

A STUDY ON THE SHOAL STRUCTURE, INTERACTION, AND BEHAVIOURAL PHENOMICS OF SOCIAL ISOLATION IN ZEBRAFISH

*Thesis submitted in partial fulfilment of the requirements for the award of the
degree of*

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the Faculty of Science

University of Calicut



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SEPTEMBER 2026



DECLARATION

I, **Aswathy Sivaraman**, hereby declare that the work embodied in this thesis entitled **"A STUDY ON THE SHOAL STRUCTURE, INTERACTION, AND BEHAVIOURAL PHENOMICS OF SOCIAL ISOLATION IN ZEBRAFISH"**, submitted to the University of Calicut in partial fulfilment of the requirements for the award of 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.

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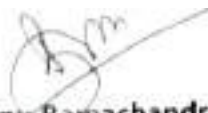
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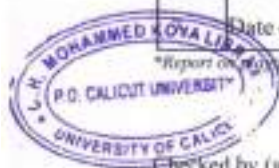




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From being a student for whom my mother searched through countless tuition centres just to help me understand Biology and secure a passing mark, to completing a Doctor of Philosophy in Zoology, this journey has been nothing short of transformative. What once felt distant and incomprehensible gradually unfolded into curiosity, then interest, and eventually deep passion. Biology evolved from a subject I struggled with into one I chose with conviction for my undergraduate studies, and that enthusiasm, combined with persistent curiosity, ultimately led me to pursue research and embrace the challenges of a PhD in Zoology. At this meaningful moment, I express my heartfelt gratitude to everyone who has contributed to this academic journey.

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Dedication

“I dedicate this thesis to Binu Sir, my research advisor, whose steadfast stewardship and encouragement transformed my long-cherished dream of a ‘first flight’ into a reality, and whose mentorship culminated in my first research publication. With that same unwavering spirit, he has steered me through this thesis journey—lifting me through challenges, inspiring me at every turn, and helping me translate aspirations into achievements.”

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Table 6.1.	Behavioural endpoints and their definitions used in the current study
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ദീർഘകാല സാമൂഹിക ഒറ്റപ്പെടൽ (chronic social isolation) ജീവികളുടെ പെരുമാറ്റത്തിലും മാനസിക ഘടനയിലും ഗൗരവമായ മാറ്റങ്ങൾ സൃഷ്ടിക്കുന്ന ഒരു പ്രധാന പരിസ്ഥിതി സമ്മർദ്ദ ഘടകമായി കണക്കാക്കപ്പെടുന്നു. എങ്കിലും, ഈ സ്വാധീനം ലിംഗ വ്യത്യാസങ്ങളിലൂടെയും സാമൂഹിക സംഘടനയുടെ വിവിധ ഘടനാതലങ്ങളിലൂടെയും എങ്ങനെ പ്രകടമാകുന്നു എന്നത് ഇതുവരെ സമഗ്രമായി വ്യക്തമാക്കിയിട്ടില്ല.

ഈ പശ്ചാത്തലത്തിൽ, *Danio rerio* (സീബ്രാഫിഷ്) എന്ന മാതൃകാ മത്സ്യത്തെ ഉപയോഗിച്ച്, ദീർഘകാല സാമൂഹിക ഒറ്റപ്പെടലിന്റെ പെരുമാറ്റപരമായ പ്രതിഫലനങ്ങളെ ബഹുതല സമീപനത്തിലൂടെ ഈ പഠനം വിലയിരുത്തുന്നു.

മാതൃകാ മത്സ്യങ്ങളിൽ നടത്തിയ പരീക്ഷണങ്ങളിലൂടെ, വ്യക്തിഗത പെരുമാറ്റപരിശോധനകളായ ഓപ്പൺ ഫീൽഡും ലൈറ്റ്-ഡാർക്ക് പരിശോധനകളും ഒറ്റപ്പെട്ടവയിൽ സഞ്ചാര പ്രവർത്തനം വർധിക്കുന്നതും ആശങ്കാസൂചക പ്രതികരണങ്ങളിൽ വ്യത്യാസങ്ങൾ ഉണ്ടാകുന്നതും വ്യക്തമാക്കുന്നു. ശ്രദ്ധേയമായി, സാധാരണ സാഹചര്യങ്ങളിൽ വ്യക്തമായിരുന്ന ലിംഗ വ്യത്യാസങ്ങൾ, ഒറ്റപ്പെടലിന്റെ സ്വാധീനത്തിൽ മങ്ങിപ്പോകുന്നതായി കണ്ടെത്തി.

തുടർന്ന്, മൂന്ന്-ചേംബർ സാമൂഹിക പരീക്ഷണങ്ങളിലൂടെ, ഒറ്റപ്പെടൽ സാമൂഹിക മുൻഗണനയെ കുറയ്ക്കുകയും, ഭീഷണിയുടെയും കണ്ണാടി ഉത്തേജനത്തിന്റെയും പശ്ചാത്തലത്തിൽ പെരുമാറ്റ പ്രതികരണങ്ങളിൽ ലിംഗാനുസൃത വ്യത്യാസങ്ങൾ സൃഷ്ടിക്കുകയും ചെയ്യുന്നതായി കണ്ടെത്തി. ഇതിൽ, ആൺ മത്സ്യങ്ങളിൽ ഭാഗികമായ സാമൂഹിക സംരക്ഷണ ശേഷി നിലനിൽക്കുമ്പോൾ, പെൺ മത്സ്യങ്ങളിൽ അതിന്റെ കാര്യക്ഷമത കുറവാണെന്ന് സൂചിപ്പിക്കുന്നു.

കൂടാതെ, കൂട്ടമായും ദ്വയമായും നടത്തിയ വിശകലനങ്ങൾ ഒറ്റപ്പെട്ട ഗ്രൂപ്പുകളിൽ സ്ഥലം പങ്കിടൽ, ചലന ഏകീകരണം, കൂടാതെ കൂട്ടത്തിന്റെ ആന്തരിക സ്ഥിരത എന്നിവയിൽ ശ്രദ്ധേയമായ വ്യതിയാനങ്ങൾ ഉണ്ടാകുന്നതായി തെളിയിക്കുന്നു.

ഇതിനൊപ്പം, ആന്തരാവയവ മൈക്രോബയോം സംബന്ധിച്ച വിവരണാത്മക വിശകലനം ലിംഗാനുസൃതമായ സൂക്ഷ്മജീവ ഘടനാ വ്യത്യാസങ്ങൾ ഉണ്ടാകുന്നതായി സൂചിപ്പിക്കുന്നു. ഇത് പെരുമാറ്റ മാറ്റങ്ങളുമായി ബന്ധപ്പെടാവുന്ന ഒരു സാധ്യതാപരമായ ജൈവ അടിസ്ഥാനം നിർദ്ദേശിക്കുന്നു.

സമഗ്രമായി നോക്കുമ്പോൾ, ദീർഘകാല സാമൂഹിക ഒറ്റപ്പെടൽ വ്യക്തിഗതവും കൂട്ടപരവുമായ പെരുമാറ്റ ഘടനകളിൽ ഉണ്ടാക്കുന്ന വ്യതിയാനങ്ങളെ ഈ പഠനം സമഗ്രമായി വെളിവാക്കുന്നു. പ്രത്യേകിച്ച്, ന്യൂറോഡെവലപ്മെന്റൽ അസ്വസ്ഥതകളും മാനസിക സമ്മർദ്ദവുമായി ബന്ധപ്പെട്ട അവസ്ഥകളും മനസ്സിലാക്കുന്നതിനുള്ള പ്രായോഗികവും ശാസ്ത്രീയവുമായ അടിത്തറയായി, ഈ പഠനം ദീർഘകാല സാമൂഹിക ഒറ്റപ്പെടലിനെ ഒരു പ്രധാന അടിസ്ഥാന മാതൃകയായി നിർദ്ദേശിക്കുന്നു.

പ്രധാന പദങ്ങൾ: സാമൂഹിക ഒറ്റപ്പെടൽ, സീബ്രാഫിഷ്, ലിംഗവ്യത്യാസങ്ങൾ, സാമൂഹിക പ്രതിരോധം, കൂട്ടമായി നീന്തുന്ന സ്വഭാവം, ദ്വയ ഇടപെടൽ, കൂടൽ സൂക്ഷ്മജീവ സമൂഹം, പരിവർത്തന നാഡീശാസ്ത്രം

Chronic social isolation is a widely used environmental stress model; however, its behavioural consequences across sexes and levels of social organisation remain insufficiently understood. The present study investigated the effects of prolonged social isolation in zebrafish (*Danio rerio*) using a multi-level behavioural framework integrating individual assays, stimulus-based social tests, collective shoaling dynamics, dyadic interactions, and descriptive gut microbiome profiling. Open field and light–dark assessments revealed increased locomotor activity and altered anxiety-like responses in isolated fish, with strong baseline sex differences becoming masked under isolation. In three-chamber paradigms, isolation reduced social preference and modified predator- and mirror-induced behaviours in a sex-dependent manner, with partial stress buffering observed in males and reduced buffering efficiency in females. Shoal and dyad analyses demonstrated disrupted spatial cohesion, alignment, and emergent state stability in isolated groups. Descriptive microbiome analysis further indicated sex-specific shifts in gut microbial composition. These findings provide translational insight into how chronic social isolation may contribute to behavioural dysregulation relevant to neurodevelopmental and stress-related conditions.

Keywords: social isolation, zebrafish, sex differences, social buffering, shoaling behaviour, dyadic interaction, gut microbiome, translational neuroscience

Chapter **1**

Introduction

1.1 Social Behaviour and Its Versatile Applications

Social behaviour is the foundation of human society, as it creates an essential framework for human connections and facilitates the maintenance of societal structures (Wilson, 1975). Understanding social behaviour is vital to unravel the complex factors that influence how people respond across different social contexts, ranging from intricate biological processes to external environmental motivations. Various research methods are involved in studying social behaviours (Rosenthal & Rosnow, 1991), such as observational, experimental (Milgram, 1963), survey (Dillman et al., 2014), and ethnographic (Atkinson & Hammersley, 1994) research. The present study includes the social behaviour of zebrafish that have undergone chronic social isolation under different social contexts, which better fits both observational and experimental approaches. Experimental studies on social behaviours are particularly relevant in social psychology, and the use of automatic tracking software helps minimize observational bias, as seen in traditional observational studies (Burghardt et al., 2012).

As per the theories of social behaviour, it is influenced by biological, psychological, and sociological perspectives. According to the biological view (Hamilton, 1964), social behaviour is partly shaped by evolution, as organisms behave in ways that improve survival and reproduction. The biological perspective is primarily grounded in natural selection, with specific theories such as kin selection explaining the evolution of social behaviours (Hamilton, 1964; Kay et al., 2019). The second, psychological perspective describes how social behaviour develops through learning and thinking processes, predominantly explained by social learning and cognitive dissonance theories. Social learning theory (Bandura & Walters, 1977), put forward by Albert Bandura, states that watching and imitating others plays a major role in learning, and it does not require direct experience; rather, observing rewards or punishments is sufficient. The second theory, proposed by Leon Festinger, explains that when discomfort (cognitive dissonance) (Festinger, 1962) develops due to conflict between thoughts and actions, individuals change their behaviour accordingly to reduce this dissonance. The third, sociological perspective explains that behaviour is shaped by society and culture, which includes symbolic interactionism (Blumer, 1986) and social constructionism (Berger & Luckmann, 1967).

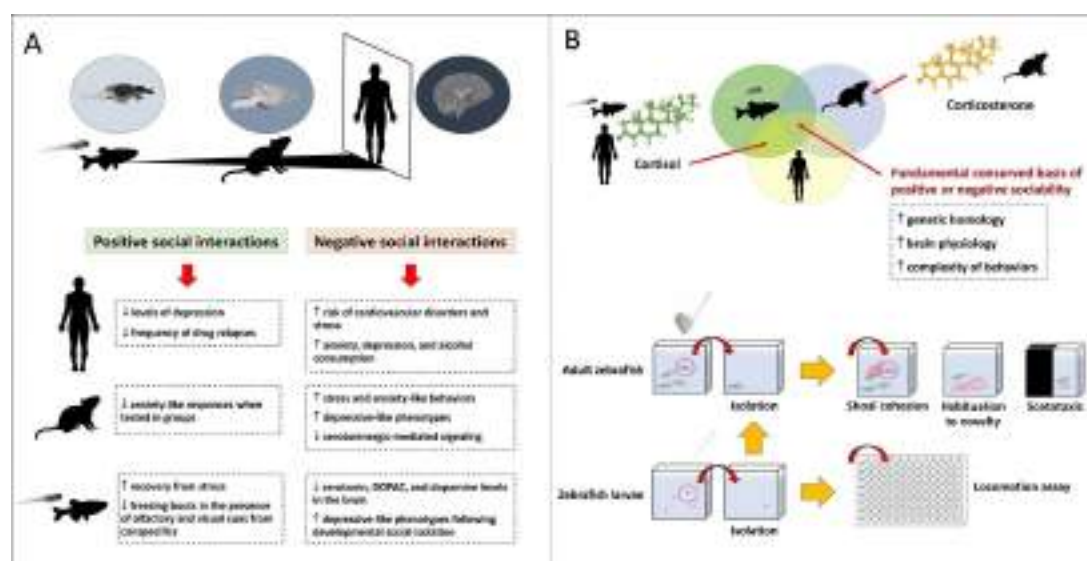
Social behaviours are generally defined as interactions between individuals of the same species and are broadly categorized into cooperative or competitive forms (Cacioppo et al., 2011; Rubenstein & Rubenstein, 2013). Cooperative behaviours along with reciprocal altruism (Trivers, 1971) allow individuals to work together to achieve shared goals and maximize mutual benefits, such as mating or resource sharing. In contrast, competitive behaviours, often observed as aggression, may arise under conditions of limited resources, where the success of one individual occurs at the expense of another (Johnson & Johnson, 1989). In humans, social behaviours include prosocial, communicative, affiliative, and aggressive patterns that emerge from complex social cognition and learning processes (Arioli et al., 2018). Multiple factors, including environmental influences and intrinsic biological traits, act as drivers of these behaviours (Decety et al., 2016). Social interactions are essential for adaptive functioning, enabling individuals to form stable relationships and maintain overall psychological and social well-being (Kakulte & Shaikh, 2023).

In this context, the lack of social interactions or social support has emerged as a significant concern (Holt-Lunstad et al., 2015). Lack of social interactions can be subjective or objective, where loneliness, considered a subjective form of social deficit, has increasingly been discussed as a global issue. The meta-analysis conducted by Holt-Lunstad et al. (2015) found that social connection increases the odds of survival and also demonstrated that there were no differences between objective and subjective social isolation. Several studies report an increased occurrence of mental health problems (Brandt et al., 2022; Bruss et al., 2024) and post-traumatic stress disorder (PTSD) in individuals experiencing prolonged social disconnection (Charuvastra & Cloitre, 2008). Similarly, studies (Timmermans et al., 2021) and one study from the 2010-2015 period marked an increase in loneliness among adults over 45 years of age. Major health organizations, including the World Health Organization (WHO), recognize lack of social interactions as a major public health concern, highlighting the need for timely interventions to improve both mental and physical health outcomes. Recent reports from the Gallup-Meta (2023) survey of 142 countries suggest that the loneliness epidemic affects one in four adults. Dr. Vivek Murthy (US Surgeon General) popularized the “15 cigarettes a day” comparison in his 2023 Advisory, *Our Epidemic of Loneliness and Isolation* (General, 2023).

The 2019-2020 coronavirus (COVID-19) pandemic further emphasized the role of social connectedness in health. Public health measures involving enforced social distancing and isolation were essential for infection control. However, several studies documented increased levels of cognitive dysfunction (Quan et al., 2023; Wu & Liu, 2025) and alcohol consumption (Botella-Juan et al., 2025; Mohamed et al., 2024) during this period, reinforcing the importance of social interaction in maintaining psychological balance. These observations collectively underline the need to understand the biological and behavioural consequences of social isolation (Ding et al., 2025). Controlled experimental models therefore become essential to dissect the mechanisms underlying social deficits and their long-term outcomes.

Rodent models have long been predominant in the study of social behaviour and isolation-induced deficits, largely due to their complex social structures, well-characterized neurobiology, and high translational value (Powell & Swerdlow, 2023). However, rodent studies are resource-intensive, require larger housing facilities, and often involve longer experimental timelines. These practical considerations, along with the need for high-throughput and system-level approaches, have encouraged the use of alternative vertebrate models in the present study (Ellenbroek & Youn, 2016).

In this regard, zebrafish (*Danio rerio*) have emerged as a valuable complementary model (Choi et al., 2021). Zebrafish are diurnal, form social groups, and display complex behavioural repertoires including shoaling, aggression, and social preference. Importantly, zebrafish possess conserved monoaminergic systems, stress-axis regulation via the hypothalamic-pituitary-interrenal (HPI) axis, which is functionally comparable to the hypothalamic-pituitary-adrenal (HPA) axis in mammals, and genetic homology relevant to neuropsychiatric research (Krylov et al., 2021; Langova et al., 2020). Previous work has established zebrafish as a translational model for social deficits. Alterations in social experience have been associated with dysregulated behaviours and neurochemical changes, some of which resemble phenotypes observed in neurodevelopmental and neuropsychiatric conditions. However, the diversity of isolation protocols and behavioural paradigms has led to heterogeneous outcomes, indicating that the effects of social isolation may depend strongly on developmental stage, duration, and contextual factors.



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Figure 1.1. Impact of social interactions and social isolation across taxa and translational relevance of zebrafish models.

(A) Zebrafish as a translational model to study behavioural and neurobiological phenotypes associated with positive social interactions and social isolation. Despite anatomical differences between fish and mammals, zebrafish possess homologous brain structures with conserved functions, enabling modelling of human social behaviour. Positive social interactions are associated with reduced risk of depression and psychiatric relapse, whereas social isolation is linked to increased risks of cardiovascular disorders, anxiety, depression, stress, and substance use. Experimental studies demonstrate reduced anxiety-like behaviour in group-housed animals and faster stress recovery in zebrafish exposed to conspecific cues. Social isolation has also been associated with alterations in neurotransmitter systems, including reduced dopamine and serotonin levels, and increased affective behavioural changes. (B) Overview of behavioural and neuroendocrine strategies used to assess social isolation effects. Zebrafish serve as an effective model due to conserved stress physiology, including cortisol as the primary stress hormone, and their suitability for studying locomotion, anxiety-like behaviour, and sociability under short- and long-term isolation conditions in both larval and adult stages.

1.2 Social Isolation in Zebrafish: Literature Review, Conceptual Gaps and Experimental Relevance

In this section, we address the important previous studies that have been conducted on social isolation using zebrafish as a model organism, emphasizing the nature of the isolation protocols established. Majorly, the work has been identified as developmental and adult social isolation, and both acute and chronic experimental

durations have also been observed. Basically, developmental social isolation focuses on the long-term impact of isolation starting from embryonic or larval stages and extending up to juvenile or adult stages. Whereas adult isolation in zebrafish is examined after they have reached sexual maturity, ranging from short durations as well as long-term analysis.

Gerlach et al. (2008) found that isolation from 0 to 21- or 30-days post fertilization (dpf) resulted in an inability to differentiate odour cues between kin and non-kin in zebrafish. This particular isolation was complete, such that fish were completely blocked from visual, olfactory, and tactile cues. Social preference and anxiety-related behavioural analysis conducted with isolation from 0 dpf to the juvenile stage in zebrafish demonstrated decreased social preference and swimming activity paired with increased freezing. The study linked these behavioural modulations to disrupted *c-fos* expression in brain regions associated with stress (Tunbak et al., 2020). Studies conducted during a chronic period of isolation beginning from 0 dpf to adulthood led to decreased social preference toward individuals with different pigment patterns (Engeszer et al., 2004), and depressive-like behavioural phenotypes were also reported (Sampaio et al., 2018). Anxiolytic drugs showed alleviation of the depressive-like phenotypes (De Melo et al., 2019). Physiological and neurochemical alterations were also observed as a result of developmental social isolation. Isolation extended from 0 dpf to 150 dpf showed reduced weight gain and reduced levels of dopamine and 5-HIAA in brain tissue (Fulcher et al., 2016). 5-HIAA is the primary metabolite of serotonin (5-HT) and is considered a marker for analyzing the serotonergic system, particularly regarding anxiety and stress conditions.

One of the major inspirational studies that led to our present study was conducted by Shams et al. (2018). The study analysed social isolation from 0 dpf to 180 dpf in zebrafish and found hyperactivity, reduced anxiety-like behavioural responses, altered shoaling, and reduced DOPAC (primary metabolite of dopamine) levels (Shams et al., 2018). One of the earlier studies conducted isolation from 6 dpf to 160 dpf and found altered neuronal plasticity, including decreased proliferating progenitor cells in sensory niches such as the olfactory bulb (Lindsey & Tropepe, 2014).

Taken together, developmental isolation studies indicate that early-life social deprivation induces persistent behavioural, neurochemical, and neuroplastic

alterations that extend into adulthood. However, the direction of behavioural outcomes, whether anxiogenic, anxiolytic, hypoactive, or hyperactive appears inconsistent across studies. These inconsistencies may not necessarily reflect contradictions but could arise from differences in isolation onset, duration, sensory cue availability, and behavioural paradigms used for assessment.

Adult social isolation studies have also demonstrated different behavioural responses in zebrafish. Short-term isolation for 1 day showed no change in locomotion and social response, but exhibited a marked decrease in dopamine, DOPAC, and 5-HIAA in the brain (Shams et al., 2017). Similarly short-term isolation extended to 3 to 5 days exhibited decreased swimming activity in social groups that mimicked the effects of MPTP, a potent neurotoxin that has been linked with Parkinson's-like symptoms (Saszik & Smith, 2018). A similar 14-day adult isolation has been conducted in studies that showed behavioural, hormonal, and neuronal alterations. Lindsey and Tropepe (2014), found suppression of cell proliferation in the vagal lobe, paired with this observation, an enhanced proportion of new neurons in the telencephalon also identified. The experimental fish exhibited anxiolytic-like behaviours, and reduced cortisol, considered the primary stress hormone, was reported in another 14-day isolation paradigm (Parker et al., 2012). As established in a prior study by Shams et al. (2015), showed the impact of 90 days of isolation in zebrafish and observed decreased anxiety-like behaviours, noted as reduced thigmotaxis behaviour (wall hugging), with unaltered general locomotory responses, supported by lower serotonin levels.

Additionally, based on previous research, vital modulating factors altering the effects of social isolation have also been noted. One of the modulating factors includes environmental enrichment, it has been shown that enrichment increases cell proliferation and cortisol levels compared to barren environments. Socially isolated fish showed preference toward enrichment, which promoted social behaviours (Von Krogh et al., 2010). Interestingly, group size has been identified to mitigate the negative impact of certain drug exposure-induced behavioural alterations when treated with an untreated group compared to isolated conditions. High caffeine-exposed fish showed reduced swimming activity, while no change in the same parameter was observed when exposed along with an untreated group of fish (Neri et

al., 2019). Similarly, alcohol-treated fish showed higher swimming activity when tested with an untreated group of fish than those tested in isolation (Ladu et al., 2014). In studies related to housing conditions and stress-related responses, Giacomini et al. (2015) showed the critical role of the social environment prior to a stressful event in modulating stress-linked physiological responses. Similarly, zebrafish subjected to 15 or 30 days of isolation exhibited a reduced cortisol response when exposed to an acute stressor. In this experiment, the stressor involved chasing the fish with a hand net for 120 seconds. Pagnussat et al. (2013), found that upon exposure to the convulsant PTZ, individual fish reached stage III seizures faster than those kept in groups.

Collectively, adult isolation studies suggest that social deprivation even after sexual maturity can rapidly alter monoaminergic system, stress physiology, and neurogenesis. However, the behavioural expression of these neurobiological changes remains variable, again pointing toward methodological heterogeneity as a major contributing factor.

Recent studies have also brought valuable insight into the field of social isolation. Daniel and Bhat (2022) analysed different modes of isolation based on the cues the fish were receiving. These included a mixed-cue group (isolated but receiving visual and olfactory cues from conspecifics), a visual-cues-only group, and a complete isolation group without cues. The study analysis was entirely based on behavioural parameters. The results showed increased stress levels in the completely isolated groups compared to the others and showed lower chances of recovery from social isolation as observed in other acute social isolation paradigms. This study found sex-specific responses to social isolation, where females were reported as resilient and males as highly vulnerable.

Wee et al. (2022) raised larval zebrafish in isolation and found that social isolation triggered increased oxytocinergic neuronal activity in the hypothalamus, and this activation was linked to nociceptive pathways. This oxytocinergic pathway activity induced behavioural outputs including increased avoidance and suppressed appetite. The circuit activity was reduced to baseline when the isolated fish were exposed to water previously occupied by other zebrafish. Whereas Dissinger et al. (2024) showed that chronic juvenile isolation from 20 dpf to 51 dpf, exhibited increased feed intake, and higher feed conversion ratio, and increased inflammatory markers, however, no

significant change in growth parameters including weight and length was observed despite increased consumption. The observations suggested a stress-induced increased feeding or absence of competition from other group members. This study contradicts with Wee et al. (2022), which found isolation suppressed appetite, suggesting the role of developmental stage and diet type in experimental modulation and responses. Fontana et al. (2025) identified that a two-week period of social isolation intensified the behavioural responses of *adgrl3* knockout zebrafish. The *adgrl3* knockout zebrafish, an established model of ADHD, showed hyperactivity and impulsivity. When these fish underwent social isolation, it further intensified the externalizing phenotypes including increased anxiety-like behaviours, hyperactivity, occurrence of abnormal repetitive patterns, reduction in working memory, and altered shoaling.

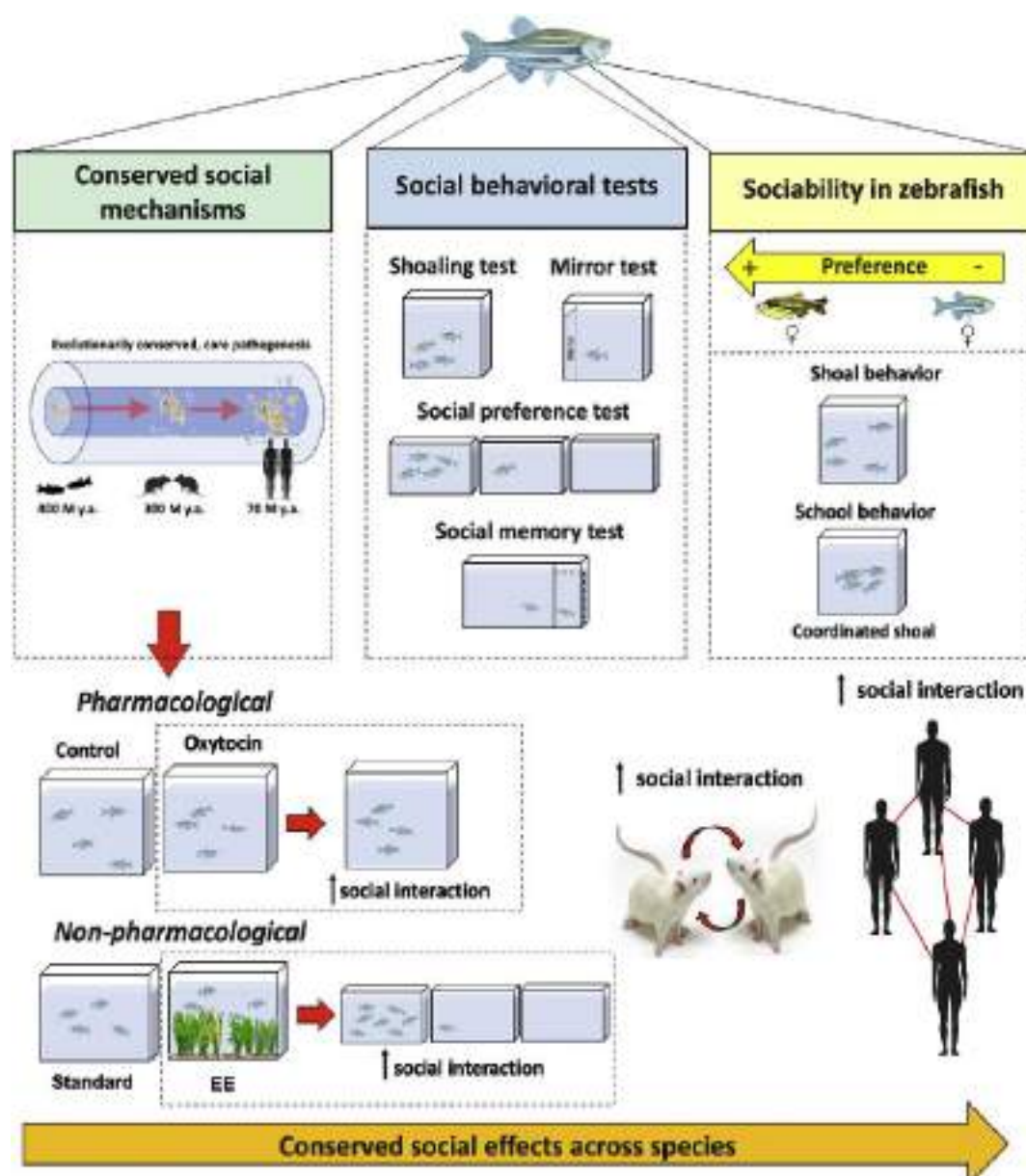
These recent studies broaden the field by linking social isolation not only to behaviour but also to neural circuitry, metabolic regulation, inflammatory processes, and genetic susceptibility. Importantly, they also introduce the dimension of sex-specific vulnerability, although this has not yet been systematically explored across chronic paradigms.

Spanning through the literature review specific to social isolation in zebrafish provides an intricate picture of the diversity of methodological protocols applied and the multiple aspects of outcomes, including behavioural, neuronal, hormonal, and genetic parameters. Similarly, the behavioural responses varied across studies, in some cases anxiogenic and reduced locomotion, in others anxiolytic and hyperlocomotion. Despite the growing body of work, a consistent and structured sex-specific analysis across chronic developmental isolation paradigms remains limited. Many studies either use mixed-sex groups without stratified analysis or report sex differences integrating them across behavioural domains. This leaves a gap in understanding whether the variability in behavioural outcomes across studies may partly arise from sex-dependent effects that are not uniformly examined.

Together, in our study, we aimed to analyse the sex-specific behavioural responses of zebrafish subjected to chronic social isolation (21 dpf -180 dpf) across multiple domains involving relevant and context-specific behavioural assays and parameters. The experimental hierarchy includes single-animal tests involving the open field and

light- dark test to evaluate the internal behavioural valence of social isolation and the external phenotype associated with it, followed by assessment of social contexts including predator exposure and mirror exposure, and how conspecific presence modulates social isolation effects. Higher-order behavioural tasks included shoaling and dyadic interactions, accompanied by descriptive sex-specific analysis of social isolation and gut microbiome composition.

Thus, the present study attempts to integrate chronic developmental isolation, multidimensional behavioural phenotyping, sex-specific analysis, and gut microbiome profiling within a single experimental framework, aiming to provide a more cohesive understanding of long-term social deprivation in zebrafish. This multi-pronged approach to social isolation is intended to provide a comprehensive narrative on sex specificity and multidimensional behavioural analysis. This may further support improved diagnostic understanding and intervention strategies to mitigate the negative impacts of social isolation.



Adopted from: Fontana et al., 2022 (DOI: 10.1016/j.pneurobio.2021.101993)

Figure 1.2. Zebrafish models and multilevel approaches for studying social interactions.

This figure presents selected examples of zebrafish models and the different levels of analysis used to investigate social behaviour. Social traits and their underlying mechanisms are evolutionarily conserved across zebrafish, rodents, and humans. These include shared pharmacological targets, such as the oxytocin system, as well as non-pharmacological interventions like environmental enrichment, both of which have been shown to enhance sociability across species. The figure also highlights well-established and sensitive behavioural paradigms used in zebrafish research to assess social phenotypes, including assays for social preference, shoaling behaviour, and social memory.

1.3 Zebrafish as a Preeminent Model Organism

The History of Zebrafish: From Ornamental Fish to a Core Pillar of Scientific Discovery

The zebrafish (*Danio rerio*) (Hamilton et al., 1822) has redefined scientific research, becoming a preeminent model organism in genome evolution (Postlethwait et al., 2004), developmental biology (Kinkel & Prince, 2009), behavioural studies (Norton & Bally-Cuif, 2010), and toxicology (Peterson & MacRae, 2012). With its transparent embryos, rapid external development, large clutch sizes, high genetic homology with humans, and regenerative capabilities, for instance, of the heart and nervous system (Ghosh & Hui, 2016), it has accelerated breakthroughs in biomedical research, especially in human diseases (Lieschke & Currie, 2007; Santoriello & Zon, 2012). The present section discusses the history of zebrafish as a research model, and its journey from natural habitat to a well-established experiment model in modern applications.

The zebrafish is a small cypriniform teleost, commonly known as a minnow, belonging to the lineage of ray-finned fishes (Actinopterygii), which is one of the dominant classes of bony vertebrates (McCluskey & Braasch, 2019). Zebrafish were originally named *Cyprinus rerio* by Francis Hamilton in 1822 and identified from the Kosi River in India. The species name is based on the local Bengali name “Dhani” (of the rice field) (Spence et al., 2008). The native range of the fish comprises the South Asian Ganges and Brahmaputra River basins of India, Bangladesh, and Nepal. They are found in different habitats, ranging from stagnant ponds and rice paddies to flowing streams, demonstrating adaptability to laboratory conditions (McCluskey & Braasch, 2019; Parichy, 2015).

Zebrafish are omnivorous and feed on insect larvae and adults, as well as small crustaceans and other zooplankton, while also consuming algae, plant material, and various detritus (McCluskey & Braasch, 2019). The presence of similar core gut bacteria was identified in both wild-caught and laboratory strains of zebrafish, reflecting the importance of a stable gut community for growth and development (Roeselers et al., 2011). Zebrafish face natural predators including snakeheads (*Channa sp.*), freshwater garfish (*Xenentodon cancila*), Catfish, Knifefish, and Indian

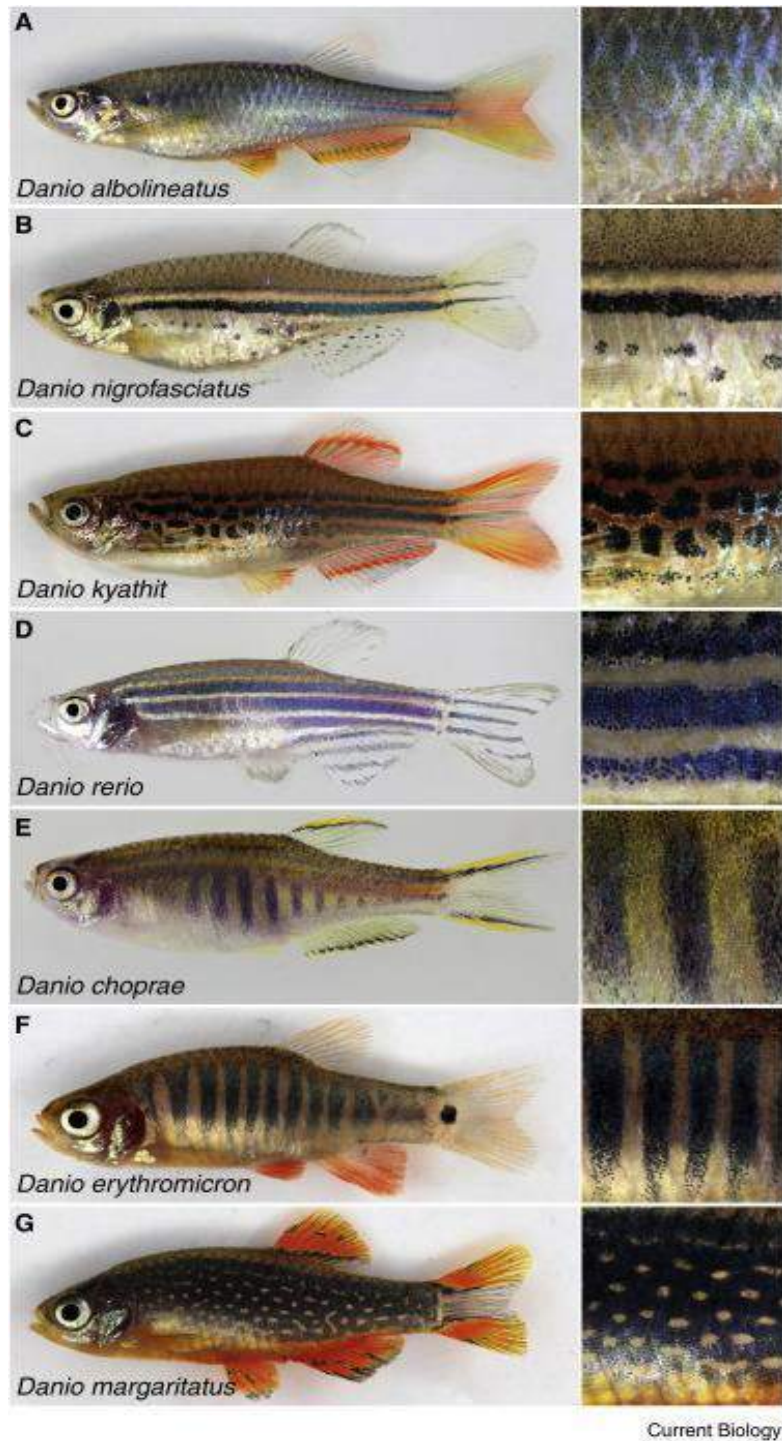
leaf fish (*Nandus nandus*), while birds such as common kingfishers and Indian pond herons also prey on them (Bass & Gerlai, 2008; Gerlai, 2009). While climbing perch (*Anabas testudineus*) and zebrafish coexist in the same geographical regions and habitats, it is not listed as a primary predator of zebrafish. However, climbing perch is a generalist predator, and studies have shown *Anabas* to act as a potential stressor of zebrafish, particularly in three-chambered tank tests (Chathooth et al., 2025). Schooling fish exhibit strong anti-predator responses including tightened shoaling, release of sensory cues (alarm substances from injured fish), freezing, change in pigmentation, and increased aggression towards group members (Gerlai, 2009; Goulart & Young, 2013).

Zebrafish are prolific breeders, and females can lay several hundred eggs at once. Breeding in the wild primarily occurs during the summer monsoons. Spawning typically occurs near dawn and is characterized by male territoriality and female selectivity regarding oviposition sites (Parichy, 2015; Spence et al., 2008). Mating behaviours observed under laboratory conditions include initial approach, chasing and circling by the male, and the female leading the male to the oviposition site. Under laboratory conditions, zebrafish can reach reproductive maturity in 4-6 weeks, and their lifespan can extend up to approximately 5 years under ideal conditions. Little is known about their lifespan in wild conditions (Kishi et al., 2009). Under laboratory breeding set-ups, successful breeding can be achieved by mimicking natural dawn conditions and maintaining proper tank and water quality for the high yields of viable eggs. After spawning, adult zebrafish exhibit no parental care and consume their own eggs or larvae if not separated by a breeding chamber. Zebrafish eggs are small, around 0.7 mm in diameter, and optically transparent, the transparency resulting from the unique yolk structure and a clear outer chorion (Hamm, 2018). The visual transparency and rapid external growth make zebrafish a vital model for developmental biology and toxicology (Spence et al., 2008).

The fish owes its common name “zebrafish” to the stereotypic pattern of longitudinal blue and yellow stripes, reminiscent of a zebra. Within the small *Danio* genus, which contains approximately 20 valid species (distinct from close relatives *Devario* and *Danionella*), significant colour diversity is found (Parichy, 2015). For example, *Danio albolineatus* exhibits almost uniformly distributed melanophores with a posterior red

stripe; *D. nigrofasciatus* displays distinct stripes; in *D. kyathit* stripes break into spots; and *D. rerio* is characterised by continuous horizontal stripes. While many species exist, *D. rerio* became the primary model for the study of vertebrate colouration due to the progress in transgenic tools, in vivo imaging, and availability of large collections of mutants (Singh & Nüsslein-Volhard, 2015).

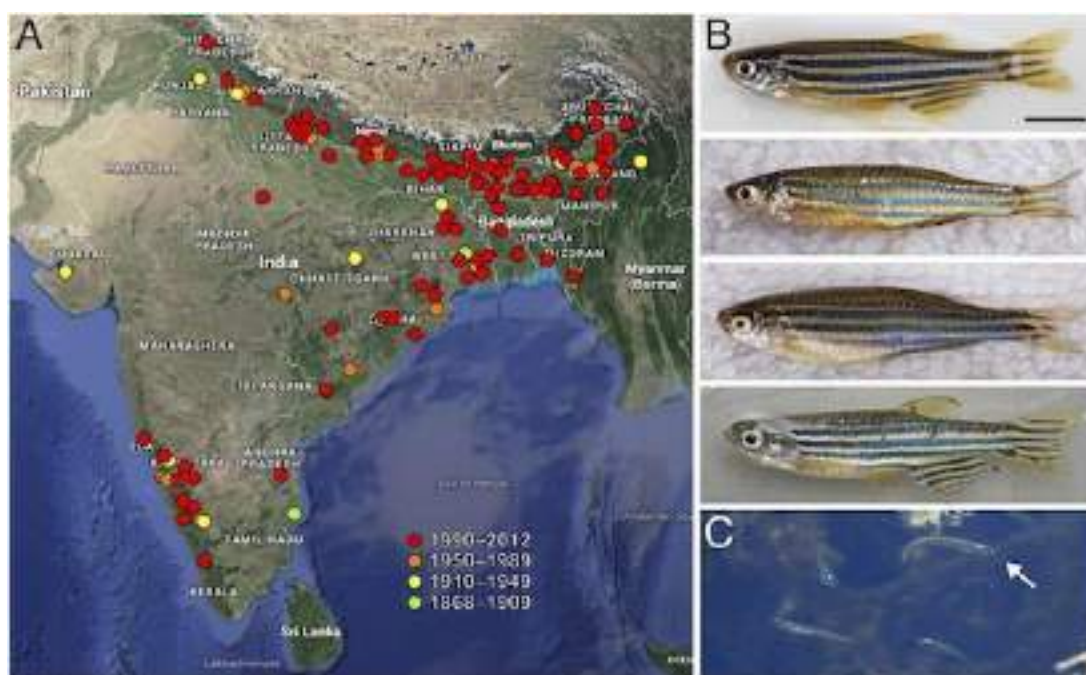
It was first introduced to the aquarium trade in 1905 and named the zebra danio. Zebrafish were formally established as a model organism by George Streisinger in the early 1980s. Working at the University of Oregon, using homozygous diploid clone methods, George Streisinger and colleagues produced one of the earliest cloned vertebrate lines of *Danio rerio* (Streisinger et al., 1981), more than a decade before the cloning of Dolly the sheep was achieved from an adult somatic cell (Williams, 2003). During the 1990s, the model's prominence rose with the identification of key developmental mutants, solidifying its role in genetics and developmental biology. The sequencing of the entire zebrafish genome was conducted during the 2000s, enabling advanced genetic research, and expanding its utility into neurobiology, cancer research, and toxicology. In modern applications, large numbers of researchers across the globe use zebrafish for studies in immunology, drug screening, regeneration, and genome editing (CRISPR). Therefore, today, the zebrafish, with approximately 70% genetic similarity to humans, is established as a cornerstone model in modern biomedical research (Howe et al., 2013; Kint et al., 2013; Priyadarshini, 2024).



Adopted from: Singh & Nüsslein-Volhard, 2015 (DOI: 10.1016/j.cub.2014.11.013)

Figure 1.3. Diversity of colour patterns among species of the genus *Danio*.

This figure illustrates the wide variation in body pigmentation patterns observed across different *Danio* species, including stripes, spots, vertical bars, and uniform pigmentation. Differences in body and fin patterning, as well as pigment cell organization, are evident across species. These variations highlight the utility of zebrafish as a model for studying vertebrate colour pattern formation.



Adopted from: Parichy, 2015 (DOI: 10.7554/eLife.05635)

Figure 1.4. Geographic distribution and natural populations of zebrafish (*Danio rerio*).

This figure illustrates the geographic range of zebrafish across South Asia, including India, Nepal, Bangladesh, and possibly Myanmar. It also presents representative individuals from natural populations, highlighting sexual dimorphism in body coloration and morphology. Additionally, zebrafish are shown in their natural habitat, demonstrating their typical shoaling behaviour in shallow freshwater environments.

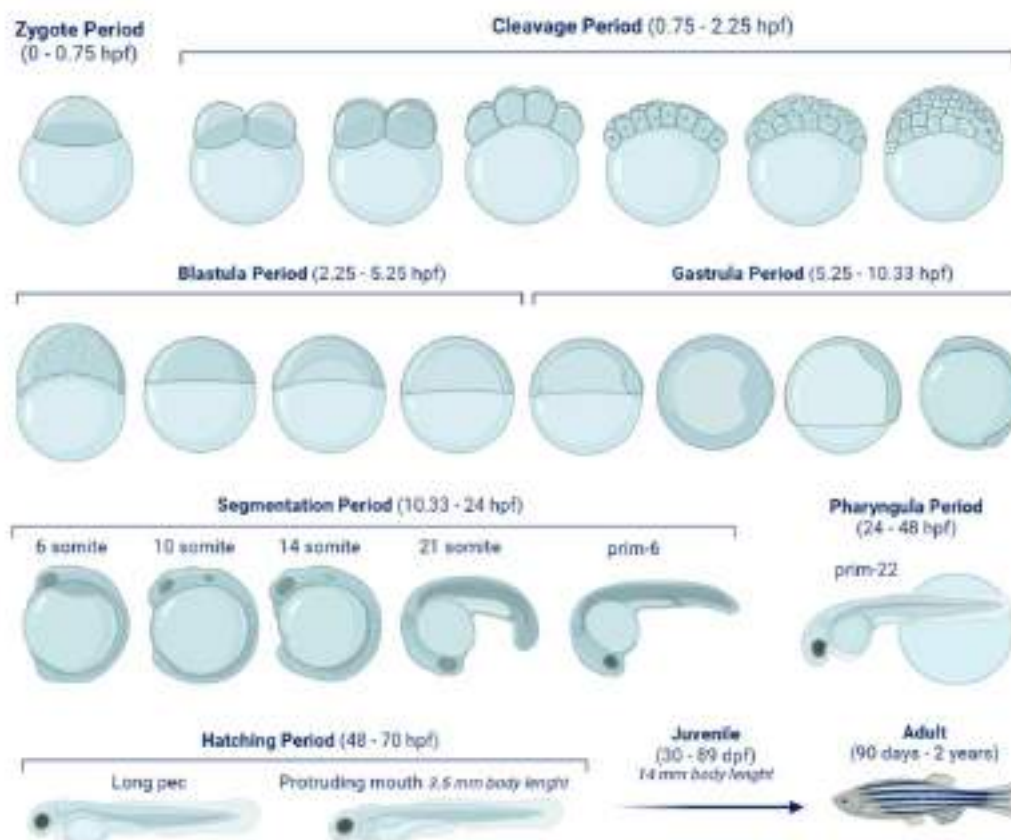
1.4 Zebrafish Male vs. Female: Relevance of Sex in Preclinical Models

Sex is an important factor affecting physiology, behaviour, and pharmacological responses across species (Bale & Epperson, 2016; Farrukh & Becker, 2025). For a long time, sex was frequently disregarded as a biological factor in preclinical research, with experiments commonly carried out using mixed or unsexed groups. Nevertheless, increasing evidence indicates that sex-specific variations can greatly influence biological mechanisms and drug responses (Hillman et al., 2025).

Zebrafish sex development begins with a common ovarian pathway, where all larvae develop ovary-like gonads known as proto-ovaries with oocytes from 10-25 days post fertilization (dpf), leading to sex differentiation. Continuation of oocyte maturation

occurs in females, while males undergo programmed cell death, known as oocyte apoptosis, making them juvenile hermaphrodites (Aharon & Marlow, 2021; Kossack & Draper, 2019). Both genetic and environmental factors influence the entire process of sex differentiation (Kossack & Draper, 2019). There are several key molecular players involved in the sex determination pathway, for instance, *sox9a*, a known male-determining gene, showing strong expression in developing testes, while Bone Morphogenetic protein 15 (BMP 15), a protein produced by oocytes, is essential for maintaining the female pathway. Next, DNA methylation has an important role in reprogramming germ cells and regulating sex determination genes (Kossack & Draper, 2019). Sex is generally maintained at the adult stage; at this stage, sex identification is possible by using external morphological features. Females are typically larger, characterized by a rounded abdomen and a prominent genital papilla, whereas males are slender with more vibrant yellow or orange pigmentation, particularly on the anal fins. Additionally, males often exhibit thicker pectoral fin rays and a more streamlined morphology (Crowder et al., 2018). These external features are highly dependent on the observer's expertise in the field; however, studies suggest that sexing adult female zebrafish on the basis of prominent genital papillae alone is a reliable technique (Yossa et al., 2013). More accurate sex identification can be performed using geometric morphometric analyses (Duff et al., 2019). It allows the analysis of the shape of anatomical structures for the detection of subtle morphological differences between males and females. This accurate testing is valuable for high-throughput screening (Duff et al., 2019).

As discussed earlier, sex-specific responses were evident across previous research, impacting brain structure, neurochemical composition, and most significantly behaviour, which directly influences clinical translational research (Genario et al., 2020). Males are generally more explorative, while females are shy or anxious in novel experimental setups. Increased aggression and boldness responses were marked in males while females exhibit greater sociability and improved memory retention in some cognitive tests. These external responses were also related to neurochemical differences; females are identified with higher dopamine levels in specific brain areas, while serotonin and its metabolites vary considerably across sexes (Genario et al., 2020).



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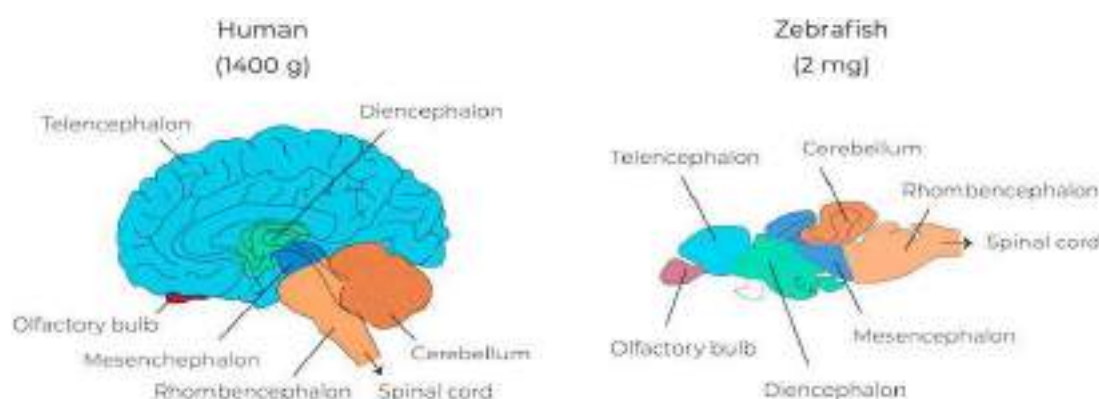
ZeClinics. <https://www.zeclinics.com/blog/understanding-zebrafish-development-stages/> and based on Kimmel et al., 1995

Figure 1.5. Developmental stages of zebrafish from fertilization to adulthood.

This figure illustrates the sequential progression of zebrafish development, including the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching stages, followed by juvenile and adult phases. Each stage is characterized by distinct morphological and developmental events that provide a framework for studying vertebrate embryogenesis.

At the physiological level, the brains of males and females exhibit unique cellular and hormonal patterns. For example, female zebrafish demonstrated enhanced neurogenesis in telencephalic areas, whereas males display greater activity in hypothalamic regions (Genario et al., 2020). Differences in hormonal profiles are also found; estradiol levels are higher in females, while increased levels of androgen 11-ketotestosterone are marked in males. Evidence from studies indicate that the estradiol exposure inhibits mating behaviour in males (De Castro Assis et al., 2018; Fuzzen et

al., 2011), and environmental estrogens altered both spermatogenesis and sexual behaviours in male and female zebrafish (Tan et al., 2024). Anxiety-related behaviours further demonstrate how sex influences zebrafish responses in preclinical research. Females are found to be more anxious than males in standard tests like the novel tank test, remaining longer at the bottom and demonstrating less exploratory behaviour than males (Amar & Ramachandran, 2023; Tao et al., 2025). These results indicate the role of sex as a biological variable in research, ranging from behavioural tests to pharmacological studies.



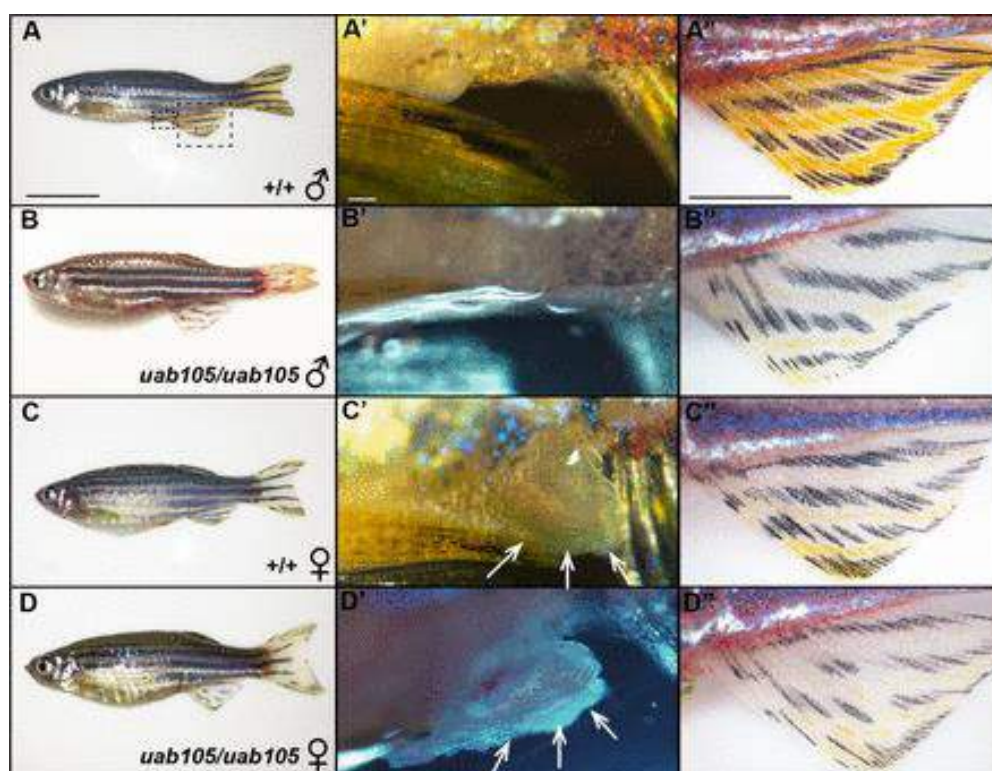
Adapted from:

ZeClinics. <https://www.zeclinics.com/services-and-solutions/neurotoxicity/>

Figure 1.6. Conserved brain structures between zebrafish and humans.

This figure presents a comparative overview of major brain regions in humans and zebrafish, highlighting conserved neuroanatomical organization across vertebrates. Key regions such as the telencephalon, diencephalon, mesencephalon, cerebellum, and rhombencephalon are identifiable in both species, supporting the use of zebrafish as a model for neurobiological research.

Therefore, understanding the vital role of sex as an additional influencing factor, especially in behavioural studies (Y. Wang et al., 2025; Zhai et al., 2022), we employed sex-specific analysis in anxiety, activity, stress, aggression, buffering, dyad and gut microbiome analysis. The collective behaviours and shoaling study have already established the role of sex in determining the spatial interactive area within a shoal, thereby we adapted mixed sex shoals for the present study. Incorporating sex as a biological variable allowed for a novel analysis of social isolation's effects, thereby improving the accuracy, reproducibility, and translational relevance of the study and increasing the plausible opportunities for targeted therapeutic interventions.



Adopted from: Crowder et al., 2018 (DOI: 10.1210/en.2018-00685)

Figure 1.7. Morphological differences between male and female zebrafish.

This figure illustrates sexually dimorphic traits in zebrafish, where males exhibit a slender body shape, absence of a prominent genital papilla, and a yellowish anal fin, while females display a rounded body with a distended abdomen, a visible genital papilla, and a paler anal fin.

1.5 Rationale of the Study

Current research on social isolation excels at elucidating its importance and impacts on cognitive distortion, biological stress, skill atrophy, and loss of support across animal models. It aligns with the biopsychosocial model, which suggests that overall health is a sum of genetics, thoughts, and social factors (such as isolation). Although existing studies sometimes are unable to address the sex-specific outcomes of the condition, there is also a discrepancy attributed to their inability to examine the effects of chronic social isolation. Moreover, the present study evaluates chronic social isolation through multi-pronged behavioural tasks arranged in a cognitive order, that is, from internal behavioural valence to higher cognitive functions such as social interactions and decision-making.

Together, the present thesis on social isolation formulates five core objectives in order to address the existing research gap and demonstrate sex-specific analysis across vital behavioural tasks, while also adding a descriptive layer of gut microbial analysis to illustrate the prominent role of the microbial-gut-brain axis in maintaining homeostasis.

Additionally, a significant challenge of the present research was the quantification of social interaction in shoaling analysis and maintaining the optimal sex ratio in order to produce maximum shoal cohesion. Consequently, an initial phase of the study was dedicated to establishing and validating a novel shoaling methodology. This methodological framework was independently published and served as the foundational study for the core analysis related to social isolation, particularly in Objectives 3 and 4. The details regarding the methodology, major outcomes, and factors contributing toward the core objectives are summarized as a separate chapter in the final sections of the thesis.

1.6 Core Objectives

1. To assess anxiety-like behaviours and activity changes under chronic social isolation.
2. To evaluate behavioural responses to multiple stimuli and examine the acute modulatory effects of a group of conspecifics on stress and aggression.
3. To analyse shoaling behaviour and emergent states under social isolation.
4. To examine the spatio-temporal dynamics of dyads under social isolation.
5. To conduct a descriptive analysis of gut microbiota abundance under social isolation with respect to sex.

1.7 Objective Narration

1. To assess anxiety-like behaviours and activity changes under chronic social isolation.

The primary objective aimed to address the internal behavioural valence of both control and isolated zebrafish with respect to sex, utilising the Open Field Tank (OFT)

test. OFT is a standard and well-validated behavioural paradigm to evaluate both the anxiety-like responses and exploratory behaviours of zebrafish. Time spent across the wall, termed thigmotaxis, is accounted for as the primary anxiety measure, and other supporting metrics are added to strengthen the behavioural readout. Various locomotor parameters were also evaluated from the same experimental design to examine the activity changes relative to chronic social isolation. The Light and Dark test (LDT) is then utilized as a confirmatory measure of anxiety, by analysing the zone preference and latency to enter the light zone. Efforts were taken to understand the sex-specific differences, as studies report strong baseline sex differences existing in the anxiety-like behaviours and exploratory nature in zebrafish. Therefore, we hypothesised a sex-dependent alteration across both behavioural domains in socially isolated zebrafish.

2. To evaluate behavioural responses to multiple stimuli and examine the acute modulatory effects of a group of conspecifics on stress and aggression.

The second objective consisted of critical layers associated with chronic social isolation and sex across different social contexts, including predator-induced stress and mirror evoked aggressive-like responses. The outcome of this objective was predicated to be critically impacted by the internal valence of behaviour identified in the primary objective. A three chambered glass tank was employed in this entire analysis, and it is a well-validated behavioural task associated with choice based behavioural experiments. We examined the acute modulatory effect of a group of conspecifics on stress and aggressive responses under social isolation. Therefore, the number of choices was dual, that justifies the use of a three chambered tank design that can hold dual choices simultaneously. The objective includes a No Stimulus (NS) condition, holding only the experimental animal in the middle chamber; this NS condition served as the baseline for the subsequent stimulus conditions and was important to omit the possibilities of side preferences existing in the experimental animals. Shoal only (SO) includes a group of conspecifics serving as the positive stimulus, to quantify the response towards a known stimulus from the zebrafish across different experimental groups. Predator only (PO) and Mirror only (MO) were the important stimulus choices to evaluate the responses against a stressor and a

competitor. Two buffering conditions were designed: Predator + Shoal (PS) and Mirror + Shoal (MS) in the dual-choice conditions. Statistical comparisons of relevant parameters across PO and PS and MO and MS were predicted to give insight on the role of conspecifics in stress and aggression buffering under social isolation. Rigorous statistical comparisons were included to check the effect of stimulus within each experimental group with respect to sex and stimulus specific between group comparisons across control and experimental conditions (CM, TM, CF, TF). As similar to baseline sex differences in novel experimental conditions, existing literature suggests strong sex differences related to stress and aggression. Therefore, we hypothesised that chronic social isolation will be sex-dependently influence the behavioural responses across multiple stimuli in single-choice experiments and that social buffering will exert a positive modulatory effect on social isolation induced behavioural changes.

3. To analyse shoaling behaviour and emergent states under social isolation.

The third objective included analysing the higher cognitive behavioural responses from socially isolated individuals within a shoal. From this objective onwards the challenging transition occurred from measuring movements from single animals to quantifying group-level dynamics. A five membered shoal was constructed for control and test individuals, and based on the previously mentioned foundational methodological study, we used 4:1 mixed sex ratio to build the shoal structure. The similar OFT tank used in the primary objective was used in the study. Previous studies reported altered shoaling impacted probably with social isolation based fundamentally on distance-based metrics. Efforts were taken to integrate multidimensional parametric analysis that could capture distance-based, alignment, and spatial orientation within the shoal and also employed established computational mathematical models to understand the transient nature and examine the different emergent states of a shoal. We hypothesized that chronic social isolation induces complete shoal disruption not only in the distance metrics but also in the alignment and orientation metrics of shoaling and differences in the occurrence of emergent states compared to the control group.

4. To examine the spatio-temporal dynamics of dyads under social isolation.

Objective 4 was carried out in the similar OFT tank used in the previous Objectives 1 and 3. Different studies including both fundamental and computational mathematical models across multi-animal interactions, have shown that interactions are formed based on pairwise interactions, and there are also studies that leverage the importance of three body interactions to form complex collective behaviours. We relied on the pairwise interactions as the basic unit in the multiple animal interactions based on the research and included extensive experimental grouping based on sex and test combinations to understand the spatio-temporal dynamics of dyads that eventually lead to complex collective behaviours. The experimental groups were broadly classified into two, homogeneous dyads (Control Male-Male, Control Female-Female, Control Male-Female, Test Male-Male, Test Female-Female, Test Male-Female) and heterogeneous dyads (Control Male-Test Male, Control Female-Test Female, Control Male-Test Female, Control Female-Test male). We gave importance to the spatio-temporal dynamics of dyads including multidimensional parameters and purposefully relied upon non-reproductive behaviours. Efforts were taken to elucidate the potential initiation of more directional turns from individuals that form leadership behaviour when two animals interact within a pair. Therefore, we hypothesized that as observed earlier in the foundational methodological study, sex will be a critical factor for dyadic interactions as well, and social isolation will induce prominent changes in the spatio-temporal dynamics of dyads, potentially affecting the initiation turns.

5. To conduct a descriptive analysis of gut microbiota abundance under social isolation with respect to sex.

Encouragingly, the microbial-gut-brain axis is found to influence the behavioural responses particularly related to neurodevelopmental disorders such as autism. By identifying the microbial shifts if any impacted from chronic social isolation with respect to sex, this will provide preliminary descriptive insight relevant to further future studies related to sex-specific therapeutic interventions. Therefore, examining microbial abundance is relevant in order to correlate a complementary biological layer

associated with chronic social isolation. We employed next-generation sequencing methods to understand the trends associated with microbial abundance across experimental groups. The samples were pooled, comprising seven gut components from each group, which justifies the presentation of data as a pilot study or descriptive in nature. As the horizontal transmission is blocked in the test groups, we hypothesized that the gut microbial abundance will be impacted by the chronic social isolation. Whereas related to sex-specificity, gut microbiome and sex specificity related literature show both sex dependent and independent trends, the hypothesis avoided prediction related to sex.

Chapter 2

Review of Literature

2.1 Bidirectional Anxiety Responses: Anxiogenic and Anxiolytic-like Behaviours

The historical and clinical evolution of anxiety builds the roots and contradicts the concept of anxiety being a modern invention, as it was, documented as Nicanor's phobia in Greco-Roman texts and identified as a medical disorder (Crocq, 2015). Similarly, ancient philosophers conceptualized ideas similar to Cognitive Behavioural Therapy (CBT), including those of Seneca and Cicero, as well as the concept of Ataraxia. Seneca and Cicero defined peace of mind as a state free from disturbances, whereas Ataraxia is an Epicurean goal of the mind to be free from negative thoughts from the past or future. The word "anxiety" reflects the physical sensation of tightness or narrowness in the chest or spirit. From the Middle Ages to the 17th century, both depression and anxiety were considered under an umbrella term, melancholia. Later, in the 18th century, panophobia was introduced, to describe panic terror often occurring at night (Crocq, 2015). Then during the 19th century, the word "neurasthenia" was used to describe broad nervous exhaustion that included anxiety, which eventually led to Freud's Anxiety Neurosis. In the current DSM era, DSM I and II considered anxiety as a core feature of psychoneurotic disorders, and in DSM III, it was separated into panic disorder (PD) and Generalized Anxiety Disorder (GAD). This distinction was pharmacologically validated using imipramine, which was found to reduce PD but not GAD. In DSM-5, the modern integration defined anxiety as anticipation of future threat and entirely distinguished it from fear, which is considered as an emotional response to a real threat. Notably, DSM-5 separated anxiety into three broader spectra including anxiety disorders, obsessive-compulsive-like disorders, and trauma/ stress-related disorders (Crocq, 2015).

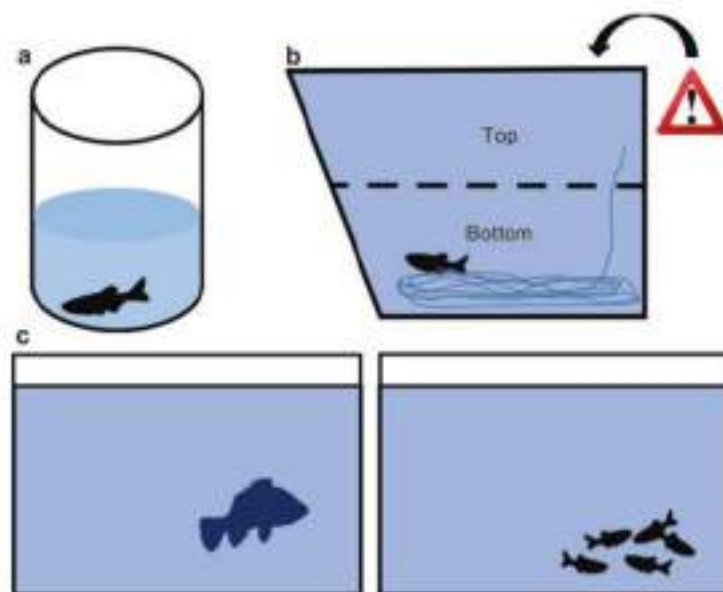
The occurrence of anxiety disorders has increased in the present time, particularly post-COVID (Burkauskas et al., 2023; Santomauro et al., 2021), thereby seriously affecting human well-being. Anxiety disorders considerably increase the chances of other comorbidities such as diabetes (Mersha et al., 2022), thyroid problems (Zhou et al., 2021), gastro-intestinal disorders (Navarro-Tapia et al., 2021), and chronic somatic disorders (Zheng et al., 2019). An estimated prevalence of anxiety disorders ranges from 7.38% to 28% and it is ranked as the sixth-largest contributor to worldwide disability by WHO (Gong et al., 2020; Santomauro et al., 2021). In the present

scenario, the pathogenesis behind anxiety is less clear; however, imbalance between brain excitatory and inhibitory neurotransmitters such as glutamate and gamma-aminobutyric acid (Chen et al., 2019; Pollack et al., 2008), and deficiencies of 5-HT and norepinephrine (Kuo et al., 2017) are some of the recognized reasons. The initial observations of Charles Darwin laid the foundation for studying animal models of psychiatric disorders, particularly in rodents, due to their similarity to human-like emotional responses toward anxiety and stress (Campos et al., 2013). In the present scenario, there are multiple important models available to induce anxiety or stress in animal models. As our study design is based on the relevant ethological models, additionally, unconditioned and conditioned models, pharmacological models, and psychosocial stress models are present.

Under the unconditioned animal models of anxiety specific to rodent models, predator stress model, social isolation model, and neonatal separation model are included. In the predator stress model, by preventing the direct attack from the predator, visual, olfactory, or acoustic stimuli related to the predator are used to evoke anti-predator stress responses from the experimental animal (Scott et al., 2022). Rodent models exhibit increased excitement upon predator presence; rigid movements, crouching, and fleeing can be observed (Scott et al., 2022). The anti-predator responses were suggested to be evoked by the activation of the hypothalamic-pituitary-adrenal axis in mammals (Zoladz et al., 2021). Rodent models exhibit group behaviour, and forced social isolation is evidenced with cognitive decline in rodent models. Social isolation and associated alterations in the levels of 5-HT and dopamine were found in rodent models (Krupina et al., 2020). The neonatal maternal separation model is used to induce early life stress in rodent models, as they exhibit strong parental care (Rosa et al., 2022). Rodents undergoing neonatal maternal separation exhibited increased anxiety-like behaviours and increased retention of memory related to fear (Farzan et al., 2023). Under conditioned animal models of anxiety specific to rodent models are include restraint stress, which can be acute or chronic, fear-conditioning anxiety model, and repeated social defeat. Drug and genetic models are additional anxiety models. Rodent-specific behavioural tests used to elucidate anxiety related behaviours include elevated plus maze, open-field test, light and dark test, elevated zero maze, and novelty-suppressed feeding test (Zhao et al., 2023). All these are characterized by being comparatively simple in design and not harmful to the animals; semi-to-fully

automated tracking results in more realistic interpretation of behavioural outcomes. Multiple factors are involved in the occurrence of psychiatric disorders such as neurotransmitter dysfunction, neuroendocrine dysfunction, immune dysfunction, neuronal plasticity, ion channels, endocannabinoid system, neural circuits, gut microbiota, mitophagy, and genetic factors (Neff, 2016; Zhao et al., 2023).

In comparison with the established rodent model of anxiety disorders, as discussed in the earlier section, currently, zebrafish are also considered well-validated animal models for the study of psychiatric disorders (Blaser et al., 2010; Sackerman et al., 2010). Comparative studies between zebrafish and rodent models using xanthotoxin have shown similar anxiety-related responses in both models, further suggesting the role of zebrafish in research on anxiety disorders (Maciąg et al., 2020). Similar to rodent models, zebrafish are also used across various anxiety models including pharmacological and ethological models to evaluate anxiety or stress responses (Bencan et al., 2009; Champagne et al., 2010; Speedie & Gerlai, 2008; Stewart et al., 2010b). Here, the use of the open-field test (OFT) and thigmotaxis as an important anxiety metric is prominent. Thigmotaxis is the wall-hugging behaviour primarily evaluated in rodent models from an open-field arena; generally, increased thigmotaxis is considered as anxiogenic and decreased thigmotaxis is evaluated as an anxiolytic response (Grossman et al., 2010; Levin et al., 2007; Stewart et al., 2010a). Interestingly, studies have demonstrated that similar thigmotaxis behaviour is associated with anxiety in the open-field test both in larval (Schnörr et al., 2012) and adult zebrafish and is considered as a valuable behavioural endpoint in anxiety disorders (Lucon-Xiccato et al., 2022). From the same experimental design, the total distance travelled, speed, and entries to specific zones can be evaluated under the exploratory activity levels of experimental animals. In addition to this, the light and dark paradigm is generally employed across zebrafish and rodent models, where the time spent in the light zone is considered as the relevant marker; increased time in the light zone can be anxiolytic and decreased time in the light zone can be anxiogenic in nature. The natural preference for dark zones as shelter-seeking behaviour, especially in adult zebrafish is termed scototaxis behaviour and can be monitored using the light and dark test (Maximino et al., 2010b; Maximino et al., 2011; Serra et al., 1999). The use of LDT has also been validated in juvenile zebrafish (Steenbergen et al., 2011).



Adopted from: Collier et al., 2017 (DOI: 10.1007/978-3-319-33774-6_3)

Figure 2.1. Experimental paradigms used to induce anxiety-like behaviours in adult zebrafish.

This figure illustrates commonly used experimental stress models, including confinement stress, alarm substance exposure, and predator exposure, which induce measurable anxiety-like behavioural responses such as avoidance, increased stress levels, and altered locomotion patterns.

In zebrafish research, the study on anxiogenic response (Baraban et al., 2005; Barcellos et al., 2007; Cachat et al., 2010; Wong et al., 2010) is extensively validated, but the study on anxiolytic responses (Tang & Davies, 2020) is comparatively scarce. Stress-associated emotion regulation is predictive, and it represents a psychophysiological adaptation (Jentsch & Wolf, 2024). Therefore, this emotional valence of an experimental animal undergoing stress can be bidirectional. The effect of anxiolytic drugs like benzodiazepines, buspirone, and ethanol has been validated in zebrafish and has shown reduced shoaling, increased exploration in the top of the tank, and increased time spent in the light zone (Gebauer et al., 2011). This pharmacological study also suggests the light and dark test as a reliable and low-cost screening test for anxiolytic drugs (Gebauer et al., 2011; Sackerman et al., 2010). Scopolamine, a muscarinic acetylcholine receptor antagonist, has been reported to exert anxiolytic-like effects in zebrafish. In contrast to rodents, where scopolamine

administration increased anxiety-like behaviour in standard behaviour tests, zebrafish exhibited anxiolytic like behaviours. Scopolamine reduced anxiety in a dose-dependent manner, peaking at 800 μ M before exhibiting a biphasic response at higher concentrations. This study further strengthens the use of zebrafish in elucidating the anxiolytic responses using zebrafish (Hamilton et al., 2017).

Together, the review summarises the history, prevalence, and current status of anxiety disorders, as well as important unconditioned models and relevant behavioural tests and endpoints used in the zebrafish and anxiety-stress research. Our present study focuses on chronic social isolation as the stressor and is dependent on the OFT and LDT for the behavioural analysis related to anxiety responses.

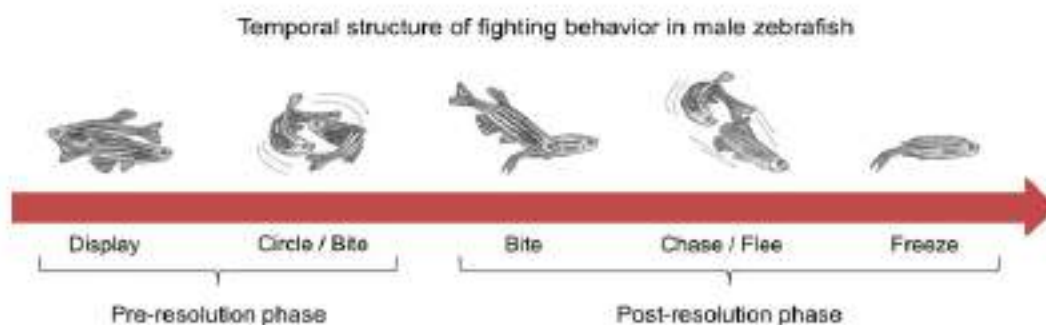
2.2 Behavioural Phenotypes in Zebrafish Models of Aggression

Aggression is a conserved adaptive behaviour across species that is fundamental to protect offspring, resource acquisition, territoriality, and for the maintenance of social hierarchies (Koolhaas et al., 1999). In humans, altered aggressive responses are connected to multiple neuropsychiatric conditions including major depressive disorder, anxiety, impulse control deficits, and borderline personality disorder (Brent, 2009; Farrington, 2004; Scott et al., 2017; Weisbrot & Ettinger, 2002). These aggression networks in mammals involve the amygdala, lateral septum, and hypothalamus, whereas, zebrafish possess conserved CNS circuits and neurotransmitter system that mirrors this mammalian aggression functions (Nelson & Trainor, 2007; Newman, 1999).

Research on aggression is predominant in rodent models (Cenci et al., 2002; De Boer, 2018; Nestler & Hyman, 2010), especially in stress induced aggression model such as social isolation (Matsumoto et al., 2005) similar to other developments in behavioural neuroscience, zebrafish are now considered an important model organism to study relevant aggressive behaviours (Gutiérrez et al., 2018; Laan et al., 2018; Way et al., 2015b)

Zebrafish demonstrate observable and measurable aggressive phenotypes spanning from behavioural tests, pharmacological, and pharmacogenetic models of aggression. The present study is dependent on behavioural tests to evaluate the aggressive responses from isolated male and female zebrafish. The relevant behavioural tests

used for aggression comprise of mirror-biting test, dyadic fight test, video stimulus test, and stress-evoked aggression (Gerlai et al., 2000; Oliveira et al., 2011; Teles & Oliveira, 2016; Way et al., 2015a). Zebrafish perceive their reflection in the mirror as a conspecific intruder or competitor and metrics including time spent in the mirror zone and number of mirror bites or direct attacks were used as the relevant behavioural metrics from the mirror-biting test (Gerlai et al., 2000).



Adopted from: Teles & Oliveira, 2016 (DOI: 10.1007/978-1-4939-3771-4_20).

Figure 2.2. Temporal structure of aggressive interactions in zebrafish.

This figure depicts the sequential phases of aggressive encounters, including an initial mutual interaction phase followed by the establishment of dominance, where one individual display dominant behaviour while the other exhibits submissive responses.

In the dyadic fight test, the direct social interaction between two fish will be monitored (Oliveira et al., 2011), whereas in the video stimulus test, a computer monitor will display aggressive conspecifics to evoke aggressive responses from the test fish (Way et al., 2015a). Studies have also used physical stressors to trigger stress induced aggressive responses (Giacomini et al., 2016). Research supports the use of zebrafish as a valid pharmacological model of aggression, thereby facilitating the screening of potential therapeutic compounds. Distinct levels of monoamines were found in the dominant and subordinate fish, where dominant fish exhibited lower serotonin turnover in the hindbrain (Dahlbom et al., 2012; Teles et al., 2013). Exposure to fluoxetine, an antidepressant identified to reduce offensive aggression in dominant fish (Theodoridi et al., 2017). Similarly, MK-801 administration was found to be associated with reduced aggressive behavioural phenotypes (Zimmermann et al., 2016) whereas ketamine exposure displayed a biphasic effect such as increasing

aggression at low doses and decreasing aggression at higher doses (Michelotti et al., 2018). Similarly, taurine-exposed fish showed increased aggression and suggested a serotonergic receptor pathway association (Mezzomo et al., 2020).

Notably, alcohol has been identified to exert an inverted U-shaped dose-response curve related to frequency of aggression (Fontana et al., 2016; Gerlai et al., 2000; Sterling et al., 2015). Studies have suggested additional pharmacological inhibitors of aggression including caffeine, sildenafil, (Gutiérrez et al., 2020) and Kava kava extracts (Wang et al., 2020). Zebrafish can be used as an effective tool for high-efficiency mutation and study of strain-specific differences in behaviour (Cade et al., 2012; Séguret et al., 2016). Studies also highlighted the potential sex-specific differences associated with aggression. Under chronic stress male zebrafish displayed increased aggression and females showed no change in aggression when compared to their respective control counterparts (Rambo et al., 2017). Other external environmental factors, such as hunger showed reduction in female aggression than male aggression (Ariyomo & Watt, 2015). Diet has also been correlated with dysregulated aggression levels in zebrafish (Sivarajan et al., 2025), such as high-fat diets are associated with increased aggression and anxiety-like phenotypes (Picolo et al., 2021).

Overall, zebrafish represent a powerful aggression model for elucidating the environmentally modulated behavioural phenotypes, subsequently making them a critical species in translational research of aggression.

2.3 Cross-Species Perspectives on Social Buffering

Social buffering signifies the alleviation of psychophysiological stress responses through the presence of conspecifics (Davitz & Mason, 1955). Social buffering is based on the principle that an organism's threat response is shaped by its social environment. As we discussed in the earlier sections, social behaviours or group living benefits survival strategies. Multimodal cooperative communication such as through vocal, visual, and chemical cues improves the detection of predators and access to resources (Kikusui et al., 2001; Seyfarth & Cheney, 2003). Social isolation is an established unconditioned stress model across different social species, and isolation is marked with heightened endocrine and autonomic reactivity, described as isolation

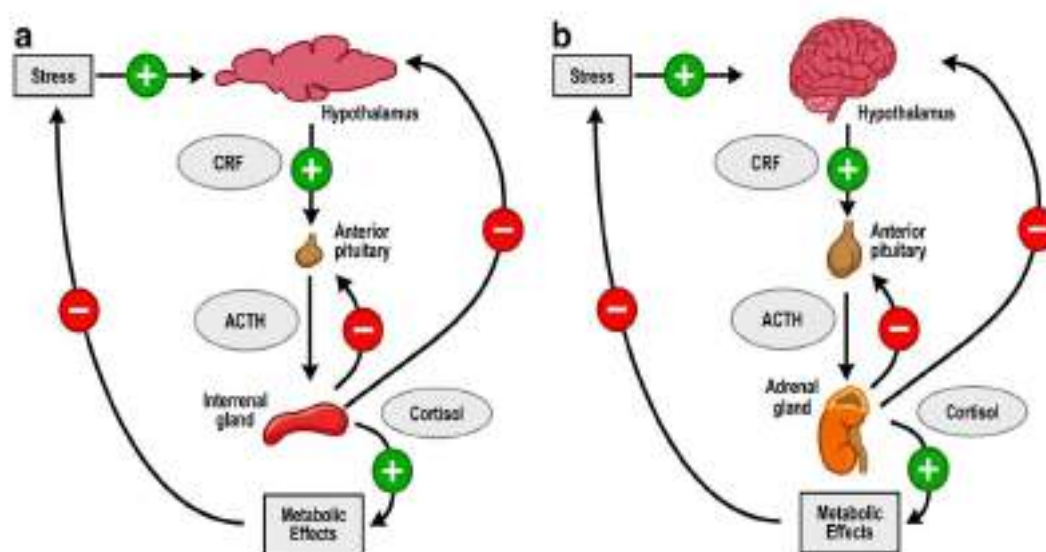
syndrome (Hatch et al., 1965; Valzelli, 1973). Here, the relevance and scope of social buffering are brought into salience as a facilitating mechanism for recovery from adverse environmental conditions (Hennessy et al., 2000).

Research in rodents demonstrates that social buffering functions at both behavioural and motivational levels. Reduction in the fear responses in rodents including freezing and withdrawal from novel areas are noted in the presence of a calm conspecific (Davitz & Mason, 1955). Similarly, heightened social contact also resulted as a stress response. Studies suggest that in rodents social interactions function as a reinforcer and buffering may engage neural systems associated with reward (Latané, 1969; Taylor, 1981). Likewise, observing a non-fearful partner can stimulate the reduction of fear (Tomizawa et al., 2013).

The vital role of the HPA axis in social buffering is prevalent at the physiological level. The presence of conspecifics can inhibit the corticotropin-releasing factor (CRF) release from the paraventricular nucleus (PVN) of the hypothalamus, thereby this facilitates the reduction of triggered release of ACTH and glucocorticoids (Hostetler & Ryabinin, 2013; Rivier & Vale, 1983; Vale et al., 1981). Compared to isolated individuals, stressed animals with conspecifics, measured with reduced c-Fos expression, a relevant marker of decreased PVN neural activity (Kiyokawa et al., 2004). At the neurotransmitter level, oxytocin is suggested as an important mediator of social buffering. Tactile stimulation can increase the oxytocin release via social bonding and subsequently inhibit the HPA activation, thereby attenuating stress response (DeVries et al., 2003; Neumann, 2002).

Sensory cues play a key role in the transmission of signals related to buffering. In rodents and primates tactile contact plays a central role (Wilson, 2001; Winslow et al., 2003). Visual exposure to familiar individuals is sufficient to reduce cortisol and autonomic stress response in sheep (Da Costa et al., 2004). In rats and pigs the chemical pheromones from mother mammals have demonstrated reduction in the tachycardia and aggressive behaviours (Kiyokawa et al., 2005; McGlone et al., 1986). Vocal communication also contributes to social buffering; following separation, hearing a call from a bonded partner showed to reduce the cortisol spike in marmosets (Rukstalis & French, 2005). The quality of social relationships and familiarity over unfamiliar conspecifics determine the effectiveness of social buffering (Coe et al.,

1978; DeVries, 2002). Likewise, the emotional state of the conspecifics also contributes; a calm companion over a stressed companion can provide higher attenuation on fear response (Davitz & Mason, 1955; Kiyokawa et al., 2004). Importantly, developmental exposure is a critical factor determining the potency of social buffering, as observed in primates subjected to maternal deprivation, which show social communication deficits and reduced responses to social buffering later in life. Therefore, social buffering is context-dependent and influenced by multiple factors across species (Winslow et al., 2003).



Adopted from: Collier et al., 2017 (DOI: 10.1007/978-3-319-33774-6_3)

Figure 2.3. Comparative organization of stress response axes in zebrafish and humans.

This figure shows the functional similarity between the zebrafish hypothalamus–pituitary–interrenal axis and the human hypothalamus–pituitary–adrenal axis, highlighting conserved hormonal signalling pathways involved in stress regulation.

Considering the scenario in fish specifically, social buffering defined as the social interaction facilitated reduction of behavioural, neural, and endocrine responses to environmental stressors (Beery & Kaufer, 2015; Kiyokawa & Hennessy, 2018; Schreck et al., 2016). Interestingly, the reduced stress hormone levels have been observed as a result of prolonged social deprivation, hence this cannot be considered as the true buffering (Hennessy et al., 2009; Jeffrey et al., 2014; Kiyokawa & Hennessy, 2018). Therefore, in fish, buffering is modulated primarily through the

activity of the hypothalamic-pituitary-interrenal axis (HPI), the functional homologue of the mammalian HPA axis (Bouyoucos et al., 2021; Denver, 2009; Hennessy et al., 2009; Hostinar et al., 2014). The key buffering indicators in fish include, reduced cortisol levels and freezing behaviour, and also changes in the neural activity patterns. Research showcases a widespread social buffering among ray-finned fishes. In sticklebacks, predator induced stress has been mitigated with conspecific presence as evidenced with the lower cortisol levels than individuals tested individually (Mommer & Bell, 2013). Similarly, thermal stress induced spike in cortisol levels also alleviated in Lake sturgeon held in groups rather than alone (Yusishen et al., 2020). Social buffering is well-validated in zebrafish; group exposure to novel environments is measured with lower cortisol level than exposed individually (Pagnussat et al., 2013). Similarly, the presence of conspecifics reduced the fear induced freezing behaviour in zebrafish (Faustino et al., 2017). Considering the efficacy of sensory cues, both visual and olfactory cues possess dominance but visual cues appear more effective for sustained cortisol reduction (Faustino et al., 2017). Studies substantiate the sex-dependent social buffering responses both in rodent and fish models (Akinrinade et al., 2023; Reichert et al., 2025).

The homologue of the mammalian PVN in fish, the preoptic area (POA) regulates the release of CRF (Hostetler & Ryabinin, 2013). Fish also possess a conserved social behavioural network, which modulates stress response, including brain regions analogous to the mammalian amygdala and prefrontal cortex (O'Connell & Hofmann, 2011; Ogawa et al., 2021; Porter & Mueller, 2020). Likewise, isotocin the teleost homologue of oxytocin identified to a key modulator in social behaviour and expressed in POA (Godwin & Thompson, 2012; Goodson et al., 2003; Herget et al., 2017; Ogawa et al., 2021; Saito et al., 2004). Elevated whole-brain isotocin levels were measured in zebrafish during stress recovery with familiar conspecifics (Mathuru et al., 2017). Together, studying social buffering in fish contributes to understanding the social regulation of stress (Gilmour & Bard, 2022).

2.4 Collective Behaviours

Collective behaviours have emerged as a fascinating area of research and have increasingly captivated the attention of scientists over years (Couzin, 2009). Flocking of birds, colonies of ants, and schooling in fishes are among the prominent collective

organisations, serving multiple survival benefits, including enhanced foraging capabilities and antipredator responses (Krause et al., 2010; Okubo, 1986). Previous research has reflected on fundamental changes in shoaling distances and has also addressed probabilistic and stochastic properties of animal behaviour, urging shifts toward mathematical models to understand the core of emerging patterns in social groups (Couzin & Krause, 2003).

In this study, we focus on zebrafish (*Danio rerio*), a highly social minnow demonstrating both phases of collective movements, including schooling and shoaling (Miller & Gerlai, 2012). In the context of these collective behaviours, schooling is defined as a more aligned and synchronised movement pattern, often associated with higher swimming speed. In contrast, shoaling refers to loose aggregation of groups with reduced alignment and calmer movement patterns (Miller & Gerlai, 2011). Grounded in the “wisdom of crowds theory” (Surowiecki, 2004), zebrafish gain advantages from schooling and shoaling through improved cognitive ability in processing information. The formation of global intelligent behaviour arises from simple local interactions, where individuals sense changes in the position and motion of immediate neighbours without a single defined leader (Pitcher, 1979). These pairwise interactions often limit awareness of the informational status of others, further emphasising the role of individual abilities in shaping the internal group structure.

Based on previous literature on zebrafish social behaviours, shoaling is an emergent developmental behaviour occurring during two to three weeks post fertilization. Some studies have reported the onset of olfactory cue-based interactions as early as 6dpf and the emergence of strong conspecific preference from 12dpf onwards, with significant robust cohesion strengthening and becoming more central during adulthood (Buske & Gerlai, 2011a; Engeszer et al., 2007). Research, notably by Dreosti et al. (2015), highlights 21 dpf as a landmark stage at which zebrafish exhibit strong social preferences and shoaling behaviour. A recent finding in *Danionella cerebrum* (Zada et al., 2024), a very close relative of zebrafish, further strengthens the developmental nature of shoaling. According to this, *D. cerebrum* begins social aggregations at around four weeks and postural alignment by six weeks, suggesting

that aggregation and alignment in collective behaviour represent two distinct developmental processes.

Major sensory mechanisms are involved in the progress of collective behaviour, comprising vision, mechanosensation, and olfaction. While collective behaviour is predominantly vision based, the lateral line system functions in maintaining alignment during schooling (Anneser et al., 2020), whereas imprinting of olfactory and visual stimuli were involved in kin and non-kin identification (Hinz et al., 2013) and predator detection via alarm substances (Mukherjee et al., 2024, Mukherjee & Bhat, 2023b; Speedie & Gerlai, 2008). The vital role of the primary visual centre of the brain, the optic tectum (Bergmann et al., 2018), in complex visually guided conspecific image processing and alignment during collective development is well validated. The optic tectum also acts as a visual-motor hub and behavioural regulator through hypothalamic neural networks (Heap et al., 2018). Research emphasising the role of the HPA axis in neurodevelopmental disorders, for instance, dysregulated HPA activity in hyperactivity associated with ADHD (Fulun et al., 2025) and HPA axis dysfunction in the pathophysiology of ASD (Sharpley et al., 2016), has been identified. This indicates a plausible association between neuronal pathways linked to visually mediated signals in the context of social environmental manipulations. Although spontaneous activity (Marachlian et al., 2018) of the optic tectum in the absence of sensory inputs has also been reported, these studies further demonstrated the importance of social experience in fine tuning social and collective behaviour.

Zebrafish behaviour in the wild varies depending on their environmental conditions. The water flow is identified as a primary driver in determining the group size and collective behaviours (Suriyampola et al., 2016; Suriyampola et al., 2017; Suriyampola et al., 2023). That is, in certain conditions like a stagnant water body, fish with generally smaller body sizes tend to form groups with fewer individuals, including up to 22 fish. Whereas in slow-flowing water, zebrafish form groups with very few individuals, including up to 4-12 fish, often observed with higher aggressive events. Whereas in fast flowing water, large zebrafish can form a group size up to 300 showing collective rheotaxis (Suriyampola et al., 2016).

Interestingly, studies showed that zebrafish can shoal with other fishes also. This might be context dependent, as in the case of higher predation risk and lower number

of conspecifics present. In some contexts, zebrafish prefer familiar heterospecific over unfamiliar conspecific fish (Mukherjee & Bhat, 2023a). Studies showed that familiarity and group size of conspecifics were also analysed as two important shoaling choices (Swaney et al., 2025). Generally, zebrafish were found to shoal with flying barbs, whitespots, and giant danios. Studies also demonstrated that zebrafish did not suffer from any foraging cost in mixed-species shoals (Mukherjee & Bhat, 2023a).

Shoaling studies related to the influence of sex are comparatively less, but when we analyse the existing literature, sex is an important biological variable in the context of shoaling. Females exhibited sex-assortative shoaling (Etinger et al., 2009), shoaling with same-sex individuals, also they prefer to shoal with established groups over newly formed ones (Velkey et al., 2022). The primary drive towards female-only shoaling indicates the chances of higher protection and survival benefits. Whereas males prefer to shoal with females, indicating the higher chances of mating. Males prioritize vegetation over a large number of female groups, again indicating the safety and successful chances of spawning offered by the vegetation (Ghoshal & Bhat, 2021). Even though on the basis of spatial cohesion, the mixed-sex shoal exhibited tighter shoal cohesion than same-sex shoals (Sivaraman et al., 2022). A recent study showed the influence of estradiol (E2) (Lindley et al., 2025) on the social preference of male and female zebrafish towards shoaling. Females blocked E2 production via fadrozole exposure showed reduced social drive towards female-only shoal, whereas males did not show any significant alteration in the social affiliation towards male-only shoal. This indicates an increased sensitivity of females to E2 in influencing the social drive towards same sex shoal than males. Whereas increasing E2 levels heightened shoaling in both sexes (Lindley et al., 2025).

Now we are moving onto one of the critical layers in this literature review, the preclinical pharmacological models used to evaluate the social deficits of neurodevelopmental disorders, especially in Autism and Schizophrenia. One of the important models is MK801 (dizocilpine), a non-competitive NMDA receptor antagonist. As it plausibly interrupts glutamate transmission, leading to significant social withdrawal and repetitive behaviours in rodent and fish models. Franscescon et al. (2021) showed that MK801 alters shoaling in zebrafish by increasing inter-

individual distances and shoal area, and further demonstrated the therapeutic effects of taurine in re-establishing the social deficits induced by MK801. The study showed that taurine acts specifically to rescue shoal dispersion but was unable to bring the inter-individual distances back to baseline. Similarly, the acute effect of MK801 in zebrafish showed hyperactivity and stereotypic behaviours, including circling, in the open field tank test; despite these behavioural disruptions, homebase retention was still present in the treated fish (Mohammed et al., 2025). Notably, an earlier zebrafish pharmacological study (Swain et al., 2004) using different doses of MK801 showed increased circling, altered swimming, and disrupted place preference. Maaswinkel et al. (2013) showed that in a heterogeneous shoal, comprising only one MK801-treated zebrafish within a control shoal, the entire shoal cohesion was altered, further explaining that perturbation at the individual level can affect group dynamics.

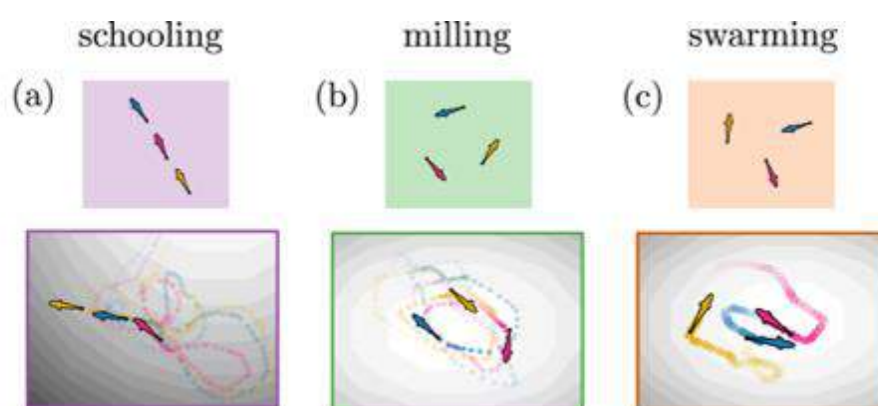
Together, these studies establish MK801 as a robust pharmacological model for disrupting social organisation and collective behaviour in zebrafish, making shoaling a sensitive behavioural output of neurodevelopmental alterations.

The next important preclinical pharmacological model used specifically in ASD is the valproic acid (VPA) model. VPA is a histone deacetylase inhibitor and antileptic drug which, when exposed prenatally, induces neurodevelopmental and behavioural alterations across animal models. This model has been identified to capture core ASD-related external symptoms, including social communication deficits, altered locomotor activity, repetitive behaviours, and changes in aggression. Zimmermann et al. (2015) and Chen et al. (2018) showed that early developmental disruption induced by VPA exposure is critical in producing behavioural and social deficits. Neelotpol et al. (2024) demonstrated ASD-like phenotypes, including repetitive behaviours, in mice, ensuring the translational validity of the VPA model across vertebrates. Li et al. (2024) showed a negative correlation between aggressive response and ASD related traits, demonstrating reduced aggressive responses in VPA-treated fish. Similarly, Dhanabal and Durairaj (2024) showed altered shoaling and reduced mirror-bite responses in the VPA-treated group, plausibly indicating reduced aggression, whereas aggression was reported to be unaltered in the study by Zimmermann et al. (2015).

Taken together, these models demonstrate that chemically induced neurodevelopmental changes reliably disrupt shoaling, social interaction, locomotor,

and aggressive responses in zebrafish. Therefore, environmental models capable of evoking comparable alterations in such behavioural responses can be considered as valid and relevant preclinical approaches for studying neurodevelopmental disorders.

Mathematical models and simulations have also been explored to better understand the mechanisms of shoaling as well as the interactions between individual shoal members. Early frameworks, including Aoki model (Aoki, 1980), self-propelled particle models (Strömbom, 2011), the Huth and Wissel model (Huth & Wissel, 1992), and the zonal model (Couzin et al., 2002; Herbert-Read et al., 2011), have established that interactions within a shoal do not require a leader; instead, collective organisation emerges from local decision-making. These models describe that a fish primarily interacts with its nearest neighbours to form a cohesive group with heading and positional alignment. Likewise, the importance of individual-level interaction was formalized by categorizing three functional zones around each fish—repulsion, attraction, and polarization, as established specifically in the zonal model proposed by Couzin et al. (2002).



Adopted from: Zampetaki et al., 2024 (DOI: 10.1038/s41467-024-46426-1)

Figure 2.4. Collective behavioural states observed in zebrafish groups.

This figure illustrates distinct patterns of group behaviour, including schooling, milling, and swarming, representing different modes of coordinated movement and social interaction among individuals.

Rosenthal et al. (2015) described the visual field model, illustrating how information propagates through the shoal, which links to the role of the optic rectum discussed earlier. More recent physics-based analyses have utilized these modelling frameworks to advance the current understanding of collective dynamics. Notably, Tunstrøm et al.

(2013) described three emergent collective states and collective memory in fish groups, including milling (circling), schooling (polarized), and swarming (disordered) states. Zampetaki et al. (2024) suggested that collective behaviour is influenced by multi-body correlations, demonstrating that three individuals are sufficient to generate multiple collective states, and that increasing group size leads to greater stability and reduced entropy. Additionally, Ito and Uchida (2024), using a physical modelling approach, argued that visual attention in fish is dynamic and adapts to neighbour movement patterns and positions, such as swimming speed, further elaborating the complex map of pairwise and three-body interactions.

In the similar way, the importance of individual traits or phenotypes in influencing group organisation has been demonstrated in social fish. According to Pike et al. (2008), a study conducted in three-spined sticklebacks showed that bolder fish tend to have more evenly distributed interactions, whereas shy individuals exhibit highly skewed interactions, associating with fewer preferred partners. In contrast to the basic pairwise interaction rules forming the foundation of collective movements, Katz et al. (2011) demonstrated that three-body interactions substantially contribute to group dynamics in golden shiners. Herbert-Read et al. (2011) further explained pairwise interaction rules, showing that fish primarily respond to their single nearest neighbour in mosquitofish. They also demonstrated that repulsion is driven by changes in speed rather than turning by neighbouring fish. Similarly, Jolles et al. (2017) showed in sticklebacks that individual differences in speed drive collective behaviour.

Specific to zebrafish studies Harpaz et al. (2017) tracked zebrafish to infer mode-dependent social interaction rules and found that fish alternate between active and passive modes; in the active mode, individuals were sensitive to neighbours, whereas in the passive mode they ignored neighbours. Likewise, Collignon et al. (2019) analysed zebrafish groups varying in size to quantify collective departure initiation and leadership, demonstrating that faster fish become leaders by attempting to initiate collective movements more frequently than slower individuals.

More recently, Nunes et al. (2020) demonstrated that zebrafish social affiliation is strongly modulated by biological motion rather than static body forms. Liu and Burbano (2025) showed in dyads and triads of zebrafish that environmental

manipulations affect group-wise exploration and dispersion, for instance under light and dark illumination conditions.

Social isolation is an extensively studied research area due to its importance in neurological function and behaviour across human and other animal models. Different methodological frameworks have been observed in the previous literature, including acute and chronic early-life isolation and acute adult isolation. Social isolation and associated altered shoaling have been reported in zebrafish, and the vital role of neurotransmitter systems, including oxytocin, dopamine, and serotonin, has been analysed over the years (Shams et al., 2018). Gemmer et al. (2022) found that social isolation mirrored the oxytocin receptor mutant in behavioural assays, including inter-individual distances in shoaling. Additionally, the study revealed that the effect of social isolation is progressive, as four-week isolated fish did not exhibit altered social behaviours, whereas eight-week isolation produced significant changes.

Similarly, Wilson et al. (2024) found that social buffering was reduced in fish administered oxytocin antagonists and showed a similar slow recovery to individuals transferred to an isolation group after alarm exposure, further elaborating the critical role of oxytocin in maintaining social preference and shoaling in zebrafish. Fontana et al. (2025) found that isolated *adgrl3.1* knockout fish showed altered shoaling. Tang et al. (2020) identified 20 genetic mutants associated with shoaling and revealed the involvement of multiple genes in this behaviour. The study also demonstrated that the same gene may not affect all parameters uniformly. Muller found that adult isolation produced altered shoaling, which was re-established after reintroduction with conspecifics, and also showed altered dopamine and serotonin systems. Chronic social isolation and shoaling were addressed by Shams et al. (2018) who showed that shoaling was altered at 180 days post-fertilization in terms of increased inter-individual and nearest neighbour distances.

2.5 Microbiota-Gut-Brain Axis (MGB) As A Potential Biomarker in Behavioural Studies

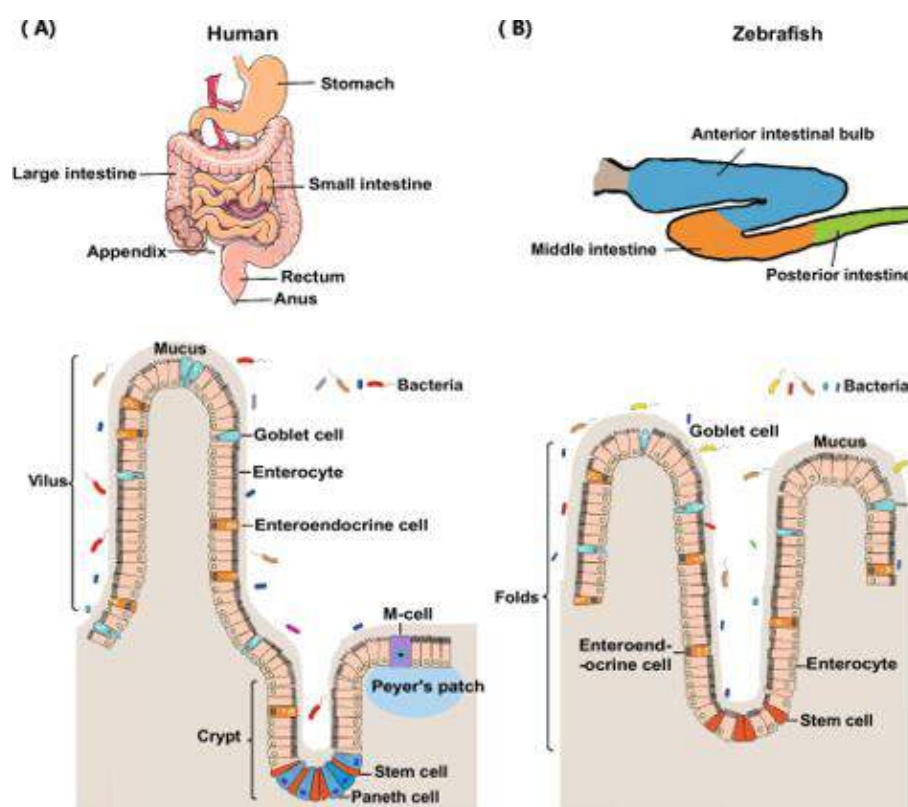
The microbiota-gut-brain axis is a bidirectional communication pathway comprising both efferent and afferent signalling between the central nervous system (CNS) and the gastrointestinal tract (Lynch & Pedersen, 2016). Neural, hormonal, and immune

pathways are actively involved in MGB functionality. While the vital role of the vagus nerve in the neural pathway has been widely studied (Bravo et al., 2011; Sampson & Mazmanian, 2015), microbial metabolites including short-chain fatty acids (SCFAs) and tryptophan derivatives alongside signalling mediated by cytokines and microbiota-activated immune cells also play a predominant role in the MGB communication network (Dalile et al., 2019; Gershon & Tack, 2007; Sharon et al., 2014; Sikander et al., 2009). Microbial imbalance, known as dysbiosis, is considered a potential biomarker linked to neurodevelopmental and neurodegenerative disorders (Morais et al., 2021). Increased gut barrier permeability and systemic inflammation are commonly observed in association with these conditions. Therefore, research into the MGB axis provides new insights into complex clinical translational studies and offers significant potential for therapeutic strategies, such as probiotic intervention.

Zebrafish serve as a relevant translational model, with 70% genetic similarity and sharing approximately 82% of human disease-related genes (Howe et al., 2013). The tractability provided by zebrafish transparent embryos, and the availability of established microbiome databases, well position zebrafish as an animal model in studies involving the MGB axis (Flores et al., 2020; Kiran et al., 2024; Melancon et al., 2017; Pham et al., 2008; Zhong et al., 2022). The structure of the digestive tract of zebrafish shows both similarities and dissimilarities with mammalian models. Structures such as the pancreas, gall bladder, liver, and intestine are present in zebrafish. Including zebrafish, approximately 15% of fish species are devoid of a stomach and pepsin, where the intestine directly joins the oesophagus. Similarly, intestinal folds are absent in zebrafish, along with Paneth cells, microfold cells, and submucosal glands. Zebrafish have enterocytes, goblet cells, and enteroendocrine cells that offer functions ranging from nutrition absorption to immune regulation (Lu et al., 2021). Also, gut epithelial cells proliferation and differentiation are controlled through microbial colonization via Delta-Notch and Wnt signalling pathways (Cheesman et al., 2011; Troll et al., 2018).

Major differences exist in the microbial composition between zebrafish and mammals, whereas the molecular responses to microbes are largely conserved (Rawls et al., 2006). The human gut predominantly harbors Bacteroidetes, Firmicutes, and Actinobacteria at the phylum level (Eckburg et al., 2005), while the dominant

microbial composition in zebrafish varies depending on developmental stages; however, it is generally considered that the zebrafish gut is dominated by Proteobacteria, Fusobacteria, and Firmicutes (Kanthar & Rawls, 2010; Li et al., 2022; Murdoch & Rawls, 2019). All these differences arise from variations in species, genetics, environment, and diet. However, the intestinal gene expression, structure, and microbiome function share similarities with humans at a fundamental level (Lu et al., 2021).



Adopted from: Xia et al., 2022 (DOI: 10.1186/s10020-022-00579-1)

Figure 2.5. Comparative intestinal anatomy of humans and adult zebrafish.

This figure presents differences and similarities in intestinal organization, including epithelial cell types and regional specialization, while highlighting the absence of certain structures such as crypts and Paneth cells in zebrafish.

The literature review now elaborates by adding relevant and recent preclinical studies conducted across zebrafish, rodents, and humans in the context of neurodevelopmental disorders. It communicates how different experimental modifications were associated with behavioural alterations and concomitant gut dysbiosis, while also exploring possible therapeutic strategies and recent advancements in the field of MGB axis research.

Research shows that the risk of neurodevelopmental disorders particularly in autism spectrum disorders (ASD), has been associated with maternal obesity during pregnancy. 16S rRNA gut microbiome analysis of male offspring from the maternal high-fat diet group exhibited social and synaptic plasticity deficits, reduced oxytocin levels, and major gut microbiome alterations, especially a reduction in *Lactobacillus reuteri*. Interestingly, the reintroduction of *L. reuteri* significantly improved social deficits and restored the oxytocin levels by activating the paraventricular nucleus through the vagus nerve (Buffington et al., 2016).

Sgritta et al. (2019) further expanded the effect of *L. reuteri* in rescuing social deficits across multiple ASD models, including environmental (maternal high fat diet), genetic (*shank 3b* mutant), idiopathic (BTBR mice), and germ-free mice. The study demonstrated that the application of *L. reuteri* significantly improved social deficits across all models regardless of causality. Additionally, vagotomised mice showed no improvement in social behaviour, further indicating the gut-brain connection through the vagus nerve, and pointing to the oxytocin-dopamine system in modulating microbiome activity.

The relationship between obesity and social deficits was further demonstrated by (Liu et al., 2021) in both human cohorts and mouse models. They showed that a maternal high-fat diet in mouse offspring replicated social and synaptic deficits and notably reduced the levels of acetate and propionate in the gut. A high-fibre diet enhanced social behaviours and restored SCFA-producing bacteria. Gut microbiome transfer from human obese individuals to germ free mice produced similar social deficits. Oral administration of acetate and propionate alone was able to mimic the effects of the high-fibre diet.

Di Gesù et al. (2022) showed that the effects of maternal high fat diet induced social deficits were intergenerational and produced sex-specific behavioural abnormalities in the F2 generation. The microbiome treatment involved using *Limosilactobacillus reuteri* increased SCFA producing taxa and improved social deficits in F2 descendants. Notably, the *L. reuteri* produced sex-specific effects, females being more malleable to the treatment, producing 38 discriminant taxa compared to only 13 in males.

Chen et al. (2026) conducted large-scale sampling across different cohorts of children, including metagenomics, genomic variants, and metabolomics, to understand the mechanisms of ASD more deeply. The researchers were able to identify a highly accurate 20-marker diagnostic panel, spanning from specific microbial genetic mutations to host neurometabolite alterations that go beyond simple microbial abundance. Additionally, the study identified a critical link involving the *BtuD* mutation, which encodes a Vitamin B12 ABC transporter. The altered Vitamin B12 uptake was connected with further imbalances in neurochemicals, such as dopamine.

A complementary perspective of Vitamin B12 was provided by Qi et al. (2023) in a zebrafish model. The study demonstrated that *Aeromonas hydrophila* infection was rescued by the addition of *Bacillus velezensis* working alongside the native *Cetobacterium*. *Cetobacterium* was found to produce Vitamin B12 *de novo*, which further strengthens gut brain barrier tight junctions, prevents pathogenic infections, and stabilizes the entire microbiome community.

Different modes of social stressors have been linked with altered social behaviour and gut microbiome alterations, ranging from social isolation to the presence of an aggressor, across zebrafish and rodent models. A study conducted in mice demonstrated that social stress induced bacterial restructuring and microbiota-activated increases in pro-inflammatory cytokines to cope with stress (Bailey et al., 2011). Similarly, previous rodent studies have shown that acute social stressors induce structural changes in colonic mucosa and reduce beneficial bacteria such as *L. reuteri* (Galley et al., 2014). Zebrafish-specific study conducted exclusively on males showed that only dominant fish were developed *Cetobacterium*, whereas other stressed groups including subordinate and isolated individuals showed reductions in beneficial and blooms of pathogenic taxa, with *Acinetobacter* being one among them (Scott et al., 2023). Research supports that chronic social isolation negatively impacts feeding behaviour in zebrafish and produces anxiety-like behaviours (Dissinger et al., 2024), this further provides plausible links between gut-microbiome mediated changes and brain responses through neurotransmitter systems, or vice versa. A recent study demonstrated that the microbiome regulates social behaviour through the hypothalamus-pituitary-adrenal (HPA) axis. The study further identified the potential probiotic role of *Enterococcus faecalis* in improving the social deficits in mice (Wu

et al., 2021). Additionally, a number of species-specific probiotic interventions have been reported to improve behavioural deficits, for example *Lactocaseibacillus rhamnosus* SL42 in improving autistic behaviour in a Wistar rat model (Ziar et al., 2025).

Both nanoplastics and microplastics have been shown to affect zebrafish microbiome composition, and in these studies Proteobacteria blooming and Firmicutes depletion were considered potential biomarkers for gut dysbiosis (Li et al., 2023). Similarly, Actinobacteria is a minor phylum that functions in maintaining the gut barrier, and depletion of Actinobacteria is also considered a marker of dysbiosis (Binda et al., 2018).

A critical sex-specific layer in the MGB axis also exists. Recent research conducted on antimicrobial chemical agents in zebrafish showed significant gut dysbiosis and led to the triggering of NF- κ B signalling and histological damage in the gut. Notably, the study found that females were more susceptible to stressors and subsequent microbial alterations than males, suggesting that MGB axis responses and mechanisms may be sex-dependent (Liu et al., 2024). Adamovsky et al. (2020) showed that the effect of phthalate-induced gut dysbiosis showed both shared effects across sexes in some taxa, whereas others showed male-specific patterns.

Together, the literature review broadly covers the basics of the MGB axis and its relevance in maintaining homeostasis across animal models and humans. Some studies, such as Stephens et al. (2016), suggest that while the zebrafish microbiome evolves as the fish matures, these changes happen consistently across both sexes, showing a lack of sex-specific differences. However, as mentioned earlier, other studies support the sex-specific outcomes associated with the gut microbiome (Martyniuk et al., 2022). Given the inconsistent finding regarding sex specificity in zebrafish gut microbiome, we conducted a pilot study on gut microbial modulation under social isolation in a sex-specific context. As primary insights from novel experiments are increasingly welcomed in this field (Menachery et al., 2026), we present our findings on trends associated with social isolation and gut microbiota while also considering both economic and ethical perspectives.

Chapter 3

Methodology

Materials and Methods

3.1 Zebrafish & Housing

We have adhered with all established ethical guidelines for laboratory animal use. All procedures outlined in this study were conducted following ARRIVE guidelines (Kilkenny et al., 2010) and in accordance with the guidelines approved by institutional animal ethics committee (IAEC) of University of Calicut (Registration No. 426/GO/Re/S/01/CCSEA). All experimental protocols were reviewed and approved by the IAEC under the proposal number IAEC/UOC/24/08.

Zebrafish (*Danio rerio*) larvae at 5dpf, derived from adult wild type AB strain broodstock, were procured from a government approved aquaculture facility (License No. KL/04/OH/264/2021) located in Alappuzha, Kerala. The animals were sourced externally, as breeding was not conducted in-house.

Upon arrival, larvae were maintained at University of Calicut in a temperature-controlled facility at 27 ± 1 °C, with a pH of 6.5-7, 14-h light and 10-h dark cycle. The zebrafish were fed with live *Paramecium* during early developmental stages. From the juvenile stage (30 dpf) onwards fish were fed twice daily, with freshly harvested live *Artemia* nauplii and a commercial dry pellet (Tiqlid nursery pellets, 0.8mm size). After the procurement, zebrafish larvae were allowed to acclimatize in the laboratory conditions for 15 days. At 21dpf, surviving and healthy individuals were selected and randomly grouped into control and test experimental conditions (Bedell et al., 2025).

3.2 Experimental Design, Sample Size & Zebrafish Sex Identification

3.2.1 Chronic Social Isolation

In this experiment, zebrafish at 21dpf were randomly assigned to two groups: control and test (Dreosti et al., 2015; Stednitz & Washbourne, 2020). The control groups were maintained in ten tanks with a water capacity of 16 L per tank, each accommodating 10-15 fish, ensuring social housing conditions. In contrast, the test group representing socially isolated Zebrafish, was transferred to individual 1L glass containers (*as per CCSEA protocols*).

In order to block all social interaction, olfactory cues were blocked, and visual cues were minimized by covering each container with white paper, leaving only the top open. A surplus of fish was maintained for the socially isolated group to accommodate the chronic and sex specific design of the study, as zebrafish larvae do not undergo sex differentiation at the time of group allocation (Shams et al., 2018). The chronic isolation period was maintained up to 180 days (Shams et al., 2018).

All other conditions, including laboratory environment, feeding regimen, water quality parameters, tank cleaning procedures, and health monitoring of fish, were kept identical across control and test groups.

3.2.2 Sex Identification

Chronic social isolation was maintained and sex differentiation carried out at 180 dpf (Shams et al., 2018), prior to the initiation of behavioural assays. Males and females were separated from both control and test groups without the use of anaesthesia, using three major morphological features, including shape of abdomen, yellow coloration and genital papillae, assessed by three independent researchers (*sex differences in detail were explained in the Introduction, section 1.1.2*). Final sex was assigned based on agreement of at least two of the three observers (Crowder et al., 2018; Yossa et al., 2013).

3.2.3 Experimental Groups and Sample Size

Final experiment groups were divided into four categories based on sex and test conditions exclusively for objective 1 and 2; Control Male (CM), Control Female (CF), Test Isolated Male (TM), and Test Isolated Female (TF), and sample size for each group were maintained 20 for all the behavioural assays conducted.

For Objective 3, the experimental groups were divided into two shoal conditions; control and test isolated shoal, each comprising five fish per shoal. Based on our preliminary study, a mixed sex ratio was maintained for each shoal at a 4:1 ratio. Accordingly, 10 male-rich and 10 female-rich shoals were composed and averaged to obtain a total of 20 samples per shoal condition.

For objective 4, the experimental groups were divided broadly into two, homogeneous and heterogeneous dyads. Homogeneous dyads consisted of pairs derived from the same experimental condition, subdivided by sex. Therefore, control homogeneous

dyads included CMM, CFF, and CMF, while test homogeneous dyads included TMM, TFF, and TMF. The sample size for each homogeneous dyad group was maintained at 15, comprising a total of 45 males and 45 females each for control and test dyads.

Heterogeneous dyads consisted of pairs with one fish from the control and one from the test group, with all possible sex combinations, namely, CM-TM, CF-TF, CF-TM, and CM-TF. Similar to homogenous dyads, the sample size for each group was maintained at 15. A total of 30 males and 30 females used this heterogeneous dyad (Bedell et al., 2025).

3.3 Habituation Period

Before beginning the behavioural assessments, each experimental fish was net-transferred to an individual pretreatment tank within the experimental room. Fish inhabited a 1.5 L polycarbonate tank from the housing system for a duration of 10 minutes. Habituation tanks were fully covered by white paper to reduce the exposure to external stimuli. The habituation period was used to acclimate the fish to the testing room conditions, and minimise handling stress. This habituation period was uniformly applied across all objectives.

3.4 Behavioural Testing & Tracking

All behavioural experiments were conducted from 9.00 AM to 4.00 PM. Morning feeding of zebrafish was completed at least 2 h prior to the behavioural testing. Illumination for all testing arena was maintained at 300 lux, and the water temperature was maintained between 26°-28°C. A high-resolution camera (Sony alfa7c, Viltrox 35mm lens) was suspended approximately 90 cm above from the testing arena. This set-up was used for open-field testing (Objective 1) as well as dyad and shoal analyses (Objective 3 and 4) at 30-50 fps. For Light and Dark testing (Objective 1) and the three chambered tank tests under Objective 2, behaviour was recorded using Logitech C270 HD 720p camera. Zebrafish movements were tracked from the recorded videos using SMART 3.0 (Panlab-Harvard Apparatus) (Won et al., 2020), tracking software for Objective 1 and 2. The multi-animal tracking for dyad and shoal experiments (Objective 3 and 4) were performed using IdTracker 2.1 tracking. Python based libraries were utilized for the data processing in multiple animals-based experiments.

3.5 Overview of Statistical Analysis

From objective 1 to 4, we employed non parametric tests to ensure uniformity across data comparisons because the study was built on a single core objective-social isolation. Normality and assumptions on equal variances varied across parameters; additionally, considering the uneven nature of behavioural data, we relied on non-parametric tests (Rowson et al., 2025). The selection of tests was completely dependent on the total number of experimental groups being compared and whether the comparisons were within group or between groups. For two independent group comparisons, Mann-Whitney U test was used. Similarly for two dependent or paired within group comparisons, the Wilcoxon paired signed-rank test was chosen. For multiple group comparisons, the Kruskal-Wallis test was used (Hagen et al., 2023), followed by pairwise comparisons with FDR correction (Benjamini, Krieger, and Yekutieli) (Haynes, 2013; Rajput et al., 2022) for independent groups and the Friedman test was used for within-group multiple comparisons.

Effect sizes (Fritz et al., 2011) were calculated from the Z values obtained from pairwise comparisons using an established online statistical tool (<https://www.statskingdom.com/effect-size-calculator.html>). All statistical comparisons were conducted using GraphPad prism version 9, except for effect size calculations. Correlation analysis was conducted using Spearman rank correlation, a non-parametric method. Objective 5 included only descriptive analysis without any statistical inference.

3.6 Objective-wise Materials and Methods

3.6.1.1 Behavioural Assays: Objective 1

(a) Anxiety and Locomotor Activity Test 1: The Open Field Test

Anxiety-like behaviours and locomotor activity in Zebrafish was quantified by using open field test (OFT), following established protocols. The OFT arena consisted of a 30*30*10 cm white acrylic tank and was filled to a depth of 6 cm with system water. Each Zebrafish was carefully introduced into the centre of the arena, and after a 1-minute acclimatization period, the fish was allowed to explore freely for 6 minutes. After each trial the arena was cleaned and system water replaced to prevent olfactory

cues from influencing subsequent fish (Borba et al., 2023; Corcoran et al., 2025; Horka et al., 2024).

During animal tracking using SMART 3.0 software (Won et al., 2020), the experimental zone was virtually divided into two unequal zones, the outer narrow width zone as thigmotaxis zone (wall hugging behaviour) and the rest of the zone as exploratory zone. Time spent in thigmotactic zone was considered as the primary measure of anxiety-like behaviours in the zebrafish behavioural studies (Kalueff et al., 2013; Resmim et al., 2023; Salazar et al., 2012; Vossen et al., 2022).

Adapted from: BioRender.com and modified by the author

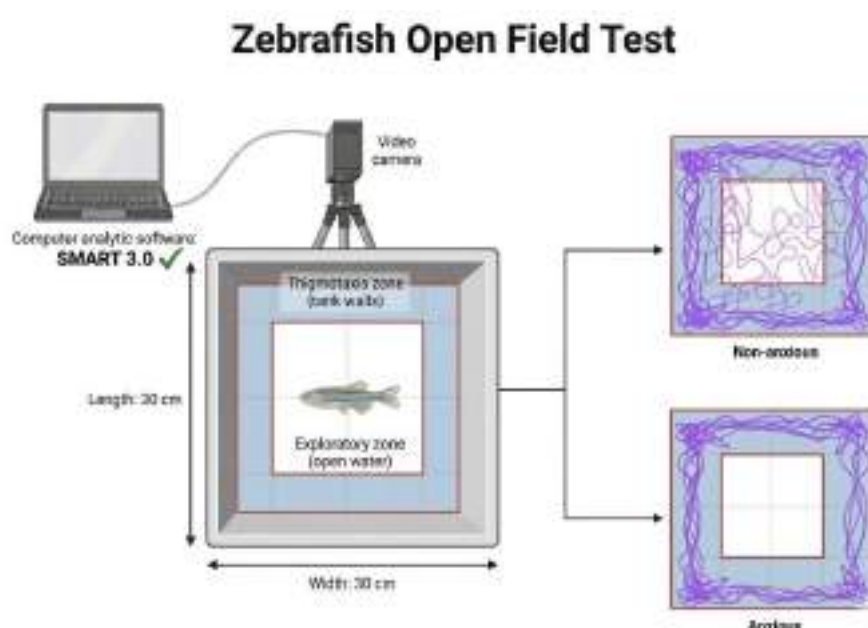


Figure 3.1. Schematic representation of the zebrafish open field test (OFT) setup and behavioural tracking.

This figure illustrates the experimental setup used to assess locomotor activity and anxiety-like behaviour in zebrafish using the open field test. The test arena consists of a square tank with defined zones, including a peripheral region adjacent to the tank walls (thigmotaxis zone) and a central exploratory region. A video camera positioned above the tank records fish movement, which is analysed using automated tracking software. Representative movement paths are shown to demonstrate behavioural patterns, where increased peripheral activity indicates anxiety-like behaviour, while greater exploration of the central area reflects reduced anxiety levels.

(b) Anxiety-The Confirmatory Test 2: The Light and Dark Test

The Light and Dark Test (LDT) serves as a confirmatory measure of anxiety-like behaviours in Zebrafish. Compared to OFT, the LDT is less influenced by locomotor activity and primarily reflects the fish's preference for light or dark zones. The apparatus consisted of a rectangular tank (45 cm × 10 cm × 15 cm; L × W × H) divided into equal light and dark zones, with transparent partitions allowing free movements between zones. Total water depth was maintained at 10 cm. Each Zebrafish gently placed in the centre of the tank, and after a 3-minute of acclimatization period, the partition was removed to allow free exploration for 6 minutes (Fontana et al., 2021; Maximino et al., 2010b).

Adapted from: Maximino et al. (2010b, 2011) (tank design)

Figure created by the author using AI-assisted tools



Figure 3.2. Schematic representation of the light–dark preference test in adult zebrafish.

This figure illustrates the experimental setup used to assess anxiety-like behaviour in adult zebrafish using the light–dark preference test. The apparatus consists of a rectangular tank divided into two equal compartments: a dark region and a light region. Zebrafish naturally exhibit scototaxis, showing a preference for darker environments. Increased time spent in the dark compartment is indicative of anxiety-like behaviour, whereas increased exploration of the illuminated region reflects anxiolytic responses. Fish movement is recorded using an overhead video camera, and behavioural parameters are analysed to evaluate zone preference and activity patterns.

3.6.1.2 Objective 2

(a) Multiple Stimulus Test (Three-Chamber Tank with Single Stimulus)

The three-chamber tank measured 60 cm × 20 cm × 20 cm (length × height × width) and was divided into three equal compartments by glass partitions that prevented olfactory exchange. The central chamber housed the experimental fish, while the left and right chambers contained the stimuli (Velkey et al., 2022). Only a single stimulus was presented during each trial, and the positions of stimuli were alternated between trials to prevent side bias.

Four conditions were tested:

1. **No Stimulus (NS)** – served as the baseline to check the possibility of tank side preference
2. **Shoal Only (SO)** – a social stimulus, used as the positive control, to measure the response against a well-known established stimulus.
3. **Predator-Stimulus Only (PO)** – stress-inducing condition using *Anabas testudineus*, a well-known predatory fish used to evoke antipredator responses in zebrafish.
4. **Mirror-Stimulus Only (MO)** – 15 cm * 15 cm mirror was positioned in one of the side chambers of the 3-chambered tank to elicit aggressive responses toward the experimental fish's own reflection.

The water level was retained at 15 cm. Each zebrafish was gently introduced into the centre of the arena and given a 1-min acclimatization period, followed by 6 min of free exploration. After each trial, the tank was cleaned and refilled with fresh system water to remove residual olfactory cues (Angiulli et al., 2020; Audira et al., 2018; Chathooth et al., 2025; Diakos et al., 2024; Gerlai et al., 2000; Ladu et al., 2015; Reichmann et al., 2022; Spinello et al., 2019).

Adapted from: previously published three-chamber zebrafish assay designs

Figure created by the author using AI-assisted tools

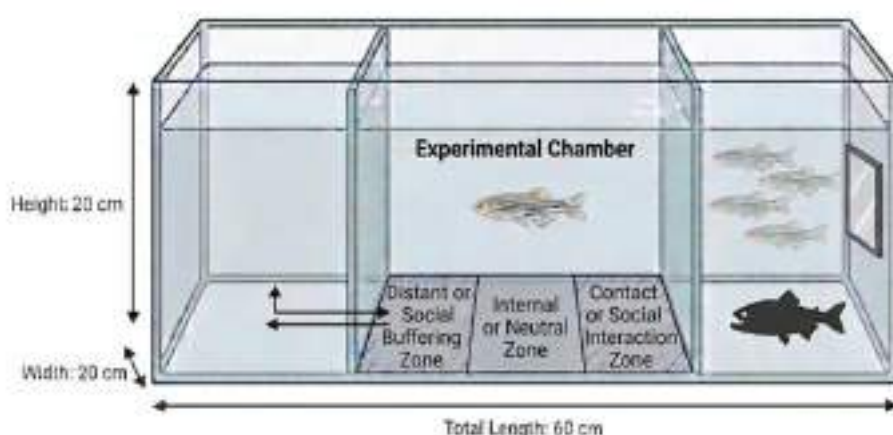


Figure 3.3. Schematic representation of the three-chamber social preference and buffering assay in zebrafish.

This figure illustrates the experimental design of the three-chamber tank used to assess social preference and social buffering behaviour in zebrafish. The apparatus consists of a central experimental chamber flanked by two lateral compartments. The middle chamber contains the test fish, while the side chambers serve as stimulus and non-stimulus zones depending on the experimental condition.

In the single-choice paradigm, one lateral chamber contains a stimulus (e.g., conspecifics, mirror image, or predator cue), while the opposite chamber remains empty and is considered the distant or non-stimulus zone. The central chamber is virtually divided into three regions: a contact zone adjacent to the stimulus chamber, an intermediate neutral zone, and a distant zone near the non-stimulus side, allowing assessment of spatial preference.

In the social buffering paradigm, the non-stimulus chamber is replaced with a shoal of conspecific zebrafish to evaluate the modulatory effects of social presence on stress or fear responses induced by aversive stimuli. Behavioural responses are quantified based on the time spent in different zones and proximity to the stimulus.

For schematic representation, the stimulus is shown on one side; however, in actual experiments, the position of the stimulus and non-stimulus chambers is alternated between trials to eliminate potential side bias.

Adapted from: previously published zebrafish behavioural assay designs

Figure created by the author using PowerPoint and AI-assisted tools

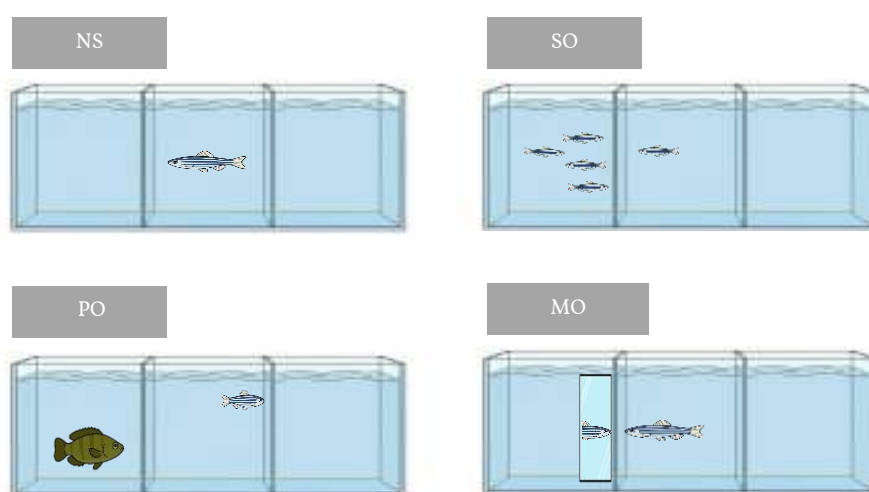


Figure 3.4. Experimental conditions used in the single-choice paradigm of the three-chamber assay.

This figure illustrates the different experimental conditions employed in the single-choice paradigm to evaluate behavioural responses in zebrafish. In all conditions, the test fish is placed in the central chamber, while one lateral compartment is used to present a specific stimulus.

The no-stimulus (NS) condition involves the absence of any stimulus in both lateral chambers and is used to assess baseline exploratory behaviour and to identify any inherent side preference. The shoal-only (SO) condition includes a group of conspecific zebrafish in one chamber and serves as a positive control to validate the experimental setup and assess social preference.

The predator-only (PO) condition presents a predator stimulus to evoke fear- and stress-related responses, while the mirror-only (MO) condition introduces a mirror stimulus to elicit aggressive or social interaction behaviours. Behavioural responses are quantified based on spatial preference and movement patterns within the experimental chamber.

(b) Social Buffering Test for Stress and Aggression (Three-Chamber Tank with Dual Stimuli)

The same chamber tank was used to examine the influence of conspecific buffering during stress and aggression. Each test involved one experimental fish and eight conspecifics used as the buffering group.

Depending on the condition, either a predator (Predator + Shoal = PS) or a mirror (Mirror + Shoal = MS) was placed in one lateral chamber, and the conspecific shoal was placed in the opposite chamber. Stimulus positions were alternated between trials. Each session lasted 6 min (representing acute social support), following a 1-min acclimatization period. The predator fish were replaced after every two trials to maintain active threat presentation.

During animal tracking using SMART 3.0 software (Won et al., 2020), the experimental arena was virtually divided into three equal zones, namely contact zone (the zone near to stimulus), internal zone (considered as the neutral or no preference zone), and distant zone (the zone away from the stimulus). The contact zone was later named according to the stimulus present in the respective condition-shoal zone for SO, predator zone for PO, and mirror zone for MO. In the dual stimulus tests (PS and MS), the distant zone was occupied by the conspecific buffering group, therefore it was referred to as the shoal zone (buffering stimulus) (Kuniyil et al., 2025).

Adapted from: previously published zebrafish behavioural assay designs

Figure created by the author using PowerPoint and AI-assisted tools

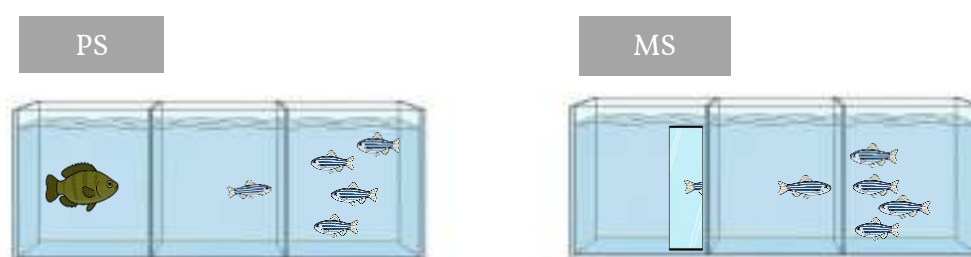


Figure 3.5. Dual-stimulus (social buffering) conditions in the three-chamber assay.

This figure illustrates the dual-stimulus experimental conditions used to evaluate social buffering effects in zebrafish. In this paradigm, the test fish is placed in the central chamber, while one lateral compartment contains an aversive or social stimulus and the opposite chamber contains a shoal of conspecific zebrafish.

In the predator-plus-shoal (PS) condition, a predator stimulus is presented in one chamber to induce fear- and stress-related responses, while the opposite chamber contains a group of conspecifics to assess the modulatory effect of social presence. In the mirror-plus-shoal (MS) condition, a mirror stimulus is used to evoke aggressive

or social interaction behaviour, with the shoal compartment serving as a buffering stimulus.

Behavioural responses are quantified based on spatial preference, proximity to each stimulus, and movement patterns within the experimental chamber, allowing evaluation of the extent to which social interaction mitigates stress or alters behavioural responses.

3.6.1.3 Objective 3 and 4

The similar open-field tank of 30 cm length, 30 cm width, 10 cm height, white polyacrylic white, used for objective 1 was used for objective 3 and 4. Objective 3, with 5 fish tracking, while objective 4 had 2 fish tracking at a time. The tank was filled with system water with a depth of 6 cm. Each Zebrafish was carefully introduced into the centre of the arena, and after a 1-minute acclimatization period, the fish was allowed to explore freely for 6 minutes. After each trial the arena was cleaned and system water replaced to prevent olfactory cues from influencing subsequent fish (Sivaraman et al., 2022).

Figure created by the author using AI-assisted tools
Behavioural tracking performed using: idTracker (version 2.1)

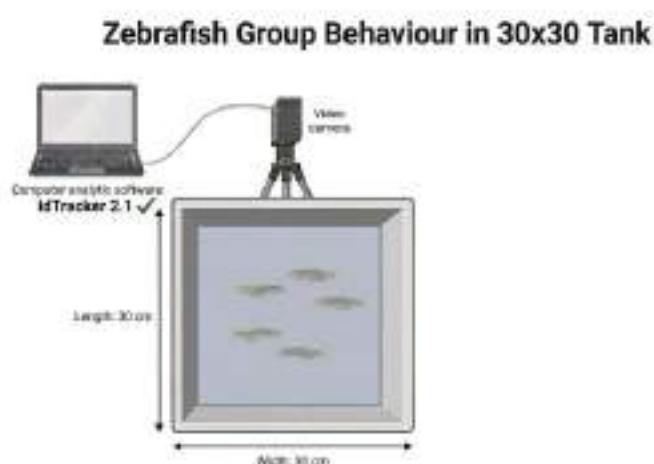


Figure 3.6. Open field test setup for shoaling behaviour analysis using multi-animal tracking.

This figure represents the open field test (OFT) setup used to analyse shoaling behaviour in zebrafish. The experimental arena is identical to that described in Figure 3.1, with recordings obtained using a top-mounted video camera. In this paradigm, a group of five zebrafish is introduced into the arena to evaluate collective movement and social interaction patterns. Behavioural tracking is performed using multi-animal tracking software, enabling the extraction of individual trajectories and group-level parameters such as inter-individual distance, cohesion, and spatial distribution.

Figure created by the author using AI-assisted tools
Behavioural tracking performed using: idTracker (version 2.1)

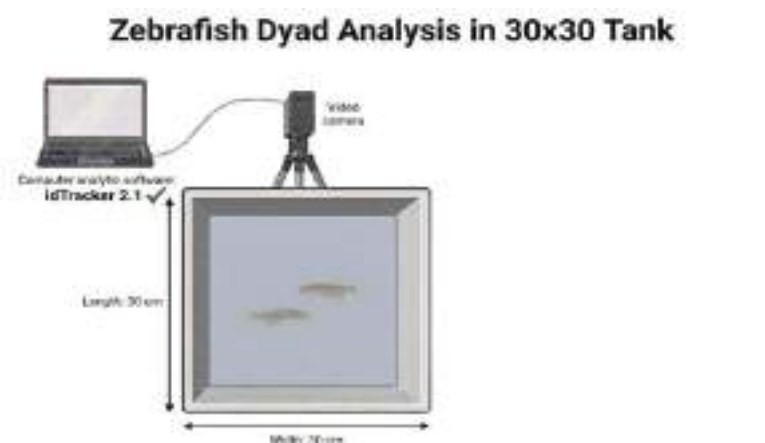


Figure 3.7. Open field test setup for spatiotemporal analysis of dyadic interactions in zebrafish.

This figure illustrates the open field test (OFT) setup used to examine spatiotemporal dynamics of zebrafish dyads under control and social isolation conditions. The experimental arrangement includes a top-mounted video camera for continuous behavioural recording, and multi-animal tracking is performed to monitor the movement of paired individuals simultaneously. The analysis focuses on interaction patterns, relative positioning, and movement coordination between individuals, providing insights into the effects of social conditions on dyadic behaviour.

3.6.2 Behavioural Endpoints

3.6.2.1 Objective 1

(a) Anxiety-Related Endpoints

Behaviours examined under the OFT analysis were zone specific time spent (Thigmotactic vs. Exploratory) within each group as the primary anxiety measure; zone specific distance travelled as a complementary measure for anxiety in OFT. Thigmotaxis, is the wall hugging behaviour of zebrafish, a behavioural endpoint used to assess the internal state of fish. A high thigmotaxis indicates shy or anxious behaviour of individual fish, induced either by novelty induced stress of open field tank (neophobia) (Gjinaj et al., 2025), environmental manipulation (social isolation) or drug (anxiolytic or anxiogenic) related response. LDT utilized as a confirmatory measure for anxiety, included time spent in light and dark zones and latency to first enter the light zones as vital anxiety markers. Time in the light zone used a well-

known marker for anxiolytic-like behavioural response in previous research as adult zebrafish prefer dark environments in a binary choice test, indicating a baseline anxious response (Kalueff et al., 2013).

(b) Activity-Related Endpoints

Zone specific entire or transitions, total rapid movements (speed > 15 cm/s), global zone transitions, global distance travelled, speed range (minimum speed subtracted from maximum speed), activity index (proportion of swimming time to total time spent) and derived activity balance index (ABI), i.e., ratio of speed range to activity index, were as the locomotor activity measures (Kalueff et al., 2013).

3.6.2.2. Objective 2

(a) Single Stimulus

1. Time spent in each zone (contact/internal/distant)
2. Zone entries and swimming speed at contact or stimulus zone
3. Freezing (speed < 1 cm/s)
4. Rapid movements (speed > 15 cm/s)
5. Mirror bites (for MO and MS, counted by three independent observers).

(Kalueff et al., 2013)

(b) Dual Stimulus

For predator-induced stress and buffering, freezing was considered the primary stress related measure and rapid movements, time spent across three zones considered as the supporting metrics to evaluate the stress buffering across PO and PS conditions. Whereas for mirror-induced aggression and buffering, mirror bites and time allocation across MO and MS were emphasized (Akinrinade et al., 2023; Faustino et al., 2017; Gerlai, 2009; Kalueff et al., 2013; Pham et al., 2012).

3.6.2.3 Objective 3

(a) Spatial Metrics:

Shoal structure was primarily assessed by estimating shoal area and defined as the spatial extent occupied by the group. The convex hull enclosing the positions of all

fish at each frame was computed, and the resulting area was converted into square centimetres. Average shoal area across the duration of the trial reported as the measure of group dispersion.

To estimate the inter-individual distances, Euclidean distances between all pairs of fish were calculated from their frame-wise positions and averaged over time. From the Euclidean distances, nearest neighbour distance (NND) and farthest neighbour distance (FND) were derived as the minimum and maximum distances observed for each individual, respectively (Kalueff et al., 2013; Sivaraman et al., 2022; Yang et al., 2022).

(b) Orientation and Alignment Metrics:

Directional alignment was computed by calculating heading angles from frame -to-frame changes in individual positions. Heading angle differences were estimated as the mean absolute angular difference between the movement directions of fish pairs. Additionally, mean cosine dissimilarity and heading correlation (r) was also computed as alignment metrics. Higher cosine values indicate reduced alignment in movement direction. Heading correlation (r) was calculated by using Pearson correlation between heading angle time series of fish pairs, providing a temporal alignment in direction (Zada et al., 2024).

(c) Speed Metrics:

Individual locomotor activity was computed from instantaneous frame-to-frame displacement of each individual and averaged across a trial². Whereas shoal level locomotor activity was dependent on the overall shoal centroid displacement over frame-to-frame. Freezing and rapid movements were expressed as frequencies per second for each individual and averaged across fish within a trial⁹. Freezing was defined as a swimming speed below 1 cm/s, whereas rapid movement was categorized as exceeding 10 cm/s (Audira et al., 2018; Félix et al., 2025; Mukherjee et al., 2024; Sivaraman et al., 2022).

(d) Two order parameters

Following established approaches for the analysis of animal collective motion, shoal level emergent states were assessed using polarization and rotational order

parameters. The same was reported as the Tunstrom-Couzin model in the result section below. Four collective states were defined using thresholds and Op-Or phase space mapping, including polarized ($Op > 0.65$ & $Or < 0.35$), milling ($Op < 0.35$ & $Or > 0.65$), swarming ($Op < 0.35$ & $Or < 0.35$), and transitional (values outside these regimens) (Tunstrøm et al., 2013). Polarization was used to measure the degree of alignment between shoal members and reflect the extent to which fish move coherently in a common direction. Higher values indicate highly ordered movement, whereas lower values indicate disordered movement. Rotational order was used to quantify coherent circular or milling behaviour, this reflects the tendency of shoal members to rotate collectively around the group centroid (Tunstrøm et al., 2013).

The outcome measure was the fraction of time spent in each state for each trial, and calculated by dividing the number of frames assigned to a given state by the total number of analysed frames (Harpaz et al., 2017; Tunstrøm et al., 2013; Zampetaki et al., 2024).

(e) Memory Time

Both polarization and rotational memory times were quantified to assess the temporal stability of emergent states. Autocorrelation functions were used to compute the respective order parameter time series. The memory time was defined as the lag at which autocorrelation decayed to 37% ($1/e$) of its initial value. Shorter memory times indicate the faster transitions between collective states and reduced temporal stability (Tunstrøm et al., 2013; Zienkiewicz et al., 2014).

3.6.2.4 Objective 4

Parameter Derivation of Dyadic Interactions

For the dyadic experiments conducted, one pair of fish were recorded simultaneously in the open field experimental arena for 6 minutes. Their X and Y coordinates were extracted using idTracker 2.1 multiple animals tracking software. The pixels to centimetres conversion of raw positional values were done using a calibrated conversion factor. Frames with identity loss were avoided before further analysis. All analyses were performed using custom made scripts in Python.

The dyadic parameters were categorized into two sets, where the first one focused on movement coordination and other behavioural metrics, while the second set analysed directional interaction and initiation patterns within the dyad.

(a) First set of Parameters

The distance between the two fish were calculated at each frame using their centroid positions. From this series, the mean inter-individual distance across the entire trial was obtained. In addition to average spacing, proximity was computed by defining a fixed spatial threshold of 5 cm. The proportion of time during which the fish remained within this range was identified. Together the mean Euclidean distance and percentage of time spent in the proximity zone was reported as spatial cohesion metrics in dyadic interactions (Lehtonen et al., 2022).

The analysis further extended to better understand the movement alignment and synchronization using path cosine dissimilarity, speed difference, acceleration correlation, and maximum velocity cross correlation. For this displacement vectors were obtained from frame-to-frame changes in position. Cosine dissimilarity was then calculated between the displacement vectors of both individuals, providing a measure of how closely their movements aligned in direction and magnitude over time (Harpaz et al., 2025; Zada et al., 2024).

For each fish, total distance travelled was calculated by adding all frame-wise displacements. Average speed was derived by dividing total distance by the trial duration. The absolute difference in average speed between the two individuals was used as an indicator of locomotor asymmetry within the dyad (PapaspYROs et al., 2024).

Acceleration was calculated from changes in velocity across consecutive frames. The magnitude of acceleration for each fish was extracted, and a Pearson correlation analysis was performed between the two acceleration time series. This provided a measure of fine-scale coordination in movement dynamics (PapaspYROs et al., 2024).

Temporal coordination was examined using velocity cross-correlation. Linear velocity traces were normalized before analysis. The cross-correlation was obtained within a lag window of ± 5 seconds. Although the time lag corresponding to the

maximum was calculated, the maximum correlation values were retained for further statistical analysis (Papaspyros et al., 2024; Stednitz & Washbourne, 2020).

Behavioural state dynamics were derived from speed thresholds. Freezing was defined as a swimming speed below 1 cm/s, whereas rapid movement was categorized as exceeding 10 cm/s (Audira et al., 2018; Félix et al., 2025; Mukherjee et al., 2024). The frequency of both states was calculated as the number of episodes occurring per second and their average values were considered for the statistical comparisons.

(b) Second set of Parameters

This section focused on the directional organization within the dyad using geometric following and directional initiation/leadership.

The geometric following was defined using both spatial and angular criteria. A following event was identified when one fish remained within two body lengths (6 BL) of the other and positioned within $\pm 45^\circ$ behind the leader's heading direction. Heading vectors were derived from velocity direction and the fraction of time spent in geometric following was calculated separately for each direction within the pair (Jolles et al., 2017).

Directional initiation leadership was assessed using time-shifted comparisons of heading direction. For each frame, the alignment between the two fish was evaluated across a time window of ± 1 second. The lag that produced the strongest alignment was identified, and leadership was assigned when alignment exceeded a predefined threshold. The fraction of time each fish initiated the leadership position was calculated and these values were further used for the statistical comparisons (Collignon et al., 2019; Nagy et al., 2013).

Although the parametric derivation was conducted in two sets of analysis, the statistical comparisons and results were merged and placed under the two experimental groups, Homogeneous and Heterogeneous dyads.

3.6.3 Data Processing and Statistical Analysis

3.6.3.1 Objective 1:

Percentage change was used to assess the change from control to test groups (TM vs. CM and TF vs. CF), while percentage difference was used to compare groups without a defined control (CF vs. CM and TF vs. TM). Accordingly, all percentage changes and differences were calculated using group medians, which provide reliable indications of central tendency under non-normal data conditions, and due to the typical characteristics of behavioural data and to maintain consistency across all group-wise analyses. Non-parametric statistical tests (Mann Whitney U Test & Wilcoxon Signed Rank Test) were employed throughout the study, using Graph Pad Prism, version 9.5 (GraphPad, San Diego, CA, USA). All results were evaluated against a significance level of 0.05. Effect sizes (r) were calculated from the Z value obtained, for all comparisons detailed in the Results section, using established online statistical tools (For ex., Statistics Kingdom, 2017) except for non-significant results in the section 1.1.5., which were avoided to prevent both overinterpretation and redundancy.

Spearman's rank correlation was used to assess associations among seven parameters related to thigmotactic and exploratory zones (time, distance, entries), and total rapid movements from OFT. Normality was tested using the D'Agostino-Pearson omnibus test ($K^2 = 20.93$, $p < 0.0001$). When pairs of highly correlated parameters (correlation coefficient > 0.80) were detected, one of the two was selected based on relevance for PCA, and the other was removed. Therefore, two parameters were excluded: entries to the exploratory zone ($r = 1$, $p < 0.0001$) due to high correlation with entries to the thigmotactic zone, and distance in the exploratory zone ($r = 0.99$, $p < 0.0001$) due to high correlation with time in the same zone. Principal Component Analysis (PCA) was conducted on the remaining parameters, for the multivariate analysis.

3.6.3.2 Objective 2:

Since the behavioural data displayed variable distribution patterns (ranging from normal to non-normal) and unequal variances, non-parametric statistical methods were applied uniformly across all analyses to ensure robustness. For the single-stimulus analysis (NS, SO, PO, MO), within-group comparisons were performed

using the Kruskal–Wallis test followed by pairwise comparisons with FDR correction (Benjamini, Krieger, and Yekutieli) (Haynes, 2013). Between-group comparisons (CM vs TM, CF vs TF, CM vs CF, TM vs TF) were analysed using Mann–Whitney U tests with Holm correction for multiple testing (Holm, 1979; McLaughlin & Sainani, 2014).

For the buffering experiments (PO vs PS and MO vs MS), behavioural measures were compared within each group using Mann–Whitney U tests, as each condition involved a single comparison. Between-group comparisons for the buffering effects followed the same test with Holm correction (Holm, 1979). The zone-specific time allocations under buffering (contact/internal/distant) were compared using the Friedman test followed by FDR correction for pairwise comparisons (Haynes, 2013). Finally, to assess social affinity modulation across contexts (SO, PS, MS), SPI values were compared within each group using the Kruskal–Wallis test with FDR correction. All statistical analyses were performed in GraphPad Prism v9 (Bedell et al., 2025).

3.6.3.3. Objective 3:

Assumptions for normal distribution and equal variance were varied between fundamental shoal level metrics. In order to maintain consistency across statistical comparisons, we followed non-parametric tests uniformly across all parameters in this objective. Fundamental shoal metrics only having two independent groups including control and test, therefore Mann-Whitney U test, followed with effect size calculation conducted for each parameter. The two order parameter analysis categorized within group and between group analysis. Within group analysis across multiple groups conducted by Friedman Test followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli for pairwise comparisons, and corrected q values were reported, whereas between group comparisons in the single parameter had eight groups, only specific comparisons were made, therefore again followed Mann Whitney U test, while holm correction applied as $m=4$. All statistical tests were carried out using GraphPad Prism, while error size reported calculated from Z values using established online statistical calculators (For e.g., Statistics Kingdom, 2017).

3.6.3.4 Objective 4:

Normality and homoscedasticity tests indicated shifts across variables; in order to maintain consistency across statistical tests performed, we employed non-parametric tests throughout the inferential analysis. In the present study, each variable was treated as a family of comparisons, and selected pairwise comparisons were analysed. In order to control the family-wise error rate, we used Holm correction on nine pairwise comparisons for each variable. Effect size (r), calculated based on the Z value, was also reported.

3.6.4 Data Presentation

3.6.4.1 Objective 1:

Data were represented as box plots with individual values, violin plots with cumulative distributions, and bar graphs (mean $\pm$ SEM) for derived measures. Heatmaps (OFT) and PCA were generated in Python, while correlations and eigen value plots were prepared in Prism. Figures were standardized with consistent colour coding and layout for clarity.

3.6.4.2 Objective 2:

This specific objective includes multiple domains of social isolation and sex specific impacts on social preference, stress, aggression, and buffering. Understanding the complexity of data with multiple parameters and several experimental groups, we attempted to ease the visual presentation of the results by sectionizing them specifically.

We presented the raw data of each group (CM, TM, CF, TF) across multiple stimuli (NS, SO, PO, MO), including descriptive and inferential statistics using column bar graphs with individual data points. This was intended to understand the stimulus-specific responses within each experimental group separately. This section also included in-depth data representations, which further enabled us to include summary heatmaps of the same data in the subsequent sections for better visualization.

In the next section, we analysed between-group differences under each stimulus condition to understand the major differences observed in isolated groups compared

to control groups. Here, we adopted heatmaps representing median values for the analysed parameters to avoid repeated presentation of the same data shown in the first section.

The third section introduced stress and buffering conditions (PS)- specific parameters including freezing, rapid movements, and zone allocation across groups. We relied on column bar graphs with all individual data points. The core of the objective was represented as difference scores of each parameter from PO to PS, using individual scatter dot plots.

The final section included mirror bites from MO and MS conditions, and MS-specific parameters were presented separately using column bar graphs with all data points across the four experimental groups. Similar difference score scatter plots were used to visualize the buffering patterns across experimental groups in MO to MS conditions. Additionally, percentage pie charts were used in the both buffering experiments to visualize changes associated with time allocation across multiple experimental groups and stimuli.

3.6.4.3 Objective 3:

Fundamental shoal level metrics were represented as violin plots with individual data points with median and 95% confidence intervals and generated in prism. Two order parameters were represented as density maps and bar graphs (mean $\pm$ SEM). Density maps were generated using python and bar graphs were generated using prism respectively.

3.6.4.3 Objective 4:

The results have been grouped into two: Homogeneous and Heterogeneous dyads. Dyadic level behavioural metrics across six pairs of zebrafish (representing all possible sex combinations within control and isolation conditions), categorized based on sex combinations, were illustrated using box and whisker plots indicating all individual data points. For the parameters showing sex specificity, these were illustrated through percentage heatmaps generated from the raw data for better visualization and to avoid overcrowding of statistical values. Statistical comparisons

exhibiting only significant p-values were represented in the figures; all descriptive and inferential statistical details have been tabulated for each parameter.

For the heterogeneous groups (that is dyads composed of individuals from different experimental conditions such as control-isolated combinations), specific parameters were analysed at the individual level across various combinations of experimental groups (CM-TM, CF-TF, CM-TF, CF-TM). Such individual level analysis and illustration were done using violin plots with all experimental data points included. All statistical comparisons were marked in the figures, including both significant and non-significant results, in order to demonstrate individual variation within each dyad.

Therefore, within dyadic experiments, comparisons were primarily conducted within homogenous groups (same experimental condition) and secondarily within homogeneous groups (mixed experimental condition). At the third level, homogeneous groups were compared with heterogeneous groups to evaluate overall similarities and dissimilarities across dyad groups. Because all raw data points and descriptive analyses were presented in the first two sections, in the third section the data were represented using bar diagrams compiling all groups and categorizing them into male-male, female-female, and mixed-sex dyads to demonstrate similarity patterns. For instance, whether heterogeneous dyads resemble control or test isolated homogeneous dyads. This comparison was performed across all dyadic level metrics.

Table 3.1. Behavioural metrics and corresponding Python libraries used for data acquisition and analysis in Objectives 3 and 4.

Metric Category	Metric	Libraries Used
Shoal Metrics	Cohesion, Alignment	Pandas, NumPy
Spatial Cohesion	Euclidean Distance	NumPy
	Proximity	NumPy
Movement Alignment	Cosine Dissimilarity	sklearn, NumPy
	Geometric Following	NumPy, SciPy (circmean)
	Speed Difference	NumPy
	Acceleration Correlation	NumPy
Synchronization	VCC Max	SciPy (Signal), NumPy
	Directional Leadership	SciPy (Signal), NumPy
State Dynamics	Freezing	NumPy, scipy. ndimage. label
	Rapid Movement	NumPy, scipy. ndimage. label

3.7 Materials and Methods: Objective 5

Pilot Study on Sex-Specific Gut Microbiome Analysis Under Social Isolation

3.7.1 Gut Dissection and Sampling

All the dissection tools were autoclaved and sterilized, and the workspace was cleaned with 70% ethanol. Fourteen control fish were selected randomly from three tanks; seven males and seven females were grouped into control males (CM) and control females (CF). Isolated males and females were also segregated into seven test males (TM) and seven test females (TF). Therefore, a total of 28 animals were used for gut microbiome analysis. Fish were then euthanized using hypothermia, and the gastrointestinal tract was carefully dissected following Gupta and Mullins (2010). Gut contents were homogenized using a new sterile motor and pestle for each sample to prevent contamination or mixing, and pooled within each group to recover from the lower DNA quantity. This ensured sufficient DNA in each sample and represented group-level microbiota. Pooled samples were transferred to DNA-free microcentrifuge tubes and stored at -20°C for the next step, DNA extraction.

3.7.2 DNA Extraction and Quality Check

Genomic DNA was extracted from pooled gut samples using a commercial kit, ORIonX Genomic DNA Kit (Origin Lab, India). All the procedures involved in extraction were followed according to the instructions provided by the kit. At the final stage, the eluted DNA was stored at -20 °C until shipment for subsequent 16s rRNA amplicon-based microbiome analysis. Prior to outsourcing, the DNA concentration in the filtrate was assessed using Nanodrop spectrophotometer (Nanodrop technologies, USA), and the concentration was ensured to be at a threshold of approximately 30 ng/μL (Sivarajan et al., 2025).

3.7.3 PCR and Library Setup

Targeting the V4 region of the 16S rRNA gene, PCR amplification was verified by examining the amplified PCR products on a 2% agarose gel. Following confirmation, the PCR products were purified according to the Illumina 16S amplicon library

preparation protocol. Library construction was conducted using the NEBNext Ultra DNA Library Preparation Kit (New England Biolabs). The quality and quantity of the prepared libraries were analysed using the Agilent 2200 TapeStation system. Sequencing was performed on the Illumina HiSeq 2500 platform, following the manufacturer's instructions, to generate high-quality reads for downstream microbiome analysis (Sivarajan et al., 2025).

3.7.4 Sequence Processing and Data Analysis

Raw reads were quality filtered and trimmed using DADA2 using R programming software. Amplicon sequence variants (ASVs) were inferred using the DADA2 error-correction model. The consensus method used to eliminate chimeras. Taxonomic assignment was further conducted by classifying ASVs using the SILVA reference database. Non-bacterial reads were removed before final dataset preparation.

Because the present study was pilot or descriptive in nature, and also considering the limitation of species level resolution for certain taxa (as some species share high sequence similarity), relative abundance profiles were generated at phylum and genus levels only. Alpha diversity indices, including Shannon, Simpson, and Chao, were tabulated. Further higher-level analyses including beta diversity and PERMANOVA were not conducted due to the limitation of biological replicates. Accordingly, the present study was designed as a pilot investigation, and the results are intended to provide preliminary observations rather than definitive conclusions (Sivarajan et al., 2025).

Figure created by the author using AI-assisted tools

Zebrafish Gut Microbiome Analysis

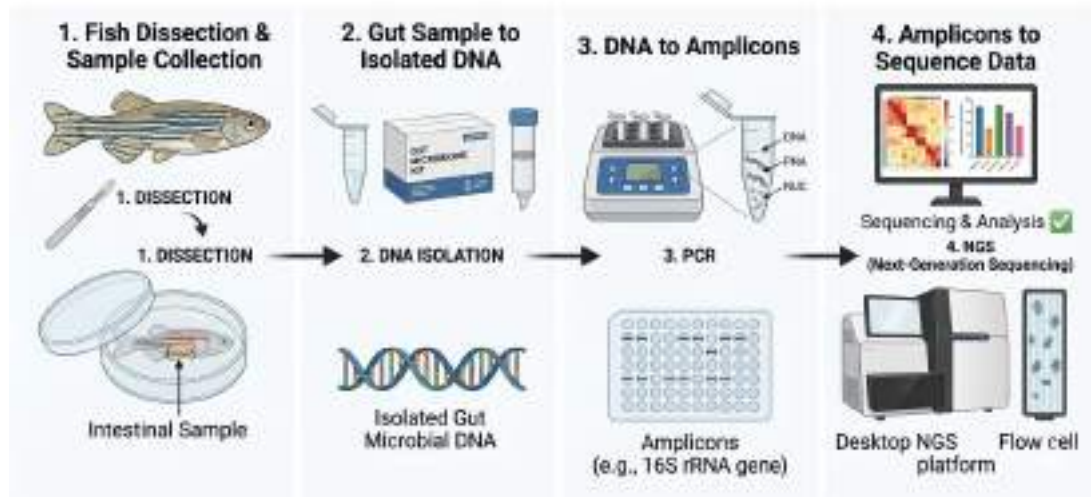


Figure 3.8. Workflow of intestinal microbiome analysis in zebrafish.

This figure presents the overall workflow of the experimental procedures used for intestinal microbiome analysis in zebrafish. The process begins with intestinal sample collection, followed by DNA isolation from the collected samples. The extracted DNA is then subjected to polymerase chain reaction (PCR) amplification targeting the 16S rRNA gene. Subsequently, the amplified products are processed for next-generation sequencing (NGS) to characterize microbial composition and diversity.

Chapter 4

Results

4.1 Objective 1: Results

4.1.2 Objective 1 Statement: Anxiety-Like Behaviours and Activity Levels Under Social Isolation

Under the first objective, we analysed the behavioural outcomes of individual zebrafish using the open field test (OFT) and light-dark test (LDT) to assess anxiety- and activity-related changes associated with chronic social isolation. We integrated both anxiety and activity parameters, including time spent in the thigmotaxis zone in the OFT and time spent in the light zone in the LDT as primary anxiety measures, followed by activity parameters including zone transitions, rapid movements, speed range, and activity balance index (ABI). Both univariate analyses and multivariate analysis of OFT parameters were employed.

The results were subcategorised based on the comparisons conducted to assess treatment effects within each sex (control males vs isolated males and control females vs isolated females), baseline sex differences under control conditions (control males vs control females), and treatment-related sex differences (isolated males vs isolated females). General behavioural trends were described using median differences, while detailed statistical outcomes are presented in the corresponding tables.

4.1.3 Behavioural Test 1: Open-Field Test

4.1.3.1 Isolation-Induced Altered Behavioural Changes in Male Zebrafish (TM vs CM)

Pairwise comparisons between TM and CM revealed that TM showed a 17.641% decrease in the time spent in the exploratory zone and 108.119% an increase in the thigmotactic zone compared to CM, both with statistically significant p-values ($p < 0.05$) and medium effect sizes ($r = 0.49$). TM exhibited an 153.165% increase in the exploratory zone entries and an 151.25% increase in the thigmotactic zone entries relative to CM, with statistically significant p-values and large ($r = 0.85$) effect sizes.

TM exhibited an 14.176% decrease in percentage distance travelled in exploratory zone and an 90.754% increase in the percentage distance travelled in thigmotactic zone relative to CM, with statistically significant p-values and medium ($r = 0.49$) effect sizes. The median rapid moments in the TM were 10.18 units higher than CM,

with statistical significance and large effect size ($r = 0.77$). TM exhibited an 44.941% increase in the speed range and an 2.086% decrease in the activity index relative to CM, with statistically significant p-values and medium (speed range: $r = 0.44$, activity index: $r = 0.41$) effect sizes.

4.1.3.2 Isolation-Induced Altered Behavioural Changes in Female Zebrafish (TF vs CF)

Pairwise comparisons between TF and CF revealed that TF showed an 87.262% increase in the time spent in the exploratory zone and 32.318% a decrease in the thigmotactic zone compared to CF, both with statistically significant p-values ($p < 0.05$) and medium effect sizes ($r = 0.44$). TF exhibited an 123.596% increase in the exploratory zone entries and an 121.111% increase in the thigmotactic zone entries relative to CF, with statistically significant p-values and large ($r = 0.61$) effect sizes.

TF exhibited an 91.625% increase in the percentage distance travelled in exploratory zone and an 36.159% decrease in the percentage distance travelled in thigmotactic zone relative to CF, with statistically significant p-values and medium ($r = 0.42$) effect sizes. The median rapid moments in the TF were 47.56 units higher than CF, with statistical significance and large effect size ($r = 0.77$). TF exhibited an 44.878% increase in the speed range and a 5.019% decrease in the activity index relative to CF, with statistically significant p-values and medium (speed range: $r = 0.46$, activity index: $r = 0.47$) effect sizes.

4.1.3.3 Behavioural Variations in Zebrafish Exploratory Activity Under Control Conditions (CF vs CM)

Pairwise comparisons between CF and CM revealed that CF showed a 104.3% difference in the time spent in the exploratory zone and a 135.47% difference in the thigmotactic zone compared to CM, both with statistically significant p-values ($p < 0.05$) and large effect sizes ($r = 0.85$). CF exhibited 11.90% difference in entries to the exploratory zone and 11.76% difference in the entries to the thigmotactic zone relative to CM, with statistically non-significant p-values and small ($r = 0.12$) effect sizes.

CF exhibited an 101.377% difference in the percentage distance travelled in exploratory zone and an 136.548% difference in the percentage distance travelled in

thigmotactic zone relative to CM, with statistically significant p-values and large ($r = 0.85$) effect sizes. The median rapid moments in the CF were 0.64 units higher than CM, with statistically non-significant p value and small effect size ($r = 0.29$). CF exhibited an 9.099% difference in the speed range and an 0.422% difference in the activity index relative to CM, with statistically non-significant p-values and small (speed range: $r = 0.23$, activity index: $r = 0.25$) effect sizes.

4.1.3.4 Comparative Analysis of Isolated Zebrafish in the Open Field Test (TF vs TM)

Paired comparisons between TF and TM revealed that TF showed a 33.22% difference in the time spent in the exploratory zone and a 51.39% difference in the thigmotactic zone compared to TM, both with statistically non-significant p-values ($p > 0.05$) and small effect sizes ($r = 0.21$). Even though the percentage difference was considerable, the comparison did not reach statistical significance under the Mann–Whitney U test, possibly due to high variability and overlapping percentile values, which reduced the differences in rank sums between groups. TF and TM had almost equal entries to zones; TF exhibited a 0.50% difference in the number of entries to the exploratory zone and a 1.0% difference in the number of entries to the thigmotactic zone compared to TM, with statistically non-significant p-values and small ($r = 0.098$) effect sizes.

TF exhibited an 31.125% difference in the percentage distance travelled in exploratory zone and an 55.8614% difference in the percentage distance travelled in thigmotactic zone relative to TM, with statistically non-significant p-values and small ($r = 0.2$) effect sizes. The median rapid moments in the TF were 33.56 units higher than TM, with statistically non-significant p value and medium effect size ($r = 0.3$). TF exhibited an 9.055% difference in the speed range and an 2.617% difference in the activity index relative to TM, with statistically non-significant p-values and small (speed range: $r = 0.14$, activity index: $r = 0.15$) effect sizes.

4.1.3.5 Cumulative Behavioural Trends and Analysis of Derived Indices

Cumulative percentage plots of four experimental groups revealed variations in the behavioural distribution of total zone transitions. A linear curve was observed with CM, an S-shaped curve in CF, a convex curve in TM, and a concave curve in TF. Despite the considerable differences observed in curve patterns among the groups,

pairwise comparisons using the Mann-Whitney U test, based on the gender (CF vs. CM, TF vs. TM, $P > 0.05$), showed no significant differences. However, comparisons based on treatment revealed a statistically significant increase in the total number of zone transitions both in TM and TF compared to their respective controls, with large effect sizes ($r = 0.85$ for TM vs. CM; $r = 0.61$ for TF vs. CF). Isolation significantly altered exploration as indicated by enhanced number of zone transitions in zebrafish, regardless of gender.

Cumulative percentage plots of total distance travelled exhibited inter-individual behavioural variation within each group. A concave curve was observed with CM, a linear to mildly convex curve in CF, an S-shaped curve in TM, and a convex curve in TF. Pairwise comparisons on the control groups (CF vs. CM) showed a statistically significant difference with a large (0.79) effect size, and the treatment groups (TF vs. TM) did not differ significantly. However, comparisons based on treatment revealed a statistically significant increase in the total distance travelled both in TM and TF compared to their respective controls, with large effect sizes ($r = 0.84$ for TM vs. CM; $r = 0.76$ for TF vs. CF). Control groups showed gender-specific locomotor patterns, which were abolished in the isolated groups. Isolation altered the locomotor activity by neutralizing baseline gender-specific differences and increasing total distance travelled in both genders.

A dual-axis bar plot was used to illustrate changes in speed range and proportion of swim time across groups. Control groups (CM and CF) showed a higher proportion of swim time and a lower speed range. In contrast, both the test groups exhibited a higher speed range and a lower proportion of swim time, indicating a shift in locomotor behaviours. This pattern was also reflected in the ABI across four groups: TM (25.17 ± 1.738) and TF (33.33 ± 3.774) showed higher mean ABI, and CM (16.45 ± 2.246) and CF (18.50 ± 1.076) showed lower mean ABI, which may reflect burst-like or hyperactive behaviours associated with isolation.

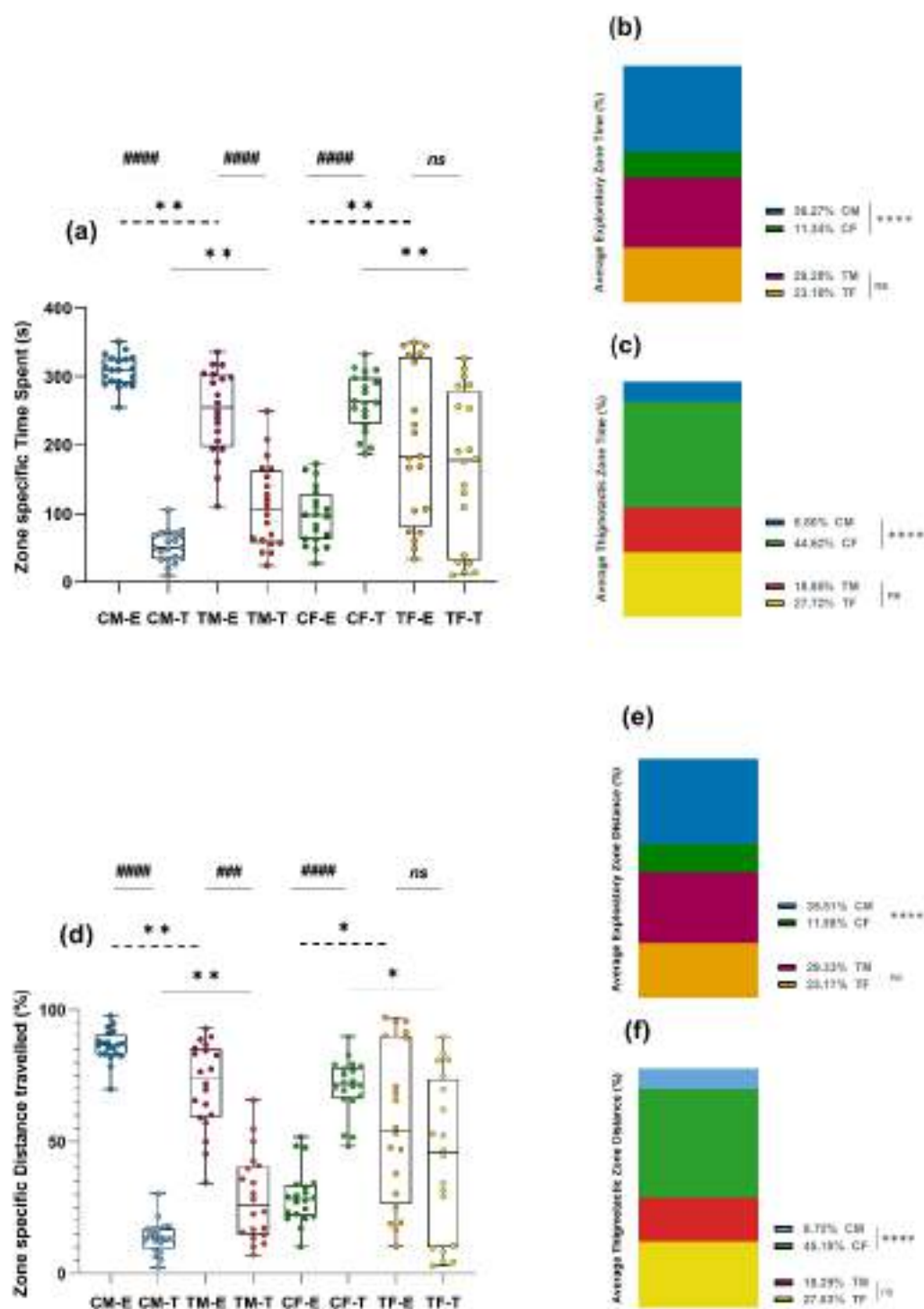


Figure 4.1. Primary and complementary anxiety-related metrics assessed in the open field test (OFT).

(a) Time spent in exploratory (E) and thigmotactic (T) zones of the OFT, representing primary anxiety-related behaviour across control males (CM), test isolated males (TM), control females (CF), and test isolated females (TF). Data are presented as box-and-whisker plots showing all individual observations ($n = 20$ per group), median

values, and 95% confidence intervals. Between-group comparisons were performed using the Mann–Whitney U test, while within-group comparisons between E and T zones were assessed using the Wilcoxon signed-rank test. Statistical significance after Holm correction is indicated by asterisks (*) for between-group comparisons and hashtags (#) for within-group comparisons; non-significant comparisons are denoted as ns.

(b–c) Percentage distribution of average time spent in exploratory (E; b) and thigmotactic (T; c) zones, illustrating sex-based differences within control and test isolated conditions. Stacked bar representations depict the proportional contribution of male and female groups within each condition. Comparisons between CM vs. CF and TM vs. TF were conducted using the Mann–Whitney U test, with significance levels reported following Holm correction.

(d–f) Complementary anxiety-related metrics depicting distance travelled within exploratory (E) and thigmotactic (T) zones across CM, TM, CF, and TF groups. These measures are included to support zone-specific anxiety assessment and are not interpreted as indices of general locomotor activity.

Overall, control males preferentially occupied the exploratory (E) zone, whereas control females showed greater thigmotactic (T) zone occupancy, indicating baseline sex differences in anxiety-related behaviour; in contrast, both test isolated males and test isolated females predominantly occupied the exploratory zone, with no significant sex differences observed between test isolated groups.

All statistical analyses including generation of figures were conducted using GraphPad Prism (version 9),

Significance thresholds: $p < 0.05$ (), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

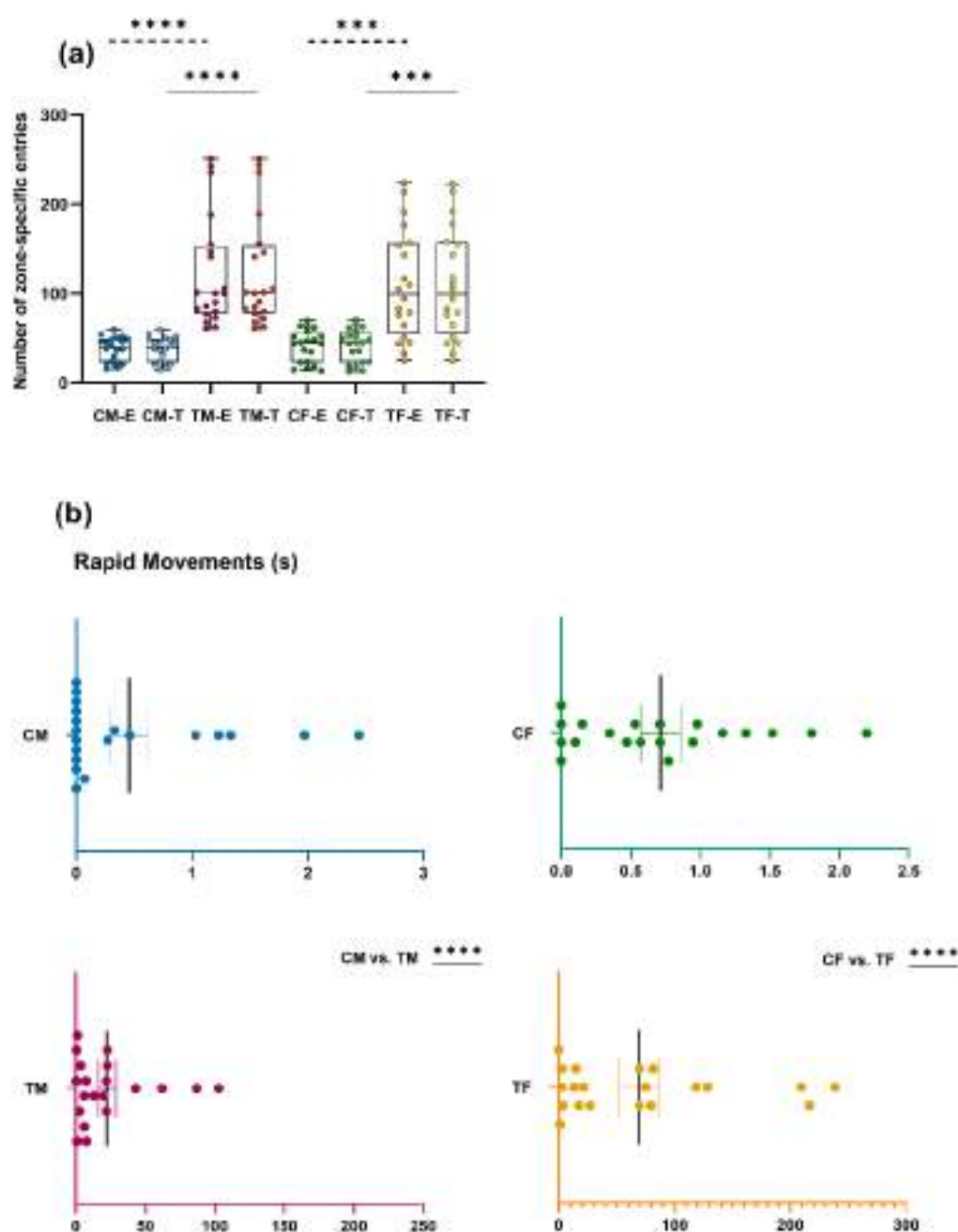


Figure 4.2. Activity-related measures assessed in the open field test (OFT).

(a) Number of zone-specific transitions between exploratory (E) and thigmotactic (T) zones across control males (CM), test isolated males (TM), control females (CF), and test isolated females (TF). Data are presented as box-and-whisker plots showing all individual observations ($n=20$), median values, and 95% confidence intervals. Between-group comparisons were conducted using the Mann–Whitney U test, with significance indicated only for comparisons that survived Holm correction. Within-

group comparisons between E and T zone transitions were non-significant and are therefore not displayed.

(b) Rapid movements, shown as scatter dot plots with individual data points depicted separately for each group (CM, TM, CF, TF). Central tendency and dispersion are indicated by median and 95% confidence interval markers. Only statistically significant between-group comparisons are annotated; comparisons that did not show sex-based differences (CM vs CF & TM vs TF) were omitted from graphical representation.

Overall, test isolated males and test isolated females exhibited a higher number of zone-specific transitions and increased rapid movements compared to their respective control groups, indicating elevated activity levels that were independent of sex.

All statistical analyses including generation of figures were conducted using GraphPad Prism (version 9).

Significance thresholds: $p < 0.05$ (), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

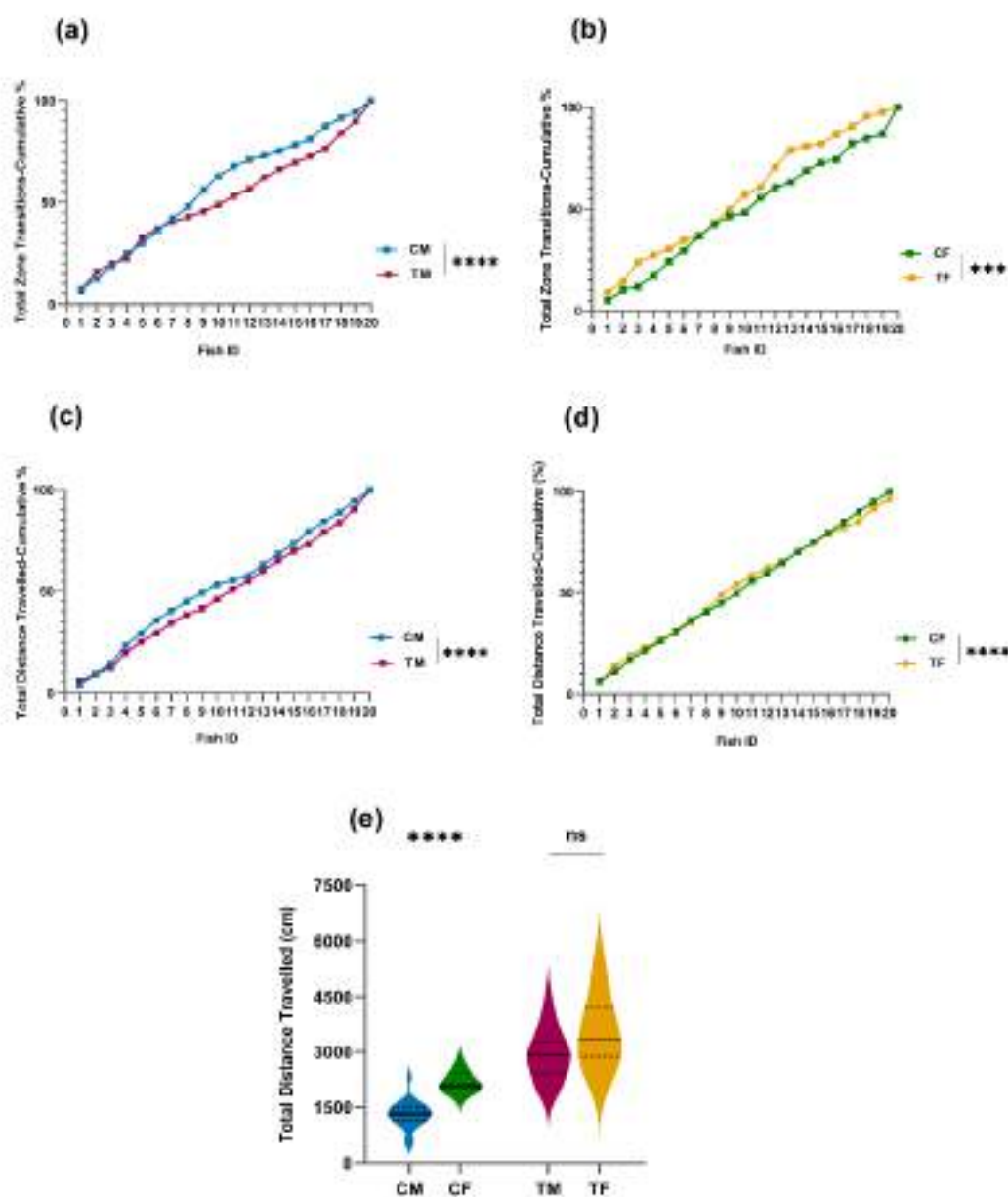


Figure 4.3. Global activity measures assessed in the open field test (OFT).

(a–b) Total zone transitions represented as cumulative percentages derived from individual observations (subjects 1–20). Panel (a) depicts control males (CM) versus test isolated males (TM), and panel (b) depicts control females (CF) versus test isolated females (TF). Statistical analysis was performed on the raw data using the Mann–Whitney U test, while cumulative representations were used exclusively for improved data visualisation. Sex-based comparisons were not statistically significant for total zone transitions and were therefore not included in the graphical representation; only treatment-related differences (CM vs. TM and CF vs. TF) are shown.

(c–d) Cumulative percentage plots of total distance travelled, presented in the same format as zone transitions. Panel (c) shows CM versus TM, and panel (d) shows CF versus TF. As above, statistical analyses were conducted on raw distance values using the Mann–Whitney U test, with cumulative plots used to facilitate comparison of group-wise activity distributions.

(e) Total distance travelled displayed as violin plots to illustrate baseline sex differences in activity. Comparisons between CM vs. CF revealed a sex-dependent difference under control conditions, whereas no significant sex differences were observed between TM and TF under test isolated conditions.

Overall, global activity measures accounted for the activity-related changes observed across experimental groups. Total zone transitions were sex-independent in both control and test isolated conditions, whereas total distance travelled showed sex dependence under control conditions but not following test isolation. Control females exhibited greater distance travelled than control males, consistent with anxiety-related activity accompanied by higher thigmotactic preference. In contrast, both test isolated males and test isolated females displayed elevated activity levels, including increased distance travelled relative to controls, indicative of hyperlocomotion.

All statistical analyses including generation of figures were conducted using GraphPad Prism (version 9).

Significance thresholds: $p < 0.05$ (), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

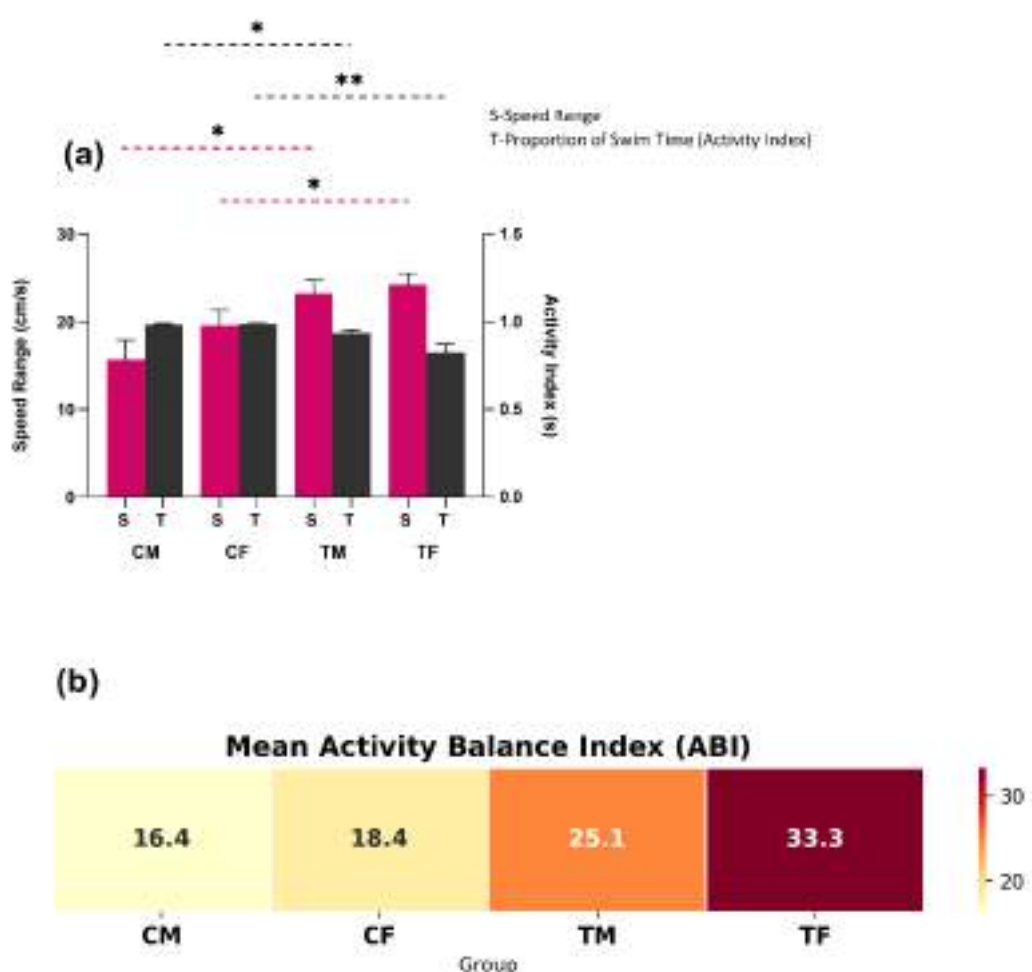


Figure 4.4. Derived locomotor stability metrics assessed in the open field test (OFT).

Speed range was defined as the variation in locomotor speed calculated as the difference between maximum and minimum swimming speed (max – min). Activity Index was defined as the proportion of time spent within the normal swimming speed range (1–10 cm/s). The Activity Balance Index (ABI) was calculated as the ratio of speed range to Activity Index ($ABI = \text{speed range} / \text{Activity Index}$), where lower ABI values indicate stable and balanced movement patterns, and higher ABI values indicate irregular or burst-like activity.

(a) Dual-axis bar plot depicting speed range (left y-axis) and Activity Index (right y-axis) across control males (CM), control females (CF), test isolated males (TM), and test isolated females (TF), presented in this order. Data are shown as mean $\pm$ SEM. Parametric between-group comparisons were performed, and statistically significant differences are indicated by asterisks (*).

(b) Heat map representation of ABI values across experimental groups, with mean ABI values displayed within each coloured cell. Control groups (CM, CF) exhibited

a higher proportion of normal swimming speeds accompanied by a lower speed range, resulting in lower ABI values indicative of stable locomotor behaviour. In contrast, test isolated males (TM) and test isolated females (TF) showed increased speed range and reduced normal-swim proportion, yielding higher ABI values consistent with hyperactive and burst-like movement patterns.

The dual-axis bar plots were generated using GraphPad Prism (version 9), and the ABI heat map was generated using Google Colab.

Significance thresholds: $p < 0.05$ (), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

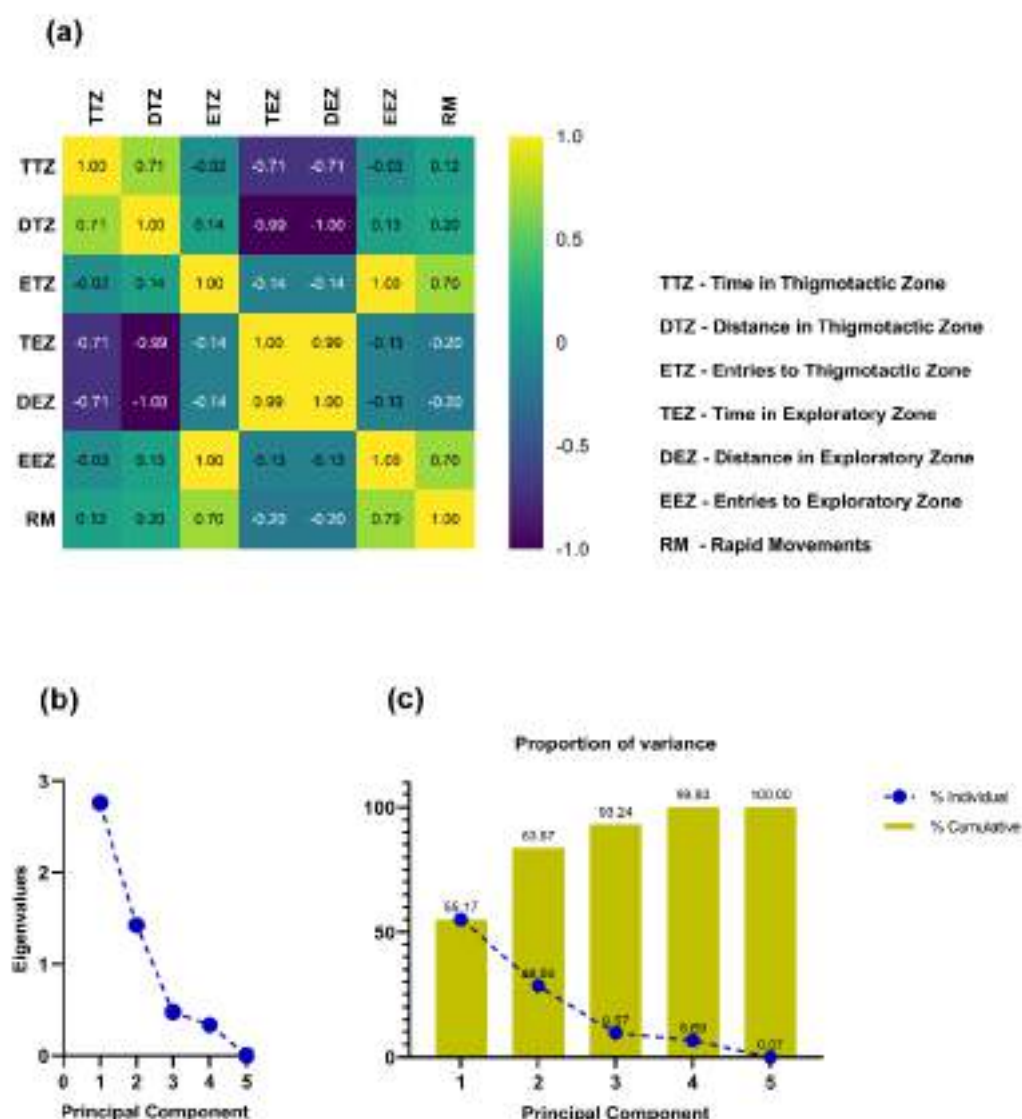
4.1.3.6 Multivariate Analysis Using Correlation and PCA

Spearman's rank correlation was used to assess associations among seven parameters related to thigmotactic and exploratory zones (time, distance, entries), and total rapid movements from OFT. Normality was tested using the D'Agostino-Pearson omnibus test ($K2 = 20.93$, $p < 0.0001$). When pairs of highly correlated parameters (correlation coefficient > 0.80) were detected, one of the two was selected based on relevance for PCA, and the other was removed. Therefore, two parameters were excluded: entries to the exploratory zone ($r = 1$, $p < 0.0001$) due to high correlation with entries to the thigmotactic zone, and distance in the exploratory zone ($r = 0.99$, $p < 0.0001$) due to high correlation with time in the same zone.

Principal Component Analysis (PCA) was conducted on the remaining parameters, and following the Kaiser-Guttman criterion, we considered the first two axes of PCA with eigenvalues of 2.758 and 1.424, respectively. These two components accounted for 55.17% and 28.50% of the total variance, respectively, amounting to a cumulative variance of 83.67%. The variables with the highest absolute contributions to each component were Time in Exploratory Zone (33.69%), Distance in Thigmotactic Zone (33.68%), and Time in Thigmotactic Zone (24.93%) for dimension 1; and Entries to Thigmotactic Zone (51.88%) and Rapid Movements (40.12%) for dimension 2. Therefore, PC1 was interpreted as representing anxiety-like behaviours, and PC2 as representing activity-related behaviours.

Based on the PC scores distribution around the space of PCA, higher PC1 scores indicated increased anxiogenic behaviours, while higher PC2 scores reflected greater activity-related behaviours. Statistical comparisons revealed that the TM had higher PC1 scores than CM ($U = 40$, $p < 0.0001$), but TF had lower PC1 scores than CF ($U =$

88, $p = 0.0019$). A comparison between CF and CM ($U = 0$, $p < 0.0001$) revealed gender-based differences, but TF and TM showed no gender-based differences ($U = 142$, $p = 0.1190$). TM ($U = 10$, $p < 0.0001$) and TF ($U = 4$, $p < 0.0001$) also showed elevated PC2 scores compared to their respective controls. The comparison between CF and CM ($U = 0$, $p < 0.0001$) revealed gender-based differences, while TF and TM showed no significant gender differences ($U = 199$, $p = 0.9893$), potentially linked to hyperactive behaviours associated with isolation.



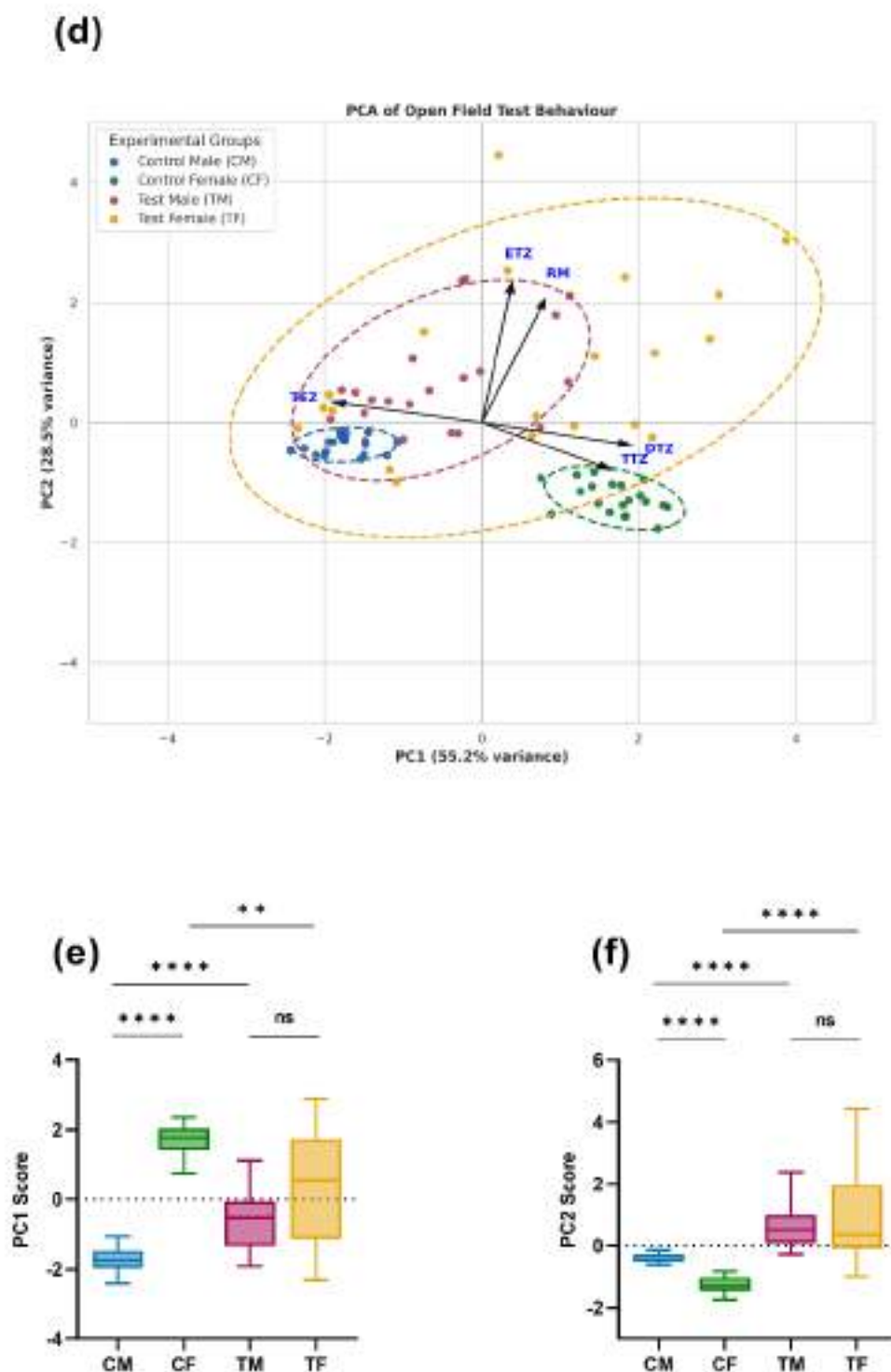


Figure 4.5. Multivariate analysis of open field test (OFT) parameters using correlation and principal component analysis (PCA).

(a) Spearman's rank correlation matrix illustrating relationships among seven OFT parameters. Parameter abbreviations are indicated adjacent to the matrix for clarity. Correlation coefficients are displayed to represent the strength and direction of monotonic associations between variables.

(b) Scree plot showing eigenvalues derived from PCA, and **(c)** proportion of variance explained by each principal component. Together, these plots were used to identify two principal components for further interpretation.

(d) PCA biplot illustrating variable loadings and group distribution along the first two principal components. Principal component 1 (PC1) was primarily associated with anxiety-related metrics, whereas principal component 2 (PC2) was predominantly associated with activity-related metrics.

(e–f) Box-and-whisker plots depicting group-wise scores along PC1 **(e)** and PC2 **(f)**. Between-group comparisons were performed using the Mann–Whitney U test, with Holm-corrected p-values indicated by asterisks where significant.

All statistical analyses including generation of figures were conducted using GraphPad Prism (version 9), except for the generation of the PCA biplot, which was performed using Google Colab.

Significance thresholds: $p < 0.05$ (), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

All statistical comparisons for the above-described open field results were tabulated in Tables 4.1 and 4.2 for between-group and within-group comparisons, respectively.

4.1.4 Behavioural Test 2: Light and Dark Test

4.1.4.1 Light and Dark Test Reveals the Anxiolytic Response from Socially Isolated Zebrafish

Within-group comparisons between the dark and light zones across the four experimental groups revealed that CM and CF exhibited 85.06% and 78.91% decreases, respectively, in the time spent in the light zone compared to the dark zone. Similarly, TM and TF exhibited a pattern opposite to the controls and showed 35.75% and 40.48% increases, respectively, in the time spent in the light zone compared to the dark zone.

Independent pairwise comparisons across the four experimental groups revealed that TM showed a 342.94% increase in the time spent in the light zone relative to CM, with statistical significance and a large effect size ($r = 0.74$). TF showed a 235.40% increase in the time spent in the light zone relative to CF, also with statistical significance and a large effect size ($r = 0.81$). A 29.04% difference in the time spent

in the light zone was reported between CM and CF, which was statistically non-significant and associated with a small effect size ($r = 0.05$). Only a 1.43% difference was found between TM and TF, which was also statistically non-significant with a small effect size ($r = 0.08$). Due to the equal dimensions of the two zones, and considering the light zone as the primary indicator of anxiety, only time spent in the light zone was discussed here. The time spent in the dark zone was included for visualising the within-group differences and is depicted in Figure 1a.

TM showed a 5.21% decrease in latency to first entry into the light zone relative to CM, and TF showed a 2.89% decrease in latency to first entry into the light zone relative to CF, both statistically significant p-values with medium effect sizes ($r = 0.41$). A 1.91% difference in latency to first entry into the light zone was reported between CM and CF, which was statistically non-significant and associated with a small effect size ($r = 0.09$). Only a 0.49% difference was found between TM and TF which was also statistically non-significant with a small effect size ($r = 0.16$), (Figure 1b).

Due to the non-normal distribution (D'Agostino & Pearson test: $K2 = 6.134$, $p = 0.0466$) of the data, Spearman's rank correlation was performed, and revealed a moderate negative monotonic correlation ($r = -0.4252$, 95% CI: -0.5941 to -0.2204) with statistical significance ($p < 0.0001$).

All statistical comparisons for the above-described light-dark test results were tabulated in Tables 4.3 and 4.4 for between-group and within-group comparisons, respectively.

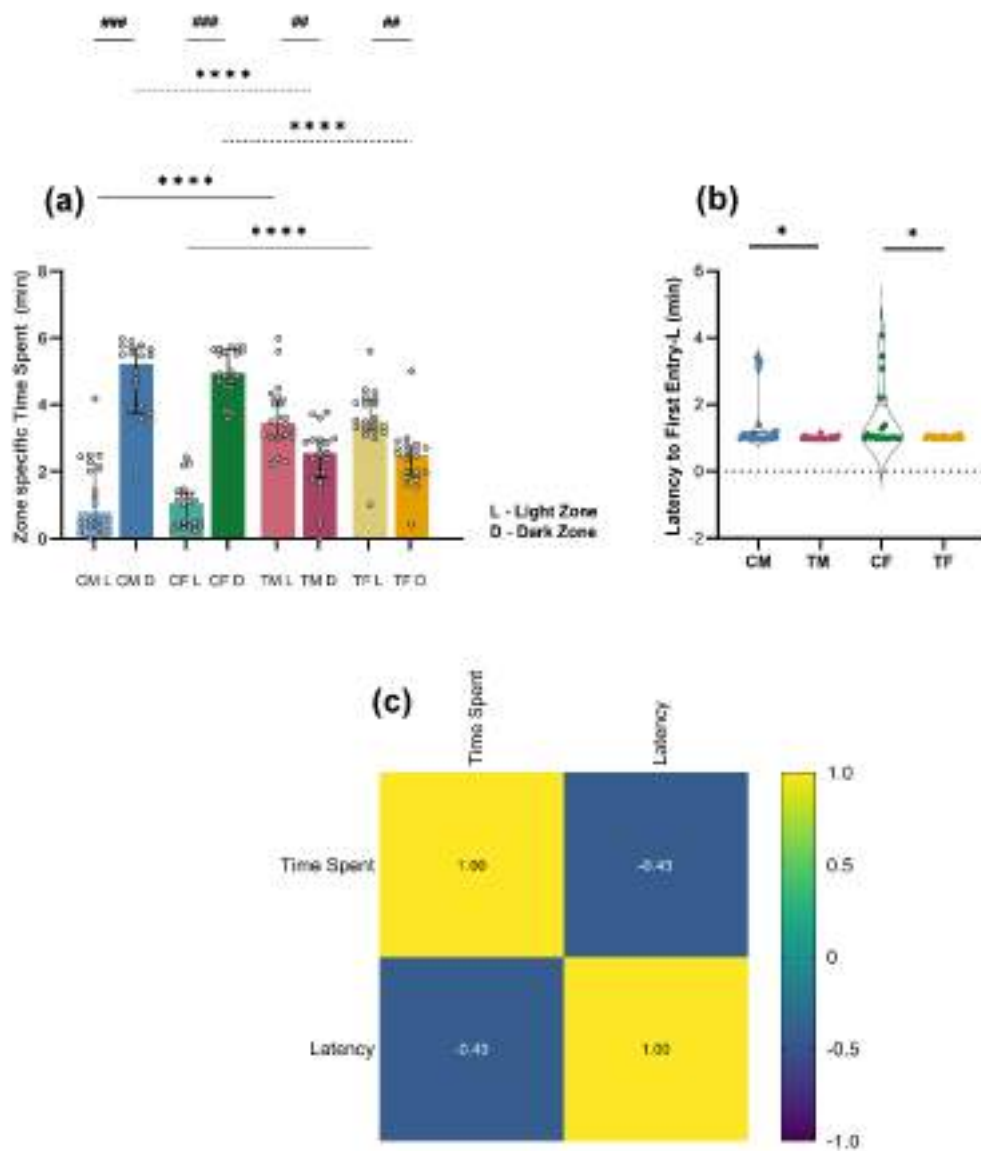


Figure 4.6. Confirmatory anxiety-related measures assessed using the light–dark test (LDT).

(a) Zone-specific time spent in the light (L) and dark (D) compartments across control males (CM), control females (CF), test isolated males (TM), and test isolated females (TF), presented in this order. Data are shown as bar plots with all individual data points overlaid, median values, and 95% confidence intervals. Within-group comparisons between L and D zones were performed using the Wilcoxon signed-rank test and are indicated by hashtags (#), while between-group comparisons were conducted using the Mann–Whitney U test and are denoted by asterisks (*). All p-values were Holm corrected. Control groups showed a preference for the dark zone independent of sex, whereas test isolated groups showed increased light zone occupancy, indicating an anxiolytic-like behavioural shift under isolation.

(b) Latency to first entry into the light zone, presented as violin plots for CM, TM, CF, and TF. Between-group comparisons were conducted using the Mann–Whitney U test, and only Holm-corrected significant comparisons are shown. Both test isolated males and test isolated females exhibited reduced latency to enter the light zone compared to their respective control groups, consistent with anxiolytic-like and hyperactivity-associated behavioural responses.

(c) Spearman’s rank correlation analysis between time spent in the light zone and latency to first light-zone entry, demonstrating a negative association between these two measures.

All statistical analyses including generation of figures were conducted using GraphPad Prism (version 9).

Significance thresholds: $p < 0.05$ (), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

Table No. 4.1

Descriptive and Inferential Statistics of Open Field Test (OFT) Comparisons (Comp.) using the Mann-Whitney U test between Independent Groups.

Parameter	Comp.	Median1 95% CI	Median2 95% CI	U	Sum of Ranks	p-Value	Holm corrected p
Time Spent- Thigmotaxis	CM vs TM	50.50 [41.41, 62.66]	105.1 [82.35, 140.4]	84	294, 526	0.0013 **	0.0039**
	CF vs TF	262.7 [244.2, 283.3]	177.8 [111.2, 216.5]	96	514, 306	0.0043 **	0.0086**
	CM vs CF	50.50 [41.41, 62.66]	262.7 [244.2, 283.3]	0	210, 610	<0.0001 ****	<0.0001****
	TM vs TF	105.1 [82.35, 140.4]	177.8 [111.2, 216.5]	150	360, 460	0.1826 ns	0.1826 ns
Time Spent- Exploratory	CM vs TM	309.5 [297.4, 318.6]	254.9 [219.6, 277.7]	84	526,294	0.0013 **	0.0039**
	CF vs TF	97.35 [76.75, 115.8]	182.3 [143.5, 248.8]	96	306, 514	0.0043 **	0.0086**
	CM vs CF	309.5 [297.4, 318.6]	97.35 [76.75, 115.8]	0	610,210	<0.0001 ****	<0.0001****
	TM vs TF	254.9 [219.6, 277.7]	182.3 [143.5, 248.8]	150	460, 360	0.1826 ns	0.1826 ns
Distance - Thigmotaxis	CM vs TM	13.52 [10.82, 16.57]	25.79 [21.04, 36.40]	84	294, 526	0.0013 **	0.0038 **
	CF vs TF	71.71 [65.96, 75.80]	45.78 [29.55, 57.84]	102	508, 312	0.0073 **	0.0146*
	CM vs CF	13.52 [10.82, 16.57]	71.71 [65.96, 75.80]	0	210, 610	<0.0001 ****	<0.0001****
	TM vs TF	25.79 [21.04, 36.40]	45.78 [29.55, 57.84]	152	362, 458	0.2012 ns	0.2012 ns
Distance - Exploratory	CM vs TM	86.48 [83.43, 89.18]	74.22 [63.60, 78.96]	84	526, 294	0.0013 **	0.0038 **
	CF vs TF	28.30 [24.20, 34.04]	54.23 [42.16, 70.45]	102	312, 508	0.0073 **	0.0146*
	CM vs CF	86.48 [83.43, 89.18]	28.30 [24.20, 34.04]	0	610, 210	<0.0001 ****	<0.0001****
	TM vs TF	74.22 [63.60, 78.96]	54.23 [42.16, 70.45]	152	458, 362	0.2012 ns	0.2012 ns

Parameter	Comp.	Median1 95% CI	Median2 95% CI	U	Sum of Ranks	p-Value	Holm corrected p
Entries- Thigmotaxis	CM vs TM	40.00 [31.47, 44.23]	100.5 [93.61, 151.6]	0	210, 610	<0.0001 ****	<0.0001****
	CF vs TF	45.00 [33.33, 50.37]	99.50 [81.04, 138.3]	58	268, 552	<0.0001 ****	0.0003***
	CM vs CF	40.00 [31.47, 44.23]	45.00 [33.33, 50.37]	172.5	382.5, 437.5	0.4649 ns	0.9296 ns
	TM vs TF	100.5 [93.61, 151.6]	99.50 [81.04, 138.3]	176.5	433.5, 386.5	0.5335 ns	0.9296 ns
Entries- Exploratory	CM vs TM	39.50 [31.55, 44.15]	100.0 [93.54, 151.4]	0	210, 610	<0.0001 ****	<0.0001****
	CF vs TF	44.50 [33.26, 49.94]	99.50 [80.84, 137.9]	56.50	266.5, 553.5	<0.0001 ****	0.0003***
	CM vs CF	39.50 [31.55, 44.15]	44.50 [33.26, 49.94]	176	386, 434	0.5245 ns	1.0000 ns
	TM vs TF	100.0 [93.54, 151.4]	99.50 [80.84, 137.9]	176.5	433.5, 386.5	0.5335 ns	1.0000 ns
Rapid Movements	CM vs TM	0.000 [0.1101, 0.8029]	10.18 [8.865, 36.31]	21	231, 589	<0.0001 ****	<0.0001****
	CF vs TF	0.6400 [0.4166, 1.013]	48.20 [33.64, 105.4]	20	230, 590	<0.0001 ****	<0.0001****
	CM vs CF	0.000 [0.1101, 0.8029]	0.6400 [0.4166, 1.013]	131.5	341.5, 478.5	0.0573 ns	1.0000 ns
	TM vs TF	10.18 [8.865, 36.31]	48.20 [33.64, 105.4]	129	339, 481	0.0550 ns	1.0000 ns
Total Zone Transitions	CM vs TM	79.50 [63.02, 88.38]	200.5 [187.1, 303.0]	0	210, 610	<0.0001 ****	<0.0001****
	CF vs TF	89.50 [66.59, 100.3]	199.0 [161.9, 276.1]	57	267, 553	<0.0001 ****	0.0003***
	CM vs CF	79.50 [63.02, 88.38]	89.50 [66.59, 100.3]	175	385, 435	0.5073 ns	1.0000 ns
	TM vs TF	200.5 [187.1, 303.0]	199.0 [161.9, 276.1]	176.5	433.5, 386.5	0.5336 ns	1.0000 ns

Parameter	Comp.	Median1 95% CI	Median2 95% CI	U	Sum of Ranks	p-Value	Holm corrected p
Total Distance Travelled	CM vs TM	1328 [1129, 1487]	2894 [2596, 3257]	4	214, 606	<0.0001 ****	<0.0001****
	CF vs TF	2096 [2044, 2314]	3367 [3059, 3975]	21	231, 589	<0.0001 ****	<0.0001****
	CM vs CF	1328 [1129, 1487]	2096 [2044, 2314]	14	224, 596	<0.0001 ****	<0.0001****
	TM vs TF	2894 [2596, 3257]	3367 [3059, 3975]	128	338, 482	0.0524 ns	0.0524 ns
Speed Range	CM vs TM	15.42 [12.41, 21.34]	22.35 [20.18, 26.35]	96	306, 514	0.0042 **	0.0126*
	CF vs TF	16.89 [16.08, 20.53]	24.47 [21.68, 26.86]	91	301, 519	0.0026 **	0.0104*
	CM vs CF	15.42 [12.41, 21.34]	16.89 [16.08, 20.53]	145.5	355.5, 464.5	0.1435 ns	0.2870 ns
	TM vs TF	22.35 [20.18, 26.35]	24.47 [21.68, 26.86]	167	377, 443	0.3797 ns	0.3797 ns
Activity Index (Proportion of swim time)	CM vs TM	0.9920 [0.9793, 0.9931]	0.9713 [0.8987, 0.9748]	103.5	506.5, 313.5	0.0082 **	0.0246*
	CF vs TF	0.9962 [0.9767, 0.9970]	0.9462 [0.7219, 0.9281]	90	520, 300	0.0023 **	0.0092**
	CM vs CF	0.9920 [0.9793, 0.9931]	0.9962 [0.9767, 0.9970]	140	350, 470	0.1065 ns	0.2130 ns
	TM vs TF	0.9713 [0.8987, 0.9748]	0.9462 [0.7219, 0.9281]	165	445, 375	0.3511 ns	0.3511 ns
Activity Balance Index (ABI)	CM vs TM	14.82 [11.75, 21.15]	23.80 [21.53, 28.81]	85	295, 525	0.0014 **	0.0042**
	CF vs TF	17.03 [16.24, 20.75]	29.96 [25.43, 41.23]	72	282, 538	0.0003 ***	0.0012**
	CM vs CF	14.82 [11.75, 21.15]	17.03 [16.24, 20.75]	135	345, 475	0.0810 ns	0.1620 ns
	TM vs TF	23.80 [21.53, 28.81]	29.96 [25.43, 41.23]	140	350, 470	0.1081 ns	0.1620 ns

PCA Axis	Comp.	Median1 95% CI	Median2 95% CI	U	Sum of Ranks	p-Value	Holm corrected p	ES
PC1	CM vs TM	-1.745 [-1.890, -1.579]	-0.5243 [-0.9539, -0.07145]	40	250, 570	<0.0001 ****	<0.0001 ****	0.68/L
	CF vs TF	1.773 [1.479, 1.894]	0.5572 [-0.4715, 1.019]	88	522, 298	0.0019 **	0.005**	0.48/M
	CM vs CF	-1.745 [-1.890, -1.579]	1.773 [1.479, 1.894]	0	210, 610	<0.0001 ****	<0.0001 ****	0.85/L
	TM vs TF	-0.5243 [-0.9539, -0.07145]	0.5572 [-0.4715, 1.019]	142	352, 468	0.1190	0.1190 ns	0.25/S
PC2	CM vs TM	-0.3232 [-0.4331, -0.2984]	0.5194 [0.3136, 1.090]	10	220, 600	<0.0001 ****	<0.0001 ****	0.81/L
	CF vs TF	-1.302 [-1.371, -1.125]	0.3534 [0.2546, 1.569]	4	214, 606	<0.0001 ****	<0.0001 ****	0.84/L
	CM vs CF	-0.3232 [-0.4331, -0.2984]	-1.302 [-1.371, -1.125]	0	610, 210	<0.0001 ****	<0.0001 ****	0.85/L
	TM vs TF	0.5194 [0.3136, 1.090]	0.3534 [0.2546, 1.569]	199	409, 411	0.9893 ns	0.9893 ns	0.002/S

Table No. 4.1 Description: The table summarises descriptive and inferential statistics of 12 behavioural parameters and the PC1 and PC2 axes derived from the open field test (OFT), analysed using the Mann–Whitney U test. Data are expressed as medians with 95% confidence intervals, with the lower 95% CI reported first followed by the upper 95% CI. Median 1 and Median 2 represent the order of comparison; for example, in CM vs TM comparison, CM represents Group 1 and TM represents Group 2. Both pairwise p values and Holm-corrected p values are reported, with $m = 4$, and the Holm-corrected p values are indicated in the corresponding figures. For PC1 and PC2, effect sizes (r) are reported in the table. Effect sizes (ES) were calculated from Z values and interpreted as S (small, ≈ 0.1), M (medium, ≈ 0.3), and L (large, ≥ 0.5).

Table No. 4.2

Inferential Statistics of Open Field Test (OFT) Comparisons using the Wilcoxon Signed-Rank test on Zone-Specific Parameters.

Parameter	Comp.	Sum of Signed Ranks	Sum of Positive & Negative Ranks	p-Value	Holm corrected p	ES
Exploratory vs. Thigmotaxis Time Spent	CM T vs CM E	-210.0	0.000, -210.0	<0.0001****	<0.0001****	1.07/L
	TM T vs TM E	-194.0	8.000, -202.0	<0.0001****	<0.0001****	0.91/L
	CF T vs CF E	210.0	210.0, 0.000	<0.0001****	<0.0001****	1.07/L
	TF T vs TF E	-26.00	92.00, -118.0	0.6477 ns	0.6477 ns	0.10/S
Exploratory vs. Thigmotaxis Distance Travelled	CM T vs CM E	-210.0	0.000, -210.0	<0.0001****	<0.0001****	1.07/L
	TM T vs TM E	-188.0	11.00, -199.0	0.0001***	0.0002***	0.87/L
	CF T vs CF E	206.0	208.0, -2.000	<0.0001****	<0.0001****	1.01/L
	TF T vs TF E	-56.00	77.00, -133.0	0.3118 ns	0.3118 ns	0.23/S
Exploratory vs. Thigmotaxis Entries	CM T vs CM E	0.000	18.00, -18.00	>0.9999 ns	1.0000 ns	0.002/ S
	TM T vs TM E	15.00	30.00, -15.00	0.5078 ns	1.0000 ns	0.31/M
	CF T vs CF E	30.00	48.00, -18.00	0.2266 ns	0.6798 ns	0.44/M
	TF T vs TF E	27.00	31.50, -4.500	0.0703 ns	0.2812 ns	0.72/L

Table No. 4.2 Description: The table summarises the inferential statistics of zone-wise comparisons conducted within groups using the Wilcoxon signed-rank test (GraphPad Prism, version 9). All differences were calculated as thigmotaxis minus exploratory values. The corresponding positive and negative sums of ranks are reported. The W value was considered as the sum of signed ranks and is indicated with sign as per the Prism reporting style. Both pairwise p values and Holm-corrected p values are reported, with $m = 4$, and the Holm-corrected p values are indicated in the corresponding figures. Effect sizes (ES) were calculated from Z values and interpreted as S (small, ≈ 0.1), M (medium, ≈ 0.3), and L (large, ≥ 0.5).

Table No. 4.3

Descriptive and Inferential Statistics of Light and Dark Test (LDT) Comparisons using the Mann-Whitney U test between Independent Groups.

Parameter	Comp.	Median1 95% CI	Median2 95% CI	U	Sum of Ranks	p-Value	Holm corrected p
Time in Light Zone (min)	CM vs TM	0.7800 [0.7163, 1.774]	3.455 [3.150, 4.072]	25.50	235.5, 584.5	<0.0001 ****	<0.0001****
	CF vs TF	1.045 [0.6794, 1.330]	3.505 [3.208, 4.026]	11	221, 599	<0.0001 ****	<0.0001****
	CM vs CF	0.7800 [0.7163, 1.774]	1.045 [0.6794, 1.330]	187	423, 397	0.7330 ns	1.0000ns
	TM vs TF	3.455 [3.150, 4.072]	3.505 [3.208, 4.026]	180	390, 430	0.5969 ns	1.0000ns
Time in Dark Zone (min)	CM vs TM	5.220 [4.226, 5.284]	2.545 [1.927, 2.849]	25.50	584.5, 235.5	<0.0001 ****	<0.0001****
	CF vs TF	4.955 [4.670, 5.321]	2.495 [1.974, 2.792]	11	599, 221	<0.0001 ****	<0.0001****
	CM vs CF	5.220 [4.226, 5.284]	4.955 [4.670, 5.321]	187	397, 423	0.7330 ns	1.0000ns
	TM vs TF	2.545 [1.927, 2.849]	2.495 [1.974, 2.792]	181	429, 391	0.6156 ns	1.0000ns
Latency to First Entry-Light Zone (min)	CM vs TM	1.055 [1.024, 1.781]	1.000 [1.000, 1.040]	110	500, 320	0.0077 **	0.0308*
	CF vs TF	1.035 [1.112, 1.994]	1.005 [1.008, 1.041]	105.5	504.5, 315.5	0.0084 **	0.0308*
	CM vs CF	1.055 [1.024, 1.781]	1.035 [1.112, 1.994]	177.5	387.5, 432.5	0.5476 ns	0.6132ns
	TM vs TF	1.000 [1.000, 1.040]	1.005 [1.008, 1.041]	166	376, 444	0.3066 ns	0.6132ns

Table No. 4.3 Description: The table summarises descriptive and inferential statistics of 3 behavioural parameters from the Light and Dark test (LDT), analysed using the Mann–Whitney U test. Data are expressed as medians with 95% confidence intervals, with the lower 95% CI reported first followed by the upper 95% CI. Median 1 and Median 2 corresponding to the order of groups compared. Both pairwise p values and Holm-corrected p values are reported, with $m = 4$, and the Holm-corrected p values are indicated in the corresponding figures.

Table No. 4.4

Inferential Statistics of Light and Dark Test (LDT) Comparisons using the Wilcoxon-Signed Rank test on Zone-Specific Parameters.

Parameter	Comparison	Sum of Signed Ranks	Sum of Positive & Negative Ranks	p-Value	Holm corrected p	Effect Size
Light vs. Dark Zone Time Spent	CM L vs CM D	-196.0	7.000, -203.0	<0.0001****	0.0001***	0.92/Large
	TM L vs TM D	128.0	159.0, -31.00	0.0079**	0.0079**	0.59/Large
	CF L vs CF D	-210.0	0.000, -210.0	<0.0001****	0.0002***	0.87/Large
	TF L vs TF D	154.0	172.0, -18.00	0.0009***	0.0018**	0.76/Large

Table No. 4.4 Description: The table summarises the inferential statistics of zone-wise comparison conducted within groups using the Wilcoxon signed-rank test (GraphPad Prism, version 9). All differences were calculated as light zone minus dark zone values. The corresponding positive and negative sums of ranks are reported. The W value was considered as the sum of signed ranks and is indicated with sign as per the Prism reporting style. Both pairwise p values and Holm-corrected p values are reported, with $m = 4$, and the Holm-corrected p values are indicated in the corresponding figures. Effect sizes were calculated based on the Z values obtained from online statistical tools.

4.2 Objective 2: Results

4.2.1 Objective 2 Statement:

This section presents a comprehensive, multi-layered statistical analysis addressing Objective 2, which examines the effects of chronic social isolation in male and female zebrafish across different social contexts and buffering conditions.

The section is categorised into five analytical components based on the statistical comparisons conducted. The first examines within-group responses of CM, TM, CF, and TF across four experimental conditions. The second component addresses between-group comparisons. The third component introduces the dual-stimulus condition involving predator and shoal (PS), followed by stress-buffering

comparisons between predator only (PO) and PS. The fourth component examines the mirror and shoal (MS) condition and subsequent aggression-buffering comparisons between mirror only (MO) and MS. The fifth component analyses social affinity shifts based on the social preference index (SPI) across shoal only (SO), PS, and MS.

The entire analysis is structured to capture the interaction effects of social isolation, multiple stimuli, and sex using non-parametric statistical approaches.

The initial result section presents the raw behavioural data for each experimental group, including time spent across three zones in the three chambered tank test, entries and speed at the contact (stimulus) zone, freezing duration, and rapid movements. The subsequent section analyses the same dataset with altered comparison direction (between-group orientation), and the data are additionally represented through heatmaps for better visualisation.

Due to the large number of multiple comparisons required, each analysis was carefully conducted with appropriate correction procedures applied, and effect sizes were reported to minimise the risk of false-positive findings.

4.2.2 Behavioural Profiling of Experimental Groups Across Multiple Stimulus: A Within Group Analysis

4.2.2.1 Control Male (CM)

CM exhibited stimulus-specific responses across contact, internal and distant zones. In the contact zone, using NS as baseline, CM showed significantly higher engagement with the shoal zone in SO, a marked reduction in time spent near the predator zone in PO, and a significant response to the mirror zone in MO indicating heightened activity near the mirror, ensuring the successful stimulus-specific response in contact zone time. SO exhibited higher time followed by MO, then NS and PO.

Relative to NS, SO showed significant reduction in the internal time spent, while other comparisons with PO and MO were non-significant. SO was observed with lower time spent among all stimuli and reported significant differences with PO and MO. PO and MO also showed non-significant time differences.

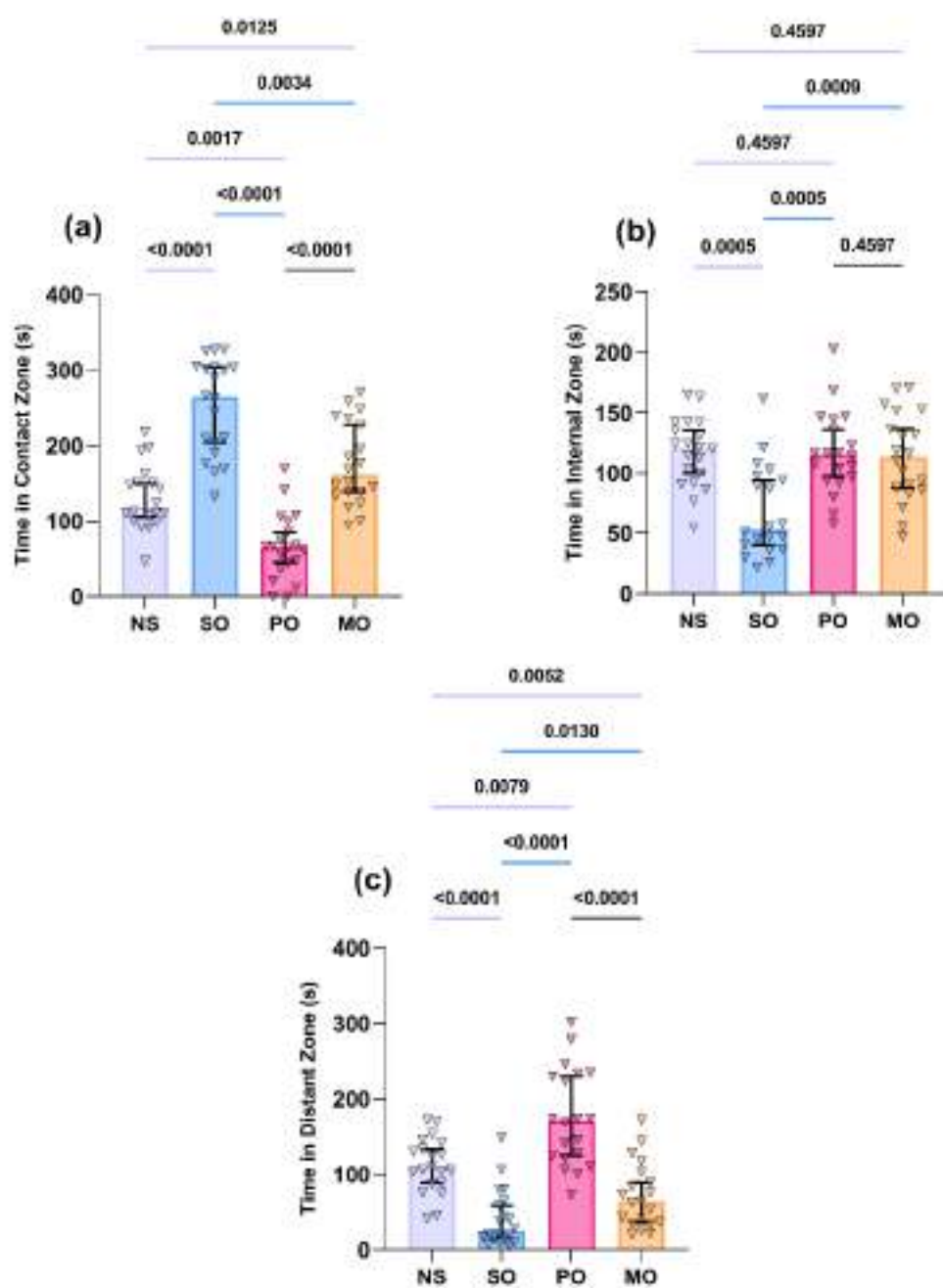
Highest distant zone time spent was reported with PO and smallest with SO relative to all other stimuli. Relative to NS, MO showed significant decrease in the distant zone time.

Time allocation heatmap analysis across the three zones revealed clear zone preferences associated with different stimuli. NS showed roughly equal time across all zones, ruling out any side preference bias in the experimental chamber. SO exhibited a clear preference for the shoal zone over the internal and distant zones, reflecting the innate social drive of zebrafish. PO showed avoidance of the predator zone and distributed time similarly between internal and distant zones. Interestingly, CM spent more time near the mirror and internal zones than the distant zone, indicating that the mirror reflection may have posed a challenging stimulus but produced an intermediate response in CM.

Analysis of motor activity metrics showed that the number of entries to the contact zone was higher in NS than all other stimuli, and comparisons between SO, PO and MO showed non-significant differences. Higher speed was observed at the contact zone in NS and PO and lower speed was observed in SO and MO. The higher and lower speed were also statistically significant. The increase in PO probably indicated the influence of stress induced by the predator and a panic-driven escape locomotion rather than controlled exploration as in NS.

Freezing in NS was lower than all other stimuli, consistent with minimal stress induction. Freezing in SO and MO was higher than NS but significantly lower than PO. The higher freezing reported with PO compared to all other stimuli indicated a stress-induced fear response. Rapid movements were lower in SO and MO than NS and PO, suggesting more stimulus-oriented interaction than exploratory activity. NS and PO showed higher rapid movements, but the difference was statistically non-significant, suggesting a non-stressed exploratory activity in NS and escape-like behaviour in PO.

The results for control males are illustrated in Figure 4.7. Statistical comparisons of CM across multiple stimuli are presented in Table 4.5, and the corresponding descriptive statistics are provided in Table 4.6.



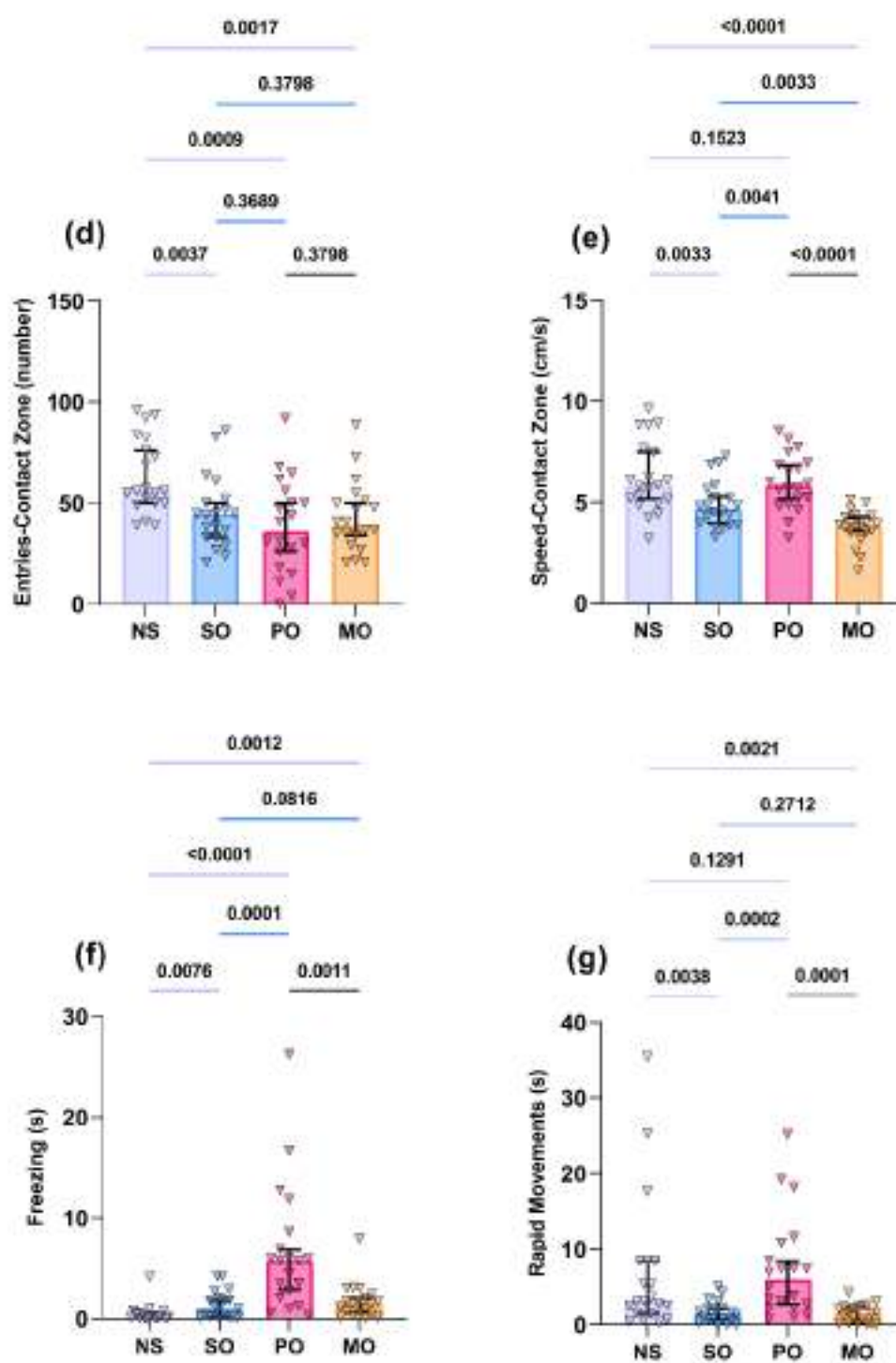


Figure 4.7. Column plots with overlaid individual data points showing seven behavioural parameters of socially housed male zebrafish (CM) across four independent stimulus conditions.

Panels (a–g) represent seven distinct behavioural metrics measured in control males under four independent stimulus conditions: NS – No stimulus (blank control), SO –

Shoal only (positive control), PO – Predator only (stressor), and MO – Mirror only (aggressor).

- (a) Time spent in the contact zone (s)
- (b) Time spent in the internal zone (s)
- (c) Time spent in the distant zone (s)
- (d) Entries to the contact zone (number)
- (e) Speed at the contact zone (cm/s)
- (f) Freezing duration (s)
- (g) Rapid movements duration (s)

Each point represents an individual fish ($n = 20$ per group). Bars indicate the median and whiskers show the 95% confidence interval. Statistical comparisons were performed using the Kruskal–Wallis test, followed by the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli to control the false discovery rate (FDR). Corrected q values are reported for all pairwise comparisons above connecting lines. Comparisons against NS are shown in pale periwinkle, and comparisons against SO are shown in bright sky blue.

4.2.2.2 Test Isolated Male (TM)

TM showed different stimulus-specific responses across three zones. Considering the contact zone, with NS as baseline, TM exhibited significant increase in the time spent near shoal zone in SO and mirror zone in MO relative to NS, while PO and NS showed comparable differences. Also, relative to MO, SO and PO showed decreased engagement in the contact zone. SO showed comparable contact zone proximity with PO.

Higher time in the internal zone was observed in NS and lower time in MO among all other stimuli. SO showed comparable internal zone preference with PO. Distant zones exhibited distinct allocation of time engagement across stimuli, where PO showed highest time and MO exhibited lowest time. NS and SO showed comparable distant zone time.

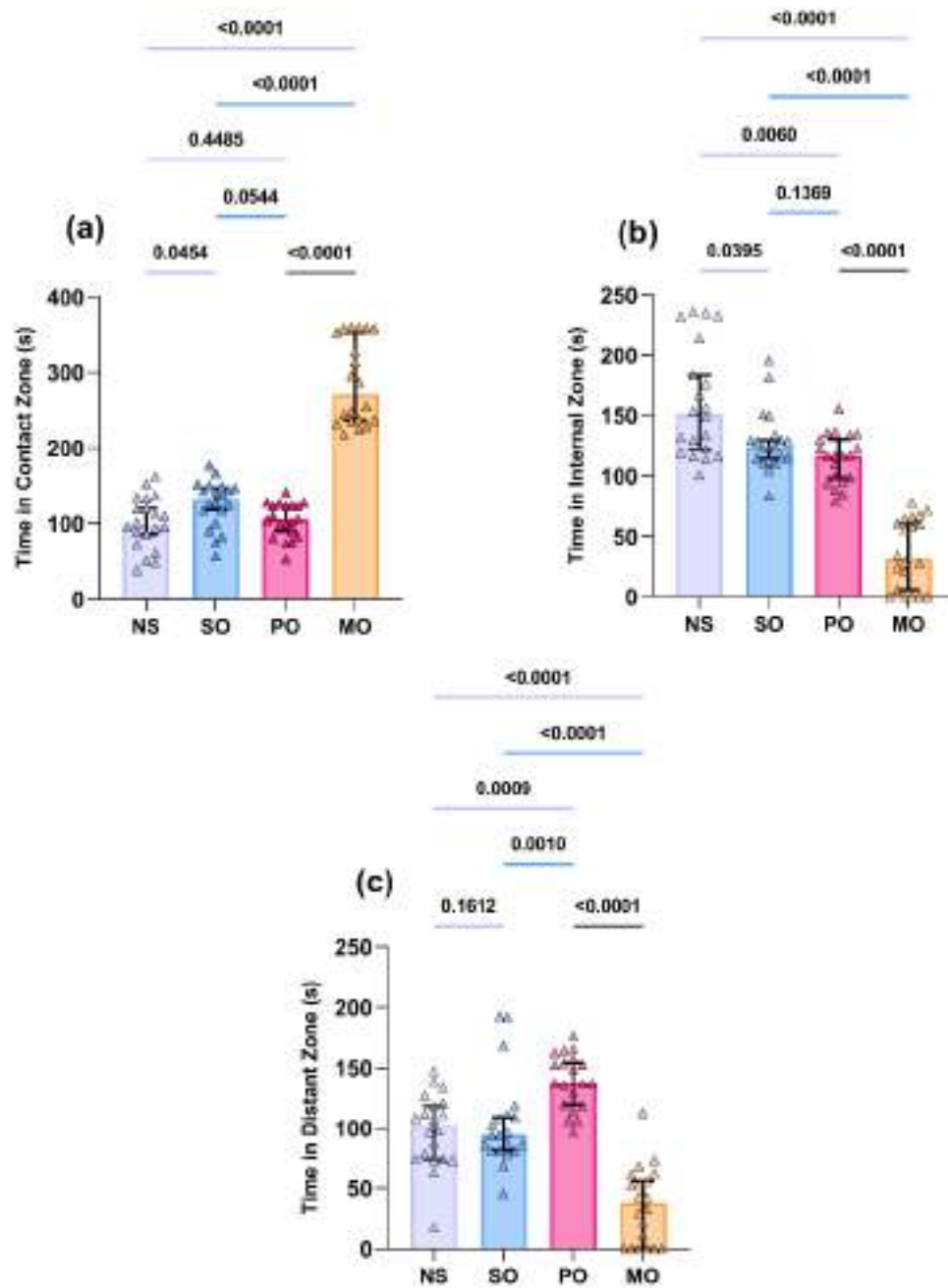
The heatmap representation of TM under NS confirmed the absence of side bias in the testing chamber but revealed an increased preference for the internal zone, reflecting heightened centre exploration, a potential indicator of moderate exploratory activity or reduced anxiety in the absence of external cues. In SO, TM allocated more

time near the shoal and internal zones compared to the distant zone, suggesting partial conservation of social attraction. In contrast, PO elicited a clear stress response in TM, with relatively high occupancy in the distant zone. A close inspection of the heatmap revealed a redistribution of time across zones (predator $\approx$ internal $<$ distant), suggesting that while stress induction was evident, the lack of a significant difference between predator and internal zones indicates that isolation might have affected the precision of spatial avoidance rather than the intensity of stress itself. In MO, the time allocation was concentrated near the mirror zone relative to the other two zones, supporting the earlier observation of a heightened mirror-induced response in TM.

Motor activity metrics in TM revealed that entries into the contact zone relative to NS were higher than in all other stimuli. In SO and PO, TM made similar numbers of entries into the shoal and predator zones. In contrast, TM made significantly fewer entries into the mirror zone compared with all other stimuli. This reduction in entries may indicate sustained engagement within the mirror zone, as reflected by the higher time allocation observed earlier. Further analysis of motor activity using speed within the contact zone showed that TM, relative to NS, SO and PO maintained similar speeds, but displayed significantly lower speed in MO. In SO and PO, the relatively higher speed may reflect transient exploration or escape-like movement rather than focused engagement within the stimulus, suggesting that isolation may have altered the motivational or attentional processing under these conditions.

Considering the salient stress-related measure, freezing behaviour, TM showed the lowest freezing durations in both NS and SO conditions, while the highest freezing was observed in PO and MO. These differences between lower and higher freezing were statistically significant. Regarding rapid movements, TM showed the highest activity in NS compared to all other stimuli, supporting greater exploratory behaviour or reduced stress under baseline conditions. SO, PO and MO showed comparable non-significant differences.

Test male results are illustrated in Figure 4.8. Statistical comparisons of TM across multiple stimuli are presented in Table 4.7, with corresponding descriptive statistics in Table 4.8.



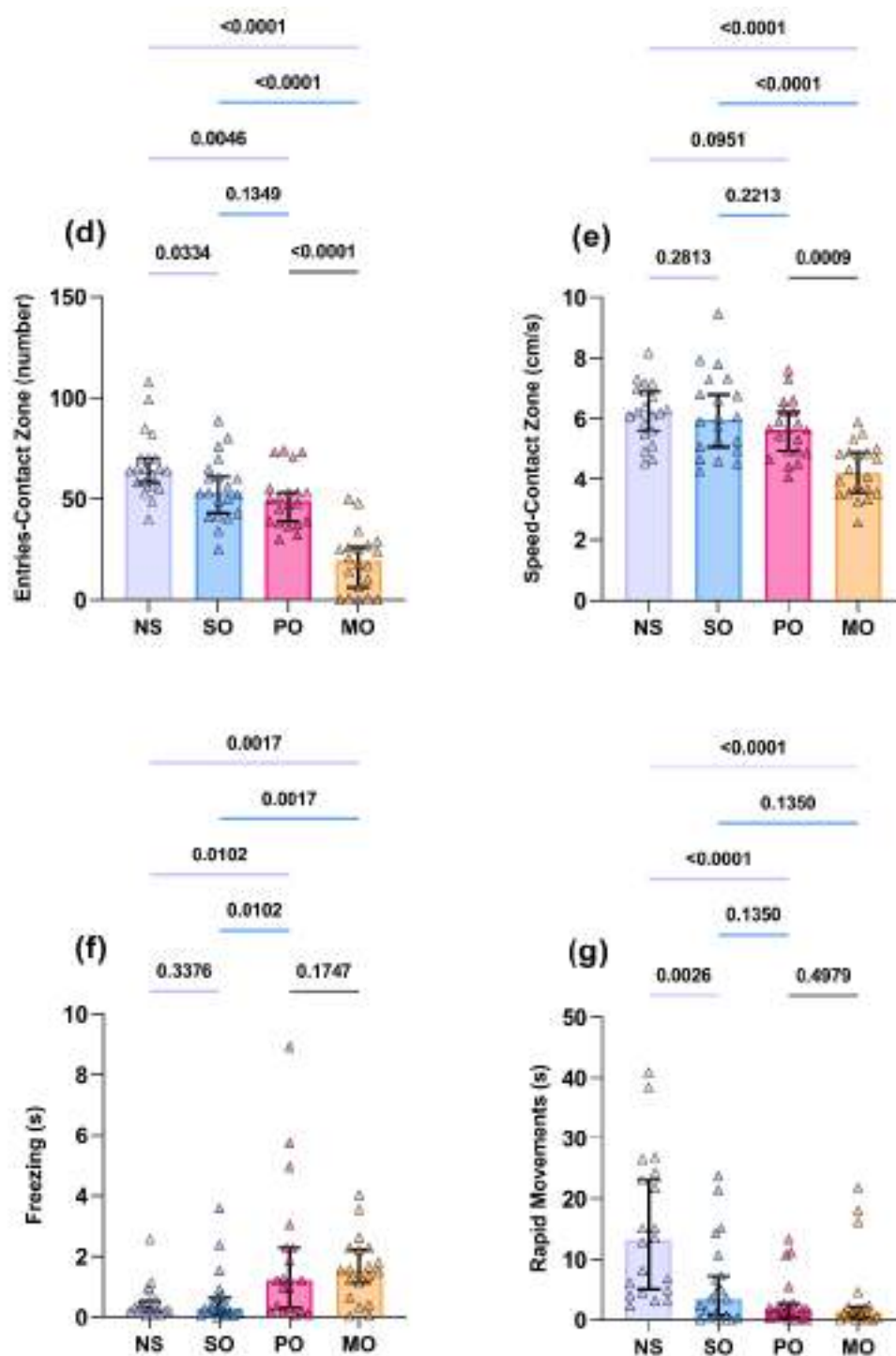


Figure 4.8. Column plots with overlaid individual data points showing seven behavioural parameters of socially isolated male zebrafish (TM) across four independent stimulus conditions.

Panels (a–g) represent the same behavioural parameters described in Figure 7, measured in test isolated males (TM) under NS, SO, PO, and MO conditions ($n = 20$ per condition). Data representation, statistical analysis, and graphical conventions are identical to those described in Figure 7.

4.2.2.3 Control Female (CF)

CF exhibited stimulus-specific responses across contact, internal and distant zones. In the contact zone, using NS as baseline, CF showed significantly higher engagement with the shoal zone in SO, a marked reduction in time spent near the predator zone in PO, which was the lowest among all stimuli, and a response to the mirror zone in MO that was statistically non-significant relative to SO, indicating heightened activity near the mirror.

Internal zone time in CF showed NS and PO as statistically non-significant and higher than SO and MO, while SO and MO were statistically non-significant and lower than NS and PO ($NS \approx PO > SO \approx MO$). These differences were statistically significant.

The distant zone in CF showed a distinct allocation pattern, with PO showing the highest time among all stimuli, especially relative to NS. SO and MO showed the lowest and statistically non-significant distant zone time, while NS remained intermediate ($PO > NS > SO \approx MO$), complementing the higher time spent in the contact zone under SO and MO conditions.

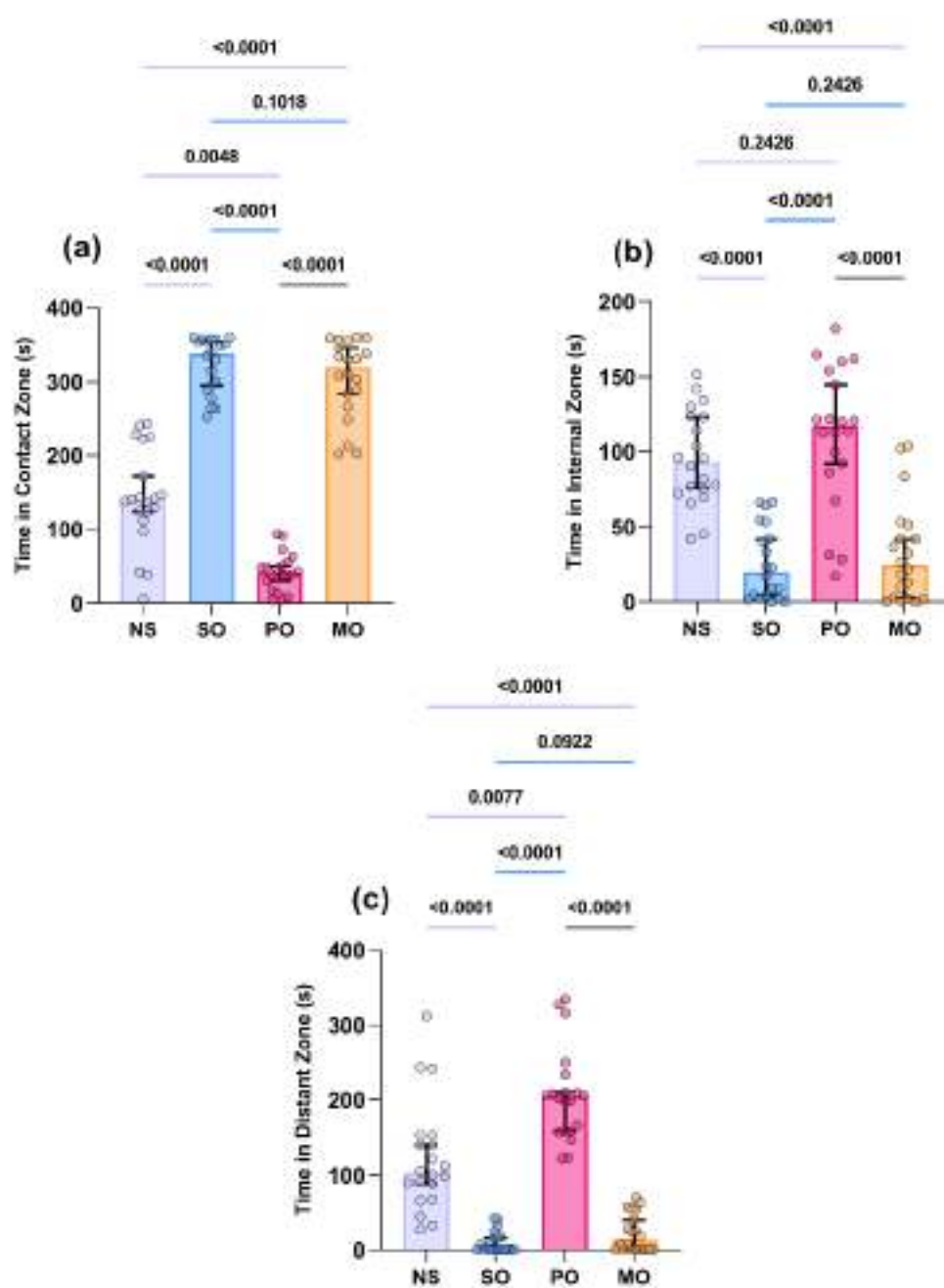
Time allocation heatmap analysis across the three zones revealed clear zone preferences associated with different stimuli. NS showed roughly equal time across all zones, ruling out any side preference bias in the experimental chamber. SO exhibited a clear preference for the shoal zone over the internal and distant zones, reflecting the innate social drive of zebrafish. PO showed a strong preference for the distant zone, completely avoiding areas near the contact zone, indicating stress-induced zone avoidance. In PO, CF exhibited a redistribution of time across zones (distant > internal > contact). Interestingly, CF spent more time near the mirror zone than the internal or distant zones, indicating that the mirror reflection may have posed a challenging stimulus and elicited heightened responses.

Analysis of motor activity metrics indicated that NS showed higher entries than all other stimuli. Comparisons involving SO, specifically with PO and MO, were statistically non-significant, although PO had higher entries than MO. Speed measured in the contact zone further revealed that NS and PO exhibited higher speeds, while SO and MO showed lower speeds. The higher entries and speed in NS suggested

non-stressed exploratory activity, whereas the higher entries and speed in PO compared to MO suggested stress-induced escape responses.

Analysis of the salient stress-related measure, the freezing behaviour, confirmed stress-induced responses in CF. PO elicited the highest freezing among all stimuli, while SO and MO also showed higher freezing than NS; however, the differences between SO and MO were statistically non-significant. Increased rapid movements were observed only in NS, supporting non-stressed, unbiased activity, while all other stimuli showed lower rapid movements relative to NS.

Control female results are illustrated in Figure 4.9. Statistical comparisons of CF across multiple stimuli are presented in Table 4.9, with corresponding descriptive statistics in Table 4.10.



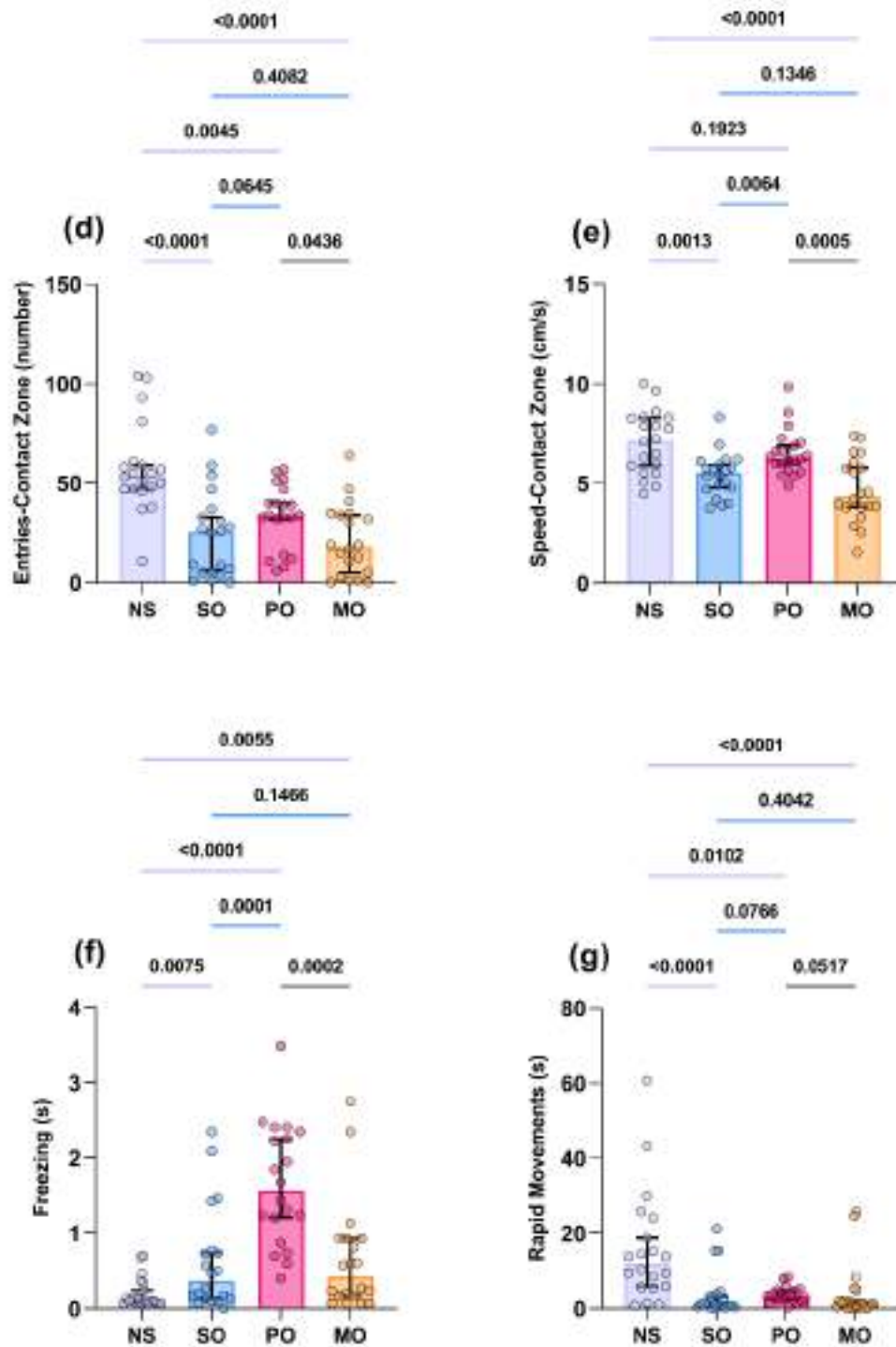


Figure 4.9. Column plots with overlaid individual data points showing seven behavioural parameters of socially housed female zebrafish (CF) across four independent stimulus conditions.

Panels (a–g) represent the same behavioural parameters described in Figure 7, measured in control females (CF) under NS, SO, PO, and MO conditions (n = 20 per condition). Data representation, statistical analysis, and graphical conventions are identical to those described in Figure 7.

4.2.2.4 Test Isolated Female (TF)

TF showed different stimulus-specific responses across the three zones. Considering the contact zone, with NS as baseline, TF exhibited a significant increase in time spent near the shoal zone in SO and the mirror zone in MO relative to NS, while PO and NS showed statistically non-significant differences. Also, SO and MO showed statistically non-significant differences, but both showed higher time than NS and PO.

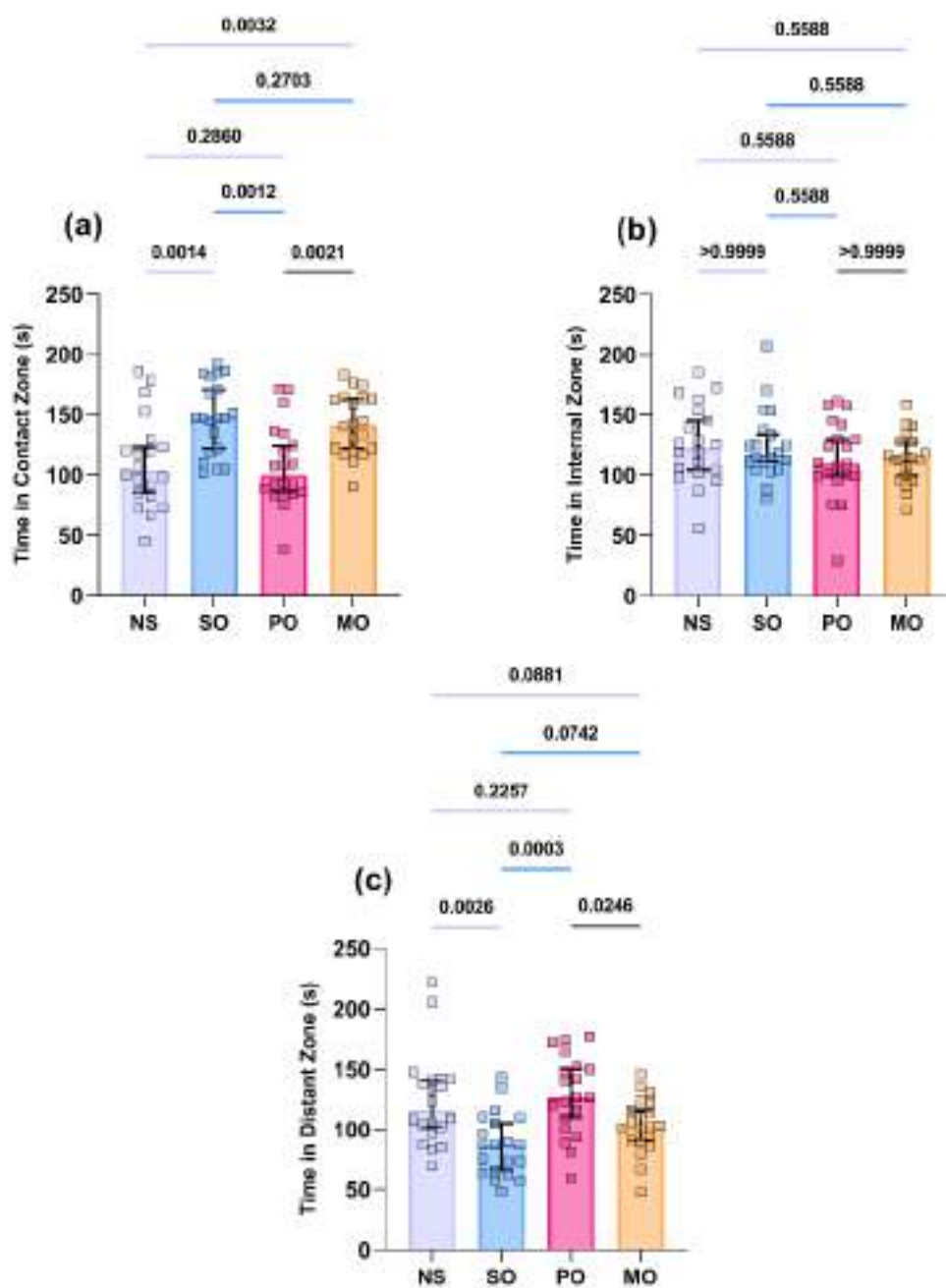
Internal zone preference was observed, with similar time spent across all stimuli.

Distant zone did not exhibit a distinct allocation of time engagement across stimuli. NS and PO showed statistically non-significant differences and higher time than SO. MO exhibited statistically non-significant differences with NS and SO, and was significantly lower than PO.

Time allocation heatmap analysis revealed NS without any zone preference, distributing time equally between the three zones, indicating absence of side bias in the experimental chamber or non-stressed exploration. In SO, the distant zone was significantly avoided, while the remaining activity time was distributed similarly between the shoal and internal zones. Interestingly, PO exhibited a time distribution pattern similar to NS, without showing any significant preference in any of the three zones. In MO, relative to the internal and distant zones, time spent in the mirror zone was higher, and between zones away from the stimulus, time distribution was comparatively similar.

Motor activity measures were also analysed. Entries to the contact zone showed no significant differences across multiple stimuli. Speed at the contact zone was significantly higher in NS than in SO and MO, while PO showed statistically non-significant speed compared to NS. Other comparisons among SO, MO, and PO showed no significant differences. Interestingly, freezing showed a similar trend across all stimuli without significant differences. Higher rapid movements were observed only in NS compared to all other stimuli.

Test female results are illustrated in Figure 4.10. Statistical comparisons across multiple stimuli are presented in Table 4.11, with corresponding descriptive statistics in Table 4.12.



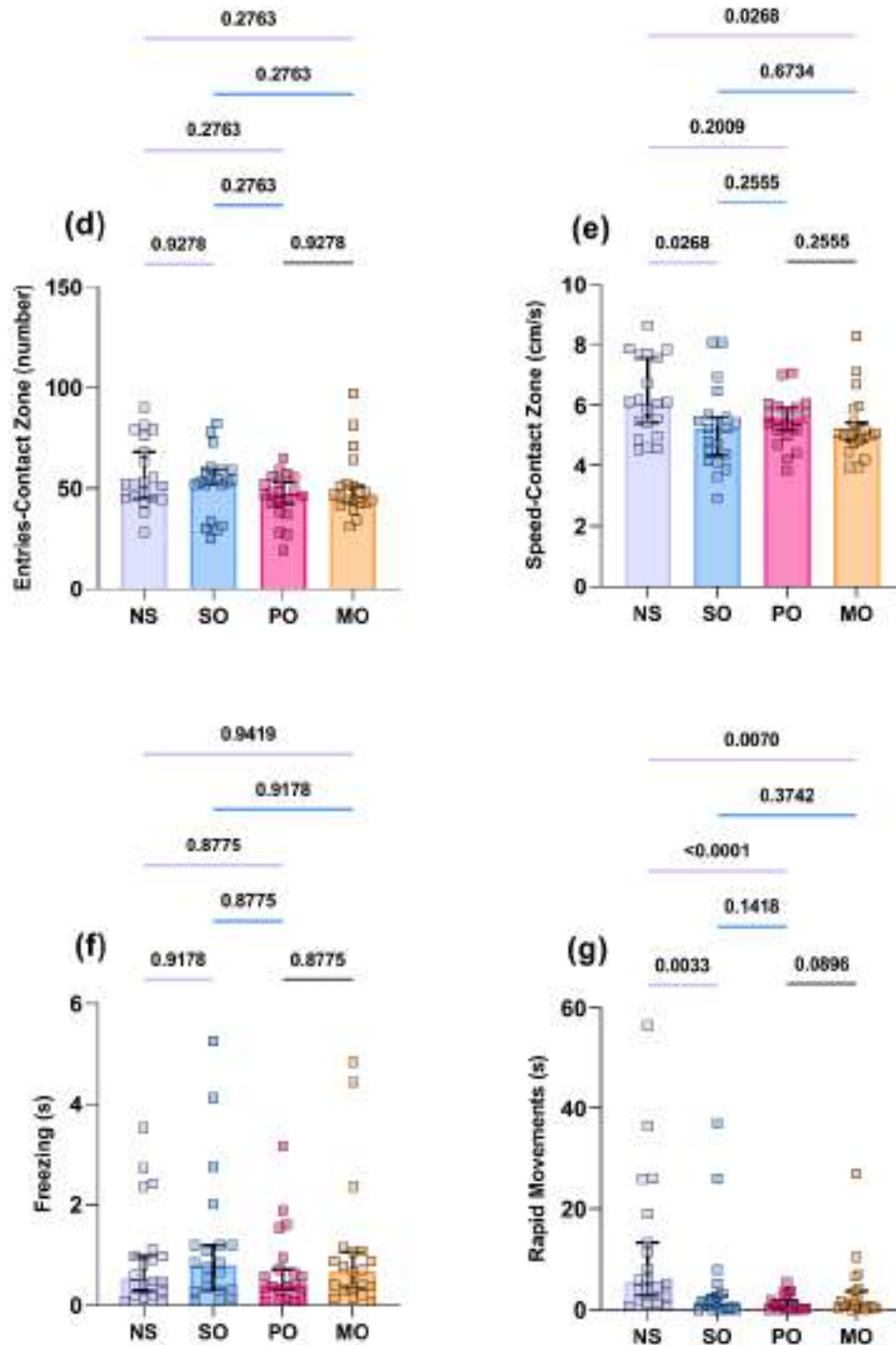


Figure 4.10. Column plots with overlaid individual data points showing seven behavioural parameters of socially isolated female zebrafish (TF) across four independent stimulus conditions.

Panels (a–g) represent the same behavioural parameters described in Figure 7, measured in test isolated females (TF) under NS, SO, PO, and MO conditions ($n = 20$ per condition). Data representation, statistical analysis, and graphical conventions are identical to those described in Figure 7.

Table 4.5. Kruskal–Wallis and FDR-Corrected Pairwise Comparisons for Behavioural Parameters in Control Males (CM)

Non-parametric comparisons were performed using the Kruskal–Wallis test. Effect size was calculated as eta-squared (η^2). Effect magnitude was interpreted using the threshold criteria ($\eta^2 = S$ (≈ 0.01), M (≈ 0.06), and L (≥ 0.14)). Post hoc comparisons were conducted using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli controlling the false discovery rate at $Q = 0.05$. A result was considered a significant discovery when $q < 0.05$.

Kruskal-Wallis				Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli:			
Parameter	H	p	η^2 & ES	Comp.	Rank Sum Diff	p	q
Time Spent in Contact Zone (s)	51.47	<0.0001	0.64 Large	NS vs SO	-30.93	<0.0001	<0.0001
				NS vs PO	20.08	0.0063	0.0017
				NS vs MO	-13.25	0.0714	0.0125
				SO vs PO	51.00	<0.0001	<0.0001
				SO vs MO	17.68	0.0162	0.0034
				PO vs MO	-33.33	<0.0001	<0.0001
Time Spent in Internal Zone (s)	19.46	0.0002	0.22 Large	NS vs SO	27.80	0.0002	0.0005
				NS vs PO	1.15	0.8756	0.4597
				NS vs MO	3.25	0.6583	0.4597
				SO vs PO	-26.65	0.0003	0.0005
				SO vs MO	-24.55	0.0008	0.0009
				PO vs MO	2.10	0.7751	0.4597
Time Spent in Distant Zone (s)	43.85	<0.0001	0.54 Large	NS vs SO	30.25	<0.0001	<0.0001
				NS vs PO	-15.28	0.0376	0.0079
				NS vs MO	17.13	0.0198	0.0052
				SO vs PO	-45.53	<0.0001	<0.0001
				SO vs MO	-13.13	0.0741	0.0130
				PO vs MO	32.40	<0.0001	<0.0001
Entries to Contact Zone (number)	16.63	0.0008	0.18 Large	NS vs SO	21.43	0.0035	0.0037
				NS vs PO	26.75	0.0003	0.0009
				NS vs MO	24.03	0.0011	0.0017
				SO vs PO	5.33	0.4685	0.3689

				SO vs MO	2.60	0.7233	0.3798
				PO vs MO	-2.73	0.7106	0.3798
Speed at Contact Zone (cm/s)	33.50	<0.0001	0.40 Large	NS vs SO	18.38	0.0124	0.0033
				NS vs PO	1.20	0.8703	0.1523
				NS vs MO	36.93	<0.0001	<0.0001
				SO vs PO	-17.18	0.0194	0.0041
				SO vs MO	18.55	0.0116	0.0033
				PO vs MO	35.73	<0.0001	<0.0001
Freezing (s)	34.45	<0.0001	0.41 Large	NS vs SO	-15.40	0.0361	0.0076
				NS vs PO	-42.55	<0.0001	<0.0001
				NS vs MO	-20.75	0.0047	0.0012
				SO vs PO	-27.15	0.0002	0.0001
				SO vs MO	-5.35	0.4665	0.0816
				PO vs MO	21.80	0.0030	0.0011
Rapid Movements (s)	23.46	<0.0001	0.27 Large	NS vs SO	19.75	0.0072	0.0038
				NS vs PO	-7.50	0.3073	0.1291
				NS vs MO	21.85	0.0029	0.0021
				SO vs PO	-27.25	0.0002	0.0002
				SO vs MO	2.10	0.7750	0.2712
				PO vs MO	29.35	<0.0001	0.0001

Table 4.6. Descriptive Statistics for Behavioural Parameters in Control Males (CM)

Median (Mdn) values with 95% confidence intervals are presented for each stimulus condition (NS, SO, PO, MO). Sample size per condition: N = 20.

Parameter	Stimulus	Mdn	95% CI Lower	95% CI Upper	Kruskal–Wallis Mean Rank
Time Spent in Contact Zone (s)	NS	118.4	111.2	150.1	34.48
	SO	264.7	221.4	280.4	65.40
	PO	65.51	46.10	87.25	14.40
	MO	161.3	149.7	201.0	47.73
Time Spent in Internal Zone (s)	NS	120.7	104.0	130.5	48.55
	SO	53.02	50.39	85.50	20.75
	PO	115.8	100.8	132.9	47.40
	MO	113.7	96.18	130.4	45.30
Time Spent in Distant Zone (s)	NS	109.0	95.06	129.2	48.53
	SO	24.82	23.36	59.00	18.28
	PO	171.6	144.5	204.8	63.80
	MO	64.21	50.91	91.41	31.40
Entries to Contact Zone (number)	NS	56.0	54.56	71.94	58.55
	SO	44.5	36.93	53.37	37.13
	PO	36.0	27.44	49.46	31.80
	MO	39.0	34.56	50.94	34.53
Speed at Contact Zone (cm/s)	NS	5.84	5.409	7.07	54.63
	SO	4.69	4.363	5.438	36.25
	PO	5.82	5.361	6.651	53.43
	MO	3.841	3.354	4.168	17.70
Freezing (s)	NS	0.23	0.1151	0.9729	20.83
	SO	1.035	0.7399	1.997	36.23
	PO	5.76	3.706	9.587	63.38
	MO	1.56	0.935	2.588	41.58
Rapid Movements (s)	NS	3.155	2.585	11.16	49.03
	SO	1.54	0.9991	2.369	29.28
	PO	5.98	4.299	10.56	56.53
	MO	1.50	0.900	2.068	27.18

Table 4.7. Kruskal–Wallis and FDR-Corrected Pairwise Comparisons for Behavioural Parameters in Test Males (TM)

Statistical analyses for test males (Tables 4.7 and 4.8) were performed using the same methods described for Table 4.5

Kruskal-Wallis				Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli:			
Parameter	H	p	η^2 & ES	Comp.	Rank Sum Diff	p	q
Time Spent in Contact Zone (s)	48.83	<0.0001	0.60 Large	NS vs SO	-13.95	0.0576	0.0454
				NS vs PO	-1.35	0.8542	0.4485
				NS vs MO	-45.10	<0.0001	<0.0001
				SO vs PO	12.60	0.0864	0.0544
				SO vs MO	-31.15	<0.0001	<0.0001
				PO vs MO	-43.75	<0.0001	<0.0001
Time Spent in Internal Zone (s)	51.08	<0.0001	0.63 Large	NS vs SO	12.30	0.0942	0.0395
				NS vs PO	18.60	0.0114	0.0060
				NS vs MO	50.30	<0.0001	<0.0001
				SO vs PO	6.30	0.3913	0.1369
				SO vs MO	38.00	<0.0001	<0.0001
				PO vs MO	31.70	<0.0001	<0.0001
Time Spent in Distant Zone (s)	47.61	<0.0001	0.59 Large	NS vs SO	-0.73	0.9214	0.1612
				NS vs PO	-21.58	0.0033	0.0009
				NS vs MO	28.80	<0.0001	<0.0001
				SO vs PO	-20.85	0.0045	0.0010
				SO vs MO	29.53	<0.0001	<0.0001
				PO vs MO	50.38	<0.0001	<0.0001
Entries to Contact Zone (number)	45.33	<0.0001	0.56 Large	NS vs SO	12.88	0.0796	0.0334
				NS vs PO	19.25	0.0088	0.0046
				NS vs MO	47.78	<0.0001	<0.0001
				SO vs PO	6.38	0.3854	0.1349
				SO vs MO	34.90	<0.0001	<0.0001
				PO vs MO	28.53	<0.0001	<0.0001
	28.51	<0.0001	0.34	NS vs SO	4.55	0.5358	0.2813

Speed at Contact Zone (cm/s)			Large	NS vs PO	11.40	0.1208	0.0951
				NS vs MO	35.95	<0.0001	<0.0001
				SO vs PO	6.85	0.3512	0.2213
				SO vs MO	31.40	<0.0001	<0.0001
				PO vs MO	24.55	0.0008	0.0009
Freezing (s)	15.98	0.0011	0.17 Large	NS vs SO	0.33	0.9647	0.3376
				NS vs PO	-17.18	0.0193	0.0102
				NS vs MO	-23.15	0.0016	0.0017
				SO vs PO	-17.50	0.0172	0.0102
				SO vs MO	-23.48	0.0014	0.0017
				PO vs MO	-5.98	0.4159	0.1747
Rapid Movements (s)	24.69	<0.0001	0.29 Large	NS vs SO	22.23	0.0025	0.0026
				NS vs PO	31.35	<0.0001	<0.0001
				NS vs MO	31.83	<0.0001	<0.0001
				SO vs PO	9.13	0.2142	0.1350
				SO vs MO	9.60	0.1913	0.1350
				PO vs MO	0.48	0.9484	0.4979

Table 4.8. Descriptive Statistics for Behavioural Parameters in Test Males (TM)

Median values with 95% confidence intervals are presented for each stimulus condition (NS, SO, PO, MO). Sample size per condition: N = 20.

Parameter	Stimulus	Mdn	95% CI Lower	95% CI Upper	Kruskal–Wallis Mean Rank
Time Spent in Contact Zone (s)	NS	98.61	84.56	117.2	25.40
	SO	132.1	111.8	141.4	39.35
	PO	105.9	93.95	115.6	26.75
	MO	271.6	260.5	312.9	70.50
Time Spent in Internal Zone (s)	NS	151.0	139.3	182.6	60.80
	SO	123.9	116.4	140.3	48.50
	PO	116.8	105.0	124.2	42.20
	MO	31.34	22.89	49.75	10.50
Time Spent in Distant Zone (s)	NS	102.5	83.74	112.6	42.13
	SO	94.37	86.34	122.3	42.85
	PO	137.3	128.1	149.4	63.70
	MO	37.94	21.21	51.23	13.33
Entries to Contact Zone (number)	NS	64.5	59.3	74.5	60.48
	SO	53.0	47.52	62.38	47.60
	PO	49.0	43.37	56.33	41.23
	MO	19.5	11.72	25.88	12.70
Speed at Contact Zone (cm/s)	NS	6.205	5.757	6.652	53.48
	SO	5.98	5.473	6.763	48.93
	PO	5.645	5.177	6.067	42.08
	MO	4.21	3.86	4.677	17.53
Freezing (s)	NS	0.285	0.2383	0.7677	30.50
	SO	0.295	0.2135	1.058	30.18
	PO	1.21	0.7981	2.936	47.68
	MO	1.545	1.091	2.084	53.65
Rapid Movements (s)	NS	13.2	9.567	20.65	61.85
	SO	3.475	2.716	9.514	39.63
	PO	1.83	1.17	4.912	30.50
	MO	1.41	0.7958	6.926	30.03

Table 4.9. Kruskal–Wallis and FDR-Corrected Pairwise Comparisons for Behavioural Parameters in Control Females (CF)

Statistical analyses for control females (Tables 4.9 and 4.10) were performed using the same methods described for Table 4.5

Kruskal-Wallis				Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli:			
Parameter	H	p	η^2 & ES	Comp.	Rank Sum Diff	p	q
Time Spent in Contact Zone (s)	62.56	<0.0001	0.78 Large	NS vs SO	-32.90	<0.0001	<0.0001
				NS vs PO	16.75	0.0226	0.0048
				NS vs MO	-28.85	<0.0001	<0.0001
				SO vs PO	49.65	<0.0001	<0.0001
				SO vs MO	4.05	0.5815	0.1018
				PO vs MO	-45.60	<0.0001	<0.0001
Time Spent in Internal Zone (s)	44.03	<0.0001	0.54 Large	NS vs SO	33.75	<0.0001	<0.0001
				NS vs PO	-4.00	0.5862	0.2426
				NS vs MO	30.85	<0.0001	<0.0001
				SO vs PO	-37.75	<0.0001	<0.0001
				SO vs MO	-2.90	0.6931	0.2426
				PO vs MO	34.85	<0.0001	<0.0001
Time Spent in Distant Zone (s)	60.46	<0.0001	0.76 Large	NS vs SO	33.40	<0.0001	<0.0001
				NS vs PO	-15.35	0.0366	0.0077
				NS vs MO	28.75	<0.0001	<0.0001
				SO vs PO	-48.75	<0.0001	<0.0001
				SO vs MO	-4.65	0.5266	0.0922
				PO vs MO	44.10	<0.0001	<0.0001
Entries to Contact Zone (number)	28.75	<0.0001	0.34 Large	NS vs SO	32.98	<0.0001	<0.0001
				NS vs PO	20.98	0.0043	0.0045
				NS vs MO	35.05	<0.0001	<0.0001
				SO vs PO	-12.00	0.1023	0.0645
				SO vs MO	2.08	0.7776	0.4082
				PO vs MO	14.08	0.0554	0.0436
	23.13	<0.0001	0.26	NS vs SO	22.80	0.0019	0.0013

Speed at Contact Zone (cm/s)			Large	NS vs PO	4.40	0.5493	0.1923
				NS vs MO	30.10	<0.0001	<0.0001
				SO vs PO	-18.40	0.0123	0.0064
				SO vs MO	7.30	0.3205	0.1346
				PO vs MO	25.70	0.0005	0.0005
Freezing (s)	33.95	<0.0001	0.41 Large	NS vs SO	-15.43	0.0355	0.0075
				NS vs PO	-42.15	<0.0001	<0.0001
				NS vs MO	-16.93	0.0210	0.0055
				SO vs PO	-26.73	0.0003	0.0001
				SO vs MO	-1.50	0.8380	0.1466
				PO vs MO	25.23	0.0006	0.0002
Rapid Movements (s)	24.62	<0.0001	0.28 Large	NS vs SO	30.38	<0.0001	<0.0001
				NS vs PO	19.00	0.0097	0.0102
				NS vs MO	32.53	<0.0001	<0.0001
				SO vs PO	-11.38	0.1216	0.0766
				SO vs MO	2.15	0.7698	0.4042
				PO vs MO	13.53	0.0657	0.0517

Table 4.10. Descriptive Statistics for Behavioural Parameters in Control Females (CF)

Median values with 95% confidence intervals are presented for each stimulus condition (NS, SO, PO, MO). Sample size per condition: N = 20.

Parameter	Stimulus	Mdn	95% CI Lower	95% CI Upper	Kruskal–Wallis Mean Rank
Time Spent in Contact Zone (s)	NS	139.3	111.5	173.9	29.25
	SO	337.7	305.7	340.9	62.15
	PO	41.32	30.34	54.31	12.50
	MO	319.8	279.8	329.7	58.10
Time Spent in Internal Zone (s)	NS	93.26	81.01	110.4	55.65
	SO	19.89	14.25	37.31	21.90
	PO	117.3	88.92	132.6	59.65
	MO	24.56	16.77	47.62	24.80
Time Spent in Distant Zone (s)	NS	103.2	87.48	155.7	52.20
	SO	2.165	4.324	17.59	18.80
	PO	204.8	178.1	235.8	67.55
	MO	13.19	11.03	34.5	23.45
Entries to Contact Zone (number)	NS	54.0	47.18	68.22	62.75
	SO	25.5	13.97	34.63	29.78
	PO	34.0	26.12	40.98	41.78
	MO	18.0	12.66	29.54	27.70
Speed at Contact Zone (cm/s)	NS	7.165	6.319	7.808	54.83
	SO	5.56	4.934	5.963	32.03
	PO	6.285	6.011	7.10	50.43
	MO	4.38	3.953	5.46	24.73
Freezing (s)	NS	0.115	0.1177	0.3043	21.88
	SO	0.37	0.3008	0.9462	37.30
	PO	1.56	1.267	2.017	64.03
	MO	0.425	0.3153	1.005	38.80
Rapid Movements (s)	NS	12.05	8.867	22.93	60.98
	SO	1.075	1.06	6.70	30.60
	PO	3.39	2.679	4.604	41.98
	MO	1.215	0.5853	7.638	28.45

Table 4.11. Kruskal–Wallis and FDR-Corrected Pairwise Comparisons for Behavioural Parameters in Test Females (TF)

Statistical analyses for test females (Tables 4.11 and 4.12) were performed using the same methods described for Table 4.5

Kruskal-Wallis				Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli:			
Parameter	H	p	η^2 & ES	Comp.	Rank Sum Diff	p	q
Time Spent in Contact Zone (s)	19.35	0.0002	0.22 Large	NS vs SO	-23.55	0.0014	0.0014
				NS vs PO	1.70	0.8170	0.2860
				NS vs MO	-20.15	0.0061	0.0032
				SO vs PO	25.25	0.0006	0.0012
				SO vs MO	3.40	0.6436	0.2703
				PO vs MO	-21.85	0.0029	0.0021
Time Spent in Internal Zone (s)	2.05	0.5632	0.01 Very Small	NS vs SO	0.90	0.9025	>0.9999
				NS vs PO	7.70	0.2947	0.5588
				NS vs MO	8.00	0.2763	0.5588
				SO vs PO	6.80	0.3548	0.5588
				SO vs MO	7.10	0.3339	0.5588
				PO vs MO	0.30	0.9674	>0.9999
Time Spent in Distant Zone (s)	18.09	0.0004	0.20 Large	NS vs SO	23.15	0.0016	0.0026
				NS vs PO	-5.80	0.4299	0.2257
				NS vs MO	10.85	0.1398	0.0881
				SO vs PO	-28.95	<0.0001	0.0003
				SO vs MO	-12.30	0.0942	0.0742
				PO vs MO	16.65	0.0235	0.0246
Entries to Contact Zone (number)	4.93	0.1771	0.02 Small	NS vs SO	1.08	0.8836	0.9278
				NS vs PO	12.90	0.0790	0.2763
				NS vs MO	11.03	0.1332	0.2763
				SO vs PO	11.83	0.1073	0.2763
				SO vs MO	9.95	0.1754	0.2763
				PO vs MO	-1.88	0.7985	0.9278
	8.50	0.0368	0.07	NS vs SO	18.65	0.0111	0.0268

Speed at Contact Zone (cm/s)			Medium	NS vs PO	10.75	0.1435	0.2009
				NS vs MO	18.30	0.0128	0.0268
				SO vs PO	-7.90	0.2823	0.2555
				SO vs MO	-0.35	0.9620	0.6734
				PO vs MO	7.55	0.3042	0.2555
Freezing (s)	1.78	0.6194	0.01	NS vs SO	-3.50	0.6337	0.9178
			Very	NS vs PO	5.95	0.4179	0.8775
			Small	NS vs MO	-0.95	0.8971	0.9419
				SO vs PO	9.45	0.1982	0.8775
				SO vs MO	2.55	0.7284	0.9178
				PO vs MO	-6.90	0.3475	0.8775
Rapid Movements (s)	19.70	0.0002	0.22	NS vs SO	22.60	0.0021	0.0033
			Large	NS vs PO	31.50	<0.0001	<0.0001
				NS vs MO	19.90	0.0067	0.0070
				SO vs PO	8.90	0.2250	0.1418
				SO vs MO	-2.70	0.7128	0.3742
				PO vs MO	-11.60	0.1138	0.0896

Table 4.12. Descriptive Statistics for Behavioural Parameters in Test Females (TF)

Median values with 95% confidence intervals are presented for each stimulus condition (NS, SO, PO, MO). Sample size per condition: N = 20.

Parameter	Stimulus	Mdn	95% CI Lower	95% CI Upper	Kruskal–Wallis Mean Rank
Time Spent in Contact Zone (s)	NS	103.3	92.83	128.4	30.00
	SO	147.3	133.4	161.6	53.55
	PO	100.2	91.95	123.8	28.30
	MO	141.2	130.0	154.0	50.15
Time Spent in Internal Zone (s)	NS	122.7	110.1	140.6	44.65
	SO	117.1	111.2	138.5	43.75
	PO	109.6	98.48	128.8	36.95
	MO	113.7	104.2	123.9	36.65
Time Spent in Distant Zone (s)	NS	116.0	106.1	142.0	47.55
	SO	87.06	74.95	99.49	24.40
	PO	126.4	112.8	143.7	53.35
	MO	102.6	92.44	114.3	36.70
Entries to Contact Zone (number)	NS	52.0	48.97	64.63	46.75
	SO	53.5	44.63	59.47	45.68
	PO	47.5	40.31	51.09	33.85
	MO	46.5	43.41	58.39	35.73
Speed at Contact Zone (cm/s)	NS	6.09	5.628	6.822	52.43
	SO	5.23	4.599	5.871	33.78
	PO	5.515	5.096	5.864	41.68
	MO	5.06	4.809	5.806	34.13
Freezing (s)	NS	0.535	0.482	1.418	40.88
	SO	0.79	0.5464	1.838	44.38
	PO	0.385	0.3442	1.077	34.93
	MO	0.675	0.4311	1.677	41.83
Rapid Movements (s)	NS	5.525	4.922	18.55	59.00
	SO	1.47	0.1821	9.148	36.40
	PO	0.385	0.4728	1.987	27.50
	MO	0.975	0.5927	6.404	39.10

4.2.3 Comparative Study - A Between-Group Analysis

4.2.3.1 Time Spent Across Three Zones: Heatmap 1 Analysis

1. No Stimulus (NS)

A significant reduction in the time spent in the contact zone was reported in both TM and TF compared to their respective controls; however, these differences did not remain significant after Holm correction, indicating only a moderate effect. Comparisons between sexes within both control and test groups showed non-significant differences, suggesting that sex did not influence contact zone proximity within NS.

A significant increase in the time spent in the internal zone was found in both TM and TF compared to their respective controls. Heightened internal zone engagement was observed in males compared to females in both control and test groups, indicating that even though isolation increased internal zone time across sexes, the innate male-biased activity was retained.

All comparisons involving time spent in the distant zone showed no significant differences within NS, indicating an unbiased side preference and non-stressed exploratory activity across sexes and test groups.

2. Shoal Only (SO)

Time spent in the shoal zone was significantly reduced in both TM and TF compared to their corresponding controls. The significantly higher social engagement noted in control females was markedly reduced in test females, consequently resulting in comparable differences between TM and TF. This suggests that isolation reduced the innate female social attraction and masked the underlying sex differences observed in the controls.

Corresponding to the pattern observed in shoal zone activity, both internal and distant zones showed higher time spent in the test groups across sexes. Control females exhibited lower time spent in both internal and distant zones, highlighting a sex difference within controls. No significant differences were observed between TM and TF.

3. Predator-Stimulus Only (PO)

A significant increase in time spent in the predator zone was observed in both TM and TF compared to their respective controls. Control females showed a reduction in predator zone activity; however, this difference did not remain significant after correction. TM and TF showed comparable predator zone time, indicating that isolation impaired stress recognition in both male and female zebrafish.

Across most group pairs, time spent in the internal and distant zones showed no significant differences, except in test females, who spent significantly less time in the distant zone compared to control females. This significant reduction in TF was observed because it contrasted with the strong stress-induced zone avoidance observed in CF. The decreased distant zone time in TF indicates a shift from fear-induced escape behaviour following prolonged isolation.

4. Mirror-Stimulus Only (MO)

Among all stimuli, the most significant level and sex interactions were reported in MO, and these differences reversed the innate sex pattern observed within controls. Time spent in the mirror zone showed an inverted pattern between test groups. TM exhibited higher and TF showed lower activity near the mirror zone than their respective controls; this resulted in reversed sex differences compared to the controls, suggesting that prolonged isolation might have modulated mirror approach behaviour in a sex-specific manner in zebrafish.

Time spent in the internal and distant zones was inversely related to time spent in the mirror zone. TM spent less time in both internal and distant zones compared to CM, complementing the higher time spent in the mirror zone. TF spent more time in both internal and distant zones compared to CF, complementing the lower time spent in the mirror zone. This resulted in sex differences in both control and test groups in the internal and distant zones, highlighted by lower TM and CF activity and higher TF and CM activity across these two zones.

The distribution of time spent across the three zones is illustrated in Figure 4.11 (Heatmap 1).

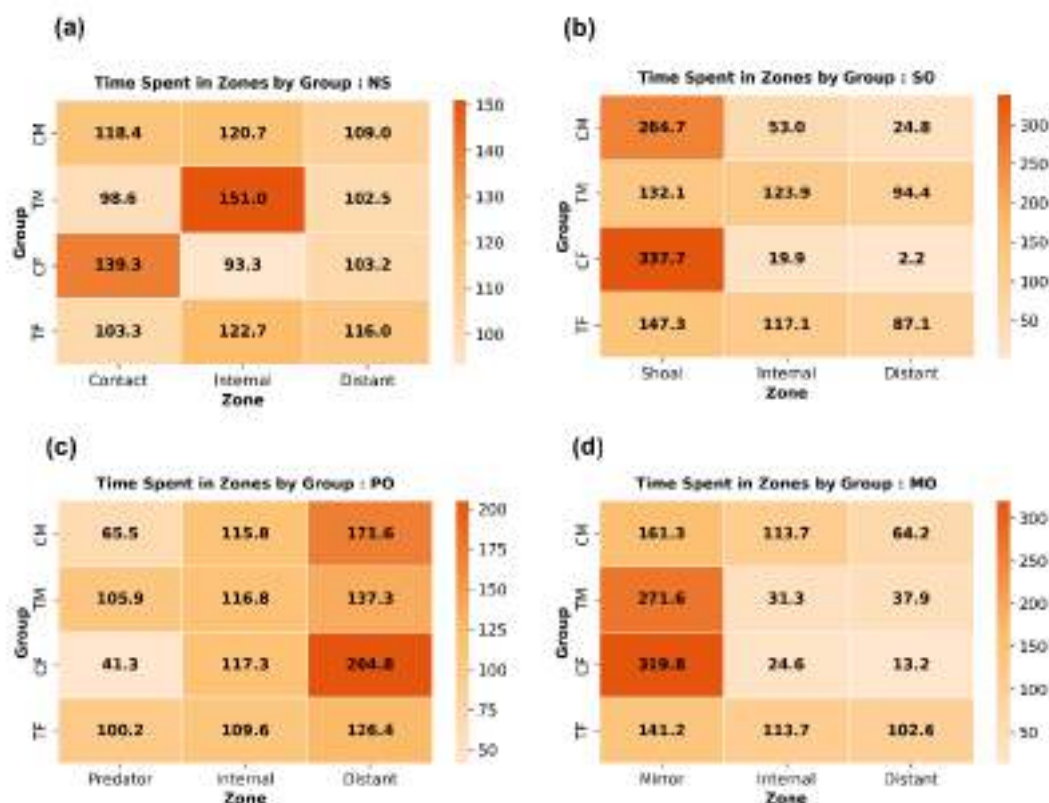


Figure 4.11. Heatmap showing median time allocation (s) of experimental groups across three zones under four stimulus conditions.

The figure consists of four heatmaps representing time spent (median values in seconds) across three zones of the three-chamber apparatus under four stimulus conditions: (a) NS – No Stimulus, (b) SO – Shoal Only, (c) PO – Predator Only, and (d) MO – Mirror Only.

In each heatmap, the X-axis (horizontal) represents the three zones specific to the stimulus condition:

- NS: Contact (stimulus zone), Internal, Distant
- SO: Shoal, Internal, Distant
- PO: Predator, Internal, Distant
- MO: Mirror, Internal, Distant

The Y-axis (vertical) represents the experimental groups: CM (Control Male), TM (Test Isolated Male), CF (Control Female), and TF (Test Isolated Female).

Each cell displays the median time (s) spent by a given group in a specific zone. Colour intensity represents magnitude of time allocation, with lighter shades indicating lower time spent and darker shades indicating higher time spent within that panel.

The heatmaps are intended to be read in two ways:

- Horizontally (left to right) to examine zone-wise allocation within a specific group.
- Vertically (top to bottom) to compare different groups within a specific zone.

Statistical analyses corresponding to these median values are presented in Tables 4.13–4.16, while between-group analysis is provided in Table 4.17.

Table 4.13. Friedman Test and Post Hoc Comparisons for Time Allocation Across Zones Under NS Stimulus

Friedman test was conducted to compare time allocation across Contact (C), Internal (I), and Distant (D) zones within each experimental group (CM, TM, CF, TF) under the No Stimulus (NS). Post hoc pairwise comparisons were performed using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. A result was considered a significant discovery when $q < 0.05$. Effect size is reported as Kendall's W. Effect size (Kendall's W) was interpreted as S (≈ 0.1), Med (≈ 0.3), and L (≥ 0.5).

Group	Friedman Statistic	p-value	Effect Size (W)	Comp.	Rank Sum Difference	Individual p-value	q-value
CM	0.7000	0.7047 ns	0.01/Very Small	C vs I	1.000	0.8744	0.9181
				C vs D	5.000	0.4292	0.8302
				I vs D	4.000	0.5271	0.8302
TM	9.700	0.0078 **	0.24 / Small	C vs I	-19.00	0.0027	0.0028
				C vs D	-5.000	0.4292	0.1502
				I vs D	14.00	0.0269	0.0141
CF	2.759	0.2516 ns	0.06/Very Small	C vs I	8.500	0.1790	0.2819
				C vs D	9.500	0.1331	0.2819
				I vs D	1.000	0.8744	0.9181
TF	1.300	0.5220 ns	0.03/Very Small	C vs I	-5.000	0.4292	0.6760
				C vs D	-7.000	0.2684	0.6760
				I vs D	-2.000	0.7518	0.7894

Table 4.14. Friedman Test and Post Hoc Comparisons for Time Allocation Across Zones Under SO Stimulus

The statistical methodology and interpretation applied are identical to those described for Table 4.13, differing only in the stimuli analysed.

Group	Friedman Statistic	p-value	Effect Size (W)	Comp.	Rank Sum Difference	Individual p-value	q-value
CM	38.10	<0.0001 ****	0.95 / Large	C vs I	21.00	0.0009	0.0014
				C vs D	39.00	<0.0001	<0.0001
				I vs D	18.00	0.0044	0.0046
TM	11.20	0.0037 **	0.28 / Small	C vs I	4.000	0.5271	0.1845
				C vs D	20.00	0.0016	0.0016
				I vs D	16.00	0.0114	0.0060
CF	39.52	<0.0001 ****	0.99 / Large	C vs I	20.50	0.0012	0.0019
				C vs D	39.50	<0.0001	<0.0001
				I vs D	19.00	0.0027	0.0028
TF	13.90	0.0010 **	0.35 / Medium	C vs I	7.000	0.2684	0.0939
				C vs D	23.00	0.0003	0.0003
				I vs D	16.00	0.0114	0.0060

Table 4.15. Friedman Test and Post Hoc Comparisons for Time Allocation Across Zones Under PO Stimulus

The statistical methodology and interpretation applied are identical to those described for Table 4.13, differing only in the stimuli analysed.

Group	Friedman Statistic	p-value	Effect Size (W)	Comp.	Rank Sum Difference	Individual p-value	q-value
CM	15.60	0.0004 ***	0.39 / Medium	C vs I	-18.00	0.0044	0.0023
				C vs D	-24.00	0.0001	0.0002
				I vs D	-6.000	0.3428	0.1200
TM	9.300	0.0096 **	0.23 / Small	C vs I	-3.000	0.6353	0.2223
				C vs D	-18.00	0.0044	0.0046
				I vs D	-15.00	0.0177	0.0093
CF	31.30	<0.0001 ****	0.78 / Large	C vs I	-22.00	0.0005	0.0008
				C vs D	-35.00	<0.0001	<0.0001
				I vs D	-13.00	0.0398	0.0418
TF	5.200	0.0743 ns	0.13 / Small	C vs I	-10.00	0.1138	0.1793
				C vs D	-14.00	0.0269	0.0846
				I vs D	-4.000	0.5271	0.5534

Table 4.16. Friedman Test and Post Hoc Comparisons for Time Allocation Across Zones Under MO Stimulus

The statistical methodology and interpretation applied are identical to those described for Table 4.13, differing only in the stimuli analysed.

Group	Friedman Statistic	p-value	Effect Size (W)	Comp.	Rank Sum Difference	Individual p-value	q-value
CM	14.70	0.0006 ***	0.37 / Medium	C vs I	9.000	0.1547	0.0542
				C vs D	24.00	0.0001	0.0002
				I vs D	15.00	0.0177	0.0093
TM	33.27	<0.0001 ****	0.83 / Large	C vs I	25.50	<0.0001	<0.0001
				C vs D	34.50	<0.0001	<0.0001
				I vs D	9.000	0.1547	0.0542
CF	33.33	<0.0001 ****	0.83 / Large	C vs I	25.00	<0.0001	<0.0001
				C vs D	35.00	<0.0001	<0.0001
				I vs D	10.00	0.1138	0.0398
TF	7.600	0.0224 *	0.19 / Small	C vs I	14.00	0.0269	0.0141
				C vs D	16.00	0.0114	0.0120
				I vs D	2.000	0.7518	0.2631

Table 4.17. Zone Allocation Across Three Zones Within Groups and Between-Group Statistical Analysis

Between-group comparisons of time spent in Contact (Shoal/Predator/Mirror), Internal, and Distant zones under each stimulus condition (NS, SO, PO, MO) were analysed using the Mann–Whitney U test. Holm correction was applied for multiple comparisons. Effect size is reported as *r*.

Stimulus	Parameter	Comparison	Sum of Ranks	U value	p value	Holm-corrected p	Effect Size (<i>r</i>)
NS	Time Spent in Contact Zone (s)	CM vs TM	488, 332	122	0.0350*	0.1400 ns	0.33 Medium
		CF vs TF	488, 332	122	0.0350*	0.1400 ns	0.33 Medium
		CM vs CF	380, 440	170	0.4291 ns	0.8582 ns	0.13 Small
		TM vs TF	388, 432	178	0.5648 ns	0.8582 ns	0.09 Small
	Time Spent in Internal Zone (s)	CM vs TM	303, 517	93	0.0032**	0.0128*	0.46 Medium
		CF vs TF	313, 507	103	0.0080**	0.0240*	0.41 Medium
		CM vs CF	490, 330	120	0.0304*	0.0310*	0.34 Medium
		TM vs TF	499, 321	111	0.0155*	0.0310*	0.38 Medium
	Time Spent in Distant Zone (s)	CM vs TM	456, 364	154	0.2211 ns	0.6633 ns	0.19 Small
		CF vs TF	384, 436	174	0.4945 ns	0.9890 ns	0.11 Small
		CM vs CF	421, 399	189	0.7788 ns	0.9890 ns	0.04 Small
		TM vs TF	341, 479	131	0.0634 ns	0.2536 ns	0.29 Small
SO	Time Spent in Shoal Zone (s)	CM vs TM	596, 224	14	<0.0001****	<0.0001****	0.79 Large
		CF vs TF	610, 210	0	<0.0001****	<0.0001****	0.85 Large
		CM vs CF	277, 543	67	0.0002***	0.0004***	0.57 Large
		TM vs TF	338, 482	128	0.0515 ns	0.0515 ns	0.31 Medium
	Time Spent in Internal Zone (s)	CM vs TM	243, 577	33	<0.0001****	<0.0001****	0.71 Large
		CF vs TF	210, 610	0	<0.0001****	<0.0001****	0.85 Large
		CM vs CF	538, 282	72	0.0003***	0.0006***	0.55 Large
		TM vs TF	434, 386	176	0.5246 ns	0.5246 ns	0.10 Small
	Time Spent in Distant Zone (s)	CM vs TM	248, 572	38	<0.0001****	<0.0001****	0.69 Large
		CF vs TF	210, 610	0	<0.0001****	<0.0001****	0.85 Large
		CM vs CF	539, 281	71	0.0003***	0.0006***	0.55 Large

		TM vs TF	461, 359	149	0.1716 ns	0.1716 ns	0.22 Small
PO	Time Spent in Predator Zone (s)	CM vs TM	291, 529	81	0.0009***	0.0027**	0.51 Large
		CF vs TF	238, 582	28	<0.0001*****	<0.0001*****	0.73 Large
		CM vs CF	484, 336	126	0.0452*	0.0904 ns	0.31 Medium
		TM vs TF	407, 413	197	0.9467 ns	0.9467 ns	0.01 Small
	Time Spent in Internal Zone (s)	CM vs TM	409, 411	199	0.9839 ns	1.0000 ns	0.002 Small
		CF vs TF	414, 406	196	0.9200 ns	1.0000 ns	0.01 Small
		CM vs CF	409, 411	199	0.9840 ns	1.0000 ns	0.002 Small
		TM vs TF	405, 415	195	0.9042 ns	1.0000 ns	0.01 Small
	Time Spent in Distant Zone (s)	CM vs TM	472, 348	138	0.0951 ns	0.2885 ns	0.26 Small
		CF vs TF	565, 255	45	<0.0001*****	0.0001****	0.66 Large
		CM vs CF	357, 463	147	0.1551 ns	0.3110 ns	0.22 Small
		TM vs TF	443, 377	167	0.3834 ns	0.3834 ns	0.14 Small
MO	Time Spent in Mirror Zone (s)	CM vs TM	253, 567	43	<0.0001*****	<0.0001*****	0.67 Large
		CF vs TF	610, 210	0	<0.0001*****	<0.0001*****	0.85 Large
		CM vs CF	231, 589	21	<0.0001*****	<0.0001*****	0.76 Large
		TM vs TF	610, 210	0	<0.0001*****	<0.0001*****	0.85 Large
	Time Spent in Internal Zone (s)	CM vs TM	592, 228	18	<0.0001*****	<0.0001*****	0.78 Large
		CF vs TF	223, 597	13	<0.0001*****	<0.0001*****	0.80 Large
		CM vs CF	587, 233	23	<0.0001*****	<0.0001*****	0.75 Large
		TM vs TF	212, 608	2	<0.0001*****	<0.0001*****	0.84 Large
	Time Spent in Distant Zone (s)	CM vs TM	506, 314	104	0.0084**	0.0084**	0.41 Medium
		CF vs TF	216, 604	6	<0.0001*****	<0.0001*****	0.83 Large
		CM vs CF	550, 270	60	0.0001***	0.0002****	0.60 Large
		TM vs TF	232, 588	22	<0.0001*****	<0.0001*****	0.76 Large

4.2.3.2 Entries & Speed at Contact Zone: Heatmap 2 Analysis

1. No Stimulus (NS)

Among all pairwise comparisons within NS on entries and speed at the contact zone, no significant differences were observed, except between TM and TF, where TM made more entries than TF; however, this was no longer significant after Holm correction.

2. Shoal Only (SO)

TM and TF made significantly more entries to the shoal zone compared to their respective controls, but the significance for TM was no longer significant after Holm correction. CM made significantly more entries to the shoal zone than CF, which can be linked to the lower time spent in the shoal zone observed in CM. TM and TF made comparable entries to the shoal zone, supporting the diminished social activity within the shoal zone. Higher speed at the shoal zone was observed in TM relative to CM. Isolated males and females initially showed a significant difference in speed, with males showing higher speed; however, this difference was no longer significant after Holm correction.

3. Predator-Stimulus Only (PO)

For both entries and speed at the predator zone, only TF showed significant differences compared to CF. TF made more entries and showed lower speed at the predator zone than CF, complementing the shift observed in the fear-induced escape behaviour in the earlier parameter analysis.

4. Mirror-Stimulus Only (MO)

Within entries to the mirror zone, all pairwise comparisons showed significant differences. Both control and isolated males and females exhibited an inverse relationship in entries: TM made lower and TF made higher entries, while CM made higher and CF made lower entries. Isolation induced a sex-specific behavioural response that was inverted relative to the control group. Interestingly, in speed at the mirror zone, only significant differences were found between isolated males and females, where TF was observed with higher speed than TM, suggesting that the difference was visible because among four experimental groups, only TF showed

lower time spent in the mirror zone, thereby exhibiting higher speed and entries, supporting more frequent exits from the mirror zone. The distribution of behavioural metrics is illustrated in Figure 4.12 (Heatmap 2).

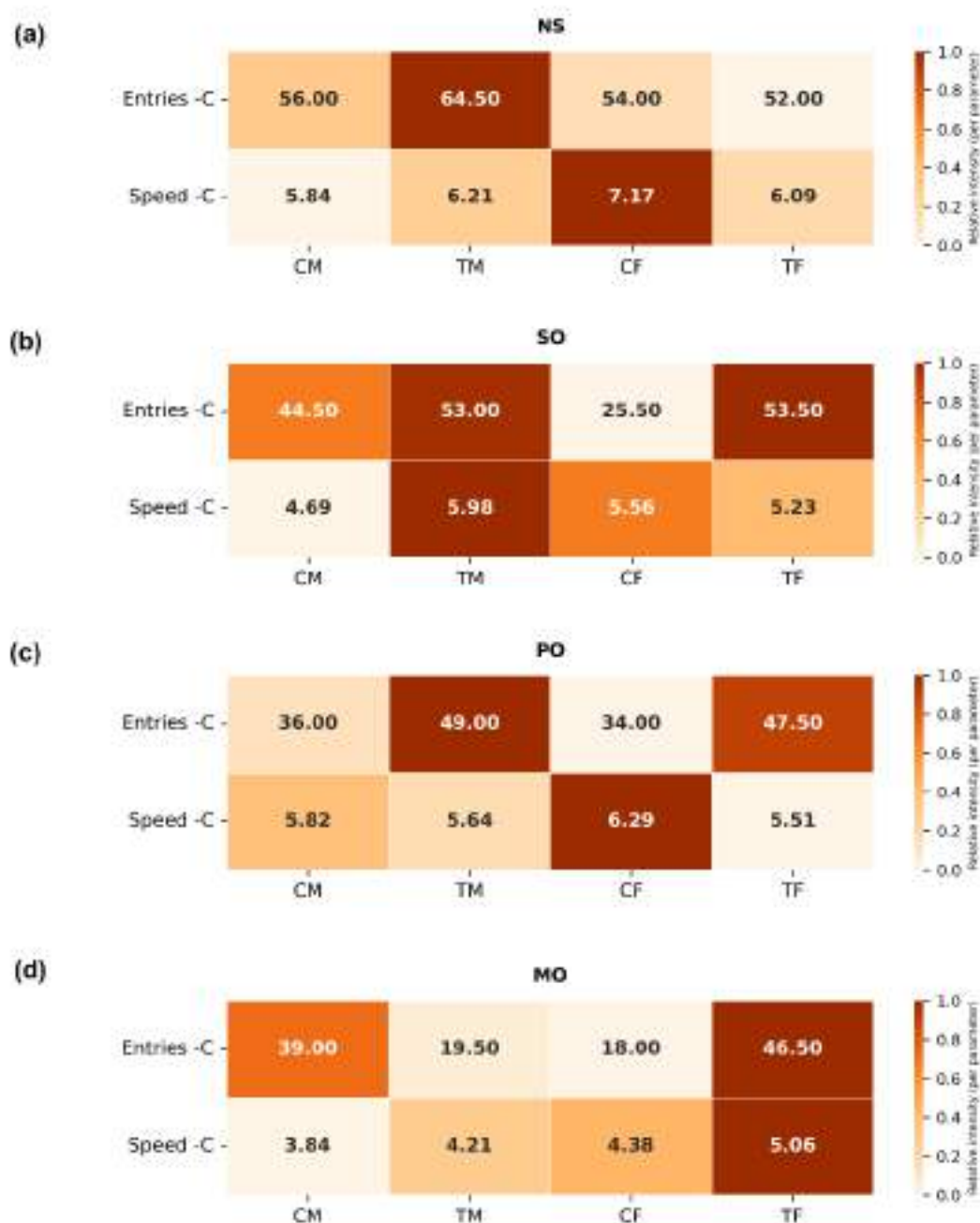


Figure 4.12. Heatmap representation of median entries to the centre and speed at the centre across groups under different stimulus conditions.

Heatmaps showing the median values of entries to the centre (number) and speed at the centre (cm/s) for four groups—control males (CM), test males (TM), control

females (CF), and test females (TF)—under each stimulus condition: NS (no stimulus), SO (shoal only), PO (predator only), and MO (mirror only).

Within each panel (a–d), groups are displayed on the X-axis in left-to-right order: CM, TM, CF, TF. The Y-axis lists the two behavioural parameters. Cell numbers represent absolute median values. The colour intensity scale bar indicates relative differences within each parameter for a given stimulus condition, with darker shades of orange representing higher relative values. This heatmap provides an alternative visual representation of the same median data previously shown in the column scatter plots.

Statistical analyses corresponding to these results are presented in Tables 4.18.

Table 4.18. Between-group statistical analysis of contact/shoal/predator/mirror zone-specific entries and speed across multiple stimuli (NS, SO, PO, MO).

Between-group comparisons were performed using the Mann–Whitney U test. Holm correction was applied for multiple comparisons. Effect size is reported as *r* and interpreted as Small (<0.3), Medium (0.3–0.5), and Large (>0.5).

Stimulus	Parameter	Comparison	Sum of Ranks	U value	p value	Holm-corrected p	ES
NS	Entries to Contact Zone (number)	CM vs TM	372, 448	162	0.3104 ns	0.9312 ns	0.16/Small
		CF vs TF	424, 396	186	0.7128 ns	0.9312 ns	0.05/Small
		CM vs CF	443.5, 376.5	166.5	0.3721 ns	0.9312 ns	0.14/Small
		TM vs TF	489, 331	121	0.0320 *	0.1280 ns	0.34/Medium
	Speed at Contact Zone (cm/s)	CM vs TM	389.5, 430.5	179.5	0.5878 ns	1.0000 ns	0.08/Small
		CF vs TF	474, 346	136	0.0846 ns	0.3384 ns	0.27/Small
		CM vs CF	354, 466	144	0.1344 ns	0.4032 ns	0.24/Small
		TM vs TF	422, 398	188	0.7532 ns	1.0000 ns	0.04/Small
SO	Entries to Shoal Zone (number)	CM vs TM	333.5, 486.5	123.5	0.0381 *	0.0762 ns	0.33/Medium
		CF vs TF	276, 544	66	0.0001 ***	0.0004 ***	0.57/Large
		CM vs CF	521.5, 298.5	88.5	0.0020 **	0.0060 **	0.48/Medium
		TM vs TF	424, 396	186	0.7126 ns	0.7126 ns	0.05/Small
	Speed at Shoal Zone (cm/s)	CM vs TM	303, 517	93	0.0031 **	0.0124 *	0.46/Medium
		CF vs TF	444.5, 375.5	165.5	0.3581 ns	0.3581 ns	0.15/Small
		CM vs CF	345, 475	135	0.0797 ns	0.1594 ns	0.28/Small
		TM vs TF	483, 337	127	0.0483 *	0.1449 ns	0.31/Medium

PO	Entries to Predator Zone (number)	CM vs TM	340, 480	130	0.0585 ns	0.1755 ns	0.29/Small
		CF vs TF	318, 502	108	0.0119 *	0.0476 *	0.39/Medium
		CM vs CF	423.5, 396.5	186.5	0.7228 ns	1.0000 ns	0.05/Small
		TM vs TF	430, 390	180	0.5966 ns	1.0000 ns	0.08/Small
	Speed at Predator Zone (cm/s)	CM vs TM	448, 372	162	0.3108 ns	0.6216 ns	0.16/Small
		CF vs TF	528.5, 291.5	81.5	0.0010 **	0.0040 **	0.50/Large
		CM vs CF	362.5, 457.5	152.5	0.2036 ns	0.6108 ns	0.20/Small
		TM vs TF	421, 399	189	0.7737 ns	0.7737 ns	0.04/Small
MO	Entries to Mirror Zone (number)	CM vs TM	555.5, 264.5	54.5	<0.0001 ****	0.0001 ***	0.62/Large
		CF vs TF	247, 573	37	<0.0001 ****	<0.0001 ****	0.70/Large
		CM vs CF	539, 281	71	0.0003 ***	0.0003 ***	0.55/Large
		TM vs TF	236.5, 583.5	26.5	<0.0001 ****	<0.0001 ****	0.74/Large
	Speed at Mirror Zone (cm/s)	CM vs TM	360.5, 459.5	150.5	0.1848 ns	0.3102 ns	0.21/Small
		CF vs TF	357, 463	147	0.1551 ns	0.3102 ns	0.22/Small
		CM vs CF	340.5, 479.5	130.5	0.0605 ns	0.1815 ns	0.29/Small
		TM vs TF	293.5, 526.5	83.5	0.0012 **	0.0048 **	0.49/Medium

4.2.3.3 Freezing & Rapid Movements: Heatmap 3 Analysis

1. No Stimulus (NS)

Among all the pairwise comparisons made, only TF showed significantly higher freezing in NS relative to CF. Novelty-induced stress is commonly observed in zebrafish, but the higher freezing reported only in TF suggests the influence of prolonged isolation. Control sex-specific significant differences were identified in male and female zebrafish, with increased rapid movements in female zebrafish; however, this difference was masked in isolated individuals. TM showed significantly higher rapid movements than CM, whereas TF and CF showed comparable differences.

2. Shoal Only (SO)

All pairwise comparisons for freezing and rapid movements showed comparable differences, except the initial significance observed in freezing between isolated male and female (TM vs TF), which was no longer significant after Holm correction. With a similar trend observed in NS, TF showed higher freezing than TM, but this difference did not remain significant after Holm correction.

3. Predator-Stimulus Only (PO)

TM and TF showed significantly lower freezing and rapid movements in PO relative to their corresponding controls, consistent with the shift associated with general fear-induced behaviour observed in the earlier analysis. Control male and female zebrafish showed a significant sex-specific pattern in freezing behaviour, with males exhibiting higher freezing than females. However, rapid movements exhibited comparable differences between control male and female. Isolated male and female showed comparable differences in both freezing and rapid movements.

4. Mirror-Stimulus Only (MO)

Freezing in MO showed sex-specific differences in control zebrafish only. Control males showed significantly higher freezing than control females. Although TM vs TF showed an initial difference, this did not remain significant after Holm correction. All pairwise comparisons made for rapid movements showed comparable differences.

The distribution of behavioural metrics is illustrated in Figure 4.13 (Heatmap 3).

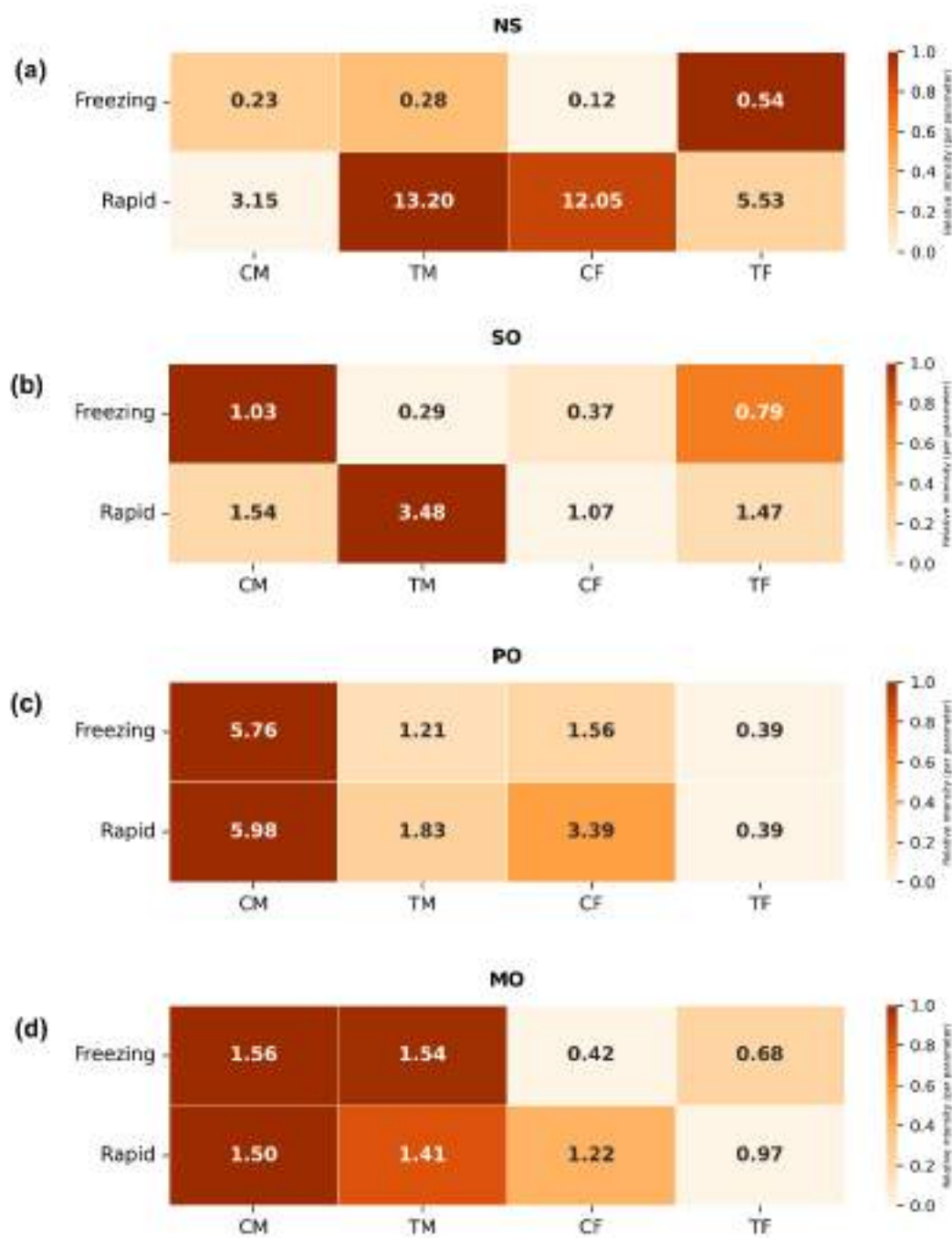


Figure 4.13. Heatmap representation of median freezing duration and rapid movements duration across groups under different stimulus conditions.

Heatmaps showing the median values of freezing duration (s) and rapid movements duration (s) for four groups-CM, TM, CF, and TF-under NS, SO, PO, and MO stimulus conditions.

Within each panel (a–d), groups are displayed on the X-axis in left-to-right order: CM, TM, CF, TF. The Y-axis lists the two behavioural parameters. Cell numbers indicate

absolute median values. The colour intensity scale bar represents relative differences within each parameter for a given stimulus condition, with darker shades of orange indicating higher relative values. This figure presents an alternative visualization of the same data shown previously in the corresponding behavioural plots. Statistical analyses corresponding to these results are presented in Tables 4.19.

Table 4.19. Between-group statistical analysis of freezing and rapid movements across multiple stimuli (NS, SO, PO, MO).

Between-group comparisons were performed using the Mann–Whitney U test. Holm correction was applied for multiple comparisons. Effect size is reported as *r* and interpreted as Small (<0.3), Medium (0.3–0.5), and Large (>0.5).

Stimulus	Parameter	Comparison	Sum of Ranks	U value	p value	Holm-corrected p	ES
NS	Freezing (s)	CM vs TM	380.5, 439.5	170.5	0.4319 ns	0.4319 ns	0.12/Small
		CF vs TF	285, 535	75	0.0004 ***	0.0016 **	0.54/Large
		CM vs CF	469, 351	141	0.1092 ns	0.3276 ns	0.25/Small
		TM vs TF	361.5, 458.5	151.5	0.1931 ns	0.3862 ns	0.21/Small
	Rapid Movements (s)	CM vs TM	304.5, 515.5	94.5	0.0036 **	0.0144 *	0.45/Medium
		CF vs TF	464, 356	146	0.1493 ns	0.2254 ns	0.23/Small
		CM vs CF	310, 510	100	0.0061 **	0.0183 *	0.43/Medium
		TM vs TF	469, 351	141	0.1127 ns	0.2254 ns	0.25/Small
SO	Freezing (s)	CM vs TM	481, 339	129	0.0548 ns	0.1924 ns	0.30/Medium
		CF vs TF	348.5, 471.5	138.5	0.0977 ns	0.1924 ns	0.26/Small
		CM vs CF	479.5, 340.5	130.5	0.0602 ns	0.1924 ns	0.29/Small
		TM vs TF	337, 483	127	0.0481 *	0.1924 ns	0.31/Medium
	Rapid Movements (s)	CM vs TM	342.5, 477.5	132.5	0.0683 ns	0.2732 ns	0.29/Small
		CF vs TF	435, 385	175	0.5068 ns	1.0000 ns	0.10/Small
		CM vs CF	392, 428	182	0.6345 ns	1.0000 ns	0.07/Small
		TM vs TF	462.5, 357.5	147.5	0.1580 ns	0.4740 ns	0.22/Small
PO	Freezing (s)	CM vs TM	540, 280	70	0.0002 ***	0.0006 ***	0.55/Large
		CF vs TF	546.5, 273.5	63.5	0.0001 ***	0.0004 ***	0.58/Large
		CM vs CF	538, 282	72	0.0003 ***	0.0006 ***	0.55/Large
		TM vs TF	474, 346	136	0.0844 ns	0.0844 ns	0.27/Small

	Rapid Movements (s)	CM vs TM	521, 299	89	0.0021 **	0.0063 **	0.47/Medium
		CF vs TF	548, 272	62	0.0001 ***	0.0004 ***	0.59/Large
		CM vs CF	470.5, 349.5	139.5	0.1036 ns	0.2072 ns	0.26/Small
		TM vs TF	469.5, 350.5	140.5	0.1075 ns	0.2072 ns	0.25/Small
MO	Freezing (s)	CM vs TM	409, 411	199	0.9840 ns	0.9840 ns	0.002/Small
		CF vs TF	366.5, 453.5	156.5	0.2443 ns	0.4886 ns	0.18/Small
		CM vs CF	511, 309	99	0.0055 **	0.0220 *	0.43/Medium
		TM vs TF	490.5, 329.5	119.5	0.0287 *	0.0861 ns	0.34/Medium
	Rapid Movements (s)	CM vs TM	396, 424	186	0.7128 ns	1.0000 ns	0.05/Small
		CF vs TF	413, 407	197	0.9413 ns	1.0000 ns	0.01/Small
		CM vs CF	406.5, 413.5	196.5	0.9305 ns	1.0000 ns	0.01/Small
		TM vs TF	410, 410	200	0.9892 ns	1.0000 ns	0.002/Small

4.2.4 Predator and Shoal (PS)

The simultaneous introduction of conspecifics along with a predator stimulus in the three-chamber experimental setup was designed to assess how the presence of conspecifics influences stress-induced behavioural responses observed under the predator-only condition. Among the measured parameters, freezing served as the primary indicator of stress, while rapid movements and zone allocation reflected escape tendencies and avoidance behaviour. These parameters were collectively used to evaluate the extent of social buffering. The following section exclusively focuses on dual-stimulus trials within each group and compares these behavioural measures between control and test groups.

In the PS condition, freezing analysed between all experimental groups showed comparable differences, including the comparison between isolated male and female, as the initial significance did not remain after Holm correction.

For rapid movements, only CF vs TF remained statistically significant after Holm correction, where TF showed significantly lower rapid movements than CF. Although significantly lower rapid movements were initially reported in TM relative to CM, this did not remain significant after Holm correction.

The third category of behavioural measures analysed was time spent across predator, internal, and shoal zones. Across all three zones, baseline sex differences were observed between CM and CF, where males exhibited significantly lower predator and internal zone preference and significantly higher shoal zone engagement than females. These innate sex differences were absent in the isolated groups. Another significant comparison was observed between isolated male and control male, where isolated male showed significantly increased preference toward predator and internal zones and significantly lower engagement in the shoal zone. Isolated females showed comparable differences relative to control females across all three zones.

Time allocation heatmap analysis revealed the zone preferences made by all groups. CM exhibited a visible zone preference, allocating the most time in the shoal zone, less time in the internal zone, and the least time in the predator zone, with all three pairwise comparisons reaching significance. Similar to CM, the other three groups, including CF, TM, and TF, showed a consistent time allocation pattern, spending the most time in the shoal zone, followed by the internal zone and the least time in the predator zone, with all pairwise comparisons reaching statistical significance after correction.

PS results and associated metrics are illustrated in Figure 4.14 (a–c), with corresponding statistical analyses presented in Tables 4.20–4.22 for (a), (b), and (c), respectively.

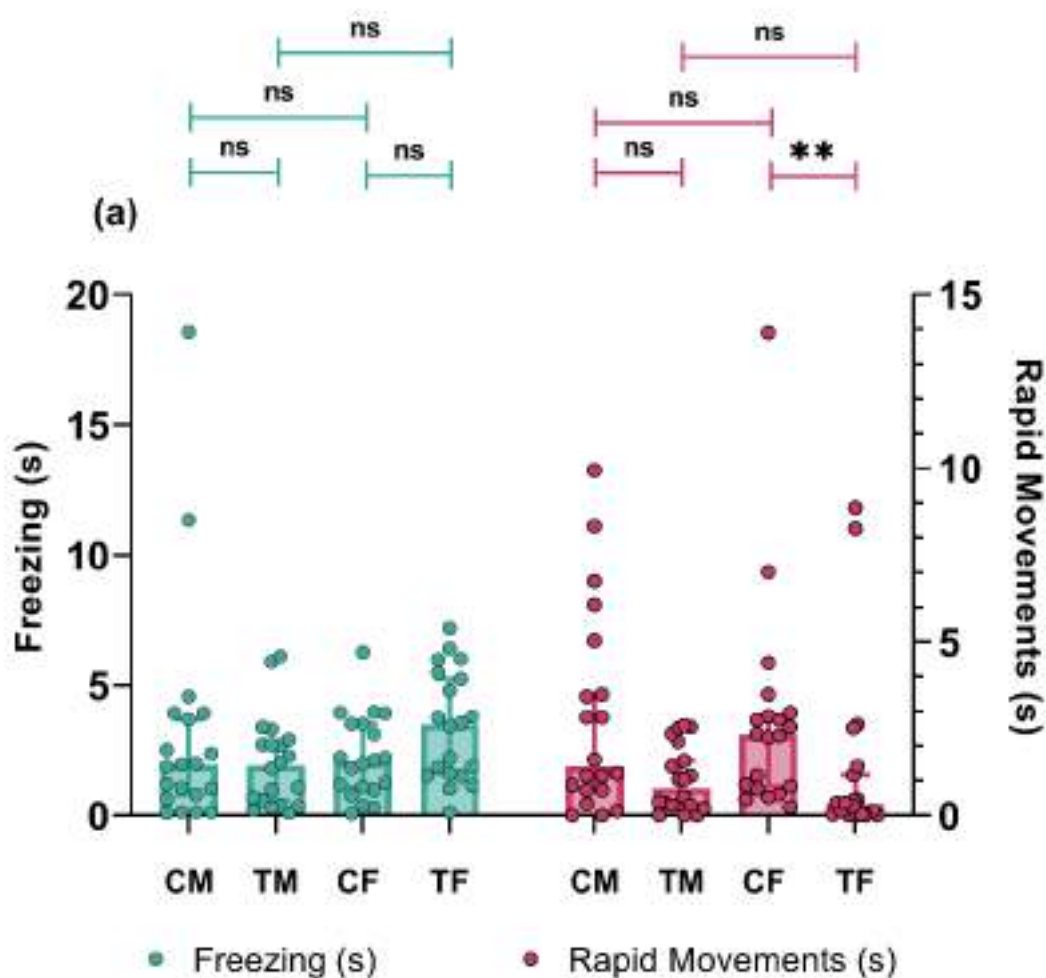
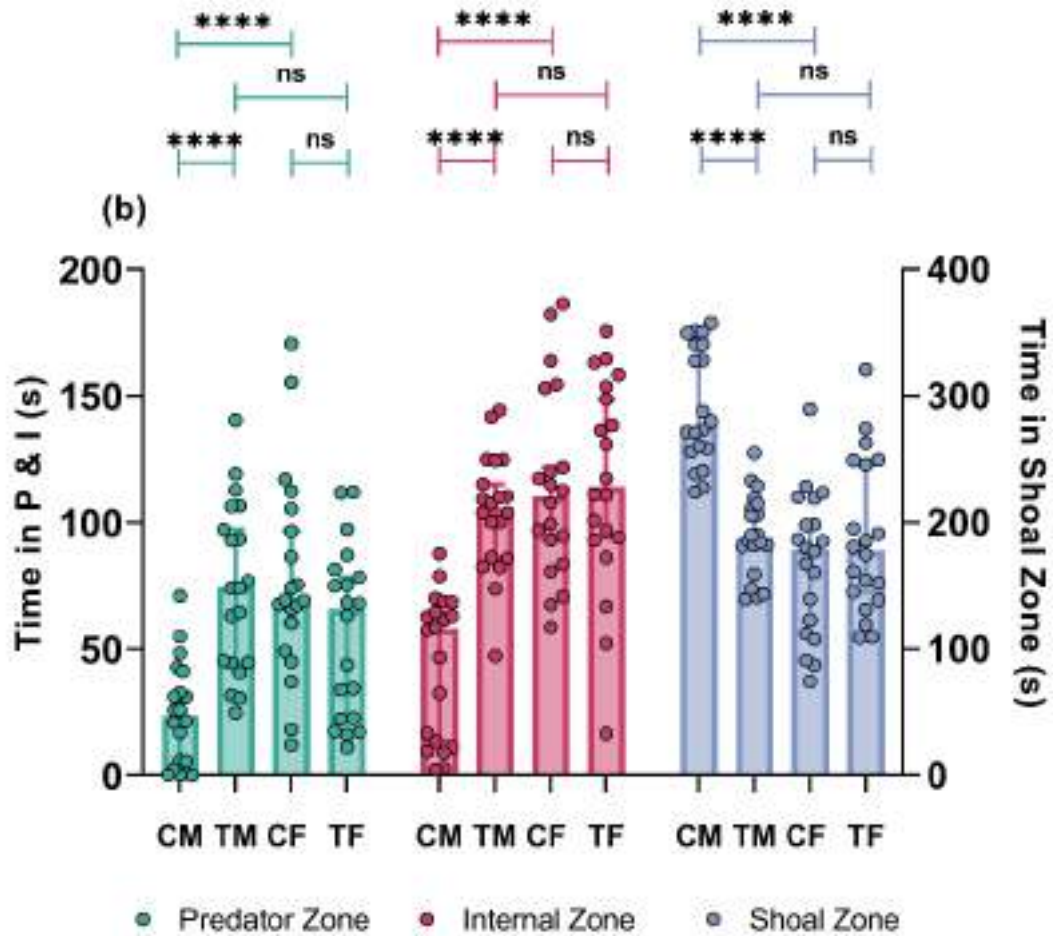


Figure 4.14. Behavioural responses and zone allocation under Predator + Shoal (PS) condition.

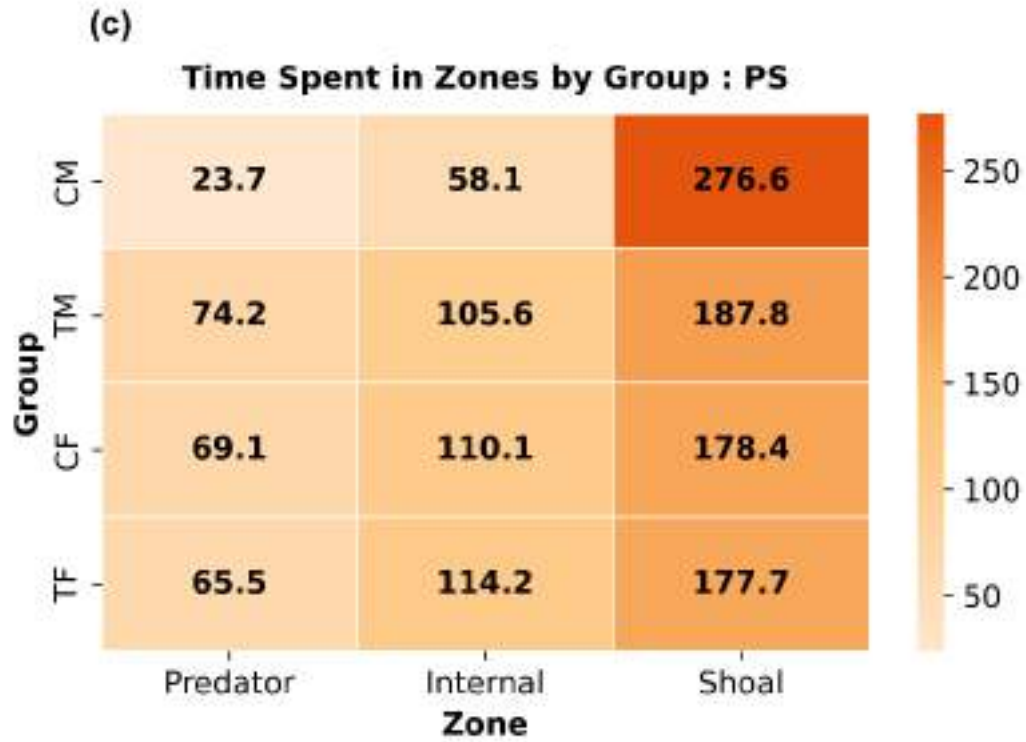
(a) Dual-axis column scatter plot showing freezing duration and rapid movements under PS.

Freezing duration (s) is plotted on the left Y-axis (green points and bars), and rapid movements duration (s) is plotted on the right Y-axis (magenta points and bars). Groups are arranged along the X-axis in the following order: control males (CM), test males (TM), control females (CF), and test females (TF). Each point represents an individual fish (n = 20 per group). Bars represent the median with 95% confidence intervals. Statistical comparisons are indicated above connecting lines (ns = non-significant; asterisks denote level of significance).



(b) Dual-axis column scatter plot showing time allocation across three zones under PS (between-group analysis).

Time spent in the predator zone (pink) and internal zone (yellow) is plotted on the left Y-axis (Time in P & I, s), while time spent in the shoal zone (green) is plotted on the right Y-axis (Time in Shoal Zone, s). Groups are displayed along the X-axis in the order CM, TM, CF, and TF for each zone category. Individual data points ($n = 20$ per group) are overlaid on median bars with 95% confidence intervals. Statistical comparisons are shown above connecting lines.



(c) Heatmap showing within-group time allocation under PS.

Heatmap illustrating median time (s) spent in predator, internal, and shoal zones for CM, TM, CF, and TF. Groups are arranged along the Y-axis and zones along the X-axis. Cell numbers indicate absolute median values. Colour intensity represents relative differences within the PS condition, with darker shades indicating higher time allocation.

Table 4.20. Between-group statistical analysis of freezing and rapid movements under Predator–Shoal (PS) condition.

Between-group comparisons were performed using the Mann–Whitney U test with Holm correction for multiple comparisons. Effect size is reported as *r* and interpreted as Small (<0.3), Medium (0.3–0.5), and Large (>0.5).

Parameter	Comp.	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p	Holm-corrected p	ES
Freezing (s)	CM vs TM	1.930 (1.025, 5.181)	1.920 (1.206, 2.856)	418, 402	192	0.8357 ns	1.0000 ns	0.03 Small
	CF vs TF	2.060 (1.488, 2.984)	3.490 (2.439, 4.414)	348, 472	138	0.0951 ns	0.2853 ns	0.26 Small
	CM vs CF	1.930 (1.025, 5.181)	2.060 (1.488, 2.984)	400, 420	190	0.7942 ns	1.0000 ns	0.04 Small
	TM vs TF	1.920 (1.206, 2.856)	3.490 (2.439, 4.414)	330, 490	120	0.0299 *	0.1196 ns	0.34 Medium
Rapid Movements (s)	CM vs TM	1.410 (1.456, 4.208)	0.7900 (0.5991, 1.481)	485.5, 334.5	124.5	0.0406 *	0.1218 ns	0.32 Medium
	CF vs TF	2.325 (1.310, 4.200)	0.3200 (0.1480, 2.571)	527.5, 292.5	82.5	0.0011 **	0.0044 **	0.50 Large
	CM vs CF	1.410 (1.456, 4.208)	2.325 (1.310, 4.200)	407.5, 412.5	197.5	0.9520 ns	0.9520 ns	0.008 Small
	TM vs TF	0.7900 (0.599, 1.481)	0.3200 (0.1480, 2.571)	448, 372	162	0.3092 ns	0.6184 ns	0.16 Small

Table 4.21. Between-group statistical analysis of time allocation across Predator–Shoal (PS) zones: Between-group comparisons were performed using the Mann–Whitney U test with Holm correction. Effect size is reported as *r*.

Parameter	Comp.	Mdn 1 (95% CI)	Mdn 2 (95% CI)	Sum of Ranks	U	p	Holm-corrected p	ES
Time Spent in Predator Zone (s)	CM vs TM	23.67 (14.36, 33.59)	74.17 (58.48, 89.85)	250, 570	40	<0.0001 ****	<0.0001 ****	0.68 Large
	CF vs TF	69.12 (58.83, 96.55)	65.63 (41.23, 72.42)	460, 360	150	0.1826 ns	0.2528 ns	0.21 Small
	CM vs CF	23.67 (14.36, 33.59)	69.12 (58.83, 96.55)	251, 569	41	<0.0001 ****	<0.0001 ****	0.68 Large
	TM vs TF	74.17 (58.48, 89.85)	65.63 (41.23, 72.42)	467, 353	143	0.1257 ns	0.2528 ns	0.24 Small

Time Spent in Internal Zone (s)	CM vs TM	58.08 (30.88, 57.68)	105.8 (92.98, 114.8)	227, 593	17	<0.0001 ****	<0.0001 ****	0.78 Large
	CF vs TF	110.1 (96.58, 131.3)	114.2 (96.38, 135.1)	398, 422	188	0.7584 ns	0.7584 ns	0.04 Small
	CM vs CF	58.08 (30.88, 57.68)	110.1 (96.58, 131.3)	228, 592	18	<0.0001* ***	<0.0001 ****	0.78 Large
	TM vs TF	105.8 (92.98, 114.8)	114.2 (96.38, 135.1)	360, 460	150	0.1803ns	0.3612 ns	0.21 Small
Time Spent in Shoal Zone (s)	CM vs TM	276.6 (270.0, 313.5)	187.8 (174.6, 205.1)	602, 218	8	<0.0001* ***	<0.0001 ****	0.82 Large
	CF vs TF	178.4 (142.0, 194.7)	177.7 (159.1, 215.8)	383, 437	173	0.4777 ns	0.9554 ns	0.11 Small
	CM vs CF	276.6 (270.0, 313.5)	178.4 (142.0, 194.7)	596, 224	14	<0.0001 ****	<0.0001 ****	0.79 Large
	TM vs TF	187.8 (174.6, 205.1)	177.7 (159.1, 215.8)	434, 386	176	0.5246 ns	0.9554 ns	0.10 Small

Table 4.22. Within-group analysis of time allocation across Predator–Shoal (PS) zones using Friedman test.

Within-group comparisons were performed using the Friedman test followed by the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (FDR $Q = 0.05$). A result was considered a significant discovery when $q < 0.05$. Effect size is reported as Kendall's W .

Group	Friedman Stat.	p	ES	Comp.	Rank Sum Diff.	p	q
CM	34.90	<0.0001****	0.87 Large	P vs I	-14	0.0269	0.0282
				P vs S	-37	<0.0001	<0.0001
				I vs S	-23	0.0003	0.0004
TM	33.60	<0.0001****	0.84 Large	P vs I	-12	0.0578	0.0202
				P vs S	-36	<0.0001	<0.0001
				I vs S	-24	0.0001	<0.0001
CF	14.40	0.0007***	0.36 Medium	P vs I	-12	0.0578	0.0404
				P vs S	-24	0.0001	0.0003
				I vs S	-12	0.0578	0.0404
TF	26.80	<0.0001****	0.67 Large	P vs I	-22	0.0005	0.0003
				P vs S	-32	<0.0001	<0.0001
				I vs S	-10	0.1138	0.0398

4.2.4.1 Role of Conspecific Buffering in Predator-Induced Stress

This section discusses the influence of conspecifics in buffering predator-induced stress within control male, female, and isolated male and female groups separately.

In the PS condition, CM exhibited significant reductions in freezing, rapid movements, time spent in the predator zone, and time spent in the internal zone, along with increased time spent in the shoal zone compared to PO, indicating a true sign of conspecific buffering of stress within male zebrafish.

Interestingly, isolated males showed no significant differences in freezing, rapid movements, and time spent in the internal zone. However, significant predator zone avoidance, reflected by lower time spent in the predator zone and higher shoal zone affiliation in PS relative to PO, was observed in TM, indicating that unlike the true buffering pattern observed within control males, isolated males only showed partial buffering through a shift in zone allocation.

CF showed no change in freezing behaviour in PS relative to PO, but a significant reduction in rapid movements was reported in PS compared to PO. Unexpectedly, CF spent significantly more time in the predator zone in PS compared to PO, indicating that females respond differently to stress and buffering pathways.

TF also responded differently than TM, with a marked increase in freezing behaviour when exposed to PS compared to PO, suggesting that the presence of conspecifics amplified the stress response under isolation. Comparable changes were found in rapid movements and time spent in the internal zone. Even though the emotional valence changed unexpectedly, TF showed a shift in zone allocation, with significantly lower time spent in the predator zone and higher time spent in the shoal zone in PS relative to PO. Because freezing increased in TF despite this zone shift, the buffering effect appears less effective or incomplete.

Although the statistical comparisons were described in Results 4.2.4.1 as per Table No.4.23, the data are represented as individual scattered plots of freezing and rapid movement difference scores (PO–PS), with red indicating higher freezing and rapid movements in PO and blue indicating higher freezing and rapid movements in PS. Zone-wise time allocation across all groups under PO and PS is further illustrated using pie charts.

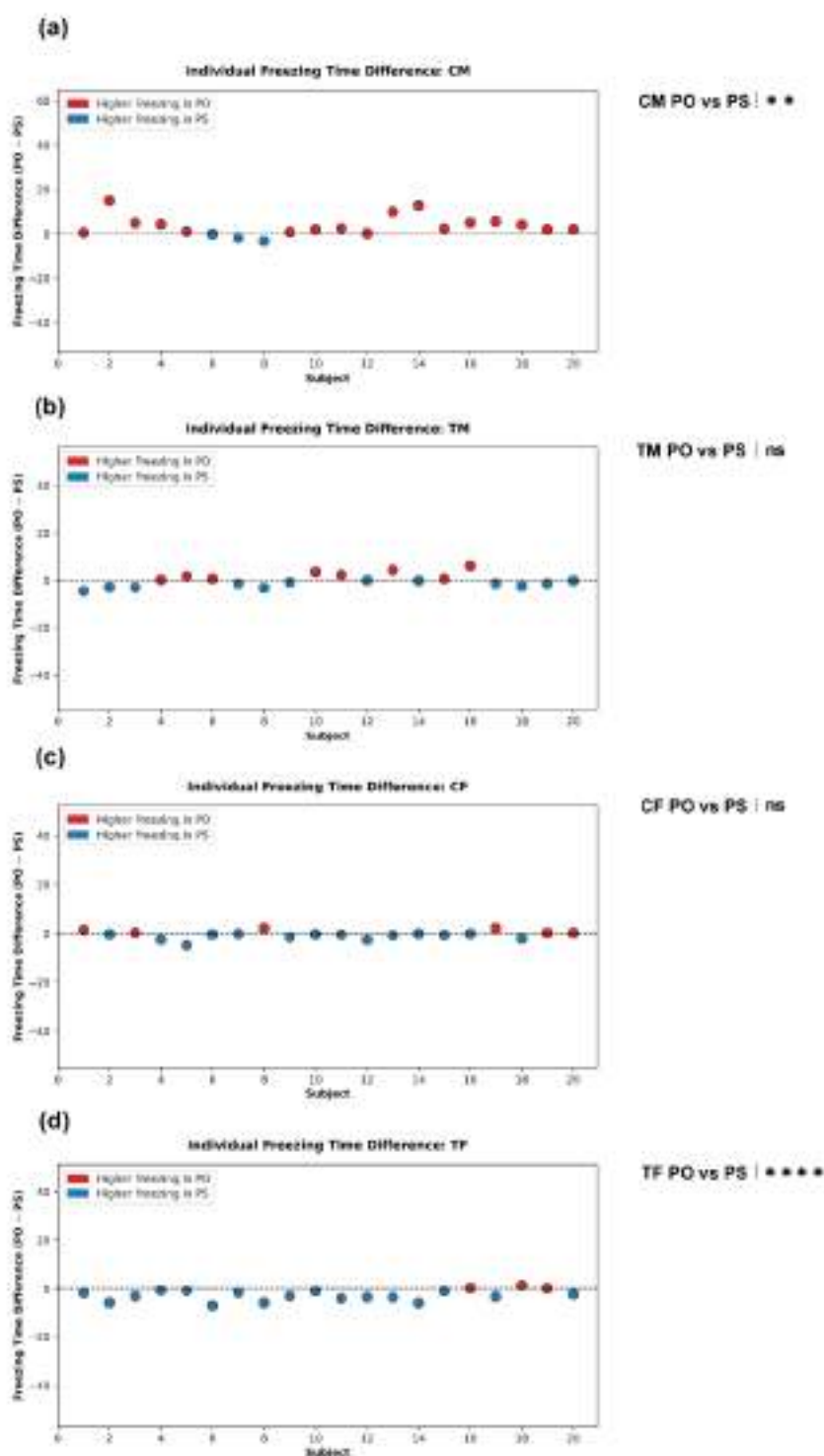


Figure 4.15. Individual Difference Scores of Freezing Behaviour Between Predator Only (PO) and Predator + Shoal (PS) Conditions

Scatter dot plots showing individual freezing time difference scores (PO – PS) for each group: (a) Control Male (CM), (b) Test Isolated Male (TM), (c) Control Female (CF), and (d) Test Isolated Female (TF). Each dot represents one subject ($n = 20$ per group). The y-axis indicates the freezing time difference (seconds), calculated as PO

minus PS. The dashed horizontal line at zero represents no change between conditions.

Red dots indicate higher freezing in PO relative to PS (positive values), whereas blue dots indicate higher freezing in PS relative to PO (negative values). Statistical analyses were conducted on the raw freezing data (not on difference scores) as described in Section 4.2.4.1 and corresponding tables. Significance levels for PO vs PS comparisons are indicated adjacent to each panel.

This visualization highlights within-group stress modulation under conspecific buffering, illustrating differential buffering efficacy across control and isolated male and female zebrafish.

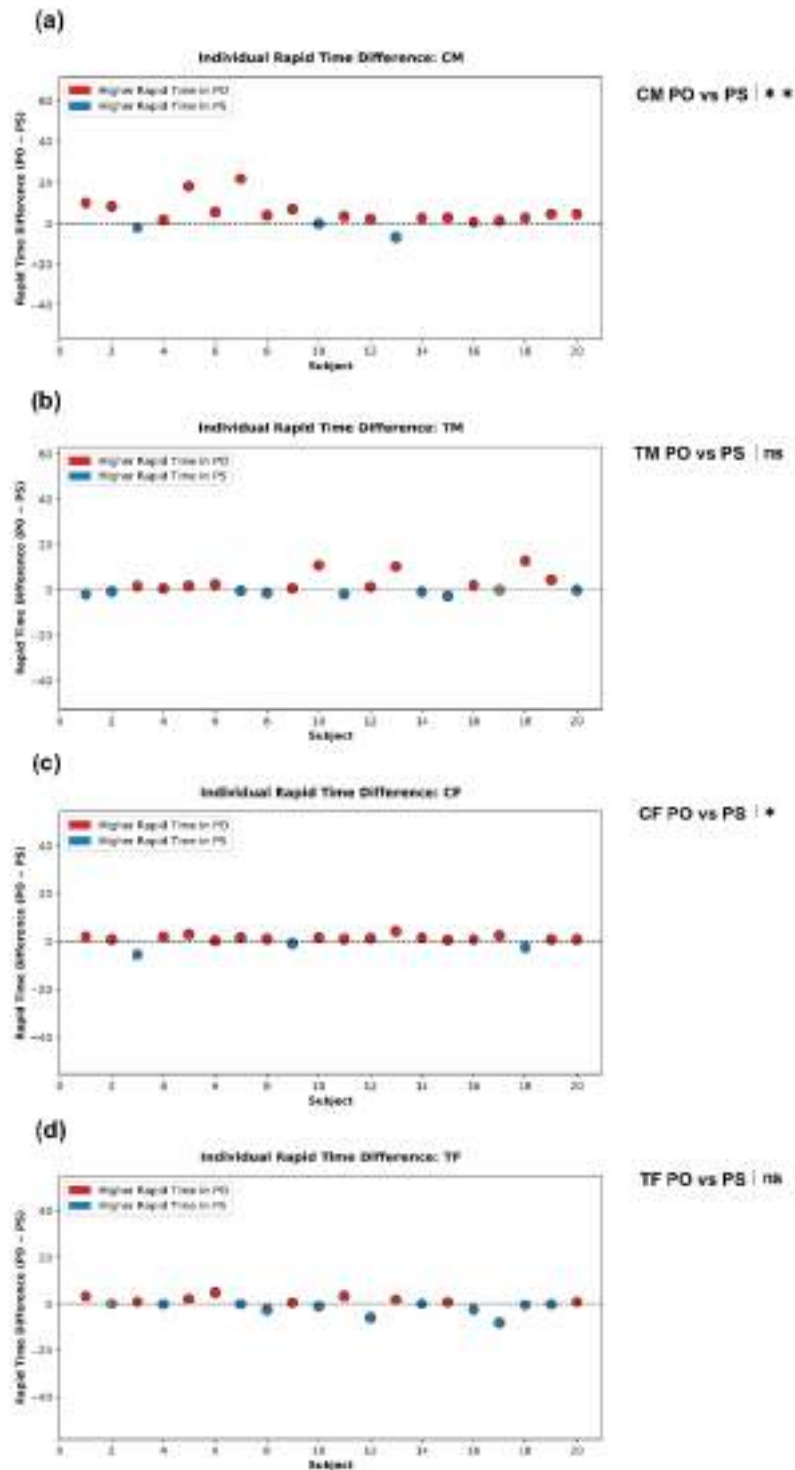


Figure 4.16. Individual Difference Scores of Rapid Movements Between Predator Only (PO) and Predator + Shoal (PS) Conditions

Scatter dot plots showing individual rapid movement time difference scores (PO – PS) for each group: (a) Control Male (CM), (b) Test Isolated Male (TM), (c) Control Female (CF), and (d) Test Isolated Female (TF). Each dot represents one subject ($n = 20$ per group). The y-axis indicates the rapid movement time difference (seconds),

calculated as PO minus PS. The dashed horizontal line at zero represents no change between conditions.

Red dots denote higher rapid movements in PO compared to PS, while blue dots denote higher rapid movements in PS compared to PO. Statistical comparisons were performed on the original raw rapid movement data, as detailed in Section 4.2.4.1 and corresponding statistical tables. Significance indicators for PO vs PS are shown adjacent to each panel.

This figure complements the freezing analysis by illustrating individual variability in hyperlocomotion-related behavioural modulation under predator exposure with and without social buffering.

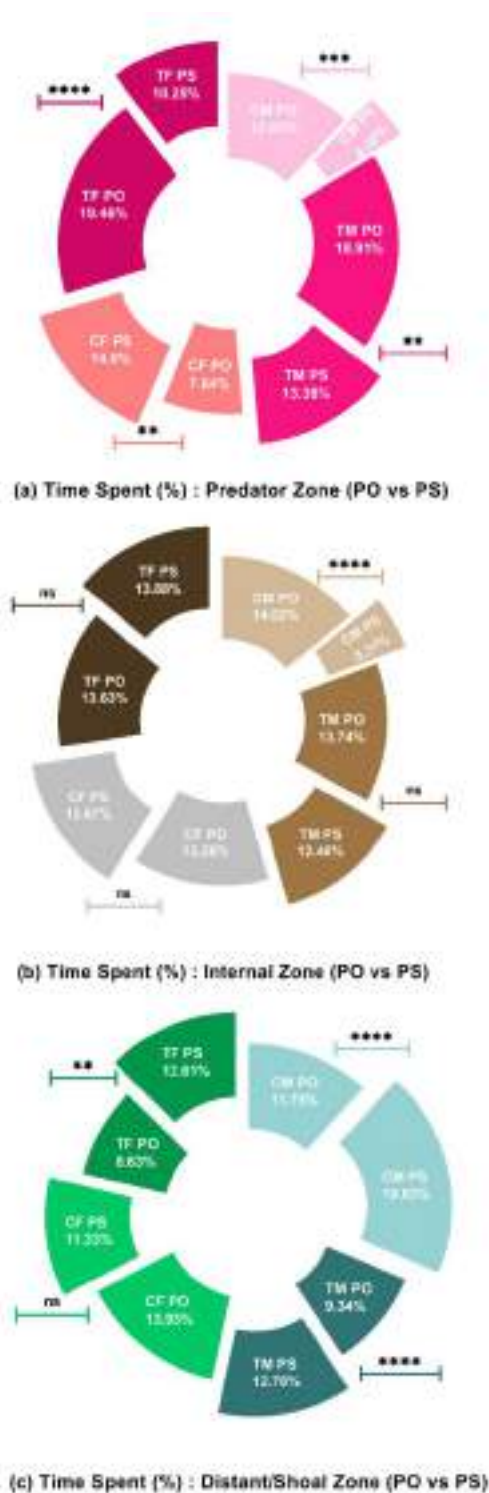


Figure 4.17. Percentage-based comparison of zone allocation between Predator Only (PO) and Predator + Shoal (PS) conditions across groups.

Exploded donut charts illustrating the percentage distribution of time spent in each zone under PO and PS conditions for control males (CM), test males (TM), control females (CF), and test females (TF). Raw time values were converted to percentage of total trial duration to enable direct proportional comparison between conditions.

Each chart represents one zone:

(a) Predator zone (contact zone)

(b) Internal zone

(c) Shoal zone (buffering zone in PS condition)

Within each donut chart:

- Each group (CM, TM, CF, TF) is represented by two adjacent segments corresponding to PO and PS conditions.
- The same colour shade is used within a group to represent its two conditions (PO vs PS), enabling direct visual comparison.
- Percentage values are indicated inside each segment.
- Segments are slightly separated (exploded format) to enhance readability and emphasize proportional differences.

Statistical comparison lines are drawn between PO and PS segments within the same group for the respective zone. Asterisks indicate the level of statistical significance (p value thresholds), while “ns” denotes non-significant differences.

Interpretation guidance:

- In panel (a), differences represent changes in predator-directed allocation when a shoal is introduced (PS) compared to predator alone (PO).
- In panel (b), values reflect redistribution toward the neutral internal zone.
- In panel (c), values indicate shoal-zone preference under buffering conditions, demonstrating social modulation of threat response.

Thus, this figure provides a proportional representation of behavioural redistribution across zones, highlighting how the presence of a shoal alters predator-associated spatial preference in a group-specific manner.

Statistical details corresponding to Figures 4.15–4.17 are presented in Table 4.28.

Table 4.23. Pairwise analysis of predator-induced stress (PO) and conspecific buffering (PS) across experimental groups.

Within-group comparisons between Predator Only (PO) and Predator–Shoal (PS) conditions were performed using the Mann–Whitney U test. Effect size is reported as *r* and interpreted as Small (<0.3), Medium (0.3–0.5), and Large (>0.5).

Group	Parameter	Comparison	Sum of Ranks	U	p value	ES
CM	Freezing (s)	PO vs PS	508.5, 311.5	101.5	0.0068 **	0.42 Medium
	Rapid Movements (s)		512.5, 307.5	97.5	0.0048 **	0.44 Medium
	Time Spent in P zone (s)		533, 287	77	0.0006 ***	0.52 Large
	Time Spent in I zone (s)		594, 226	16	<0.0001 ****	0.78 Large
	Time Spent in D/S zone (s)		242, 578	32	<0.0001 ****	0.72 Large
TM	Freezing (s)	PO vs PS	376, 444	166	0.3650 ns	0.14 Small
	Rapid Movements (s)		467.5, 352.5	142.5	0.9750 ns	0.24 Small
	Time Spent in P zone (s)		520, 300	90	0.0023 **	0.47 Medium
	Time Spent in I zone (s)		458, 362	152	0.1987 ns	0.20 Small
	Time Spent in D/S zone (s)		250, 570	40	<0.0001 ****	0.68 Large
CF	Freezing (s)	PO vs PS	379, 441	169	0.4096 ns	0.13 Small
	Rapid Movements (s)		490, 330	120	0.0299 *	0.34 Medium
	Time Spent in P zone (s)		296, 524	86	0.0016 **	0.49 Medium
	Time Spent in I zone (s)		422, 398	188	0.7531 ns	0.04 Small
	Time Spent in D/S zone (s)		473, 347	137	0.0897 ns	0.27 Small
TF	Freezing (s)	PO vs PS	248.5, 571.5	38.5	<0.0001 ****	0.69 Large
	Rapid Movements (s)		412.5, 407.5	197.5	0.9516 ns	0.008 Small
	Time Spent in P zone (s)		556, 264	54	<0.0001 ****	0.62 Large
	Time Spent in I zone (s)		397, 423	187	0.7381 ns	0.05 Small
	Time Spent in D/S zone (s)		292, 528	82	0.0010**	0.50/Large

4.2.5 Mirror and Shoal (MS)

Mirror bites, the salient indicator of aggression, observed under Mirror Only (MO) and Mirror and Shoal (MS) conditions are discussed here.

Within MO, TM showed increased and TF showed decreased mirror bites compared to their respective controls, supporting the pattern of time spent within the mirror zone observed earlier in isolated groups. Baseline sex differences observed within CM and CF showed an inverted pattern of mirror bites in TM and TF.

Considering MS, consistently higher mirror bites were observed in TM relative to CM. TF and CF showed comparable differences in mirror bites. Baseline sex differences were also consistent within MS, where CF reported higher bites than CM. Interestingly, sex differences were not visible between TM and TF in mirror bites under MS.

Considering zone time allocation within MS, no sex differences were found between control and isolated groups across the mirror, internal, and shoal zones. TM and TF showed significantly higher time in the mirror zone than their respective controls; however, TF did not reach the significance threshold after Holm correction. Similarly, TM and TF showed significantly higher time in the internal zone than their respective controls, and these differences remained significant after Holm correction. TM and TF were observed with significantly lower time in the shoal zone than their corresponding control groups.

The zone allocation within MS is further explained through a heatmap, which revealed that isolated groups, including TM and TF, showed equal preference across the three zones, resulting in comparable differences that were not statistically significant. CM and CF showed a similar zone allocation pattern with statistically higher preference towards the shoal zone relative to both mirror and internal zones, but largely similar preference towards mirror and internal zones, resulting in a non-significant difference between them.

MO and MS results and associated metrics are illustrated in Figure 4.18 (a–c), with corresponding statistical analyses presented in Tables 4.24–4.26 for (a), (b), and (c), respectively.

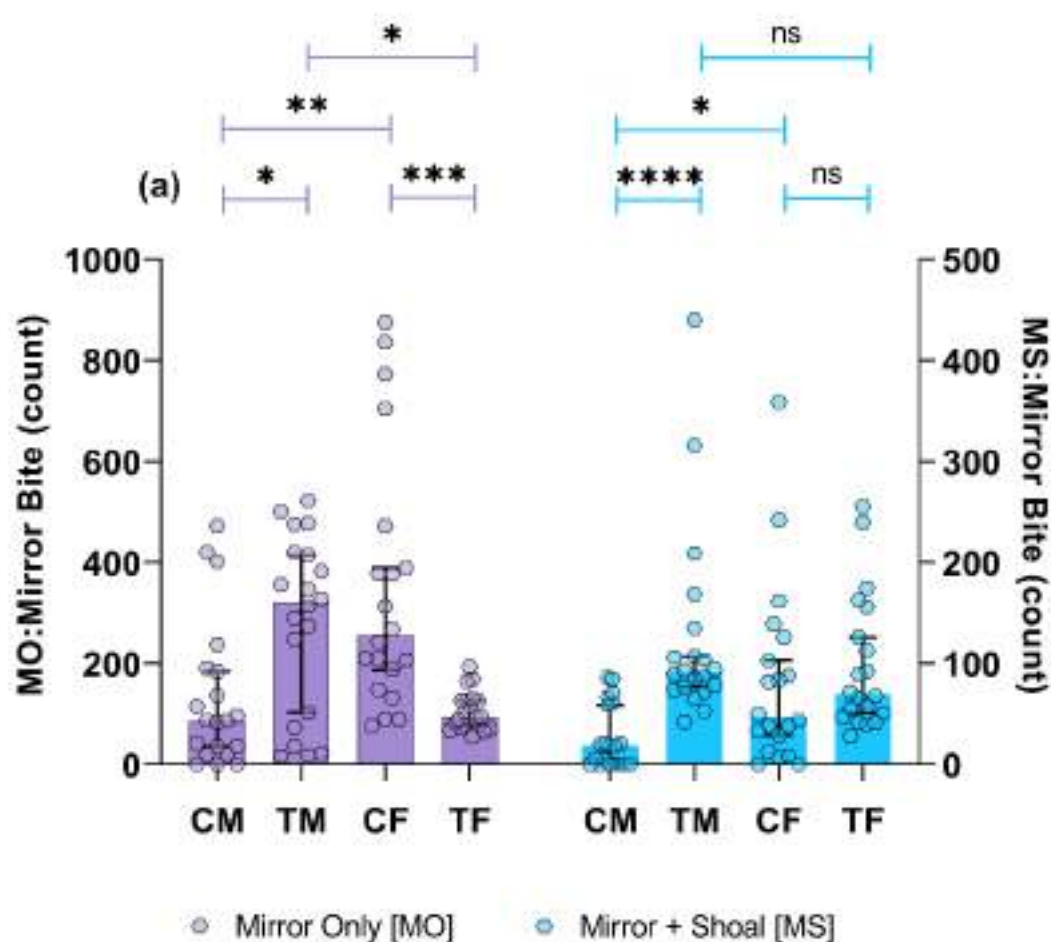


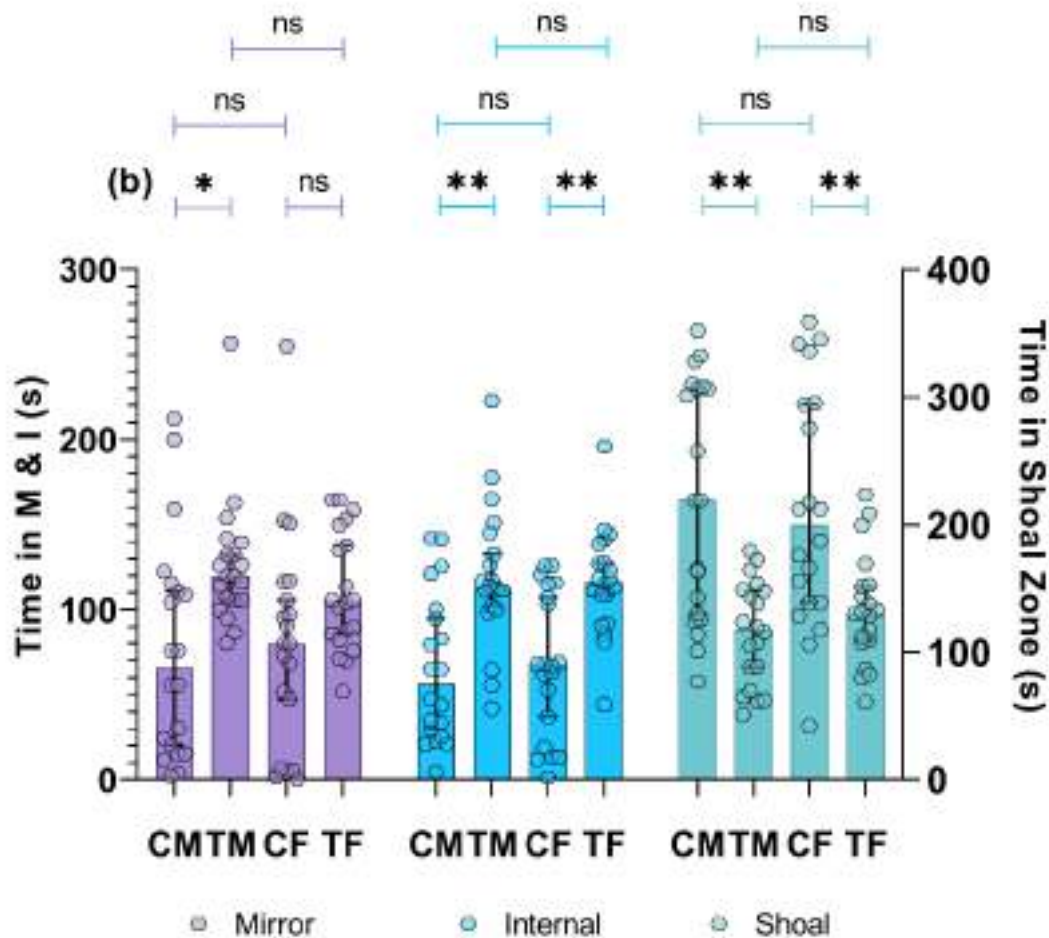
Figure 4.18. Behavioural responses and zone allocation under Mirror + Shoal (MS) condition.

(a) Dual-axis column scatter plot showing mirror-directed biting behaviour under MO and MS conditions.

Mirror-directed bites (number) are plotted for Mirror Only (MO) on the left Y-axis and for Mirror + Shoal (MS) on the right Y-axis. Groups are arranged along the X-axis in the following order: control males (CM), test males (TM), control females (CF), and test females (TF). For each group, MO and MS are presented as separate adjacent columns to allow direct comparison of aggression under isolated mirror exposure versus conspecific buffering.

Each point represents an individual fish ($n = 20$ per group). Bars indicate the median with 95% confidence intervals. Statistical comparisons between MO and MS within each group are shown above connecting lines (ns = non-significant; asterisks denote

level of significance). Detailed statistical outcomes are provided in the corresponding tables.



(b) Dual-axis column scatter plot showing time allocation across three zones under MS (between-group analysis).

Time spent in the mirror zone (M) and internal zone (I) is plotted on the left Y-axis (Time in M & I, s), while time spent in the shoal zone (S) is plotted on the right Y-axis (Time in Shoal Zone, s). Groups are displayed along the X-axis in the order CM, TM, CF, and TF within each zone category.

Individual data points (n = 20 per group) are overlaid on median bars with 95% confidence intervals. Statistical comparisons are indicated above connecting lines, with significance levels marked accordingly. Complete statistical details are reported in the tabulated results.



(c) Heatmap showing within-group time allocation under MS.

Heatmap illustrating median time (s) spent in mirror, internal, and shoal zones for CM, TM, CF, and TF. Zones are arranged along the X-axis (Mirror, Internal, Shoal), and groups are arranged along the Y-axis from top to bottom in the order: CM, TM, CF, TF.

Cell numbers represent absolute median values. Colour intensity reflects relative differences within the MS condition, with darker shades indicating higher time allocation. The heatmap provides a visual summary of within-group spatial preference under mirror-induced aggression with conspecific buffering. Detailed statistical comparisons corresponding to these patterns are presented in the associated tables.

Table 4.24. Between-group analysis of mirror-directed bites under Mirror Only (MO) and Mirror + Shoal (MS) conditions (Figure a).

Between-group comparisons were performed using the Mann–Whitney U test with Holm correction for multiple comparisons. Effect size is reported as *r* and interpreted as Small (<0.3), Medium (0.3–0.5), and Large (>0.5).

Stimuli	Comp.	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p	Holm-corrected p	ES
MO	CM vs TM	86.50 (65.60, 201.4)	321.5 (199.2, 363.4)	320, 500	110	0.0140 *	0.0170 *	0.38 Medium
	CF vs TF	256.0 (229.6, 468.7)	93.00 (87.04, 123.0)	566, 254	44	<0.0001 ****	0.0001 ***	0.67 Large
	CM vs CF	86.50 (65.60, 201.4)	256.0 (229.6, 468.7)	288.5, 531.5	78.5	0.0007 ***	0.0021 **	0.52 Large
	TM vs TF	321.5 (199.2, 363.4)	93.00 (87.04, 123.0)	506, 314	104	0.0085 **	0.0170 *	0.41 Medium
MS	CM vs TM	19.00 (12.33, 41.77)	91.50 (79.59, 170.1)	235.5, 584.5	25.5	<0.0001 ****	<0.0001 ****	0.75 Large
	CF vs TF	46.50 (39.95, 124.5)	70.00 (67.54, 130.3)	353.5, 466.5	143.5	0.1291 ns	0.2582 ns	0.24 Small
	CM vs CF	19.00 (12.33, 41.77)	46.50 (39.95, 124.5)	317, 503	107	0.0107 *	0.0321 *	0.40 Medium
	TM vs TF	91.50 (79.59, 170.1)	70.00 (67.54, 130.3)	460.5, 359.5	149.5	0.1760 ns	0.2582 ns	0.21 Small

Table 4.25. Between-group analysis of time allocation across three zones under MS (Mirror + Shoal) condition (Figure b). Between-group comparisons were performed using the Mann–Whitney U test with Holm correction.

Parameter	Comp.	Mdn 1 (95% CI)	Mdn 2 (95% CI)	Sum of Ranks	U	p	Holm-corrected p	ES
Time Spent in Mirror Zone (s)	CM vs TM	65.73 (45.85, 106.1)	119.9 (108.5, 143.5)	303, 517	93	0.0031 **	0.0124 *	0.46 Medium
	CF vs TF	79.92 (50.30, 109.6)	104.4 (93.91, 126.7)	336, 484	126	0.0452 *	0.1356 ns	0.31 Medium
	CM vs CF	65.73 (45.85, 106.1)	79.92 (50.30, 109.6)	409, 411	199	0.9840 ns	0.9840 ns	0.002 Small
	TM vs TF	119.9 (108.5, 143.5)	104.4 (93.91, 126.7)	458, 362	152	0.2012 ns	0.4024 ns	0.20 Small

Time Spent in Internal Zone (s)	CM vs TM	55.98 (44.57, 85.35)	112.9 (99.43, 138.2)	287, 533	77	0.0006***	0.0020**	0.52 Large
	CF vs TF	67.55 (48.13, 87.69)	116.2 (101.9, 131.0)	285, 535	75	0.0005***	0.0020**	0.53 Large
	CM vs CF	55.98 (44.57, 85.35)	67.55 (48.13, 87.69)	404.5, 415.5	194.5	0.8883 ns	1.0000 ns	0.02 Small
	TM vs TF	112.9 (99.43, 138.2)	116.2 (101.9, 131.0)	409, 411	199	0.9893 ns	1.0000 ns	0.002 Small
Time Spent in Shoal Zone (s)	CM vs TM	219.0 (175.6, 262.7)	117.8 (96.30, 134.2)	535, 285	75	0.0005***	0.0020**	0.53 Large
	CF vs TF	199.8 (168.8, 255.5)	130.7 (113.2, 153.4)	518, 302	92	0.0029**	0.0087**	0.46 Medium
	CM vs CF	219.0 (175.6, 262.7)	199.8 (168.8, 255.5)	414, 406	196	0.9201 ns	0.9201 ns	0.01 Small
	TM vs TF	117.8 (96.30, 134.2)	130.7 (113.2, 153.4)	374, 446	164	0.3408 ns	0.6816 ns	0.15 Small

Table 4.26. Within-group analysis of time allocation across three zones under MS (Mirror + Shoal) condition using Friedman test (Figure c).

Within-group comparisons were performed using the Friedman test followed by the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (FDR $Q = 0.05$). Effect size is reported as Kendall's W .

Group	Friedman Stat.	p	ES	Comp.	Rank Sum Diff.	p	q
CM	14.80	0.0006***	0.37 Medium	M vs I	-2	0.7518	0.2631
				M vs S	-22	0.0005	0.0005
				I vs S	-20	0.0016	0.0008
TM	0.7000	0.7047ns	0.01 Very Small	M vs I	4	0.5271	0.8302
				M vs S	-1	0.8744	0.9181
				I vs S	-5	0.4292	0.8302
CF	16.90	0.0002***	0.42 Medium	M vs I	1	0.8744	0.3060
				M vs S	-22	0.0005	0.0003
				I vs S	-23	0.0003	0.0003
TF	1.900	0.3867ns	0.04 Very Small	M vs I	-8	0.2059	0.4227
				M vs S	-7	0.2684	0.4227
				I vs S	1	0.8744	0.9181

4.2.5.1 Role of Conspecific Buffering in Mirror-Induced Aggression

This section discusses the influence of conspecifics in buffering mirror-induced aggression within control male, female, isolated male, and isolated female groups separately.

In the MS condition, CM exhibited significant reductions in mirror bites, time spent in the mirror zone, and time spent in the internal zone, along with increased time spent in the shoal zone compared to MO, indicating a true sign of conspecific buffering of aggression within male zebrafish.

Interestingly, isolated males also showed reductions in mirror bites and time spent in the mirror zone, along with higher time spent in the shoal zone in MS relative to MO. Time spent in the internal zone in MS was significantly higher than MO. Considering the pattern of time allocation within MS, this suggests that TM was impacted by the presence of conspecifics by reducing mirror bites, but not through a complete zone shift towards conspecifics.

CF showed significantly lower mirror bites, lower time spent in the mirror zone, and higher time spent in the shoal zone in MS relative to MO. CF also showed increased time spent in the internal zone in MS relative to MO. Considering the zone allocation pattern with higher affinity towards the shoal zone in MS, the behavioural response of CF indicates a true conspecific buffering effect under mirror-induced aggression.

TF responded differently than TM, with no significant differences in mirror bites and time spent in the internal zone in MS relative to MO. TF showed a shift in zone allocation, with significantly lower time spent in the mirror zone and higher time spent in the shoal zone in MS relative to MO. However, considering the largely equal distribution of time allocation across the three zones under MS, the behavioural response of TF suggests that buffering was less effective or incomplete.

Although the statistical comparisons were described in Results **4.2.5.1** as per Table No. 4.27, the data are represented as individual scatter plots of mirror bite difference scores (MO-MS) in Figure 4.19, with red indicating higher mirror bites in MO and

blue indicating higher mirror bites in MS. Zone-wise time allocation across all groups under MO and MS is further illustrated using pie charts (Figure 4.20).

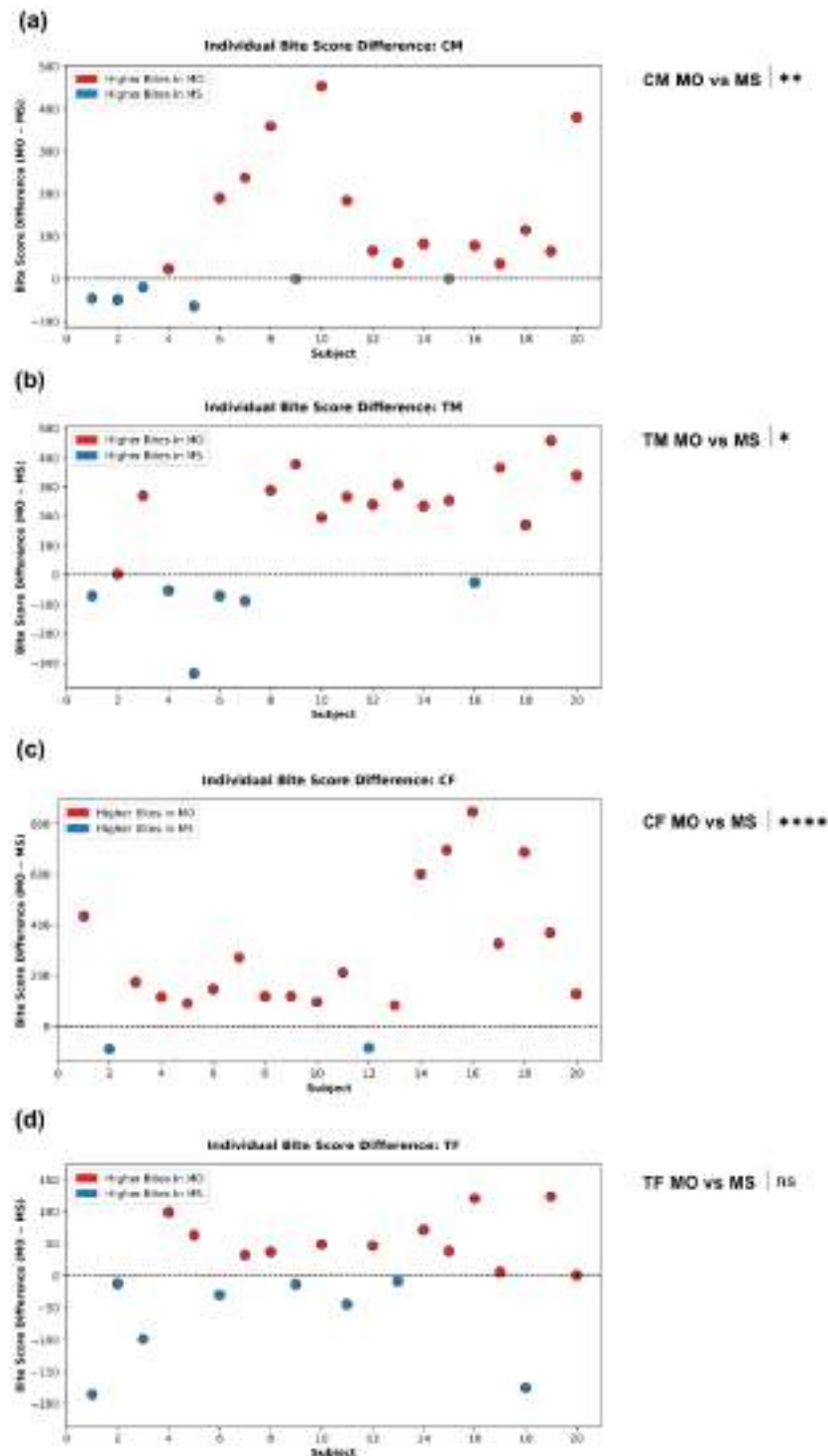


Figure 4.19. Individual mirror bite difference scores (MO-MS).

Scatter plots showing individual mirror bite difference scores (MO–MS) for (a) CM, (b) TM, (c) CF, and (d) TF. Each point represents one fish ($n = 20$ per group). The Y-axis shows the difference score (MO minus MS). Values above zero (red points) indicate higher mirror bites in MO. Values below zero (blue points) indicate higher mirror bites in MS. The dotted horizontal line at zero represents no change between conditions.

Statistical significance (MO vs MS) is indicated adjacent to each panel (ns = non-significant; asterisks denote level of significance). Statistical analyses were conducted on the raw mirror bite data; the difference plots are presented solely to visually illustrate the direction and magnitude of individual behavioural shifts.

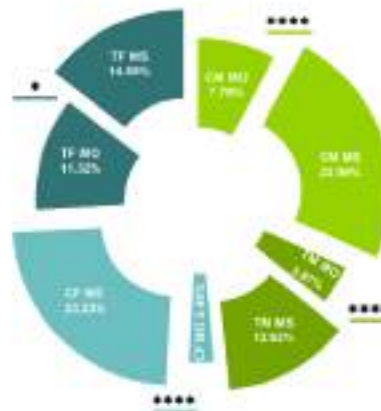
This representation allows visualization of: Consistency of behavioural change across individuals, directionality of aggression modulation, and within-group buffering patterns under MS relative to MO.



(a) Time Spent (%): Mirror Zone (MO vs MS)



(b) Time Spent (%): Internal Zone (MO vs MS)



(c) Time Spent (%): Shoal Zone (MO vs MS)

Figure 4.20. Zone-wise percentage allocation under MO and MS (exploded pie charts).

Exploded pie charts illustrating percentage time allocation across zones under Mirror Only (MO) and Mirror + Shoal (MS) conditions for CM, TM, CF, and TF.

Panels represent: (a) Mirror zone (aggression zone), (b) Internal zone, and (c) Shoal zone (buffering zone)

Time spent in each zone was converted to percentage values to standardize comparisons across groups. Within each panel: Each group has two adjacent segments representing MO and MS. Identical colours within a group correspond to MO and MS for that specific group. Percentage values are displayed within each segment. Statistical comparison lines between MO and MS within each group indicate whether zone allocation significantly changed (ns = non-significant; asterisks denote significance level). Compare MO vs MS within the same group to evaluate buffering shifts. Larger MS shoal-zone percentages indicate stronger conspecific affiliation. Reductions in mirror-zone percentage under MS reflect aggression buffering. Internal-zone changes indicate redistribution rather than direct buffering.

These pie charts provide a proportional visualization of spatial redistribution patterns under aggression buffering conditions.

Table 4.27. Pairwise analysis of mirror-induced aggression and conspecific buffering across experimental groups.

Within-group comparisons between Mirror Only (MO) and Mirror + Shoal (MS) conditions were performed using the Mann–Whitney U test. Effect size is reported as *r* and interpreted as Small (<0.3), Medium (0.3–0.5), and Large (>0.5).

Group	Parameter	Comparison	Sum of Ranks	U	p value	ES
CM	Mirror Bites (count)	MO vs MS	524.5, 295.5	85.5	0.0014 **	0.49 Medium
	Time Spent in M zone (s)		561, 259	49	<0.0001 ****	0.64 Large
	Time Spent in I zone (s)		530, 290	80	0.0008 ***	0.51 Large
	Time Spent in D/S zone (s)		238, 582	28	<0.0001 ****	0.73 Large
TM	Mirror Bites (count)	MO vs MS	492.5, 327.5	117.5	0.0249 *	0.35 Medium
	Time Spent in M zone (s)		600, 220	10	<0.0001 ****	0.81 Large
	Time Spent in I zone (s)		232, 588	22	<0.0001 ****	0.76 Large
	Time Spent in D/S zone (s)		236, 584	26	<0.0001 ****	0.74 Large
CF	Mirror Bites (count)	MO vs MS	571, 249	39	<0.0001 ****	0.69 Large
	Time Spent in M zone (s)		606, 214	4	<0.0001 ****	0.84 Large
	Time Spent in I zone (s)		305, 515	95	0.0038 **	0.45 Medium
	Time Spent in D/S zone (s)		215, 605	5	<0.0001 ****	0.83 Large
TF	Mirror Bites (count)	MO vs MS	463, 357	147	0.1550 ns	0.22 Small
	Time Spent in M zone (s)		518, 302	92	0.0029 **	0.46 Medium
	Time Spent in I zone (s)		400, 420	190	0.7994 ns	0.04 Small
	Time Spent in D/S zone (s)		319, 501	109	0.0132 *	0.39 Medium

4.2.6 Social Affinity Shifts Under Buffering (SO vs PS & SO vs MS)

A Kruskal–Wallis test was conducted to identify differences in the social preference index (SPI) among the three stimulus conditions, namely SO, PS, and MS, within each group.

In CM, the test revealed a statistically significant difference in median ranks, $H(2) = 7.728$, $p = 0.0210$, with a mean rank score of 27.85 for SO, 39.15 for PS, and 24.50 for MS, and an observed effect size ($\eta^2 = 0.1$) indicating a medium magnitude of difference among the conditions. The highest median SPI was 0.5367 for PS, followed by 0.4108 for SO, and the lowest was 0.2172 for MS. Pairwise post hoc comparisons, conducted using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (with FDR correction applied), showed that the mean rank differences between SO and PS ($q = 0.0428$) and PS and MS ($q = 0.0168$) were statistically significant at $Q = 0.05$. The results indicate that the social preference of control males in SO was positively modulated in the PS.

In TM, the test revealed a statistically significant difference in median ranks, $H(2) = 30.20$, $p < 0.0001$, with a mean rank score of 23.60 for SO, 47.90 for PS, and 20.00 for MS, and an observed effect size ($\eta^2 = 0.49$) indicating a large magnitude of difference among the conditions. The highest median SPI was 0.03344 for PS, followed by -0.2648 for SO, and the lowest was -0.3454 for MS. Pairwise post hoc comparisons, conducted using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (with FDR correction applied), showed that the mean rank differences between SO and PS ($q < 0.0001$) and PS and MS ($q < 0.0001$) were statistically significant at $Q = 0.05$. The results indicate that the social preference of test males in SO was positively modulated in the PS, whereas in MS it declined relative to PS.

In CF, the test revealed a statistically significant difference in median ranks, $H(2) = 29.00$, $p < 0.0001$, with a mean rank score of 47.10 for SO, 18.40 for PS, and 26.00 for MS, and an observed effect size ($\eta^2 = 0.47$) indicating a large magnitude of difference among the conditions. The highest median SPI was 0.8763 for SO, followed by 0.1098 for MS, and the lowest was -0.008655 for PS. Pairwise post hoc comparisons, conducted using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (with FDR correction applied), showed that the mean rank

differences between SO and PS ($q < 0.0001$) and SO and MS ($q < 0.0001$) were statistically significant at $Q = 0.05$. The results indicate that in CF, the SPI values for PS and MS were markedly decreased from SO. SPI values were highest under SO and comparatively lower under PS and MS.

In TF, the test revealed a statistically significant difference in median ranks, $H(2) = 9.780$, $p = 0.0075$, with a mean rank score of 29.10 for SO, 39.75 for PS, and 22.65 for MS, and an observed effect size ($\eta^2 = 0.14$) indicating a medium magnitude of difference among the conditions. The highest median SPI was -0.01279 for PS, followed by -0.1805 for SO, and the lowest was -0.2740 for MS. Pairwise post hoc comparisons, conducted using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (with FDR correction applied), showed that the mean rank difference between PS and MS ($q = 0.0041$) was statistically significant at $Q = 0.05$. The results indicate that in TF, the SPI significantly declined from PS to MS, indicating reduced social affinity under MS relative to PS.

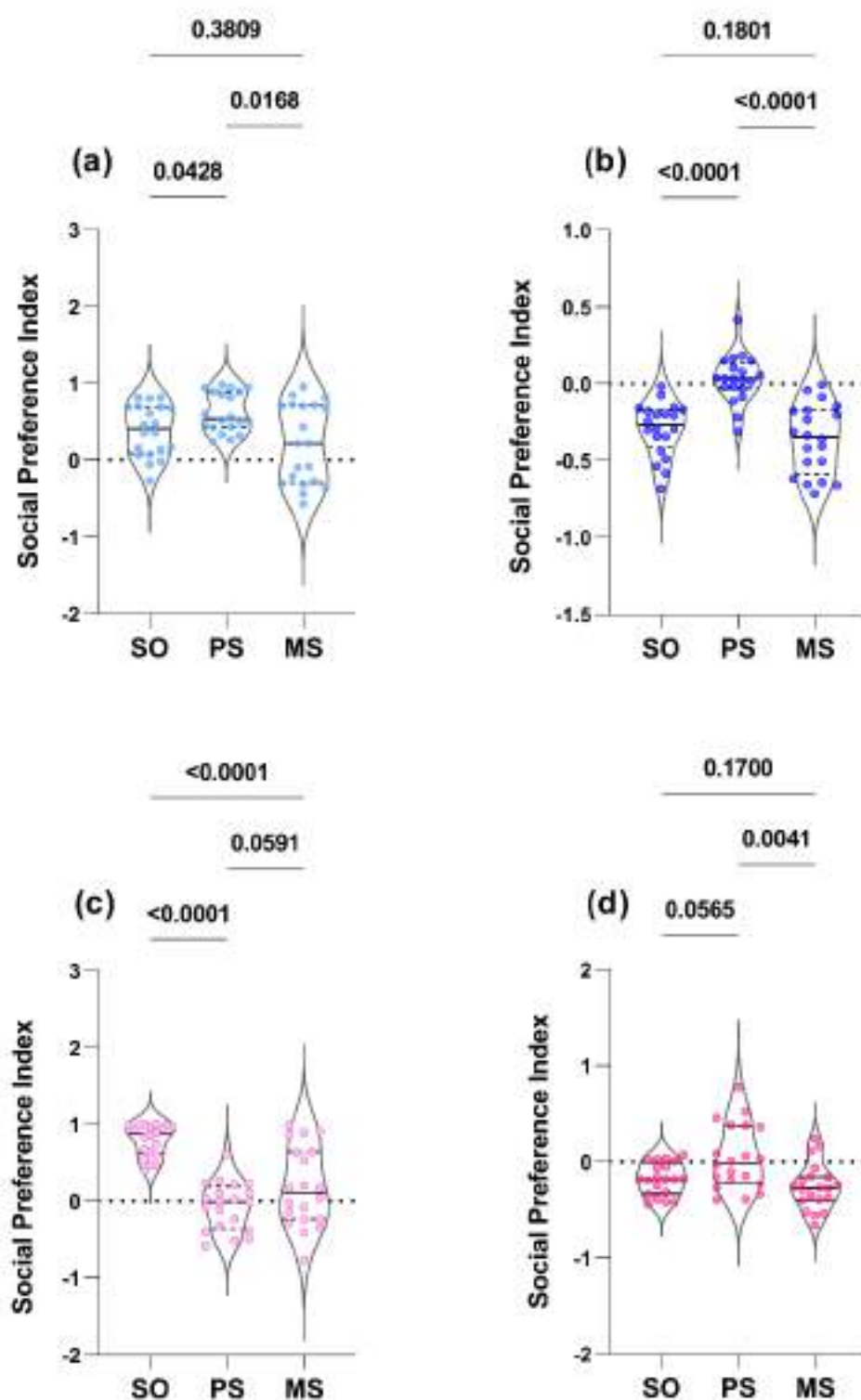


Figure 4.21. Social Preference Index (SPI) across SO, PS, and MS.

Violin plots showing Social Preference Index (SPI) across three social conditions: SO (Shoal Only), PS (Predator + Shoal), and MS (Mirror + Shoal)

Panels represent: (a) CM, (b) TM, (c) CF, and (d) TF

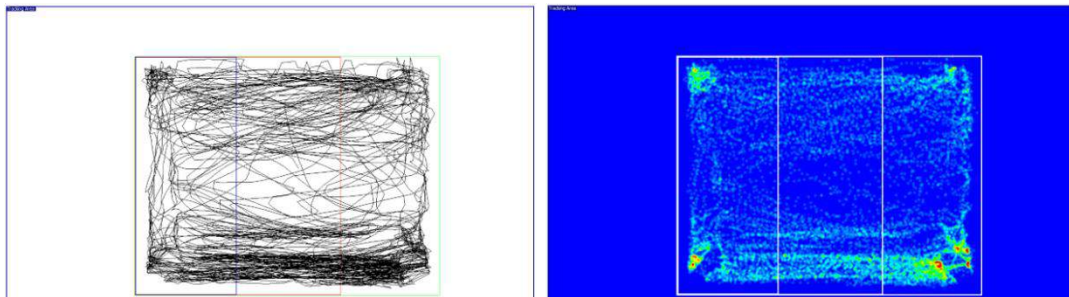
Individual data points were represented ($n = 20$ per group). The central line represents the median. Violin width reflects distribution density. The dotted horizontal line at zero indicates neutral preference. Statistical comparisons (Kruskal–Wallis followed by FDR-corrected post hoc testing) are displayed above the violins. Both significant and non-significant pairwise differences are marked with corresponding q values.

SPI was calculated as the difference between time spent in the shoal zone and time spent in the non-shoal zone(s), divided by the total observation time (360s).

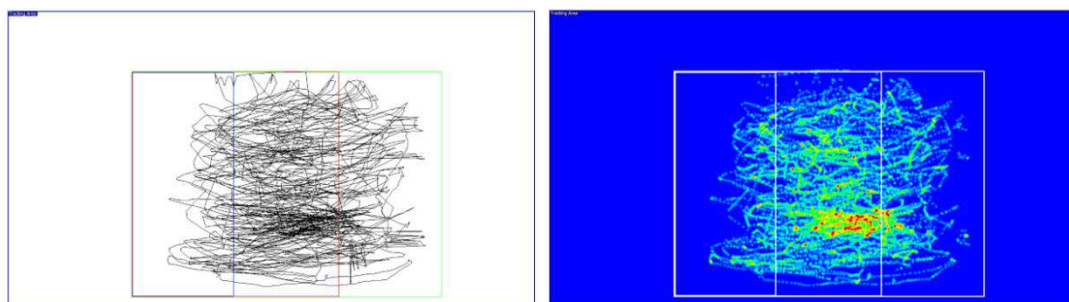
The definition of non-shoal zones varied by condition: In SO, the non-shoal zone refers to the internal (neutral) zone and the distant zone. In PS, the non-shoal zones include the predator zone and the internal zone. In MS, the non-shoal zones include the mirror zone and the internal zone.

Positive SPI values indicate social preference toward the shoal stimulus. Negative SPI values indicate avoidance or reduced affiliation. Differences between SO, PS, and MS reflect modulation of social affinity under stress (PS) and aggression (MS) buffering contexts.

(a) Control Male: No Stimulus (NS)



(b) Test Male: No Stimulus (NS)



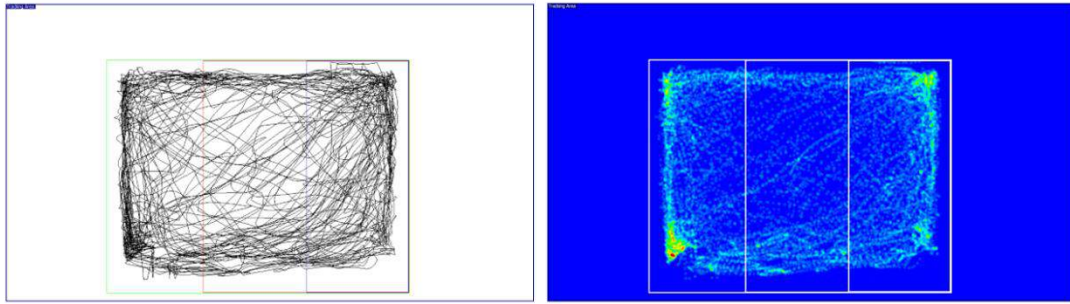
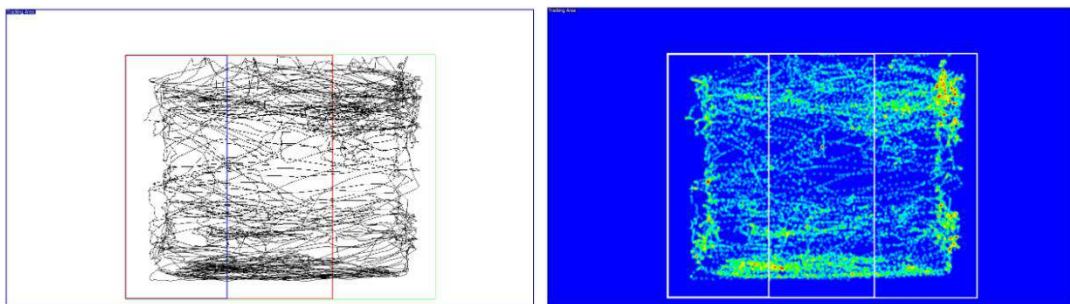
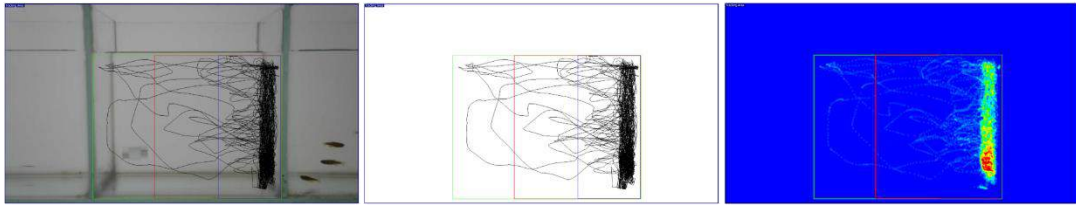
(c) Control Female: No Stimulus (NS)**(d) Test Female: No Stimulus (NS)**

Figure 4.22. Heat map representation of locomotor activity in the No-Stimulus (NS) condition

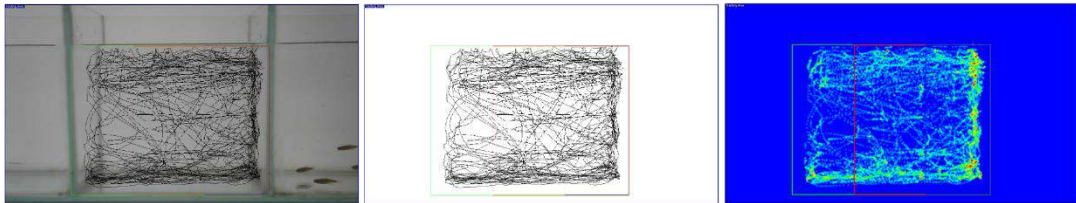
Heat map visualization of zebrafish locomotor activity in the three-chamber apparatus under the No-Stimulus (NS) condition, arranged in the order (a) Control Male (CM), (b) Test Male (TM), (c) Control Female (CF), and (d) Test Female (TF). Heat maps were generated using SMART 3.0 tracking software.

Across all groups, locomotor activity was distributed across the chamber without a pronounced side bias, indicating the absence of directional preference when no stimulus was present. However, TM exhibited relatively higher activity within the internal zone, suggesting increased exploratory behaviour compared to the other groups.

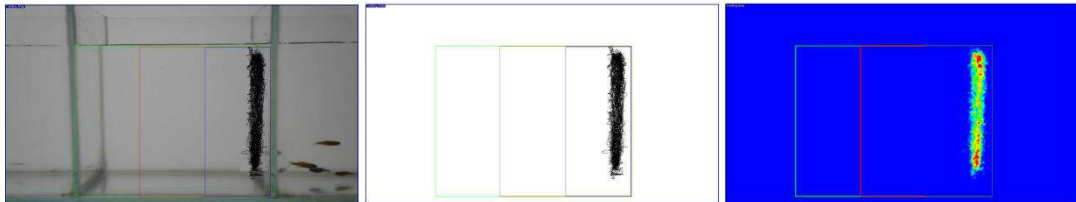
(a) Control Male: Shoal Only (SO)



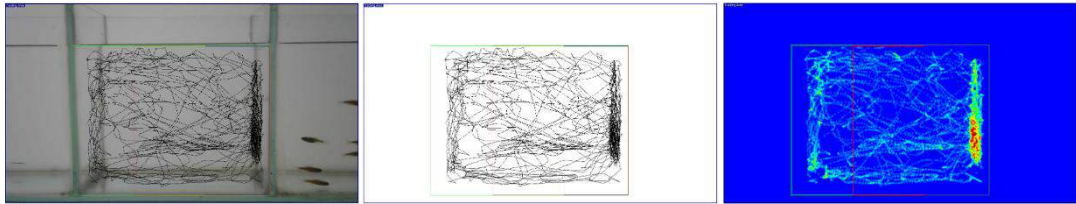
(b) Test Male: Shoal Only (SO)



(c) Control Female: Shoal Only (SO)



(d) Test Female: Shoal Only (SO)

**Figure 4.23. Heat map representation of shoaling preference**

Heat map showing the spatial distribution of zebrafish activity during the shoaling test in the order (a) CM, (b) TM, (c) CF, and (d) TF.

In this paradigm, the green zone represents the distant zone, whereas the blue zone represents the shoal stimulus zone.

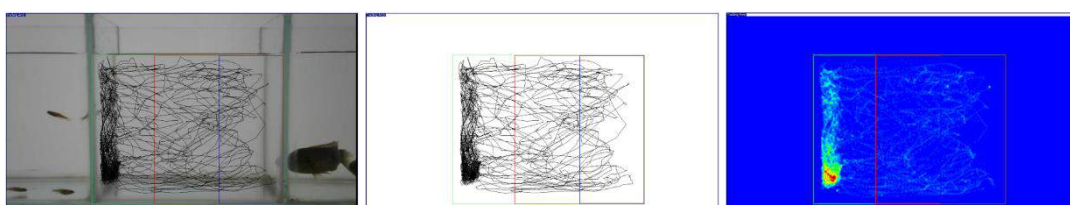
All groups demonstrated social preference by spending more time near the shoal zone, although behavioural patterns differed between control and test groups. Control groups showed enhanced preference toward the shoal stimulus, whereas test groups displayed relatively reduced shoaling tendency, suggesting a mild social deficit compared to controls.

- **Panel 1**

(a) Control Male: Predator Only (PO)

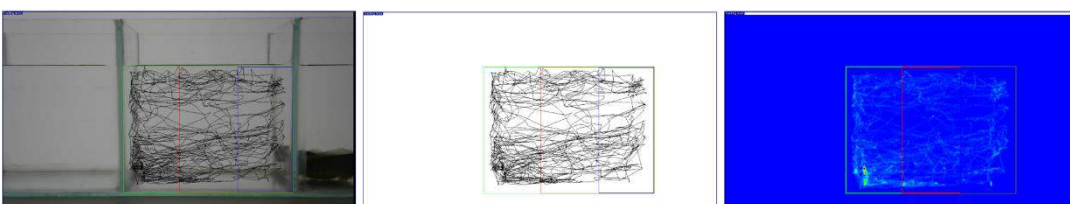


(b) Control Male: Predator + Shoal (PS)

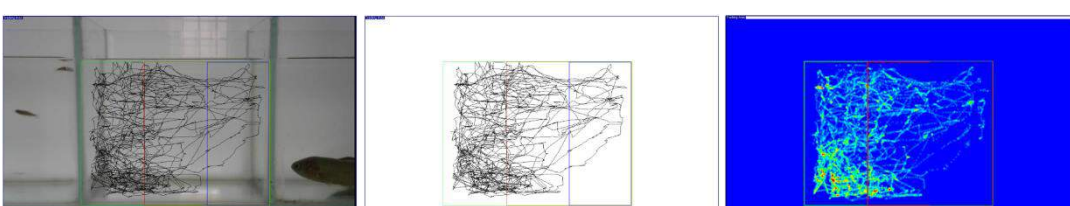


- **Panel 2**

(a) Test Male: Predator Only (PO)

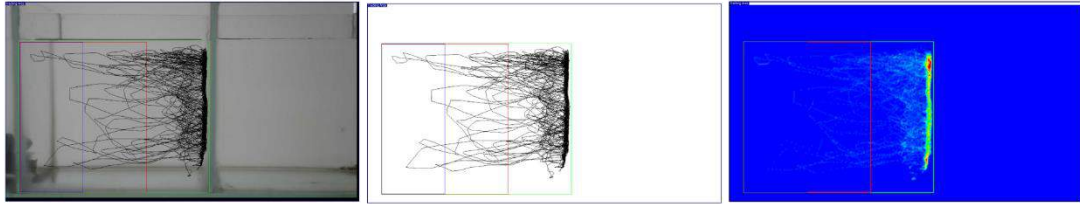


(b) Test Male: Predator + Shoal (PS)

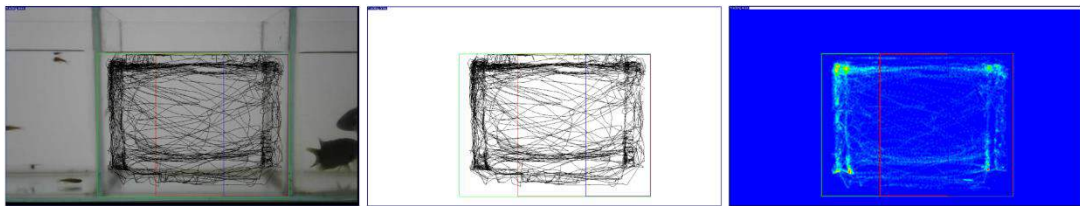


- **Panel 3**

(a) Control Female Predator Only (PO)

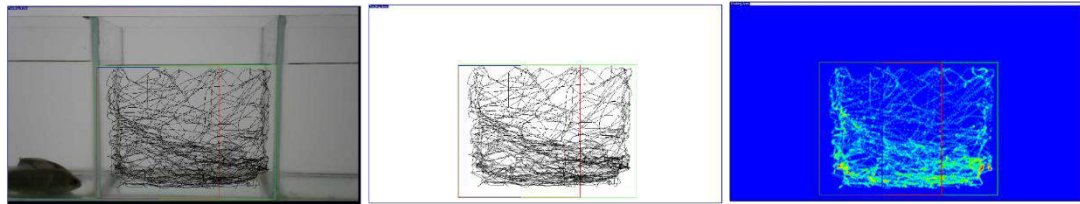


(b) Control Female Predator+ Shoal (PS)



- **Panel 4**

(a) Test Female Predator Only (PO)



(b) Test Female Predator + Shoal (PS)

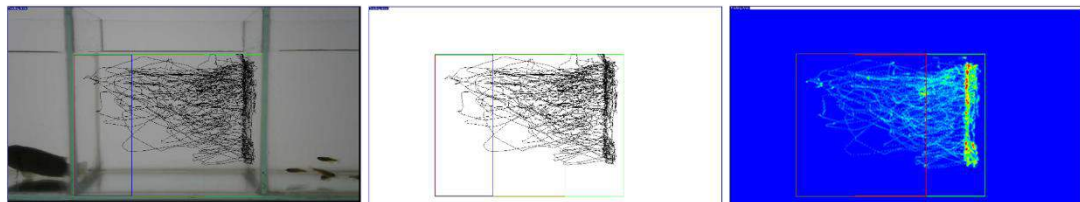


Figure 4.24. Heat map representation of Predator Only (PO) and Predator + Shoal (PS) conditions

Heat map representation of zebrafish locomotor activity under Predator Only (PO) and Predator + Shoal (PS) conditions. Within-group comparisons are arranged sequentially as Panel 1: (a) CM-PO and (b) CM-PS; Panel 2: (a) TM-PO and (b) TM-PS; Panel 3: (a) CF-PO and (b) CF-PS; Panel 4: (a) TF-PO and (b) TF-PS.

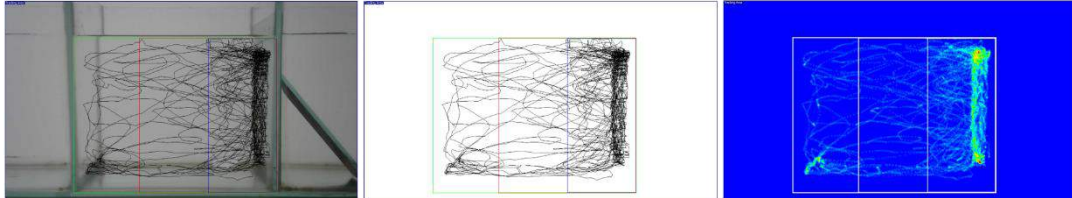
In the PO condition, the blue zone represents the predator zone, while the green zone represents the distant zone. CM and CF exhibited predator avoidance behaviour, spending more time in the distant zone away from the predator stimulus.

In contrast, TM and TF displayed increased exploratory activity across zones, indicating altered threat-avoidance responses.

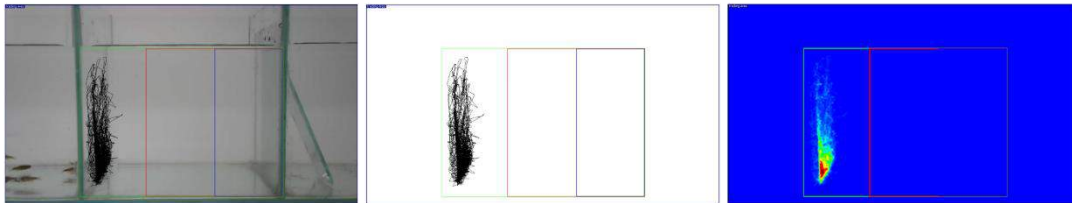
In the PS condition, the blue zone represents the shoal stimulus zone. CM demonstrated enhanced social preference, whereas CF showed increased exploration near the predator zone than PO. TM and TF exhibited social preference in PS.

- **Panel 1**

(a) Control Male Mirror Only (MO)

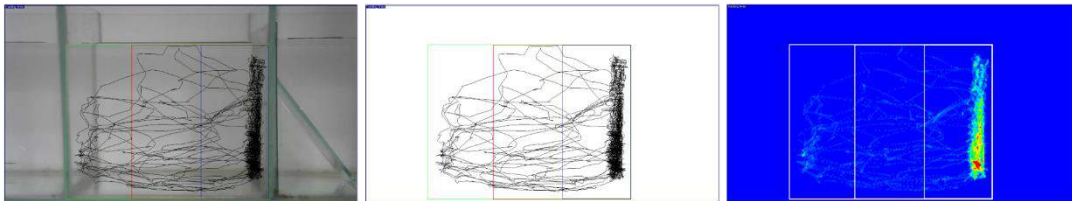


(b) Control Male Mirror + Shoal (MS)

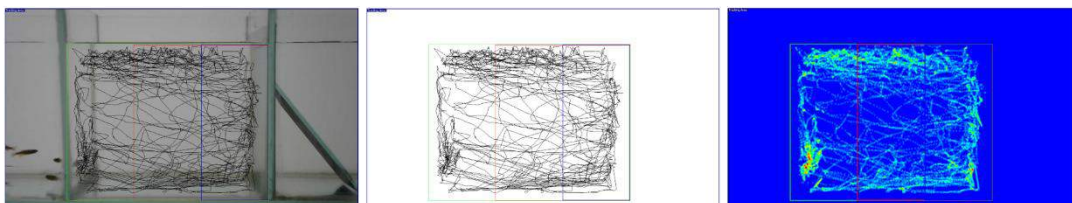


- **Panel 2**

(a) Test Male Mirror Only (MO)

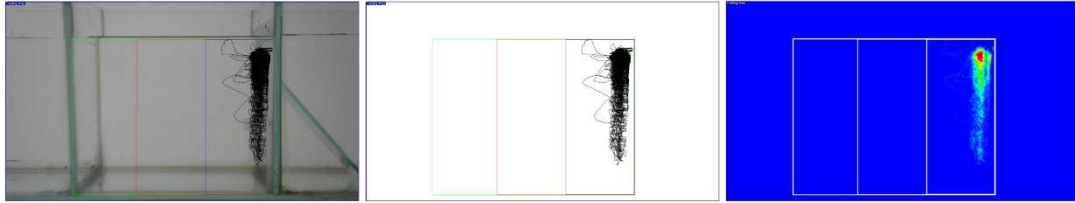


(b) Test Male Mirror + Shoal (MS)

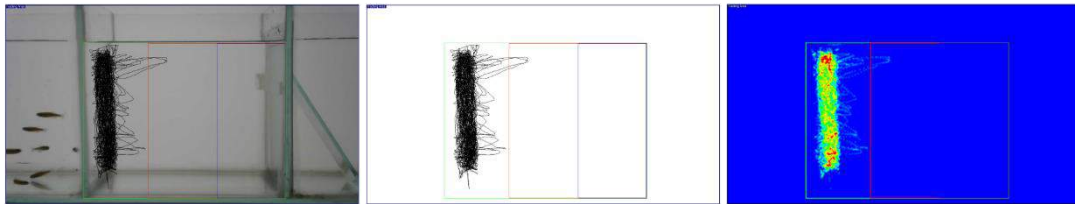


- **Panel 3**

(a) Control Female Mirror Only (MO)

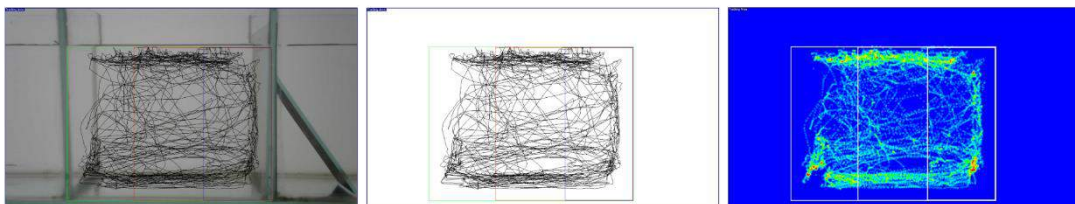


(b) Control Female Mirror + Shoal (MS)



- **Panel 4**

(a) Test Female Mirror Only (MO)



(b) Test Female Mirror + Shoal (MS)

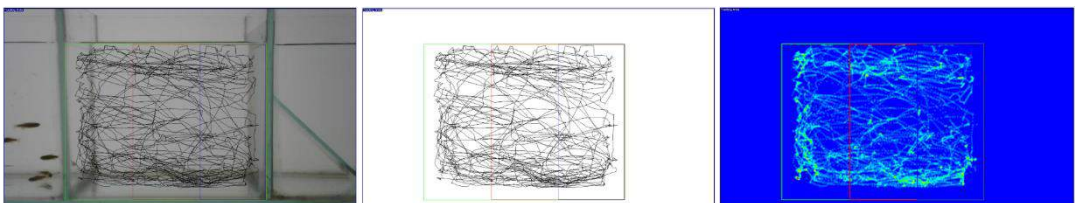


Figure 4.25. Heat map representation of Mirror Only (MO) and Mirror + Shoal (MS) conditions

Heat map visualization of zebrafish activity during Mirror Only (MO) and Mirror + Shoal (MS) conditions in the order CM, TM, CF, and TF.

Panel 1: (a) CM-MO and (b) CM-MS; Panel 2: (a) TM-MO and (b) TM-MS; Panel 3: (a) CF-MO and (b) CF-MS; Panel 4: (a) TF-MO and (b) TF-MS.

In MO, the blue zone represents the mirror zone, while the green zone represents the distant zone. CM and CF exhibited mirror-directed preference, with CF displaying higher mirror zone activity than CM. Among the test groups, TM showed greater mirror zone activity than TF, whereas TF exhibited exploratory behaviour across the three zones.

In MS, the green zone represents the shoal stimulus zone. CM and CF exhibited increased activity near the shoal zone, indicating enhanced social attraction. In contrast, TM and TF displayed broader exploratory behaviour with comparatively reduced shoal-directed preference.

For the mirror-based assays (MO and MS), the mirror stimulus was positioned adjacent to the chamber to obtain a complete reflective image of the fish. An auxiliary supporting mirror was used solely to stabilize the primary mirror and ensure proper alignment, thereby preventing distortion of the reflected stimulus and avoiding misinterpretation of behavioural responses.

4.3 Objective 3: Results

4.3.1 Objective Statement

The present objective section was to investigate the effect of chronic social isolation on shoaling organization, spatial structure, alignment dynamics, locomotor activity, and collective state transitions in adult zebrafish using descriptive and inferential statistics.

4.3.2 Chronic developmental isolation disrupts shoaling structure and induces hyperlocomotion

Alterations in the fundamental shoaling structure across multiple levels were observed in zebrafish subjected to chronic developmental isolation. The descriptive and inferential statistical results, including effect sizes, are explained in this section.

(1) Spatial Metrics

Shoal area and inter-individual distances were the major spatial metrics considered below.

Isolated shoals (ISO) maintained a significantly greater median shoal area (CON: 84.51 cm², [95% CI 71.69 - 105.5] vs ISO: 125.6 cm², [95% CI 111.8 - 144.3]; Mann-Whitney U = 86, p = 0.0016, r = 0.49, Medium), indicating greater spatial expansion and reduced social bonding within isolated shoals.

This reduced social bonding was further evidenced across three distance metrics. Isolated shoals exhibited increased mean Euclidean distances (CON: 10.55 cm, [95% CI 9.724, 11.52] vs ISO: 12.75 cm, [95% CI 11.93, 13.59]; Mann-Whitney U = 78, p = 0.0007, r = 0.52, Large), increased nearest neighbour distances (NND) (CON: 9.507

cm, [95% CI 8.740 - 10.51] vs ISO: 11.90 cm, [95% CI 10.87, 12.41]; Mann-Whitney $U = 81$, $p = 0.0009$, $r = 0.51$, Large), and increased farthest neighbour distances (FND) (CON: 11.97 cm, [95% CI 11.23, 12.71] vs ISO: 13.98 cm, [95% CI 13.02, 14.86]; Mann-Whitney $U = 80$, $p = 0.0008$, $r = 0.51$, Large).

Therefore, isolation disrupts the baseline spatial proximity observed in controls, causing shoal members to spread out and maintain larger distances from each other.

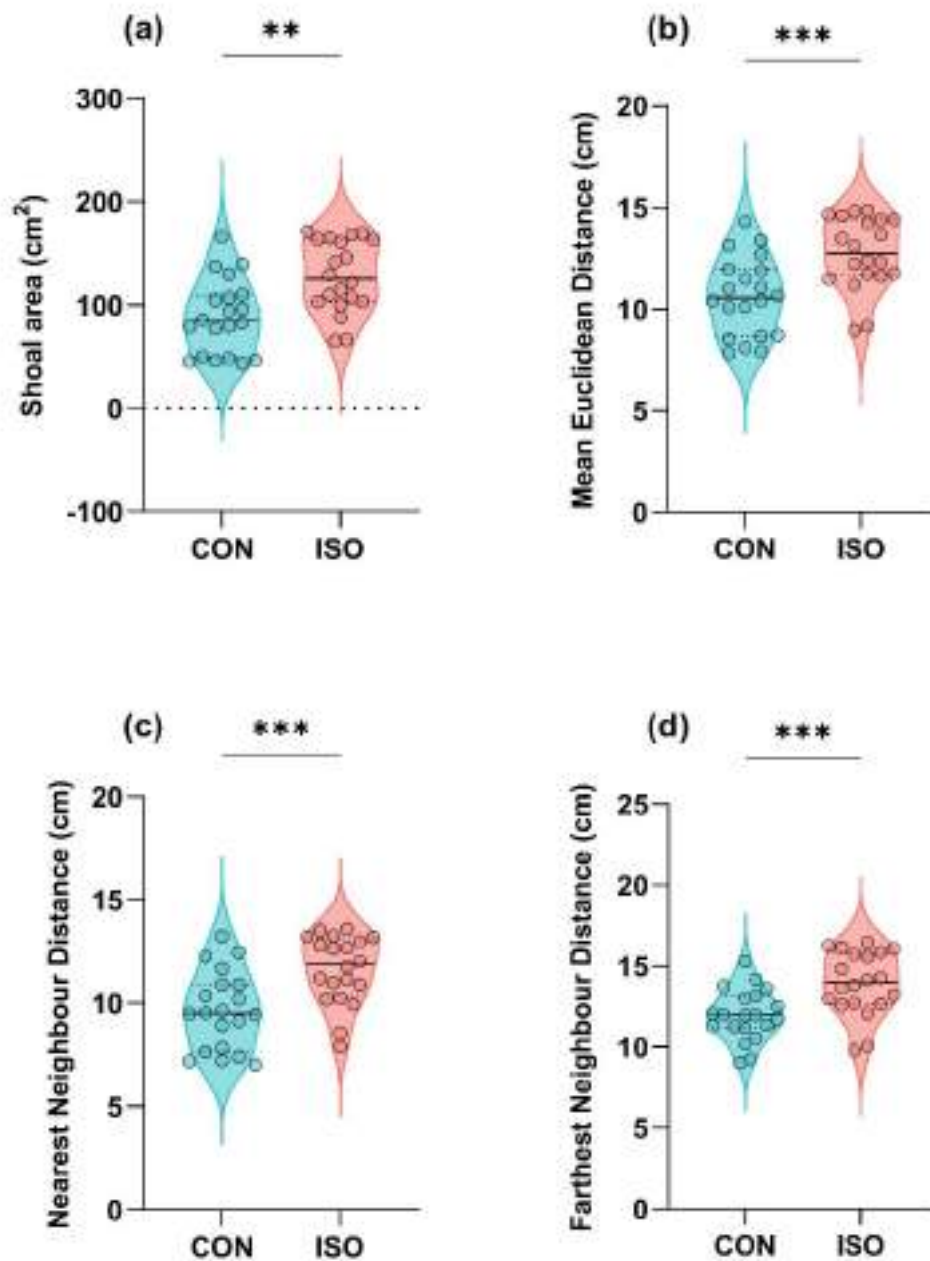


Figure 4.26. Spatial Metrics of Shoal Organization in Control and Isolated Groups

Violin plots depicting spatial cohesion parameters in control (CON) and isolated (ISO) shoals ($n = 20$ per group). Each violin illustrates the distribution of values with kernel density estimation, overlaid with individual data points to show within-group variability. Central lines indicate the median with 95% confidence intervals.

Panels are organized as follows: **(a)** Shoal area, **(b)** mean Euclidean distance, **(c)** mean nearest neighbour distance (NND), and **(d)** mean farthest neighbour distance (FND).

Across spatial metrics, isolated shoals demonstrate a general trend toward spatial expansion relative to controls. This pattern is reflected by increased shoal area and greater inter-individual distances, indicating reduced group compactness and weakened spatial cohesion following isolation exposure. In contrast, control shoals exhibit tighter clustering and reduced spacing among individuals, consistent with intact social organization.

(2) Orientation and Alignment Metrics

We analysed three orientation and alignment metrics: mean heading angle differences, mean cosine dissimilarity and mean heading correlation (r).

Heading angle differences for both CON and ISO shoals were clustered between 60-90° without wrap-around near 0° or 360°. This distribution permitted the use of the Mann-Whitney U test for statistical comparisons.

Mean heading angle differences were significantly higher in ISO compared to CON (CON: 79.59°, [95% CI 76.84 - 81.45] vs ISO: 83.13°, [95% CI 81.28 - 84.56]; Mann-Whitney $U = 108$, $p = 0.0119$, $r = 0.39$, Medium), indicating looser directional coordination compared to tightly aligned controls.

This alignment disruption was further evidenced by two additional alignment metrics. ISO shoals showed higher mean cosine dissimilarity (CON: 0.8252, [95% CI 0.7703 - 0.8579] vs ISO: 0.8996, [95% CI 0.8814 - 0.9179]; Mann-Whitney $U = 83$, $p = 0.0011$, $r = 0.49$, Medium) and lower mean heading correlation, r , (CON: 0.06449, [95% CI 0.05797 - 0.08219] vs ISO: 0.05064, [95% CI 0.03914 - 0.06214]; Mann-Whitney $U = 121$, $p = 0.0326$, $r = 0.34$, Medium).

Therefore, isolation leads to increased divergence in movement vectors and decreased temporal correlation in heading.

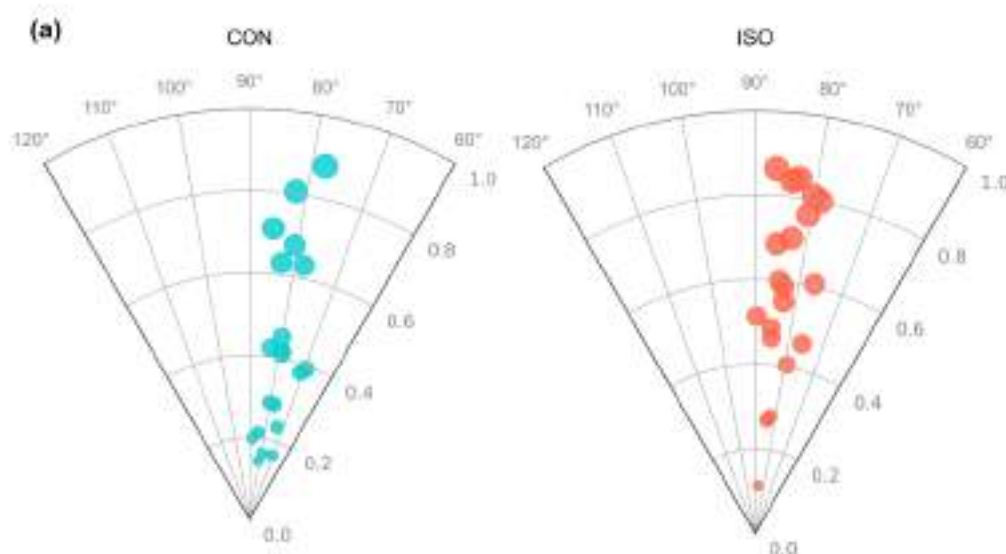
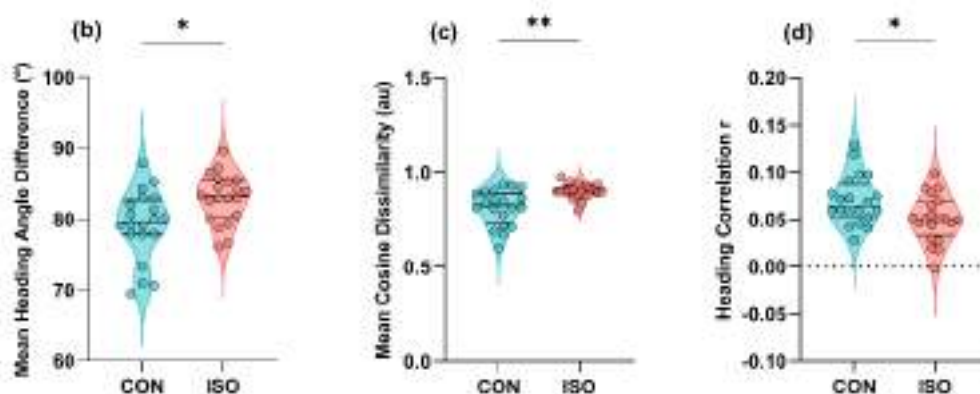


Figure 4.27. Orientation and Alignment Metrics in Control and Isolated Shoals

Violin plots and angular representations illustrating directional coordination in control (CON) and isolated (ISO) shoals ($n = 20$ per group). Individual data points are overlaid on violin distributions, which represent kernel density estimation. Central lines denote medians with 95% confidence intervals. Statistical comparisons were performed using the Mann–Whitney U test, as heading differences did not exhibit circular wrap-around behaviour (values clustered between 60–90°, with no observations near 0° or 360°).

(a) Polar Plot of Heading Angle Difference (°).

Half-polar plots depict the distribution of mean inter-individual heading angle differences. Radial lines originate from the centre (0.0), and the angular distribution curve spans approximately 60–120°. Control shoals show tighter clustering within a narrower angular range, indicating stronger directional alignment. In contrast, isolated shoals display a broader angular spread, reflecting increased divergence in movement vectors and reduced directional cohesion.



(b) Mean Heading Angle Difference (°).

Violin plots illustrate group-wise distributions of mean heading differences. Isolated shoals demonstrate a general upward shift in angular separation compared to controls, indicating reduced synchronization of movement direction.

(c) Mean Cosine Dissimilarity.

Cosine-based dissimilarity values quantify deviation in orientation between individuals (higher values indicate lower alignment). Isolated shoals show increased dissimilarity, consistent with weakened directional coherence.

(d) Heading Correlation Coefficient (r).

Temporal correlation of heading direction between individuals is shown as a measure of collective alignment strength. Control shoals exhibit stronger positive heading correlations, reflecting coordinated movement. Isolated shoals display comparatively reduced correlation, indicating diminished synchronization and disrupted social alignment.

(3) Speed Metrics

Speed metrics were analysed through shoal speed and shoal distance travelled (indicating coordinated movement as a unit), average individual speed and average individual distance travelled (representing mean values of individual activity independent of the shoal).

Both elevated shoal speed (CON: 4.110 cm/s, [95% CI 3.687 - 4.513] vs ISO: 5.263 cm/s, [95% CI 4.977 - 6.750]; Mann-Whitney $U = 88$, $p = 0.0020$, $r = 0.48$) and shoal distance travelled (CON: 1480 cm, [95% CI 1327 - 1625] vs ISO: 1895 cm, [95% CI 1792 - 2430]; Mann-Whitney $U = 88$, $p = 0.0020$, $r = 0.48$) were noted in the isolated shoal compared to the control with medium effect sizes.

The subsequent parameters, including average individual speed (CON: 7.349 cm/s, [95% CI 6.511 - 7.614] vs ISO: 9.717 cm/s, [95% CI 9.156 - 11.83]; Mann-Whitney $U = 50$, $p < 0.0001$, $r = 0.64$) and average individual distance travelled (CON: 2646 cm, [95% CI 2344 - 2741] vs ISO: 3498 cm, [95% CI 3296 - 4259]; Mann-Whitney $U = 50$, $p < 0.0001$, $r = 0.64$), also showed a significant increase in isolated shoals, with large effect sizes.

Speed metrics were further expanded with the analysis of freezing episodes and rapid movements in isolated and control shoals, where isolated shoals exhibited fewer freezing episodes (CON: 0.7500 /s, [95% CI 0.6963 - 1.033] vs ISO: 0.4656 /s, [95% CI 0.3917 - 0.6005]; Mann-Whitney $U = 78$, $p = 0.0006$, $r = 0.52$) and more rapid movements (CON: 2.475 /s, [95% CI 2.100 - 2.729] vs ISO: 3.317 /s, [95% CI 2.972 - 3.544]; Mann-Whitney $U = 69$, $p = 0.0002$, $r = 0.56$), both with large effect sizes compared to controls.

Correlation analysis was conducted within isolated and control conditions between freezing and rapid movements. Isolated shoals exhibited a weak negative correlation (Spearman's $\rho = -0.07068$, 95% CI -0.5081 to 0.3958) that was statistically non-significant ($p = 0.7672$), in contrast to the strong, statistically significant ($p < 0.0001$) negative (Spearman's $\rho = -0.8120$, 95% CI -0.9250 to -0.5673) correlation observed in the control shoal.

Together, increased individual-level speed and distance indicate heightened individual activity in ISO, thereby elevating shoal-level speed metrics and reflecting hyperactive group movement, independent of shoal coordination. Impaired behavioural organization and dysregulated movement patterns were observed from the weak correlation between freezing and rapid episodes in ISO.

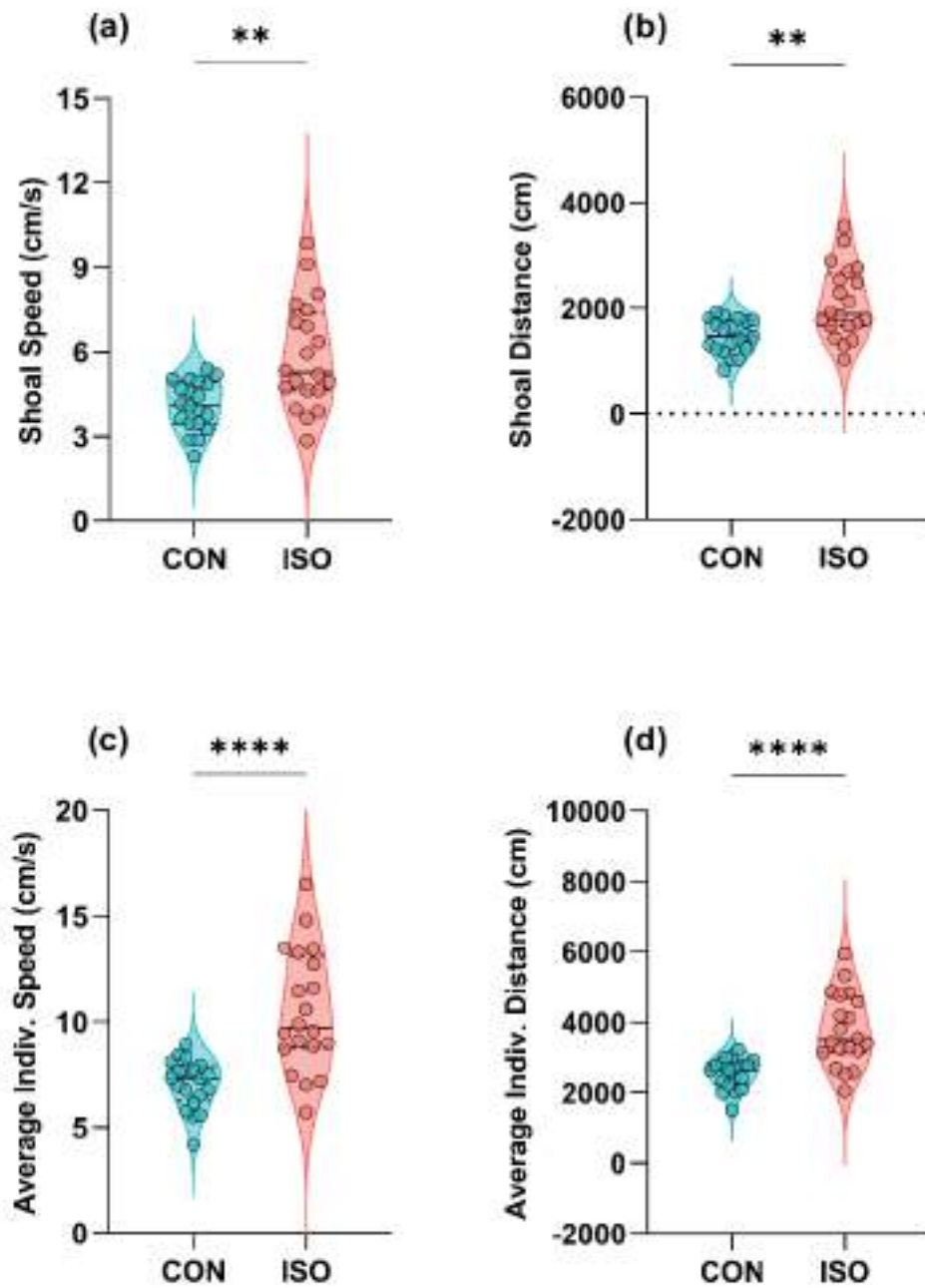


Figure 4.28. Speed Metrics I: Shoal- and Individual-Level Locomotor Activity

Violin plots showing locomotor parameters in control (CON) and isolated (ISO) shoals (n = 20 per group). Distributions are presented with overlaid individual data points; central lines represent medians with 95% confidence intervals.

Panels:

- (a) Shoal-level mean speed,
- (b) Shoal-level total distance travelled,
- (c) Individual-level average speed,
- (d) Individual-level average distance travelled.

Overall, isolated shoals show elevated locomotor activity at both shoal and individual levels compared to controls, indicating increased movement output following isolation.

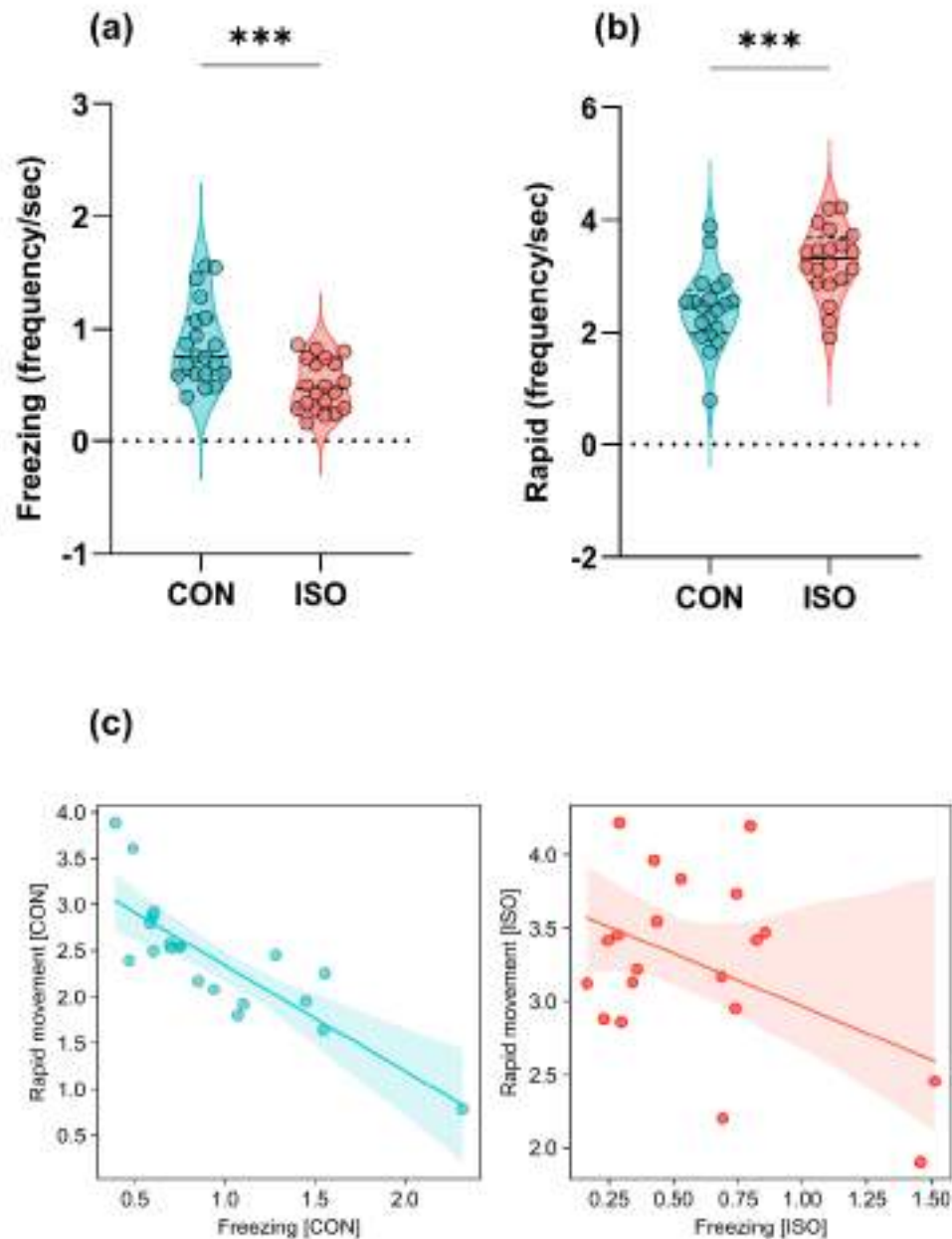


Figure 4.29. Speed Metrics II: Freezing and Rapid Movement Dynamics

Violin plots and correlation analysis of movement state transitions in control (CON) and isolated (ISO) shoals ($n = 20$ per group).

Panels:

- (a) Freezing frequency (events/s; violin plot),
- (b) Rapid movement frequency (events/s; violin plot),

(c) Correlation plots (separate for CON and ISO) with freezing frequency on the x-axis and rapid movement frequency on the y-axis.

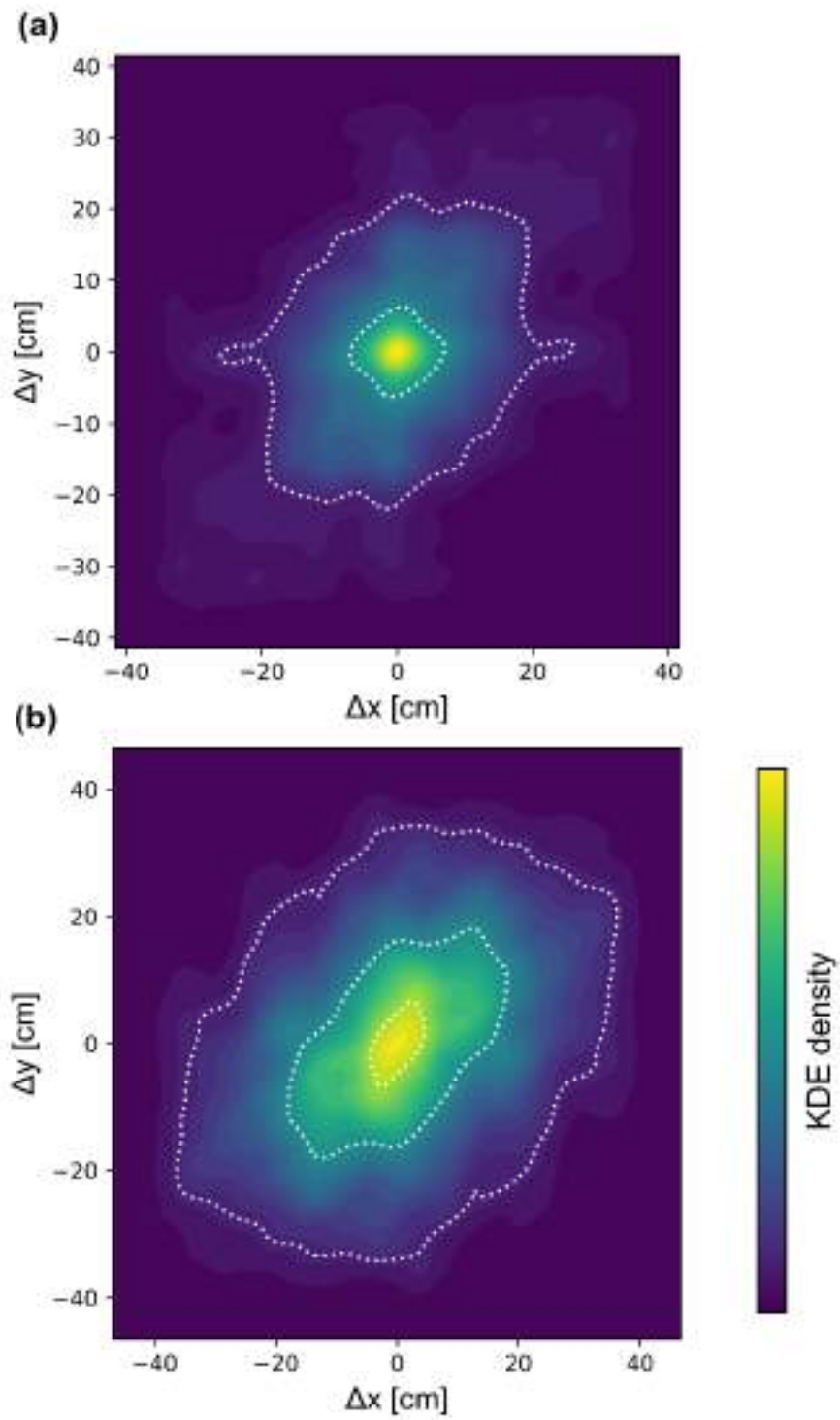
Isolated shoals display reduced freezing and increased rapid movements. Control shoals show a clear negative relationship between freezing and rapid states, whereas this relationship appears attenuated in isolated shoals.

(4) Representative Neighbour Density and Heading Alignment Maps

Neighbour Density Map: The focal fish was positioned at [0,0] pointing upwards, and the map showed density of the neighbouring fish relative to focal fish. Control shoals exhibited smaller kernel density estimation (KDE) distribution circles, while isolated shoals showed larger KDE distribution circles.

Heading Alignment Map: The map showed the alignment of neighbouring fish relative to focal fish motion. The colour scale zero indicates perfect alignment. Control shoals showed smaller alignment values, while isolated shoals showed larger alignment values.

Taken together, isolated shoals showed expanded neighbour density and higher directional misalignment compared to controls, reflecting altered shoal structure.



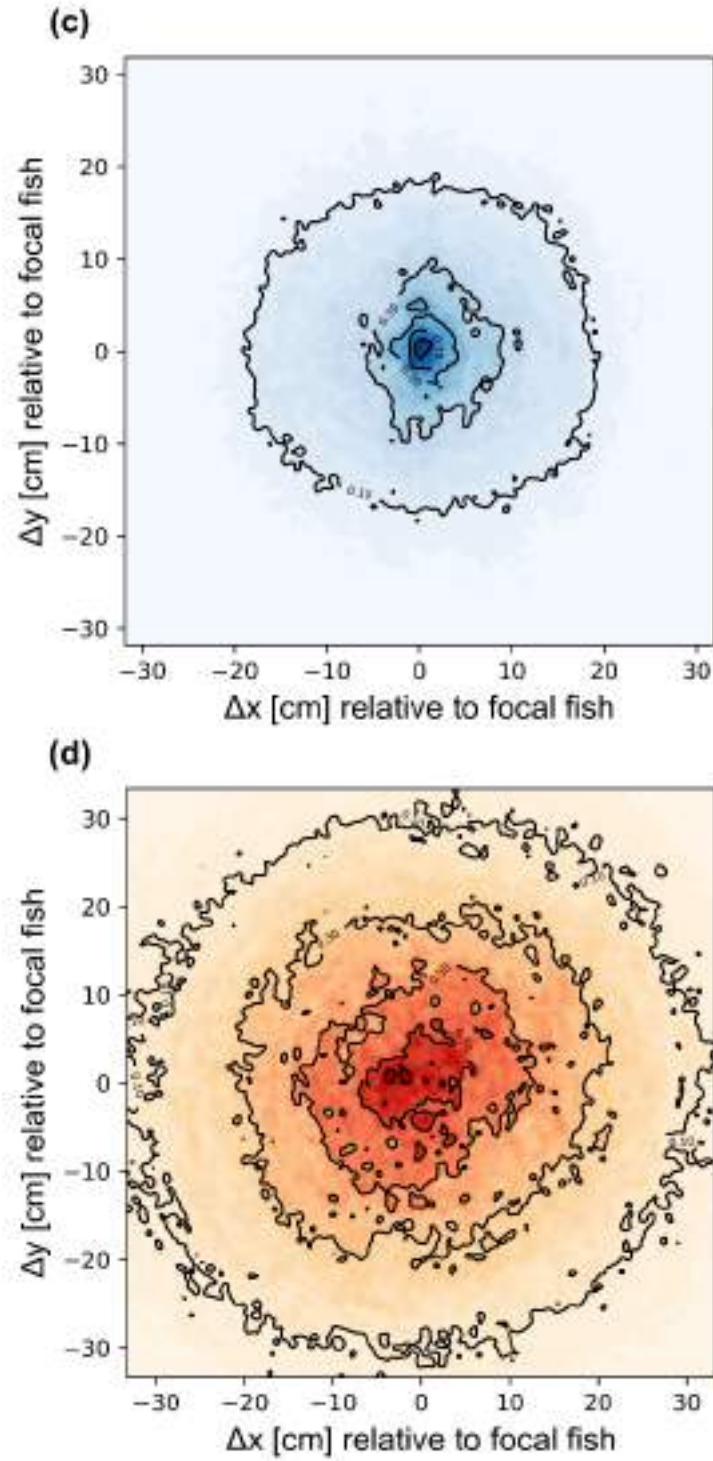


Figure 4.30. Representative Neighbour Density [(a) Control Shoal and (b) Isolated Shoal] and Heading Alignment Maps [(c) Control Shoal and (d) Isolated Shoal].

4.3.3 Dynamical model of Isolated Shoal - “The Tunstrom-Couzin model”

(1) Two Order Parameters and Collective States in Shoals

The collective emergent states within CON and ISO were evaluated using two order parameters, polarization (Op) and rotation (Or), followed by the Tunstrom-Couzin model. Density distribution heatmaps of Op and Or across shoals were observed with the presence of similar patterns of collective states involving milling, polarization and swarming.

However, the density heatmap indicated a shift in the distribution of Op and Or values between the two shoals. Therefore, the quantitative assessment of the fraction of time spent in each state was carried out both within and between groups. This was calculated as the proportion of total frames satisfying the threshold values of each collective state (the milling state (M) $Op < 0.35$ and $Or > 0.65$; polarized state (P) $Op > 0.65$ and $Or < 0.35$; and the swarm state (S) $Op < 0.35$ and $Or < 0.35$; transitional regime (T) - outside these ranges).

Although the within group analysis revealed the presence of all three collective states in two groups, the statistical significance varied in the test group compared to control shoal. In CON shoals, the Friedman test ($p < 0.0001$, $Fr = 53.34$) followed by Two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli pairwise comparisons revealed that, M vs P ($q < 0.0001$), M vs S ($q = 0.0116$), M vs T ($q < 0.0001$), P vs S ($q = 0.0013$), P vs T ($q = 0.0078$), S vs T ($q < 0.0001$). Whereas in ISO shoals, the Friedman test ($p < 0.0001$, $Fr = 46.98$) followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli pairwise comparisons revealed that, M vs P ($q = 0.0007$), M vs S ($q = 0.0496$), M vs T ($q < 0.0001$), P vs S ($q = 0.0278$), P vs T ($q = 0.0008$), S vs T ($q < 0.0001$). Together, these results indicate that, all three major dynamical states (M, P, and S) were observed in both CON and ISO shoals, but the intensity and dominance differed significantly.

We compared between groups to analyse the dominance and stability of fraction of time spent in each state across CON and ISO. Although a total of eight groups were present under the single parameter, the total number of selected multiple comparisons

was four. Therefore, we used Mann-Whitney U test followed by Holm Correction with $m = 4$.

Two important dynamical state shifts were evidenced: first, isolated shoals showed a marked increase in the milling behaviour (CON: 0.08071, [95% CI 0.07124 - 0.09323] vs ISO: 0.1081, [95% CI 0.09369 - 0.1270]; $U = 98$, $p = 0.0050$, Holm $p = 0.0200^*$, $r=0.43$), second, isolated shoals showed a significant reduction in polarization (CON: 0.2707, [95% CI 0.2456 - 0.3168] vs ISO: 0.2209, [95% CI 0.1948 - 0.2424]; $U = 100$, $p = 0.0060$, Holm $p = 0.020^*$, $r = 0.43$), compared to controls and both with medium effect sizes. Together, these reductions in polarization and increases in milling indicate a shift toward repetitive, circular motion of swimming and reduced collective order in ISO.

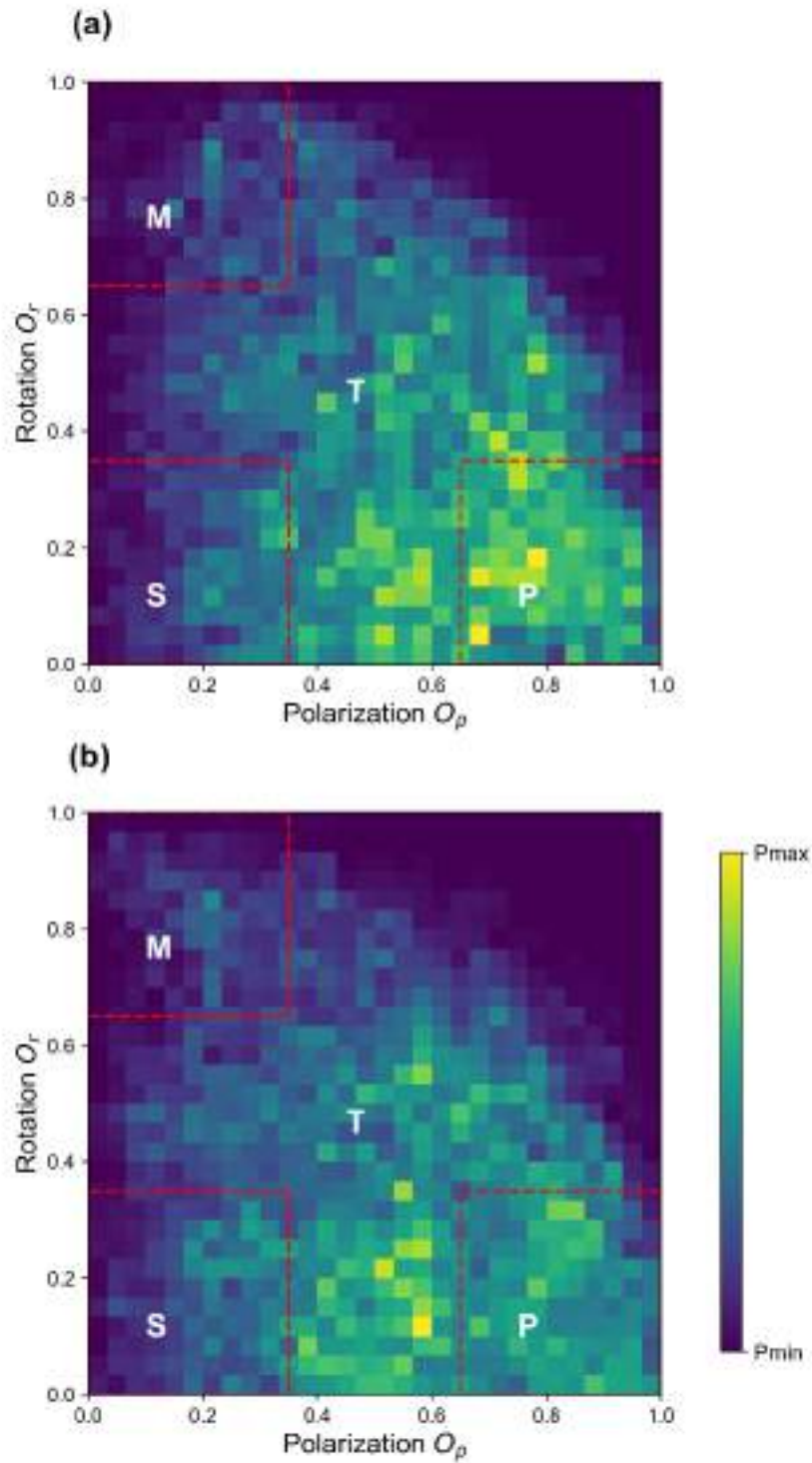


Figure 4.31 Order Parameters and Collective Phase Mapping of Shoal Behaviour and Fraction of Time Spent in Collective Behavioural States.

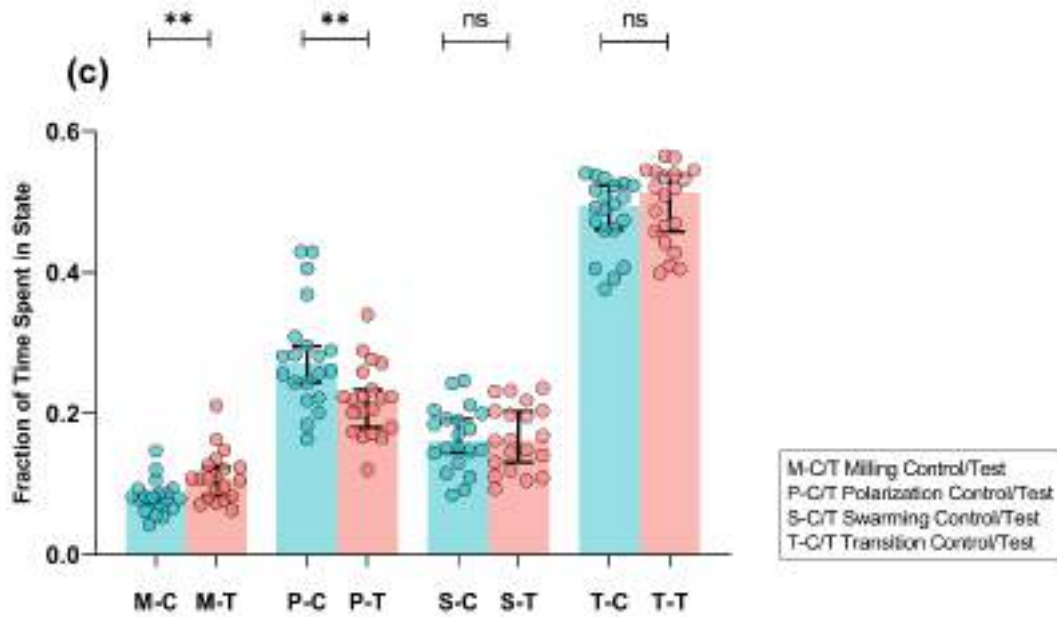
Phase-space density maps illustrating collective behavioural states in **(a)** control (CON) and **(b)** isolated (ISO) shoals ($n = 20$ per group).

Shoal-level collective dynamics were quantified using two order parameters: **Polarization (O_p)**, representing directional alignment and cohesive straight-line motion, and **Rotation (O_r)**, representing circular coherence characteristic of milling behaviour. Each video frame was projected into O_p – O_r phase space, and kernel density estimation (KDE) was applied to generate probability density heatmaps.

Collective states were operationally defined using threshold criteria:

- **Milling (M):** $O_p < 0.35$ and $O_r > 0.65$
- **Polarized (P):** $O_p > 0.65$ and $O_r < 0.35$
- **Swarm (S):** $O_p < 0.35$ and $O_r < 0.35$
- **Transitional (T):** values outside these defined regimes

Both control and isolated shoals occupied milling, polarized, and swarm states within the phase space. However, the density distribution and dominance of these states differed between groups, indicating altered collective organization following isolation exposure.



(c) Fraction of Time Spent in Collective Behavioural States

Column bar plots showing the fraction of time spent in each collective state by control (CON) and isolated (ISO) shoals ($n = 20$ per group). Individual data points are overlaid on each bar to illustrate within-group variability.

States are arranged sequentially as: Milling–Control (M-C), Milling–Isolated (M-ISO); Polarized–Control (P-C), Polarized–Isolated (P-ISO); Swarm–Control (S-C), Swarm–Isolated (S-ISO); Transitional–Control (T-C), Transitional–Isolated (T-ISO).

Isolated shoals spent a greater fraction of time in the milling state (higher O_r , lower O_p) and a reduced fraction in the polarized state compared to controls. This pattern reflects a shift from aligned collective motion toward more rotational group configurations. Overall, isolation biases collective dynamics toward less coordinated, circling-type states and away from sustained directional alignment.

(2) State Stability and Memory Time

Temporal stability of collective states was assessed using autocorrelation decay of polarization (O_p) and rotation (O_r) time series. This reflects the time each shoal maintains its current state before transitioning to another state. Isolated shoals showed reduced polarization (CON: 0.7400 s, [95% CI 0.6661 - 0.7919] vs ISO: 0.4800 s, [95% CI 0.4476 - 0.5324]; $U = 24$, $p < 0.0001$, $r = 0.75$) and rotational (CON: 0.7600 s, [95% CI 0.7286 - 0.9754] vs ISO: 0.5800 s, [95% CI 0.5317 - 0.7503]; $U = 80.50$, $p = 0.0008$, $r = 0.51$) memory time compared to controls. Together, these results indicate that isolated shoals exhibit less stable collective states overall and more frequent temporal variability across polarization, milling, and other states.

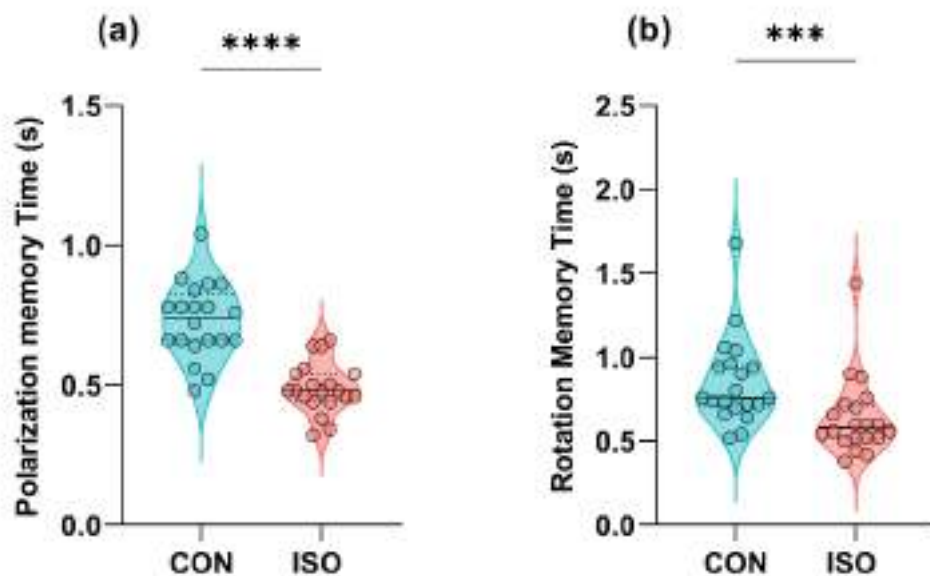


Figure 4.32. State Stability and Collective Memory Time

Violin plots illustrating the temporal stability of collective states in control (CON) and isolated (ISO) shoals ($n = 20$ per group). Individual data points are overlaid, and statistical significance is indicated on the plots.

Temporal stability was quantified using autocorrelation decay of the Polarization (O_p) and Rotation (O_r) time series. For each trial, the autocorrelation function $R(\tau)$ was computed across increasing time lags (τ). The characteristic decay time (“memory time”) was defined as the time lag at which autocorrelation decreased to $1/e$ (~ 0.37) of its initial value. This measure reflects the duration for which a shoal maintains a given collective organization before transitioning to another state.

Panels depict:

- (a) Polarization memory time,
- (b) Rotation memory time.

Isolated shoals exhibit shorter polarization and rotational memory times compared to controls, indicating reduced stability of both aligned and milling configurations. Overall, isolation is associated with diminished persistence of collective states and increased temporal variability in shoal organization.

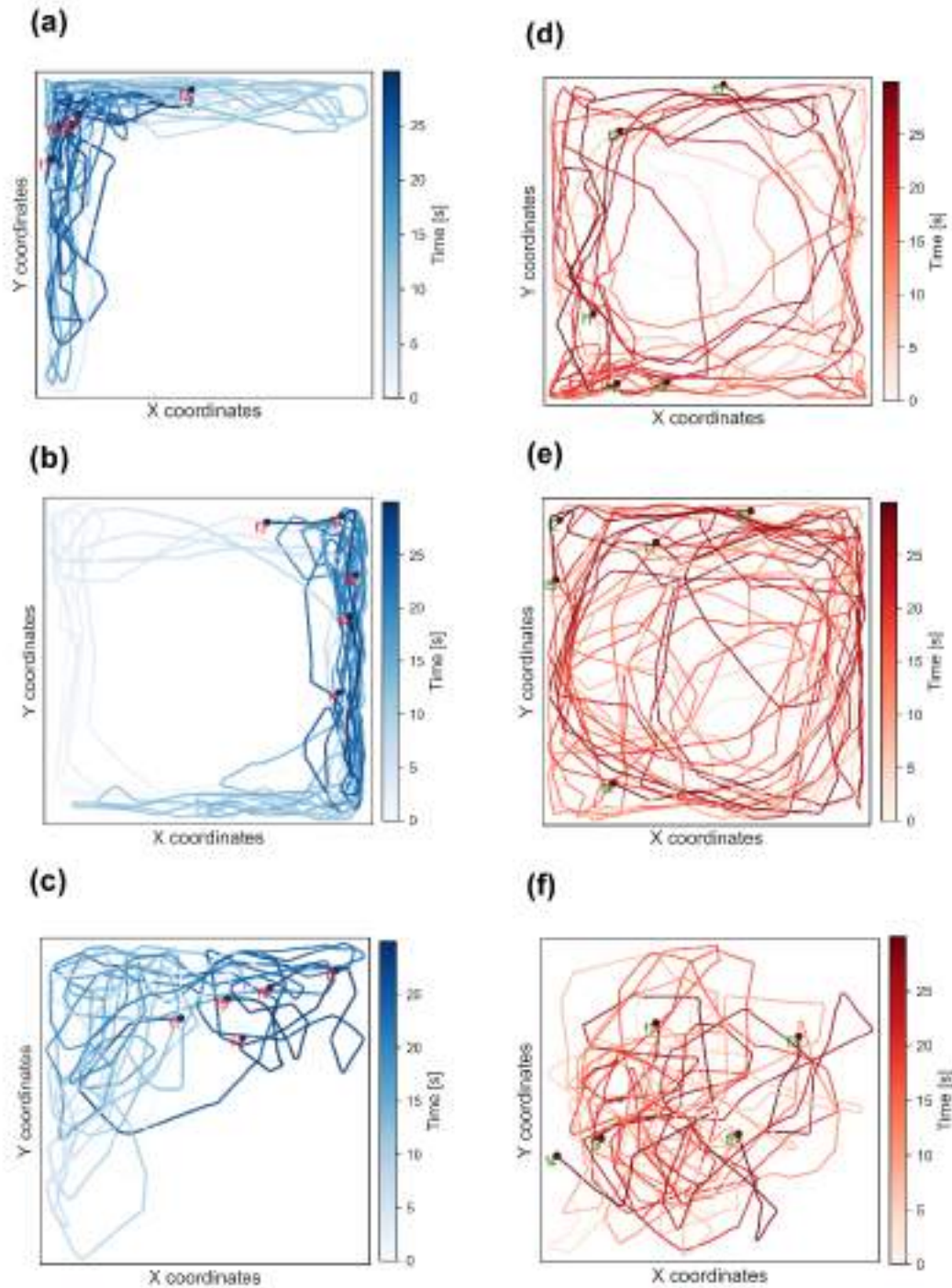


Figure 4.33. Representative Track Paths of Control and Isolated Shoals (Initial 30 s)

Representative movement trajectories derived from idTracker 2.1 using Cartesian (X–Y) coordinates, illustrating the first 30 seconds of recording for control (CON) and isolated (ISO) shoals.

Control shoal trajectories (blue tracks) display condensed, high-density movement patterns, indicating spatially restricted and cohesive exploration (a, b, & c). In contrast, isolated shoal trajectories (red tracks) exhibit dispersed and expanded paths

covering a larger proportion of the arena, reflecting increased movement spread and broader spatial utilization (d, e, & f).

4.4 Objective 4: Results

4.4.1 Objective statement

Spatio-temporal dynamics of homogeneous and heterogeneous dyads under chronic social isolation have been analysed separately below. The first deals with the nine dyadic metrics in homogeneous groups across control and test. The second section deals with the important individual-level metrics within a heterogeneous dyad. The third and fourth sections are exclusively for the between and within-group comparisons, such as between homogeneous vs heterogeneous and within multiple groups under heterogeneous dyads focusing on specific dyad-level metrics.

Normality and homoscedasticity tests indicated shifts across variables; in order to maintain consistency across statistical tests, we employed non-parametric tests throughout the inferential analysis. In the present study, each variable was treated as a family of comparisons, and selected pairwise comparisons were analysed. In order to control the family-wise error rate, we used Holm correction on nine pairwise comparisons for each variable. Effect size (r), calculated based on the Z value, was also reported.

4.4.2 Spatio-Temporal Dynamics of Homogeneous Dyads Composed of Control-Control and Isolated-Isolated Individual Pairs

1. Mean Euclidean Distance (cm)

The male-male (MM), female-female (FF), and male-female (MF) dyads under control and isolated groups exhibited significant differences in the mean Euclidean distance measured. Isolated dyads in each sex pair showed significantly higher Euclidean distances compared to their respective control counterparts. All other comparisons between control and isolated groups showed no significant patterns, except TFF and TMF ($p = 0.0408$). However, this significance did not remain after the Holm correction was applied ($m = 9$), indicating that isolation caused altered Euclidean distances independent of sex combinations in zebrafish dyads.

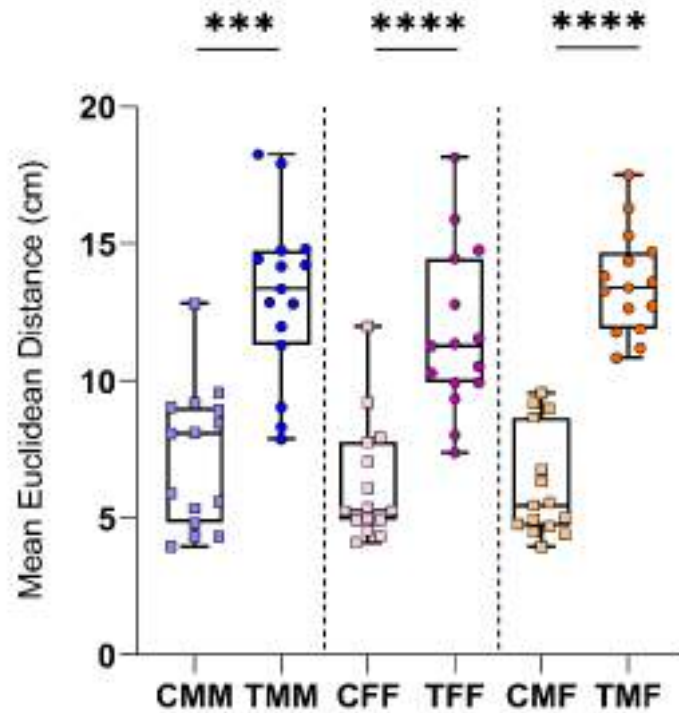


Figure 4.34. Mean Euclidean Distance (cm)

Box-and-whisker plot showing the mean Euclidean distance across six zebrafish dyad pairs. Treatment-wise comparisons were grouped to illustrate control versus isolated differences. Only statistically significant comparisons (Holm-corrected) are depicted. Significance levels are indicated as **** $p < 0.0001$ and *** $p < 0.001$. Square symbols (■) represent control dyads, and circle symbols (●) represent isolated dyads. Statistical analysis was performed using the Mann–Whitney U test. The plot was generated using GraphPad Prism v9.

Table 4.28. Between-group comparison of Mean Euclidean Distance (cm) using Mann–Whitney U test (Dyad Analysis).

Pairwise comparisons were performed using the Mann–Whitney U test with Holm correction for multiple comparisons. Effect size is reported as *r* and interpreted as Small (<0.3), Medium (0.3–0.5), and Large (>0.5). Significance levels: $p < 0.05$ (*), <0.01 (**), <0.001 (***), <0.0001 (****); ns = not significant.

Comparison	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p Value	Holm Corrected p Value	ES
CMM vs TMM	8.074 (5.823–8.643)	13.36 (11.38–14.76)	140, 325	20	<0.0001 ****	0.0002 ***	0.70/Large
CFF vs TFF	5.277 (5.045–7.461)	11.27 (10.05–13.36)	134, 331	14	<0.0001 ****	<0.0001 ****	0.74/Large
CMF vs TMF	5.468 (5.101–7.271)	13.40 (12.52–14.59)	120, 345	0	<0.0001 ****	<0.0001 ****	0.85/Large
CMM vs CFF	8.074 (5.823–8.643)	5.277 (5.045–7.461)	258, 207	87	0.3046 ns	1.0000 ns	0.19/Small
CMM vs CMF	8.074 (5.823–8.643)	5.468 (5.101–7.271)	255, 210	90	0.3669 ns	1.0000 ns	0.17/Small
CFF vs CMF	5.277 (5.045–7.461)	5.468 (5.101–7.271)	233, 232	112	>0.9999 ns	1.0000 ns	0.00/Small
TMM vs TFF	13.36 (11.38–14.76)	11.27 (10.05–13.36)	263, 202	82	0.2169 ns	1.0000 ns	0.23/Small
TMM vs TMF	13.36 (11.38–14.76)	13.40 (12.52–14.59)	230, 235	110	0.9349 ns	1.0000 ns	0.01/Small
TFF vs TMF	11.27 (10.05–13.36)	13.40 (12.52–14.59)	183, 282	63	0.0408*	0.2450ns	0.37/Medium

2. Proximity Time (%)

The male-male (MM), female-female (FF), and male-female (MF) dyads under control and isolated groups exhibited significant differences in the proximity time maintained. Isolated dyads in each sex pair showed significantly lower proximity time compared to their respective control counterparts. All other comparisons between control and isolated groups showed no significant patterns, indicating that isolation caused a reduction in the proximity time, independent of sex combinations in zebrafish dyads.

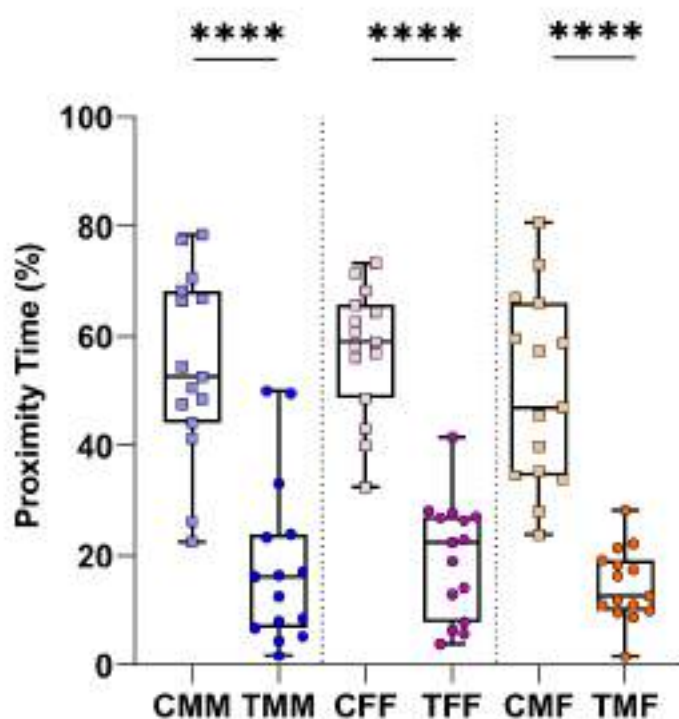


Figure 4.35. Proximity Time (%)

Box-and-whisker plot showing proximity time (%) across six zebrafish dyad pairs. Treatment-wise comparisons were grouped to illustrate control versus isolated differences. Only statistically significant comparisons (Holm-corrected) are displayed. Significance levels are marked as **** $p < 0.0001$ and * $p < 0.05$. Square symbols (■) denote control dyads, and circle symbols (●) denote isolated dyads. Statistical analysis was conducted using the Mann–Whitney U test. Generated using GraphPad Prism v9.

Table 4.29. Between-group comparison of Proximity Time (%) using Mann–Whitney U test (Dyad Analysis).

Pairwise comparisons were performed using the Mann–Whitney U test with Holm correction for multiple comparisons. Effect size is reported as *r* and interpreted as Small (<0.3), Medium (0.3–0.5), and Large (>0.5).

Comparison	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p Value	Holm Corrected p Value	ES
CMM vs TMM	52.59 (44.96–63.82)	16.10 (9.955–26.87)	329, 136	16	<0.0001 ****	<0.0001 ****	0.73/Large
CFF vs TFF	59.01 (50.84–63.85)	22.38 (13.55–25.47)	343, 122	2	<0.0001 ****	<0.0001 ****	0.83/Large
CMF vs TMF	46.96 (40.37–59.64)	12.62 (10.90–18.29)	343, 122	2	<0.0001 ****	<0.0001 ****	0.83/Large
CMM vs CFF	52.59 (44.96–63.82)	59.01 (50.84–63.85)	222, 243	102	0.6827 ns	1.0000 ns	0.07/Small
CMM vs CMF	52.59 (44.96–63.82)	46.96 (40.37–59.64)	251, 214	94	0.4610 ns	1.0000 ns	0.14/Small
CFF vs CMF	59.01 (50.84–63.85)	46.96 (40.37–59.64)	261, 204	84	0.2496 ns	1.0000 ns	0.21/Small
TMM vs TFF	16.10 (9.955–26.87)	22.38 (13.55–25.47)	217, 248	97	0.5393ns	1.0000ns	0.11/Small
TMM vs TMF	16.10 (9.955–26.87)	12.62 (10.90–18.29)	232, 233	112	>0.9999 ns	1.0000 ns	0.00/Small
TFF vs TMF	22.38 (13.55–25.47)	12.62 (10.90–18.29)	264, 201	81	0.2017 ns	1.0000 ns	0.23/Small

3. Path Cosine Dissimilarity (au)

The male-male (MM), female-female (FF), and male-female (MF) dyads under control and isolated groups exhibited significant differences in the mean cosine distance obtained. Isolated dyads in each sex pair showed significantly higher cosine distances compared to their respective control counterparts. All other comparisons between control and isolated groups showed no significant patterns, indicating that isolation caused altered cosine distances independent of sex combinations in zebrafish dyads.

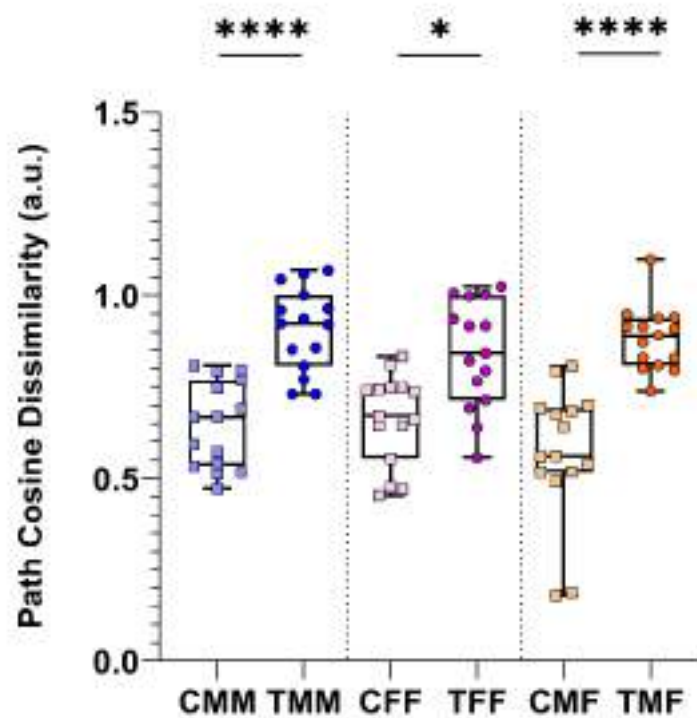


Figure 4.36. Path Cosine Dissimilarity (au.)

Box-and-whisker plot illustrating path cosine dissimilarity across six dyad pairs. Treatment-wise comparisons were grouped to highlight control versus isolated differences. Only significant comparisons (Holm-corrected) are shown. Significance levels are indicated as **** $p < 0.0001$ and * $p < 0.05$. Square symbols (■) represent control dyads, and circle symbols (●) represent isolated dyads. Mann–Whitney U test was applied. Generated using GraphPad Prism v9.

Table 4.30. Between-group comparison of Mean Cosine Distance (au) using Mann–Whitney U test (Dyad Analysis).

Pairwise comparisons were performed using the Mann–Whitney U test with Holm correction for multiple comparisons. Effect size is reported as *r* and interpreted as Small (<0.3), Medium (0.3–0.5), and Large (>0.5).

Comparison	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p Value	Holm Corrected p Value	ES
CMM vs TMM	0.6678 (0.5796– 0.7106)	0.9241 (0.8451– 0.9711)	134, 331	14	<0.0001 ****	<0.0001 ****	0.74/Large
CFF vs TFF	0.6719 (0.5940– 0.7302)	0.8420 (0.7608– 0.9232)	161, 304	41	0.0023 **	0.0161 *	0.54/Large
CMF vs TMF	0.5613 (0.4677– 0.6719)	0.8908 (0.8336– 0.9300)	124, 341	4	<0.0001 ****	<0.0001 ****	0.82/Large
CMM vs CFF	0.6678 (0.5796– 0.7106)	0.6719 (0.5940– 0.7302)	227, 238	107	0.8381 ns	1.0000 ns	0.03/Small
CMM vs CMF	0.6678 (0.5796– 0.7106)	0.5613 (0.4677– 0.6719)	252, 213	93	0.4363 ns	1.0000 ns	0.14/Small
CFF vs CMF	0.6719 (0.5940– 0.7302)	0.5613 (0.4677– 0.6719)	263, 202	82	0.2169 ns	1.0000 ns	0.23/Small
TMM vs TFF	0.9241 (0.8451– 0.9711)	0.8420 (0.7608– 0.9232)	264, 201	81	0.2017 ns	1.0000 ns	0.23/Small
TMM vs TMF	0.9241 (0.8451– 0.9711)	0.8908 (0.8336– 0.9300)	253, 212	92	0.4124 ns	1.0000 ns	0.15/Small
TFF vs TMF	0.8420 (0.7608– 0.9232)	0.8908 (0.8336– 0.9300)	221, 244	101	0.6529 ns	1.0000 ns	0.08/Small

4. Geometric Following: Fraction of Time

The male-male (MM), female-female (FF), and male-female (MF) dyads under control and isolated groups exhibited significant differences in the average following time, represented here as the fraction of time spent in following. Isolated dyads in each sex pair showed significantly lower average following compared to their respective control counterparts. All other comparisons between control and isolated groups showed no significant patterns, indicating that isolation reduced coordination with the partner's movement, irrespective of sex combinations.

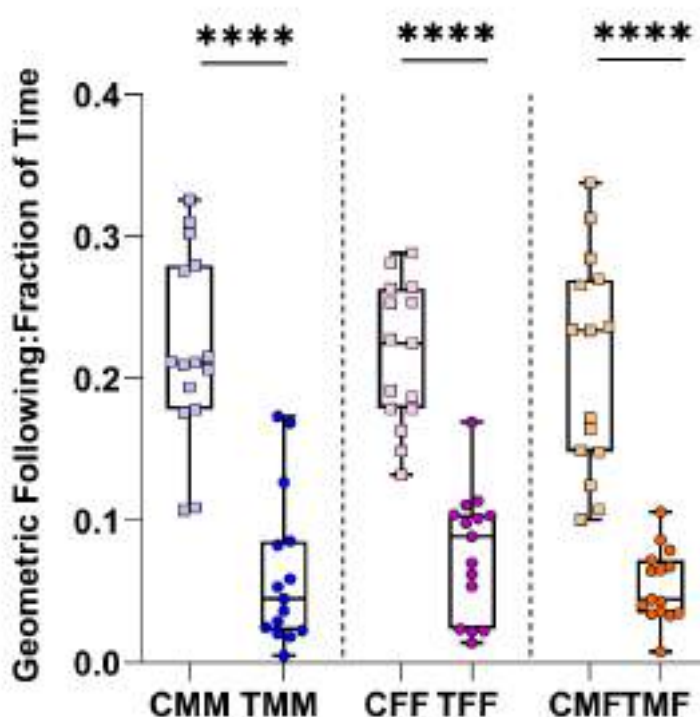


Figure 4.37. Average Fraction of Time in Following

Box-and-whisker plot showing the fraction of time spent in following behaviour across six dyad pairs. Treatment-wise comparisons were grouped to illustrate control versus isolated differences. Only significant Holm-corrected comparisons are displayed (**** $p < 0.0001$). Square symbols (■) indicate control dyads, and circle symbols (●) indicate isolated dyads. Statistical analysis was performed using the Mann-Whitney U test. Generated using GraphPad Prism v9.

Table 4.31. Fraction of Time in Following Across Groups

Pairwise comparisons of fraction of time spent in following behaviour between groups using the Mann–Whitney U test with Holm correction.

Comparison	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p Value	Holm Corrected p Value	ES
CMM vs TMM	0.2112 (0.1838–0.2578)	0.04488 (0.0334–0.0933)	339, 126	6	<0.0001 ****	<0.0001 ****	0.80 / Large
CFF vs TFF	0.2249 (0.1878–0.2437)	0.08871 (0.05267–0.1016)	342, 123	3	<0.0001 ****	<0.0001 ****	0.83 / Large
CMF vs TMF	0.2338 (0.1670–0.2517)	0.04434 (0.04004–0.06842)	344, 121	1	<0.0001 ****	<0.0001 ****	0.84 / Large
CMM vs CFF	0.2112 (0.1838–0.2578)	0.2249 (0.1878–0.2437)	239, 226	106	0.8063 ns	1.0000 ns	0.04 / Small
CMM vs CMF	0.2112 (0.1838–0.2578)	0.2338 (0.1670–0.2517)	243, 222	102	0.6827 ns	1.0000 ns	0.07 / Small
CFF vs CMF	0.2249 (0.1878–0.2437)	0.2338 (0.1670–0.2517)	239, 226	106	0.8063 ns	1.0000 ns	0.04 / Small
TMM vs TFF	0.04488 (0.0334–0.0933)	0.08871 (0.05267–0.1016)	207, 258	87	0.3046 ns	1.0000 ns	0.19 / Small
TMM vs TMF	0.04488 (0.0334–0.0933)	0.04434 (0.04004–0.06842)	225, 240	105	0.7748 ns	1.0000 ns	0.05 / Small
TFF vs TMF	0.08871 (0.05267–0.1016)	0.04434 (0.04004–0.06842)	266, 199	79	0.1736 ns	1.0000 ns	0.25 / Small

5. Speed Difference (cm/s)

The male-male (MM), female-female (FF), and male-female (MF) dyads under control and isolated groups exhibited significant changes in speed difference. Isolated dyads in each sex pair showed significantly higher speed difference compared to their respective control counterparts. All other comparisons between control and isolated groups showed no significant patterns, indicating that isolation caused heightened speed difference between individuals within pairs, irrespective of sex combinations.

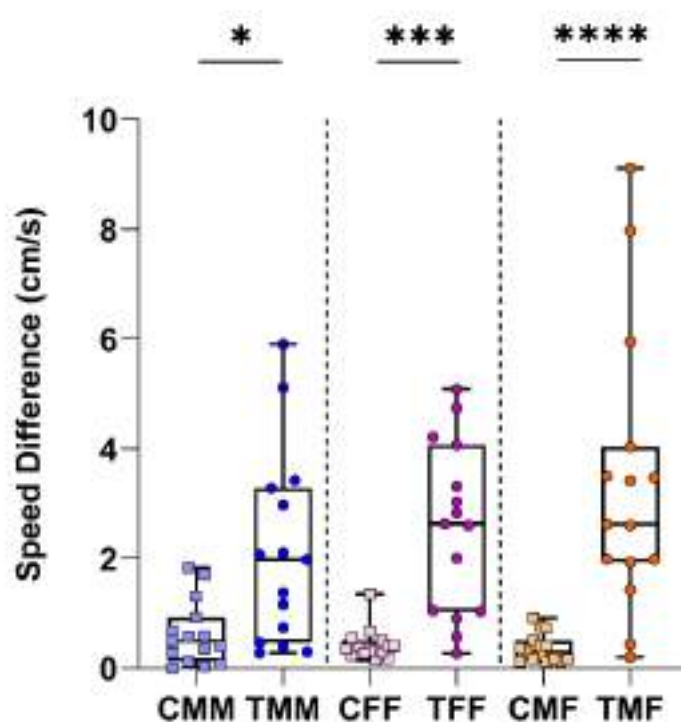


Figure 4.38. Speed Difference (cm/s)

Box-and-whisker plot depicting speed difference between dyad partners across six dyad pairs. Treatment-wise comparisons were grouped to illustrate control versus isolated differences. Only significant Holm-corrected comparisons are shown. Significance levels are marked as **** $p < 0.0001$, *** $p < 0.001$, and * $p < 0.05$. Square symbols (■) represent control dyads, and circle symbols (●) represent isolated dyads. Mann-Whitney U test was conducted. Generated using GraphPad Prism v9.

Table 4.32. Speed Difference (cm/s) – Between-group comparison using Mann–Whitney U test (Dyad Analysis).

Comparison	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p Value	Holm Corrected p Value	ES
CMM vs TMM	0.4963 (0.3026– 0.9470)	1.981 (1.136– 3.073)	167, 298	47	0.0057 **	0.0399 *	0.49/Medium
CFF vs TFF	0.3504 (0.2659– 0.5867)	2.633 (1.695– 3.418)	137, 328	17	<0.0001 ****	0.0001 ***	0.72/Large
CMF vs TMF	0.2944 (0.2241– 0.5117)	2.625 (1.966– 4.786)	133, 332	13	<0.0001 ****	<0.0001 ****	0.75/Large
CMM vs CFF	0.4963 (0.3026– 0.9470)	0.3504 (0.2659– 0.5867)	251, 214	94	0.4610 ns	1.0000 ns	0.14/Small
CMM vs CMF	0.4963 (0.3026– 0.9470)	0.2944 (0.2241– 0.5117)	253, 212	92	0.4124 ns	1.0000 ns	0.15/Small
CFF vs CMF	0.3504 (0.2659– 0.5867)	0.2944 (0.2241– 0.5117)	252, 213	93	0.4363 ns	1.0000 ns	0.14/Small
TMM vs TFF	1.981 (1.136– 3.073)	2.633 (1.695– 3.418)	213, 252	93	0.4363 ns	1.0000 ns	0.14/Small
TMM vs TMF	1.981 (1.136– 3.073)	2.625 (1.966– 4.786)	197, 268	77	0.1485 ns	0.8910 ns	0.27/Small
TFF vs TMF	2.633 (1.695– 3.418)	2.625 (1.966– 4.786)	221, 244	101	0.6529 ns	1.0000 ns	0.08/Small

6. Acceleration correlation (r)

The male-male (MM), female-female (FF), and male-female (MF) dyads under control and isolated groups exhibited significant differences in acceleration correlation. Isolated dyads in each sex pair showed significantly lower acceleration correlation compared to their respective control counterparts. All other comparisons between control and isolated groups showed no significant patterns, indicating that isolation disrupted synchronisation in acceleration bursts, irrespective of sex combinations.

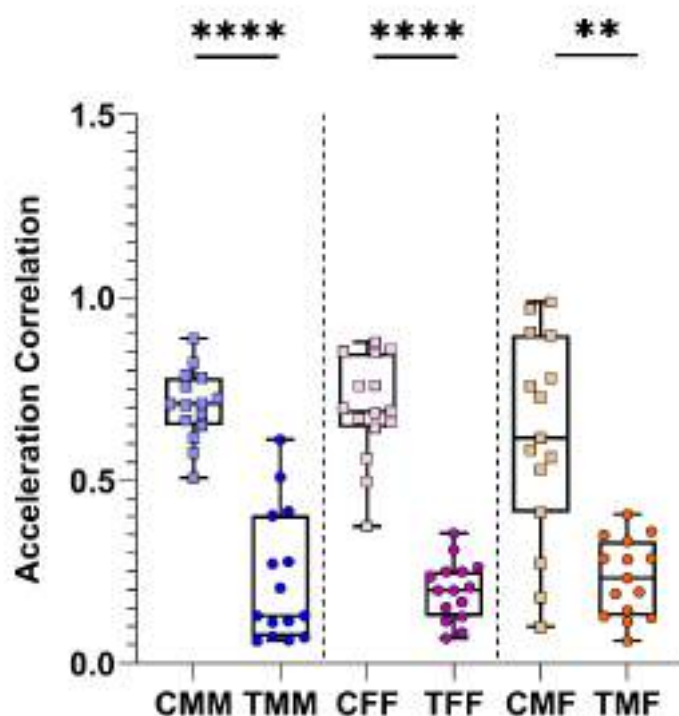


Figure 4.39. Acceleration Correlation (r)

Box-and-whisker plot showing acceleration correlation coefficients across six dyad pairs. Treatment-wise comparisons were grouped to illustrate control versus isolated differences. Only statistically significant comparisons (Holm-corrected) are depicted. Significance levels are marked as **** $p < 0.0001$ and ** $p < 0.01$. Square symbols (■) denote control dyads, and circle symbols (●) denote isolated dyads. Statistical analysis was performed using the Mann–Whitney U test. Generated using GraphPad Prism v9.

Table 4.33. Acceleration Correlation Across Groups

Pairwise comparisons of acceleration correlation between groups using the Mann–Whitney U test with Holm correction for multiple comparisons.

Comparison	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p Value	Holm Corrected p Value	ES
CMM vs TMM	0.7085 (0.6509– 0.7584)	0.1296 (0.1311– 0.3286)	342, 123	3	<0.0001 ****	<0.0001 ****	0.83 / Large
CFF vs TFF	0.6879 (0.6162– 0.7734)	0.1990 (0.1538– 0.2443)	345, 120	0	<0.0001 ****	<0.0001 ****	0.85 / Large
CMF vs TMF	0.6158 (0.4622– 0.7744)	0.2329 (0.1744– 0.2914)	314, 151	31	0.0004 ***	0.0027 **	0.61 / Large
CMM vs CFF	0.7085 (0.6509– 0.7584)	0.6879 (0.6162– 0.7734)	236, 229	109	0.9025 ns	1.0000 ns	0.02 / Small
CMM vs CMF	0.7085 (0.6509– 0.7584)	0.6158 (0.4622– 0.7744)	247, 218	98	0.5668 ns	1.0000 ns	0.11 / Small
CFF vs CMF	0.6879 (0.6162– 0.7734)	0.6158 (0.4622– 0.7744)	244, 221	101	0.6529 ns	1.0000 ns	0.08 / Small
TMM vs TFF	0.1296 (0.1311– 0.3286)	0.1990 (0.1538– 0.2443)	229, 236	109	0.8941 ns	1.0000 ns	0.02 / Small
TMM vs TMF	0.1296 (0.1311– 0.3286)	0.2329 (0.1744– 0.2914)	216, 249	96	0.5125 ns	1.0000 ns	0.12 / Small
TFF vs TMF	0.1990 (0.1538– 0.2443)	0.2329 (0.1744– 0.2914)	214, 251	94	0.4545 ns	1.0000 ns	0.14 / Small

7. Maximum Velocity Cross-Correlation

The male-male (MM), female-female (FF), and male-female (MF) dyads under control and isolated groups exhibited significant differences in the maximum velocity cross correlation (VCC_{max}). Isolated dyads in each sex pair showed significantly lower VCC_{max} compared to their respective control counterparts. All other comparisons between control and isolated groups showed no significant patterns, indicating that isolation desynchronised velocity changes between individuals within pairs, irrespective of sex combinations.

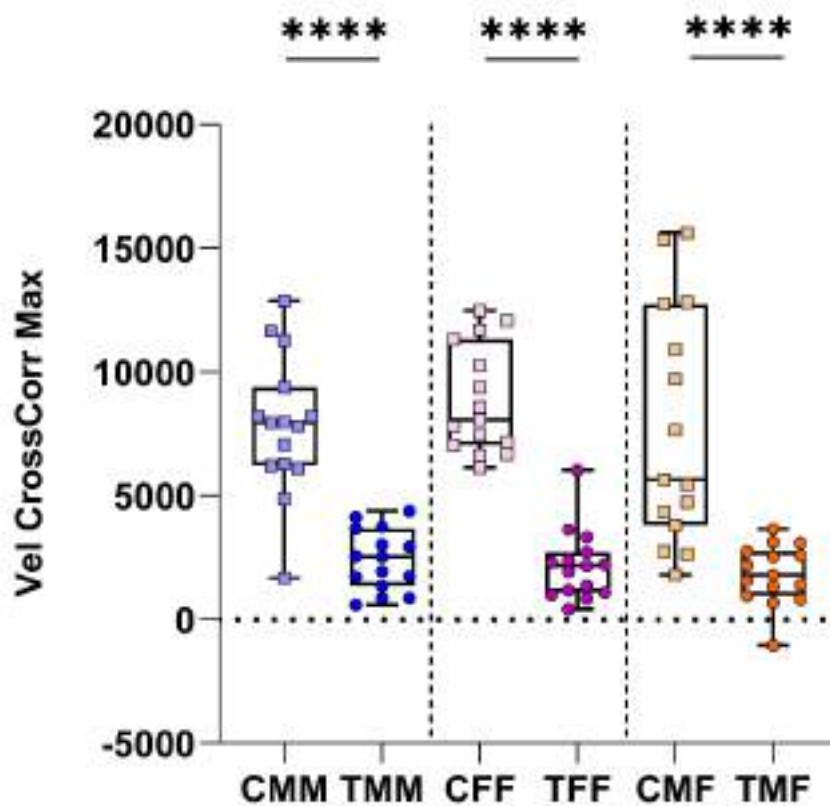


Figure 4.40. Maximum Velocity Cross-Correlation (VCC_{max})

Box-and-whisker plot illustrating maximum velocity cross-correlation (VCC_{max}) across six dyad pairs. Treatment-wise comparisons were grouped to highlight control versus isolated differences. Only significant Holm-corrected comparisons are displayed (**** $p < 0.0001$). Square symbols (■) represent control dyads, and circle symbols (●) represent isolated dyads. Mann–Whitney U test was conducted. Generated using GraphPad Prism v9.

Table 4.34. Velocity Cross-Correlation (Maximum) Across Groups

Pairwise comparisons of maximum velocity cross-correlation between groups using the Mann–Whitney U test with Holm correction.

Comparison	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p Value	Holm Corrected p Value	ES
CMM vs TMM	7952 (6280– 9378)	2524 (1718– 3081)	334, 131	11	<0.0001 ****	<0.0001 ****	0.76 / Large
CFF vs TFF	8070 (7636– 10057)	2174 (1392– 2956)	345, 120	0	<0.0001 ****	<0.0001 ****	0.85 / Large
CMF vs TMF	5650 (5094– 10354)	1809 (1154– 2497)	330, 135	15	<0.0001 ****	<0.0001 ****	0.73 / Large
CMM vs CFF	7952 (6280– 9378)	8070 (7636– 10057)	210, 255	90	0.3669 ns	1.0000 ns	0.17 / Small
CMM vs CMF	7952 (6280– 9378)	5650 (5094– 10354)	247, 218	98	0.5668 ns	1.0000 ns	0.11 / Small
CFF vs CMF	8070 (7636– 10057)	5650 (5094– 10354)	258, 207	87	0.3046 ns	1.0000 ns	0.19 / Small
TMM vs TFF	2524 (1718– 3081)	2174 (1392– 2956)	251, 214	94	0.4610 ns	1.0000 ns	0.14 / Small
TMM vs TMF	2524 (1718– 3081)	1809 (1154– 2497)	257, 208	88	0.3245 ns	1.0000 ns	0.18 / Small
TFF vs TMF	2174 (1392– 2956)	1809 (1154– 2497)	238, 227	107	0.8381 ns	1.0000 ns	0.03 / Small

8. Average Freezing Frequency per Second

The male-male (MM), female-female (FF), and male-female (MF) dyads under control and isolated groups exhibited significant differences in the freezing frequency. Isolated dyads of each sex pair showed significantly lower freezing frequency compared to their respective control counterparts. All other comparisons between control groups did not differ significantly, indicating that the freezing levels were comparable across sex combinations in the control dyads (CMM, CFF, and CMF). In contrast, isolated MM dyads showed significantly higher freezing than the other two isolated dyads. Because the isolated FF dyad exhibited significant reduction in freezing, the isolated mixed dyads showed higher freezing than TFF and lower than TMM. However, the statistical significance observed between TFF and TMF did not

remain after Holm correction. Hence, these results indicate that under isolation, freezing in dyads showed sex-specific variations across the three groups.

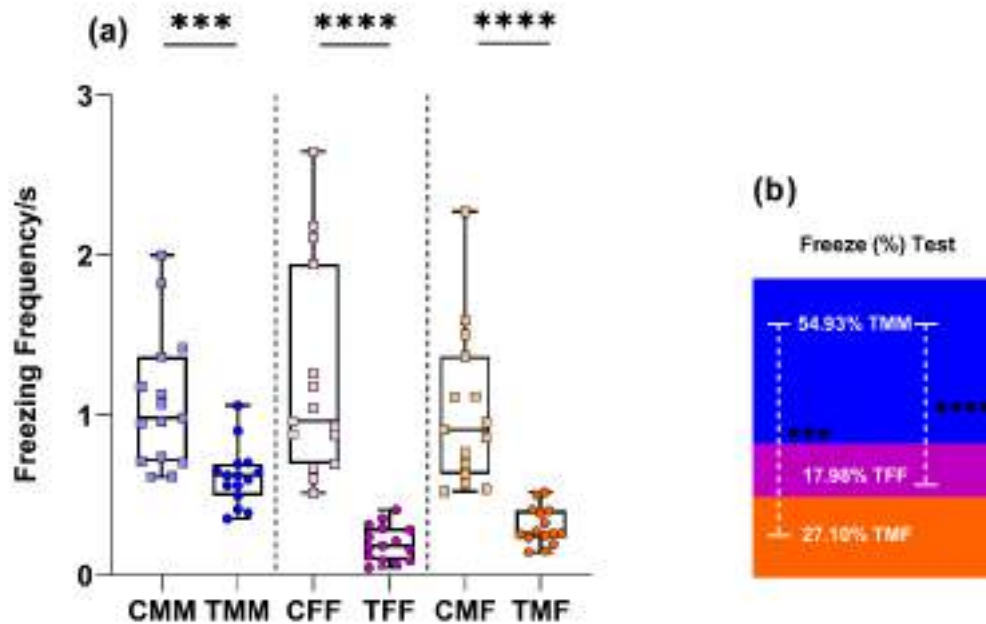


Figure 4.41. Freezing Frequency Dynamics

Box-and-whisker plot showing average freezing frequency (events/s) across six dyad pairs, grouped by treatment to illustrate control versus isolated differences. Only statistically significant comparisons following Holm correction are displayed (**** $p < 0.0001$, *** $p < 0.001$). Square symbols (■) represent control dyads, and circle symbols (●) represent isolated dyads. Statistical analysis was conducted using the Mann–Whitney U test. Generated using GraphPad Prism v9.

Accompanying heatmaps depict the temporal distribution and intensity of freezing events, separately visualizing sex-specific patterns within isolated condition.

Table 4.35. Freezing Frequency (events/second) – Between-group comparison using Mann–Whitney U test (Dyad Analysis).

Comparison	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p Value	Holm Corrected p Value	ES
CMM vs TMM	0.9819 (0.8513–1.317)	0.6236 (0.5152–0.7198)	319, 146	26	0.0001 ***	0.0005 ***	0.65/Large
CFF vs TFF	0.9625 (0.8671–1.601)	0.1833 (0.1410–0.2632)	345, 120	0	<0.0001 ****	<0.0001 ****	0.85/Large
CMF vs TMF	0.9097 (0.7592–1.298)	0.2611 (0.2402–0.3690)	345, 120	0	<0.0001 ****	<0.0001 ****	0.85/Large
CMM vs CFF	0.9819 (0.8513–1.317)	0.9625 (0.8671–1.601)	230, 235	110	0.9349 ns	1.0000 ns	0.015/Small
CMM vs CMF	0.9819 (0.8513–1.317)	0.9097 (0.7592–1.298)	249, 216	96	0.5125 ns	1.0000 ns	0.12/Small
CFF vs CMF	0.9625 (0.8671–1.601)	0.9097 (0.7592–1.298)	251, 214	94	0.4610 ns	1.0000 ns	0.14/Small
TMM vs TFF	0.6236 (0.5152–0.7198)	0.1833 (0.1410–0.2632)	342, 123	3	<0.0001 ****	<0.0001 ****	0.83/Large
TMM vs TMF	0.6236 (0.5152–0.7198)	0.2611 (0.2402–0.3690)	332, 133	13	<0.0001 ****	0.0002 ***	0.75/Large
TFF vs TMF	0.1833 (0.1410–0.2632)	0.2611 (0.2402–0.3690)	182, 283	62	0.0367 *	0.1468 ns	0.38/Medium

9. Average Rapid Frequency per Second

The male-male (MM), female-female (FF), and male-female (MF) dyads under control and isolated groups exhibited significant differences in the rapid movement frequency. Isolated dyads of each sex pair showed significantly higher rapid movements compared to their respective control counterparts. Although the p-value significance between CFF and TFF did not remain after Holm correction, the reported medium effect size ($r = 0.42$) indicates potential practical or biological relevance that may have been masked by the correction stringency. Between control group comparisons, significant reduction in the rapid movements was observed only in control MF dyads relative to control FF dyads. In contrast, isolated MM dyads showed significantly higher rapid movements than the other two isolated dyads. Because the isolated FF dyad exhibited a significant reduction in rapid movements, the isolated

mixed dyads showed higher rapid movements than TFF and lower than TMM. However, the statistical significance observed between TFF and TMF did not remain after Holm correction, though a medium effect size was reported, suggesting a possible biological relevance in this comparison. Hence, these results indicate that under isolation, rapid movements in dyads showed altered sex-specific variations across the three groups compared to the controls.

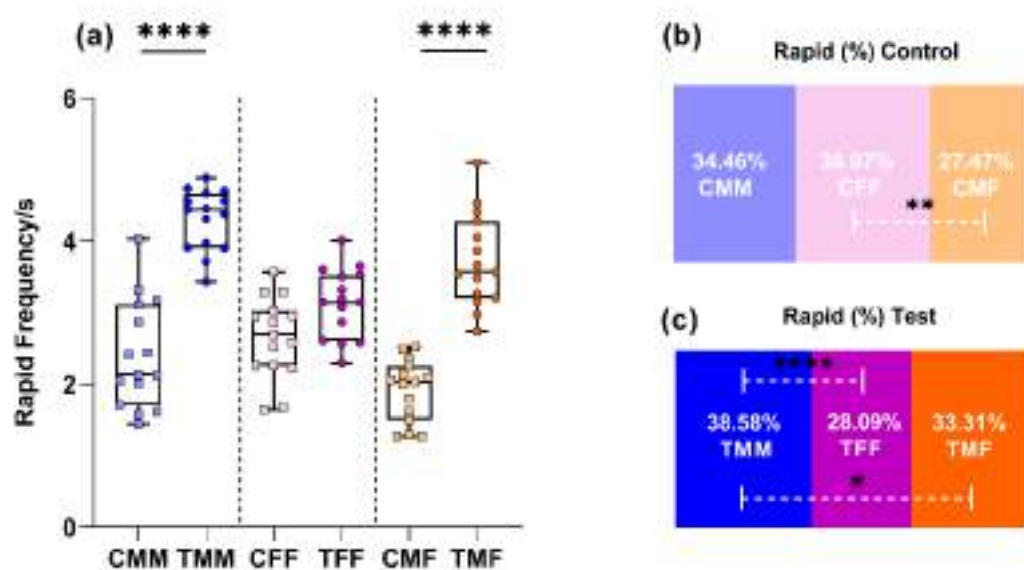


Figure 4.42. Rapid Movement Frequency Dynamics

Box-and-whisker plot illustrating rapid movement frequency (events/s) across six dyad pairs, grouped by treatment to compare control and isolated dyads. Only statistically significant Holm-corrected comparisons are shown (**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$). Square symbols (■) denote control dyads, and circle symbols (●) denote isolated dyads. Mann-Whitney U test was performed. Generated using GraphPad Prism v9.

Corresponding heatmaps display the temporal distribution and intensity of rapid movement events, separately depicting sex-specific activity patterns within control and isolated conditions to enable comparison of male and female dyads under each treatment.

Table 4.36. Rapid Frequency (events/second) – Between-group comparison using Mann–Whitney U test (Dyad Analysis).

Comparison	Median 1 (95% CI)	Median 2 (95% CI)	Sum of Ranks	U	p Value	Holm Corrected p Value	ES
CMM vs TMM	2.132 (1.979–2.816)	4.438 (4.071–4.542)	125, 340	5	<0.0001 ****	<0.0001 ****	0.81/Large
CFF vs TFF	2.694 (2.334–2.963)	3.142 (2.868–3.403)	177, 288	57	0.0209 *	0.0627 ns	0.42/Medium
CMF vs TMF	2.026 (1.672–2.152)	3.565 (3.359–4.079)	120, 345	0	<0.0001 ****	<0.0001 ****	0.85/Large
CMM vs CFF	2.132 (1.979–2.816)	2.694 (2.334–2.963)	202, 263	82	0.2169 ns	0.2169 ns	0.23/Small
CMM vs CMF	2.132 (1.979–2.816)	2.026 (1.672–2.152)	272, 193	73	0.1064 ns	0.2128 ns	0.29/Small
CFF vs CMF	2.694 (2.334–2.963)	2.026 (1.672–2.152)	313.5, 151.5	31.5	0.0004 ***	0.0024 **	0.61/Large
TMM vs TFF	4.438 (4.071–4.542)	3.142 (2.868–3.403)	336, 129	9	<0.0001 ****	<0.0001 ****	0.78/Large
TMM vs TMF	4.438 (4.071–4.542)	3.565 (3.359–4.079)	298, 167	47	0.0057 **	0.0285 *	0.49/Medium
TFF vs TMF	3.142 (2.868–3.403)	3.565 (3.359–4.079)	173, 292	53	0.0128 *	0.0512 ns	0.45/Medium

4.4.3 Spatial-Temporal Dynamics of Heterogeneous Dyads Composed of Control and Isolated Individual Pairs

1. Directional Initiation Leadership and Geometric Following: Fraction of Time

The heterogeneous dyads consisting of CMTM, CFTF, CFTM, and CMTF showed a similar pattern in the fraction of time spent in directional initiation and geometric following. Across heterogeneous dyads, test individuals showed higher initiation fractions and control individuals initiated fewer directional changes. Subsequently, control individuals showed higher following fraction compared to test fish, indicating less frequent follower zone alignment in isolated individuals. In the homogeneous groups, isolated pairs showed a lower average fraction of time spent in following, complementing the pattern observed in heterogeneous groups.

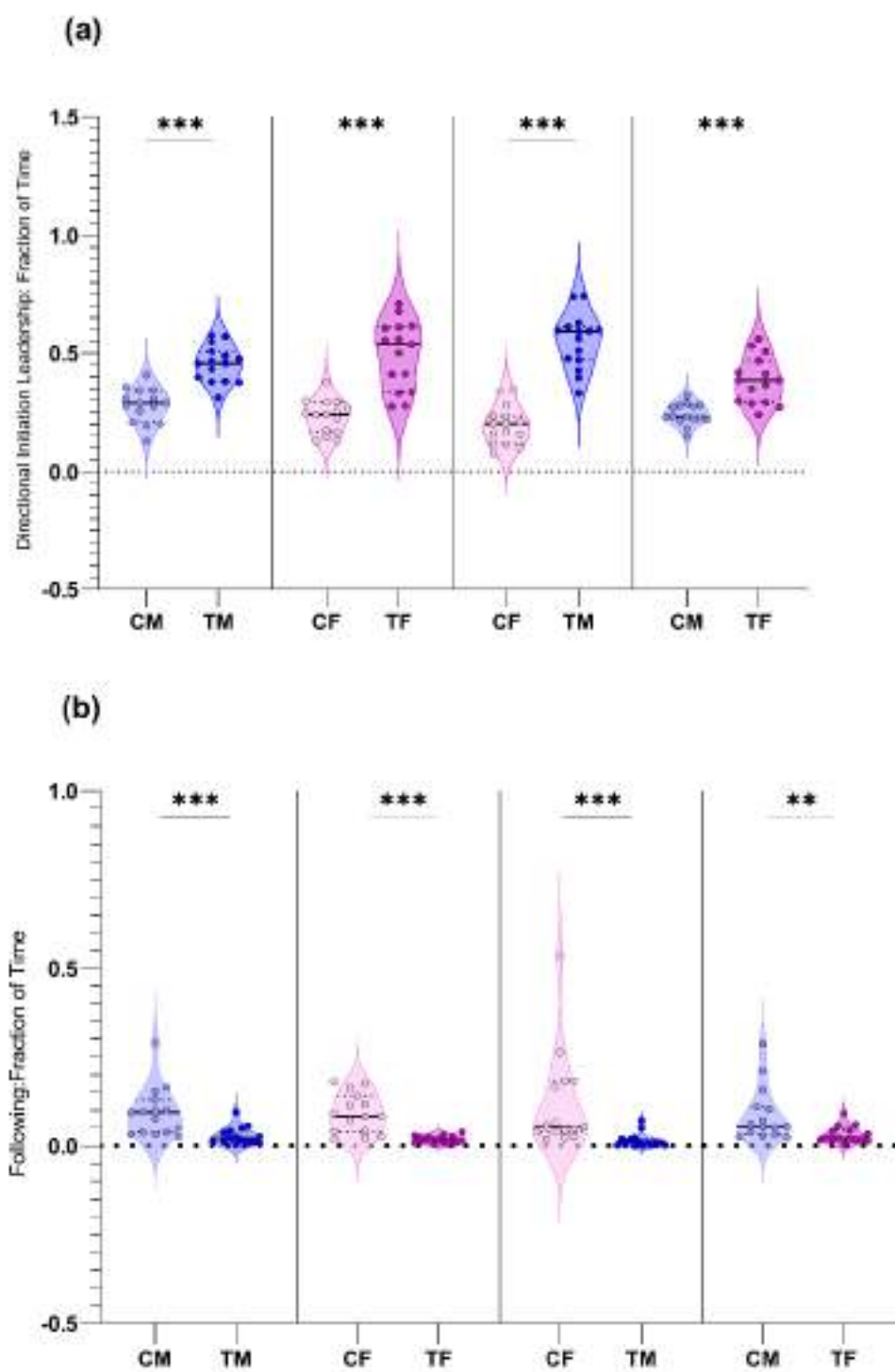


Figure 4.43. Directional Initiation Leadership and Comparison of Following Behaviour in Heterogeneous Zebrafish Dyads

(a) Directional Initiation Leadership in Heterogeneous Zebrafish Dyads.

Violin plots representing the fraction of time spent in directional initiation leadership during dyadic interactions between control and socially isolated zebrafish. Each plot

illustrates individual behavioural differences within the following sex combinations: (a) CM vs. TM, (b) CF vs. TF, (c) CF vs. TM, and (d) CM vs. TF. In all pairings, socially isolated individuals (TM and TF) demonstrate a significant increase in leadership frequency compared to their respective control counterparts. Statistical significance was determined using the Wilcoxon signed-rank test, with triple asterisks (***) denoting $p < 0.001$. These results indicate that social isolation consistently enhances directional leadership initiation, regardless of the sex of the interacting partner.

(b) Comparison of Following Behaviour in Heterogeneous Zebrafish Dyads.

Violin plots representing the fraction of time spent in following behaviour during dyadic interactions between control and socially isolated zebrafish across four sex combinations: (a) CM vs. TM, (b) CF vs. TF, (c) CF vs. TM, and (d) CM vs. TF. In stark contrast to leadership trends, socially isolated individuals (TM and TF) demonstrate a significant reduction in following behaviour compared to their respective control partners (CM and CF). Statistical significance was determined using the Wilcoxon signed-rank test, with asterisks denoting high levels of significance (*** $p < 0.001$; ** $p < 0.01$). These results indicate that social isolation induces a behavioural shift toward active initiation and a concurrent withdrawal from social following, independent of the sexual dimorphism of the dyad.

Table 4.37. Wilcoxon Signed-Rank Test for Initiating and Following in Heterogeneous Dyads

Within-pair comparisons of initiating and following behaviour using the Wilcoxon signed-rank test with Holm correction.

Group	Param.	Comparison	Median 1 (95% CI)	Median 2 (95% CI)	W	p	Holm p	ES
CMTM	Init.	CM vs TM	0.2881 (0.2383-0.3191)	0.4571 (0.4126-0.4980)	-114	0.0003 ***	0.0006 ***	0.93 L
	Follow	CM vs TM	0.0946 (0.0558-0.1334)	0.0213 (0.0143-0.0417)	120	<0.0001 ****	0.0002 ***	1.04 L
CFTF	Init.	CF vs TF	0.2409 (0.1920-0.2710)	0.5373 (0.4145-0.5740)	-114	0.0003 ***	0.0006 ***	0.93 L
	Follow	CF vs TF	0.0837 (0.0600-0.1222)	0.0166 (0.0117-0.0231)	120	<0.0001 ****	0.0002 ***	1.04 L
CFTM	Init.	CF vs TM	0.2015 (0.1501-0.2414)	0.5927 (0.4873-0.6169)	-120	<0.0001 ****	0.0002 ***	1.04 L
	Follow	CF vs TM	0.0523 (0.0395-0.1924)	0.0083 (0.0045-0.0269)	120	<0.0001 ****	0.0002 ***	1.04 L
CMTF	Init.	CM vs TF	0.2318 (0.2176-0.2662)	0.3861 (0.3299-0.4398)	-118	0.0001 ***	0.0003 ***	0.99 L
	Follow	CM vs TF	0.0549 (0.0448-0.1298)	0.0213 (0.0188-0.0446)	106	0.0012 **	0.0012 **	0.84 L

2. Swimming Speed Dynamics: Rapid and Freezing within Heterogeneous Dyads

When paired with either control males or females, isolated males (TM) showed no significant differences in rapid movements per second. However, a significant reduction in freezing frequency was observed only when TM was paired with control males (CMTM), while no such difference was found when paired with control females (CFTM). In contrast, isolated females (TF) consistently exhibited significantly higher rapid movements and lower freezing frequency, irrespective of the sex of their control partner.

Recalling the homogeneous dyads, TMM earlier showed both higher rapid movements and higher freezing compared to other isolated pairs. In the current case, when TM was paired with controls (CM or CF), the control individuals may have adjusted their movement speeds to match TM, leading to the non-significant

difference in rapid movements observed in both CMTM and CFTM dyads. Similarly, the control female might have modulated her freezing behaviour when paired with TM, resulting in the non-significant difference seen in freezing in CFTM.

Overall, these results indicate that in isolated males, dyadic dynamics were altered in heterogeneous groups, possibly because the control partners adjusted their responses to the isolated individual. In contrast, isolated females maintained the pattern of higher rapid movements and lower freezing observed in homogeneous dyads, suggesting that such interactions might not favour mutual coordination between test and control individuals.

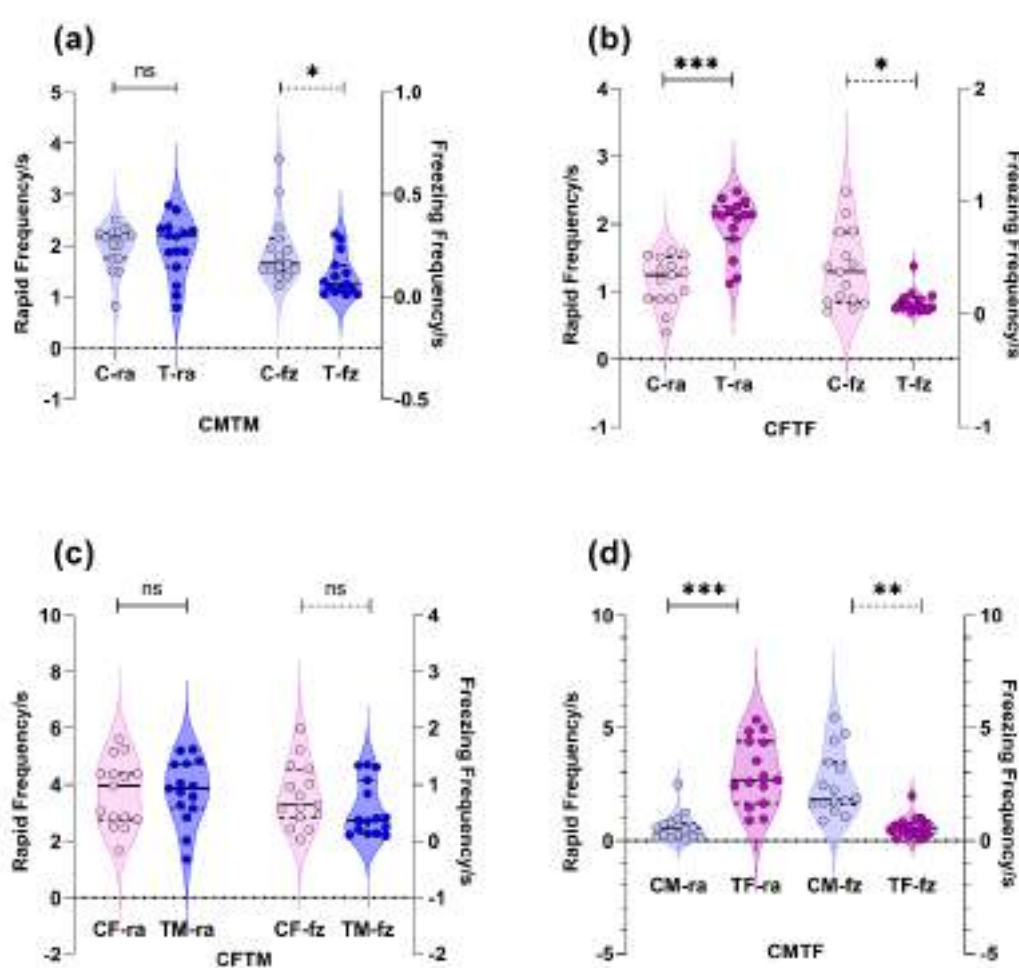


Figure 4.44. Dual-Axis Behavioural Metric Analysis of Rapid Movement and Freezing Frequency

Violin plots illustrating the frequencies of (left axis) rapid movements and (right axis) freezing behaviour per second across four heterogeneous zebrafish dyads: (a) CM-TM, (b) CF-TF, (c) CF-TM, and (d) CM-TF (n=15 per group). This dual-metric

approach allows for a synchronized evaluation of high-arousal and defensive behaviours within the same social context. Statistical significance was determined using the Wilcoxon signed-rank test, with asterisks denoting high levels of significance (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, non-significant).

Table 4.38. Wilcoxon Signed-Rank Test for Rapid and Freezing Frequency Between Paired Groups

Within-pair comparisons of rapid and freezing frequency (per second) using the Wilcoxon signed-rank test with Holm correction.

Group	Param.	Comparison	Median 1 (95% CI)	Median 2 (95% CI)	W	p	Holm p	ES
CMTM	Rapid	CM vs TM	2.164 (1.728–2.208)	2.178 (1.636–2.282)	2.0	0.9780 ns	1.0000 ns	0.007 VS
	Freeze	CM vs TM	0.1667 (0.1364–0.3231)	0.0667 (0.0466–0.1570)	98	0.0034 **	0.0102 *	0.76 L
CFTF	Rapid	CF vs TF	1.247 (0.9501–1.350)	2.131 (1.751–2.212)	-114	0.0003 ***	0.0009 ***	0.93 L
	Freeze	CF vs TF	0.3778 (0.2163–0.5848)	0.0639 (0.0480–0.1598)	92	0.0067 **	0.0134 *	0.70 L
CFTM	Rapid	CF vs TM	3.958 (3.040–4.358)	3.861 (3.145–4.392)	-12	0.7615 ns	1.0000 ns	0.07 VS
	Freeze	CF vs TM	0.6444 (0.5011–1.116)	0.3639 (0.2874–0.8244)	60	0.0946 ns	0.0946 ns	0.43 M
CMTF	Rapid	CM vs TF	0.5111 (0.2501–0.9477)	2.683 (2.209–3.844)	-118	0.0001 ***	0.0004 ***	0.99 L
	Freeze	CM vs TF	1.867 (1.743–3.315)	0.5472 (0.3394–0.8687)	114	0.0003 ***	0.0012 **	0.93 L

4.4.4 Dyadic Metric Comparisons Across Homogeneous and Heterogeneous Dyads

Because the evidence suggested that mutual response induced altered behaviour in individual positioning and movement speed, we further expanded the comparisons towards the dyadic metrics to analyze which homogeneous group the heterogeneous group closely resembled. This approach was used to reveal the influencing factor, whether it is the control or isolated individual within a pair. All heterogeneous dyads showed resemblance to the isolated dyads in the homogeneous group in terms of Euclidean distance, cosine distance, proximity time, and speed difference, indicating that the dynamics of the dyad were largely influenced by the presence of the isolated individual, and the control partner likely adapting to it.

However, when considering acceleration correlation, all heterogeneous dyads continued to show resemblance to isolated homogeneous dyads, even though the statistical significance between CMF and CMTF did not remain after correction, leaving a medium effect size ($r = 0.42$). In contrast, maximum velocity cross-correlation revealed that CFTM resembled CMF rather than TMF, suggesting that the opposite-sex partner (control female) influenced movement synchronization in isolated males. When isolated males were paired with control males (CMTM), they showed resemblance to TMM—the isolated male–male homogeneous dyads. Conversely, in CMTF, the isolated female resembled TMF, but the dissimilarity observed with CMF lost significance after correction, despite a medium effect size ($r = 0.38$). This pattern indicates that opposite-sex influence on movement synchronization was effective in isolated males but not in isolated females, where only medium effect sizes supported the trend without corrected statistical significance.

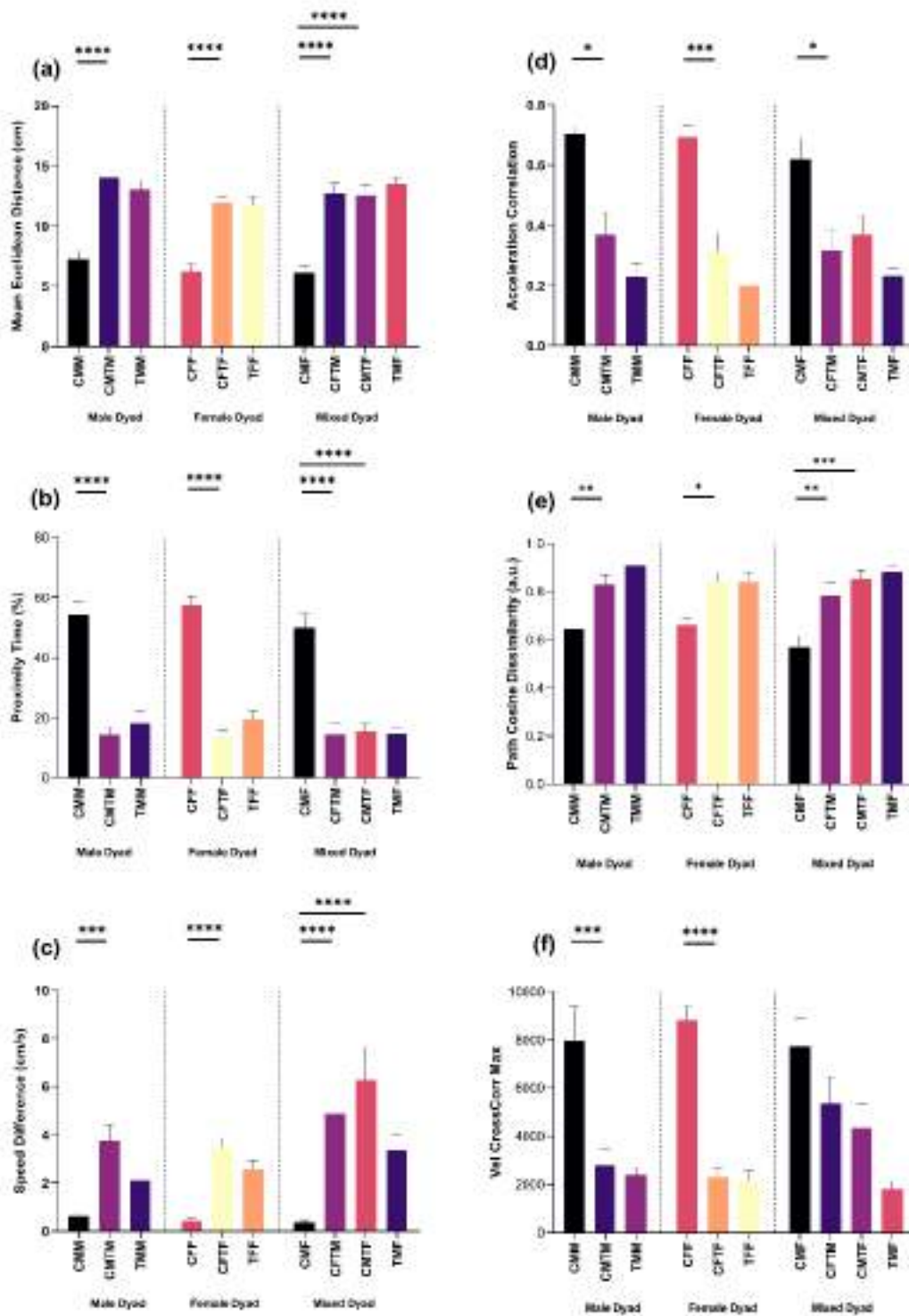


Figure 4.45. Comparison of Dyadic Behavioural Metrics Across Homogeneous and Heterogeneous Zebrafish Groups

A comprehensive analysis of six dyadic-level metrics comparing heterogeneous pairs to their respective control and isolated homogeneous counterparts ($n=15$ per group). Statistical significance was determined using the Mann-Whitney U test with a Holm-

Bonferroni correction for multiple comparisons ($m=9$ per metric). Symbols denote significance levels: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

- (a) Mean Euclidean Distance (cm)
- (b) Proximity Time (%)
- (c) Speed Difference (cm/s)
- (d) Acceleration Correlation
- (e) Path Cosine Dissimilarity (a.u.)
- (f) Maximum Velocity Cross-Correlation.

Table 4.39. Pairwise Comparisons Across Six Behavioural Parameters Using Mann–Whitney U Test with Holm Correction

Between-group comparisons for Mean Euclidean Distance, Mean Cosine Distance, Proximity Time, Speed Difference, Acceleration Correlation, and Maximum Velocity Cross-Correlation. Holm-adjusted p-values were used to correct for multiple testing.

Parameter	Comparison	Sum of Ranks	U	p Value	Holm p	Effect Size
Mean Euclidean Distance (cm)	CMM Vs CMTM	126,339	6	<0.0001 ****	<0.0001 ****	0.80/Large
	TMM vs CMTM	218,247	98	0.5668 ns	1.0000 ns	0.11/Small
	CFF vs CFTF	129,336	9	<0.0001 ****	<0.0001 ****	0.78/Large
	TFF vs CFTF	217,248	97	0.5393 ns	1.0000 ns	0.11/Small
	CMF vs CFTM	130,335	10	<0.0001 ****	<0.0001 ****	0.77/Large
	CMF Vs CMTF	128,337	8	<0.0001 ****	<0.0001 ****	0.79/Large
	TMF vs CFTM	245,220	100	0.6236 ns	1.0000 ns	0.09/Small
	TMF Vs CMTF	252,213	93	0.4363 ns	1.0000 ns	0.14/Small
Mean Cosine Distance (au)	CMM vs CMTM	157,308	37	0.0012 **	0.0072 **	0.57/Large
	TMM vs CMTM	263,202	82	0.2169 ns	0.8068 ns	0.23/Small

	CFF Vs CFTF	162,303	42	0.0027 **	0.0133 *	0.53/Large
	TFF vs CFTF	237,228	108	0.8702 ns	1.0000 ns	0.03/Small
	CMF vs CFTM	156,309	36	0.0010 ***	0.0070 **	0.58/Large
	CMF vs CMTF	141,324	21	<0.0001 ****	0.00035 ***	0.69/Large
	TMF vs CFTM	264,201	81	0.2017 ns	0.8068 ns	0.23/Small
	TMF vs CMTF	233,232	112	>0.9999 ns	1.0000 ns	0.00/Small
Proximity Time (%)	CMM vs CMTM	341,124	4	<0.0001 ****	<0.0001 ****	0.82/Large
	TMM vs CMTM	244,221	101	0.6529 ns	1.0000 ns	0.08/Small
	CFF Vs CFTF	345,120	0	<0.0001 ****	<0.0001 ****	0.85/Large
	TFF vs CFTF	269,196	76	0.1370 ns	0.5480 ns	0.27/Small
	CMF vs CFTM	332,133	13	<0.0001 ****	<0.0001 ****	0.75/Large
	CMF vs CMTF	336,129	9	<0.0001 ****	<0.0001 ****	0.78/Large
	TMF vs CFTM	260,205	85	0.2671 ns	0.8013 ns	0.20/Small
	TMF vs CMTF	245,220	100	0.6236 ns	1.0000 ns	0.09/Small
Speed Difference (cm/s)	CMM vs CMTM	146,319	26	0.0001 ***	0.0007 ***	0.65/Large
	TMM vs CMTM	192,273	72	0.0975 ns	0.3901 ns	0.30/Medium
	CFF Vs CFTF	122,343	2	<0.0001 ****	<0.0001 ****	0.83/Large
	TFF vs CFTF	193,272	73	0.1064 ns	0.3901 ns	0.29/Small
	CMF vs CFTM	120,345	0	<0.0001 ****	<0.0001 ****	0.85/Large

	CMF vs CMTF	121,344	1	<0.0001 ****	<0.0001 ****	0.84/Large
	TMF vs CFTM	213,252	93	0.4363 ns	0.4363 ns	0.14/Small
	TMF vs CMTF	197,268	77	0.1485 ns	0.3901 ns	0.27/Small
Acceleration Correlation	CMM vs CMTM	302,163	43	0.0032 **	0.0220 *	0.52/Large
	TMM vs CMTM	198,267	78	0.1607 ns	0.4821 ns	0.26/Small
	CFF Vs CFTF	320,145	25	0.0001 ***	0.0009 ***	0.66/Large
	TFF vs CFTF	212,253	92	0.4063 ns	0.8134 ns	0.15/Small
	CMF vs CFTM	296,169	49	0.0075 **	0.0452 *	0.48/Medium
	CMF vs CMTF	288,177	57	0.0209 *	0.1045 ns	0.42/Medium
	TMF vs CFTM	224,241	104	0.7437 ns	0.8134 ns	0.06/Small
	TMF vs CMTF	194,271	74	0.1160 ns	0.4640 ns	0.29/Small
Maximum Velocity Cross- Correlation	CMM vs CMTM	324,141	21	<0.0001 ****	0.0003 ***	0.69/Large
	TMM vs CMTM	243,222	102	0.6827 ns	1.0000 ns	0.07/Small
	CFF Vs CFTF	345,120	0	<0.0001 ****	<0.0001 ****	0.85/Large
	TFF vs CFTF	225,240	105	0.7748 ns	1.0000 ns	0.05/Small
	CMF vs CFTM	272,193	73	0.1064 ns	0.3192 ns	0.29/Small
	CMF vs CMTF	283,182	62	0.0367 *	0.1836 ns	0.38/Medium
	TMF vs CFTM	173,292	53	0.0128 *	0.0768 ns	0.45/Medium
	TMF vs CMTF	188,277	68	0.0675 ns	0.2700 ns	0.33/Medium

4.4.5. Dyadic Metric Comparisons Between Heterogeneous Dyads

Consistent with the statistical analysis strategy we have carried out with homogeneous dyads, the between-group comparison in heterogeneous dyads was also done at the dyadic level. Since we illustrated the data representation of heterogeneous dyads earlier in the dyad resemblance analysis section, here we used a statistical table that provided both inferential and descriptive statistics on the four heterogeneous groups we analysed. All dyad-level metrics, including Euclidean distance, cosine distance, proximity time, speed difference, acceleration correlation, and maximum velocity cross-correlation, showed non-significant differences after the multiple-group analysis. Therefore, no pairwise comparisons were needed after the non-significant p-value obtained from the Kruskal–Wallis test.

Table 4.40. Kruskal–Wallis Test Results for Dyadic Interaction Parameters Across Heterogeneous Dyads

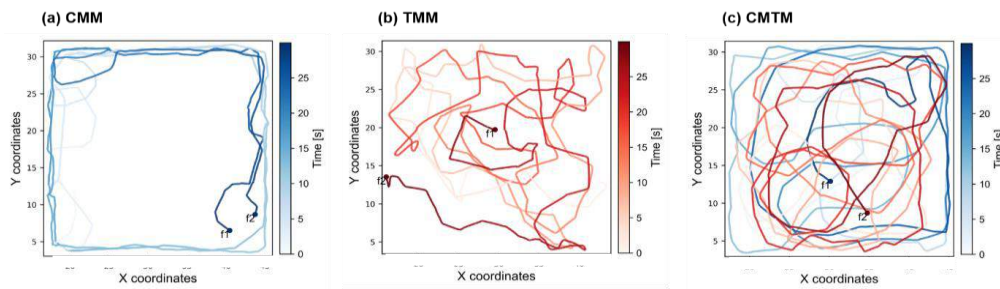
This table presents the Kruskal–Wallis test statistics comparing four dyadic groups (CMTM, CFTF, CFTM, and CMTF) across multiple behavioural parameters, including Euclidean distance, cosine distance, proximity time, speed difference, acceleration correlation, and maximum velocity cross-correlation. For each parameter, the H statistic, p-value, group-wise descriptive statistics (Mean, SEM, Median, and 95% Confidence Interval), and mean ranks are reported.

Parameter	Test Statistics	CMTM	CFTF	CFTM	CMTF
Euclidean Distance (cm)	H = 3.042 p = 0.3852 (ns)	Mean = 14.03 SEM = 0.7917 Mdn = 14.11 95% CI: 12.33–15.73 Mean Rank = 36.40	Mean = 11.94 SEM = 0.5211 Mdn = 12.42 95% CI: 10.83–13.06 Mean Rank = 25.33	Mean = 12.76 SEM = 0.8007 Mdn = 12.67 95% CI: 11.04–14.48 Mean Rank = 30.33	Mean = 12.59 SEM = 0.7923 Mdn = 12.76 95% CI: 10.89–14.29 Mean Rank = 29.93
Cosine Distance (au)	H = 1.045 p = 0.7904 (ns)	Mean = 0.8314 SEM = 0.0396 Mdn = 0.8432 95% CI: 0.7465– 0.9163	Mean = 0.8406 SEM = 0.0351 Mdn = 0.8967 95% CI: 0.7651– 0.9160	Mean = 0.7829 SEM = 0.05188 Mdn = 0.8507 95% CI: 0.6716– 0.8941	Mean = 0.8540 SEM = 0.03620 Mdn = 0.9054 95% CI: 0.7763– 0.9316

		Mean Rank = 31.20	Mean Rank = 30.87	Mean Rank = 26.80	Mean Rank = 33.13
Proximity Time (s)	H = 1.007 p = 0.7995 (ns)	Mean = 14.30 SEM = 2.391 Mdn = 12.21 95% CI: 9.176–19.43 Mean Rank = 31.27	Mean = 13.74 SEM = 1.849 Mdn = 14.84 95% CI: 9.772–17.70 Mean Rank = 31.53	Mean = 14.49 SEM = 3.608 Mdn = 8.290 95% CI: 6.752–22.23 Mean Rank = 26.67	Mean = 15.44 SEM = 2.650 Mdn = 12.30 95% CI: 9.755–21.12 Mean Rank = 32.53
Speed Difference (cm/s)	H = 1.919 p = 0.5893 (ns)	Mean = 3.749 SEM = 0.7011 Mdn = 3.614 95% CI: 2.246–5.253 Mean Rank = 27.80	Mean = 3.483 SEM = 0.4039 Mdn = 3.338 95% CI: 2.617–4.349 Mean Rank = 27.87	Mean = 4.889 SEM = 1.001 Mdn = 3.301 95% CI: 2.744–7.035 Mean Rank = 30.87	Mean = 6.280 SEM = 1.355 Mdn = 4.474 95% CI: 3.373–9.187 Mean Rank = 35.47
Acceleration Correlation	H = 0.8973 p = 0.8261 (ns)	Mean = 0.3700 SEM = 0.0719 Mdn = 0.2714 95% CI: 0.2156–0.5243 Mean Rank = 31.53	Mean = 0.3117 SEM = 0.0634 Mdn = 0.2482 95% CI: 0.1757–0.4478 Mean Rank = 28.07	Mean = 0.3171 SEM = 0.06664 Mdn = 0.2623 95% CI: 0.1741–0.4600 Mean Rank = 28.93	Mean = 0.3704 SEM = 0.05980 Mdn = 0.3223 95% CI: 0.2421–0.4986 Mean Rank = 33.47
Maximum Velocity Cross-Correlation	H = 6.865 p = 0.0763 (ns)	Mean = 2811 SEM = 658.4 Mdn = 1600 95% CI: 1399–4223 Mean Rank = 24.07	Mean = 2309 SEM = 362.1 Mdn = 1923 95% CI: 1532–3086 Mean Rank = 25.60	Mean = 5371 SEM = 1106 Mdn = 3726 95% CI: 2998–7744 Mean Rank = 38.40	Mean = 4327 SEM = 1025 Mdn = 3020 95% CI: 2128–6526 Mean Rank = 33.93

4.4.6: Dyad Representative Track Paths

- Panel 1



- Panel 2

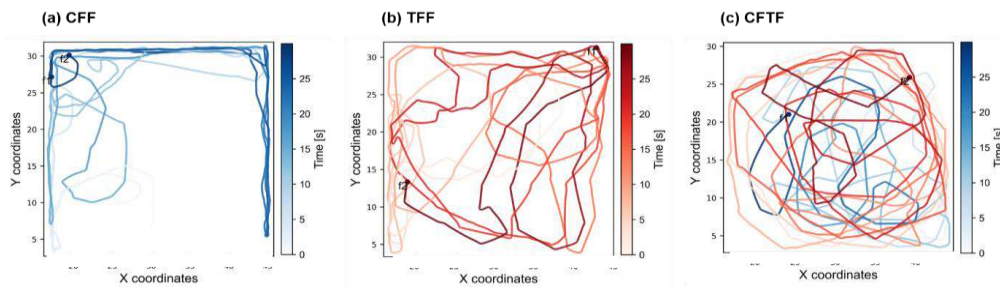


Figure 4.46. Spatiotemporal Trajectories and Exploration Patterns in Zebrafish Dyads.

Representative track paths generated from Cartesian coordinates (x and y) over the initial 30 seconds of interaction, illustrating distinct locomotive strategies across homogeneous and heterogeneous dyads.

Panel 1:

(a) Control Male-Male (CMM): Trajectories (blue) demonstrate highly synchronized, aligned movement characterized by wall-hugging (thigmotaxis).

(b) Test Male-Male (TMM): Trajectories (red) exhibit an explorative phenotype with reduced directional alignment.

(c) Control Male-Test Male (CMTM): In the heterogeneous dyad (blue and red), the overall movement pattern more closely resembles the explorative behaviour of the isolated homogeneous group.

Panel 2: (a) Control Female-Female (CFF), (b) Test Female-Female (TFF), and (c) Control Female- Test Female (CFTF).

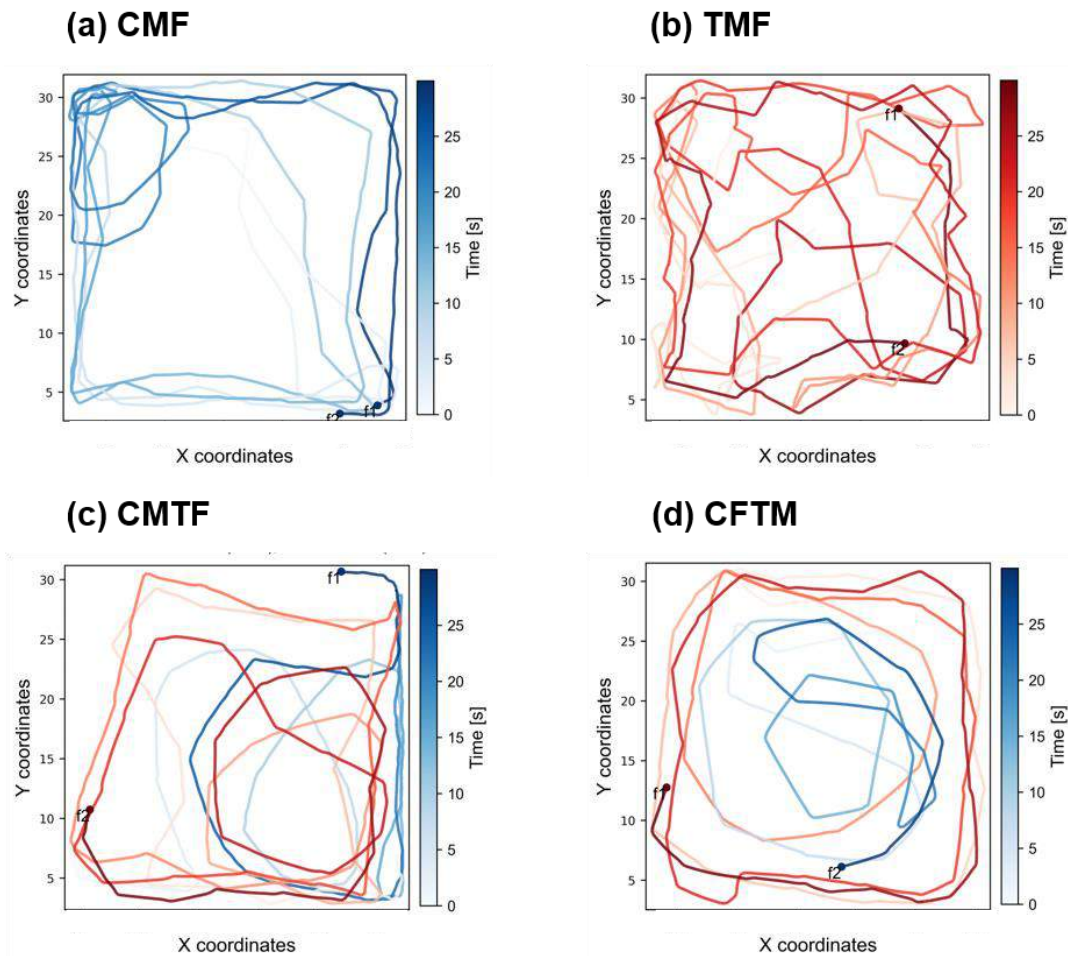


Figure 4.47. Spatiotemporal Trajectories and Exploration Patterns in Zebrafish Mixed Dyads.

(a) Control Male-Control Female (CMF), (b)Test Male -Test Female (TMF), (c) Control Male – Test Female (CMTF), and (d) Control Female – Test Male (CFTM)

4.5 Objective 5: Results

4.5.1. Objective statement

The objective proposed to investigate the alterations in the gut microbial diversity associated with chronic social isolation across male and female zebrafish. Next Generation Sequencing (NGS) was employed and the relative frequency of microbial diversity was analysed and reported at phylum, order, and genus levels descriptively. Firmicutes/Proteobacteria ratio was used to understand the microbial imbalance, calculated manually from the total abundance obtained from the NGS report. Alpha diversity was used to quantify the community structure and the outcomes were tabulated.

Table 4.41. Summary of Next-Generation Sequencing (NGS) Data Processing and Quality Control.

ID	Input Reads	Filtered Reads	% Passed Filters	Denoised	Merged	% Merged	Non-Chimeric	%Non-Chimeric
CM	81165	42950	52.92	38843	28085	34.6	6922	8.53
CF	54030	25617	47.41	22984	19073	35.3	5428	10.05
TM	62189	29851	48	26766	23470	37.74	3982	6.4
TF	75048	32647	43.5	30983	28537	38.02	2967	3.95

Table Description

Table 2. Summary of Next-Generation Sequencing (NGS) Data Processing and Quality Control. High-throughput sequencing depth per sample ranged from approximately 54,000 to 81,000 raw reads. Following the DADA2 pipeline for filtering and denoising, high-confidence Amplicon Sequence Variants (ASVs) were generated, with non-chimeric sequences representing 3.95% to 10.05% of the total input reads. Control groups (CM and CF) exhibited a slightly higher percentage of non-chimeric reads compared to isolated test fish. The successful removal of low-quality and chimeric sequences ensured the biological validity of downstream alpha diversity and relative abundance calculations.

Table 4.42. Alpha Diversity Metrics Quantifying Gut Microbial Community Structure

Alpha Diversity Metric	Control Male	Control Female	Test Male	Test Female
Shannon Entropy	4.53	4.41	4.58	4.17
Simpson Index	0.95	0.95	0.95	0.94
Chao1 (Estimated Richness)	30	26	31	23

Table Description

Table 1. Alpha Diversity Indices for Gut Microbial Communities in Male and Female Zebrafish. Comparison of Shannon Entropy, Simpson Index, and Chao1 estimated richness across control and isolated cohorts. The Test Male (TM) group exhibits the highest community complexity and taxonomic diversity, characterized by peak Shannon entropy and Chao1 richness values. Conversely, the Test Female (TF) group represents the most simplified community, demonstrating the lowest diversity and richness indices. Notably, the Simpson Index remains consistently high across all groups, indicating that the bacterial populations maintain a stable degree of evenness despite isolation-induced shifts in species richness.

4.5.2 Phylum-Level Bacterial Community Structure and Analysis of F/P Ratio

Control baseline gut microbiome descriptive analysis exhibited a distinct pattern in the microbiome abundance, thereby establishing a sexually dimorphic baseline outcome. Control males showed the presence of three major phyla including Firmicutes, Proteobacteria, and Fusobacteria, and two minor phyla including Actinobacteria and Planctomycetes. The F/P ratio of CM was 1.94, suggesting a Firmicutes-dominated stable healthy gut environment. In contrast, control females were observed with Firmicutes, Proteobacteria, and Fusobacteria as identified phyla, with an F/P ratio of 0.33, indicating a higher Proteobacteria enrichment relative to Firmicutes. However, the control female gut environment was approximately 40% inhabited by Fusobacteria, leading to a Fusobacteria-dominated healthy and stable microbiome.

Under isolation, the baseline sexually dimorphic difference was observed in both male and female zebrafish. Test isolated males were marked by increased Proteobacteria and an F/P ratio of 0.17, a shift from the baseline pattern, suggesting dysbiosis.

Reduced relative frequency of Firmicutes and Fusobacteria were also found in TM. The complete absence of Actinobacteria, an increase in Planctomycetes, and the exclusive presence of the opportunistic pathogen Chlamydiae indicate a compromised gut condition in isolated males.

The F/P ratio in test isolated females was identified as 1.18, a descriptive increase in Firmicutes relative to Proteobacteria. However, dysbiosis in isolated females was marked with a complete collapse of Fusobacteria, which constituted about 40% in the control females. The increased Firmicutes suggests the possible outgrowth in the vacant niche following Fusobacteria collapse. Hence, all these descriptive results suggest a sexually dimorphic gut microbiome alteration associated with prolonged isolation.

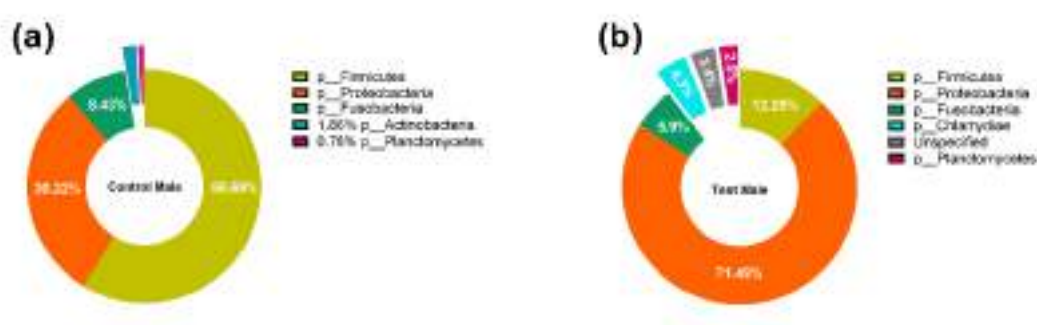


Figure 4.48. Relative Frequency (%) of Phyla in Male Zebrafish.

Ring shaped pie charts representing the microbial composition in (a) Control Male (CM) and (b) Isolated Male (TM). The (a) CM group is dominated by *Firmicutes* (58.69%), whereas the (b) TM group exhibits a significant *Proteobacteria* bloom (71.49%) and *Firmicutes* depletion (12.25%). Notably, the TM profile shows a complete absence of *Actinobacteria* (1.86% in CM) and the novel presence of *Chlamydiae* (4.3%). These indicators underscore a shift from a balanced community to a *Proteobacteria*-dominant dysbiosis in isolated males.

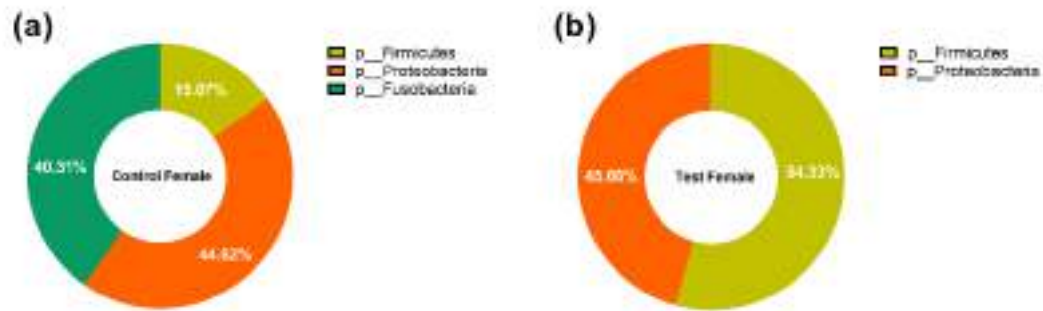


Figure 4.49. Relative Frequency (%) of Phyla in Female Zebrafish.

Ring-shaped pie charts representing the microbial composition in (a) Control Female (CF) and (b) Isolated Female (TF). The (a) CF group is characterized by a high abundance of *Fusobacteria* (40.31%) and *Proteobacteria* (44.62%), with *Firmicutes* constituting 15.07%. In contrast, the (b) TF group exhibits a significant *Firmicutes* bloom (54.33%) and a complete collapse of *Fusobacteria* (from 40.31% to 0%). These indicators highlight a distinct taxonomic shift toward *Firmicutes* dominance and a reduction in phylum-level diversity in isolated females.

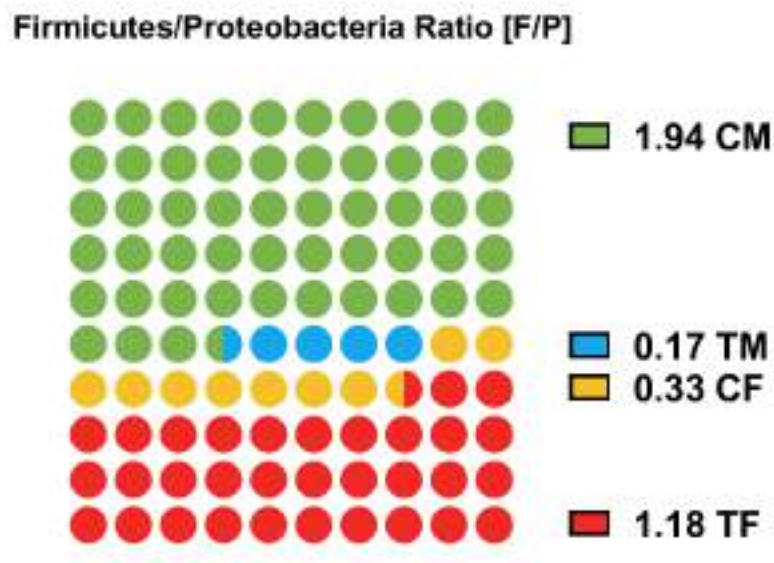


Figure 4.50. Comparison of Firmicutes/Proteobacteria (F/P) Ratios.

Stacked bar chart illustrating the F/P ratios across all experimental groups. The Control Male (CM) group exhibits the highest ratio (1.94), which undergoes a sharp decline in the Isolated Male (TM) group (0.17), reflecting the isolation-induced *Proteobacteria* bloom. Conversely, the Control Female (CF) group displays a low baseline ratio (0.33) that increases significantly in the Isolated Female (TF) group

(1.18) due to Firmicutes expansion. These divergent shifts indicate that social isolation induces sex-specific polarities in gut microbial homeostasis.

4.5.3 Functional Loss of Beneficial Firmicutes in Test Isolated Male (TM) Zebrafish: Genus-Level Descriptive Analysis

The sharp descriptive loss of phylum Firmicutes in TM was further evident from the complete loss of beneficial genera including *Bacillus*, *Paenibacillus* and *Brevibacillus*, which were reported as 44.2%, 11.08% and 3.35% in CM respectively. CF was identified with the genus *Paenibacillus* (15.07%) only, in contrast TF was observed with genera *Bacillus* (28.24%), *Paenibacillus* (12.84%) and unspecified (13.24%) under the phylum Firmicutes, further suggesting an increase in the Firmicutes diversity under Fusobacteria collapse.

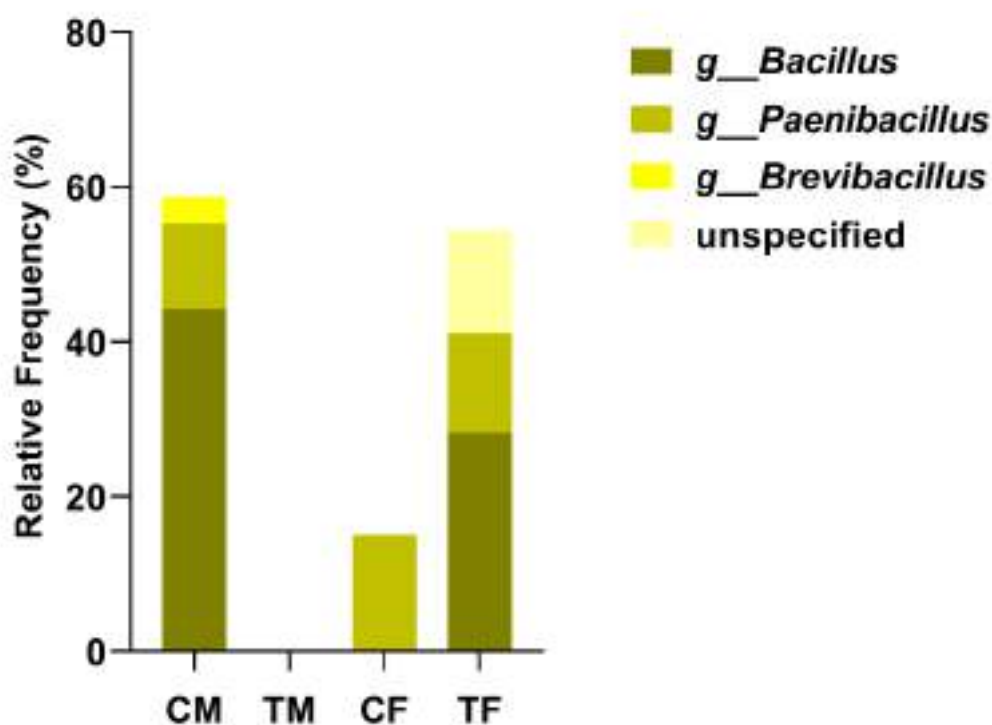


Figure 4.51. Relative Abundance of Genera within the Phylum Firmicutes.

Stacked bar chart illustrating the distribution of Firmicutes genera across the experimental groups. A critical observation in the Isolated Male (TM) group is the complete absence of beneficial *Bacillus* genera, as well as *Paenibacillus* and *Brevibacillus*, following social isolation. These findings suggest that isolation-induced stress leads to the selective elimination of potentially probiotic taxa in males.

4.5.4 Collapse of Dominant Phylum Fusobacteria in Test Isolated Female (TF) Zebrafish: Order-level Descriptive Analysis

Even though the exact genera under Fusobacteria were mentioned as unspecified in the NGS output, the order *Fusobacteriales* was consistently identified across CM (8.43%), TM (5.09%), and CF (40.03%). The phylogenetic tree suggested the presence of *Cetobacterium* in two divisions, a beneficial genus, which was absent in TF and reduced in TM.

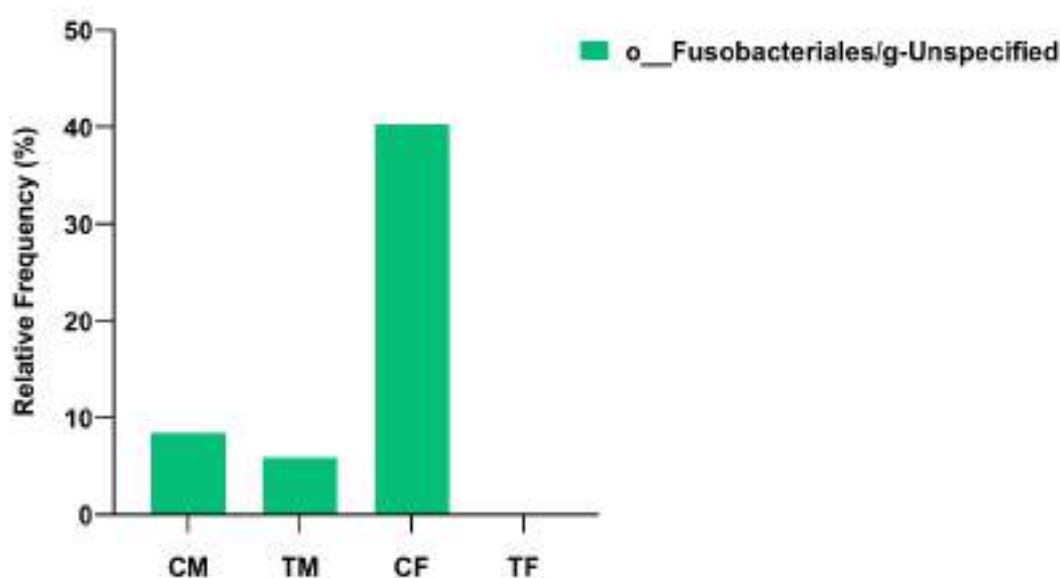


Figure 4.52. Relative Abundance of Taxa within the Phylum Fusobacteria.

Grouped bar chart representing the relative frequency (%) of the order Fusobacteriales across experimental groups. High-throughput Next-Generation Sequencing (NGS) identified these taxa exclusively at the order level. The Isolated Female (TF) group exhibits a complete collapse of Fusobacteriales (0%). A marginal reduction is observed in Isolated Male (TM). This total loss in isolated females underscores a severe, sex-specific depletion of this taxonomic group in response to social isolation.

4.5.5 Phylum Proteobacteria: Genus-level Analysis and Presence of Chlamydiae

The phylum Proteobacteria showed enhanced diversity. TM was observed with the stress marker *Acinetobacter*, along with environmental opportunists including *Legionella*, *Caulobacter*, and *Gulbenkiania*. TF was identified with *Brevundimonas* and *Filomicrobium*. Other genera under Proteobacteria were shared among all the groups, as shown in the graphical representation. The exclusive presence of

Acinetobacter in TM and other opportunists in TM and TF indicates increased vulnerability to environmental opportunists under isolation. The exclusive presence of the pathogenic genus *Simkania* under the phylum Chlamydiae in TM further strengthens the possibility of an altered gut microbiome and vulnerability under isolation.

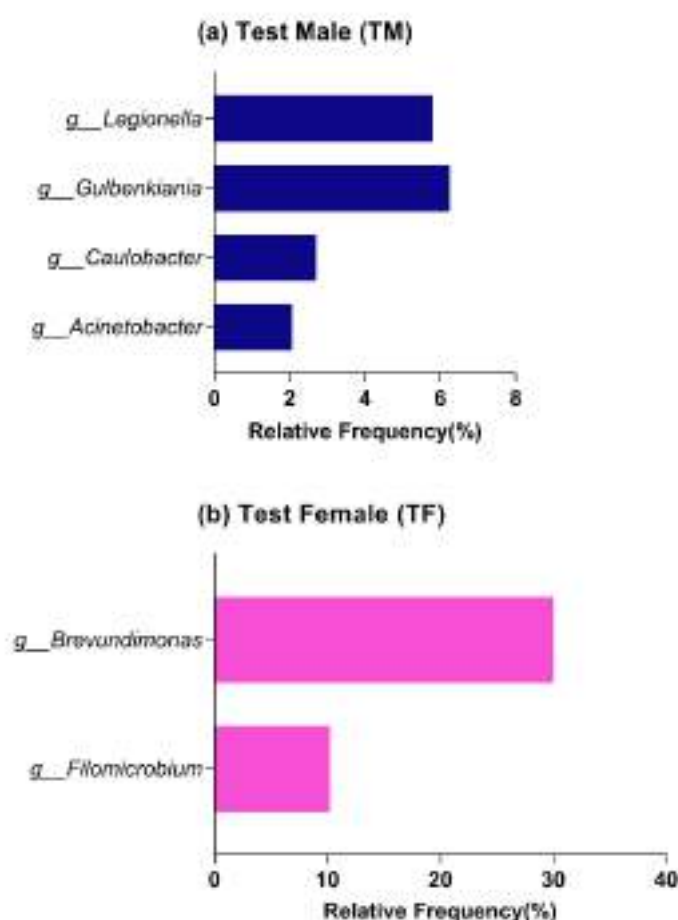


Figure 4.53. Sex-Specific Genera Distribution within the Phylum Proteobacteria.

Horizontal bar charts illustrate the relative frequency (%) of Proteobacteria genera present exclusively under social isolation in (a) Isolated Male (TM) and (b) Isolated Female (TF). High-throughput Next-Generation Sequencing (NGS) reveals a distinct sex-specific signature in the proteobacterial community. The (a) TM group is characterized by the presence of *Legionella*, *Gulbenkiana*, *Caulobacter*, and *Acinetobacter*. In contrast, the (b) TF group displays a unique profile dominated by *Brevundimonas* and *Filomicrobeum*. These findings indicate that while both sexes experience a proteobacterial shift, the specific taxonomic recruitment is highly sexually dimorphic.

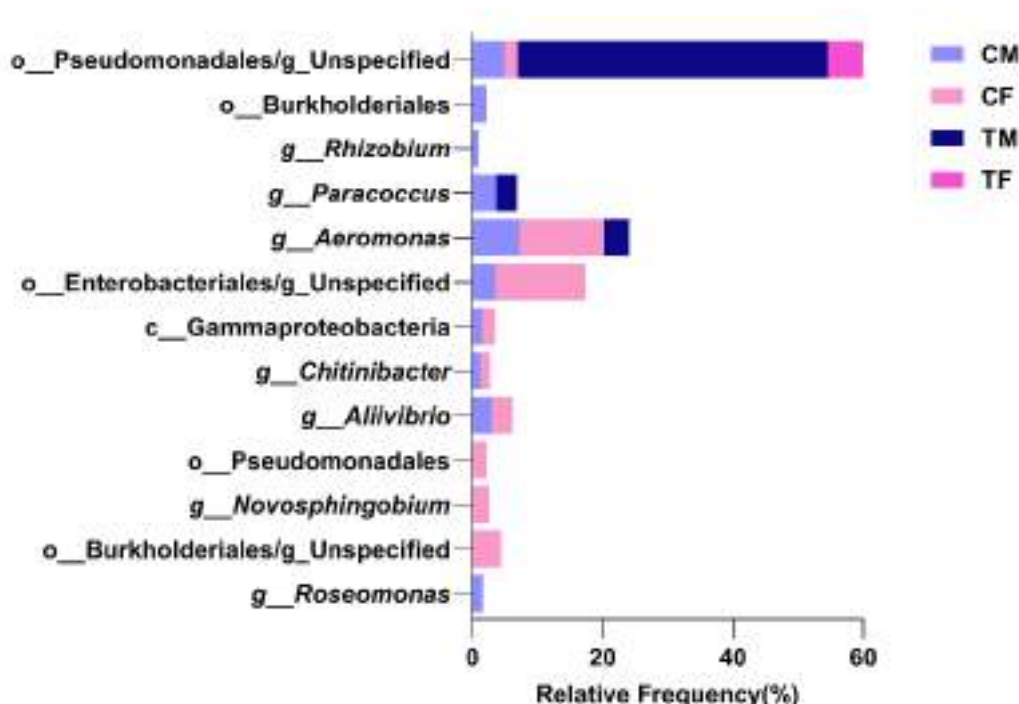


Figure 4.54. Relative Abundance of Proteobacteria Taxa Across Experimental Groups.

Stacked bar chart representing the distribution of Proteobacteria at the order and genus levels across all cohorts. High-throughput Next-Generation Sequencing (NGS) identified O_Pseudomonadales as the most prevalent group, which contains taxa with both beneficial and opportunistic pathogenic potential. This specific group is overwhelmingly dominated by the Isolated Male (TM) group, representing a significant expansion compared to others. In contrast, the Control groups (CM and CF) maintain a more balanced community profile, featuring beneficial or environmental strains such as *Rhizobium* and *Paracoccus*, alongside baseline levels of opportunistic pathogens like *Aeromonas* and O_Enterobacteriales. These results indicate that social isolation in males triggers a disproportionate surge in potentially pathogenic Pseudomonadales.

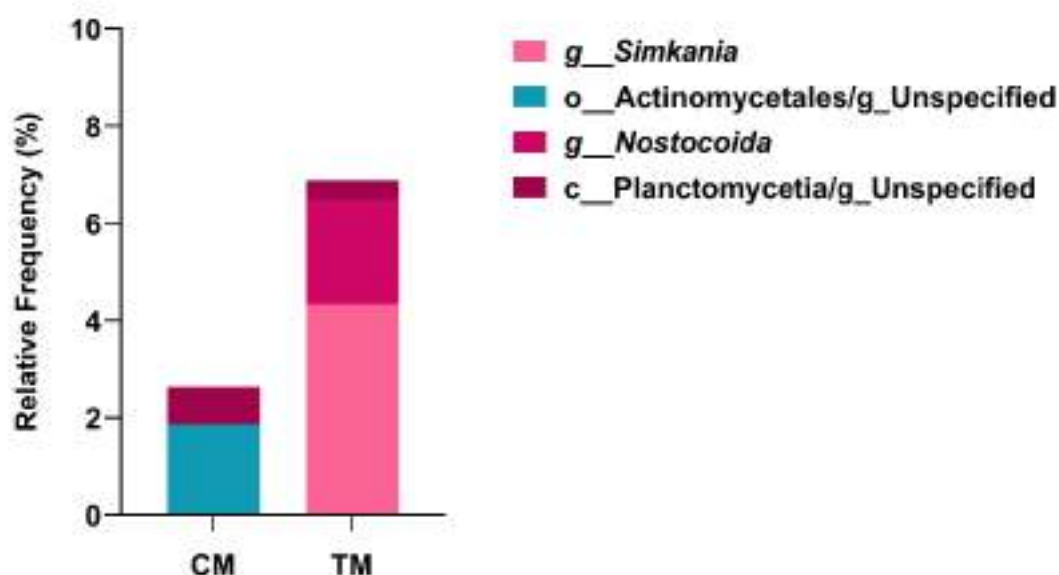


Figure 4.55. Relative Abundance of Taxa within Minor Phyla: Chlamydiae, Actinobacteria, and Planctomycetes.

Stacked bar chart illustrating the relative frequency (%) of minor bacterial phyla in Control Male (CM) and Isolated Male (TM) zebrafish. (1) The CM group exhibits a balanced baseline distribution of Actinomycetales (unspecified genus within *Actinobacteria*) and Planctomycetes. (2) In contrast, the TM group shows a higher relative abundance of *Simkania* (phylum *Chlamydiae*), while Actinomycetales was not detected. (3) Additionally, specific Planctomycetes groups, including *Nostocoida*, were identified in the isolated cohort. These shifts indicate a significant alteration in low-abundance taxa and the emergence of potentially pathogenic groups following social isolation. No representative data for these taxa were recorded across the CF and TF groups.

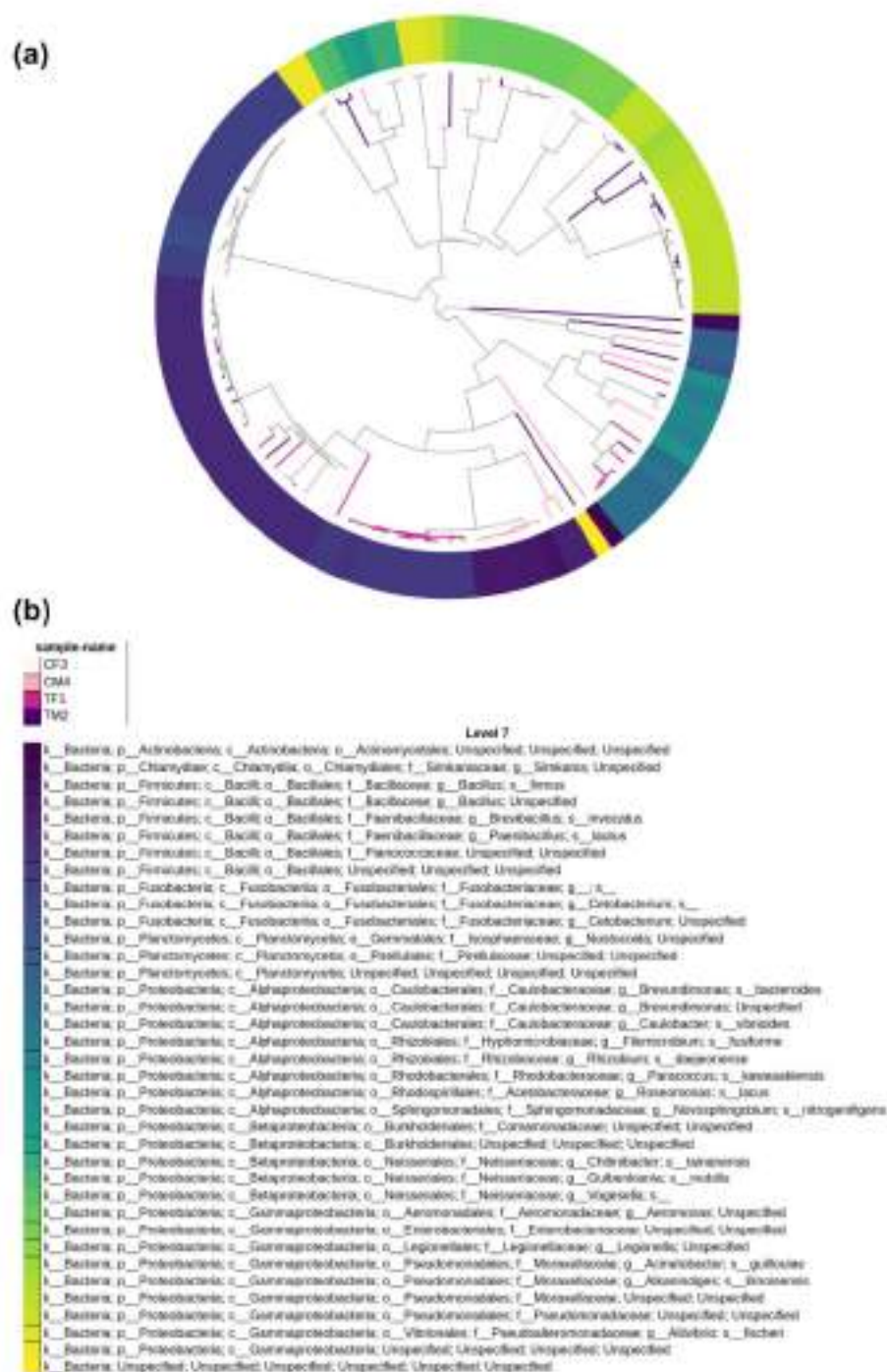


Figure 4.56. Circular Phylogenetic Tree of Gut Microbiota in Zebrafish.

A Level 7 taxonomic representation illustrating the phylogenetic relationships between identified bacterial taxa across all experimental groups. The tree highlights the presence of the genus *Cetobacterium* (Phylum: Fusobacteria) within the order Fusobacteriales. The color-coded outer ring confirms that these taxa are prevalent in control cohorts but undergo a significant reduction or complete collapse in the Isolated Female (TF1) and Isolated Male (TM2) groups, aligning with the observed phylum-level dysbiosis.

Chapter 5

Discussion

5.1 Objective 1: Discussion

5.1.1 Sex Independent Anxiolytic-Like Behavioural Responses Under Social Isolation

Chronic developmental social isolation in zebrafish has long been associated with alterations in anxiety-like behaviours and locomotor activity levels (Shams et al., 2015). Behavioural responses in zebrafish are often interpreted through measures of boldness and anxiety, depending on the context of the test. Boldness is generally characterised by high activity and exploration in areas that are inherently risky or non-preferred, such as the centre of the open field arena, the open half of the shelter test, the top zone of the novel tank, or the white compartment in the scototaxis test. Conversely, anxiety-like or shy behaviour is associated with thigmotaxis, bottom-dwelling, or preference for sheltered or dark zones (Mustafa et al., 2019b).

Studies including Dahlbom et al. (2011) and Roy and Bhat (2018) have reported sex-dependent behavioural differences in zebrafish, with males exhibiting greater boldness and dominance-related behaviours or stronger shoal association under predator exposure than females. However, other studies have demonstrated contrasting individual differences in boldness-related behaviours in females, where bold females showed enhanced centre exploration in novel environments (Beigloo et al., 2024). In some contexts, bold females were also more likely to shoal, indicating that boldness can form part of a behavioural syndrome depending on the context and social cue (Mustafa et al., 2019b; Way et al., 2015b). These contrasting observations make sex-specific associations with boldness difficult to generalise across studies.

In the present study, control males (CM) displayed greater centre exploration in the open field test (OFT), consistent with boldness as previously reported in male zebrafish. In contrast, control females (CF) spent more time in the perimeter of the tank, reflecting a more anxiety-like response. Zebrafish males exposed to social isolation (TM) displayed a reduction in exploratory drive in the novel environment compared to socially housed control males, although preference for the exploratory zone was still preserved. This suggests that isolation exerts a mild inhibitory effect on the innate exploratory bias in male zebrafish. However, confirmation of an anxiety-like emotional state was not possible, as TM maintained exploratory preference.

Interestingly, isolated females (TF) deviated from the baseline behavioural pattern of CF by showing enhanced centre exploration, suggesting that chronic social isolation may differentially modulate exploration-related behaviour in females. Despite these sex-specific baseline shifts, group-level comparisons between isolated males and females converged, resulting in non-significant differences in time spent in the thigmotactic zone when analysed exclusively using open field data. Studies have shown that in zebrafish, boldness and exploratory behaviours are closely linked and form a behavioural syndrome. In novel environment tests, these traits are associated with anxiety-related measures such as time spent in the thigmotaxis zone. Therefore, anxiety-related behaviour under chronic social isolation appeared sex independent, indicating that prolonged social deprivation may override or mask baseline behavioural syndromes related to exploration and anxiety (Conrad et al., 2011; Santos et al., 2023).

Thigmotaxis has been widely used as an anxiety-related behavioural measure in both rodent and zebrafish models (Kalueff et al., 2013). In the present study, chronic social isolation resulted in reduced thigmotaxis in both males and females, indicating that anxiety-related behaviour under isolation was largely sex independent. This pattern mirrors behavioural responses reported in pharmacological studies using known anxiolytic drugs such as chlordiazepoxide (Johnson et al., 2023) or diazepam (Richendrfer et al., 2012; Schnörr et al., 2012) which act by enhancing GABAergic signalling, and are associated with reduced wall-hugging behaviour.

At the same time, recent work by Rajput et al. (2022) described multiple behavioural domains in adult zebrafish and categorised a subset of AB strain females as “wall huggers,” without interpreting this behaviour as a shy or anxiety-like response. Their findings caution against using thigmotaxis alone as a direct indicator of anxiety or predator avoidance, and warn against overinterpretation of this single parameter in behavioural studies. In line with this perspective, anxiety-related behaviour in the present study was interpreted using multiple behavioural parameters, and the light–dark test was included as a confirmatory assay, strengthening the interpretation of anxiety-related outcomes following social isolation.

We next analysed zone-specific distance travelled as a complementary measure of time spent in zones. The pattern of zone-specific distance resembled the time in zone

preference data, such that zones where animals spent more time were also associated with greater distance travelled.

The light–dark test (LDT) was used as a confirmatory assay to extend these findings under a different anxiety-related paradigm. In agreement with previous studies, the LDT revealed a clear confirmation of the emotional valence of isolated zebrafish. Both isolated males and females spent more time in the light zone compared to their respective controls, and the latency to first entry into the light zone was significantly reduced. In contrast, control males (CM) and control females (CF) exhibited an innate preference for the dark compartment (scototaxis), without detectable sex differences.

These findings are consistent with Santos et al. (2021), who employed a three-day behavioural test battery and reported that light–dark zone preference was sex independent on day 1. By day 3, sex differences emerged, with females spending more time in the dark zone, a pattern that parallels the present open field results, where control females displayed higher anxiety-like behaviour than control males. Similarly, Fontana et al. (2019) reported higher anxiety in female zebrafish, reflected by increased scototaxis and geotaxis. Previous studies have extensively examined the effect of housing conditions in fish, either for better management in aquaculture or for evaluating behavioural responses to validate experimental conditions and avoid misinterpretation. Collymore et al. (2015) resembles our social isolation design in the single barren-housed condition but differs in the time and duration of isolation. They reported higher entries into the light zone than the dark zone and interpreted this response as anxiogenic, which contrasts with our interpretation. We have shown that socially isolated zebrafish spent higher time in the light zone with lower latency to enter it, and this clear preference for the light zone over the dark zone inclined us towards an anxiolytic interpretation. Similarly, Jorge et al. (2023) employed a comparable housing concept using barren group-housed and enriched group-housed conditions, and both groups showed higher time spent in the dark zone than the light zone, which is consistent with our baseline responses in both male and female zebrafish.

According to Onarheim et al. (2022), isolated zebrafish exhibited more entries into the white compartment than group-housed fish when the LDT was conducted first, but this preference was diminished when the LDT was administered second, following

the NTT. Additionally, isolated fish displayed a blunted whole-body cortisol response. These findings on LDT-higher light zone entries and lower cortisol levels-were broadly consistent with our observation of anxiolytic-like responses in isolated fish and also highlight the importance of test order. However, in the present study, each assay was conducted on independent samples, and test battery order was not a primary research concern.

There is a fine line between avoidance behaviours following threat perception and the maladaptive behaviours characteristic of anxiety disorders. Research has shown that animals typically respond to visual or threat-related cues by freezing (Rösler & Gamer, 2019). Studies also have shown that administration of glutamatergic and serotonergic hallucinogenic agents can induce marked anxiolytic-like behaviours in zebrafish without altering freezing frequency (Neelkantan et al., 2013), which further supporting the context specificity of behavioural metrics. Therefore, in the present objective, freezing was not considered as an anxiety-related parameter, as it represents a defensive response to an acute or visible threat rather than a generalised anxiety state. Instead, this objective utilised parameters such as time and distance spent in the thigmotactic zone in the OFT and preference for the light zone in the LDT as more dependable indicators of anxiety-like behaviour.

The discussion also poses the question of what underlying mechanism may be responsible for these sex dependent and independent responses. Recent work highlights a strong role of hormonal pathways. For example, endogenous estrogen levels, specifically, 17 β -estradiol (E2) were identified to be higher in females than males, a plausible regulatory mechanism to the sex differences in zebrafish anxiety. Females were reported with reduced brain serotonin and dopamine levels, acting through the HPI axis, further explains sexually dimorphic anxiety responses in zebrafish (Tao et al., 2025).

Social isolation and anxiety are a large research area of interest. Even though studies are based on animal models, differences in duration and timing, as well as variations in the methodological framework, have resulted in largely varied outcomes. Studies including Amiri et al. (2017), Cacioppo et al. (2011), Daniel & Bhat (2022), Lukkes et al. (2009), Santini et al. (2020), Santos et al. (2010), Yasuda et al. (2016), and showed social isolation-associated anxiogenic responses. Furthermore, other studies

showed anxiolytic responses associated with early-life chronic social isolation (Giacomini et al., 2015; Parker et al., 2012; Shams et al., 2015). The current methodological design favours chronic early-life social isolation and is consistent with anxiolytic responses, also adding a sex-specific layer and suggesting a plausible sex-independent effect of social isolation.

5.1.2 Sex Independent Hyperactivity-Like Behavioural Responses Under Social Isolation

Having established the anxiety-like behavioural responses associated with chronic social isolation, the analysis was extended to the second dimension of the study, the locomotor activity. Multiple parameters including, zone transitions, rapid movements (speed > 15cm/s), speed range, activity index and ABI were used.

All of the locomotor parameters showed increases in the test groups compared to their respective controls, and the results were sex independent in both experimental conditions. Notably, the ABI data indicated that the locomotor activity in isolated groups was fragmented rather than continuous, with swimming velocity shifts from high to low. Therefore, this trend suggests that chronic developmental social isolation not only increases the overall activity but also disrupts the organization of motor behaviour, leading to a dysregulated, hyperactive phenotype. These results are consistent with previous research showing that chronic social stress can change activity levels in zebrafish (Giacomini et al., 2015; Onarheim et al., 2022).

Similarly, high speed swimming (>15 cm/s) was classified as hyperlocomotion rather than anxiety. Consistent with Kysil et al. (2017), we interpreted higher rapid swimming in the isolated groups as increased motor activity rather than anxious response. This is further supported by the recent pharmacological evidence on oxazepam, a widely prescribed anxiolytic drug, showed increased swimming activity and speed, further indicating the anxiolytic behaviour associated with hyperlocomotion, in contrast to anxiogenic escape-driven hyperlocomotion (Herrera et al., 2025).

Under control conditions, the sex specific differences were observed in total distance travelled, with females travelling greater distance than males. Importantly, when this behavioural measure was considered together with thigmotaxis, the total distance in

CF likely reflecting to anxiety-driven escape response rather than hyperactive phenotype, consistent with previous reports connecting distance travelled in the perimeter with anxiety in zebrafish (Egan et al., 2009; Maximino et al., 2010a; Borba et al., 2025).

In contrast, test males and test females both exhibited distances exceeding their respective controls, with the increase being comparable across sexes, indicating sex independent hyperlocomotion in isolated Zebrafish. Therefore, based on the context, the distance travelled reflects either exploration, stress escape, or motor dysregulation (Fitzgerald et al., 2019; Kysil et al., 2017; Stewart et al., 2012). This interpretation is further supported by the study done by Kozol et al. (2015) in *syngap1b* and *shank3a* models of larval zebrafish. The hyper-excitable behaviours and increased locomotion observed was interpreted as a state of hyperactivity rather than anxiety induced escape response.

5.1.3 Integrating Both Anxiety and Activity Domain Under Social Isolation

The analysis of behavioural responses usually involves a large set of variables that contribute to the phenotypes assessed. In the present study using OFT, we conducted analysis of anxiety and activity-related domains independently by analysing multiple variables independently. In order to simplify and reduce the complexity of high-dimensional behavioural data into a few key dimensions, we employed principal component analysis (PCA) in OFT. The variables were selected for the PCA following Spearman's rank correlation on a total of seven parameters. Note that the total distance travelled generally applied for the locomotion metric was avoided because of the different interpretation across control females (might indicate anxiety-driven escape behaviour) and test fish groups (hyperlocomotion). The finalised anxiety-related metrics were zone preferences and distance travelled in the thigmotactic zone. Whereas, the activity-related metrics selected were rapid movements and zone transitions. This ensured that the PCA was performed on these selected variables that captured specifically exploration, anxiety, and activity-specific behavioural measures (Quadros et al., 2016).

Two principal components were extracted, which were associated with anxiety-related and exploratory variables loaded in PC1 and activity variables loaded in PC2. Consistent with the univariate analysis, both PC1 and PC2 in the test isolated groups exhibited sex-independent outcomes in contrast to the control groups. Under PC2, control groups showed significant sex-dependent results that were not apparent from univariate analysis. This pattern strongly suggests that chronic social isolation masks and overrides the baseline sex specificity observed in both the anxiety and activity-related measures. Both PC1 and PC2 outcomes were consistent with the univariate analysis. Due to the strong baseline differences in PC1, test males exhibited reduced exploration, while test females exhibited heightened exploration, even though the differences between TM and TF were statistically non-significant. In PC2, both TM and TF uniformly exceeded the baseline activity levels, even though the control groups showed sex-specific results, where CM were more active than CF (Genario et al., 2020). Therefore, these results highlight the improved sensitivity of PCA in identifying the multivariate behavioural patterns that were invisible in univariate analysis.

Shams et al. (2017) found that social isolation reduced dopamine turnover (DOPAC) during exposure to social cues in zebrafish. In the present study, chronic social isolation may have created a socially deprived state and primed the dopaminergic system to produce amplified surges when triggered with external stimuli such as novelty (Roitman & McCutcheon, 2025). Together, these studies suggest that dopaminergic modulation under social isolation might not be uniform but context dependent. The amperometry technique revealed the real-time dopamine release in the nucleus accumbens during a challenge in mice, and social isolation evoked the dopamine release in the adult and middle-aged mice. Thereby, this study suggests that social deprivation induces neurochemical hypersensitivity in the mesolimbic pathway during adulthood in mice (McWain et al., 2022). Pharmacological studies have also connected the role of the dopamine surge in anxiety and stress. Administration of β -carboline (FG 7142), an anxiogenic drug into freely moving rats produced increased dopamine in the prefrontal cortex, and this was further blocked by benzodiazepine antagonists (Bradberry et al., 1991). Recent studies on a similar arena further revealed that FG 7142 modulated fear but not anxiety in zebrafish (Scatterty & Hamilton, 2024) showing that the high-speed movement is distinct from anxiety-driven avoidance.

However, the present study showed an inclination toward anxiolytic behaviour with the zone preference in the OFT and LDT, supported by a similar anxiolytic response in the novel tank from socially isolated zebrafish (Li et al., 2025). The higher speed with exploratory nature observed under social isolation creates a paradoxical outcome, further leading to the ADHD-associated hyperlocomotion or hyper arousal like (Isaac et al., 2024) state in zebrafish. The zebrafish dopamine transporter (DAT) mutants led to persistent hyperlocomotion and repetitive behaviours such as digging in the bottom of the tank, also rescued by clozapine, which reduces dopaminergic activity, agreeing with the role of dopamine in hyperactive and repetitive behaviours (Wang et al., 2019). Similarly, *lphn3.1* zebrafish larvae morphants also exhibited significant hyperactivity, suggesting a saturated dopaminergic signalling (Lange et al., 2018). Therefore, the social isolation might have evoked a dopamine surge when exposed to OFT and LDT and created an ADHD-like hyper arousal state and the isolated groups became more exploratory and hyperactive, resembling the anxiolytic state. Though the lack of real-time measure of dopamine release limits the current interpretation, pharmacological studies clearly support the interpretation. Genetic factors are also involved, as *adgrl3.1* knockout fish changed cortisol regulation and produced both anxiety-like and hyperactive behaviours in two-week isolated zebrafish (Fontana et al., 2025). The study indicates that the genetic predispositions interact with social isolation, an environmental stressor to intensify both externalising (hyperactivity and impulsivity) and internalising (anxiety) symptoms.

Pharmacological research on anxiolytic drugs cross-validated the behavioural responses examined in our test group, specifically the increased centre exploration and hyperactivity in the earlier discussion. While we also consider the potential effects of habituation and re-exposure to the experimental arena in reducing neophobia, particularly in control females. CF exhibited increased thigmotaxis and perimeter distance travelled, classic anxiogenic response during the acute 6-minute OFT trial. In line with the study by Resmim et al. (2023), the high anxiety zebrafish (LEO) exhibited increased centre exploration and hypolocomotion upon re-exposure to the OFT. This likely reflects the increased habituation and reduced novelty-induced stress in fish. This suggests re-exposure produces behavioural patterns different from those seen in acute exposure, thereby warranting the need for future studies employing repeated trial paradigms to make this distinction.

Therefore, these studies suggest that the behavioural patterns we observed particularly the masking of baseline sex differences under isolation and the emergence of sex independent hyperactivity may reflect deeper changes in neuroendocrine and molecular pathways. The mounting evidence also highlights the importance of sex as a biological variable in behavioural studies.

5.2 Objective 2: Discussion

5.2.1 Anti-Predator Responses Under Social Isolation in the Absence and Presence of Conspecifics

Zebrafish exhibit a range of anti-predator responses, including freezing, erratic movements, hiding in vegetation and heightened shoaling, in response to visual and olfactory cues from live or robotic predators (Gerlai et al., 2009). Predator detection and successive evasion are largely dependent on visual and olfactory cues, as described earlier. However, studies have also noted the importance of the lateral line system in detecting the hydrodynamic disturbances generated by predators, particularly in zebrafish larvae (Stewart et al., 2013). The internal state of the animal strongly influences the external anti-predator responses, for example, a state of hunger has been shown to override fear avoidance toward a live predator in zebrafish (Chathooth et al., 2025). In the present study, male and female zebrafish, under social isolation exhibited attenuated anti-predator responses, as evidenced primarily by higher predator-zone time spent, lower rapid movements compared to the baseline no stimulus condition, respective to their control counterparts. Although freezing was reported in lower levels than in the control groups, isolated males showed higher freezing compared to the within-group no-stimulus condition. Interestingly isolated females exhibited similar levels of freezing in all stimulus conditions, suggesting a blunted or altered stress response in zebrafish under isolation rather than a sensory deficit.

Animal models including rodents and non-human primates are known to remember past threat related events and demonstrate threat memory thereby avoiding similar conditions later. Zebrafish also exhibit fear generalization to past experiences and individuals from high predation risk areas showed increased anxiety-like behaviours (Jiang et al., 2025). This observation complements the dangerous-niche hypothesis,

with such fish exhibiting predator odour neophobia (Thapa et al., 2024). The experimental design in the current study might not favour fear generalization from threat related incidents, thereby limiting the emergence of effective antipredator responses in isolated groups and contrasting with the patterns observed in control groups. Control groups showed lower time spent in the predator zone, within this, males avoided the predator zone but distributed themselves between internal and distant zones, indicating innate boldness or predator inspection to gather further information for survival. In females the avoidance was more evident, as they avoided the nearest predator and internal zones and moved predominantly to the distant zone, likely mimicking the shelter seeking or hiding anti-predator responses (Mukherjee & Bhat, 2024) commonly observed in wild zebrafish.

A pivotal recent study has shown that learning of predator avoidance occurs early and rapidly in zebrafish, beginning from 5 days post-fertilization (dpf) onwards and developing within approximately one minute of exposure (Zocchi et al., 2025). This outcome points towards early learning of the surrounding environment in zebrafish (Martin & Lovett-Barron, 2025), whereas social isolation in the present study began from 21 dpf. Taken together, the chronic isolation window might have influenced the stress modulation and subsequent behavioural responses during predator exposure in the test groups. Notably isolated females exhibited a heightened sensitivity, as reflected by the similar distribution of time across zones when presented with the predator stimulus.

Predator inspection, even in the presence of freezing, a reliable fear indicator, has been reported in zebrafish, particularly in the presence of conspecifics. Such cooperative predator inspection (Pimentel et al., 2021) may explain the approach behaviour displayed by control females towards the predator when exposed to both predator and conspecific stimuli. When transitioning from the single predator condition to the dual stimulus condition, control females showed increased time in the predator zone under dual stimuli compared to the predator only condition. Notably, control females exhibited similar levels of freezing in both the single and dual conditions (PO and PS), indicating that the presence of conspecifics altered approach behaviour without changing the underlying fear response. As a highly social species, zebrafish exhibit shoaling as a key anti-predator response characterised by increased polarization,

velocity and decreased inter-individual distances (Mukherjee et al., 2024, Mukherjee & Bhat, 2023b). Recalling the social deficit observed in isolated groups, the reduction in shoal zone time when presented only with the conspecific cue highlights the possibility of attenuated group or shoal seeking behaviour under predator threat. In contrast to this observation, both males and females from the isolated groups showed a higher preference gradient towards conspecifics than predator zone when presented with both stimuli. However, when compared with their respective control counterparts, isolated males showed reduced time in the conspecific and increased time in the predator zones, while isolated females showed comparable time in the conspecific time, likely due to the shift in approach behaviour observed in control females. Considering freezing behaviour under the dual stimulus condition, isolated males displayed freezing in both single and dual conditions without significant reduction. In contrast, isolated females showed heightened freezing when the conspecific was present, pointing towards the increased sensitivity in females associated with fear response under social isolation.

Pharmacological studies with oxazepam, a known anxiolytic drug have shown increased inspection of caged predators in the cyprinid fish *Rutilus rutilus* (Herrera et al., 2025). Notably, the anxiolytic behavioural profile observed in objective 1, together with the disrupted anti-predator responses reported in the present objective mirrors findings from the established anxiolytic pharmacological studies. This suggests that an anxiolytic internal state may influence the fear related responses in isolated groups.

Cross species studies in fish have also shown the relationship between habitat complexity and antipredator responses. Coral reef fish from low complexity, barren habitat exhibited reduced antipredator escape response along with increased cortisol levels, whereas fish from high complexity habitat showed more effective escape responses and lower cortisol levels (Fakan et al., 2023). Consistent with these findings, chronic social isolation in the present study may mimic a lower complexity environment, and lead to reduced anti predator responses, primarily reflected by increased predator zone time and altered freezing in both single and dual conditions. Secondary response changes including reduced erratic movements and altered speed within the predator zone further supports this interpretation.

Social animals across mammalian models, specifically in non-human primates (Smith et al., 1998; Stanton et al., 1985), rats (Kiyokawa et al., 2014; Wiedenmayer et al., 2003), guinea pigs (Graves & Hennessy, 2000; Hennessy et al., 2008) have been widely demonstrated stress alleviation in the presence of social partners or groups, this phenomenon is termed as social buffering, a conserved evolutionary mechanism. In aquatic environments, enhanced sociability has been found to reduce the duration of stress response (Allen et al., 2009) and decrease the metabolic costs linked to predator threats (Chrétien et al., 2021; Culbert et al., 2019).

Therefore, the discussion now shifts to findings regarding social buffering (Faustino et al., 2017) of predator induced stress in zebrafish, focusing on how the presence of conspecifics mitigates the behavioural stress responses particularly in freezing, rapid movements and time spent in the predator and shoal zones across predator only (PO) and predator plus shoal (PS) conditions. According to Faustino et al. (2017), visual cue of shoal was more effective in reducing freezing when the focal fish was exposed with an alarm substance, than olfactory cues.

Under social isolation, male zebrafish showed no change in freezing and rapid movements when comparing the PO and PS, but showed a spatial preference shift towards the shoal zone in the PS condition. This interaction remained statistically significant when comparing the time spent in the distant zone during PO with time spent in the shoal zone during the PS condition. Therefore, this trend in males was interpreted as partial buffering, as the shoal influenced spatial preference but lacked statistical evidence for the reduction in fear associated behaviours. In contrast, isolated females exhibited a major alteration characterized by enhanced freezing in the presence of conspecifics in the PS, which was interpreted as a failure of social buffering, and further supported by unchanged rapid movements.

Notably, a spatial preference shift similar to that observed in isolated males was also evident in isolated females. The increased freezing may indicate social contagion (Akinrinade et al., 2023), reflecting the transfer of fear cues from stressed conspecifics, however, the extent of predator threat perception and stress activation within the conspecific group warrants further investigation. If social contagion were the primary driver of fear enhancement in isolated females, a similar response would be expected in control females as well. However, control females showed a different

response, displaying enhanced cooperative predator inspection (Pimentel et al., 2021) without any change in freezing across the PO and PS conditions. thereby, highlight the heightened sensitivity associated with social isolation in females, characterised by a blunted stress response and failed buffering. Consistent with previous studies, control males exhibited classic social buffering supported by reduced freezing (Akinrinade et al., 2023) and rapid movements in the PS compared to PO, along with a clear shift towards the shoal zone in the PS. Therefore, zebrafish under social isolation are able to detect the social stimulus when paired with a stressor but they are unable to effectively reduce fear.

5.2.2 Mirror Induced Aggressive Responses and Conspecific Buffering Under Social Isolation

Agonistic behaviour comprises the entire spectrum of both aggressive and submissive behaviours (Zumpe & Michael, 2001) that have a vital role in shaping social hierarchy and social cohesion in animals. In zebrafish, aggressive behaviours include complex actions like chasing, biting, fin flaring and territorial displays to establish dominance. In contrast, subordinates often display freezing or sudden escape responses (Larson et al., 2006). At the behavioural level, aggressive behaviours are studied by observing the interactions between fish dyad or mirror tests. In the mirror test, fish respond to their own reflection, which they interpret as a competitor. Both the tests serve different purposes and outcomes, as dyadic test is intended to produce a social hierarchy, while the mirror test measures aggression potential via spent near the mirror zone, entries and mirror bites. Therefore, mirror induced aggression test acts as a reliable primary screening test for aggression prior to dyadic encounters, as this test does not physically harm the fish, unlike the dyad test (Reichmann et al., 2022). Consistent with previous research, sex acts as a profound factor in the intensity and duration of aggressive responses evoked by the mirror. Predominantly, a male biased trend is observed (Mustafa et al., 2019a). Nevertheless, research on zebrafish supports that, both males and females exhibit territorial aggressive responses (Ariyomo & Watt, 2013). Females were also exhibited baseline heightened aggressive responses than males in mirror test (Rambo et al., 2017). This sex-based variability in aggression makes zebrafish a critical model for studying agonistic behaviours, also it is well suited to assess the changes induced by experimental alterations.

The primary aim of our present study was how the prior social experience, specifically, chronic developmental social isolation, alters the aggressive behaviour in the presence of a mirror-stimulus within a three chambered tank. Secondly, how presence of conspecifics modulates the mirror induced responses, both in males and females. The primary question was addressed using multiple behavioural metrics, comparing them against the baseline ‘NS’ condition (No Stimulus). The time spent across the mirror, internal, and distant zones revealed the patterns of mirror attraction or avoidance across control and test individuals. Control males and females displayed mirror engagement by spending higher time near the mirror zone and lower time near the distant zone, but, the pattern of time allocation across the three zones differed. That is, control males engaged similarly with mirror and internal zones, spending less time in the distant zone. In contrast, control females showed a strong, consistent gradient: highest engagement with the mirror, then the internal, then the distant zone. Significant sex specific differences were observed in controls, with males being less aggressive than females in terms of time spent in the mirror zone and mirror bites. Likewise, entries to the mirror zone were consistent with time spent there. Both control males and females exhibited fewer entries than in the NS baseline, which correlates with their higher time spent in the mirror zone.

In socially isolated groups, the patterns differed significantly. Notably, a sex-inverted trend was observed compared to controls for both time spent and mirror bites. Test isolated males spent more time in the mirror zone than control males, while test isolated females spent less time there than control females. Interestingly, isolation did not merely alter responses but flipped them in a sex specific manner, indicating the profound impact of prior social experience in the behavioural output. This pattern was also visible across mirror bites; TM showed more bites than CM and TF showed fewer bites than CF. An important parameter shift was identified for freezing, all experimental groups exhibited freezing in the MO condition compared to NS. However, in-depth statistical comparison revealed a relevant change in the test groups: their freezing suggested altered behavioural responses associated with isolation. Freezing is a normal behavioural response in an agonistic behaviour context, but both test groups showed freezing in MO that was comparable to PO (predator only condition), indicating an inability to differentiate between a competitor and a stressor. In contrast, both control groups showed marked differences in freezing across stimuli,

mirror induced freezing was higher than NS but lower than PO, suggesting successful discrimination between a mirror image and a potent stressor, resulting in context-specific outcomes.

In the conspecific buffering experiments, results revealed sex-dependent outcomes of the behavioural responses in isolated individuals. The presence of conspecifics alleviated aggression both in control males and females, primarily by reducing mirror bites in mirror + shoal (MS) condition compared to mirror only (MO). A significant shift in zone allocation from the mirror zone to the shoal zone was also evident in control groups. In the test groups, these changes were sex-dependent, where males showed a clear zone shift and reduction in heightened aggressive scores in the presence of conspecifics, while females showed a different pattern, pointing to a diminished effect of conspecifics on aggression. Plausible reasons could be, one, females expressed lower aggressive responses in the MO, so there were little chances of further reduction in MS, second, reduced social drive-in isolated females could blunt the response. This is consistent with the statistically non-significant pattern of time allocation in MS for test females. Third, the females may be more sensitive to the experimental condition employed, as indicated by statistically comparable freezing across all single-stimulus conditions. Previous research (Rambo et al., 2017) has shown that female zebrafish subjected to unpredictable chronic stress do not exhibit heightened cortisol levels, suggesting a blunted HPI axis response. Based on this understanding, unlike the stress battery used by Rambo et al., (2017), we propose that, chronic social isolation may impact behavioural response more intensely. Our results indicate that isolation not only promotes a blunted state but also disrupts neural circuits governing and stimulus discrimination and social behaviour in a sex-inverted manner, ultimately leading to a passive, withdrawal state rather than a hyper-arousal state, particularly in females.

5.2.3 Social Preference Shift Across Stimulus Conditions

Social interaction deficits associated with environmental alterations, especially isolation is an extensively studied research area across animal models (Groneberg et al., 2020; Hermes et al., 2011). Zebrafish Autism models primarily focus on the social deficits along with repetitive and other behaviours (Washbourne, 2023). Zebrafish have been subjected to both acute and chronic (Engeszer et al., 2004; Shams et al.,

2017; Tunbak et al., 2020) social isolation has been observed to exhibit reduced social interaction with conspecific fish. The three-chambered tank test was the most commonly employed paradigm (Ariyasiri et al., 2019; Ogi et al., 2021).

Existing research has highlighted the role of environmental, genetic and pharmacological manipulations affecting social deficits in zebrafish. Embryonic alcohol exposure (Fernandes et al., 2019) resulted in social deficits in adult zebrafish. Following this exposure, both dopaminergic and serotonergic neurotransmitter systems were found to be affected (Buske & Gerlai, 2011b). Similarly, genetic mutations associated with autism, such as *Chd8* (H. Wang et al., 2025), and *Shank3b* (Liu et al., 2018), also lead to social interaction deficits. Oxytocin receptor mutation has also been found to be associated with the altered social preference maintenance in zebrafish (Gemmer et al., 2022). Additionally, oxytocin administration showed to increase gene expressions of *Shank 3a*, *Shank 3b*, and oxytocin receptors, and observed improvements of autistic behaviours, specifically in the social responses of zebrafish larvae (Rahmati-Holasoo et al., 2023).

Similarly, research using a zebrafish model of CLN8, a group of inherited neurodegenerative disorders, revealed social and emotional decline, particularly in social preference and shoaling test, and this study offered a therapeutic intervention using trehalose, to improve the autophagy of cellular aggregates, thereby providing a neuroprotective effect (Licitra et al., 2025). Cross-species studies, for instance in ants, have linked social isolation to negative impacts including hyperactivity and short lifespan, which are associated with cellular oxidative stress (Koto et al., 2023). In contrast to that, *Drosophila*, exhibited increased longevity when isolated (Lin et al., 2022). This indicates that *Drosophila* might benefit from social isolation due to reduced competition over resources. But being social species like ants and zebrafish, reduced interaction suggests that the isolated brain acts as a stressed brain (Makinodan et al., 2016) and that alters behavioural responses.

Although social interaction deficits associated with multiple factors were well documented in the previous research, here our present study attempts to fill the existing gaps. Here, we employed the conspecific stimulus (SO) primarily to assess test validity under single stimulus choice test, to compare the interaction of fish with a well-validated known stimulus. Secondly, the sex specific effects of chronic

social isolation on the conspecific preference remains unclear. Therefore, the conspecifics in the current test, serve dual purposes, first as a positive control with baseline 'NS', second, to assess the sex specific interactions of individuals with conspecifics. Research has been conducted on the duration (Kuniyil et al., 2025) and number of conspecifics (Faustino et al., 2017; Saverino & Gerlai, 2008) needed to evoke a better reciprocal response from the focal fish. Here, we employed 6 minutes (an acute one) exposure to eight conspecifics, in a single stimulus choice test and also as the social stimulus in the two buffering conditions (PS and MS).

The results demonstrated that the social stimulus effectively acted as a positive control on both control males and females. Controls showed increased interaction time in the shoal zone and reduced time in the distant zone. Other supporting measures including entries, speed at shoal zone, and total rapid movements were reduced in controls complementing the time spent and suggesting more focused engagement with the conspecifics. For freezing behaviour, controls exhibited freezing significantly higher than NS and comparatively lower than other two stimuli, PO and MO respectively. This may be indicative not of a fear response, but rather of a generalized stimulus specific response in controls. Furthermore, complementing previous reports, social preference in control groups was sex-specific, where females exhibited higher social drive (Velkey et al., 2022), as analysed by group comparisons of zone-specific time spent by males and females.

Under social isolation, social interaction time was higher than the NS. However, in isolated groups, interaction time in the shoal was significantly reduced compared to their respective control counterparts. The social deficit was also evident as the time spent in distant zones was comparable to the NS. Additionally, females exhibited comparable internal zone preferences across all stimulus conditions. Freezing in SO was absent in males, which contrasted with females, who exhibited comparable freezing across all stimuli. Rapid movements followed a similar pattern of reduction compared to NS as was observed in controls. Notably, the primary measure, contact zone time, was sex-independent in test groups. This further correlates with the existing literature showing prolonged social isolation negatively impacts social interaction abilities in zebrafish.

The purpose of conspecifics was then used in the buffering experiments, including stress (PS) and aggression (MS). We then analysed the social preference index (SPI) shifts across the three social conditions, namely, shoal only (SO), Predator + Shoal (PS), and Mirror + Shoal (MS). This analysis was intended to identify whether social preference increased or decreased based on experimental context. Control males showed an increase in SPI in the PS compared to SO, corroborating the buffering pattern observed in the earlier discussion. However, SPI was stable and comparable between SO and MS, indicating that the experimental context (Predator vs. Mirror) differently modulated social preference.

In contrast, control females exhibited an amplified social drive in the SO, which led to a statistically significant reduction in social preference in the PS and MS conditions. Furthermore, the PS and MS conditions were statistically comparable to each other. This response might be due to the high social drive in SO, or potentially to cooperative predator inspection (Pimentel et al., 2021) behaviour in PS condition. Our study does not interpret this as a maladaptive behaviour but rather as a sex specific behavioural response across different contexts, consistent with the findings that sex specificity is context and parameter dependent in zebrafish (Hillman et al., 2025).

Social isolation also produced the sex-dependent social preference shift in test groups but different from controls. Test males showed heightened social preference shift in the PS than the SO, again complementing the zone shift observed in the PS buffering condition. This aligns with the presence of conspecific recognition within test males, but the engagement became compromised (*comparing patterns observed in the previous sections too*). In contrast, isolated females exhibited no significant SPI (i.e., consistently low preferences) across different social contexts, including SO, PS, and MS. It is also important to recall that the higher freezing in the PS condition in test females. Therefore, the combination of the comparable SPI and elevated freezing in PS suggests failure of social buffering. The significant difference in SPI in PS compared to MS likely reflects to a fear driven response, not a true shift in social preference. This response is analogous to conditions like social anxiety disorder (SAD), where heightened social fear is observed with higher heart rate, and increased amygdala related neuronal activity, an important centre for fear responses (Toth et al., 2013). In this view, the increased freezing might be analogous to the social fear

generated as a result of environmental manipulation. In some contexts, this social fear was considered as the adaptive response towards social stress. Therefore, considering all external behavioural evidence, the prolonged social isolation impacts females more negatively than males, similar to the freezing response towards pain stimulus in female zebrafish (Marcon et al., 2022).

5.2.4 Conclusion

Our results demonstrate that chronic developmental isolation induces sex-specific maladaptive behavioural responses in zebrafish, characterized by impaired threat discrimination, failure of social buffering that is more severe in females. As per the findings on the sex independent anxiolytic and hyperactive internal states of zebrafish from objective 1 might be resulted in the complex sex-specific shifts associated with the social responses, including aggressive behaviours. Males became more aggressive while females became less reactive, both with reduced social preferences. Therefore, multiple domain impact of social isolation is very relevant to current research, as a disrupter of specific neural circuits affecting social behaviours. This study also addresses the importance of studying sex differences in stress models. Future research should emphasize the specific neuroendocrine mechanisms, particularly, dopamine, serotonin, oxytocin, cortisol, etc. to understand the sex-specific blunted response of social deprivation.

5.3 Objective 3: Discussion

5.3.1 Disrupted Collective Movements Under Socially Isolated Shoal

Zebrafish shoaling has been altered using environmental, pharmacological, and genetic modes due to its validity in clinical translational studies (Fernandes & Gerlai, 2009; Oliveira, 2012; Toni et al., 2025; Yang et al., 2024). Quantification of shoaling has been done using automated tracking systems and analysed majorly through distance metrics and time involved in freezing (Butail et al., 2013; Miller & Gerlai, 2007; Sivaraman et al., 2022). Here, in the present study, we incorporated social isolation, a well-established stressor, to analyse the modulations that occurred in collective behaviour within a five-membered zebrafish shoal. In addition to the existing literature, we employed both fundamental shoal metrics spanning from distance to orientation, alignment, and locomotor endpoints. Novel findings on the emergent states and their persistence under socially isolated shoals are also discussed in the section below.

Multiple behavioural responses were able to be interpreted from this single shoaling test, including social communication deficit, locomotor deficit, emergence of repetitive behaviours, and reduction of memory time of each collective state. Unlike previous behavioural tests that used only single animals at a time, the experimental design of shoaling allows the propagation of multimodal sensory cues and active transmission of information between individuals (Mukherjee & Bhat, 2024; Ward & Mehner, 2010).

Here, in the present study, shoals of socially isolated zebrafish were identified with reduced spatial, orientation-alignment, and locomotor deficits compared to control shoals. This further leads to plausible chances of reduced social information transfer. A notable behavioural readout was the locomotor deficit, both at the shoal and individual level, isolated shoals showed hyperactive movements, including higher speed, higher distance travelled, higher rapid movements, and lower freezing episodes. According to previous research on mechanosensory social signalling via the lateral system, *pth2* was identified as a key modulator (Anneser et al., 2020). Social isolation showed decreased *pth2* levels in zebrafish, and water perturbations alone were able to rescue *pth2* expression (Anneser et al., 2020). In the current context, with

increased locomotor movements within a shoal system, fish lacking social communication suggest an altered mechanosensory system due to isolation. Similarly, higher inter-individual distances may reduce the chances of collection or transfer of information from shoal mates (Abril-De-Abreu et al., 2015).

In earlier observations from Objective 1, individual zebrafish showed sex-independent hyperlocomotion. In a shoaling system, this behavioural phenotype of isolated individuals overrides the ability to remain in a group and to receive or transfer information through visual, olfactory, or mechanosensory pathways. Proper heading alignment and orientation effectively require visual processing, and if social stress were the reason for disrupted shoaling, stress-related responses such as freezing would be expected (Zhao et al., 2025). However, isolated groups showed lower freezing than controls; therefore, all these observations point towards hyperlocomotion induced by social isolation overriding collective behaviours in zebrafish. Previous studies have also noted locomotor changes associated with isolation (Shams et al., 2018).

The Tunstrøm-Couzin mathematical model employed in the present study showed that socially isolated shoals exhibited multiple emergent states similar to controls (Tunstrøm et al., 2013). However, the polarized state was reduced in isolated shoals. Linked to the altered shoal metrics, this indicates that polarization requires multi-level acquisition and processing of information, which the isolated group failed to achieve. Notably, the milling state was higher than in control groups, which plausibly represents stereotypic circling behaviour observed in other models of neurodevelopmental disorders rather than actual shoal circling around a centroid (Kalueff et al., 2013; Razali et al., 2022). This interpretation was further supported by the state persistence or memory time of each state in socially isolated shoals. Both polarization and rotational memory times were reduced, suggesting that even though multiple emergent states formed in isolated shoals, these states were short-lived or switched abruptly, indicating dysregulated collective movements under social isolation.

Altered behaviour associated with social isolation has been identified as progressive, as four weeks of isolation showed social preference, whereas eight weeks of isolation produced social behaviour deficits associated with reduced formation of polarized states and involvement of altered oxytocin signalling (Gemmer et al., 2022).

Considering the experimental protocol of the current study, prolonged six months of social isolation might have drastically affected decision-making, driving individuals toward a hyperarousal state. Similarly, the 21 dpf critical window might also be involved in heightened disruption of group dynamics within isolated shoals (Dreosti et al., 2015). The state of polarization is dependent on time and duration, and schooling formation may reduce with increased habituation and experimental duration (Miller & Gerlai, 2012). This was further evidenced from the track paths obtained from the two groups. Track paths were identified as highly cohesive and thigmotactic in the initial 30 seconds of shoaling in the control group, whereas they were exploratory dispersive in isolated shoals.

A similar study conducted in *Danionella cerebrum* also exhibited altered shoaling metrics associated with isolation, even though the experimental duration differed from the present study (Zada et al., 2024). The study further demonstrated that optic tectum development is involved in proper formation of collective behaviour and that this process was altered following isolation (Zada et al., 2024). The shoal test conducted in the present study revealed three major behavioural domains associated with neurodevelopmental disorders: social communication deficits reflected by dispersed group structure, locomotor deficits reflected by a hyperactive phenotype as observed in ADHD models (Norton et al., 2017), and stereotypic repetitive behaviours reflected by circling behaviour previously identified in *shank3b* mutants of autism (Liu et al., 2018) and abnormal repetitive behaviours (ARB) (Wang et al., 2019). The internal state of the individual was again observed to influence group dynamics within a shoal (Jolles et al., 2017; Pike et al., 2008). Taken together, zebrafish social isolation disrupts the internal state of fish, transforming it into a hyper locomotor external phenotype that further disrupts stress, aggression (Objective 2), and shoaling systems. Based on the fundamental rule of pairwise interaction as the core of group dynamics (Couzin et al., 2002; Herbert-Read et al., 2011), the next objective will discuss interactions between pairs from both homogeneous and heterogeneous dyads.

5.4 Objective 4: Discussion

5.4.1 Altered Spatio-Temporal Dynamics Across Homogeneous and Heterogeneous Dyads Under Social Isolation

Dyadic interactions serve as the building blocks of social cohesion and collective behaviour in social animals, including fish models. In the current study, we examined shoaling behaviour and emergent states within control and isolated groups, and subsequently explored the socio-temporal dynamics of dyads across all possible sex combinations. The primary focus of this study was on non-reproductive behaviours, including social proximity, movement coordination, synchronization, alignment, and front-follow positioning.

The analysis of spatio-temporal dynamics showed alterations in the homogeneous dyads of isolated pairs, with higher Euclidean and cosine distances and lower proximity time between individuals. The sexual identity of the dyad partners was not an influencing factor in either control or isolated groups. Previous studies have reported altered shoaling in zebrafish under social isolation (Shams et al., 2017). Socially isolated zebrafish have also been found to show reduced DOPAC levels, which may underlie the reduced social responsiveness observed in individuals within dyads. Dopaminergic control over social affiliative behaviours was further supported by pharmacological studies showing that blocking D1 receptors reduces social preference in zebrafish (Scerbina et al., 2012). Moreover, dysfunction of the dopaminergic-oxytocinergic circuitry has been associated with social deficits in autism spectrum disorders (ASD) (Kohls et al., 2012). Also, in Parkinson's Disease (PD) models, rotenone induced inhibition on dopamine release and uptake exhibited reduced time near the five-stimulus fish reward chamber in zebrafish (Hettiarachchi et al., 2022). Altogether, these findings support a neurochemical basis for the altered socio-temporal dynamics observed within isolated dyads.

Freezing behaviour, being a vital stress marker in behavioural studies, showed a consistent reduction across isolated dyads compared to their respective controls. However, the male–male isolated dyads were observed with higher freezing than female–female and mixed isolated dyads. Sex differences were observed in the stress regulation mechanism through the hypothalamic-pituitary-interrenal (HPI) axis (Lee

et al., 2024); females were higher in anxiety-like and stress-like behaviours than males (Amar & Ramachandran, 2023; Fontana et al., 2019), although the opposite trend was also reported. Dyadic encounters analysed in the novel tank test showed increased anxiety with higher freezing in subordinate partners irrespective of sex (Bozi et al., 2021; Tran & Gerlai, 2016). The subordinate males were also observed with elevated plasma cortisol, a stress hormone (Tea et al., 2019). Moreover, some reports indicate that lower anxiety is associated with higher aggression. This suggests that under isolation, the lower freezing profile detected across all homogeneous dyads compared to controls might be due to other behavioural changes, as we observed with the higher mirror aggression in isolated males than controls in Objective 2. In contrast, isolated females were observed with lower mirror aggression, suggesting that isolation dampened both stress levels and aggression in females. Supporting this, a study by Rambo et al. (2017) found that unpredictable stress caused higher aggression and elevated cortisol levels in stressed males, but not in females, and this interpreted as higher resilience of females under stress. But in our study, isolated females showed opposite patterns from the control females, suggesting a plausible altered behavioural response of females under chronic social isolation not as resilience. Thus, the overall reduction in freezing in isolated dyads might be due to isolation or a behavioural shift—for example, a shift from stress to aggression—whereas the higher freezing identified in isolated male-male dyads compared to other test dyads could reflect a stress-linked response similar to that of subordinate males.

The higher-speed swimming, considered as rapid movements per second, showed sex-specific patterns in both control and isolated homogeneous dyads. Isolated male-male dyads were observed with higher rapid movements than other isolated dyads, and also higher than control male-male dyads, indicating an altered and heightened exploratory drive, or higher aggression in the dyad-induced movement patterns in isolated male-male dyads. Previous studies have supported sex-specific differences in activity and boldness in zebrafish, where males are generally more active than females (Mustafa et al., 2019b). In contrast, this sex-specific pattern was not visible in control male-male and female-female dyads, possibly indicating that the interactions within dyads did not produce any behavioural differences across sexes, as supported by Mukherjee & Bhat (2021), where same-sex dyads did not differ in aggressive interactions temporally. However, in control mixed dyads, rapid movements were considerably

lower than in other control dyads. This might be due to the different sex-dimorphic behaviours under mixed-sex dyads (Ghoshal et al., 2019). Isolated mixed dyads showed higher rapid movements than control mixed dyads, possibly because the hyperactive male influenced the overall dynamics of the dyad, favouring the ‘fast-inaccurate driver’s phenotype explained by Wang et al. (2015). Also, due to reduced social orientation in isolated females. Supported by the study done by Dahlbom et al. (2011), in dyadic contexts, the bolder individuals were more likely to become dominant. The higher rapid movements, lower freezing, and higher mirror aggression (Obj. 2) found in isolated males might have enhanced the hyper-dominance within male-male and male-female dyads, thereby indicating that the altered dyad dynamics were isolated-male driven. Therefore, isolation amplified the baseline sex differences observed in zebrafish: males became more exploratory-active and females showed lower social orienting (Stednitz et al., 2018).

Speed-accuracy trade-off is well studied in humans but less explored in animal models. It plays a vital role in decision making among individuals (Kuroda et al., 2025; Wang et al., 2015). Under isolation, higher speed difference was observed sex-independently across all homogeneous dyads, and the acceleration correlation and maximum velocity cross-correlation were also reduced. This indicates weak coordination between individuals under isolation and disordered dyad dynamics. Such alterations might affect decision-making quality and collective movements. The very fundamental dyad interaction was altered under isolation, providing a plausible explanation for the altered collective behaviour seen in Objective 3.

Dyad-level asymmetry was further identified in both control and isolated dyads, with a lower fraction of time in geometric following. Under isolation, the following time markedly reduced. Preliminary within-group analysis (CMM vs CFF; TMM vs TFF, individual-wise) showed no significant differences in fraction of time spent in geometric following. This further validates that male and female individuals follow at comparable rates under the same treatment condition. Since CMF and TMF may include reproductive cues, role asymmetry, and identity-dependent behaviours, these dyads were excluded from individual-wise sex comparisons. Therefore, dyad-level averaging was chosen as the most conservative and unbiased approach for analysing geometric following. Higher following in control dyads reflects that they maintain

more coordinated movement trajectories, with both members adjusting direction relative to each other, whereas reduced following in test dyads indicates a shift toward more independent, less aligned paths under isolation. This suggests that the position-switching observed in controls was largely abolished under isolation. This supports Pike et al. (2008), who showed that behavioural phenotype affects social interaction structure in animal networks. Fish with low social affiliation swim faster and shape leadership and coordination dynamics within groups (Jolles et al., 2017). The isolated individuals in our study had reduced social preference and higher activity levels in previous experiments, suggesting a strong potential for monopolising the front position due to these traits. Control fish, with high social affiliation and baseline activity, were more likely to follow the fish leading in front.

In the heterogeneous dyads, a consistent pattern was observed: the isolated fish showed higher directional initiation than the control partner, and the subsequent higher follow time in control indicates a plausible following of control towards the isolated individual, irrespective of the partner's sex. Front position is determined by multiple factors, including internal state (e.g., starvation), body size (larger individuals often follow), sex, age, etc. The fish in front typically determines the group's decisions and coordinates effective collective movement, aiding in predator avoidance, shelter seeking, and foraging. In our study, the isolated fish showed higher directional initiation that shifted the dyad dynamics to resemble isolated homogeneous dyads, showing reduced coordination and synchronisation. This indicates that the isolated individuals exhibited a dominant behavioural phenotype and primarily drove the dyad's dynamics, irrespective of the sex of the control partner.

Consistent with previous behavioural studies, sex-specificity in our results was also parameter-dependent. Some metrics showed sex differences only in isolated dyads, while others showed differences in both control and isolated dyads, although the patterns never matched the baseline control differences. Heterogeneous dyads largely resembled the isolated phenotype, indicating the dominance of the isolated behavioural state in pair interactions. However, for maximum velocity cross-correlation, a subtle deviation was observed: control females appeared to influence isolated males, producing a partial improvement in alignment. Although this trend did not reach statistical significance, it suggests the possibility that opposite-sex partners

may transiently buffer or modulate certain impaired coordination features in isolated males-a pattern not observed in isolated females. Previous study done in cichlids (Ligocki et al., 2015) has shown that dominant and sub-ordinate female interaction and cortisol levels depend on the affiliative behaviour with the dominant and conflict between the dominants. This highlights that the female roles within an interaction are largely context specific.

Overall, dyadic interactions revealed sex-specific responses in some parameters, but high-velocity movements, reduced freezing, altered alignment, and disrupted synchrony were consistently present across both sexes and all dyad types. Within isolated dyads, we also noted subtle but meaningful sex-dependent variations in rapid movements, freezing behaviour, and time spent in the front position. These appeared as amplified expressions of the natural baseline tendencies-higher activity in males and reduced social orientation with higher resilience in females, alongside their stronger context-dependent behavioural modulation. Collectively, these findings underscore sex as a critical biological variable influencing how individuals respond to social isolation and how these individual phenotypes shape dyadic communication and coordination. Similarly, this analysis of dyadic interaction through extensive experimental grouping provides a clear idea on the plausible reasons that might have produced the disrupted collective behaviours as observed in the Objective 3, it might be the disrupted speed trade off that lead to the shifting of monopolisation to initiate higher turns in an interaction, disrupting entire spatio-temporal dynamics of that pairwise interaction. The higher-level propagation of such alterations leads to disrupted collective movements as a shoal affected information transfer and decision making in zebrafish subjected to chronic social isolation.

5.5 Objective 5: Discussion

5.5.1 Sex- Specific Gut-Microbiome Dysbiosis Under Social Isolation

Microbiota-Gut-Brain axis (MGB) is a bidirectional communication network, having both afferent and efferent connections, providing benefits ranging from maintaining homeostasis and physiological functions to mental state regulation (Wan et al., 2025). In the present pilot study conducted on sex-specific analysis on zebrafish social isolation and gut-microbiome through NGS have provided an opportunity to explore further research in this area. Previous microbiome studies involving zebrafish include

two opposite observations regarding sex-dependent and sex-independent microbiome diversity (Liu et al., 2024; Stephens et al., 2016). Respecting the biological complexity existing in any system, sex specificity may depend on multiple factors including methodological design of the study, age of the experimental animal, parameter analysed, etc. Therefore, we employed sex-specific analysis to understand the outcome based on our current experimental design.

As gut diversity formation is largely dependent on diet, environment, and transmission between individuals, we ensured these factors are uniform across control and test groups. Only the experimental manipulation was different across groups, that is the social isolation, that is purposefully blocked horizontal transmission from the 21st dpf onwards to 180 days. The present study employed only control and isolated individuals directly for NGS, no behavioural responses were recorded for the 28 individuals used for NGS, as behavioural responses were recorded from the other individuals from the same batch.

Previous studies reported feeding behavioural alterations in socially isolated groups (Dissinger et al., 2024). Similarly, different feed types have been demonstrated to produce varied responses in different domains including exploratory and aggressive behaviours (Sivarajan et al., 2025). Major gut dysbiosis indicators are also reported in the literature, for instance, overgrowth of Proteobacteria, depletion of Firmicutes, and changes in minor phyla (Morais et al., 2021; Xavier et al., 2024).

The current study observed notable shifts in gut microbiome diversity across control and test groups, sex-dependent manner. Males and females showed distinct microbiome profiles in both experimental groups. A Firmicutes-dominated profile along with the presence of Proteobacteria, Fusobacteria, Actinobacteria, Planctomycetes, was identified in control males, whereas, control females were identified with the presence of Firmicutes, Proteobacteria, and Fusobacteria.

Isolated males were marked by Proteobacteria bloom, Firmicute depletion, and the absence of Actinobacteria as major indicators of gut dysbiosis at the Phylum level (Xavier et al., 2024). The presence of Chlamydiae, an opportunistic phylum, was also evident in isolated males. In contrast, isolated females were identified with microbial restructuring, indicated by increased Proteobacteria, and Firmicutes and most

importantly, complete loss of Fusobacteria at the phylum level. Further trend analysis at the genus level identified marked suppression of Firmicute-associated genera, including *Bacillus*, *Paenibacillus*, and *Brevibacillus* which were detected in other groups. Similarly, a notable absence of Fusobacteriales (order level) was identified in test females, which were present in other groups. Under the Phylum Chlamydiae, the genus *Simkania* was identified in test males. Exclusive presence of genera under Proteobacteria was also found in both test males and test females, whereas control groups included balanced representations of opportunistic and beneficial genera under Proteobacteria.

As the gut microbiome profile of zebrafish is reportedly dominated by Proteobacteria, over- blooming of the phylum considered as a dysbiosis marker, as this might favour the over population of opportunistic groups and depletion of other beneficial taxa, invading the gut barrier, and occurrence of inflammatory responses within the host (Lin et al., 2025; Shen et al., 2025). When complemented with the shared behavioural alteration in both isolated groups, including heightened exploratory behaviour in open field tank test, altered stress response, and reduced social preference from three-chambered tank test, and altered collective movements, these findings plausibly link with gut dysbiosis. Because the mechanism is bidirectional, brain function challenged by isolation could affect gut diversity, and the altered gut profile might further influence CNS activity, contributing to behavioural alterations.

We present this gut dysbiosis not as the primary cause of the behavioural responses reported previously but as the potential modulator that was altered by social isolation and could have influenced behavioural outcomes.

As discussed in the earlier, the MGB axis plays an important modulatory role because altered gut microbiome might affect the neuromodulators, SCFAs, and gut barrier integrity, thereby exerting indirect effects on behaviour (Dalile et al., 2019; Gershon & Tack, 2007; Sharon et al., 2014; Sikander et al., 2009). The Proteobacteria overgrowth and absence of Actinobacteria in test males, could have possibly open a niche exploration by the pathogenic invaders, as observed with the presence of *Simkania*. Previous studies reported the beneficial role of actinobacteria in maintaining gut integrity (Binda et al., 2018). Similarly, the loss of genera under Firmicutes indicates potential loss of genera providing probiotic benefits and playing

a crucial role in maintaining gut health and homeostasis (Elshaghabee et al., 2017). *Fusobacteria* also have a prominent role in gut health, including *Cetobacterium*, a vitamin B12 producer known for its vital role in maintaining gut barrier health and reported to associate with other probiotic groups in fighting against pathogenic invaders (Qi et al., 2023). The complete disappearance of *Fusobacteria* further adds to the negative impact of social isolation on gut microbiomes.

The role of the MGB axis has currently transitioned from a basic hypothesis to a potential biomarker system, as also evidenced by behavioural alterations in rodent models. Germ free mice showed reduced sociability and repetitive behaviours when transplanted with gut microbiota from ASD donors, and behaviour was restored after recolonization (Sharon et al., 2019).

The present study includes certain delimitations and limitations. This study was designed to cover the sex-specific aspect of a chronic isolation model, which was a novel area within the current research scenario. Limitations include the pilot nature of the study due to the gut pooling adopted in the methodology, which does not address individual variability across different isolation batches. At the present stage, the study is unable to provide histological analysis indicating gut barrier changes associated with the opportunistic pathogenic taxa observed in isolated groups.

Therefore, our study supports a multifactorial model in which social isolation likely drives behavioural responses directly and indirectly while restructuring the gut microbiome community, making dysbiosis a contributing mechanism for the observed behavioural alterations. Further analysis across multiple replicates of social isolation, considering sex as an important biological variable are necessary to draw final conclusions. Nevertheless, the current study offers a new perspective on social isolation and sex-specific gut dysbiosis, linking it with possible rescue strategies using targeted taxa known for major SCFA production and probiotic properties.

Chapter **6**

**Foundational Methodological Study
on Multiple Animal Experiments
and Shoal Analysis**

Foundational Methodological Study on Multiple Animal Experiments and Shoal Analysis

‘The established methodological framework underlying multiple animal tracking is presented here as a separate chapter in order to preserve the readability and continuity of the core question raised in this research, which was initially presented across five objectives.’

This section deals with the detailing of a previously peer-reviewed published study focused on the establishment of a methodological framework necessary for multiple animal tracking using open software and R-based programming of the fundamental shoal metrics, mainly distance-based. Additionally, this was important to understand the sex composition that should be followed in a multiple animal study to establish a socially cohesive shoal in order to rule out the external factors affecting the core objective of our study, which was chronic social isolation. We have adhered to the methodological framework established, mainly in the videography protocol and sex ratio of the isolated shoal.

idTracker 2.1 is an established open software for multiple animal tracking, but the exclusive use of this software in shoaling studies in zebrafish was limited at the onset of our study. Previous research was more dependent on manual annotation and using ImageJ software and screenshots to determine the distance differences in each frame. Similarly, the dichotomous choice test was widely used rather than free-swimming analysis of fish groups. Therefore, in the initial period, it was necessary to build a methodological framework answering each question related to multiple animal tracking.

1. To establish a suitable videography setup to track multiple animals for behavioural analysis.
2. To evaluate the applicability of idTracker 2.1 in multiple animal tracking.
3. To establish foundational distance-based metrics in shoaling analysis.
4. To determine the optimum sex composition that favors better shoal cohesion in terms of spatial metrics.
5. To complete and report this methodological framework prior to the initiation of social isolation and shoaling analysis.

Materials & Methods

Experimental Design

The experimental tank was a square glass tank measuring 50 cm in length, 50 cm in width, and 15 cm in height, with a system water level of 6 cm to facilitate the two-dimensional tracking of animals.

A high-resolution camera, Canon EOS R (Mirrorless, Canon Inc., Ota, Tokyo, Japan), was placed perpendicularly to the tank approximately 60 cm above and was used to record the behavioural videos at 30 fps, ensuring precise tracking and animal identification. The total duration of recording was kept at 6 minutes.

Adult zebrafish (*Danio rerio*), measuring around 4–5 cm in length, were selected for behavioural experiments. Prior to commencing all behavioural experiments, the fish were divided by sex and placed into distinct pre-holding tanks with a total capacity of 16 L. To minimise observer error, zebrafish sex identification was based on the consensus reached by multiple researchers from the same field. At the time of the experiment, fish were randomly chosen from the pre-holding tanks and moved to a pretreatment tank (30 × 20 × 20 cm), where they were allowed to acclimatize for five minutes. The fish were subsequently moved to the experimental tank and given 3 minutes to acclimatize. After each experiment, the system water was replaced to prevent olfactory signals between test individuals.

None of the fish were reused and were tested only once. All fish were fed two hours prior to the beginning of the experiments and after the completion of the experiments. The behavioural recording time was maintained between 9:00 am and 4:00 pm IST.

Experimental groups were divided into four based on the different sex identities of shoal members, namely: Male Only (five male zebrafish shoal), Female Only (five female zebrafish shoal), Male-Rich (3 male and 2 female zebrafish shoal), and Female-Rich (3 female and 2 male zebrafish shoal), abbreviated as MO, FO, MR, and FR, respectively. The total shoal sample size for each group was kept at 10 shoals.

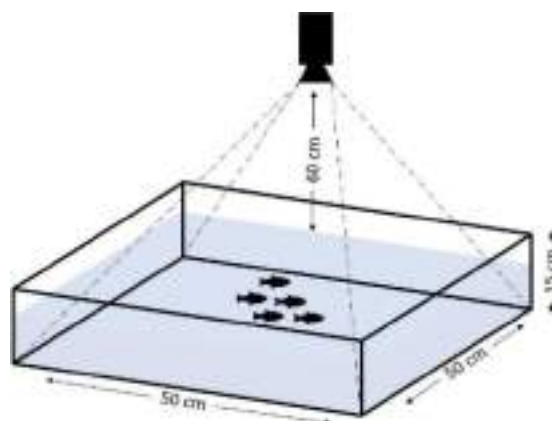


Figure 6.1. Experimental Design

Statistical Analysis and Derivation of Shoal Spatial Metrics

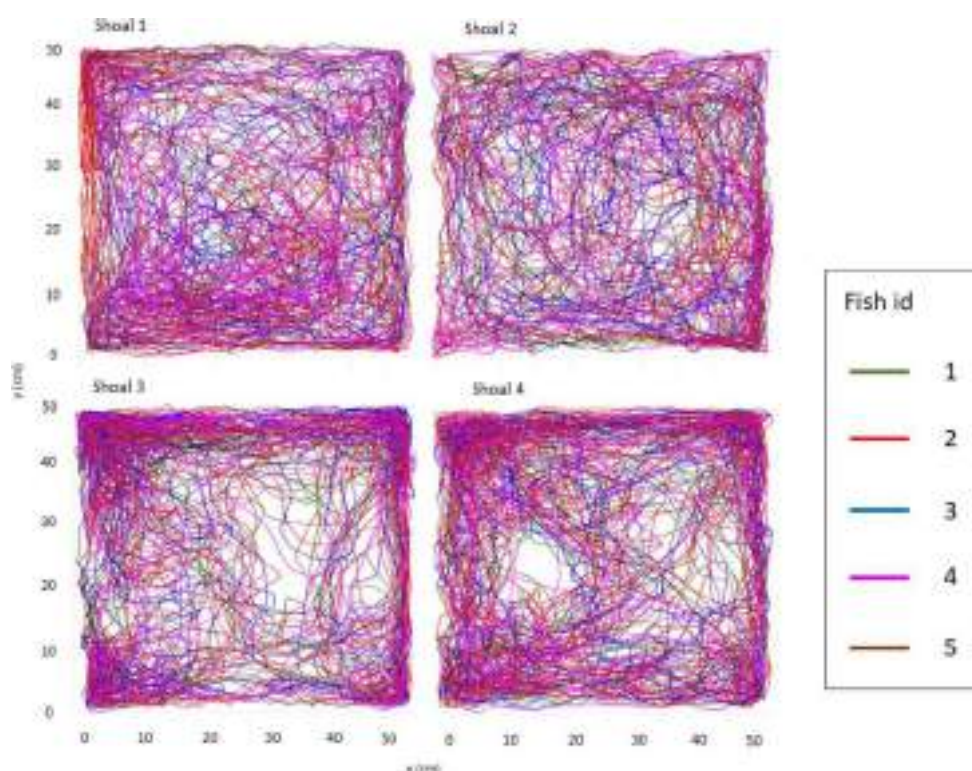
IdTracker 2.1 was used for multiple animal tracking and resulted in X and Y coordinates of each fish within a shoal, depicting the relative position of fish in the experimental tank. We categorized the behavioural metrics into two groups: (1) shoal cohesion metrics, including shoal area, inter-fish distances, nearest neighbour distances, and farthest neighbour distances; and (2) activity metrics, including thigmotaxis and total distance travelled.

Considering the comparatively small sample size and the nature of the behavioural data, we followed the Mann–Whitney U test for statistical comparisons across experimental groups in the published paper using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). However, in the present thesis section, the same dataset was re-analysed using the Kruskal–Wallis test, followed by FDR correction (Haynes, 2013), in order to align the analysis uniformly with the statistical analyses conducted throughout the thesis. There were no deviations in the results or interpretations from the published work, as the re-analysis provided the same direction as the published study (Sivaraman et al., 2022).

Statistical significance was defined as $p < 0.05$, with levels of significance denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

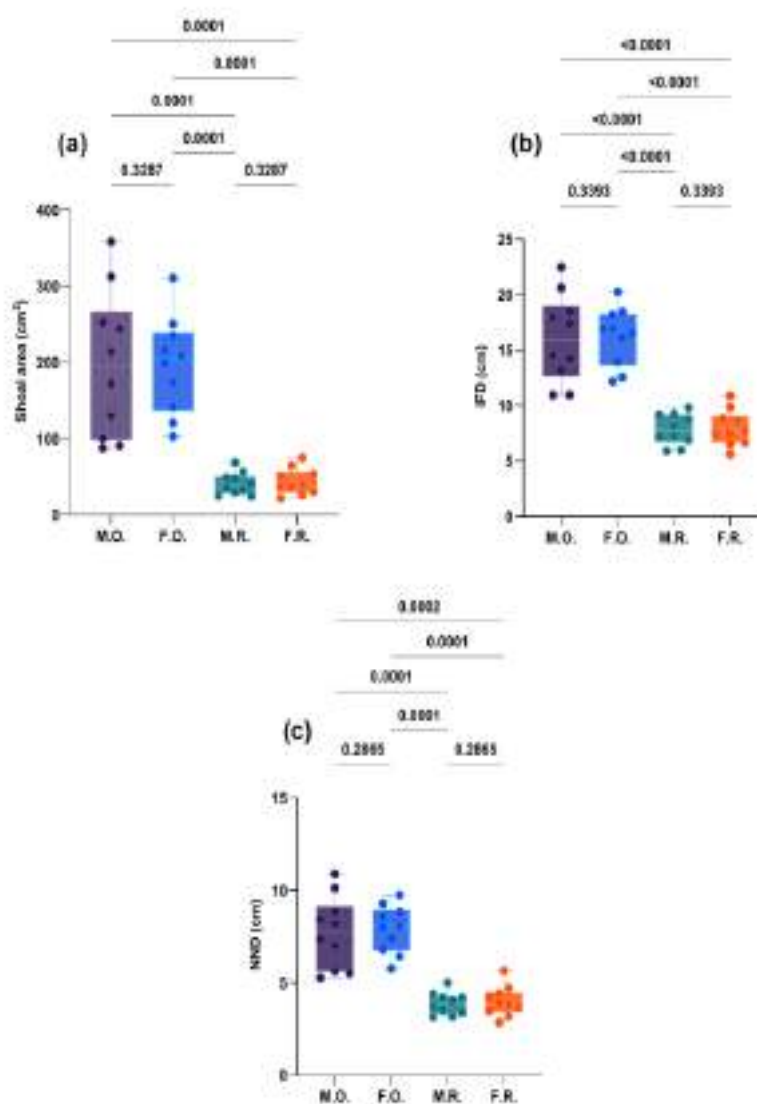
Table 6.1. Behavioural endpoints and their definitions used in the current study

Parameter	Abbreviation	Definition
Shoal area	SA	Area of the smallest polygon that contains all the fish at a given time
Inter-fish distance	IFD	Distance between the body centres of each shoal member
Nearest neighbour distance	NND	Distance between each fish's body centre and its nearest neighbouring fish
Farthest neighbour distance	FND	Distance between each fish's body centre and its farthest neighbouring fish
Total distance travelled	TDT	Total distance travelled by the zebrafish in the experimental tank
Thigmotaxis	TS	Average distance between the individuals of the shoal and the tank's centre

**Figure 6.2. Representative Tracks of Fish in Shoals**

Results

The study utilized a Kruskal-Wallis H test to determine if sex ratio (MO, FO, MR, FR) significantly influenced shoaling parameters. The analysis revealed a high statistical significance across all primary shoal cohesion metrics ($p < 0.0001$): Shoal Area ($H(3)=29.31$), Inter-fish Distance ($H(3)=29.28$), Nearest Neighbour Distance ($H(3)=28.51$), and Farthest Neighbour Distance ($H(3)=28.72$). Activity metrics also showed significant effects of sex ratio, with Total Distance Travelled ($H(3)=12.25$, $p=0.0066$) and Thigmotaxis ($H(3)=8.929$, $p=0.0302$). To understand the specific pairwise differences, we utilized the Two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli with FDR correction applied, and the resulting corrected q values were used to identify significance.



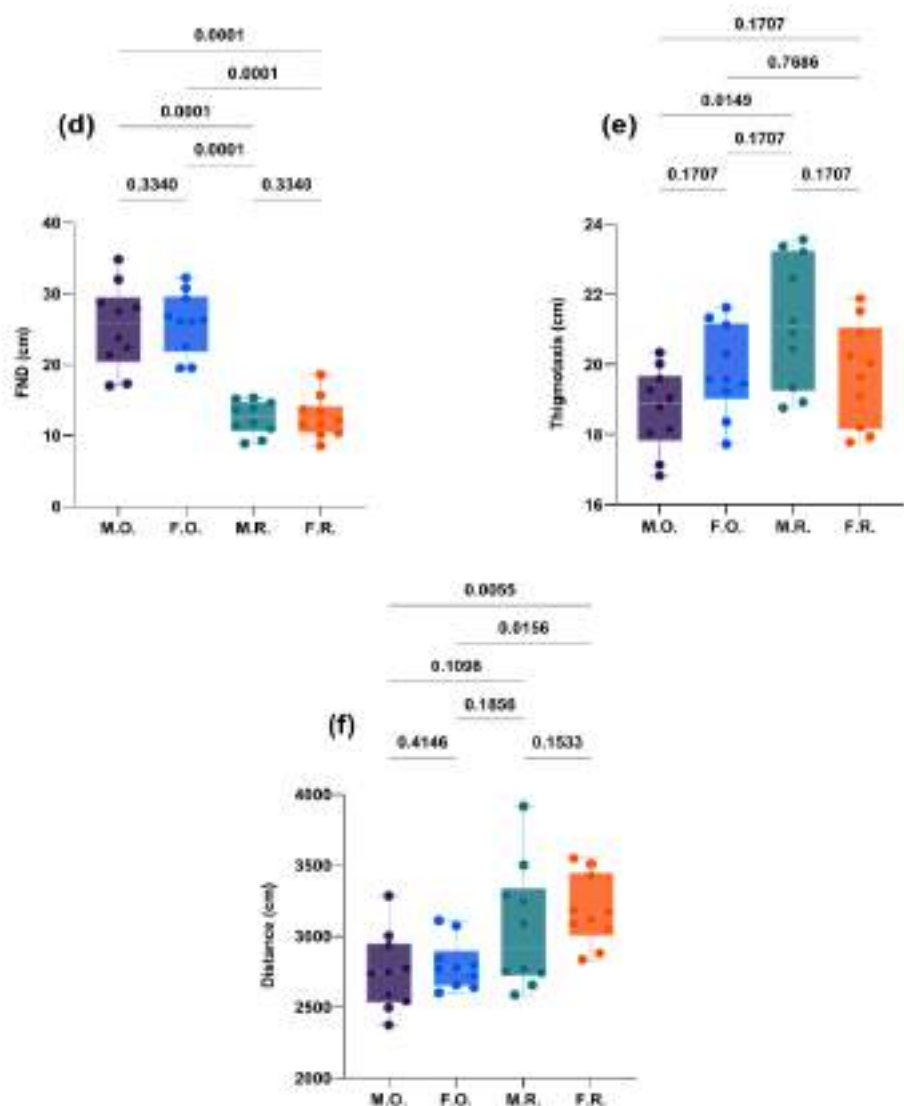


Figure 6.3. Distribution of shoaling and activity-related metrics across different shoal compositions.

Box-and-whisker plots illustrating the distribution of (a) shoal area, (b) inter-fish distance (IFD), (c) nearest neighbour distance (NND), (d) farthest neighbour distance (FND), (e) thigmotaxis, and (f) total distance travelled across different shoal compositions: male-only (M.O.), female-only (F.O.), male-rich (M.R.), and female-rich (F.R.) groups. Each box represents data from individual shoals (n = 10 per group), with median values, interquartile ranges, and full data dispersion shown.

Shoaling-related spatial metrics indicated an influence of sex ratio on group structure, with mixed-sex shoals (M.R. and F.R.) exhibiting greater shoal cohesion compared to same-sex shoals. Activity-related metrics showed selective group differences, with statistically significant comparisons highlighted in the figure. False discovery rate (FDR) correction was applied to account for multiple comparisons, and adjusted q-

values are indicated in the figure. Comparisons with $q < 0.05$ were considered statistically significant.

Table 6.2. Post Hoc Multiple Analysis for Pairwise Comparisons of Shoal and Activity Parameters

Parameter	Pairwise comparison	Mean Rank 1	Mean Rank 2	Individual p value
Shoal Area	M.O. vs. F.O.	30.30	30.70	0.9390
	M.O. vs. M.R.	30.30	10.00	0.0001
	M.O. vs. F.R.	30.30	11.00	0.0002
	F.O. vs. M.R.	30.70	10.00	<0.0001
	F.O. vs. F.R.	30.70	11.00	0.0002
	M.R. vs. F.R.	10.00	11.00	0.8483
Inter-fish Distance	M.O. vs. F.O.	30.60	30.40	0.9695
	M.O. vs. M.R.	30.60	10.30	0.0001
	M.O. vs. F.R.	30.60	10.70	0.0001
	F.O. vs. M.R.	30.40	10.30	0.0001
	F.O. vs. F.R.	30.40	10.70	0.0002
	M.R. vs. F.R.	10.30	10.70	0.9390
Nearest Neighbour Distance	M.O. vs. F.O.	29.75	30.95	0.8185
	M.O. vs. M.R.	29.75	10.00	0.0002
	M.O. vs. F.R.	29.75	11.30	0.0004
	F.O. vs. M.R.	30.95	10.00	<0.0001
	F.O. vs. F.R.	30.95	11.30	0.0002
	M.R. vs. F.R.	10.00	11.30	0.8036
Farthest Neighbour Distance	M.O. vs. F.O.	30.00	30.80	0.8784
	M.O. vs. M.R.	30.00	10.75	0.0002
	M.O. vs. F.R.	30.00	10.45	0.0002
	F.O. vs. M.R.	30.80	10.75	0.0001
	F.O. vs. F.R.	30.80	10.45	<0.0001
	M.R. vs. F.R.	10.75	10.45	0.9542

Parameter	Pairwise comparison	Mean Rank 1	Mean Rank 2	Individual p value
Total Distance Travelled	M.O. vs. F.O.	13.30	16.10	0.5923
	M.O. vs. M.R.	13.30	22.50	0.0785
	M.O. vs. F.R.	13.30	30.10	0.0013
	F.O. vs. M.R.	16.10	22.50	0.2209
	F.O. vs. F.R.	16.10	30.10	0.0074
	M.R. vs. F.R.	22.50	30.10	0.1460
Thigmotaxis	M.O. vs. F.O.	12.65	20.95	0.1124
	M.O. vs. M.R.	12.65	28.25	0.0028
	M.O. vs. F.R.	12.65	20.15	0.1514
	F.O. vs. M.R.	20.95	28.25	0.1626
	F.O. vs. F.R.	20.95	20.15	0.8784
	M.R. vs. F.R.	28.25	20.15	0.1213

Table description: The following table summarizes all pairwise comparisons for the shoaling and activity parameters resulting from the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. The FDR-corrected q values are noted in the figures.

Discussion

The study emphasizes that the spatial structure of the shoal was determined by the presence of both sexes, regardless of their relative proportions within a group. Notably, the study attributes the higher shoal cohesion observed in the male-rich and female-rich shoals to the higher female preference established in the previous literature. Interestingly, this female preference was absent in the female-only shoals and was statistically non-significant in the male-only shoals. Other activity metrics, including thigmotaxis and distance, showed only specific pairwise significance. Distance was higher in the female-rich shoal than in the male-only shoal, whereas thigmotaxis was higher in the male-rich shoal than in the male-only shoal.

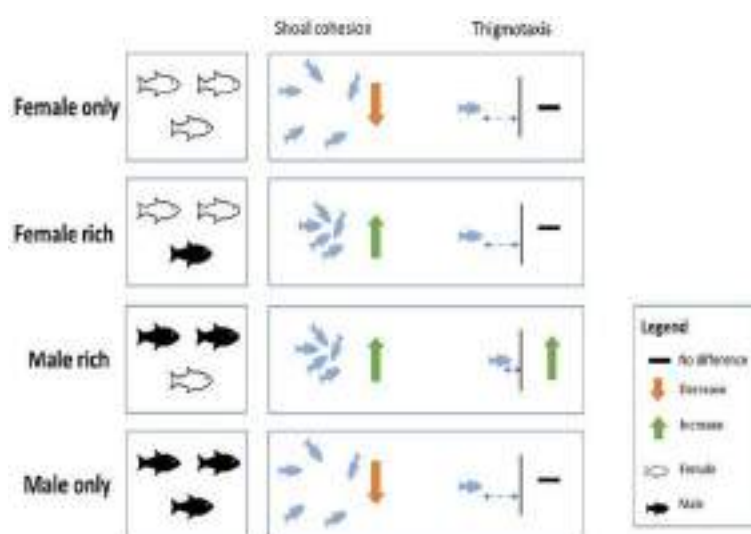


Figure 6.4. Summary of sex composition effects on shoal cohesion and thigmotaxis in zebrafish.

This figure presents the overall trends observed in shoal cohesion and thigmotaxis across different group compositions, including female-only, female-rich, male-rich, and male-only shoals. Arrows indicate the direction of change in behavioural responses, where upward arrows represent an increase, downward arrows indicate a decrease, and horizontal lines denote no significant difference. The results highlight variations in group cohesion depending on sex composition.

This work was established as a foundational methodology for the analysis of the core objectives of the study. We extended this study to design the conspecific ratio of the control and isolated shoals. It included both male-rich and female-rich shoals in a 4:1 ratio. As no statistically significant differences were observed between the male-rich and female-rich shoals in the present study, we combined them to improve the shoal sample size from ten to twenty. This strategy helped us incorporate more robust statistical analyses into the core objectives. Similarly, with respect to the videography experimental setup, we used IdTracker 2.1 as in the current study, whereas the R software used for parameter derivation was modified, and Python libraries were incorporated in the core objectives. Together, this foundational methodological study validated the subsequent social isolation experiments conducted on an established, methodologically reliable, and biologically cohesive shoal structure, which helped minimize confounds unrelated to isolation.

Chapter 7

Summary, Conclusion & Recommendations

Overall Results Summary: Objective - wise

Objective 1: Anxiety-Like Behaviours and Activity Levels Under Social Isolation

Socially housed control zebrafish showed clear baseline sex differences in open-field exploration. Control males spent more time and covered more distance in the central/exploratory zone, whereas control females spent more time and distance in the thigmotaxis (wall-hugging) zone, a primary indicator of anxiety-like behaviour. These zone preferences were consistent with the sex-independent number of entries and rapid movements (speed higher than 10 cm/s) observed in control groups. A sex difference emerged in the global locomotor output, where control females travelled a greater total distance than control males. Relative to these baseline patterns, socially isolated (test) fish showed an exploratory bias in both time and distance measures (Wilcoxon test). However, because the direction of baseline differences was opposite in males and females, the expression of anxiety-like behaviour after isolation became confounded. Test males reduced exploration compared to control males, while test females showed increased exploration compared to control females. As a result, test males and test females did not differ significantly from each other, indicating a sex-independent outcome of isolation in the OFT. Zone transitions, rapid movements, and total distance travelled were all elevated in isolated fish, again in a sex-independent manner. Derived locomotor metrics-including speed range (max–min), activity index (proportion of normal swimming time), and activity balance index ($ABI = \text{speed range}/\text{activity index}$)-consistently indicated higher activity levels in the isolated groups, with no-sex differences across either control or test conditions. Multivariate OFT analysis supported the univariate findings. Spearman correlations and PCA-revealed sex-specific baseline activity patterns in controls, where males were more active, largely reflected along PC2. Anxiety-related loadings on PC1 aligned with the univariate results. The Light-Dark Test (LDT), used as a confirmatory assessment of anxiety, showed that control groups preferred the dark zone, while isolated test groups preferred the light zone. This pattern was supported by total time and latency measures: controls displayed longer latencies to enter light, whereas test fish entered light faster. No sex differences were observed in LDT outcomes. Together, these findings suggest that chronic social isolation induces increased activity, higher

exploratory tendencies, and altered anxiety-like behaviour in both sexes, leading to sex-independent behavioural outcomes despite their distinct baseline profiles.

Objective 2: Multiple stimuli and Acute Modulatory Effect of Shoal on Stress and Aggression Under Social Isolation

In this study, we used a three-chambered tank to assess the behavioural responses of zebrafish and to understand how the shoal, as a buffering unit, modulates predator-induced stress and mirror evoked aggression across both sexes under isolation. All four experimental groups-control male (CM), control female (CF), test male (TM), and test female (TF)-were analysed in two sections. First, the behavioural responses to single stimuli were examined; then dual-stimulus conditions were introduced to analyse stress buffering and aggression buffering using context-specific metrics. In the single-stimulus conditions, No Stimulus (NS) served as the baseline control and Shoal Only (SO) as the positive control. Predator-Stimulus Only (PO) and Mirror-Stimulus Only (MO) were used to evoke stress and aggression, respectively. *Anabas testudineus* was used as the predator stimulus. Across all groups, NS showed no side-chamber bias. Control males and females responded to the other three stimuli while showing their baseline sex differences. Females exhibited higher sociality, higher mirror-induced aggression, and higher stress responses than males based on time spent in the contact (near stimulus) and distant (away from stimulus) zones. Both isolated males and females spent reduced time in the shoal contact zone, indicating a sex independent social deficit after isolation. TM and TF spent more time in the predator contact zone, suggesting altered threat perception; however, males also showed higher freezing in PO than in NS. Females displayed a blunted stress response, showing consistent freezing across all four conditions. In the MO condition, sex-dependent and opposite baseline patterns emerged: isolated males were more aggressive than isolated females, indicating context-specific sex differences under isolation.

In the second section, stress and social buffering were examined. Predator + Shoal (PS) was introduced and compared with PO using stress-related metrics, including time spent in predator and shoal zones, and changes in freezing and rapid movements. Control males showed reduced freezing and rapid movements along with a shift toward the shoal zone, indicating true social buffering. Test males showed no change in freezing or rapid movements but did shift toward the shoal zone, suggesting a

choice-based shift and partial buffering. Control females showed no change in freezing but a reduction in rapid movements. They spent more time in the predator zone in PS compared to PO. Test females showed increased freezing and no change in rapid movements in PS, indicating a heightened vigilance state; because freezing increased, a classic stress marker, test females showed failed buffering. In aggression buffering, CM, TM, and CF showed reduced aggression in the mirror bite score in Mirror + Shoal (MS) compared to MO and also displayed higher shoal-zone shift in MS than MO. In TF, due to their low mirror-induced behavioural response and the absence of a specific stimulus-oriented zone allocation in MS, the buffering effects appeared masked. For social affinity comparisons, PS and SO, as well as MS and SO, were analysed. CM showed higher Social Preference Index (SPI) in PS than SO and stable SPI between SO and MS. TM displayed a similar pattern, with higher SPI in PS and comparable SPI in MS relative to SO, suggesting predator-induced stress-driven social affinity increases-consistent with the buffering observed earlier. In CF, due to their high contact-zone time in SO and low time in the predator zone, the SPI was higher in SO and lower in PS and MS. In TF, SPI changes were visible only between PS and MS. However, despite shoal-zone shifts in PS, freezing did not reduce, suggesting a plausibly altered stress response in isolated females. Overall, these results suggest that the effects of social isolation and sex specificity are context- and experiment-dependent, which can mask or alter baseline sex differences.

Objective 3: Shoal Behaviour and Emergent States Under Social Isolation

This study's methodology was adapted from our preliminary shoal study. In that earlier work, we asked how conspecific identity and sex ratio influence social cohesion in spatial metrics. We analysed four groups of 5-member shoals comprising male-only (MO), female-only (FO), male-rich (MR), and female-rich (FR) groups, where MR and FR included a 3:2 sex ratio. Using R-based mathematical computations, we assessed spatial metrics such as shoal area, inter-fish distance (IFD), nearest neighbour distance (NND), farthest-neighbour distance (FND) as interactive spatial metrics, and thigmotaxis as an anxiety-related measure. We found that mixed-sex shoals (MR, FR) showed higher social cohesion-lower NND, FND, IFD, and shoal area-compared to MO and FO shoals. This indicated that sex composition within a shoal influences interaction strength and social cohesion, with mixed-sex shoals

exhibiting the strongest cohesion without significant differences between them. This study was published in ACS Omega (2022).

The current study examined shoaling and emergent states in 5-member isolated shoals, adapting the preliminary design in terms of sex composition. Here, we used both male-rich and female-rich shoals in a 4:1 sex ratio, with 20 shoals per group. We found that isolated shoals showed altered spatial metrics, including increased NND, FND, shoal area, and Euclidean distance. Orientation alignment measures-heading angle difference, cosine dissimilarity, and heading correlation (r)-were all disrupted in isolated shoals, marked by higher angular deviations and reduced alignment. Swimming dynamics (shoal speed and shoal distance travelled) were higher in isolated shoals, accompanied by reduced freezing and increased rapid movements. We analysed three emergent states-milling, swarming, and polarised states in both control and isolated shoals. Although both groups expressed all three states, the intensity and retention (memory) time differed markedly. Milling behaviour was higher in isolated shoals, suggesting a circling-type movement pattern. In contrast, the polarised state was reduced in isolated shoals, and both polarisation and rotation memory times were lowered, indicating consistent alterations in emergent states and disrupted shoal dynamics under social isolation.

Objective 4: Spatio-Temporal Dynamics of Dyads Under Social Isolation

In this objective, we analysed the spatio-temporal dynamics of dyads under social isolation. Two major groups were formed on the basis of the experimental conditions and the sex of the dyad partners: homogeneous and heterogeneous. The homogeneous groups included control male-male (CMM), control female-female (CFF), control male-female (CMF), and the corresponding isolated dyads-test male-male (TMM), test female-female (TFF), and test male-female (TMF). These groups were analysed to understand the fundamental differences between control and isolated dyads, based on sex identity and the effects of social isolation. The heterogeneous groups consisted of control-test combinations: control male-test male (CMTM), control female-test female (CFTF), control female-test male (CFTM), and control male-test female (CMTF). These were used to explore individual variability and dyadic-level dynamics, focusing on how the behavioural phenotype of one fish shapes the pairwise interaction that eventually contributes to collective behaviour. Homogeneous dyads

showed clear isolation-induced alterations. Spatial metrics were disrupted in isolated dyads, characterised by higher Euclidean distances and reduced proximity time within the 5 cm interaction range. Alignment-related metrics were also altered in test dyads, with higher cosine dissimilarity, greater speed differences, reduced acceleration correlation, lower maximum velocity cross-correlation, and reduced average following time. Sex differences within the homogeneous dyads were evident only in freezing and rapid movement frequency. Globally, freezing was lower in the isolated groups compared to control, but within the isolated category, TMM showed higher freezing than TFF and TMF. Similarly, although rapid movements were higher in all isolated dyads, the highest rapid movement frequency was observed in TMM, indicating altered locomotor dynamics specifically in isolated males. In heterogeneous dyads, the primary focus was on geometric following and directional initiation. Across all combinations, isolated individuals consistently showed lower geometric following and higher directional initiation, indicating a stronger tendency to lead the interaction. Subtle sex-associated patterns were visible in state dynamics such as freezing and rapid movement. Test males, when paired with either CM or CF, showed no significant changes in rapid movement frequency, whereas test females showed significantly higher rapid movements when paired with CM or CF, suggesting an altered speed trade-off between partners. Freezing patterns also varied: test males exhibited lower freezing when paired with CM but not with CF, indicating a possible influence of opposite-sex presence on alignment. Test females, however, consistently showed lower freezing regardless of partner sex, which may reflect greater female sensitivity to social isolation. When comparing dyad-level metrics across homogeneous and heterogeneous categories, most heterogeneous dyads resembled isolated homogeneous dyads. This pattern indicates that the higher initiation and lower geometric following shown by the isolated individuals made them the effective decision-makers in the pair, driving the dyad dynamics. Their higher speed supported this shift, where the isolated fish led and the control partner followed. As a result, heterogeneous dyads displayed behavioural organisation similar to isolated homogeneous dyads. An exception was observed in the velocity cross-correlation (VCCmax), where the CFTM dyad aligned more closely with the control CMF dyad rather than the TMF dyad. This suggests a possible opposite-sex influence on

alignment in TM. However, the observed difference did not remain significant after Holm correction.

Objective 5: Descriptive Analysis of Gut Microbiota Abundance and Sex Specificity Under Isolation

In this study, the descriptive analysis of the gut microbiota was performed to understand the effect of social isolation and sex-specific shifts in microbial abundance. Because pooled samples were used for sequencing, the results are interpreted at a descriptive and non-inferential level without statistical comparison. The primary focus is on identifying phylum- and genus-level differences across isolated males and females. We found that control males and control females differed in microbial abundance at the phylum level. Interestingly, isolation shifted the gut microbial abundance in TM and TF also in a sex-specific manner. TM were identified with gut dysbiosis, including Firmicutes depletion, Proteobacteria bloom, loss of Actinobacteria, reduction in Fusobacteria, and the presence of opportunistic pathogenic phyla such as Chlamydiae. TF were identified with a complete loss of Fusobacteria, Firmicutes bloom, and an increase in Proteobacteria. At the genus level, *Bacillus* was absent in TM, along with a sex-specific presence of genera under Proteobacteria in both TM and TF. These patterns indicate the existence of baseline sex differences in the gut microbial community and sex-specific gut dysbiosis under isolation.

Conclusion

The present study adopted a multi-pronged approach to elucidate the multiple layers of chronic social isolation and its impact across behavioural and gut microbial responses in zebrafish (*Danio rerio*). Inclusion of multiple behavioural assays to deepen the understanding of sex-specific analysis of social isolation by conducting tests including open-field and light and dark test for anxiety and activity like parameters, then transitioned to how the internal state of each fish modifies its behaviour towards different stimuli, by using three chambered tank test, acute social buffering as an additional key dimension. The study further progressed to collective and fundamental pairwise analysis through shoal and dyad analysis, incorporating established computational models. Also included a descriptive layer of gut

microbiome alteration as a potential biomarker of social isolation. Therefore, this multi-pronged approach further strengthens the understanding of behavioural consequences of social isolation.

As intended objective 1 clearly indicated the behavioural valence of test animals towards a hyperactive like phenotype as evidenced from higher locomotor metrics. The anxiolytic response was suggested from these tests, but we are unsure to report this as a true anxiolytic due to the strong baseline sex differences observed in the control groups and hyperactivity driven movement patterns of test groups. Based on the existing literature and strong bias towards the centre of the OFT and light zone strongly suggesting anxiolytic responses from the test groups. The key finding was the strong sex specificity noted within the control groups was masked in the isolated groups. hyperactivity overdrove the baseline sex differences in isolated males and females.

Therefore, we interpreted the behavioural valence of isolated fish as hyperactive and anxiolytic in nature, and we further considered other objectives to evaluate how this specific behaviour modulates the subsequent experimental designs. Objective 2, provided multiple layers of analysis using three chamber tank tests. Some responses from test groups were sex dependent in the same direction like controls, interestingly some are in opposite directions compared to controls. Reduced social preference were observed in both test males and females. Predator induced freezing was detected in test males, but females showed blunted stress response, as they showed similar levels of freezing across multiple stimuli. This response was sex dependent in the same direction with controls, males showed predator avoidance by translocating themselves between internal and distant zones (just away from the predator) but females showed complete avoidance of predators as they stayed in the distance zone majorly. Mirror induced aggressive responses were higher in test males and lower in test females, interestingly these responses were higher in control females and lower in control males. This response strongly suggests the magnitude of social isolation and how sex of the animal being a critical factor in this. Mirror induced aggression was present in isolated males, but we are again cautious to report true buffering in females due to the blunted responses and similar zone preferences observed across mirror only (MO) and mirror plus shoal (MS) conditions. Predator induced social buffering was partial in

test males and failed in test females, as observed with increased freezing in females when paired with predator and conspecifics together. The objective 2 strengthened, simultaneously added the possibilities of higher female susceptibility towards social isolation, as males also affected but indicated chances of recovery in the presence of conspecifics.

After this section we have done multiple animal-based analysis to understand the collective behaviour and paired interactions, and also added mathematical models established by Tunstrøm-Couzin. As hypothesised shoaling was altered in isolated shoals, but the novelty relied on the emergent states analysis. Isolated shoal showed multiple emergent states similar to control but showed variability in the fraction of time that occurred. Polarisation was lower and milling was higher in isolated groups, suggesting the spontaneous nature of state formation and transitions between multiple states. In the study we speculate the higher milling was correlated with the circling behaviour studied in the neurodevelopmental and Abnormal repetitive models. Paired analysis strengthened the possibilities of what caused the collective behaviour disruption in the socially isolated groups. The results suggested that spatial, locomotor and alignment metrics were broken in the isolated groups, affecting them negatively in maintaining aligned movements with the other individual. Most importantly, we suggest the hyperactive state of isolated fish identified from objective 1 was evident in the both multiple animal experiments, again overdriving the collective behaviours. Similarly other most notable responses were identified in the heterogeneous dyads of control and isolated pairs. Isolated individuals initiated higher turns and transitioned the heterogeneous dyad to a test homogeneous dyad type, leading to the disrupted information transfer and movements even though a control partner was present within a heterogeneous dyad. These tests again confirmed the possibilities of higher female sensitivity and chances of reversal of behavioural alterations in males within the heterogeneous test groups.

The behavioural findings, together with the gut microbiome analysis, provided a broader understanding of how chronic social isolation affects behaviour and gut microbial composition. As hypothesised, microbial shifts were evident in isolated zebrafish, with distinct sex-specific changes. The complete absence of Fusobacteria in test females, along with a Proteobacteria bloom and Firmicutes depletion in test

males, further strengthened the descriptive analysis and highlighted sex-specific microbial responses to isolation. Overall, the findings suggest that social isolation may alter the gut microbiome, while microbiome changes may also be associated with behavioural alterations, indicating a possible bidirectional relationship between social isolation, the gut microbiome, and behaviour.

Recommendation

Behavioural responses, apart from physiological changes in an organism, interpret the possible internal dysregulation of a system, which may arise from neural, genetic, or environmental factors. Understanding these aspects should incorporate and position behavioural analysis as a fundamental test, which can then be integrated into deeper approaches comprising advanced molecular and biochemical technologies. It is important to recognize that research using *in vivo* calcium imaging, including fibre photometry, has conducted multiple behavioural readouts in rodent models (Bukalo et al., 2026). This approach may eventually increase the wider acceptance and importance of behaviour-based outputs.

Considering this, research should focus on the critical variables that affect behaviour. Our study focused on the sex of the experimental animal and recommends that in future research, sex should be considered a vital biological variable. Both dependent and independent outcomes related to sex matter in research outcomes and therapeutic interventions.

The present study mainly relied on the impact of chronic social isolation on behavioural outputs across various domains. Only acute exposure to conspecifics was introduced to analyse their role in reducing fear and aggression. Therefore, the current study did not explore rescue strategies at a higher level. The acute exposure in buffering and dyadic experiments still provides indications of potential improvement in isolation-linked behavioural alterations in both parameter-dependent and sex-dependent ways, such as reduced partial stress and aggression in buffering, and improvement in velocity alignment and locomotor alterations in dyadic experiments observed in male isolated zebrafish.

Although advanced approaches related to gut microbiome analysis are available, the interaction between sex specificity and social isolation remains underexplored. Therefore, gut microbiome analysis using multiple biological replicates, with special focus on genera including *Cetobacterium* and *Bacillus*, is recommended considering their potential roles in gut integrity and probiotic integration.

Previous research has established social isolation as a well-recognised environmental stress model. This study further strengthens the relevance of chronic social isolation as an environmental model, as it can evoke behavioural responses resembling features of neurodevelopmental disorders, including social communication deficits and repetitive patterns observed in autism, as well as impulsivity associated with ADHD. Therefore, chronic social isolation may be considered a potential environmental model for investigating neurodevelopmental disorders, alongside pharmacological and pharmacogenetic models.

Limitations

This study has certain limitations that should be considered when interpreting the findings. The gut microbiome analysis was based on pooled and unreplicated samples, which limited the assessment of individual-level microbial variation. A formal a priori power analysis was not performed. Sex was determined based on morphological characteristics rather than genetic methods, although assessments were independently performed by three observers with majority agreement. In addition, only one duration of social isolation (180 days) was examined, and developmental time-course changes were not assessed. These limitations are acknowledged and provide important directions for future studies to further validate and extend the present findings.

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List of Presentations and Publications

Oral Presentations in International and National Forums

- **Zebrafish as a model for neurodevelopmental disorders** *International Webinar and Symposium on Trends in Modern Biology*, University of Calicut, Kerala, India | **August 27, 2021**
 - **Frontiers in Chemical Sciences (Oral Session)** *International Conference on Frontiers in Chemical Sciences (FCS 2022)*, University of Calicut, Kerala, India | **March 5, 2022**
 - **Shoal identity determines interactive pattern in zebrafish** *Indian Zebrafish Investigators Meeting (IZIM) 2022*, Pune, India | **September 23, 2022**
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Research Publications

Peer-Reviewed Journal Articles

- **Sivaraman, A., Nandakumar, R., & Ramachandran, B. (2022). Conspecific identity determines interactive space area in zebrafish shoal. *ACS Omega*, 7(42), 37351–37358.**
 - o DOI: [10.1021/acsomega.2c03815](https://doi.org/10.1021/acsomega.2c03815)

Book Chapters

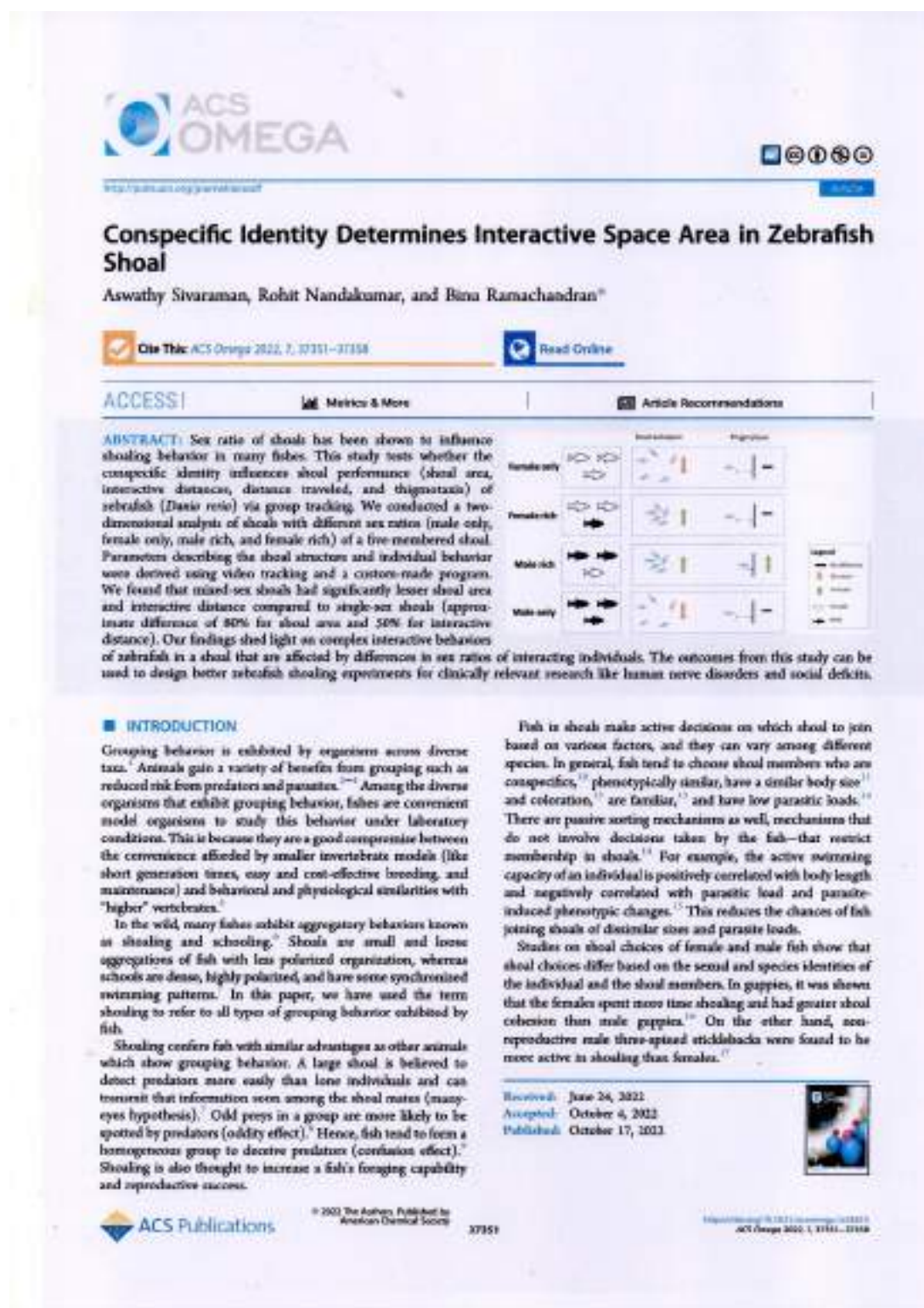
- **Sivaraman, Aswathy & Ramachandran, Binu. (2024). Zebrafish: A promising model in scientific research.** In P. Kumar Sunil (Ed.), *Reproductive Physiology and Aquaculture* (Chapter 13, pp. 105-132).
 - o **Publisher:** Astral International Pvt. Ltd.
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Previous shoaling studies on the effect of sex have largely dealt with a dichotomous shoal-choice experimental setup. In this type of study design, preference for an empty compartment of the tank and a compartment with stimulus fish shoals was inferred from the amount of time spent by the fish near each compartment.¹² But there are very few studies that look at how individuals of different sex freely interact in a shoal and affect the shoal structure as a whole. These types of studies are important because they create shoaling conditions that are closer to natural conditions, with the fish having access to social cues other than just visual.¹³

Zebrafish (*Danio rerio*, Hamilton, 1822) are model organisms widely used in various biological research fields. Their known genome, ease of genetic manipulation, and cost-effective breeding and maintenance make them attractive for laboratory experiments.^{14,15} Zebrafish exhibit sexual dimorphism after reaching adulthood,¹⁶ with male zebrafish being smaller and thinner than females and with yellowish color patterns on their bodies. Female zebrafish are more bluish and have a characteristic enlarged belly.¹⁷ The ease of identifying the sexes along with the rising popularity of zebrafish in a variety of laboratory research across diverse biological fields makes them a suitable organism for investigating how fish of different sex affect the shoal structure and the general activity patterns in the shoal.

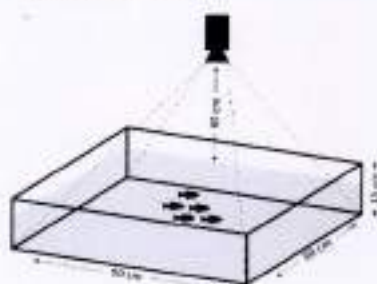
Our study looks at how shoal structure and the activity levels of individuals in them vary with differing sex ratios. We used zebrafish shoals with varying numbers of male and female fish and derived shoal-level and individual-level parameters. We used interfish distance (IFD), farthest neighbor distance (FND), nearest neighbor distance (NND), and shoal area as parameters that would describe the structure of the shoal as a whole. Total distance traveled (TOT) and thigmotaxis (wall-hugging) were used as parameters describing individual activity patterns within the shoal.

MATERIALS AND METHODS

Ethical Statement. All research involving animals was in accordance with the Ethics committee of the University of Calicut. All efforts were made to minimize the number of animals used and their suffering.

Experimental Setup. A custom-made experimental tank with a measurement of 50 cm × 50 cm × 15 cm (length × width × height) was made to analyze the zebrafish shoaling behavior (Scheme 1). A high-resolution camera (50 fps) was placed 60 cm (focal length) perpendicular to the arena. The tank was

Scheme 1. Experimental Setup



designed to have two dimensions with a length that was ten-times the average body length of an individual fish. This allowed the fish to freely explore two dimensions. The experimental shoal size was kept at five fish.¹¹ Adult zebrafish having a body length of approximately 4–5 cm were taken for the behavioral experiments.

Experimental Protocol. Before starting all behavioral experiments, the male and female fish were separated and put into separate preholding tanks. Preholding tanks had a capacity of 16 L, which could hold around 20–25 fish at a time. To reduce observer error, male and female zebrafish were identified based on a consensus arrived at by multiple researchers working on zebrafish. Five zebrafish were randomly selected at the time of the experiment from the preholding tanks and transferred to the pretreatment tank having a measurement of 30 cm × 20 cm × 20 cm and allowed to acclimatize for 5 min. The fish were then transferred to the experimental tank and allowed 5 min for acclimatization. The system water was used and maintained at a level of 6 cm from the bottom of the experimental tank. The water was changed after every experiment to avoid the subsequent subjects from being affected by the olfactory cues from the previous subjects.

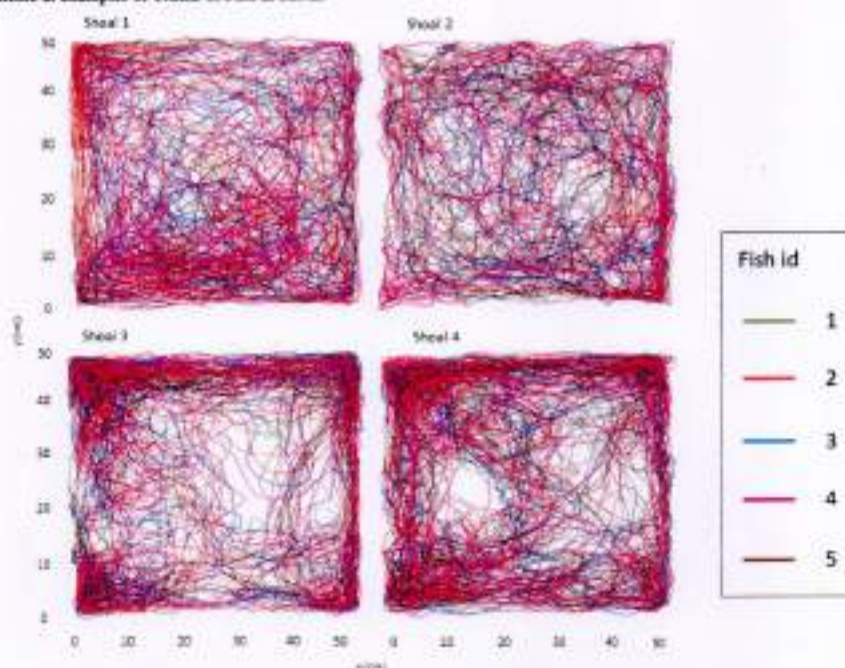
The experiments were divided into four treatments: female-only (FO), female-rich (FR), male-only (MO), and male-rich (MR). FO shoals contained five female fish, FR shoals had three females and two males, MR had two females and three males, and MO had only males (Table 2). Each treatment group contained ten shoals.

Videos of each shoal were recorded at 30 frames per second for 6 min for each experiment using a high-resolution camera Canon EOS R (Mirrorless, Canon Inc., Ota, Tokyo, Japan). The camera was placed perpendicular to the arena and 60 cm above the arena. Four 10 W LED light plates with an antiglare diffuser were placed approximately 18 cm away from the four walls of the experimental tank to attain the uniform diffuse lighting necessary for accurate tracking. A total of 100 male and 100 female fish were used for all the behavioral experiments combined. None of the fish was used for more than one experiment. All fish were fed 2 h before the start of experiments and just after the completion of experiments (at 7.00 am and 4.00 pm). The behavioral experiments were conducted from 9.00 am to 4.00 pm IST.

Statistical Analysis and Derivation of Shoal Parameters. The recorded videos were compressed using Adobe premiere pro-2020 (version 14.0, Adobe Inc., San Jose, California, US). The compressed videos were analyzed in idTracker 2.1.¹⁸ idTracker is an automatic animal tracking software that is capable of identifying individuals and tracking the movements of multiple animals simultaneously using video recordings of animal groups. The output of the software is a spreadsheet with x and y coordinates of the fish in each frame of the video and a unique identifying number for each individual. Scheme 2 shows examples of 6 min tracks of four shoals. Shoal level parameters like shoal area (SA), interfish distance (IFD), farthest neighbor distance (FND), nearest neighbor distance (NND), total distance traveled (TOT), and thigmotaxis (Table 1) for each shoal were derived from the track data using statistical programming package, R.¹⁹

We used Mann–Whitney U test to do a pairwise comparison of each of the parameters across the four treatments. A p-value less than 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).

Scheme 2. Examples of Tracks of Fish in Shoals*



*Each plot represents the tracks of positions of the fish in a shoal. Each color code represents an individual fish in the shoal. The shoal was allowed to explore for 5 min in the open tank (five fish per shoal).

Table 1. Behavioral End Points and Explanations Used in the Current Study

parameters	definition
shoal area (SA)	area of the smallest polygon that contains all the fish at a given time
interfish distance (IFD)	distance between each shoal member's body center
farthest neighbor distance (FND)	distance between each fish's body center and the farthest neighboring fish
nearest neighbor distance (NND)	distance between each fish's body center and the nearest neighboring fish
total distance traveled (TDT)	total distance traveled by the zebrafish in the experimental tank
thigmotaxis (TS)	average distance between the individuals of the shoal and the tank's center

RESULTS

Our results show that MR and FR (mixed-sex shoals) were not significantly different from each other for any of the variables that were taken into consideration. Comparisons between MO and FO (same-sex shoals) also showed similar results. On the other hand, paired comparison between the mixed-sex shoal treatments and the same-sex shoal treatments (comparison between MR–MO, MR–FO, FR–MO, and FR–FO) revealed a significant difference between the treatments for many of the variables (Table 2, Table 3).

Among the variables that were considered, shoal area showed the highest percentage difference (between 77% and 79%) and the lowest *p* value between the different treatments. Figure 2 represents the shoal area as a radar plot for better comprehension of the variation of shoals in different treatments, while Figure 3 is a network representation of four example shoals representing associations between individual zebrafish within a shoal. IFD, NND, and FND exhibited similar percentage differences among each other (between 48% and 52%) but less than that of the shoal area (Figure 3).

Since IFD, NND, FND, and shoal area describe similar behavioral end points (i.e. shoal cohesion), we refer to them collectively as "shoal cohesion" in this paper. The results also suggest that those parameters are correlated (but see ref 29 for an example where these parameters showed different patterns).

We obtained more complicated results for TDT and thigmotaxis. In general, mixed-sex shoals tended to travel less distance compared to same-sex shoals, but the differences in TDT were low (6–10%). TDT was significantly different only for MO–MR and FO–MR. FR had a lower but nonsignificant TDT compared to MR, and this might be the reason why pairs involving FR and same-sex shoals did not show any significant differences. On the other hand, thigmotaxis was generally higher for mixed-sex shoals with MR having the highest levels of

Table 2. Percentage Differences between the Pairs of Treatments Compared^a

PARAMETERS		TREATMENT COMBINATIONS					
		MO - MD	MD - FB	MO - FB	FO - MD	FO - FB	MD - FB
Shoal cohesion	Shoal size	78.07	77.77	-79.04	77.77	-5.87	0.16
	SVI	51.25	50.48	-52.65	50.58	-1.58	-0.01
	SDVI	48.8	48.72	-48.98	48.72	-3.05	-2.15
	PNV	29.84	30.32	-31.82	31.45	-0.63	-2.42
	Distance	-6.37	-8.95	6.74	-8.99	-2.84	0.5
Shoal stability	Dispersal	-51.81	-5.12	6.57	8.51	7.09	-5.60

^aGreen colored cells have values that were significantly different between the treatments compared.Table 3. Showing p Values of Each Shoal Comparison^a

Treatment	Statistical parameter	MO - FO	MO - MR	MO - FB	FO - MR	FO - FB	MR - FB
SA	p-value	0.9718	<0.0001	<0.0001	<0.0001	<0.0001	0.7394
	Exact/approximate	Exact	Exact	Exact	Exact	Exact	Exact
	Sum of ranks	103, 107	155, 55	155, 55	155, 55	155, 55	103, 107
	Mann-Whitney U	48	0	0	0	0	45
IFD	p-value	0.9705	<0.0001	<0.0001	0.0002	0.0002	0.8108
	Exact/approximate	Exact	Exact	Exact	Gaussian	Gaussian	Exact
	Sum of ranks	106, 104	155, 55	155, 55	155, 55	155, 55	103, 107
	Mann-Whitney U	49	0	0	0	0	48
FND	p-value	0.8734	<0.0001	<0.0001	<0.0001	<0.0001	0.8708
	Exact/approximate	Exact	Exact	Exact	Exact	Exact	Gaussian
	Sum of ranks	102, 108	155, 55	153, 57	155, 55	155, 55	107.5, 102.5
	Mann-Whitney U	43	0	2	0	0	47.5
NMD	p-value	0.7823	<0.0001	<0.0001	<0.0001	<0.0001	0.7344
	Exact/approximate	Gaussian	Exact	Exact	Exact	Exact	Exact
	Sum of ranks	100.5, 109.5	155, 55	152, 58	155, 55	155, 55	108, 110
	Mann-Whitney U	43.5	0	3	0	0	40
TDT	p-value	0.5787	0.2479	0.0433	0.4813	0.0347	0.315
	Exact/approximate	Exact	Exact	Exact	Exact	Exact	Exact
	Sum of ranks	95, 113	88, 121	58, 135	95, 115	73, 137	91, 119
	Mann-Whitney U	42	34	25	40	18	36
TM	p-value	0.6962	0.0052	0.1431	0.3655	0.9709	0.0962
	Exact/approximate	Gaussian	Exact	Exact	Exact	Exact	Gaussian
	Sum of ranks	82.5, 127.5	68, 141	85, 125	86, 124	106, 104	127.5, 82.5
	Mann-Whitney U	27.5	14	30	31	48	27.5

^aThe p values were calculated using a two-tailed Mann-Whitney U test, graph pad prism (n = 40). Statistically significant values are highlighted (green). Gaussian refers to p values estimated using Gaussian approximation.

thigmotaxis, except for MO-MR, none of the pairwise differences was significant (Figure 4). (Statistical comparison of shoals is given in Table 3).

DISCUSSION

Previous studies that looked at shoaling behavior of zebrafish in a similar free-shoaling setup have found that zebrafish prefer to shoal with conspecifics compared to heterospecifics.¹⁰ Our experiments suggest that in zebrafish, the sex ratio of the conspecific shoals influences shoal cohesion. Mixed-sex shoals tend to have higher shoal cohesion parameters than same-sex

shoals, but the relative number of males and females in the mixed shoals did not make a difference. Similarly, the sexual identity of same-sex shoals also did not significantly affect shoal cohesion parameters. Regardless of the sexual identity or the relative proportion of the sexes, whether a shoal is mixed-sex or not is what determines shoal cohesion.

A similar study using only mixed-sex shoals found that females in shoals preferred to swim close to other females, but males did not show any such preference.¹⁰ Hence, increased shoal cohesion in mixed-sex shoals in our study might be because of the female preference to be with individuals of the same sex. But

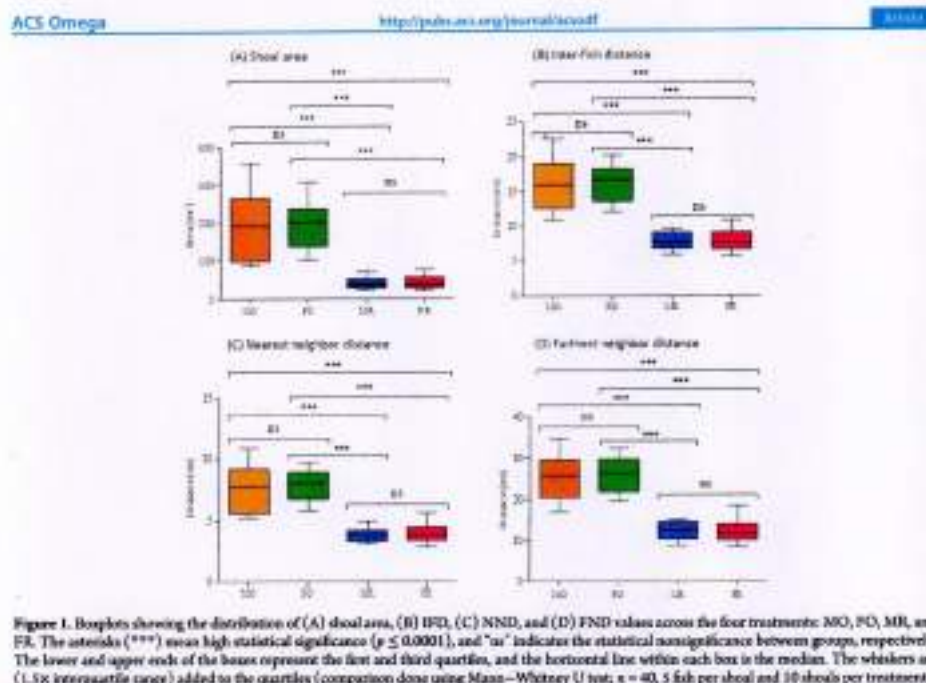
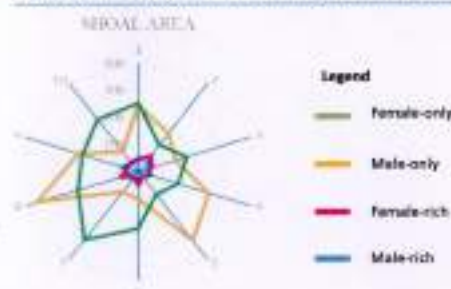


Figure 2. Radar plot of shoal area: all 10 axes in the radar graph represent the 10 shoal observations of the four different shoals. A total of 40 observations were done for the analysis. The center point of the radar starts from a value of zero and all the values fall within a maximum value of 400. The highest shoal area was reported in the male-only (MO) and female-only (FO) shoal and the lowest shoal area is the male-rich (MR) and female-rich (FR) shoal.

It is interesting to note that the female–female cohesion decreases in an all-female shoal.

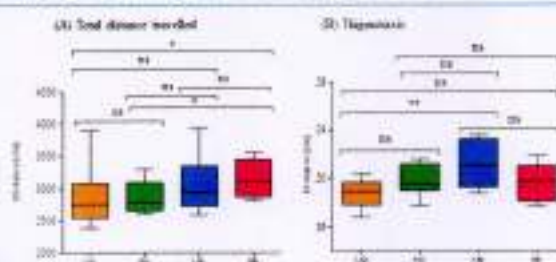
Zebrafish interfish distances vary with environmental conditions and physiology.² Interfish distance tends to be positively correlated with food availability and negatively correlated with predator threat.³⁰ In general, tightening the shoal by reducing shoal area, and reduced IFD, NND, and FFD are related to anxiety and stress.³¹ This has also been validated

by studies that show that shoal cohesion parameters and thigmotaxis increase when treated with anxiolytic drugs and reduce when treated with anxiogenic drugs.³²

The increased shoal cohesion in mixed-sex shoals could also be a result of increased stress levels, though the reason for an increase in stress is open to speculation. It is suggested that a lot of group-living animals exhibit sexual segregation because of differences in predation risk, required resources, and in general a lack of synchrony in activities.³³ In many taxa, sexual segregation happens also due to sexual harassment of females by males.³⁴ Hence, the mixed-sex shoals might be under higher stress because a male:female ratio almost equal to one might be unusual.

Contrary to the findings of this study, Way et al. found no effects of sex on shoaling behavior in zebrafish in a dichotomous shoal preference test.³⁵ The contradiction between the two studies might be due to the difference in study designs. The dichotomous preference test tells us the preference of an individual fish to interact with a shoal, whereas interactions in a free-swimming shoal test tell us about the behavior of the shoal as a whole. This underlines the importance of behavioral assays conducted with the least amount of restrictions and as close to natural conditions as possible.

Increased investment in foraging might be necessary for females because of the higher energy costs associated with tissue generation.³⁶ Hence, one might expect females to have a higher activity level in general, with TDT being a proxy for fish activity. Studies on the cichlid fish *Pelvicachromis taeniatus* found that shoals with a higher proportion of females engaged in shoaling had higher shoal activity in terms of swimming speed and



Changes in shoal cohesion parameters and activity levels have behavioral consequences on reeffish. Increased IFD may limit the intrashoal transfer of cues, which in turn will affect the stability of the shoal.³⁵ Shoal cohesion represents a trade-off

Though this paper has used zebrafish as a model organism to study behavioral, we believe that the results of this study could have wider implications for biomedical, genetic, molecular, and neurological research. Human neurological disorders associated with social communication deficits including autism spectrum disorders, schizophrenia, etc. are widely investigated based on the shoaling behavior of zebrafish.²⁹ Increased shoal area and interindividual distance can be correlated with shoal disruption and social communication in the case of neurological disorders.

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Notes

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