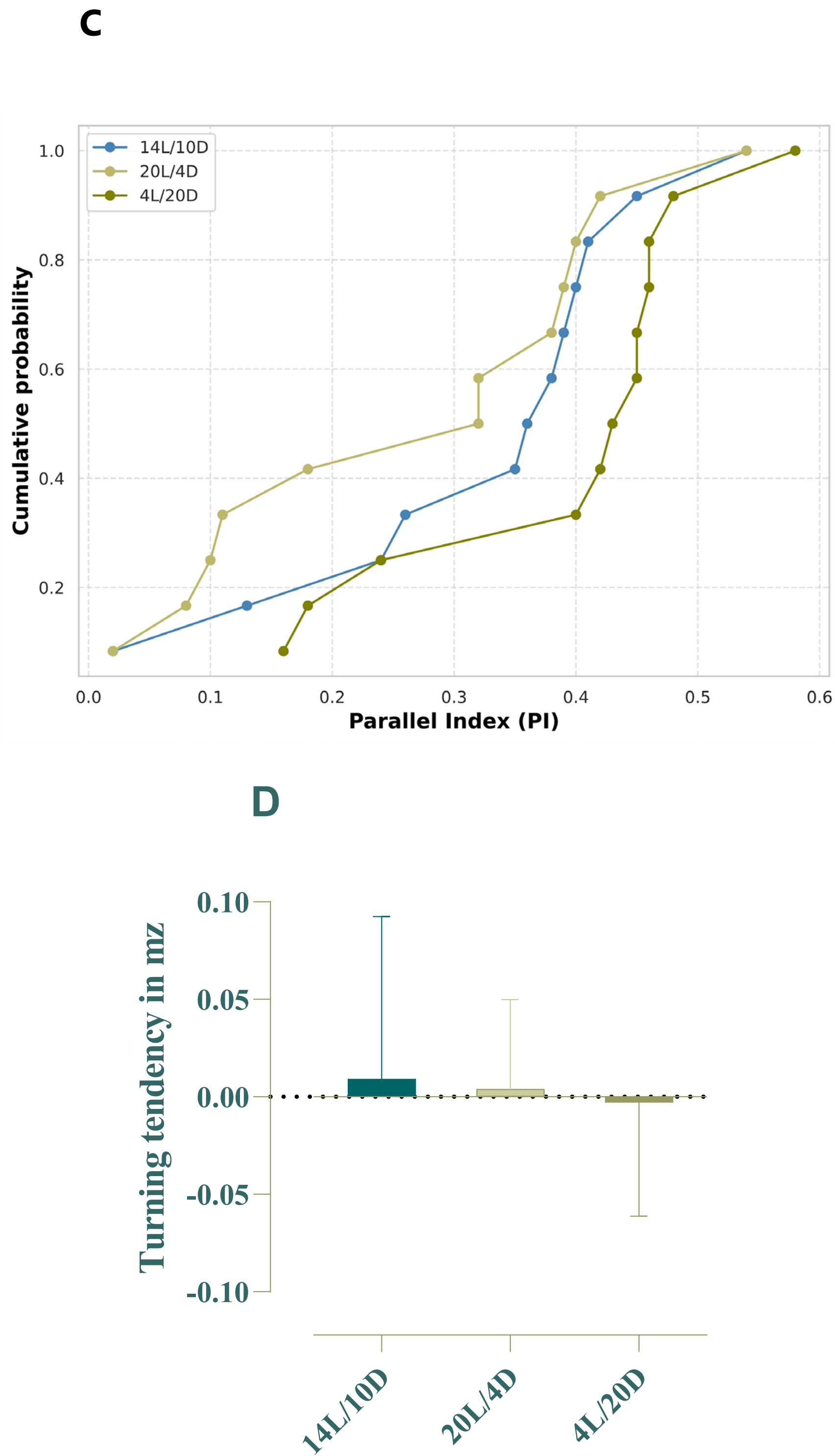
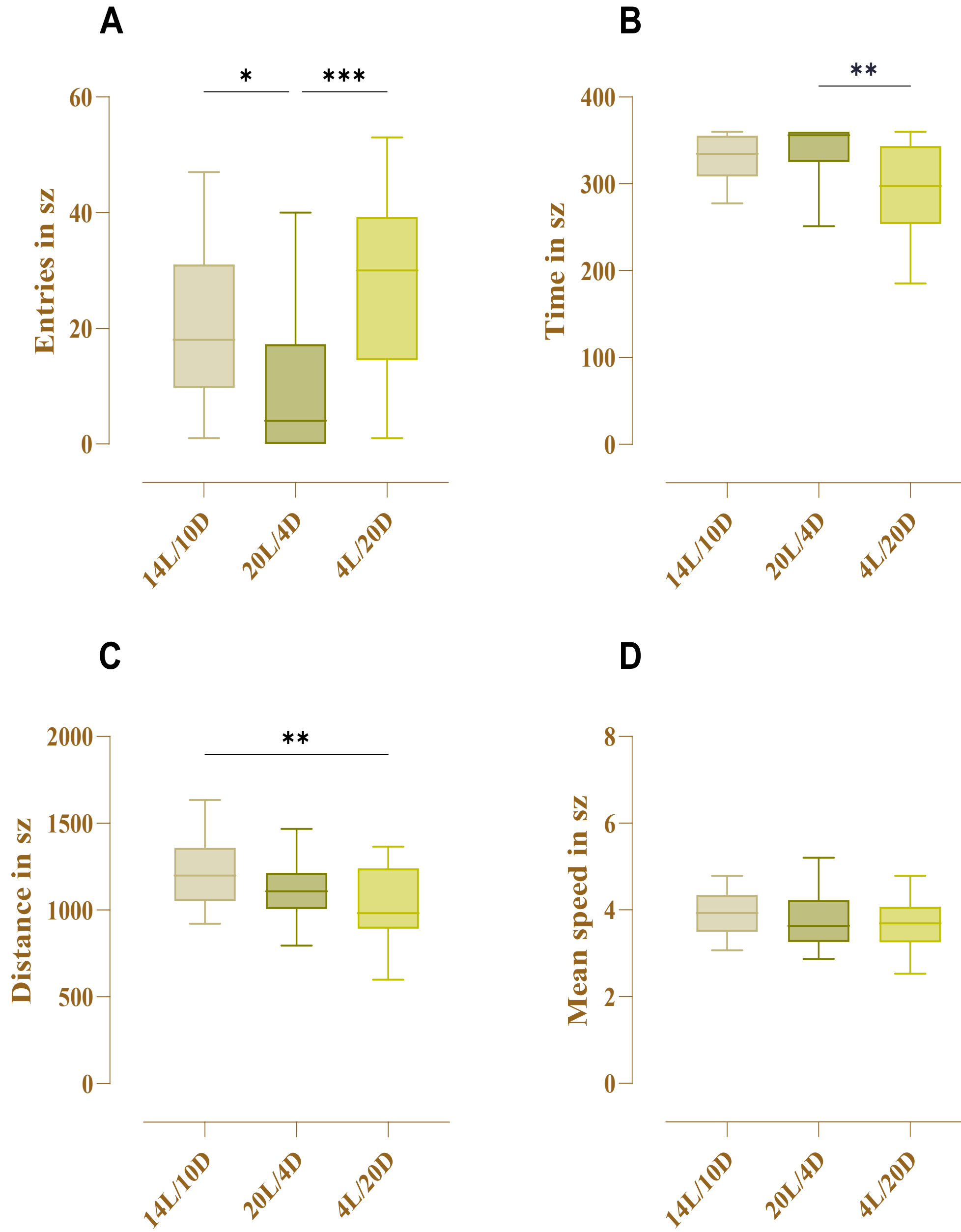


Figure 4.7: *Mirror Biting test, (A) The dotted line plot representing rate of aggression, it was higher in 20L/4D. (B) The before-after plot displaying parallel orientation of zebrafish in the non-mirror zone (NMZ) and mirror-zone (MZ) with in group. The parallel orientation (parallel index) reduced irrespective of condition in MZ (C) The multiple line plot showing parallel orientation in MZ between groups. (D) Turning tendency, bar plot showing turning tendency values with left and right*

*ward direction. The data is represented as Mean ± SEM. The statistically significant differences are indicated by **(P<0.01) and ****(p<0.0001).*



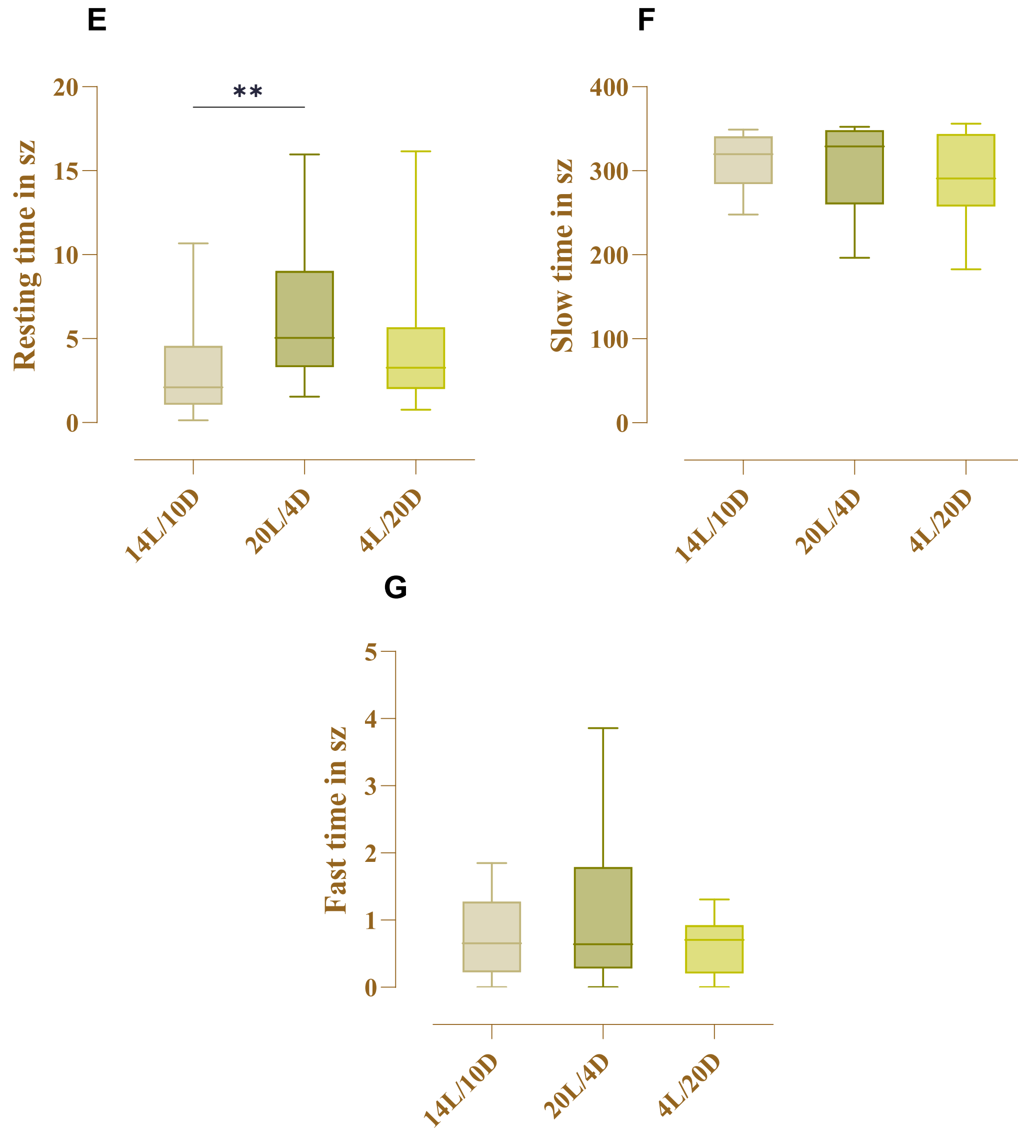
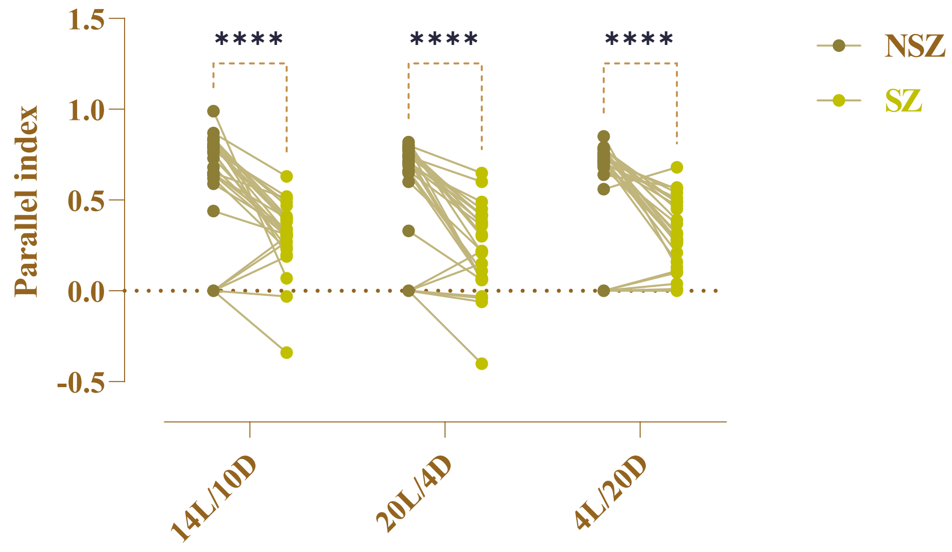
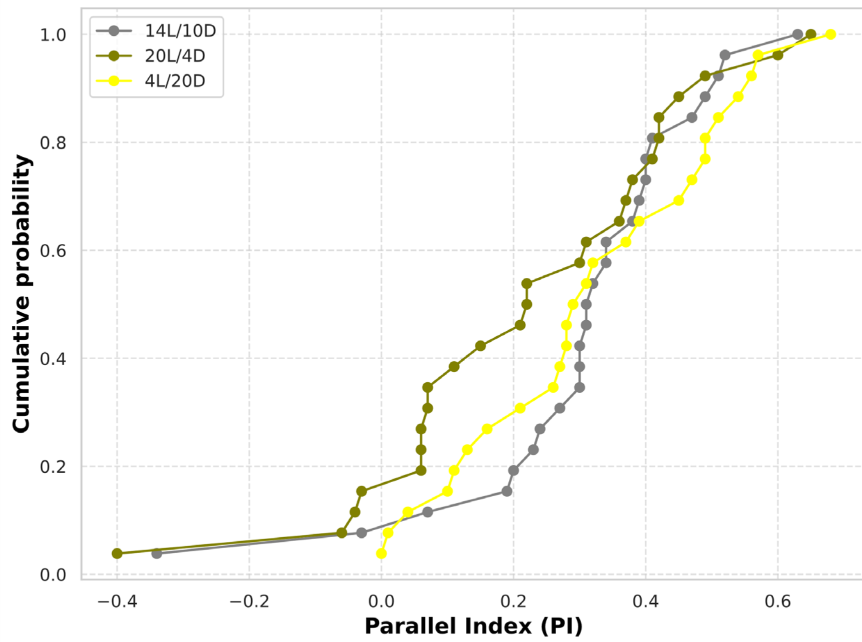


Figure 4.8: *The box plot represents behavioural endpoints from social preference test. (A) Number of entries to social zone, lower in 20L/4D. (B) Time spent in social zone, lower in 4L/20D compared to 20L/4D. (C) Distance in social zone (D) Mean speed in social zone (E) Resting time in social zone. Higher in 20L/4D. (F) Slow time in social zone (G) Fast time in social zone. The data is represented as Median IQR. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), and ***(P<0.001), n=25).*


A
Parallel index
1.5
1.0
0.5
0.0
-0.5
****
****
****
NSZ
SZ
14L/10D
20L/4D
4L/20D


B
14L/10D
20L/4D
4L/20D
Cumulative probability
Parallel Index (PI)
1.0
0.8
0.6
0.4
0.2
0.0
−0.4
−0.2
0.0
0.2
0.4
0.6

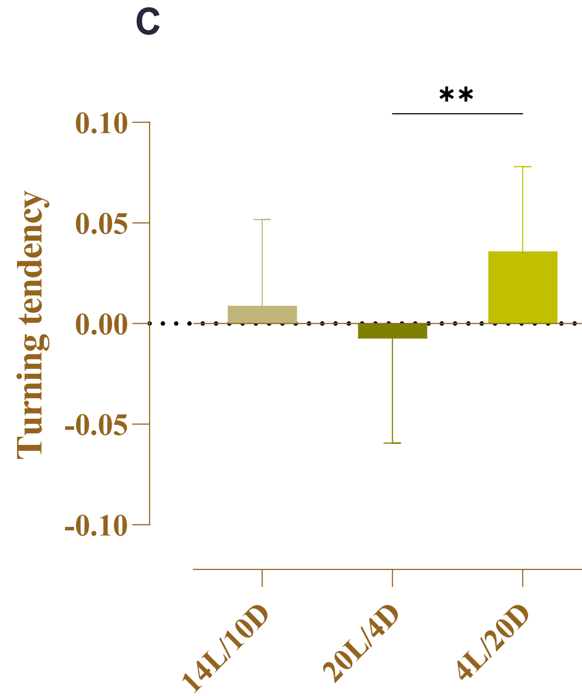


Figure 4.9: *Social preference test, (A) The before-after plot displaying parallel orientation of zebrafish in the non-social zone (NSZ) and social-zone (SZ) with in group. The parallel orientation (parallel index) reduced in all condition in sz. (B) The multiple line plot showing parallel orientation in sz between groups. (C) Turning tendency, Polar plot displaying resultant unit vectors and bar plot showing turning tendency values with left and right ward direction, 4L/20D group exhibit more rightward turns. The data is represented as Mean ± SEM. The statistically significant differences are indicated by **(P<0.01) and ****(p<0.0001).*

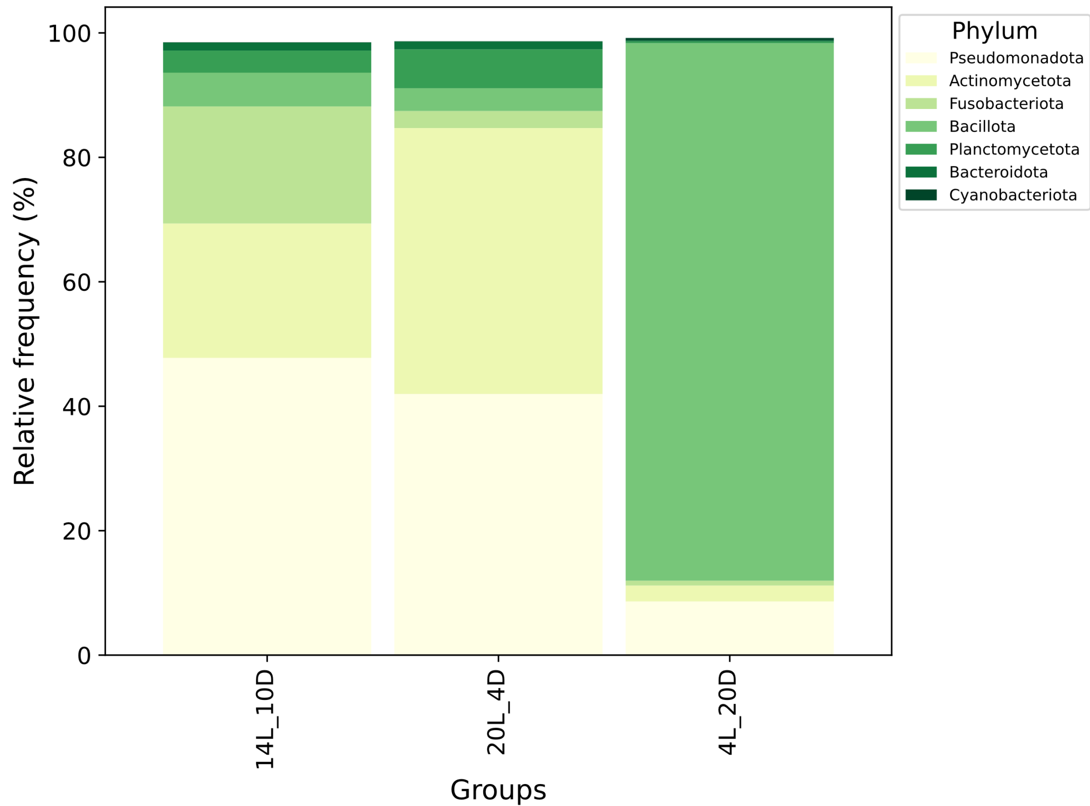


Figure 4.10A: *Gut microbiome analysis, the stacked bar plot displays the relative frequency of microbial abundance of three different conditions, Phylum-level.*

Name	14L/10D	20L/4D	4L/20D
Bacillota	5.42%	3.66%	86.38%
Pseudomonadota	47.77%	41.97%	8.63%
Actinomycetota	21.60%	42.74%	2.55%
Fusobacteriota	18.82%	2.74%	0.78%
Cyanobacteriota	0.04%	0.04%	0.37%
Planctomycetota	3.55%	6.24%	0.28%
Bacteroidota	1.29%	1.28%	0.20%

Table 4.1: *Relative abundance of top seven phyla present in different photoperiod groups.*

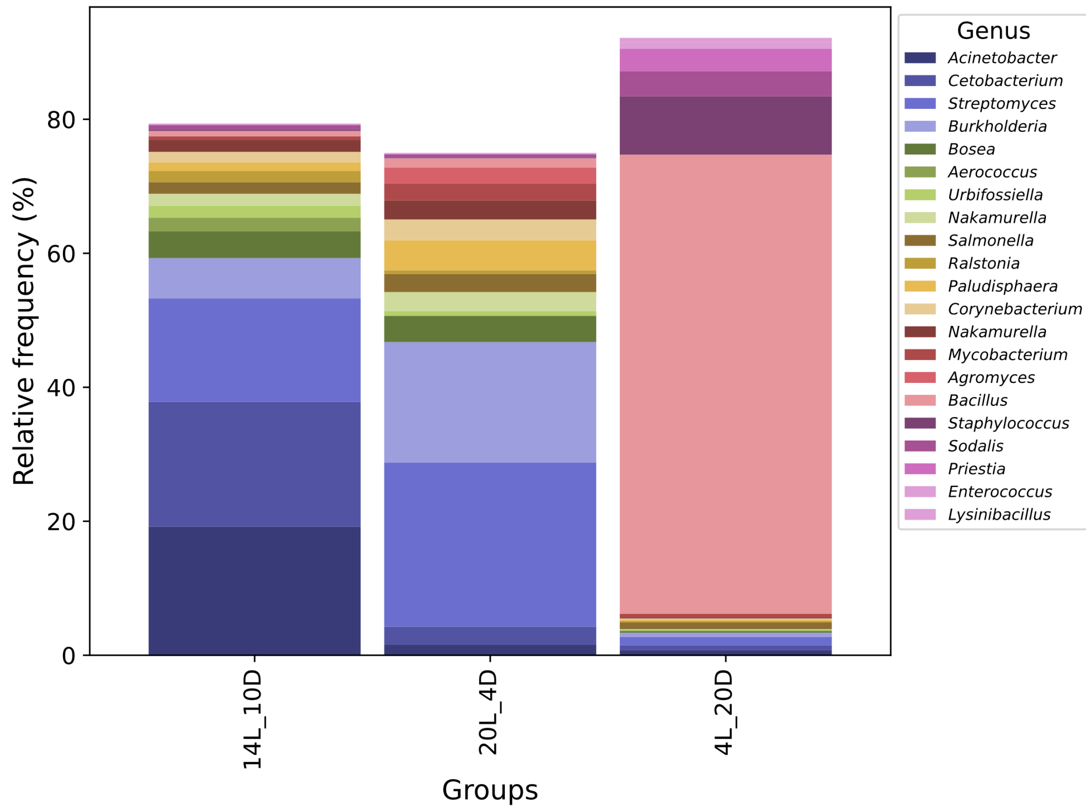


Figure 4.10B: *Gut microbiome analysis, the stacked bar plot displays the relative frequency of microbial abundance of three different conditions, Genus-level.*

Name	14L/10D	Name	20L/4D	Name	4L/20D
Acinetobacter	19.20%	*Streptomyces*	24.52%	*Bacillus*	68.51%
Cetobacterium	18.62%	*Burkholderia*	17.97%	*Staphylococcus*	8.68%
Streptomyces	15.43%	*Paludisphaera*	4.48%	*Sodalis*	3.79%
Burkholderia	6.04%	*Bosea*	3.86%	*Priestia*	3.36%
Bosea	4.00%	*Corynebacterium*	3.11%	*Streptomyces*	1.22%
Aerococcus	2.03%	*Nakamurella*	2.83%	*Salmonella*	1.00%

Table 4.2*: Relative abundance of top six genus present in different photoperiod groups.*

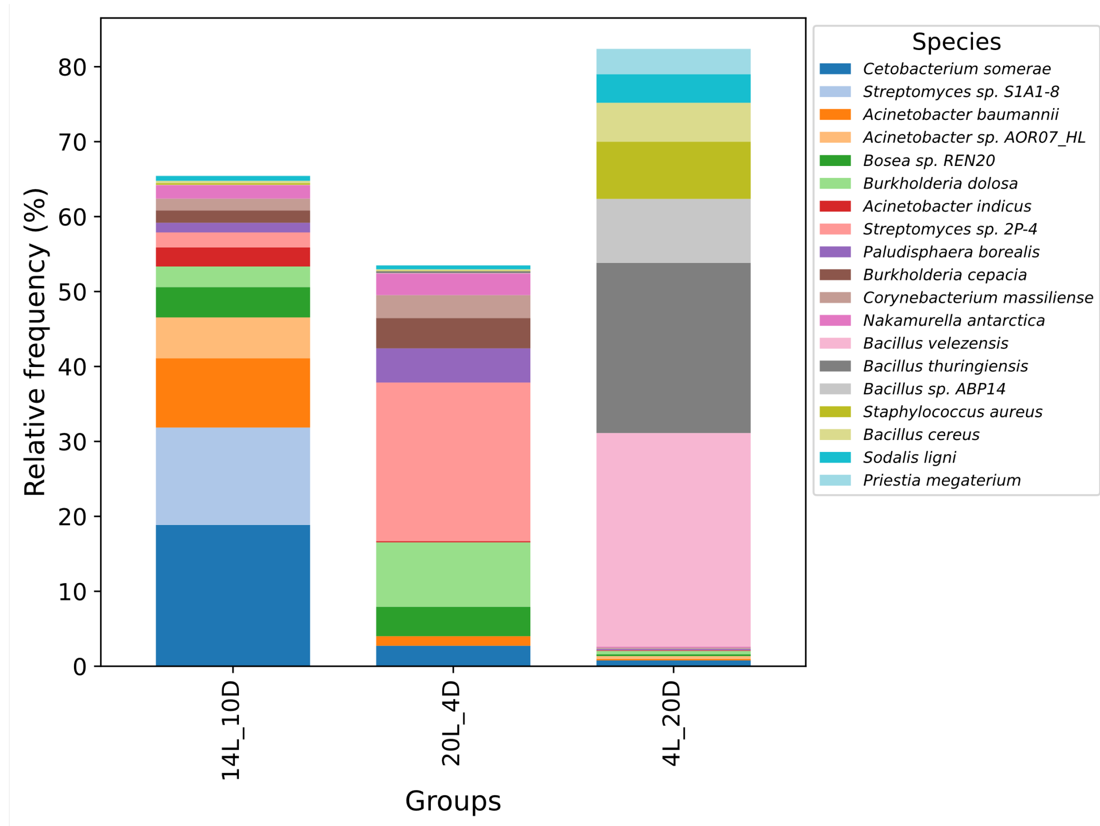


Figure 4.10C: *Gut microbiome analysis, the stacked bar plot displays the relative frequency of microbial abundance of three different conditions, Species-level.*

Name	14L/10D	Name	20L/4D	Name	4L/20D
Cetobacterium somerae	18.86%	*Streptomyces sp. 2P-4*	21.16%	*Bacillus velezensis*	28.52%
Streptomyces sp. S1A1-8	12.99%	*Burkholderia dolosa*	8.59%	*Bacillus thuringiensis*	22.71%
Acinetobacter baumannii	9.25%	*Paludisphaera borealis*	4.56%	*Bacillus sp. ABP14*	8.53%
Acinetobacter sp. AOR07_HL	5.45%	*Burkholderia cepacia*	4.05%	*Staphylococcus aureus*	7.63%
Bosea sp. REN20	4.05%	*Bosea sp. REN20*	3.93%	*Bacillus cereus*	5.20%
Burkholderia dolosa	2.74%	*Corynebacterium massiliense*	3.05%	*Sodalis ligni*	3.82%

Table 4.3: *Relative abundance of top six species present in different photoperiod groups.*

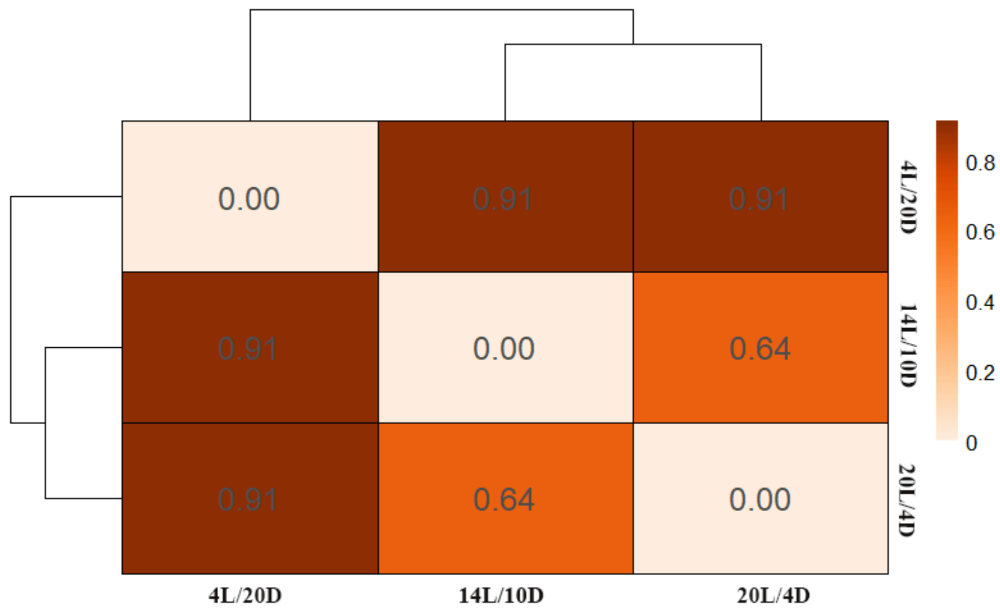


Figure 4.10D: *Gut microbiome analysis, Beta diversity. Bray Curtis Dissimilarity Matrix between species present in each sample.*

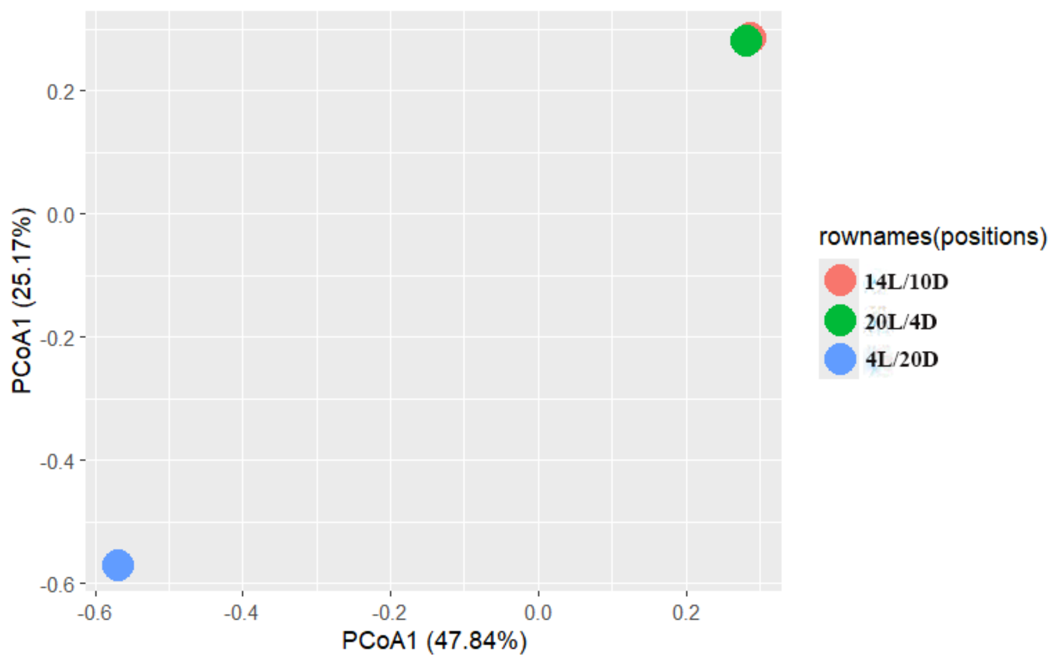


Figure 4.10E: *Gut microbiome analysis, Principal Coordinates Analysis (PCoA) plot illustrates the beta diversity or dissimilarities among samples based on their microbiota composition.*

Group	Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
14L/10D	606	828.514	45.2217	814.715	14.9543	3.71055	0.92956	14.1954	92.5042
20L/4D	708	993.338	54.3997	936.561	15.6372	3.8615	0.93493	15.3688	108.412
4L/20D	472	596.592	31.6132	582.87	12.2095	2.7474	0.8479	6.57449	64.7448

***Table 4.4**: Alpha Diversity indices of photoperiod groups.*

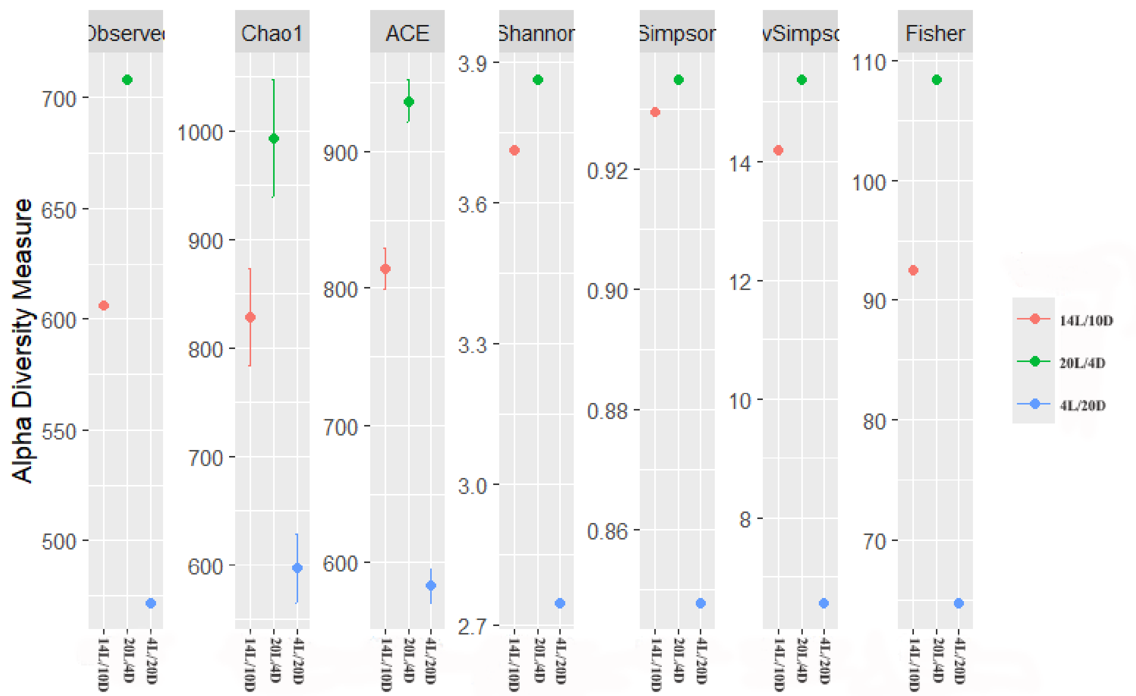
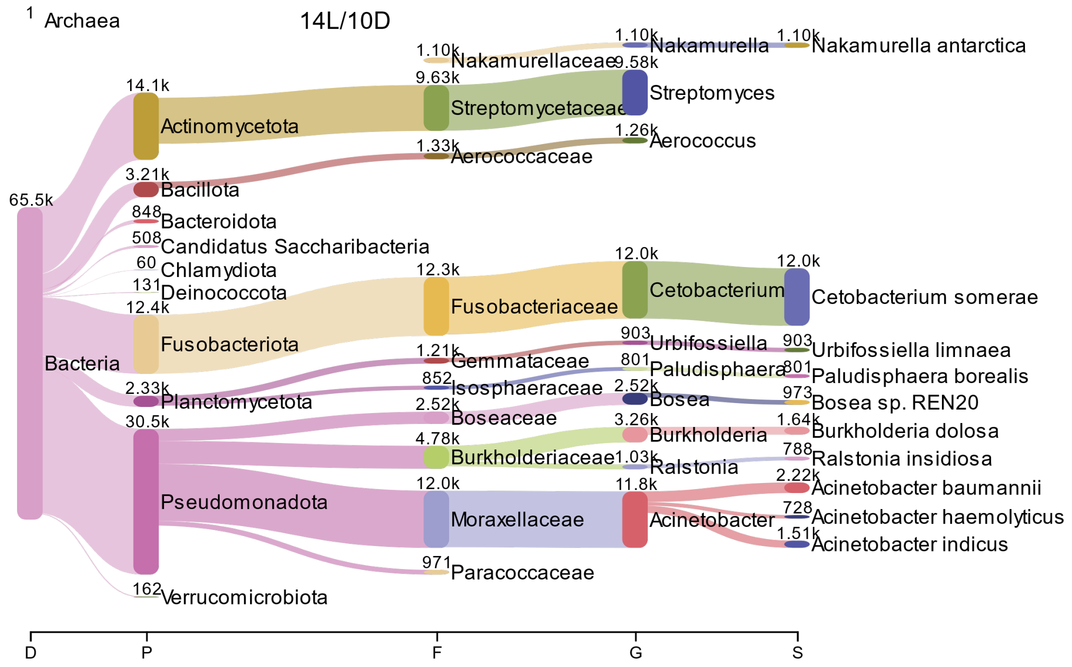
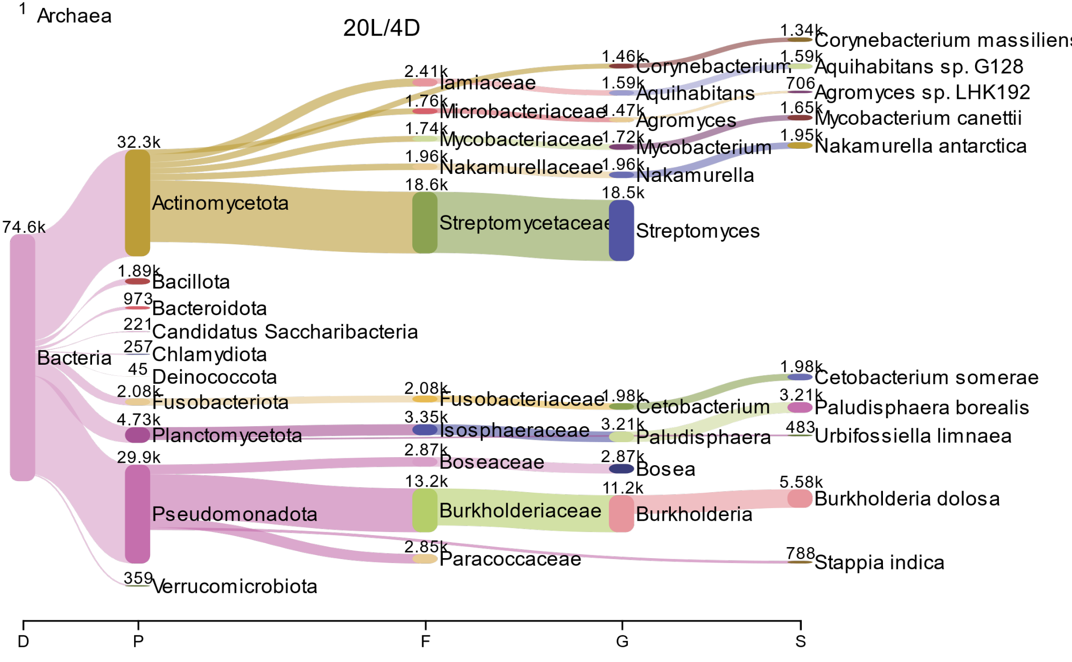


***Figure 4.10F:** Gut microbiome analysis, Alpha Diversity indices.*


14L/10D
1 Archaea
65.5k Bacteria
14.1k Actinomycetota
3.21k Bacillota
848 Bacteroidota
508 Candidatus Saccharibacteria
60 Chlamydiota
131 Deinococcota
12.4k Fusobacteriota
2.33k Planctomycetota
30.5k Pseudomonadota
162 Verrucomicrobiota
1.10k Nakamurellaceae
9.63k Streptomycetaceae
1.33k Aerococcaceae
12.3k Fusobacteriaceae
1.21k Gemmataceae
852 Isosphaeraceae
2.52k Boseaceae
4.78k Burkholderiaceae
12.0k Moraxellaceae
971 Paracoccaceae
1.10k Nakamurella
9.58k Streptomyces
1.26k Aerococcus
12.0k Cetobacterium
903 Urbifossiella
801 Paludisphaera
2.52k Bosea
3.26k Burkholderia
1.03k Ralstonia
11.8k Acinetobacter
1.10k Nakamurella antarctica
12.0k Cetobacterium somerae
903 Urbifossiella limnaea
801 Paludisphaera borealis
973 Bosea sp. REN20
1.64k Burkholderia dolosa
788 Ralstonia insidiosa
2.22k Acinetobacter baumannii
728 Acinetobacter haemolyticus
1.51k Acinetobacter indicus
D
P
F
G
S


20L/4D
1 Archaea
74.6k Bacteria
32.3k Actinomycetota
1.89k Bacillota
973 Bacteroidota
221 Candidatus Saccharibacteria
257 Chlamydiota
45 Deinococcota
2.08k Fusobacteriota
4.73k Planctomycetota
29.9k Pseudomonadota
359 Verrucomicrobiota
2.41k Iamiaceae
1.76k Microbacteriaceae
1.74k Mycobacteriaceae
1.96k Nakamurellaceae
18.6k Streptomycetaceae
2.08k Fusobacteriaceae
3.35k Isosphaeraceae
2.87k Boseaceae
13.2k Burkholderiaceae
2.85k Paracoccaceae
1.46k Corynebacterium
1.59k Aquihabitans
1.47k Agromyces
1.72k Mycobacterium
1.96k Nakamurella
18.5k Streptomyces
1.98k Cetobacterium
3.21k Paludisphaera
2.87k Bosea
11.2k Burkholderia
1.34k Corynebacterium massiliense
1.59k Aquihabitans sp. G128
706 Agromyces sp. LHK192
1.65k Mycobacterium canettii
1.95k Nakamurella antarctica
1.98k Cetobacterium somerae
3.21k Paludisphaera borealis
483 Urbifossiella limnaea
5.58k Burkholderia dolosa
788 Stappia indica
D
P
F
G
S

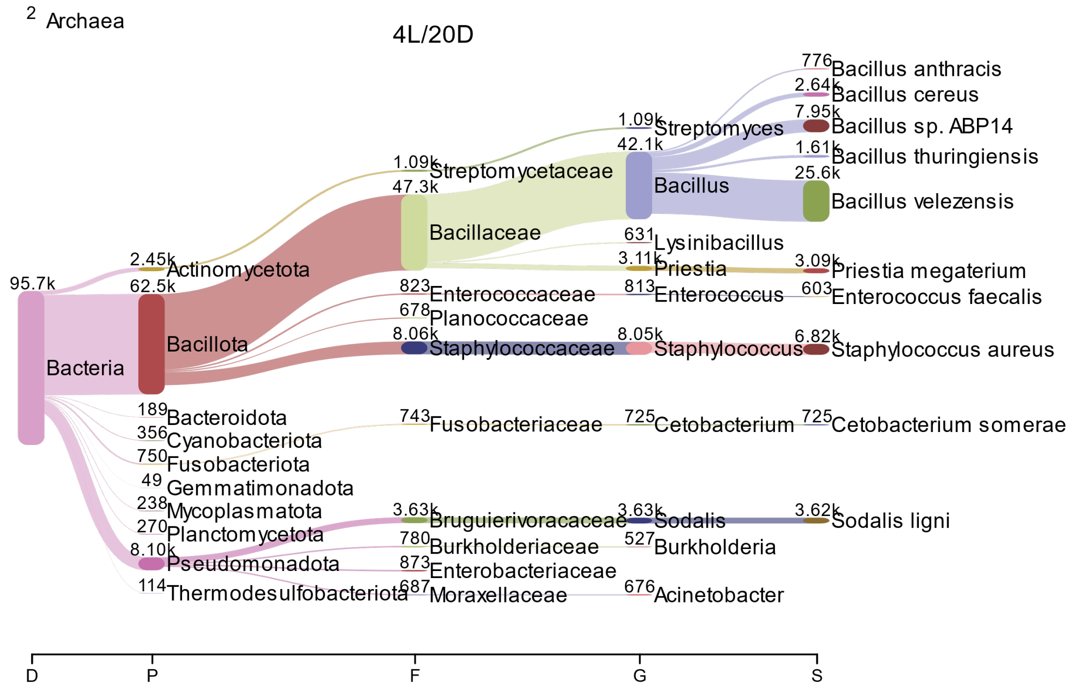


Figure 4.10G: *Gut microbiome analysis, Sankey plots- 14L/10D, 20L/4D, and 4L/20D.*

Figure 4.11A: *Brain transcriptomics, PCA Biplot showing log-transformed gene expression data in 14L/10D, 20L/4D, and 4L/20D.*

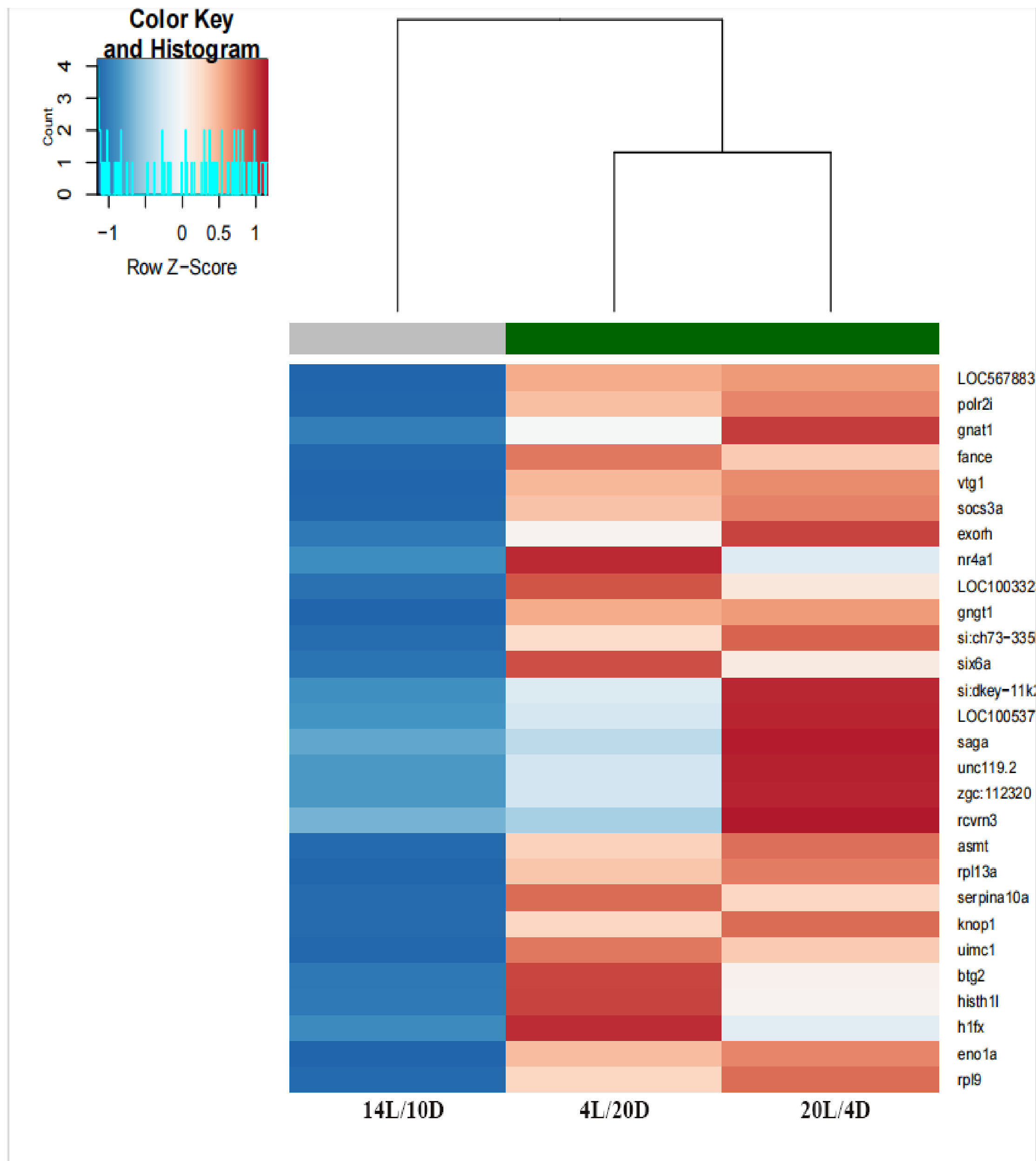


Figure 4.11B: *Brain transcriptomics, Heatmap representing upregulated DE genes.*

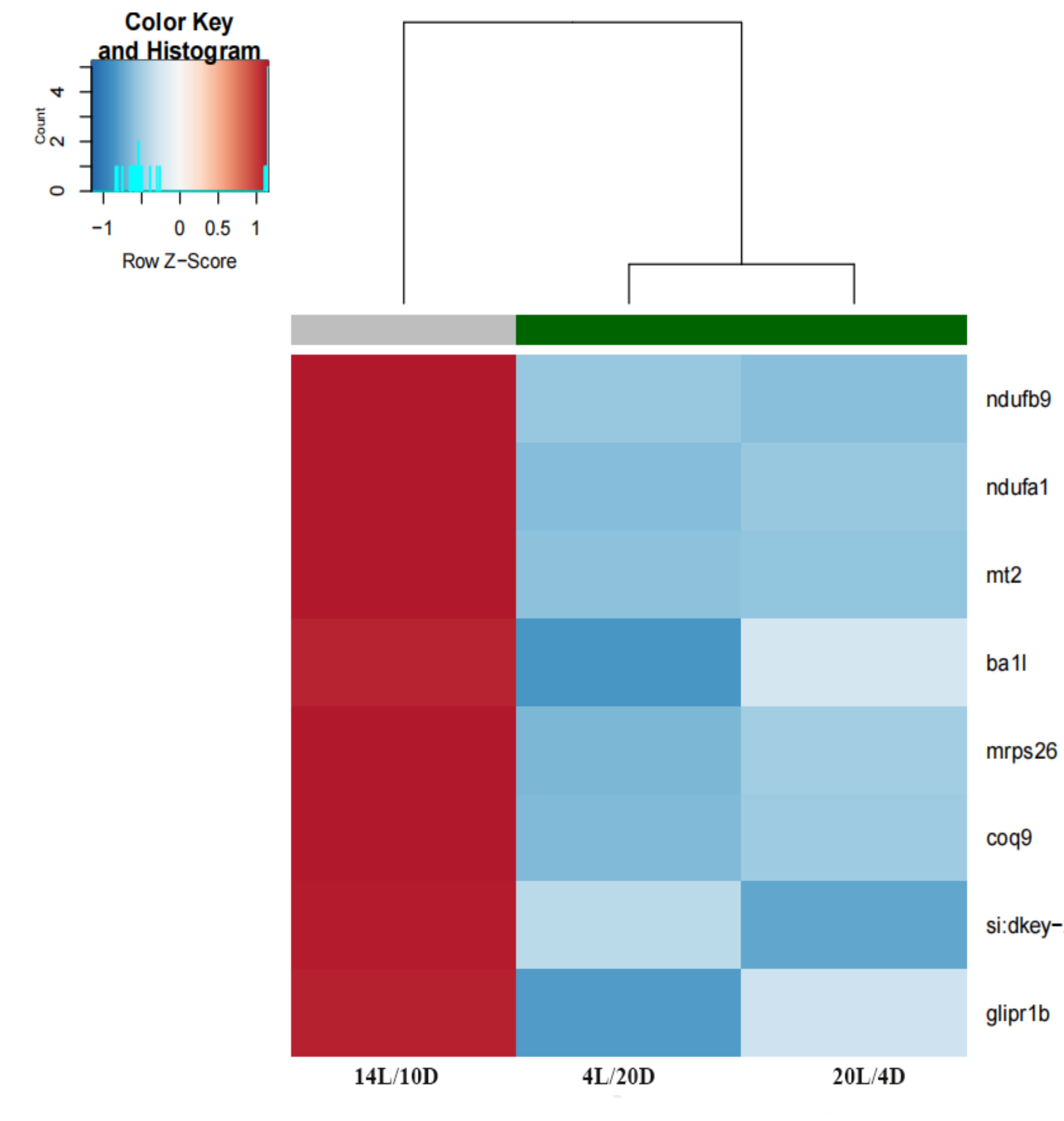


Figure 4.11C: *Brain transcriptomics, Heatmap representing downregulated DE genes.*

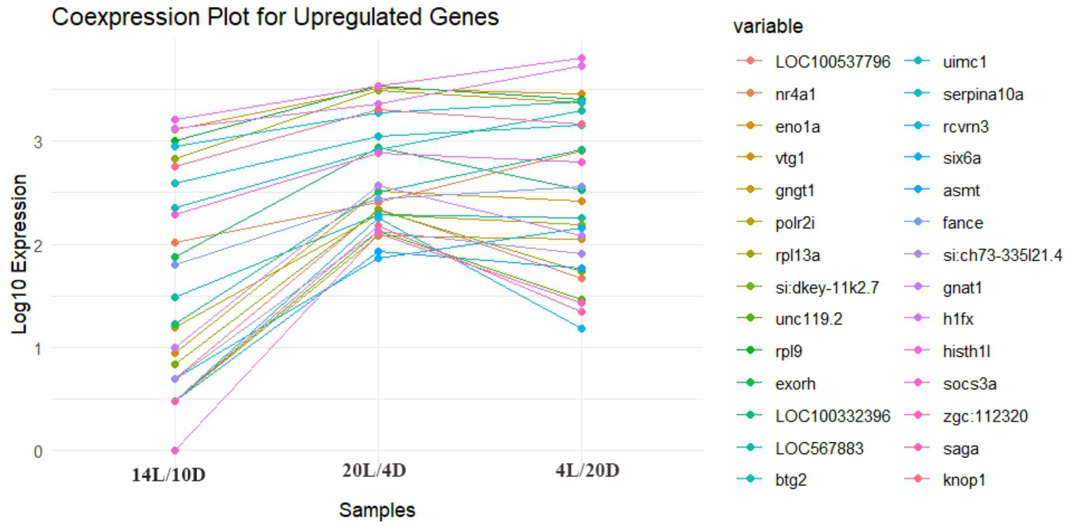


Figure 4.11D: *Brain transcriptomics, Coexpression plot for upregulated genes across three samples.*

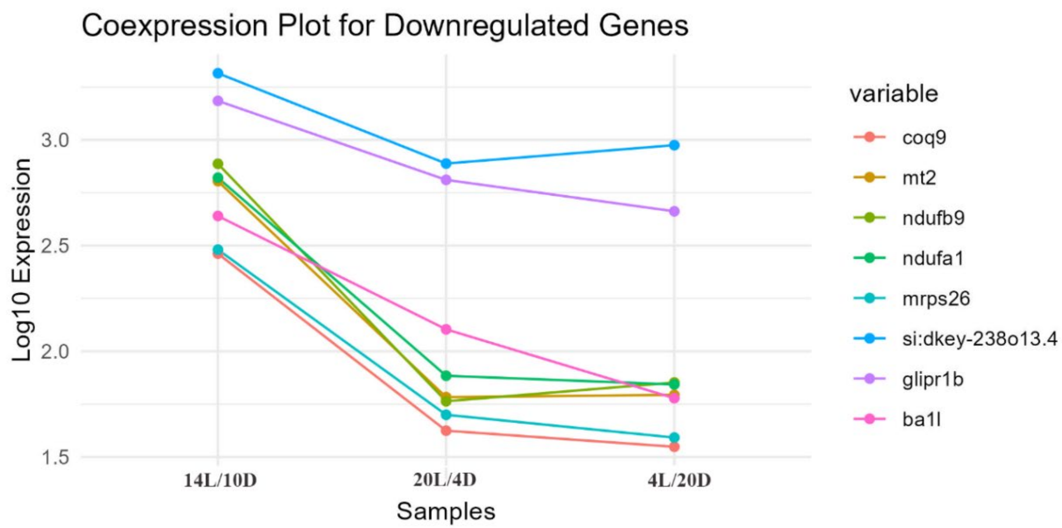


Figure 4.11E: *Brain transcriptomics, Co-expression plot for downregulated genes across three samples.*

14L/10D vs. 20L/4D

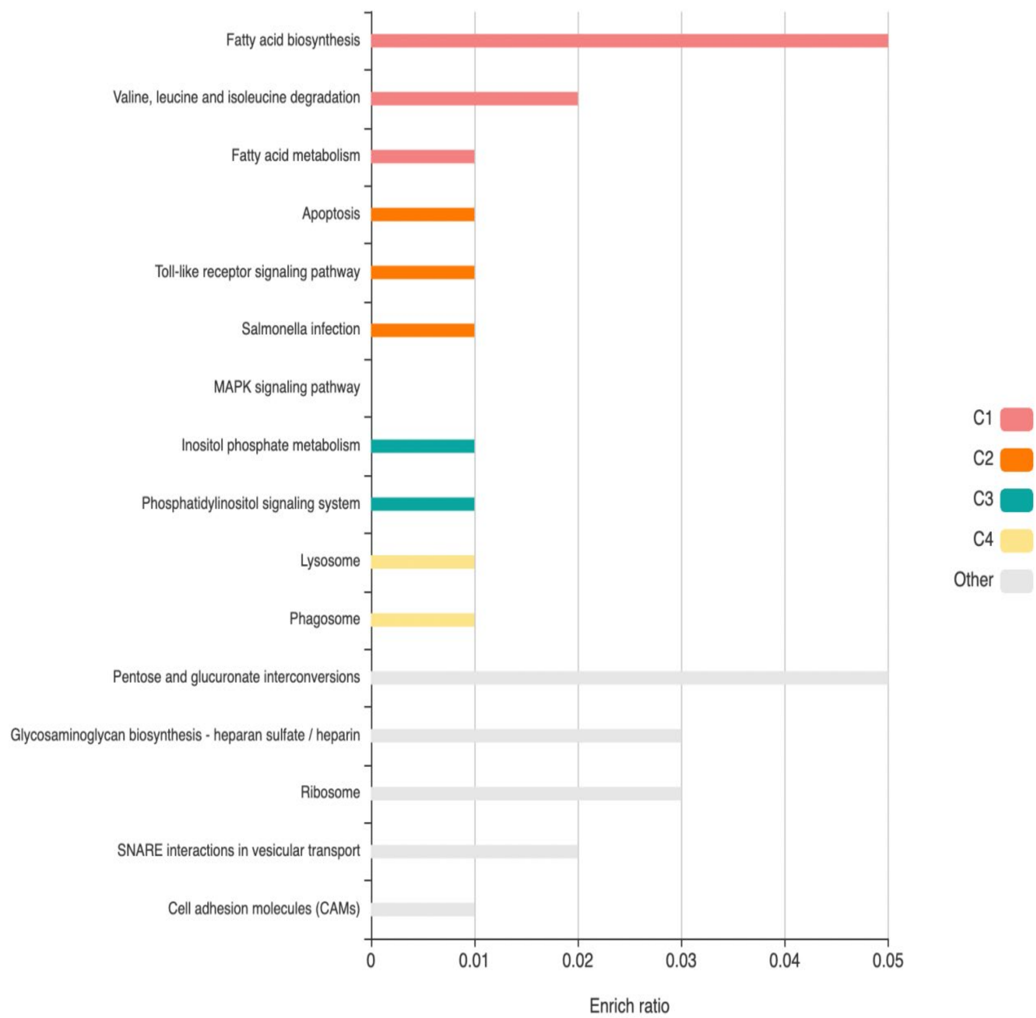


Figure 4.11F: *Brain transcriptomics, Enrichment barplot for DE genes between 14L/10D and 20L/4D.*

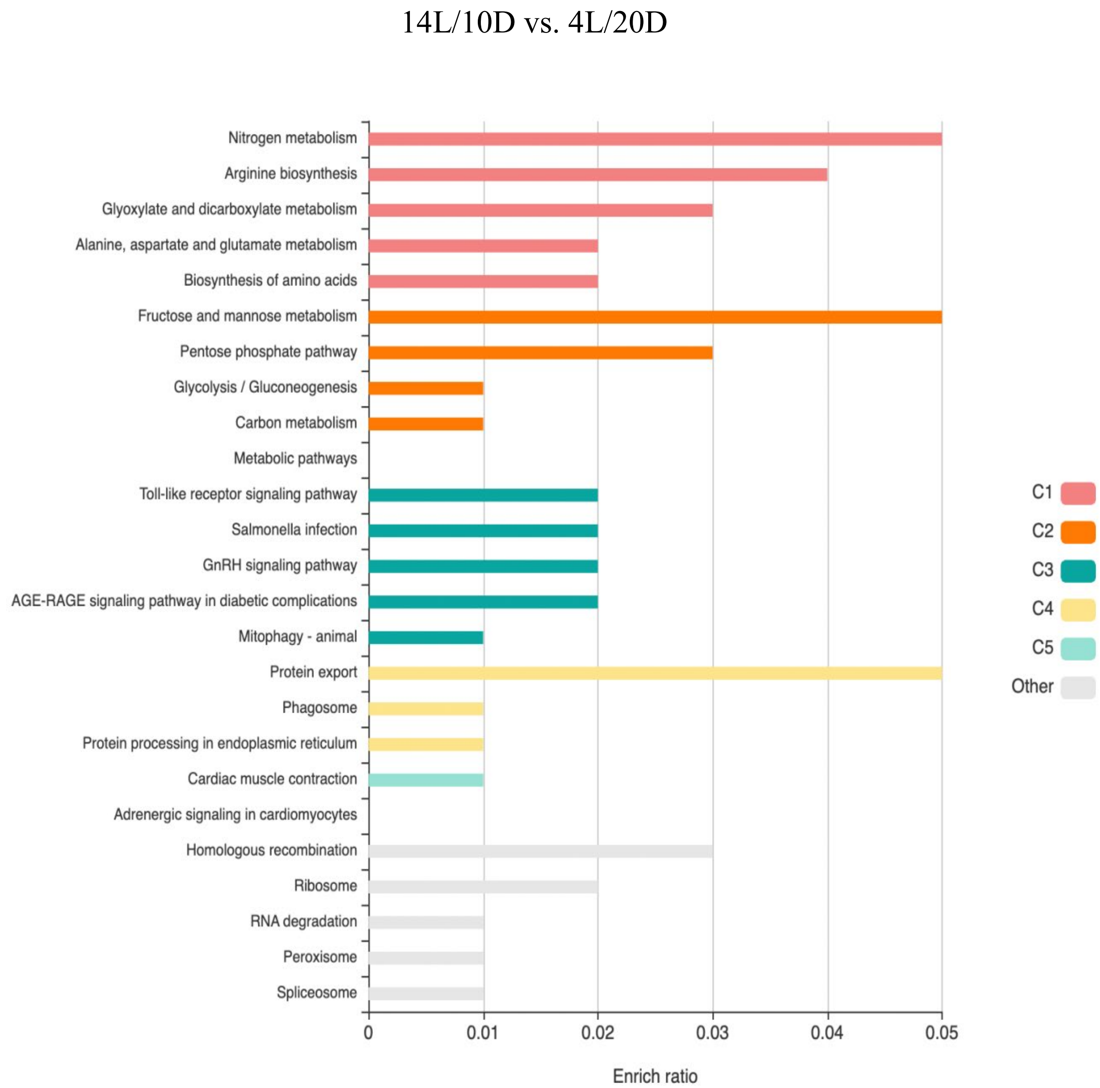


Figure 4.11G: *Brain transcriptomics, Enrichment bar plot for DE genes between 14L/10D and 4L/20D.*

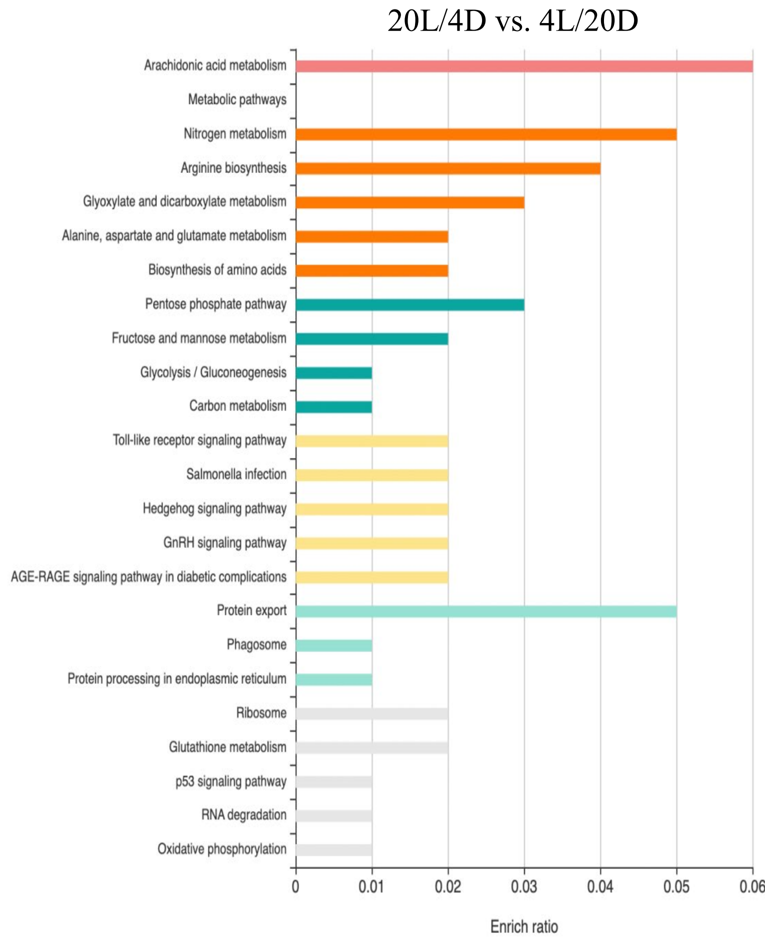


Figure 4.11H: *Brain transcriptomics, Enrichment bar plot for DE genes between 20L/4D and 4L/20D.*

4.5. Discussion

In the present study, we tried to evaluate the influence of different photoperiods, including extended light (20L/4D) and dark (4L/20D), on zebrafish behaviour, gut microbiota, and brain transcriptome profiles. Chronic exposure to different light conditions started from the early larval stage, and the influences were measured at the adult stage.

4.5.1. The extended light (20L/4D) and dark (4L/20D) induced behavioural changes in zebrafish.

Analysis of the novel tank test revealed that the group subjected to extended darkness (4L/20D) exhibited an earlier entrance and a greater number of entries into

the top zone compared to the extended light group (20L/4D), suggesting an eagerness to explore the newly introduced environment. The time spent in the top zone was higher in the 4L/20D group. And the anxiety index derived from both the novel tank test and the light-dark test further corroborates the reduced anxiety observed in the 4L/20D group. Previous research has demonstrated that shortened light duration (6L/18D) mitigated anxiety-like behaviours in diabetic rats. Also associated with decreased lipid peroxidation in the cortex, hippocampus, and thalamus. Additionally, serum fatty acids, including oleic, vaccenic, dihomo-γ-linolenic, and docosahexaenoic acids, were found to be elevated (Vasovic et al., 2023). Another study indicated that a short photoperiod regime over 21 days significantly reduced anxiety-like behaviours in rats along with ventral subicular lesions and a reduction in plasma corticosterone levels. This suggests that short photoperiods may modulate the hypothalamic-pituitary-adrenal axis (Subhadeep et al., 2020). In contrast, gerbils and fat sand rats exposed to short daylight periods of 8L/16D and 5L/19D exhibited disrupted locomotor activities and anxiety-depressive behaviours, highlighting species-specific responses to photoperiod changes (Juarez-Tapia et al., 2015; Ashkenazy et al., 2009). Also, there are studies linking greater night-time light exposure with increased risk of anxiety and other psychiatric disorders, and suggest that greater daytime light exposure is associated with a reduced risk (Burns et al., 2023). Consequently, excessive light exposure may disrupt circadian rhythms, leading to adverse psychological and physiological states (Regmi et al., 2023).

Furthermore, the 20L/4D group in the mirror-biting test exhibited increased fast time, mean speed, and mirror bites, indicating heightened aggression. The prolonged light exposure might be responsible for these increased aggression levels. A study

on psychiatric patients found that aggression and mood disorders were associated with changes in photoperiod, and higher aggression was observed during more extended daylight periods (Roitman et al., 1990). Also, the night-time exposure to blue light increased stress-induced aggression in male Sprague-Dawley rats, linked to enhanced brain-derived neurotrophic factor signalling and synaptic plasticity in the basolateral amygdala (Li et al., 2024). Furthermore, higher light intensity rather than duration boosted aggression in Nile tilapia without compromising social stability (Carvalho et al., 2013). However, because of changes in oestrogen receptor expression, shorter days seemed to be associated with higher aggression in Peromyscus mice (Trainor et al., 2007).

The 20L/4D group showed reduced entries and increased resting time in the social zone, but no decrease in time spent, suggesting that extended light duration causes a stressed approach towards social groups. A study on bright nights and social interactions, an overlooked issue by Kurvers and Holker (2015), predicted that night-time light could impact social processes such as social structure, synchrony, social information, communication, and competence. Consequently, the extended light period is likely to impair the ability to recall past dominance interactions and acquire social learning, leading to social stress (Taborsky & Oliveira, 2012). Prolonged exposure to daylight and evening lighting may delay the desired sleep onset time, particularly for individuals who remain awake later in the day. This shift could indirectly influence social behaviour and decision-making (Li et al., 2023). Continuous light exposure during early developmental stages in marmosets results changes in psychosocial behaviours, including increased alertness and hyperactivity, along with reduced vocalisations related to social interaction, displaying distinct behavioural patterns compared to those raised under a standard light-dark cycle

(Senoo et al., 2011). Wild-caught female sticklebacks preferred males under UV light conditions (Hiermes et al., 2021). Even maternal exposure to dim light at night can disrupt social preference in adolescent and adult offspring in Wistar rats (Gonzalez-Gonzalez et al., 2025).

The 4L/20D group showed increased entries but decreased time spent in the social zone compared to the 20L/4D group, and their distance in the social zone was also reduced compared to the control, suggesting that extended dark adaptation decreased the preference for social groups. In female *Physalaemus pustulosus,* light level variations influence mate choice. The females tend to commit to an initial mate choice under dim light, regardless of attractiveness, indicating that ambient light affects decision-making (Baugh et al., 2010). Research in psychological science highlighted that prolonged darkness increases self-serving behaviour and may reduce social preference (Zhong et al., 2010). Therefore, increased light and darkness differently affect social preference behaviour in zebrafish, suggesting that a proper sleep-wake cycle is essential for better social connections.

4.5.2. Shifted gut microbial composition in association with varied photoperiod

The zebrafish raised under different light-dark cycles exhibited a definitive shift in their gut microbial communities. In the standard light-dark condition (14L/10D), phyla such as Pseudomonadota, Actinomycetota, and Fusobacteriota were predominant, forming a core part of the zebrafish gut microbiome. These core microbes play a crucial role in maintaining gut homeostasis and are involved in metabolic functions like carbohydrate, amino acid, and lipid metabolism (Kayani et al., 2022). Although Pseudomonadota is sometimes linked to infections or parasitic burdens (Gaulke et al., 2016; Gaulke et al., 2019), this phylum also includes a wide variety of beneficial bacteria. Many of its members are commensals that assist in

digestion and provide protection against pathogens (Rawls et al., 2004). Additionally, the presence of Bacillota, Planctomycetota, and Bacteroidota is considered advantageous for zebrafish immune response, metabolism, and homeostasis (Willms et al., 2021; Pardue et al., 2023; Green et al., 2024). *Acinetobacter*, *Cetobacterium*, and *Streptomyces* were the dominant genera in 14L/10D, followed by *Burkholderia*, *Bosea*, and *Aerococcus*. The top six bacterial species in the standard photoperiod condition included *Cetobacterium somerae*, *Streptomyces sp*., *Acinetobacter baumannii*, other *Acinetobacter sp*., *Bosea sp*., and *Burkholderia dolosa*. *Cetobacterium somerae* increases zebrafish's antiviral immunity (Liang et al., 2024), improves gut and liver health, lowers intestinal inflammation, increases antioxidant capacity, and improves lipid metabolism (Xie, 2022). It also contributes to gut microbiota homeostasis, which is important for sustaining a healthy gut and preventing dysbiosis (Li et al., 2023) by producing vitamin B12 (Tsuchiya et al., 2008; Qi et al., 2023). *Streptomyces* produce antimicrobial compounds that can strengthen the immune response and reduce infection severity (Cheepurupalli et al., 2017), which is vital for the host organism's health. Although a small percentage of pathogenic species like *Acinetobacter baumannii* (Neto et al., 2023; Ketter et al., 2018) were present, other species in this genus are well-suited to the gut environment and stimulate pro-inflammatory cytokines (Glover et al., 2022). Research also indicates that zebrafish with bold phenotypes are dominated by *Burkholderia* species (Ayayee & Wong, 2024). Even if specific studies on *Burkholderia dolosa* in the zebrafish gut are scarce, the broader impact of *Burkholderia* species on gut health and disease suggests they may have both positive and negative effects, depending on the context (Gigan et al., 2024; Li et al., 2022). Similarly, although studies have not specifically addressed the role of

Bosea species in the gut microbiome, these Gram-negative, rod-shaped bacteria are primarily known for their association with plant root nodules, particularly in legumes (De Meyer & Willems, 2012).

Under the extended light condition of 20L/4D, there was an equal presence of Actinomycetota and Pseudomonadota, while other phyla such as Planctomycetota, Bacillota, Fusobacteriota, and Bacteroidota appeared in much smaller amounts, suggesting an adaptation to prolonged light exposure. Continuous light exposure in zebrafish has been connected with increased cortisol levels and reduced intestinal motility. It can be utilised as a stress model that affects gut health (Lee et al., 2024). This stress can disrupt circadian rhythms and help create a more favourable environment for certain bacteria. This may potentially boost the presence of Actinomycetota and Pseudomonadota. This change in microbial composition may influence behavioural phenomics and cause more stress-like behaviours and aggression. At the genus level, *Streptomyces* and *Burkholderia* were predominant, followed by *Paludisphaera*, *Bosea*, and *Corynebacterium*. Among these, *Streptomyces sp*. was the most prevalent, followed by *Burkholderia dolosa*, *Paludisphaera borealis*, and *Burkholderia cepacia*. *Streptomyces sp*. from marine sources showed probiotic quality, and zebrafish exposed to *Streptomyces* demonstrate increased resistance to pathogenic infections (Liang et al., 2022; Cheepurupalli et al., 2017). Within *Burkholderia*, *Burkholderia cepacia* is known to be pathogenic in various hosts (Coenye & Vandamme, 2003; Gigan et al., 2024). Although specific increases in *Paludisphaera borealis* in the gut are not directly addressed in studies, research highlights that gut microbiota is highly responsive to internal and external stimuli, including changes in photoperiod.

In contrast, the extended dark condition of 4L/20D selectively supported the phylum Bacillota, with Pseudomonadota and Actinomycetota present in smaller quantities. Bacillota can combat pathogenic species, enhance immune response, and maintain gut health (Karvonen et al., 2021; Wedekind et al., 2010; Sharpton et al., 2021). At the genus level, *Bacillus* was dominant, with *Bacillus velezensis* and other *Bacillus sp*. being greatly favoured by the extended dark environment. Supplementing zebrafish with *B. velezensis* has been reported to improve gut barrier function, which is crucial for developing resistance against pathogens and improving gut health. This effect is through influencing genes related to the gut barrier and reducing the expression of pro-inflammatory genes in the gut (Qi et al., 2024; Byun et al., 2023). Several species of this genus also possess probiotic properties (Wu et al., 2022). Thus, the extended dark condition shifted the microbiome to select a particular group, which is more stable and beneficial for gut health. Although species diversity is higher in 20L/4D, it also favoured some pathogenic species, potentially leading to increased aggression. There are shared species between the control and extended light groups, but the extended dark group strongly favoured *Bacillota*, possibly contributing to lower anxiety and aggression levels. The supplementation of probiotics resulted in a significant increase in Firmicutes and a decrease in Proteobacteria, indicating that specific changes in the microbial community can influence the production of neuroactive molecules and behaviour (Borrelli et al., 2016). In the previous chapter with exposure to natural environmental enrichment also solely favoured Bacillota with decreased microbial diversity.

4.5.3. Varied photoperiod and brain transcriptomic profile

In the 20L/4D condition, genes such as *gnat1*, *exorh*, *LOC100537* (*plcb4a*), *saga*, *zgc:112320* (*pde6ga*), *rcvrn3*, *asmt*, *rpl13a*, *knop1*, *eno1a*, and *rpl9* are significantly

upregulated. The genes such as *gnat1*, *saga*, and *pde6ga* mediate signal transduction, which is notably influenced by extended light exposure. The *gnat1* gene encodes the G protein alpha transducing activity polypeptide 1, involved in the adult zebrafish brain's signal transduction processes including pathways related to light stimulus detection, visual perception, and phototransduction. It is expressed in the head, particularly in the pineal complex and visual system (ZFIN). These genes transmit signals from various receptors to intracellular effectors, impacting numerous physiological processes (Wang et al., 2022; Millett et al., 2024). KEGG pathway analysis also shows increased intracellular signalling pathways and cellular degradation processes. The increased expression of *gnat1* in the zebrafish brain may be linked to biological functions or responses to environmental factors, such as stress or injury, which could activate molecular pathways related to neural plasticity and regeneration (Cosacak et al., 2015; Eachus et al., 2019). The *saga* gene, also known as s-antigen, retina and pineal gland arrestin a, is predicted to facilitate G-protein coupled receptor binding activity (ZFIN). It is involved in light-induced signal transduction in photoreceptor cells (Craft et al., 1990), and prolonged light exposure leads to the upregulation of arrestin proteins in these cells (Codega et al., 2009). Whereas PDE6 is recognised as a crucial enzyme in the phototransduction pathway. It plays a major role in the hydrolysis of cGMP in photoreceptors, essential for transmitting visual signals (Lagman et al., 2016). Mutations in *pde6* genes, such as *pde6ga*, can result in photoreceptor degeneration and are linked to inherited retinal disorders (Collery & Kennedy, 2010; Stearns et al., 2007). The increased expression of *pde6ga* might be a compensatory response to stress or damage in photoreceptors, as observed in models of retinal degeneration where cGMP levels are disrupted (Iribarne & Masai, 2017). Light exposure has been associated with

photoreceptor cell death and the development of retinitis pigmentosa (RP). The photoreceptor cell death in the *ovl* zebrafish model with outer segment deficiency is linked to light exposure (Nakao et al., 2012). The *asmt* gene is found to be the final enzyme in the melatonin biosynthesis pathway, converting N-acetylserotonin to melatonin, a process crucial for maintaining circadian rhythms and other melatonin-mediated physiological functions (Rath et al., 2016). The *exorh* (extraocular rhodopsin) gene in adult zebrafish is associated with deep brain photoreceptors, playing a crucial role in non-visual light detection. This gene is part of a broader system that allows zebrafish to respond to light stimuli and is thought to participate in various physiological processes and behavioural responses related to changes in environmental light and neural regeneration (Kojima et al., 2000; Fernandes et al., 2013). The signal transduction pathways, like MAPK and PKA, are triggered by exposure to light. These pathways are involved in the regulation of gene expression in photoreceptor cells (Yu et al., 2007). Consequently, alterations in light conditions may lead to the increased expression of the *exorh* gene. Additionally, the heightened expression of *Exorh*- might be linked to behavioural adaptations to light, such as shifts in activity patterns or stress responses, which are vital for survival in different light environments (Fernandes et al., 2013). Moreover, exo-rhodopsin is believed to facilitate nonvisual photodetection in zebrafish (Mano et al., 1999; Vigh et al., 2002; Kokel et al., 2013). The gene *plcb4a* is expected to involve phosphatidyl inositol phospholipase C activity (ZFIN). PLCβ4 gene expression is crucial for mammals in regulating calcium balance and supporting neurogenesis. Conditions like starvation and oxidative stress, which are linked to sustaining calcium homeostasis and regulating neurogenesis, lead to the upregulation of PLCβ4 (Chen et al., 2022). The *rpl13a* gene belongs to a larger group of ribosomal proteins essential for ribosome

formation and may play roles beyond protein synthesis, such as affecting neural plasticity and regeneration. The increased expression of *rpl13a* in zebrafish indicates a possible mechanism for promoting neural repair and regeneration (Provost et al., 2013; Cosacak et al., 2015; Xing et al., 2023). Mutations in ribosomal protein genes, including those akin to *rpl9*, have been associated with growth defects and a predisposition to tumours in zebrafish, suggesting that upregulation might serve as a compensatory response to mitigate these issues (Lai et al., 2009). The upregulation of ribosomal proteins has been linked to axon regeneration following injury, implying that heightened expression of *rpl13a* and *rpl9* could be part of a brain regenerative process (Xing et al., 2023). Knop1, a lysine-rich nucleolar protein, is vital for ribosome biogenesis, which is crucial for cell proliferation and survival, especially in rapidly dividing neural progenitor cells. The upregulation of such genes often indicates increased cellular activity, potentially in response to developmental signals or environmental changes (Brombin, 2015; Liang et al., 2023; Feijoo et al., 2009). An important enzyme in the glycolytic pathway, enolase1a, helps transform 2-phosphoglycerate into phosphoenolpyruvate. Since neurones mainly depend on glucose metabolism for survival and function, this step is essential for energy production in cells (Butterfield Lange, 2009; Jiang et al., 2019). It is also implicated in neurodegenerative diseases like Alzheimer's, where its increased expression is associated with altered glucose metabolism and oxidative stress. This suggests that elevated *eno1a* expression in zebrafish might be a compensatory mechanism to sustain neuronal function under stress (Butterfield & Lange, 2009). KEGG analysis also correlates with the gene expression that altered pathways involve lipid processing, amino acid and carbohydrate metabolism, immune and signalling pathways, cellular degradation, and vesicular transport. Therefore, zebrafish exposed

to the 20L/4D photoperiod condition exhibit transcriptomic profiles indicative of a metabolic shift, likely driven by increased energy demands. Additionally, evidence of neuro-immune activation suggests a stress response, accompanied by cellular reorganisation, enhanced plasticity, and stress-associated alterations at both metabolic and molecular levels. The genes that are significantly upregulated in the 4L/20D group include *nr4a1*, *six6a*, *h1fx*, *LOC100332*, *btg2*, *histh1l*, *fance*, *serpina10a*, and *uimc1*. The gene *nr4a1* is recognised for its role in mediating anti-inflammatory responses in the brain. It controls the activation of microglia, which are the central nervous system's resident immune cells, keeping them in a non-inflammatory state. This regulation is vital to prevent excessive neuroinflammation, which can result in neurodegenerative diseases (Liu et al., 2023; Liu, 2022; Rothe et al., 2017). Additionally, *nr4a1* is involved in the differentiation of neuronal progenitors, especially within the dopaminergic system. It contributes to the development of striatal structures and affects dopamine receptor signalling, which is crucial for normal neuronal function and behaviour (Cirnaru et al., 2019; Chen et al., 2013). In medaka, *nr4a1* has been associated with behaviour resembling seasonal depression. Its suppression during winter conditions is linked to heightened depression-like symptoms, whereas activating *nr4a1* can counteract these effects, highlighting its role in mood regulation (Nakayama et al., 2023). The upregulation of *nr4a1* is linked to the increased expression of neuroprotective genes, which enhances neuronal survival under stress. This suggests that *nr4a1* mediates neuroprotection and may provide therapeutic benefits for diseases like epilepsy and stroke (Liu et al., 2023; Zhang et al., 2016; Volakakis et al., 2010). The *six6a* gene plays a crucial role in the formation of the zebrafish eye. Experiments involving the knockdown of *six6b*, a gene similar to *six6a*, in zebrafish have demonstrated that

decreased expression leads to smaller eyes with poorly developed lenses and a narrower optic nerve. This indicates that *Six6a* is crucial for eye morphogenesis and optic nerve development. Increased expression correlates with increased *cdkn2b* levels, affecting cell cycle balance. *Six6a*'s expression also impacts brain development, as it is active in neural development areas, indicating a wider role in forming neural structures (Iglesias et al., 2014). The upregulation of *six6a* might impact forebrain development, similar to other homeobox genes like *six3*, which are involved in the enlargement and organisation of the forebrain (Kobayashi et al., 1998). This suggests that *six6a* could similarly influence brain patterning and growth. The increased expression of *btg2* suggests its role in limiting cell growth and encouraging differentiation, which is essential for sustaining normal brain function and development. The heightened expression is associated with various cellular reactions, including those to genotoxic stress, where *btg2* is activated through a mechanism dependent on p53 (Patel et al., 2023; Puisieux & Magaud, 1999). The upregulation of *histh1l* may indicate a greater demand for chromatin remodelling to support gene expression changes necessary for brain development or adaptation to new environments (Cavalieri & Cavalieri, 2020; Izzo et al., 2008). As a member of the Fanconi anaemia (FA) complementation group, the *fance* gene plays a crucial role in DNA repair mechanisms, which are essential for maintaining genomic stability. The *fance* genes are expressed in high-proliferation brain regions in zebrafish, suggesting a role in neurodevelopment and possibly in preventing conditions like microcephaly, which is a common characteristic in FA patients. The increased expression of *fance* in zebrafish brain tissues points to its involvement in DNA repair during rapid cell division and neurogenesis, which are crucial for proper brain development and function (Titus et al., 2009). In various cancers, *fance*

expression is elevated, correlating with tumour progression and prognosis. This indicates that *fance* could serve as a biomarker for cancer prognosis and might predict responses to cancer immunotherapy (Zhou et al., 2023). *Uimc1* is also anticipated to participate in double-strand break repair and the positive regulation of DNA repair (ZFIN). The gene *serpina10a*, also referred to as neuroserpin, is a member of the serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin). Neuroserpin plays a protective role in the nervous system by reducing excessive proteolytic activity, which can otherwise result in neuronal harm (Miranda & Lomas, 2006; Galliciotti, 2006). The neuroserpin expression is notably high during the later phases of neuronal development and continues to be significant in the adult brain, underscoring its importance in sustaining synaptic plasticity (Man & Ma, 2012; Galliciotti, 2006). An adaptive mechanism to improve synaptic connectivity and plasticity, which are essential for cognitive processes, may be indicated by an increase in neuroserpin expression in the brain of zebrafish (Reumann et al., 2017; Man & Ma, 2012). In transgenic models, changes in neuroserpin expression have been associated with variations in emotional behaviour and cognitive abilities, highlighting its vital role in mood regulation and learning (Man & Ma, 2012; Galliciotti, 2006). According to transcriptomic data, such as KEGG analysis, extended exposure to the 4L/20D photoperiod causes the zebrafish brain to undergo a prolonged neuroimmune and metabolic reaction. Despite the activation of protective mechanisms such as nr4a1-mediated anti-inflammatory regulation, the simultaneous enhancement of immune signalling and metabolic reprogramming pathways indicates a shift from adaptive to stress. Compensatory responses, which may alter circadian stability and neural homeostasis. The comparison of pathways between 20L/4D and 4L/20D indicates that zebrafish under

extended light have elevated metabolic rates, increased immune responses, and oxidative stress adaptation, which could be relevant to circadian biology and stress resistance. The genes that are downregulated in both conditions include *ndufb9*, *ndufa1*, *mt2*, *ba1l*, *mrps26*, *coq9*, *sidkey2*, and *glipr1b*. The downregulation of *ndufb9* and *ndufa1* may result in mitochondrial dysfunction, leading to decreased ATP production and heightened oxidative stress, which are linked to various neurodegenerative illnesses such as Parkinson's and Alzheimer's. This is attributed to the mitochondria's role in energy production, calcium balance, and apoptosis regulation (Chen et al., 2024; Smeitink et al., 2004; Loeffen et al., 1998). *Coq9* is also related to mitochondria, maintaining the stability and functionality of *CoQ10*, a vital electron carrier in the mitochondrial respiratory chain. A deficiency in *Coq9* can cause a drop in *CoQ10* levels, impairing mitochondrial bioenergetics and reducing ATP production (García-Corzo et al., 2013). According to Hroudová et al. (2014), the downregulation of mitochondrial protein S26 may result in impaired oxidative phosphorylation and elevated aerobic glycolysis, which could interfere with neural proliferation and contribute to neurodevelopmental disorders. The downregulation of *mt2* in neuropathic pain and neurodegenerative disease models, the susceptibility to oxidative stress and inflammation is increased (Huang et al., 2021; Najib et al., 2021). Although *mt2* is crucial for neuroprotection and stress response, its downregulation alone does not determine neurodegenerative outcomes. The general neural health of zebrafish is also influenced by additional variables, including genetic predispositions, environmental exposures, and the presence of additional protective or detrimental proteins. Though the specific role of *ba1l* in the zebrafish brain is not clearly detailed in the provided studies, the general understanding of globin gene function and regulation offers insights into its potential

importance. The downregulation of *ba1l* might indicate an adaptive response to environmental stressors like hypoxia, affecting oxygen transport, and metabolic processes in the brain. Further research is needed to clarify the exact mechanisms and implications of *ba1l* downregulation in zebrafish brain function (Roesner et al., 2006). Under prolonged photoperiod stress, the collective downregulation of mitochondrial genes suggests a weakened neuro-energetic and protective capacity in the zebrafish brain. Potentially making neural tissue more susceptible to oxidative damage, metabolic imbalance, and impaired neurodevelopment.

In conclusion, the shift in photoperiod from the early larval stage to adulthood in zebrafish affects their behavioural repertoire along with gut metagenomics and brain transcriptomics. Despite a decrease in gut microbial diversity, the prevalence of the beneficial phylum Bacillota in the gut and the upregulated expression of genes related to neuroprotection and plasticity in the brains of zebrafish in the 4L/20D group presumably contributed to their lower anxiety and reduced aggression. Conversely, the 20L/4D group, with its higher microbial diversity and more pathogenic species, along with the upregulation of genes associated with increased energy demands, neuroimmune activation, and stress adaptation, likely experienced mild stress-like symptoms and heightened aggression. Social preference was expressed differently but diminished in both extended light and dark conditions. Additionally, the downregulation of mitochondrial genes impacts the neuroenergetic and protective capacity in the brains of zebrafish exposed to altered light-dark cycles, 20L/4D and 4L/20D. However, zebrafish raised under extended light conditions are more susceptible to adverse changes in behaviour, gut microbial composition, and brain transcriptomic profiles.

4.6. References

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CHAPTER 5

Influence of environmental changes on avoidance learning behaviour in zebrafish

Rationale: The influence of dietary and environmental factors including antibiotic exposure, environmental enrichment, and photoperiod on behaviours such as anxiety, aggression, social preference, and gut microbiota composition has been established in previous chapters. Building on this foundation, investigating how these variables affect fear-induced avoidance learning could further advance our understanding of the impact of dietary and environmental factors on cognitive performance.

5.1. Graphical Abstract

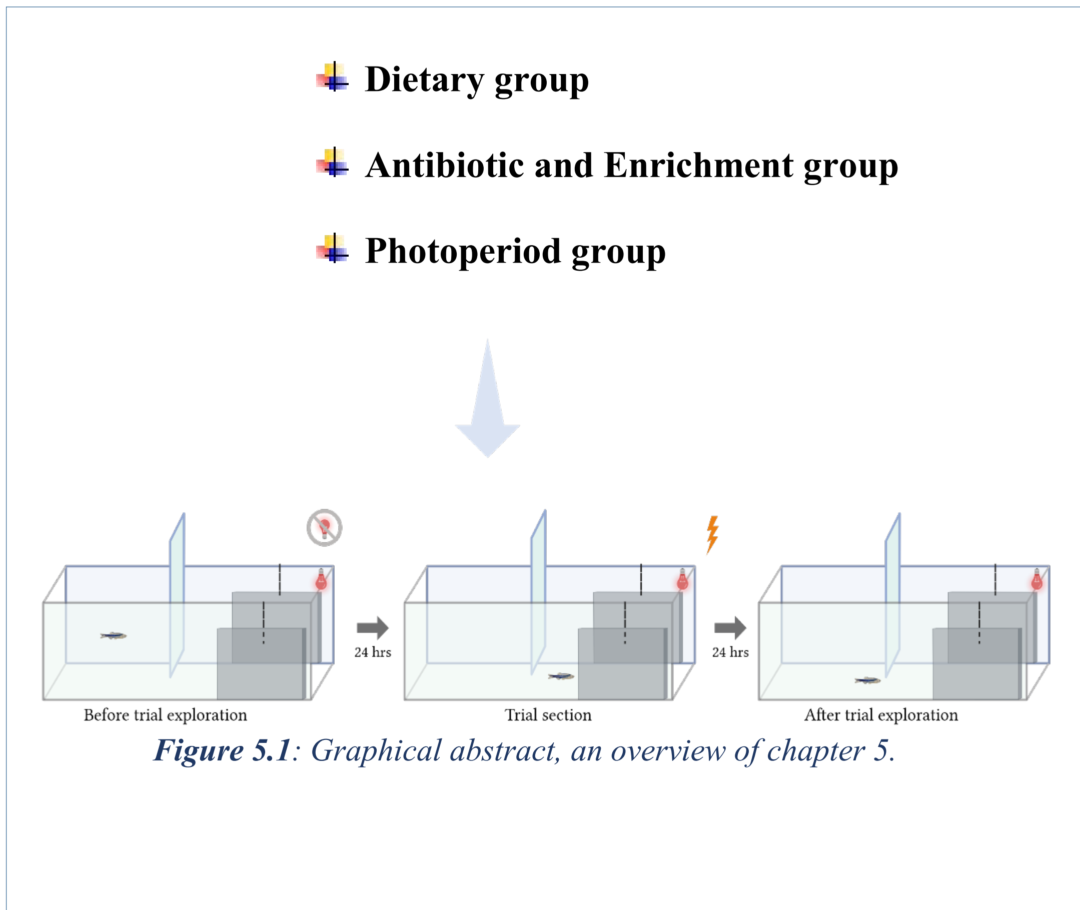


***Figure 5.1**: Graphical abstract, an overview of chapter 5.*

5.2. Introduction

Learning and memory are the processes that permit an organism to adapt to dynamic environments. Associative learning is considered one of the advanced forms of learning and memory. In associative learning, animals learn the association between two stimuli; one is the conditioned stimulus (CS), and the other is the unconditioned stimulus (US). Here, CS occur before US and serve as a predictor of the US (Howard & Eichenbaum,2015; Gerlai, 2016). Neurodegenerative conditions, including Alzheimer's disease, cause dysregulations in associative learning ability (Dickerson & Eichenbaum,2010). The conserved neural transmission circuits, comparable electrical activity patterns and shared neurotransmitter system with mammals, the teleost fish, especially zebrafish, are considered a valuable model in investigating learning and memory (Wullimann et al., 2002; Nam et al., 2004; Blank et al., 2009). There are various methods developed to assess complex behavioural phenotypes in zebrafish (Gerlai, 2012). The learning test protocols in zebrafish derived from rodent experiments include the Y-maze test, the T-maze test, and different avoidance paradigms (Cognato et al., 2012; Aoki et al., 2015; Meguro et al., 2019; Manuel et al., 2014).

In zebrafish, one of the main tests that measures learning and memory is the avoidance learning test. Inhibitory avoidance and active avoidance procedures are in use, the learning and memory associated with fear and anxiety (Stewart et al., 2012; Manuel et al., 2014). Inhibitory avoidance is determined by the time taken by an individual to reach or enter into the area that is conditioned with an aversive stimulus (electric shock). The test utilises an apparatus with black and white compartments, in which the black compartment is equipped with electrode plates (Manuel et al., 2014). Whereas the active avoidance test utilises shuttle boxes with a

hurdle that creates two compartments and an LED light as CS, followed by a mild electric shock as US (Morin et al., 2013; Meguro et al., 2019). Studies revealed that learned behaviours in zebrafish required simultaneous activation of neural groups in the telencephalon region (Aoki et al., 2013). The cholinergic receptor blockage and NMDA receptor antagonism are found to inhibit cognitive performance. Therefore, glutamate, glycine, and acetylcholine neurotransmitters are mainly involved in cognitive functioning (Braida et al., 2014; Ng et al., 2012; Sison & Gerlai, 2011; Cognato et al., 2012). Also found that dysfunction of ribosomal translation or impairment of cell adhesion affects memory formation or consolidation (Pradel et al., 1999; Agranoff et al., 1967).

Here we combined the protocol from both the active and inhibitory avoidance tests. A white acrylic rectangular box with a central sliding door is made, and one compartment is equipped with electrode plates and one LED bulb. The experiment contains 3 phases: before trial learning session, trial learning, and after trial learning. Manuel et al. tested the impact of various shock intensities, including 1, 3, and 9V, in which the response was apparent in 3V, but 9V affected zebrafish muscle control. Therefore, we selected 4.5V to generate an aversive response in zebrafish (Manuel et al., 2014). The avoidance response of dietary exposure to antibiotics, enriched environment, and varied photoperiod groups is discussed in this chapter. How these different environmental paradigms influence the learning process, especially the fear-related learning and memory through the avoidance test, is investigated.

5.3. Materials and methods

5.3.1. Zebrafish under variable treatment groups

The dietary, antibiotic, and enriched groups and the varied photoperiod groups were subjected to avoidance learning tests. Their maintenance is described in previous chapters.

5.3.2. Avoidance learning test

The avoidance learning test in zebrafish is used to determine how successfully zebrafish can avoid electric shock and learn to associate pain with conditioned stimuli. It can also assist in determining the mechanisms underpinning cognitive and stress-related behaviours in zebrafish, which are similar to those in mammals (Buccafusco, 2000). A white acrylic tank with an opaque white sliding partition in the centre was used to test the avoidance learning. One chamber is equipped with electrode plates connected to a 4.5-volt battery (unconditioned stimulus) and a red LED bulb (conditioned stimulus), and the other is a free chamber. First, we exposed zebrafish individually to explore the apparatus, a two-minute acclimatization period in the free chamber was given, then slightly lifted the sliding door (about one cm) and allowed 3 minutes to explore the tank (Exploration- Before Trial Learning- BTL) and the video was recorded. After 24 hours, each fish is subjected to a trial learning session of 20 minutes, in which 2-minute acclimatization in the free chamber followed by the opening (lifting by 1cm) of the sliding door, when the fish entered into the electrode compartment immediately closed the sliding door and LED bulb on for 10-seconds followed by 6-second electric shock. After that, the sliding door was opened and the same procedure was followed if entered again. The maximum number of times each fish the electric shock was given 6 times during the 20-minute trial session. 24 hours followed by the trial session; each fish was again tested for its learning ability (Exploration- After Trial Learning- ATL). Each fish was first introduced into the free chamber and given a 2-minute acclimatization period followed by the opening of the sliding door. It remained open and the video was recorded for 3 minutes. If the fish made entry to the electrode compartment, only the LED bulb was on and no electric shock was given (Manuel et al., 2014;

Meguro et al., 2019). The recorded session was then analysed using Smart 3.0 software.

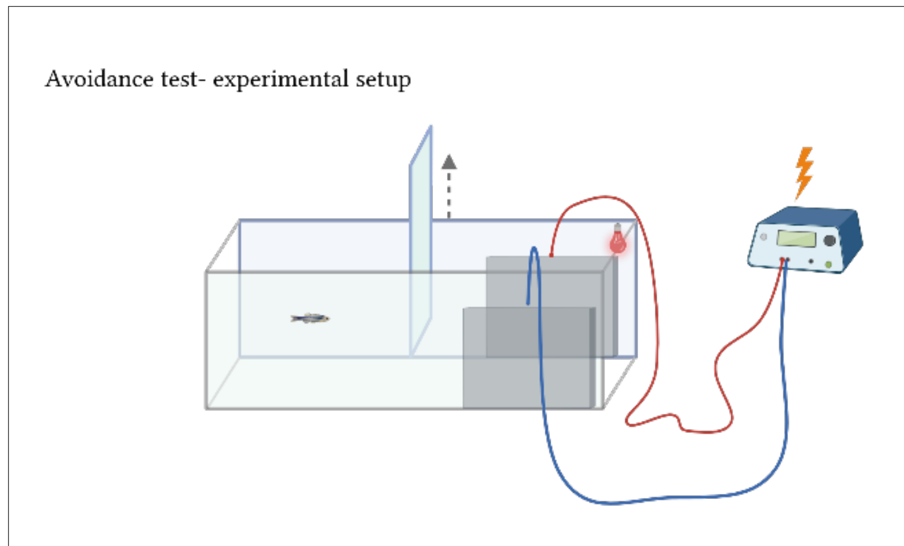


***Figure 5.2**: Avoidance test experimental setup*

5.3.3. Statistical analysis

To compare difference between group means, Kruskal-Wallis test was applied, followed by Dunn's multiple comparison test. Within-group changes were measured using Wilcoxon-matched pairs signed rank test. GraphPad Prism (version 9.5), and Microsoft Excel were used for all statistical analyses and data visualization. Throughout the study, a significance level of $p < 0.05$ was maintained.

5.4. Results

5.4.1. Effect of dietary modulation on cognitive function in avoidance learning test.

The results revealed that different dietary conditions have influence on avoidance learning (Fig. 5.3). Considering endpoints derived from avoidance test before and after trial learning (BTL & ATL), the number of entries towards electrode compartment (EC) markedly decreased after trial in APS ($p = 0.000084$), P ($p < 0.000001$), and S ($p = 0.002283$) compared to entries before trial. No significant

changes observed in AP and A groups ($p > 0.05$). When comparing between groups after trial P group showed prominent reduction of entries towards EC compared to APS ($p = 0.0247$). Significant increase in latency to enter EC were observed within APS ($p = 0.001238$), P ($p = 0.000004$), and S ($p = 0.036649$) groups after trial compared to before trial, but the changes were not statistically significant in AP and A. When considering between groups the latency was greater in P compared to APS ($p = 0.0330$) and S ($p = 0.0220$). However, time spent in EC after trial, an important endpoint marks the avoidance was reduced in APS ($p < 0.000001$), AP ($p = 0.000015$), A ($p = 0.000250$), and P ($p < 0.000001$), but the reduction was not strong enough in S group ($p = 0.05$). When comparing between groups the reduction in time spent was more prominent in P compared to APS ($p = 0.0221$), AP ($p = 0.0489$), and A ($p = 0.0062$). Whereas the distance traveled was lowered in all the groups; APS ($p < 0.000001$), AP ($p = 0.000038$), A ($p = 0.000075$), P ($p < 0.000001$), and S ($p = 0.002025$). Among them P group displayed reduced distance compared to AP ($p < 0.0001$) and A ($p < 0.0001$), APS ($p = 0.0038$) and S ($p = 0.0051$) showed distance lower than AP. No difference observed between APS, P, and S ($p > 0.05$). Similarly, no changes in distance found between AP and A ($p > 0.05$). The overall resting time or freezing was found to be lowered in APS ($p = 0.034220$) while increased in P ($p = 0.033287$) after trial with in groups, though slight increase in A and S is apparent but not statistically significant ($p > 0.05$). And it was obvious between group comparison found that P group exhibit greater resting time compared to APS ($p = 0.0004$) and S ($p = 0.0070$), also resting period in A group was higher with that of APS ($p = 0.0403$). Another endpoint describing normal swimming, the Slow swimming time in EC was decreased in all groups after trial; APS ($p < 0.000001$), AP ($p = 0.000054$), A ($p = 0.001027$), P ($p < 0.000001$),

and S ($p = 0.008069$). When taking between groups the decrease was less prominent in AP compared to other groups APS ($p = 0.0161$), A ($p = 0.0274$), P ($p < 0.0001$), and S ($p = 0.0097$). The fast-swimming duration in EC also decreased after trial in APS ($p = 0.001160$), AP ($p = 0.026047$), A ($p = 0.000556$), and P ($p = 0.013428$), no difference in S group because the fast-swimming behaviour was nearly zero before and after trial. Among APS, AP, A, and P, the fast swimming was greater in A (APS/A - $p = 0.0003$; AP/A - $p = 0.0128$; P/A - $p < 0.0001$). And the total mean speed significantly lowered in APS ($p = 0.041592$) and P ($p = 0.000010$) after trial and P showed lower speed with that of APS ($p = 0.0002$) (Fig. 5.4). The quantity changes in behaviour due to learning was assessed using time-based learning index by comparing time spent in EC before and after trial. And the result revealed that learning index was greater in P group compared to AP ($p < 0.0001$), A ($p < 0.0009$) and S ($p < 0.0001$). No significant difference between APS and P ($p > 0.05$) (Fig. 5.5).

The turning tendency in EC before trial was rightward in APS (+ve) and leftward in AP, A, P, and S (-ve). Whereas, after trial it was shifted slightly leftward in APS, while A and S remain their turning tendency towards left. However, AP and P equalized their turning behaviour as their values approaching nearly zero (Fig. 5.6).

5.4.2. Effect of antibiotics and enrichment on cognitive function in avoidance learning test.

The modulation of zebrafish environment with antibiotic exposure (PG and CPFX - 10mg/L) and enrichment with natural plants and stones significantly influenced their avoidance learning ability (Fig. 5.7). Regarding number of entries towards EC after trial with that of before trial considerable reduction found in C ($p = 0.002302$) and EE ($p < 0.000001$). In contrast increased entries observed in PG ($p = 0.000030$) and no differences in CPFX ($p > 0.05$). Between group comparison after trial confirmed

that entries were higher in PG compared to C ($p = 0.0062$) and EE ($p < 0.0001$) whereas, no difference between C and EE ($p > 0.05$). The latency to enter EC was prominently increased in C ($p = 0.000212$) after trial, no statistically significant increase in PG, CPFX and EE ($p > 0.05$). After trial comparisons showed similar latency between C and EE groups, as well as between PG and CPFX groups ($p > 0.05$). Considering time spent and distance traveled, similar trend observed in C ($p < 0.000001$) and EE ($p < 0.000001$) having substantial reduction in their values after trial compared to before trial. No differences between C and EE, as well as PG and CPFX ($p > 0.05$).

Though the overall resting or freezing was increased in PG ($p = 0.004701$) and EE ($p = 0.000029$) after trial with that of before trial, no variations were found between groups ($p > 0.05$). Slow time or normal swimming time was reduced in C ($p = 0.000005$), PG ($p = 0.001816$) and EE ($p = 0.000012$) but not in CPFX after trial with that of before trial ($p > 0.05$). And comparing across groups exhibit no differences between C and EE, as well as PG and CPFX ($p > 0.05$) but with a greater degree of variation in PG and CPFX with that of C ($p < 0.0001$) and EE ($p < 0.0001$). The fast time or fast swimming time was decreased in C ($p = 0.001027$), CPFX ($p = 0.007204$), and EE ($p < 0.000001$) when comparing within groups before and after trial, but no variation was observed between group comparison after trial ($p > 0.05$). However, the total mean speed was reduced only in EE ($p < 0.000001$) after trial with that of before trial. Inter group comparison displayed higher mean speed in C compared to others ($p < 0.0001$) (Fig. 5.8). The avoidance duration in EC before and after trial was utilised in calculating time-based learning index which was lower in PG and CPFX compared to C ($p < 0.0001$) and EE ($p < 0.0001$) (Fig. 5.9). Before trial C group was showed tendency to turn rightward (+ve) and PG, CPFX, and EE

towards left (-ve) whereas, after trial C group shifted their turns toward left while others persist leftward turning behaviour (Fig. 5.10).

5.4.3. The avoidance learning was not affected by photoperiod modulation.

The zebrafish raised in different photoperiod such as 14L/10D, 20L/4D and 4L/20D have exhibit good avoidance learning behaviour (Fig. 5.11). As evidenced by the results, the entries towards EC were decreased after trial with in groups; 14L/10D ($p = 0.025019$), 20L/4D ($p = 0.000043$) and 4L/20D ($p = 0.000036$) and no variation was found between groups after trial learning ($p > 0.05$). Also, the latency towards EC found to be increased in all the groups after trial compared to before trial; 14L/10D ($p = 0.000023$), 20L/4D ($p = 0.001238$) and 4L/20D ($p = 0.000650$). No statistically significant variation across groups after trial ($p > 0.05$). The time spent and distance traveled in EC appeared parallel trends in control and test groups. Reduced time and distance traveled in EC after trial with in 14L/10D, 20L/4D and 4L/20D groups compared to before trial ($p < 0.000001$). Though the resting time or freezing was appeared increased after trial in 14L/10D and 4L/20D compared to before trial, it was not statistically significant within and between groups ($p > 0.05$). Reduced slow time after trial with that of before trial within groups; 14L/10D ($p = 0.000001$), 20L/4D ($p < 0.000001$) and 4L/20D ($p < 0.000001$). No variation was found between groups when comparing after trial ($p > 0.05$). Fast swimming time also reduced in 14L/10D ($p = 0.000885$), 20L/4D ($p = 0.004972$) and 4L/20D ($p = 0.000305$) groups after trial with that of before trial. When comparing between groups after trial the fast-swimming time was significantly reduced in 4L/20D group compared to 14L/10D ($p = 0.0023$). In contrast mean speed in EC was increased in 20L/4D ($p = 0.002211$) and 4L/20D ($p = 0.004175$) after trial with that of before trial, however no change observed in control ($p > 0.05$). Whereas in fact the mean

speed exhibited by 20L/4D ($p = 0.0172$) and 4L/20D ($p = 0.0079$) were lower compared to 14L/10D (Fig. 5.12).

Since key parameters defining avoidance such as latency, time spent and distance traveled were markedly lower in control and test groups, as well as no significant variation between groups. This confirms that irrespective of change in photoperiod the test groups 20L/4D and 4L/20D did not affect the ability to learn to avoid electric shock therefore no difference between groups observed in learning index ($p > 0.05$) (Fig. 5.13). The turning tendency before trial showed 14L/10D group having rightward direction (+ve) while 20L/4D and 4L/20D having leftward direction (-ve). Whereas, after trial learning 14L/10D group shifted their turns more towards left while 4L/20D altered their turning tendency towards right but 20L/4D equalized their left and right turns so exhibit zero values (Fig. 5.14).

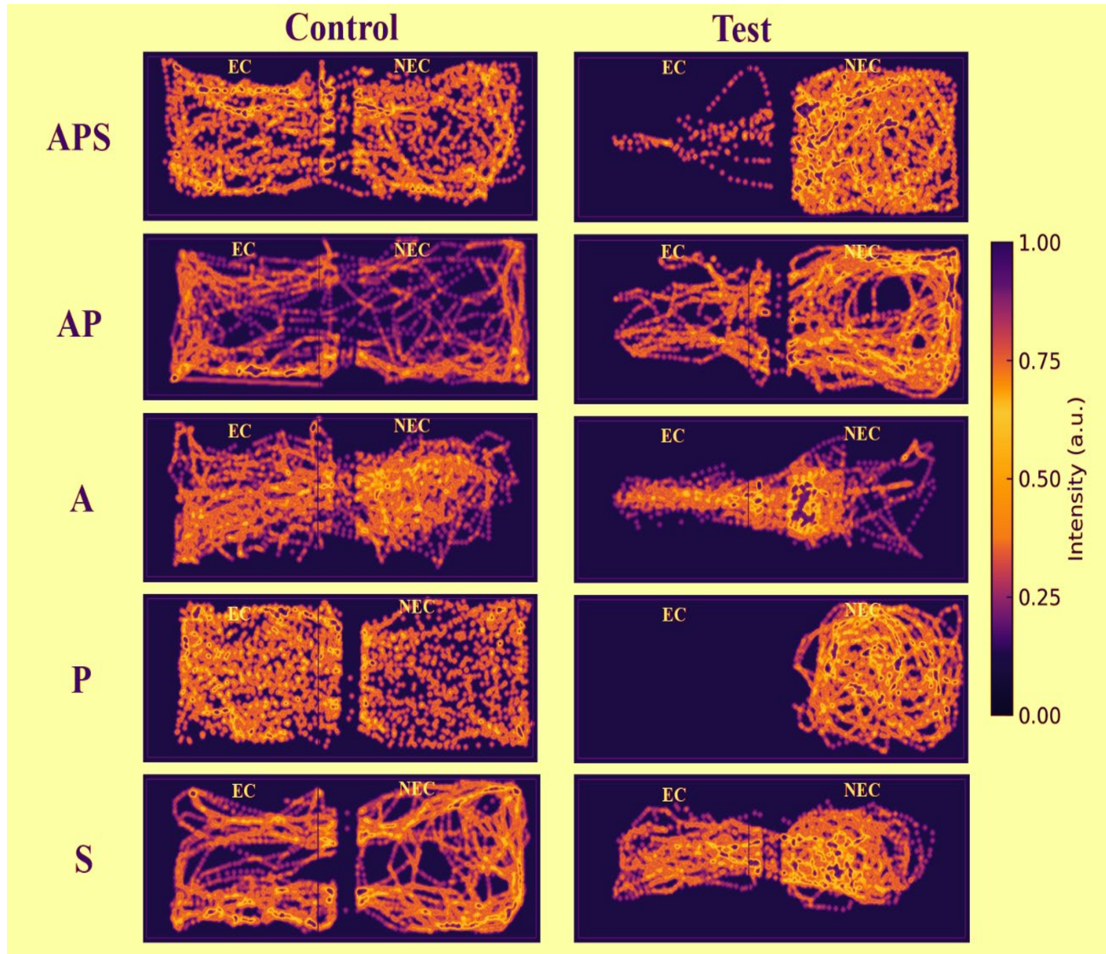
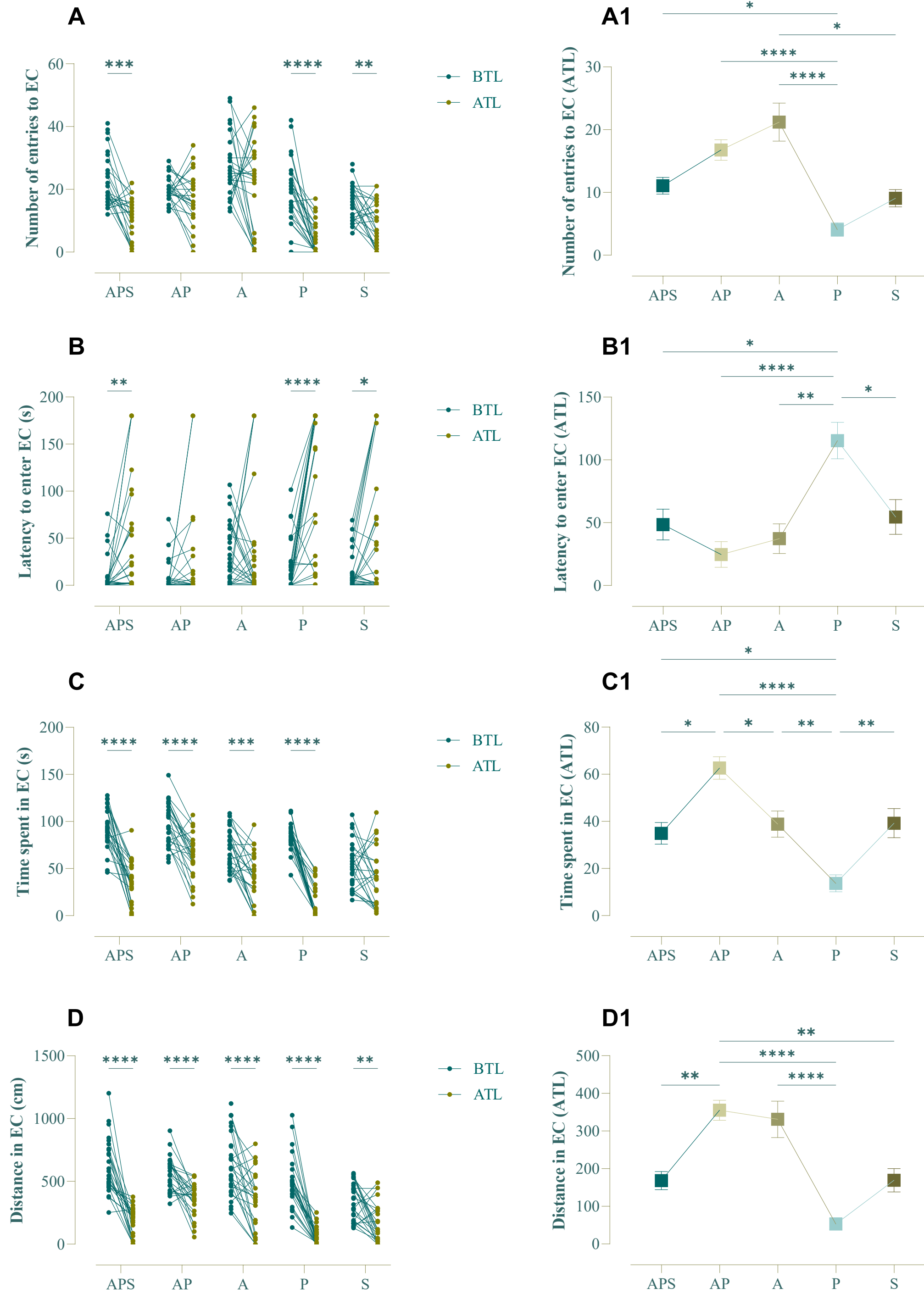


Figure 5.3: *Representative heatmaps illustrating zebrafish exploratory behaviour before (Control) and after (Test) the learning trial under different dietary conditions, generated using Smart 3.0 software. Dietary groups include APS (Artemia + Pellet + Spirulina), AP (Artemia + Pellet), A (Artemia), P (Pellet), and S (Spirulina). Notably, zebrafish in the P and APS groups exhibited increased avoidance behaviour after the trial.*


A
A1
B
B1
C
C1
D
D1
BTL
ATL
Number of entries to EC
Number of entries to EC (ATL)
Latency to enter EC (s)
Latency to enter EC (ATL)
Time spent in EC (s)
Time spent in EC (ATL)
Distance in EC (cm)
Distance in EC (ATL)
APS
AP
A
P
S

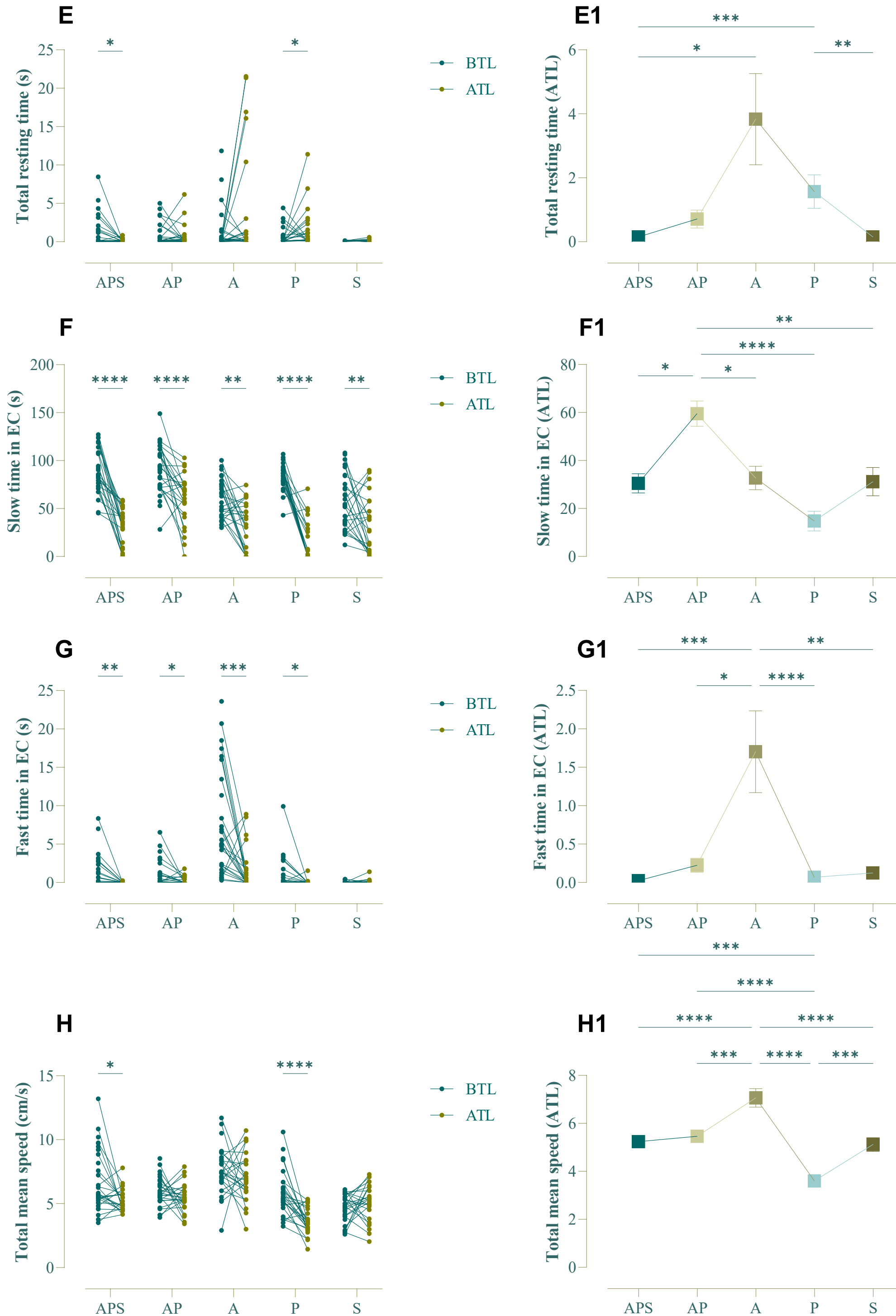


Figure 5.4: *Under dietary modulation, the before–after plots illustrate within-group variations, while the dotted-line plots depict between-group comparisons. (A) Number of entries into the EC before vs. after the trial (within-group). (A1) Number of entries into the EC after the trial (between groups). (B) Latency to enter the EC before vs. after the trial (within-group). (B1) Latency to enter the EC after the trial (between groups). (C) Time spent in the EC before vs. after the trial (within-group).*

*(C1) Time spent in the EC after the trial (between groups). (D) Distance traveled in the EC before vs. after the trial (within-group). (D1) Distance traveled in the EC after the trial (between groups). (E) Total resting time before vs. after the trial (within-group). (E1) Total resting time after the trial (between groups). (F) Duration of slow movement in the EC before vs. after the trial (within-group). (F1) Duration of slow movement in the EC after the trial (between groups). (G) Duration of fast movement in the EC before vs. after the trial (within-group). (G1) Duration of fast movement in the EC after the trial (between groups). (H) Total mean speed before vs. after the trial (within-group). (H1) Total mean speed after the trial (between groups). The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001), (n=25).*

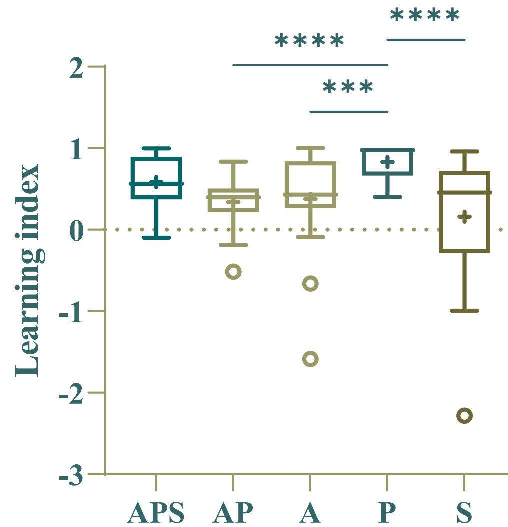


Figure 5.5: *The box plot illustrates the learning index based on the time spent avoiding the EC after the trial compared to before the trial under dietary modulation.*

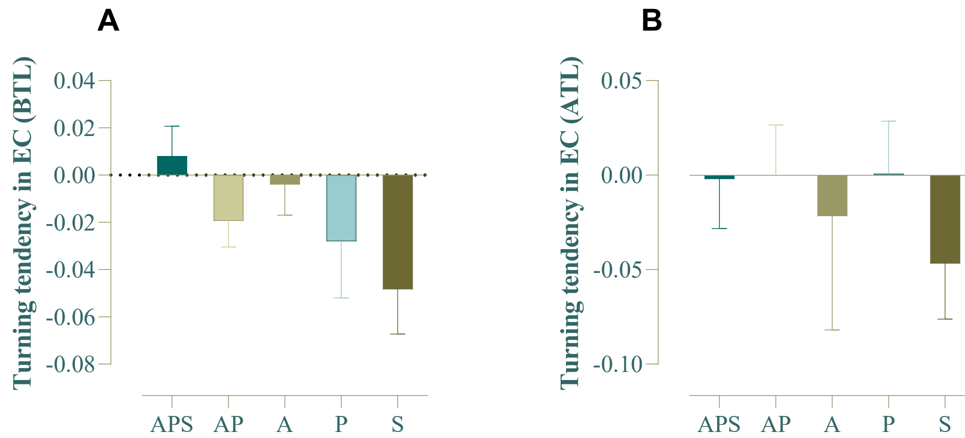


Figure 5.6: *Bar plots representing the turning tendency or directional preference of zebrafish in different dietary groups within the EC, before and after the trial under dietary modulation.*

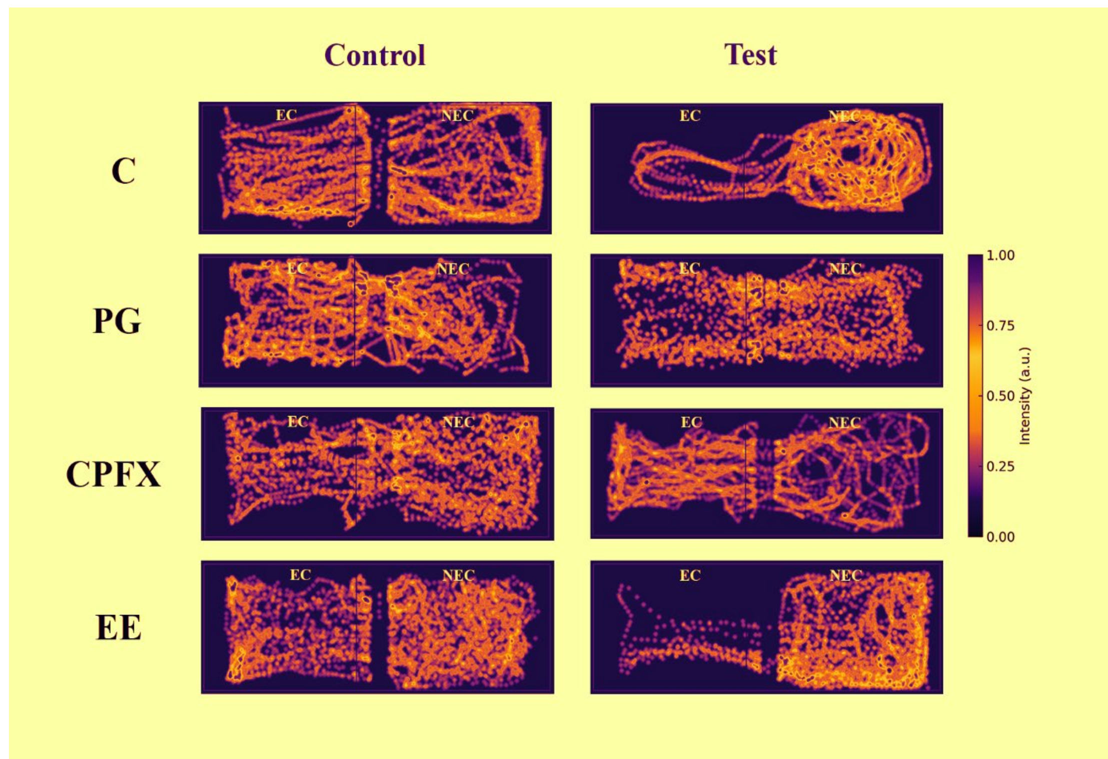
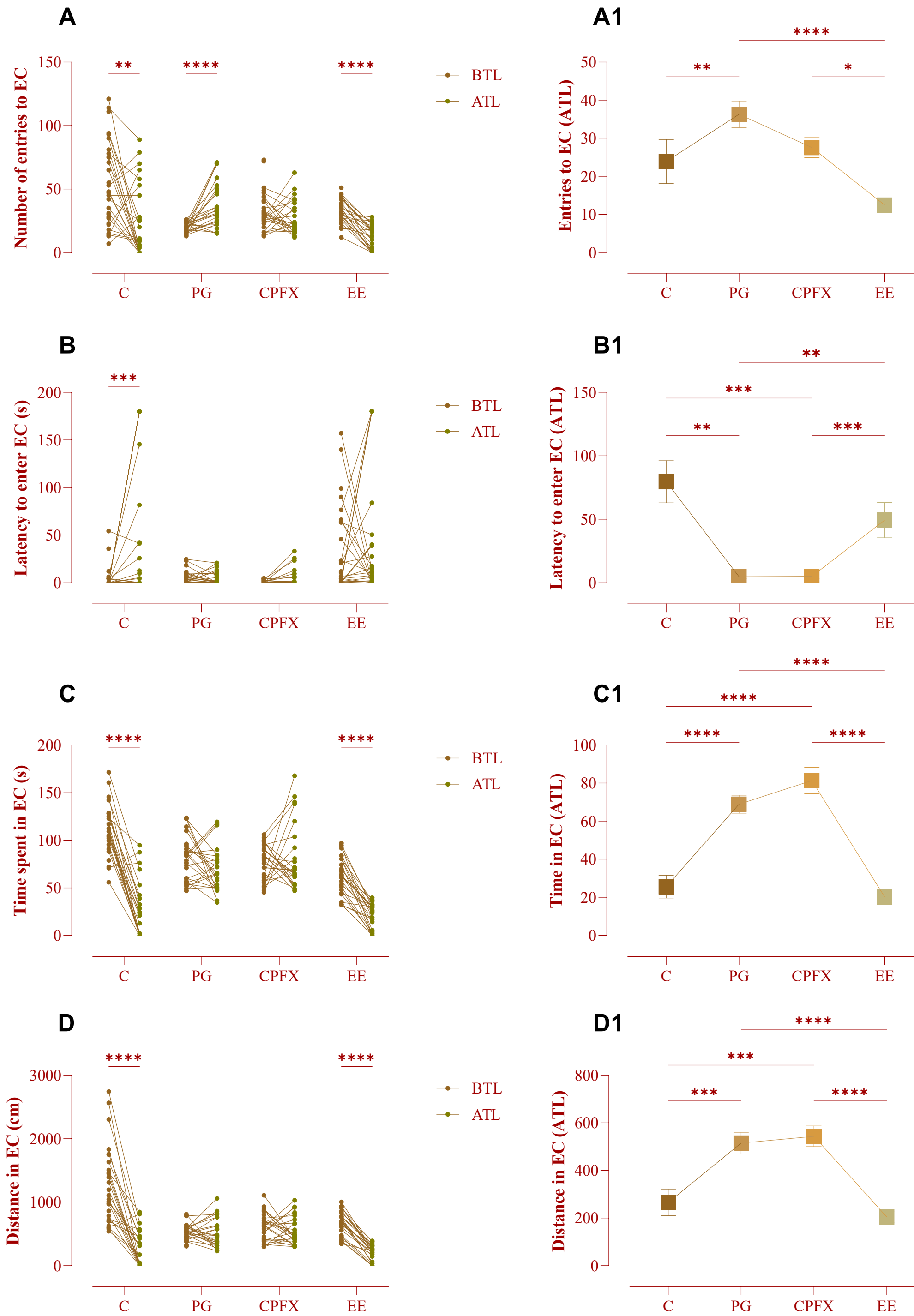


Figure 5.7: *Environmental modulation using antibiotics and enrichment. Representative heatmaps of zebrafish exploratory behavior before (Control) and after (Test) the learning trial, generated using Smart 3.0 software. Experimental groups include: (C) Control, (PG) Penicillin (10 mg/L), (CPFX) Ciprofloxacin (10 mg/L), and (EE) Enriched Environment.*


A
Number of entries to EC
C
PG
CPFX
EE
BTL
ATL
A1
Entries to EC (ATL)
B
Latency to enter EC (s)
B1
Latency to enter EC (ATL)
C
Time spent in EC (s)
C1
Time in EC (ATL)
D
Distance in EC (cm)
D1
Distance in EC (ATL)

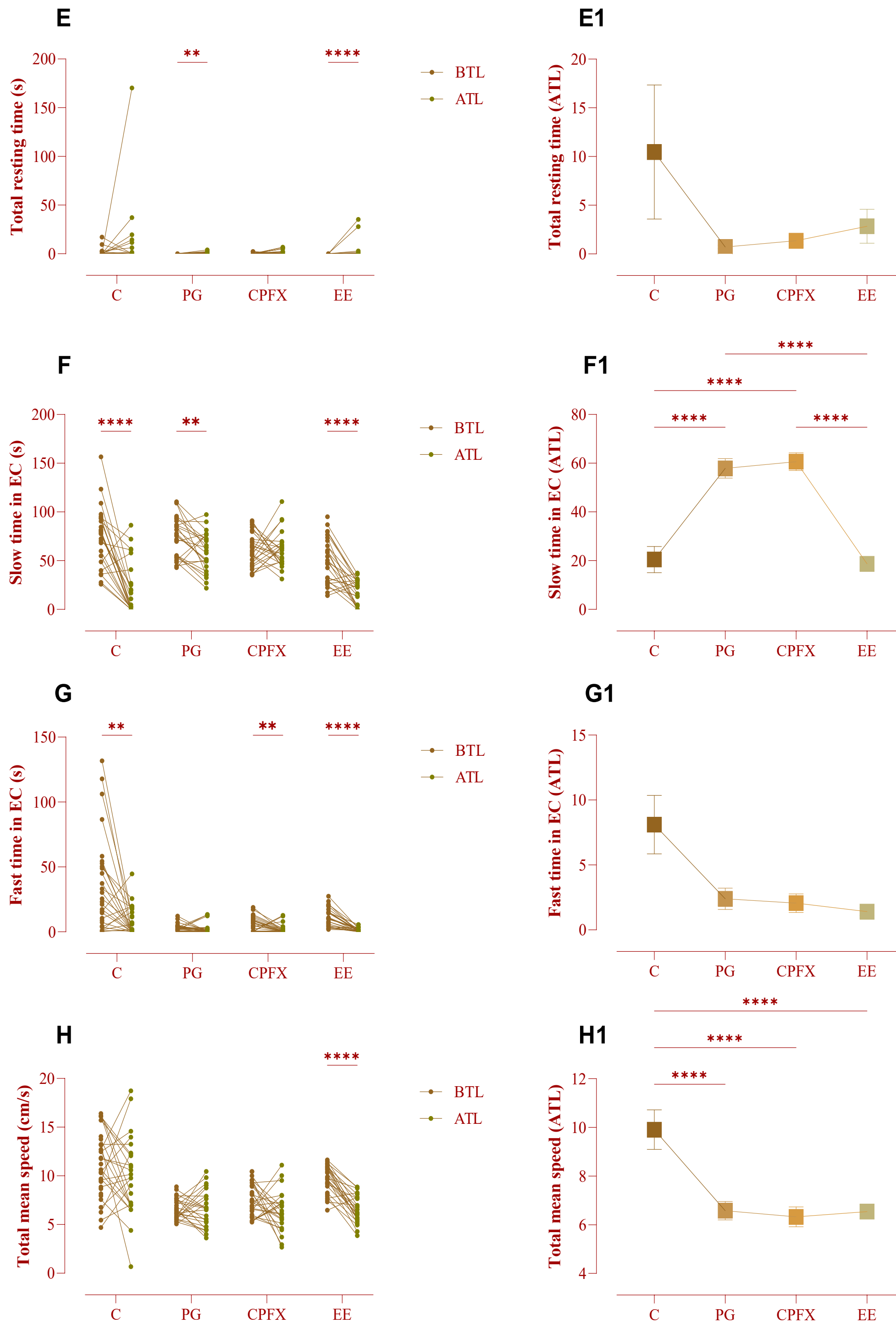


***Figure 5.8**: Environmental modulation with antibiotics and enrichment, the before-after plot describes variation with group and dotted-line plot comparing between groups. (A) Number of entries to EC before vs. after trial with in group. (A1) Number of entries to EC after trial between groups. (B) Latency to enter EC before vs. after trial with in group. (B1) Latency to enter EC after trial between groups. (C) Time spent in EC before vs. after trial with in group. (C1) Time spent in EC*

*after trial between groups. (D) Distance in EC before vs. after trial with in group. (D1) Distance in EC after trial between groups. (E) Total resting time before vs. after trial with in group. (E1) Total resting time after trial between groups. (F) Slow time in EC before vs. after trial with in group. (F1) Slow time in EC after trial between groups. (G) Fast time in EC before vs. after trial with in group. (G1) Fast time in EC after trial between groups. (H) Total mean speed before vs. after trial with in group. (H1) Total mean speed after trial between groups. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001), (n=30).*

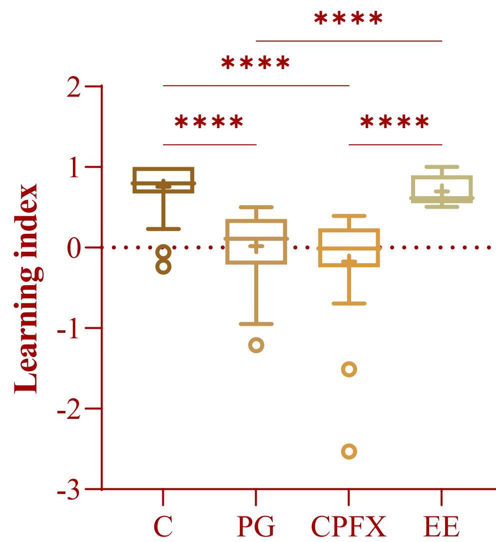


Figure 5.9: *Environmental modulation with antibiotics and enrichment, the box plot displays learning index based on time avoiding the EC after trial with that of before trial.*

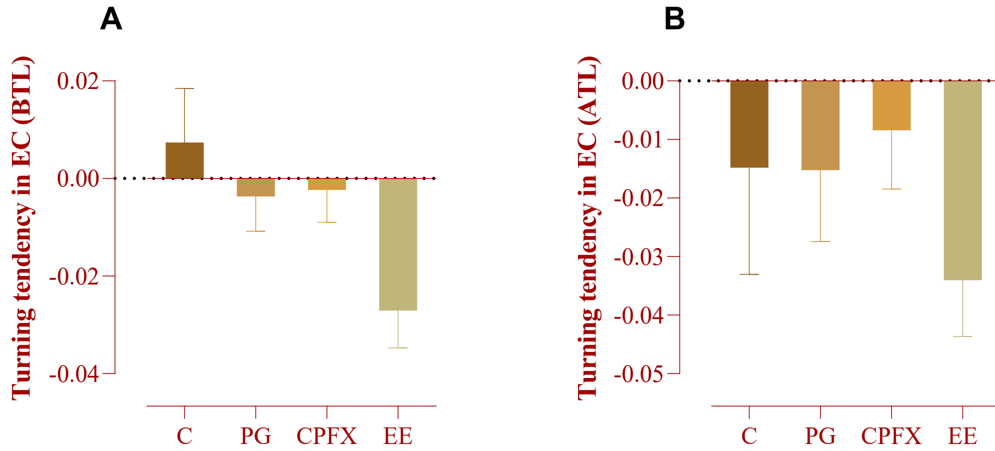


***Figure 5.10:** Environmental modulation with antibiotics and enrichment, the bar plot showing turning tendency or direction of antibiotic and enrichment groups in EC before trial and after trial.*

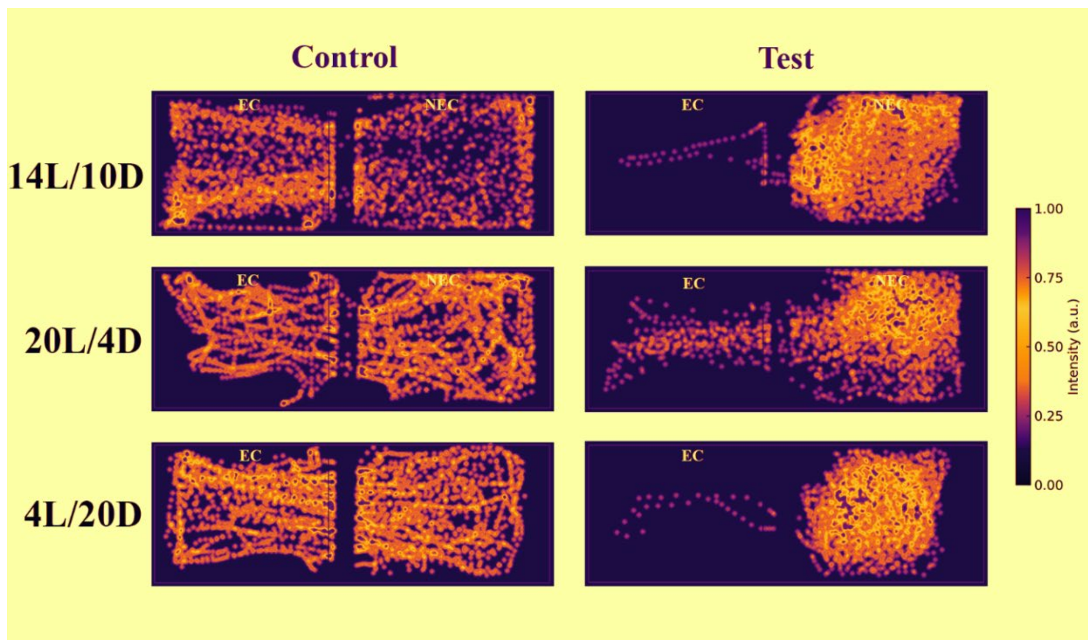
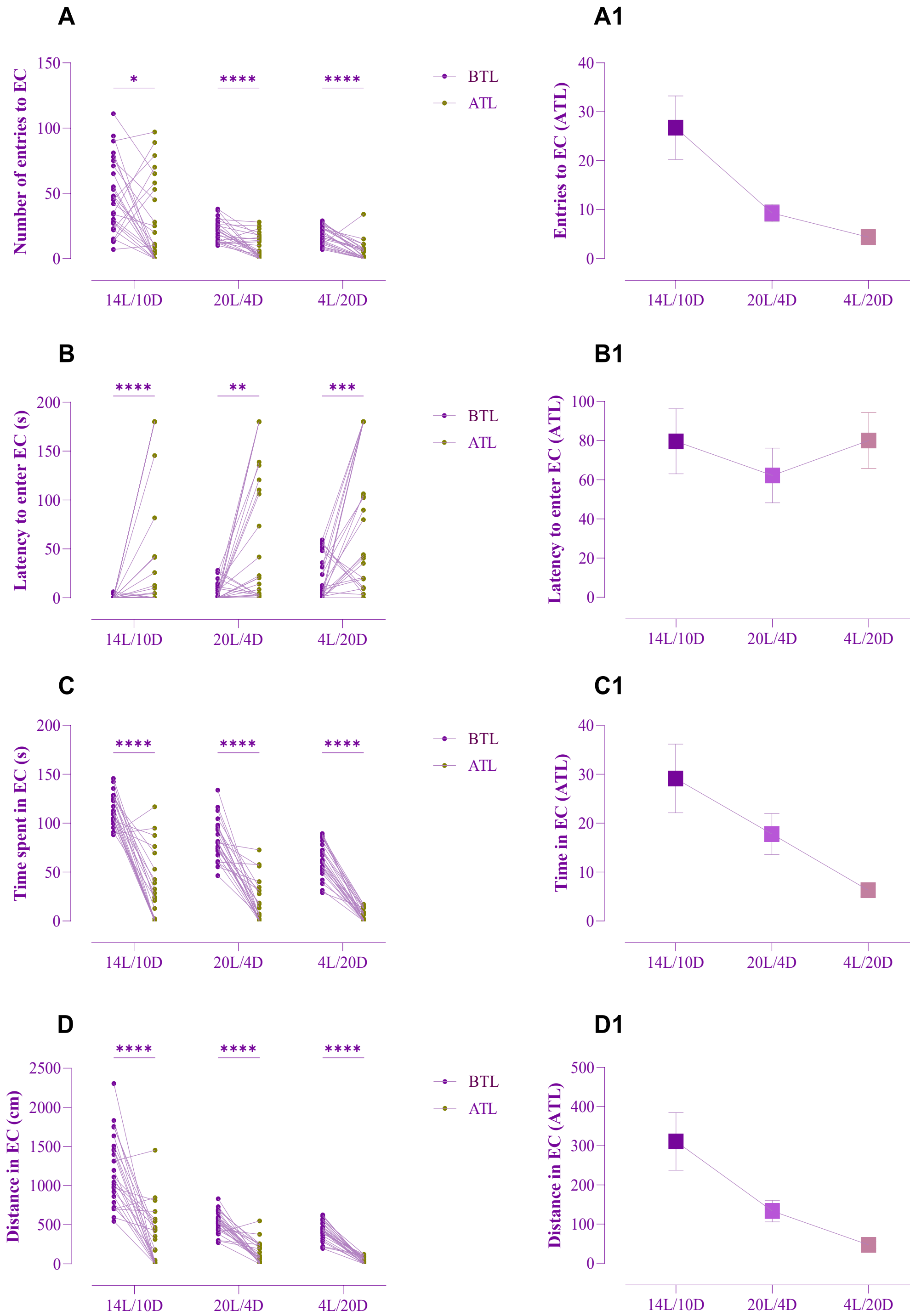


***Figure 5.11:** Photoperiod modulation, representative heatmap of zebrafish exploration before trial learning (Control) and after trial learning (Test) obtained from Smart 3.0 software. (14L/10D) Control group (20L/4D) Extended light group (4L/20D) Extended dark group.*


A
A1
Number of entries to EC
Entries to EC (ATL)
BTL
ATL
*
****
14L/10D
20L/4D
4L/20D
B
B1
Latency to enter EC (s)
Latency to enter EC (ATL)
**
***
C
C1
Time spent in EC (s)
Time in EC (ATL)
D
D1
Distance in EC (cm)
Distance in EC (ATL)

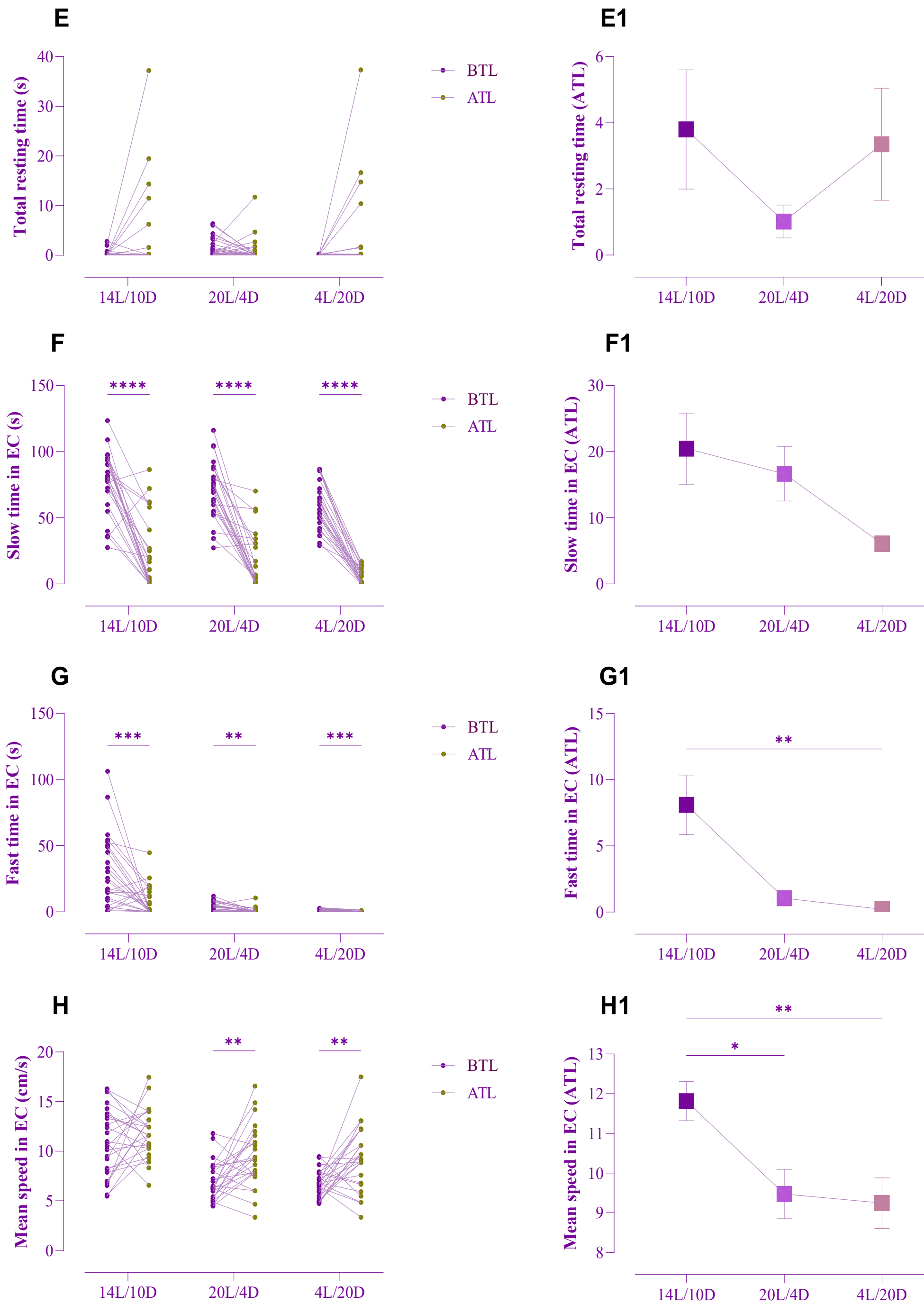


Figure 5.12: *Photoperiod modulation, the before-after plot describes variation with group and dotted-line plot comparing between groups. (A) Number of entries to EC before vs. after trial with in group. (A1) Number of entries to EC after trial between groups. (B) Latency to enter EC before vs. after trial with in group. (B1) Latency to enter EC after trial between groups. (C) Time spent in EC before vs. after trial with in group. (C1) Time spent in EC after trial between groups. (D) Distance in EC before vs. after trial with in group. (D1) Distance in EC after trial between groups.*

*(E) Total resting time before vs. after trial with in group. (E1) Total resting time after trial between groups. (F) Slow time in EC before vs. after trial with in group. (F1) Slow time in EC after trial between groups. (G) Fast time in EC before vs. after trial with in group. (G1) Fast time in EC after trial between groups. (H) Mean speed in EC before vs. after trial with in group. (H1) Mean speed in EC after trial between groups. The statistically significant differences are indicated by *(P<0.05), **(P<0.01), ***(P<0.001), and ****(p<0.0001), (n=25).*

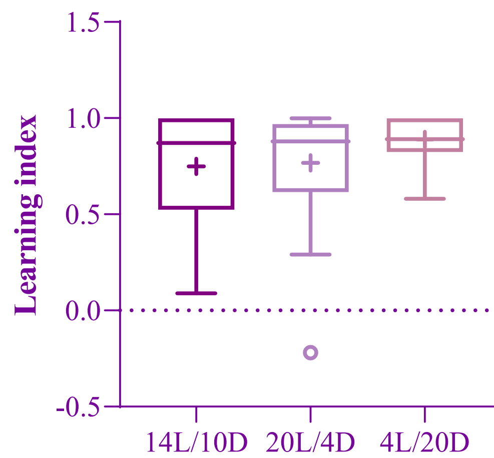


Figure 5.13: *Photoperiod modulation, the box plot representing learning index based on time avoiding the EC after trial with that of before trial.*

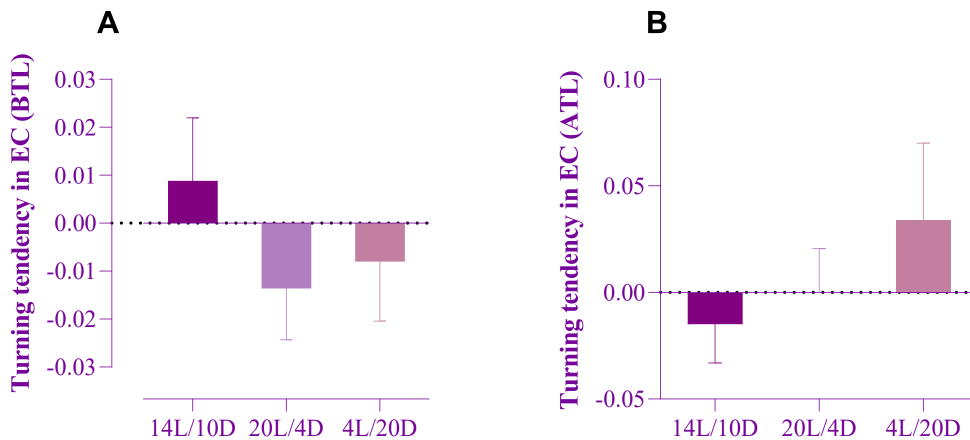


Figure 5.14: *Photoperiod modulation, the turning tendency or direction of extended light and dark groups in EC before trial and after trial.*

5.5. Discussion

Memory is an important cognitive function, and without memory, we can only perform basic reflex actions and repetitive behaviours. We gain memory through experience, and learning is the process that helps us attain memory. Because of that, the study of learning and memory is one of the most meticulously researched topics in neuroscience (Okano et al., 2000). And now zebrafish is considered the most relevant alternative vertebrate model to investigate cognitive research (Tan et al., 2022). Therefore, we chose zebrafish to learn avoidance behaviour in response to different environmental changes, including different dietary regimes, exposure to antibiotics, enriched environment and varied photoperiod. Our results revealed that the fear-induced learning was found to be apparent in all diet groups, including APS, AP, A, P, and S; however, it was more prominent in the P and APS groups. It indicates a protein-rich diet contributes more towards learning and memory because a protein-deficient or low-protein diet resulted in poor episodic or recognition memory in mice (Pillay et al., 2016). Another study in mice found that changes in gut bacteria caused by diet are associated over time with memory and learning in mice (Li et al., 2009). Independent of microbial shifts, all diet groups exhibit fear-induced avoidance learning; however, the microbial composition in P and APS diets substantially aided the learning process. The P diet group predominated with *Paenibacillus polymyxa* and *Brevibacillus choshinensis*, whereas the APS group dominated with *Streptococcus thermophilus* and *Cetobacterium somerae* (Chapter 1).

The avoidance learning was greatly reduced in PG and CPFX-treated groups; the increased presence of pathogenic bacteria and dysbiosis in these groups might contribute to the reduced learning behaviour in zebrafish. In mice, antibiotic

treatment diminished object recognition memory; however, spatial memory was unaffected. Additionally, it was found that gut-microbiota loss due to antibiotic treatment from early life stages can weaken cognitive function in mice (Mosaferi et al., 2021). Another study from the UK Biobank Database suggests that Prolonged or repeated antibiotic use during childhood might increase the probability of cognitive decline in adult life stages (Liu et al., 2022). A comprehensive review and meta-analytic approach on the impact of persistent antibiotic usage on cognitive function suggests that antibiotic treatment impaired the cognitive outcomes (Ye et al., 2024). Whereas zebrafish raised in a naturally enriched environment showed learning similar to that of controls, this implies that natural enrichment with stones and plants has a neutral effect on fear-related avoidance learning. In mice, environmental enrichment enhances inhibitory avoidance behaviour, whereas in rats, such enrichment diminishes it (Kazlauckas et al., 2011; Garrido et al., 2012). In contrast, "A study on the influence of environmental enrichment and age-related variations on inhibitory avoidance in zebrafish" revealed that environmental enrichment reduced inhibitory avoidance behaviour in zebrafish (Manuel et al., 2015). Whereas, recognition memory in zebrafish larvae bred in an environment with visual enrichment is found to be greater compared to larvae reared in a barren environment (Gatto et al., 2022; De Pasquale et al., 2021). The Change in photoperiod also did not affect avoidance learning significantly; however, the learning index was more consistent in the 4L/20D group than in the 14L/10D and 20L/4D groups.

In summary, this chapter deals with the assessment of avoidance learning behaviour in response to environmental changes with dietary variation, antibiotic exposure, enrichment and varied photoperiod. Considering dietary groups, the zebrafish raised using the P-only diet and mixed diet APS efficiently responded to the fear-related

learning process. Whereas, antibiotic-treated zebrafish significantly failed to avoid the fear stimulus, while the avoidance behaviour of zebrafish exposed to enrichment seemed to have no significant difference compared to the control group. Similarly, photoperiod variation also has no statistically significant effect on avoidance learning; however, the learning index of the extended dark exposure group seemed to be greater.

5.6. References

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SUMMARY

Behaviour represents the manifestation of an organism's response to its internal and external environments. Any modification in the environment, therefore, is reflected in behavioural changes. This study investigates how dietary and environmental factors-including antibiotic exposure, environmental enrichment, and photoperiod-affect zebrafish behaviour and gut microbiota. Research on the gut-brain axis offers a promising approach to understanding the bidirectional relationship between the gastrointestinal tract and the central nervous system. Increasing evidence reinforces the critical role of the gut microbiota in regulating neuronal functions and influencing central nervous system activity.

While mammalian models such as rodents have contributed significantly to this field, zebrafish (*Danio rerio*) are now emerging as a valuable translational model. Owing to their conserved neuroanatomical structures and functions, combined with advantages such as body transparency, rapid reproductive cycle, relatively low maintenance requirements, and greater ethical flexibility, zebrafish are increasingly used in microbiome research. However, a significant gap exists in understanding how dietary and environmental factors affect gut microbiota composition and behavioural phenotypes in zebrafish.

To address this gap, the present study was designed with three main objectives:

1. To segregate and establish different zebrafish populations under varied dietary regimes, antibiotic treatments, enriched environmental settings, and distinct photoperiod schedules.
2. To examine gut microbial composition and dynamics in zebrafish under different environmental conditions.

3. To explore how environmental modulation influences behavioural phenomics in zebrafish.

These objectives are presented and discussed across five distinct chapters. To investigate dietary effects, five different feeding regimes were implemented: three monotypic diets-*Artemia*, pellet, and spirulina-and two combination diets, *Artemia* + pellet, and *Artemia* + pellet + spirulina. Observations revealed that monotypic diets were suboptimal, whereas combination diets mitigated the nutrient limitations of monotypic feeding. This was reflected in well-balanced, active behavioural patterns, including reduced anxiety, lower aggression, and enhanced learning ability, along with a healthier gut microbiome profile.

Another important aspect of gut microbiome disruption is antibiotic exposure. With increasing environmental antibiotic contamination, including in aquatic habitats, this study examined whether acute high-dose and chronic low-dose exposures to Penicillin G and Ciprofloxacin affect zebrafish behaviour. Acute high-dose treatment induced an anxiety-like phenotype with hyperactivity, exacerbated proconvulsive effects, and significantly increased seizure frequency and reduced seizure onset latency in the pentylenetetrazole (PTZ) induced seizure model. In contrast, chronic low-dose exposure reduced novelty-induced anxiety but increased aggression and impaired fear-induced avoidance learning. In addition to behavioural changes, chronic antibiotic exposure altered intestinal villus morphology, caused neuronal cell loss and clustering in the brain, promoted the proliferation of pathogenic species, and induced gut dysbiosis. These findings reinforce that antibiotic overuse and environmental contamination can negatively modulate the gut–brain axis.

In contrast, zebrafish raised in a naturally enriched environment containing plants, stones, and algal growth exhibited behavioural patterns closely resembling wild phenotypes and showed protective effects on intestinal villi and brain cells. Although microbial diversity was lower, these fish harboured beneficial bacterial taxa.

Considering that a balanced diet and healthy environment are essential for neurological well-being. The sleep-wake cycle is also playing a key role. To assess its influence, photoperiod modulation experiments were conducted. Extended dark conditions reduced anxiety levels, whereas extended light conditions induced stress and aggression. Both conditions altered social preference behaviour, although learning ability was largely unaffected; zebrafish in the extended dark group showed more consistent learning performance. Photoperiod alterations also shifted gut bacterial communities and brain transcriptomic profiles. Though prolonged darkness provided certain benefits, maintaining a natural sleep-wake cycle was important for positive social interactions and neurological health.

Overall, this study demonstrates that dietary composition, antibiotic exposure, environmental enrichment, and photoperiod collectively shape gut microbiota and behavioural phenotypes in zebrafish. These findings provide valuable insights into the gut-brain-behaviour axis and strengthen the translational relevance of zebrafish for microbiome-neurobiology research.

CONCLUSION

The gut-brain communication pathway links the intestinal microbiota with the host's neural network. Perturbations in this pathway can alter brain chemistry, which in turn manifests as changes in behavioural processes. This study investigated the influence of physiological and environmental factors on the gut-brain axis. Due to their genetic and physiological similarity to humans, including conserved neurotransmitter systems and gut–brain axis mechanisms, zebrafish have emerged as a valuable alternative translational model in neuroscience and microbiome research. In this work, we used zebrafish to examine the effects of diet, antibiotics, environmental enrichment, and photoperiod on host gut microbiota and behaviour. The findings are presented across five chapters.

Chapter 1 examined the effects of different dietary regimes-*Artemia*, pellet, spirulina, and two combinations (*Artemia*+Pellet [AP] and *Artemia*+Pellet+Spirulina [APS])-on zebrafish behaviour and gut microbiota dynamics. Results showed that monotypic diets were suboptimal, whereas combining dietary components enhanced both gut microbiota composition and behavioural performance. *Artemia*-only diets were associated with increased anxiety and reduced exploratory behaviour. While *Artemia* contributes to immunomodulatory effects, visual stimulation, and sensory enrichment, its exclusive use lacks essential nutrients. This may facilitate colonization of pathogenic species such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*, and *Streptococcus agalactiae*. These pathogens may induce inflammation, which could underlie the observed behavioural changes.

Pellet diets-whether nursery pellets or spirulina-enriched pellets-are generally considered nutritionally complete in aquaculture and promote growth. However, exclusive pellet feeding increased body protein and fat content, potentially

predisposing fish to obesity. Pellet-fed fish showed a gut microbiota dominated by *Acinetobacter* spp., whereas spirulina diets shifted the community towards Bacillota with enrichment of *Brevibacillus*. Both groups exhibited moderate anxiety, but aggression was markedly higher in pellet-fed fish, suggesting spirulina may mitigate aggression. These results indicate that excess dietary protein or fat can contribute to obesity, which may further drive neurobehavioural changes such as heightened aggression.

Mixed diets revealed that novelty-induced anxiety was higher in the AP group compared to APS. APS-fed fish displayed a more balanced gut microbiota and exhibited calmer, more active behaviour. This may be mediated via microbial-derived metabolites such as short-chain fatty acids (SCFAs), which possess neuromodulatory effects. Overall, the findings demonstrate that diet strongly influences the gut–brain–behaviour axis, and that a balanced diet supports healthier behavioural phenotypes.

Chapter 2 investigated antibiotics as modulators of the gut-brain axis. Acute exposure to high concentrations (25 mg/L) of Penicillin G (PG) or Ciprofloxacin (CPFX) induced hyperactivity and anxiety. Given that stress increases seizure susceptibility and that certain antibiotics possess neurotoxic and proconvulsant properties, we assessed the impact of antibiotic treatment on pentylenetetrazole (PTZ)-induced seizures. Both PG- and CPFX-treated fish exhibited increased seizure frequency, earlier seizure onset, and prolonged seizure duration, indicating proconvulsive effects potentially mediated by microbiota disruption.

Chapter 3 evaluated the long-term effects of low-dose (10 mg/L) chronic antibiotic exposure and natural environmental enrichment. Simulating antibiotic-polluted and natural conditions, we found that chronic exposure to PG and CPFX reduced

novelty-induced anxiety. Light-induced anxiety decreased in PG-treated fish but increased slightly in CPFX-treated fish, likely due to CPFX phototoxicity. Both groups exhibited elevated aggression, while sociability remained unchanged. In enriched environments, zebrafish showed neophobia and greater light–dark aversion, consistent with wild-type behaviour.

Histological analysis revealed that antibiotic treatment damaged the intestinal epithelium, altered villus morphology, and caused neuronal loss and clustering in the mesencephalon. Microbiota analysis showed that PG treatment increased diversity but favoured pathogenic taxa, notably *Salmonella enterica*, while CPFX treatment caused more pronounced dysbiosis, with dominance of antibiotic-resistant *Burkholderia* spp. Enriched environments favoured Bacillota, reduced diversity, and promoted beneficial but limited bacterial communities, supporting intestinal and neural health.

Chapter 4 examined photoperiod modulation (20 h light/4 h dark and 4 h light/20 h dark) on zebrafish behaviour, microbiota, and brain transcriptomics. Extended darkness reduced anxiety-like behaviour, whereas extended light increased stress and aggression. Extended darkness reduced social preference, while extended light induced stressed social interactions. Microbiota composition differed, with extended light supporting equal proportions of Actinomycetota and Pseudomonadota, and extended darkness favouring Bacillota. Transcriptomic profiling showed that extended light activated neuroimmune pathways and metabolic reorganization. While extended darkness upregulated genes related to mood regulation, neuroprotection, brain patterning, and synaptic plasticity. Both conditions downregulated mitochondrial genes, indicating circadian and energy metabolism disruption.

Chapter 5 assessed cognitive performance using an avoidance learning paradigm linking a light stimulus to a mild electric shock. Fear-conditioned learning occurred across all diet groups but was strongest in the P and APS groups, suggesting protein-rich diets may enhance learning and memory. Learning was impaired by antibiotic exposure, likely due to pathogenic overgrowth and dysbiosis, but remained unaffected in enriched environments. Photoperiod changes did not significantly impair learning, although extended darkness produced more consistent learning scores.

Further biochemical and molecular studies are warranted to elucidate how these variables modulate the gut-brain-behaviour axis. In particular, characterizing bacterial metabolites such as SCFAs and their neurochemical actions will deepen understanding of microbiota-mediated neural modulation.

RECOMMENDATIONS

This study evaluated dietary and environmental paradigms influencing gut microbiota and behaviour. Future research should adopt more targeted experimental designs and advanced technologies to elucidate gut-brain mechanisms underlying behaviour, thereby enhancing translational relevance.

- We assessed the effects of monotypic and combined fish diets; future investigations should include additional dietary interventions such as prebiotics, probiotics, synbiotics, and personalized dietary regimens to promote a healthy microbiome and mental well-being.
- Longitudinal tracking of microbiome composition and behavioural changes across developmental stages would help in understanding temporal dynamics and developmental influences.
- Employing gnotobiotic or germ-free zebrafish models would better establish causal relationships between specific microbial communities and behavioural phenotypes.
- Integrated multi-omics approaches (metagenomics, transcriptomics, metabolomics, proteomics) are recommended to gain deeper insights into the functional mechanisms linking microbiota and brain function.
- Electrophysiological recordings and detailed neurochemical profiling could be applied to connect neurotransmitter alterations with behavioural changes.

- Advanced technologies, including faecal microbiota transplantation (FMT) from healthy to diseased models, could be used to explore causal links between microbiome composition and behavioural outcomes.
- Increased studies in this field will help in identifying specific microbial biomarkers associated with distinct behavioural phenotypes, guiding targeted therapeutic strategies for conditions linked to dysbiosis, disrupted sleep cycles, and poor dietary habits.
- Large-scale investigations on the effects of other environmental variables-such as temperature, pH, and additional water quality parameters-on gut microbiota composition and behaviour would provide a more comprehensive understanding of multifactorial influences on the gut-brain axis.
- Exploring gut-brain axis modulation as an early intervention strategy for neurodevelopmental and psychiatric disorders is recommended. Zebrafish represent a valuable model for studying microbiota-driven psychiatric conditions, with potential to inform future therapeutic development.

PRESENTATIONS AND PUBLICATIONS

- **Dhanusha Sivarajan**, Dr. Binu Ramachandran (2023), Antibiotics-induced behavioral Plasticity in zebrafish. 42nd Annual Conference of the Society of Toxicology India (STOX), UNIVERSITY OF CALICUT. 23-25 November 2023.
- **Dhanusha Sivarajan**, Dr. Binu Ramachandran (2022), Antibiotics as potential seizure modulators in zebrafish. Indian Zebrafish Investigators Meeting (IZIM), IISER PUNE. September 21-23 2022.

Article published

- **Sivarajan, D**., Pothayi, V., Devasia, S. C., & Ramachandran, B. (2026). Impact of dietary composition on behavioural expression and gut microbiome dynamics in zebrafish. *Pflügers Archiv-European Journal of Physiology*, *478*(1), 13. https://doi.org/10.1007/s00424-025-03139-8
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- Nair, J. S., Olary, L., **Sivarajan, D**., Amar, A., & Ramachandran, B. (2025). Caffeine bidirectionally regulates social preference and anxiety-like behavior

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- **Sivarajan D**, Ramachandran B. Antibiotics modulate frequency and early generation of epileptic seizures in zebrafish. Exp Brain Res. 2023 Feb;241(2):571-583. doi: 10.1007/s00221-023-06546-4. Epub 2023 Jan 10. PMID: 36625966.

Article under review

- Photoperiod Driven Modulation of Behavior, Gut Microbiota, And Brain Transcriptomics in Zebrafish.

Article in preparation

- Antibiotics Differentially Regulate Gut Microbiota Composition and Behavioral Expression in Zebrafish.
- Influence of environmental changes on avoidance learning behaviour in zebrafish.