UNIVERSITY of HULL

Role of serotonin in crayfish anxiety and effect of pH on anti-predatory behaviour in crayfish *Pontastacus leptodactylus*

being a thesis submitted in fulfilment of the

requirements for the degree of

Doctor of

Biological Sciences

in the University of Hull

by

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April 2025

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Dedication

This thesis is dedicated to my parents, Azam Khan and Gulraiz Jahan and my brother Yasar for never giving up despite all the testing times life brought upon us. I also want to dedicate this thesis to my supervisor Dr. Thomas Breithaupt for his endless support and guidance in the research, writing and mentoring of this work. A very important dedication to Gabi, who motivated me so much on a personal level and played a very important part in helping me keep my spirit and mental health up during this entire journey. This doctorate belongs to all of us together and it would not have been here without the support I got from each of the people mentioned.

Acknowledgement

I am filled with a deep sense of gratitude as I reflect on this incredible PhD journey, and there are many people who have made this achievement possible.

To my dearest parents and my brother, Yasar, you have been my rock through thick and thin. Your unwavering support and strength have carried me through every challenge, and I truly believe that this doctorate belongs to all of us.

A heartfelt and profound thanks to my supervisor, Dr. Thomas Breithaupt, whose constant inspiration, guidance, and encouragement have been the cornerstone of this journey. Your belief in my abilities and your patient mentorship has been a light in moments of doubt, and I am forever grateful for the impact you've had on both my professional and personal growth.

To Gabi, your kindness and support have meant the world to me. Adjusting to a new world and learning to navigate life on my own would have been impossible without you. You have been my lifeline, and I cannot express enough how deeply thankful I am for everything you've done for me.

A special mention goes to my best friend, Malak, who, despite being far away, has been a source of emotional strength and constant reassurance. Your care and encouragement have been a true gift.

I would also like to extend my gratitude to my research group, the Happy Chemical Cluster, and my friends for their understanding, camaraderie, and support. Your presence has made this journey so much easier and more enjoyable.

Finally, I offer my heartfelt thanks to my Almighty Lord, who has been my ultimate source of strength and grace. None of this would have been possible without Him, and I am eternally grateful for all I have received and achieved.

Publications and Conferences

Publications

Kaur, D., Khan, A., Kubec, J., Breithaupt, T. and Buřič, M. (2025). Efficacy of Administration Routes in Crayfish: Comparative Analysis of Intracoelomic and Intrapericardial Techniques Using Fluorescein Dye. Journal of experimental zoology. Part A, Ecological and integrative physiology, [online] 343(1), pp.95–104. doi:https://doi.org/10.1002/jez.2872

Conferences

1. Presented a talk titled "Role of RNA Editing in Adaptation" at the 24th Symposium of the International Association of Astacology in Zagreb, Croatia, from September 16th to 20th, 2024.

2. Presented a talk titled "Role of RNA Editing in Adaptation" at the 5th DKFZ-ZMBH Alliance Kloster Schöntal Final Retreat in Schöntal, Germany, from June 26th to 28th, 2024.

3. Presented a talk titled "Role of Serotonin in Crayfish Anti-predatory and Anxiety-like Behaviour" at the Division of Epigenetics at the German Cancer Research Institute in Heidelberg, Germany, on January 19th, 2024.

4. Attended and presented a talk titled "Role of Biogenic Amines: Serotonin and Dopamine in Anxiety-like Behaviour of Crayfish (Pontastacus leptodactylus)" at the SEB Centenary Conference held in Edinburgh from July 3rd to 7th, 2023, organized by the Society for Experimental Biology.

5. Attended and presented a talk titled "Impact of Short-Term pH Change on Crayfish Anti-predatory Behaviour" at the Animal Behaviour Early Career Researcher Symposium held in Finland from October 3rd to 7th, 2022, organized by the Society for Experimental Biology.

6. Presented a talk titled "Impact of Short-Term pH Change on Crayfish Anti-predatory Behaviour" at the 23rd Symposium of the International Association of Astacology in the Czech Republic from June 20th to 25th, 2022.

7. Presented a poster titled "Impact of Short-Term pH Change on Crayfish Anti-predatory Behaviour" at the Annual Postgraduate Research Day Conference in 2022 at the University of Hull.

8. Presented a poster titled "Role of Serotonin in Crayfish Behaviour" at the Annual Postgraduate Research Day Conference in 2021 at the University of Hull.

Abstract

Encountering threatening situations is a common occurrence in the living world. When exposed to danger for extended periods, behavioural response manifests as stress-induced sustained alertness, which is known as anxiety. It is essential to understand that anxiety can have long-lasting effects on an organism's normal behaviour and interactions. This thesis aims to understand the role of serotonin in stress responses among crayfish. Crayfish are a keystone species in an aquatic ecosystem. Changes in their behaviour can negatively affect their inter- and intra-specific interactions. This can disrupt their role in the normal functioning of the ecosystem. The research involves three studies targeting stress under different conditions: exposure to low pH, the presence of fluoxetine, and injection of exogenous serotonin, dopamine, and their blockers. The first study focuses on the impact of freshwater acidification on crayfish response to alarm order (blood from conspecifics), and if this response is mediated via serotonin. The study reports that animals exposed to pH6 exhibit a significantly reduced behavioural response to alarm odour compared to the control (pH8) group. In addition, the animals do not show an elevated level of haemolymph glucose. The latter is mediated via the Crustacean Hyperglycaemic Hormone (CHH) which has been linked to serotonin changes and is an indicator of stress. The study shows that freshwater acidification can impair crayfish olfaction, leading to reduced anti-predatory responses and the behavioural response may be influenced by serotonin. The second study investigates the impact of fluoxetine, a pharmaceutically active compound (PhAC), on anxiety response in crayfish. Fluoxetine is an SSRI (Selective Serotonin Reuptake Inhibitor) which elevates serotonin levels by preventing its reuptake in the synapse. Animals were exposed to three different levels of fluoxetine (high, low, and intermediate doses) and exhibited an anxious response by spending more time with slower active speeds in the dark zones of the plus-maze behaviour setup when compared to controls. The crayfish that were exposed to Fluoxetine

had elevated haemolymph glucose levels which suggested that serotonin is involved in the anxiogenic response. The study highlights the potentially harmful effects of pharmaceuticals in freshwater ecosystems, including changes in behaviour that can impact survival sustainability, and species interaction. The final study took a more direct approach regarding the impact of serotonin on crayfish anxiety-like behaviour (ALB). Animal groups were injected with physiological saline, serotonin, and dopamine at 2 doses each (1 µM and 10 µM) alone and in combination with their respective blockers (50 µM maleate salt and 10 µM methergine), in the pericardial sinus. Dopamine at 10 µM dose while serotonin at both tested concentrations, caused ALB among crayfish. The anxiogenic effect was successfully blocked when administered with either of the blockers. However, 1 µM dose of Dopamine did not affect behaviour but it appeared to have an anxiogenic effect when administered with methergine. Overall, our results emphasize the involvement of a complex functional interplay among biogenic amines in ALB in crayfish with blockers showing effects on more than one biogenic amine. It also leaves an open-ended question on the role of dopamine in anxiety responses which needs to be further investigated.

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Ethics Statement

The research presented in this thesis adheres to the ethical guidelines and principles set forth by the University of Hull. In designing the experiments, particular consideration was given to the welfare of the crayfish used. Sample sizes were determined based on a balance between statistical power and the minimization of harm to the animals. The number of individuals used in each experiment was kept as low as possible while ensuring the validity of the findings, following the principle of the 3Rs (Replacement, Reduction, and Refinement). Power analyses were done during the experiments to minimise animal use.

The experiments were designed to minimize stress and discomfort for the crayfish, with a focus on their natural behaviours and environmental conditions. Efforts were made to avoid unnecessary handling or exposure to potentially harmful situations such as inconsistent temperature, over-exposure to chemicals or amateur injection and handling techniques. Crayfish were housed in appropriate environments that mimicked their natural habitat, and the conditions were regularly monitored to ensure that the animals' physical and psychological needs were met.

In recent years, there has been growing discussion in the scientific community regarding the capacity of crayfish and other invertebrates to experience pain. While the evidence for pain perception in crayfish remains an area of active research with too many loose ends, the consensus is that caution should be exercised in experimental design to avoid inducing undue suffering. This thesis reflects a commitment to the ethical consideration of these animals and follows the current thinking in the field that emphasizes minimizing harm and enhancing our understanding of invertebrate welfare.

The experiments outlined in this thesis were approved by the University of Hull Ethics Review Board under approval number FEC_2023_46 (FEC_2022_98). All procedures

were conducted in compliance with the ethical guidelines for the use of animals in research.

Chapter 1 Introduction

Human activities have introduced numerous pollutants into aquatic environments, with far-reaching consequences for human health and ecosystem stability. While antidepressants and other pharmacologically active substances are vital for managing mental health disorders, their presence in water systems contributes to a growing crisis of chemical pollution (Tabak and Bunch, 1970, Fent et al., 2006; Santos et al., 2013; Hossain et al., 2019b). These contaminants, alongside other anthropogenic stressors such as acidification from industrial emissions and agricultural runoff (Galloway et al., 1983, Psenner, 1994), alter the delicate balance of freshwater ecosystems. Acidification can weaken aquatic organisms by disrupting their physiological and chemosensory processes (Leduc et al., 2013), while pharmaceuticals target neurochemical pathways, compounding their effects in non-target organisms (Hossain et al., 2019b). To better understand these interactions and their ecological implications, model organisms like crayfish provide a critical window into how such pollutants affect physiology, behaviours, and ecosystem dynamics (Evans-White et al., 2003). As keystone species, crayfish play a vital role in nutrient cycling and habitat structuring, making them essential indicators of pollution's broader impacts (Evans-White et al., 2003, Creed and Reed, 2004, Alp et al., 2016).

1.1 Crayfish Biology and life history traits

Crayfish are decapod crustaceans that thrive in various aquatic environments, including clear streams, murky ponds, wetlands, and marshes (Evans-White et al., 2003). These animals are primarily bottom dwellers, spending most of their time hidden in burrows or under rocks to protect themselves from predators (Ferro and Moore, 2014). As nocturnal creatures, crayfish are most active at night, when they emerge to forage for food (Bojsen et al., 1998). Their diet is omnivorous, consisting of plant materials such as algae and detritus, as well as small invertebrates and fish (Creed and Reed, 2004; Alp et al., 2016).

Crayfish undergo a series of moults throughout their life cycle as they grow, with some species living for several years (Willig and Keller, 1973). They require calcium to maintain their shells, and their survival rates decrease at pH levels below 5-6 (Weber and Pirow, 2009). Their reproductive habits are also fascinating; females carry fertilized eggs beneath their abdomen until they hatch into juvenile crayfish, which are initially very vulnerable to predation (Galeotti et al., 2006). Additionally, crayfish exhibit aggressive behaviours, and the outcomes of fights among them help establish social hierarchies (Huber et al., 1997).

1.2 Diversity and Relationship Between Crayfish Species

Freshwater crayfishes represent a remarkably diverse assemblage of decapod crustaceans, encompassing over 640 recognized species distributed North America, Europe, Asia, and even parts of Africa and Australia, with the greatest diversity existing in North America, particularly in the southeastern United States (Crandall and Buhay, 2007). They inhabit four predominant ecological niches: primary burrowers, which spend their entire life cycle in burrows and can excavate to the water table; stream-dwelling species; inhabitants of ponds, lakes, and large rivers; and stygobitic species, which are obligate inhabitants of subterranean environments.

Crayfish have a monophyletic origin and hence despite their differences, the species share a fairly similar genetic makeup (Crandall et al., 2000). Certain species of crayfish such as *Orconectes rusticus* and *Pacifastacus leniusculs*, are highly invasive and have been wreaking havoc in freshwater environments (Crandall and Buhay 2007, Vaessen and Hollert, 2015). There is also a parthenogenetic species of crayfish, *Procambarus virginalis* which originated from a parent species *Procambarus fallax*, a native to Florida, United States and has invaded across Europe, Asia and Madagascar (Maiakovska et al., 2021).

1.3 Anxiety

Exposure to threatening situations leads to the appearance of a fear response, which is a primary and spontaneous emotion (Fossat et al., 2014). The fear response is often accompanied by an immediate and involuntary fight or flight reaction such as fleeing for safety or displaying aggression to confront or deter the threat (Fossat et al., 2014). Such rapid response is crucial to managing and reacting to acute stress and ensuring safety (Fossat et al., 2014). However, prolonged exposure to stressful situations can result in the development of anxiety (Fossat et al., 2014). Fossat et al. (2014) describe anxiety as a state of alertness that persists even in the absence of a stressor. It is a behavioural outcome of stress and is a deep-seated fear response to previous encounters with threats (Fossat et al., 2014).

Anxiety is very well characterised in humans and some vertebrates (Graeff et al., 2003, Grillon et al., 2007, Maximino et al., 2010). This secondary emotion has been extensively studied in humans (Siepmann and Joraschky, 2007, Andreatta et al., 2015, Marcinkiewcz et al. 2016), rodent (Walf and Frye, 2007) and zebrafish (Egan et al., 2009, Maximino et al., 2010, Blaser and Rosemberg, 2012) models. There is recent evidence that even invertebrates can have anxiety-like responses (Fossat et al., 2014, Mohammad et al., 2016). Most animals have an inherent tendency to respond to fearful situations and develop some degree of adaptive defensive strategies (Fossat et al., 2014). The anticipatory nature of anxiety can have adaptive purposes such as fostering vigilance and preparedness (Fossat et al., 2014). However, it can become maladaptive when it is excessive, persistent, or disproportionate to the actual threat (Meacham and Bergstrom, 2016, van Meurs et al., 2017). Hence, extending the concept of anxiety to invertebrates and addressing the causes and triggers motivated the research for my PhD since this knowledge will be crucial in understanding the long-term behavioural effects of

pharmaceutically active compounds and their wider implications on species interactions and survival.

Anxiety-like behaviour (ALB) has been reported in invertebrates like *Drosophila* (Mohammad et al., 2016) and crayfish (Fossat et al., 2014, Fossat et al., 2015, Bacqué-Cazenave et al., 2017). Some previous studies suggested that a functional equivalent of fear exists in invertebrates (Walters et al., 1981, Bateson et al., 2011). For example, aversive conditioning in *Aplysia* enhanced defensive responses, including increased head and siphon withdrawal reflexes, inking, and escape locomotion, while simultaneously suppressing feeding behaviour (Walters et al., 1981). These effects resemble how conditioned fear stimuli in advanced mammals intensify defensive behaviours while inhibiting non-essential actions like eating, indicating an adaptive strategy to prioritize survival when faced with threats (Walters et al., 1981). Similarly to vertebrates, honeybees have been found to exhibit cognitive components of emotion (Bateson et al., 2011). This is evidenced by their pessimistic bias and reduced levels of biogenic amines following periods of agitation (Bateson et al., 2011). These findings suggest that honeybees may experience anxiety-like states, thereby expanding the concept of anxiety beyond just vertebrates (Bateson et al., 2011).

My PhD research focuses on the causes and potential environmental triggers of ALB in crayfish. Fossat et al. (2014) showed that crayfish exhibited light avoidance and limited activity during stress-induced anxiogenic response. Such an effect when translated to the aquatic environment can negatively affect individuals' fitness and survival as stress responses often entail high metabolic costs (Musil et al., 2023). Crayfish naturally tend to explore their surroundings (Fossat et al., 2014). Exploratory drive and enhanced activity are linked to fitness as they facilitate habitat expansion (Galib et al., 2022) and are necessary for foraging (Atkinson et al., 2023, O'hea Miller et al., 2024) and inter- and intra-specific interactions (Galib et al., 2022). Crayfish are keystone species in the aquatic

environment (Weinländer and Füreder, 2016). They are major consumers of leaf litter, biofilms and small benthic invertebrates (Evans-White et al., 2003, Reisinger et al., 2021). Even minor changes in their behaviour can affect essential aquatic processes such as the breakdown of leaf litter, trophic maintenance, and recycling of nutrients (Evans-White et al., 2003, Creed and Reed, 2004, Alp et al., 2016, Reisinger et al., 2021). Hence, it is crucial to understand the long-term effects of anxiety in aquatic ecosystems and how the resulting behavioural alterations may impact community dynamics and ecological balance in the future.

Moreover, this research has broader implications for understanding how stress-induced anxiety can influence behaviour and physiology across diverse animal taxa, paving the way for innovative approaches in neurobiology, behavioural science, and conservation. Investigating anxiety in crayfish can help understand the neurobiological foundations of stress and sentience across this species. Crayfish, as invertebrates with relatively simple neural architectures (Herberholz, 2022), offer a unique opportunity to study the mechanisms underlying anxiety-like behaviours in a model that can help bridge the gap between vertebrates and invertebrates. By exploring how crayfish respond to stress and develop adaptive or maladaptive behaviours, we can gain valuable insights into the universality of emotional states and their impacts on survival, species interactions, and ecological balance.

1.3.1 Role of Serotonin in anxiety-like behaviour among crayfish

The monoamine serotonin (5-HT) is widespread across the animal kingdom (Bacqué-Cazenave et al., 2020). This neuromodulator has been linked to play an important role in various behavioural and cognitive functions among vertebrates and invertebrates (Bacqué-Cazenave et al., 2020). Anxiety is one of the behavioural tendencies that is linked to 5-HT (Fossat et al. 2014, Bacqué-Cazenave et al., 2017). Under stressful conditions, 5-HT mediates a rapid mobilisation of carbohydrate reserves via the

Crustacean Hyperglycaemic Hormone (CHH) (Webster, 1996, Santos et al., 2001, Lorenzo et al., 2005, Aquiloni et al., 2012, Fossat et al., 2015, Rajendran and Vasudevan, 2020). This leads to a rise in haemolymph glucose levels to provide energy for flight and flight response (Lorenzo et al., 2005, Aquiloni et al., 2012, Fossat et al., 2014, Rajendran and Vasudevan, 2020). Stress induces anxiogenic response and 5-HT has been characterized to play a role in these responses (Maximino et al., 2013, Fossat et al., 2014, Fossat et al., 2015). For example, Maximino et al. (2013) observed a positive relationship between 5-HT levels and anxiogenic response in zebrafish. Fossat and co-workers discovered a positive correlation between 5-HT and the intensity of stress and highlighted the involvement of 5-HT in anxiety-like behaviour (ALB) among crayfish (Fossat et al., 2014, Fossat et al., 2015). Crayfish injected with 5-HT exhibited light avoidance, a characteristic of ALB (Fossat et al., 2014). Chlordiazepoxide, an anxiolytic that modulates vertebrate GABA-A receptors, was successful in reversing the anxiogenic response induced by 5-HT (Fossat et al., 2014). This draws parallels with vertebrate anxiety and highlights that 5-HT can trigger ALB among crayfish by interacting with GABA signalling (Fossat et al. 2014).

Crayfish with their well-organised brain structure (Herberholz, 2022), have the potential to be the ideal model organisms for studying ALB and exploring the conserved role of 5-HT in anxiogenic response. Understanding the role of 5-HT in ALB could benefit broader studies on stress, resilience, and behaviour in other species, including humans. It could also provide valuable insights into how environmental stressors impact aquatic ecosystems by influencing the behaviour and survival strategies of key species like crayfish. Hence, exploring the role of 5-HT in crayfish anxiety and investigating if other biogenic amines such as Dopamine (DA), can affect these responses via a possible cross-communication, motivated my PhD research.

1.3.2 Cross-communication among biogenic amines

The effects of biogenic amines are seldom observed in isolation, as these neuromodulators frequently interact in intricate and dynamic manners, thereby influencing one another's functions in the regulation of behaviour and physiological processes (Sánchez-Jiménez et al., 2013, Ibuchi and Nagayama, 2021). Research indicates that intercommunication among biogenic amines, including serotonin (5-HT), dopamine, and octopamine, is integral to the modulation of responses to both stressors and environmental cues (Tricarico and Gherardi, 2007, Ibuchi and Nagayama, 2021). For example, fluctuations in the concentration of one amine can evoke downstream effects on the activity or efficacy of others, culminating in a networked system that regulates a range of behaviours, such as aggression (Ibuchi and Nagayama, 2021). Recent research by Ibuchi and Nagayama revealed that DA has dose-dependent effects on crayfish aggression, with its facilitating influence at higher doses, diminishing when a 5-HT1 receptor antagonist is present (Ibuchi and Nagayama, 2021). This suggests that 5-HT and DA may interact through parallel pathways, indicating potential cross-communication between biogenic amines in aggressive responses (Ibuchi and Nagayama, 2021).

The complex interaction between biogenic amines hints at the possibility that DA may also influence the anxiogenic effects of 5-HT. This potential interaction deserves further investigation, especially concerning anxiety-like behaviour in crayfish. Studying how DA affects 5-HT-induced anxiety responses could uncover new insights into how neuromodulators interact, shaping stress and emotions. This research could enhance our understanding of the fundamental mechanisms of anxiety across different species.

1.3.3 Fluoxetine in freshwater systems: a potential trigger of anxiety

Pharmaceutically Active Compounds (PhACs) have been a growing concern in aquatic ecosystems since their first detection in freshwater environments in the 1970s (Tabak and Bunch, 1970). These compounds enter water bodies predominantly through human and

animal excretion, runoff from medical, agricultural, and industrial activities, as well as insufficiently treated or untreated wastewater (Fent et al., 2006; Santos et al., 2013; Hossain et al., 2019b). Among the various PhACs, those targeting neuroendocrine systems are particularly alarming due to their potential to disrupt the physiology and behaviour of aquatic organisms.

Selective Serotonin Reuptake Inhibitors (SSRIs) are a widely prescribed class of PhACs (Hossain et al., 2019b). SSRIs target the neuromodulator serotonin (Hossain et al., 2019b). They function by binding to pre-synaptic reuptake transport proteins, thereby preventing serotonin (5-HT) reabsorption in the synaptic clefts and increasing its concentration in the haemolymph (Kreke and Dietrich, 2008; Ford and Fong, 2016; McDonald, 2017; Kellner et al., 2018; Venkatachalam et al., 2023).

Fluoxetine, commonly marketed as Prozac®, is one of the most frequently prescribed SSRIs (Henry et al., 2004; Hamilton et al., 2016; Kubec et al., 2019; Hossain et al., 2019b). Like other SSRIs, Fluoxetine elevates 5-HT levels in the haemolymph (Hossain et al., 2019a). Chronic exposure to Fluoxetine can disrupt the serotonergic system by altering 5-HT release, modifying 5-HT receptor gene expression, and affecting the function of other neurotransmitters (Theodoridi et al., 2017; Cunha et al., 2018; Yamindago et al., 2021). Its widespread use has made Fluoxetine one of the most prevalent SSRI contaminants in aquatic environments, with concentrations typically ranging from 0.012 to 1.4 μg/L in freshwater systems (Webb, 1999; Christensen et al., 2009).

As discussed previously, 5-HT has been found to influence anxiety responses in both vertebrates and invertebrates (Bonhomme and Esposito, 1999, Lillesaar 2011, Fossat et al., 2014, Fossat et al., 2015, Tu et al., 2020). Zebrafish anxiogenic response was found to be proportionally related to the level of 5-HT (Maximino et al., 2013). Crayfish subjected to a high-intensity electric field were found to have elevated brain 5-HT levels, and exogenous 5-HT injection into crayfish led to anxiety-like behaviour (ALB) (Fossat

et al., 2015). The anxiolytic drug, chlordiazepoxide, reversed the effect (Fossat et al., 2014).

Hence, exposure to contaminants like Fluoxetine which target 5-HT, can ensue behavioural alterations among non-target aquatic organisms such as crayfish. Since Fluoxetine works by causing a rise in 5-HT levels, the exposure was observed to cause an upregulation of CHH, leading to a rise in haemolymph glucose levels in two species of decapod crustaceans, *Chasmagnatus granulate* and *Orconectes limosus* (Santos et al., 2001). It was also found to affect anxiety-like behaviour among crabs (Hamilton et al., 2016). Potential maladaptive anxiety induced by exposure to SSRIs like Fluoxetine, even in the absence of a stressor can affect exploratory drive (Galib et al., 2022), foraging, inter- and intra-specific interactions and species survival in the long-term (Atkinson et al., 2023, O'hea Miller et al., 2024). Given the significant role of crayfish as ecosystem engineers, even minor behaviour alterations can have far-reaching negative effects, potentially impacting nutrient cycling, decomposition of leaf litter, and population dynamics (Evans-White et al., 2003, Creed and Reed, 2004, Alp et al., 2016, Reisinger et al., 2021).

Studying the effects of Fluoxetine on anxiety-like behaviour in crayfish is crucial because it provides insights into how pharmaceutical pollutants influence non-target organisms at both individual and ecosystem levels. Understanding how exposure to SSRIs like Fluoxetine alters behaviour can reveal the broader ecological consequences of pharmaceutical contamination, shedding light on potential disruptions to species interactions and ecosystem functionality. Such research is essential for informing conservation strategies and establishing guidelines to mitigate the environmental impacts of these contaminants.

1.4 Acidification of freshwater bodies: an emerging environmental threat

The acidification of freshwater bodies is a significant environmental concern that threatens aquatic ecosystems globally. This process is primarily driven by anthropogenic factors, with acid rain being one of the most critical contributors (Psenner, 1994). Acid rain results from elevated concentrations of sulfur and nitrogen oxides emitted from industrial activities, residential areas, and vehicles (Galloway et al., 1983, Psenner, 1994). These oxides interact with atmospheric moisture to form sulfuric and nitric acids, precipitating as acid rain, and reducing pH levels in freshwater systems (Psenner, 1994).

In addition to atmospheric acid deposition, agricultural runoff containing chemical fertilizers, pesticides, and insecticides further exacerbates the acidification of freshwater environments (Henry et al., 2013). Prolonged exposure to these conditions diminishes the capacity of aquatic ecosystems to neutralize acids, rendering them increasingly susceptible to changes in pH (Leduc et al., 2013). Future projections suggest that rising levels of atmospheric CO_2 will continue to lower freshwater pH, compounding the issue of acidification (Weiss et al., 2018).

One of the most critical implications of freshwater acidification is its potential to interfere with chemosensory communication among aquatic organisms (Tierney and Atema, 1987, Allison et al., 1992, Cothran et al., 2021). In poorly illuminated aquatic habitats, animals rely heavily on chemical signals to perceive and respond to their environment (Dodson et al., 1994, Franklin et al., 2018, Breithaupt et al. 2016). Disruptions to chemosensory cues can significantly affect essential decision-making processes, including mating, foraging, habitat selection, and predator detection (Breithaupt et al. 2016). Consequently, alterations in olfactory-mediated responses can have profound impacts on species interactions, ecological dynamics, and the life histories of numerous aquatic organisms (Leduc et al., 2013).

1.4.1 Predatory Information Cues in Aquatic Ecosystems

Aquatic organisms utilize a myriad of cues—including chemical, mechanical, visual, and auditory signals—to navigate their environments and assess the risk of predation (Breithaupt and Thiel, 2011, Lukas et al., 2021). For example, bird predation is a common occurrence in freshwater fish *Poecilia sulphuraria* (Lukas et al., 2021). The fish employs a multi-sensory strategy involving visual and acoustic cues to gauge imminent risk and seek refuge in deeper waters (Lukas et al., 2021). However, when it comes to navigating in the often murky and dimly lit aquatic environments, visual cues are of limited use (Dodson et al., 1994, Franklin et al., 2018). In this environment, mechano- and chemosensory cues and signals facilitate the survival of the animal (Breithaupt et al. 2016).

Chemosensory cues in aquatic environments are composed of various chemical signals emitted by predators, conspecifics, and prey (Breithaupt et al. 2016). Many species of fish within the superorder Ostariophysi, such as minnows, zebrafish and catfish, possess specialized epidermal alarm cells that release alarm substances upon skin rupture due to predation (Korpi and Wisenden, 2001; Chivers et al., 2007, Halbgewachs et al., 2009). These chemical cues then diffuse through the water and bind to olfactory receptors in conspecifics, thereby alerting them of the presence of predators (Hintz et al., 2017). Additionally, predators may release digestive cues after consuming a conspecific, which naïve fish can recognize and respond to (Sutrinso et al., 2013). For example, naïve minnows recognized and reacted to ancestral and derived predators fed on minnow diets (Sutrinso et al., 2013).

Similarly, crustaceans such as spiny lobsters, crayfish, and hermit crabs also utilize haemolymph from freshly injured conspecifics (alarm odour) as chemosensory signals indicating predation risk (Hazlett, 1994; Hazlett and McLay, 2005; Hazlett, 2007; Briones-Fourzán et al., 2008). Upon detecting these alarm cues, aquatic animals deploy

various anti-predatory strategies, including hiding, burrowing, or locomoting away (Snyder and Snyder, 1970, Zimmer-Faust et al., 1985, Hazlett, 1994, Jacobsen and Stabell, 2004; Fleming et al., 2007, Shabani et al., 2008).

Upon successful detection of these alarm cues, animals employ defensive anti-predatory strategies to safeguard themselves. These include hiding, burrowing, and locomoting away Hazlett 1994, Jacobsen and Stabell 2004, Fleming, Muller et al. 2007, Shabani, Kamio et al. 2008), crypsis and aggregation (in fish) (Smith, 1993), and sea anemone tentacle retraction Howe and Sheikh, 1975, Howe, 1976)

1.4.2 pH-Mediated Olfactory Disruption

Since aquatic animals largely rely on chemosensory signals to perceive and react to their environment (Breithaupt and Theil, 2011), any changes that could potentially disrupt their transmission and detection can have a negative impact. Anthropogenic acidification of freshwater bodies is one such risk factor. Previous studies have demonstrated that under weakly acidic conditions, the ability of freshwater fish to detect alarm cues could be compromised (Leduc et al., 2003). The adaptive anti-predatory response to conspecific and heterospecific alarm cues was found to be significantly diminished under mild acidic conditions (pH6) in juvenile pumpkinseed *Lepomis gibbosus* (Leduc et al., 2003). Similar observations were made in the case of fathead minnows (*Pimephales promelas*) and finescale dace (*Phoxinus neogaeus*) (Brown et al., 2002). The two species failed to respond to natural and artificial alarm pheromones when exposed to low pH (Brown et al., 2002).

Under field conditions, experiments have demonstrated an episodic loss of chemosensory abilities under mildly acidic conditions (Leduc et al., 2009). Brook trout (*Salvelinus fontinalis*) (Leduc et al., 2013) and Atlantic salmon *(Salmo Salar)* (Leduc et al., 2009, Elvidge et al., 2012) did not effectively respond to conspecific alarm signals in weakly acidic streams.

pH-mediated disruption of communication in aquatic environments was also found to interfere with anti-predatory responses in freshwater snails (Cothran et al., 2021). It can also impact feeding behaviour thereby implying serious ecological consequences on population dynamics and trophic structure. For instance, a significant loss of responsiveness to basic food stimuli was observed under low pH conditions in fathead minnows (*Pimephales promelas*) (Dennis Lemly and Smith, 1985), Atlantic salmon (*Salmo salar*) (Royce-Malmgren and Watson, 1987), and crayfishes *Faxonius virilis*, *Procambarus acutus* (Tierney and Atema, 1986, Tierney and Atema, 1987) and *Cambrus baroni* (Allison et al., 1992). These experiments emphasize that chemosensory responses in aquatic animals are highly sensitive to pH and even small fluctuations can have dramatic effects.

1.4.3 Mechanism of pH-based olfactory disruption

There are a few possible mechanisms which could explain the pH-dependent loss of chemosensory abilities. One possibility by which pH changes could interrupt olfactory ability is by altering the molecular structure of alarm odour. For instance, earlier research in Ostariophysan fish indicated Hypoxanthine-3(N)-oxide (H3NO), a purine derivative, as the active ingredient of epidermal alarm cells (Chivers et al., 2007). Further research confirmed the ability of this compound to induce alarm response (Pfeiffer et al., Brown et al., 2000). Information from analytical chemistry research suggests that under acidic conditions, this compound undergoes a molecular change and is converted to 6,8-dioxypurine with a loss of the 3-*N*-oxide functional group (Woelcke and Brown, 1969, Kawashima and Kumashiro, 2006, Leduc et al. 2013). Brown et al. (2012) observed strong anti-predatory behaviour when minnows were exposed to untreated H3NO and conspecific skin extract. However, when H3NO and untreated skin extract were acidified to pH6 followed by rebuffering to pH7.5, it failed to elicit a response (Brown et al., 2002). This indicates that low pH leads to an irreversible covalent modification in the alarm

substance which cannot be restored by buffering back to normal pH (Brown et al., 2002). This irreversible change in the chemical constitution of the active compound at low pH could render the alarm odour ineffective or non-functional (Brown et al., 2002). However, the lost anti-predatory response among Atlantic salmon in under acidified conditions was regained when normal pH (pH7.5) was restored (Antoine et al., 2006, Elvidge et al., 2012). The experimental observations indicate that exposure to low pH for a short period does not lead to permanent olfactory impairment. Thus, under low pH conditions, alarm molecules undergo chemical changes that either render them ineffective or cause the concentration of the active ingredient to fall below the threshold required to elicit an anti-predatory response (Leduc et al., 2013).

Another explanation for diminished response to chemical cues is pH-mediated physiological disruption of olfactory receptors. Research on fish demonstrates that pH changes alter the state of ionisation of protein receptor sites (Tierney and Atema 1988, Moore 1994) This can affect the binding affinity of the receptor sites and render them incapable of binding to the alarm molecules (Tierney and Atema 1988). This would result in diminished anti-predatory response due to neurological change in stimulus-receptor binding (Tierney and Atema 1988, Moore 1994).

Acidification can also impact central nervous processing and affect chemosensory abilities. The mechanism for pH-dependent chemosensory impairment in freshwater and marine fish has been linked to GABA-A signalling. Acidification can disrupt normal Cl^- and/or HCO_3^- gradients over neuronal membranes, leading to depolarisation of some GABA-A receptors (Nilsson et al., 2012). This ionic imbalance during high CO2 exposure (low pH) causes putative changes in the brain leading to dramatic shifts in chemosensory abilities (Nilsson et al., 2012). Disruption of GABAergic neurotransmission when exposed to near-future CO_2 levels has been accounted for sensory impairment in both freshwater fish like three-spined sticklebacks (Lai et al., 2016)

as well as marine fish such as larval coral reef fish (Nilsson et.al., 2012). It was also linked to increased anxiety response in Rockfish (Hamilton et al., 2014).

1.4.4 Future implications of freshwater acidification

It is important to understand the effects of water acidification on aquatic ecosystems to develop effective management strategies for the conservation of aquatic organisms in the future. Low freshwater pH can impact species interactions and interspecific competition thereby potentially causing an imbalance in aquatic community dynamics. It can affect crucial behavioural responses which depend upon the transmission of chemical cues like anti-predatory behaviour and hence threaten the survival of species in the projected future (Leduc et al., 2013). Ineffective response to predatory cues can cause a significant rise in prey mortality rates, potentially disturbing population dynamics. For instance, in staged encounters, the trout predation rate by largemouth bass was significantly higher when trout alarm odour was introduced at low pH (pH6) than at neutral pH (Leduc et al., 2009). In future scenarios, acidification can lead to naïve prey due to lack of conditioning with alarm odour and hence lead to a greater predation cost to prey populations (Leduc et al., 2013). pH-mediated disruption of communication in aquatic environments was also found to interfere with anti-predatory responses in freshwater snails (Cothran et al., 2021).

In addition to predator-prey interactions, acidification has also been shown to affect a range of fitness and survival-enhancing activities. Freshwater acidification was also found to interfere with acid-base homeostasis in the Chinese mitten crab *Eriocheir sinensis* and crayfish *Faxonius virilis*, which can in turn adversely impact calcification, metabolic efficiency and ethology thereby potentially threatening survival in future (Quijada-Rodriguez et al., 2021, Malley 1980). It can also impact feeding behaviour thereby implying serious ecological consequences on population dynamics and trophic structure (Dangles and Guerold, 1999, Ninokawa and Ries, 2022). Exposure to weakly

acidic conditions has diminished spawning, upstream migration, and nesting behaviour in sockeye Salmon *Oncorhynchus nerka* (Kitamura and Ikuta, 2000, Ikuta et al., 2003).

Hence, lowering of freshwater pH can affect important behavioural tendencies mediated via chemosensory signalling. This can potentially interfere with life history traits, trophic maintenance, population structure, and ecological roles of species, thereby raising ecological concerns (Tierney and Atema, 1987, Leduc et al., 2013). In summary, the interplay between anthropogenic acidification and chemical communication in aquatic ecosystems reveals critical vulnerabilities. Understanding these dynamics is essential for developing strategies to mitigate the impacts of environmental changes on aquatic organisms and their habitats.

1.5 Research aims and approach

Many studies have focused on the role of biogenic amines in modulating behaviour, including their involvement in stress responses and predator-prey dynamics. This work seeks to adopt an interdisciplinary approach by integrating behavioural neuroscience with biochemical and ecological perspectives to understand the mechanisms underlying anxiety-related behaviours and their modulation by biogenic amines. Additionally, the work investigates how environmental factors, such as acidification, affect anti-predatory behaviour independent of anxiety and serotonin pathways.

The overarching aim of this thesis is to explore the complex interplay between biogenic amines and behaviour, with a specific focus on anxiety, pharmacological pollution, and the effects of environmental changes.

This PhD thesis is structured into three data chapters, each presenting a background introduction, specific research questions, detailed methods, and results followed by a discussion. While Chapters 2 and 3 delve into the neurochemical causes and

pharmacological underpinnings of anxiety, Chapter 4 broadens the scope to examine environmental effects on behaviour beyond neurochemical pathways.

Chapter 2: Role of biogenic amines Serotonin and Dopamine in anxiety-like behaviour of crayfish *Pontastacus leptodactylus*

The first data chapter investigates the role of the biogenic amines serotonin and dopamine in anxiety-like behaviour in crayfish and their potential crosstalk in shaping these responses. Behavioural assays are used to quantify anxiety-related behaviours in crayfish models under different treatment conditions. The injection of biogenic amines and their blockers in different combinations and concentrations allows for the assessment of specific amines and their interactions as well as cross-communication. By exploring how biogenic amines interact to influence anxiety, this chapter aims to provide a mechanistic understanding of their role in behavioural regulation, which sets the stage for pharmacological interventions in later chapters.

Chapter 3: Effect of Fluoxetine on the anxiety-like behaviour in crayfish *Pontastacus leptodactylus*

The second data chapter examines the effects of fluoxetine, a selective serotonin reuptake inhibitor (SSRI), on anxiety-like behaviour (ALB) on crayfish. It also investigates if the effect is translated on a biochemical level by measuring haemolymph glucose concentration. This chapter builds on findings from Chapter 1 to explore how modulating serotonin levels with fluoxetine can potentially alter anxiety responses. Behavioural and physiological assessments are performed for different doses of Fluoxetine to establish a dose-related correlation with ALB. This chapter offers insights into the effect of pharmaceutical pollution of aquatic ecosystems and its effect on anxiety responses for broader ecological or evolutionary implications.

Chapter 4: Acidification and Its Effects on Anti-Predatory Behaviour

The third data chapter shifts focus to the impact of environmental acidification on anti-predatory behaviour, independent of anxiety or serotonin pathways. Using crayfish as a model aquatic species, this chapter investigates how changes in environmental pH influence predator-prey interactions and survival strategies. Behavioural assays are designed to assess responses to predator cues under varying acidification scenarios. A physiological assay based on the measurement of haemolymph glucose levels is used to quantify stress response. Mechanistic studies explore whether acidification alters chemical signalling pathways or sensory perception, providing insights into how organisms adapt to changing environmental conditions. The independence from anxiety and serotonin pathways is explicitly addressed, highlighting the distinct focus of this chapter.

Chapter 2 Role of biogenic amines Serotonin and Dopamine on anxiety-like behaviour in crayfish *Pontastacus leptodactylus*

2.1 Abstract

Biogenic amines are widely known to influence behaviour among invertebrates. In this study, I investigated the role of two biogenic amines, serotonin (5-HT) and dopamine (DA) in crayfish's anxiety-like behaviour (ALB). Previous studies have indicated the involvement of 5-HT in ALB, but the role of DA is still unclear. I analysed crayfish behaviour in a plus-maze arena having light (70lux) and dark (10lux) zones. The time spent and active speed in the two zones were used as behavioural indicators. Animals displaying ALB are expected to spend more time with low active speeds in the dark zones. Serotonin injection triggered ALB at the two concentrations tested (1µM and 10µM) while dopamine appeared to have a dose-dependent effect with only 10µM concentration leading to ALB. The anxiogenic effect of 5-HT and 10µM DA disappeared when administered together with either 5-HT1 receptor antagonists (50µM maleate salt) or DA blocker (10µM methylergonovine or methergine) suggesting that a parallel pathway could be involved in 5-HT and DA activation. Surprisingly, co-injection of 1µM DA with DA blockers led to ALB rather than to the bold behaviour displayed in all other blocker administrations. In contrast, co-administration of 1µM DA with 5-HT1 blocker did not have an effect which could be a result of different receptor sites being responsive in the pathway. Overall, our results emphasize the involvement of a complex functional interplay among biogenic amines in anxiety-like behaviour in crayfish with blockers showing effects on more than one biogenic amine.

2.2 Introduction

Encounters with dangerous situations occur very frequently in the living world. Hence, all living organisms can develop coping strategies against stressful and potentially threatening situations. Prolonged encounters with danger can facilitate the development of long-term behavioural adaptation (Fossat et al., 2015). This can result in a sustained state of alertness that persists even in the absence of stressors. It is elicited by previous encounters with dangerous situations (Fossat et al., 2015). This state of sustained apprehension is called anxiety and is a behavioural outcome of stress (Fossat et al., 2015). Anxiety has been well-characterized in humans (Graeff et al., 2003, Grillon et al., 2007, Siepmann and Joraschky, 2007, Andreatta et al., 2015, Marcinkiewcz et al. 2016) and some vertebrates like Zebrafish (Egan et al., 2009, Maximino et al., 2010, Blaser and Rosemberg, 2012). However, there is still a very limited understanding of anxiety in invertebrates. Recent studies have shown that crayfish exhibit anxiety-like behaviour in stressful situations (Fossat et al., 2014, Fossat et al., 2015).

Biogenic amines especially serotonin (5-HT) and dopamine (DA) have been found to influence anxiety responses in mammals (Bonhomme and Esposito, 1998, Graeff et al., 2003, Mosienko et al., 2012, Yu et al., 2014, Zangrossi and Graeff, 2014, Hjorth et al., 2021). In crayfish and other decapod crustaceans, 5-HT has been shown to play an important role in the coordination of stress responses (Huber et al., 1997, Huber and Delago, 1998, Teshiba et al., 2001, Fossat et al., 2014, Bacque-Cazenave et al., 2017). 5-HT influences stress by upregulating crustacean hyperglycemic hormone (CCH) leading to a heightened haemolymph glucose level (Webster, 1996, Santos et al., 2001, Lorenzon et al., 2005, Fossat et al., 2014, Rajendran and Vasudean, 2020) whereas DA can have the opposite effect (Sarojini et al., 1995). However, DA has also been observed to elicit a hyperglycemic effect in tiger shrimp (Kuo et al, 1995). As such, the literature provides

some conflicting observations concerning the role of DA which needs to be further investigated.

In crayfish, 5-HT has been shown to trigger anxiety-like behaviour (ALB) (Fossat et al., 2014, Fossat et al., 2015). Crayfish display anxiety-like behaviour after a previous encounter with a strong aversive stimulus (electric shock) by remaining preferentially in dark arms and avoiding illuminated arms of the plus-maze behaviour arena (Fossat et al., 2014). The stressed animals were found to exhibit higher brain 5-HT levels. The behaviour was abolished when animals were injected with chlordiazepoxide (CDZ), a drug used to treat human anxiety (Fossat et al., 2015). 5-HT injection mimicked the effect of the aversive stimulus by causing ALB that was abolished by CDZ (Fossat et al., 2015). In contrast, DA injection did not elicit anxiety-like behaviour in crayfish (Fossat et al., 2015).

5-HT has been associated with aggressive motivation in decapod crustaceans including crayfish (Huber et al., 1997). Panksepp et al. (2003) observed that acute serotonin treatment in crayfish alters the decision-making underlying retreat, enabling subordinate animals to persist in risky, prolonged fights against larger opponents. Injection of 5-HT into subordinate crayfish led to prolonged and more intense fights with larger opponents, characterized by a reduced tendency to retreat. This effect was not observed with saline controls and persisted for a long period, suggesting that 5-HT might lead to a lasting alteration in retreat decisions.

In recent investigations by Ibuchi and Nagayama (Ibuchi and Nagayama, 2021), DA was found to exert dose-dependent opposing effects on crayfish aggression. The facilitating effect of DA on agonistic encounters was found to disappear when injected with 5-HT1 receptor antagonist. This suggests that 5-HT and DA activate each other through parallel pathways. It also points to the possibility of cross-communication between biogenic amines where one pathway can be influenced by the other in orchestrating stress

responses. Thus, there is a possibility that DA can also impact the anxiogenic effects of serotonin in crayfish in a dose-dependent manner. This needs to be further investigated.

The first aim of this study is to understand the roles of 5-HT and DA in ALB in crayfish *Pontastacus leptodactylus*. For this purpose, I subjected the animals to exogenous 5-HT and DA injections. The bioamine injections were administered at two different concentrations each (1µM and 10µM) to assess if there is a dose-dependent effect of 5-HT and DA on ALB responses. Additionally, I used 5-HT and DA blockers (maleate salt as a 5-HT1 receptor antagonist and methylergonovine (methergine) as a nonspecific DA antagonist) to verify if any behavioural effects are caused by the specific bioamine pathways. Lastly, I employed a cross-combination of blockers to assess the possibility of cross-communication and parallel pathways of activation between the two biogenic amines.

2.3 Materials and methods

2.3.1 Experimental animals

150 naïve male Turkish crayfish (*Pontastacus leptodactylus*) individuals were selected from the aquarium culture and used in the study. The animals were sourced from South Norwood Lake, London, England (Crayaway crayfish removals, Bob Ring). 25 crayfish that moulted during the study were removed from the experimental trials. The animals used in the study had mean body weight and postorbital carapace length with heir standard deviation respectively being 50.35 ± 10 g and 41.9 ± 8.52 mm. There was no statistically significant difference in the weight (see Table 2.1) and carapace length (see Table 2.2) between the animals in different experimental groups.

Table 2.1: Summary of weight across different groups

Group	Weight ± sd	Comparison	Tukey HSD p adj.
Control	48.19 ± 10.92	Dopamine-Control	0.86
Serotonin	49.78 ± 8.73	Serotonin-Control	0.61
Dopamine	51.19 ± 10.89	Serotonin-Dopamine	0.74

Table 2.2: Summary of carapace length across different groups

Group	Length ± sd	Comparison	Tukey HSD p adj.
Control	38.81 ± 9.57	Dopamine-Control	0.72
Serotonin	40.85 ± 7.84	Serotonin-Control	0.97
Dopamine	39.42 ± 8.95	Serotonin-Dopamine	0.65

Before behaviour testing, crayfish were isolated for 7 days at 14°C in individual 1.5L Tupperware boxes with a 12:12 hour light: dark cycle. Crayfish are nocturnal animals; hence experiments were performed during their normal active times. Each such box was fitted with a flow-through siphoning system to eliminate any manual interferences during water changes which were performed every 24 hours. The isolation room was temperature controlled electronically to avoid any fluctuations in temperature during the isolation period as well as during behaviour trials. The animals were fed cooked prawns (1

medium-sized prawn per animal) a day before isolation and no feeding was done during the isolation period. An opaque cotton sheet (4mx4m) was hung on the frame in front of the isolation set-up to avoid any visual disturbance to isolated animals from outside.

2.3.2 Behaviour arena

The arena for behaviour trials comprised of a plus maze- a 63.5x63.5 cm square tank with arms of 23 cm length and 12 cm width and a central buffer zone of 12 cm length and 12 cm width with alternate arms in the shape of symbol '+' (Figure 2.1). Its two opposite arms received illumination from power-driven white LED strip lights running along the edges 1/3rd from the top of each light arm, to give the required illumination of 70lx in the light zone. Power-operated red LEDs were fixed vertically at the ends of two opposite arms (dark zone) and were wrapped with a single layer of translucent diffusing sheet to produce 10lx illumination in the dark zone. Light intensity was monitored before each trial using lux meters (Hanna instruments HI 97500). The maze was filled with 12 litres of demineralised water at pH8 maintained uniformly at 14°C throughout the trial. All the water in the maze was renewed after each trial.

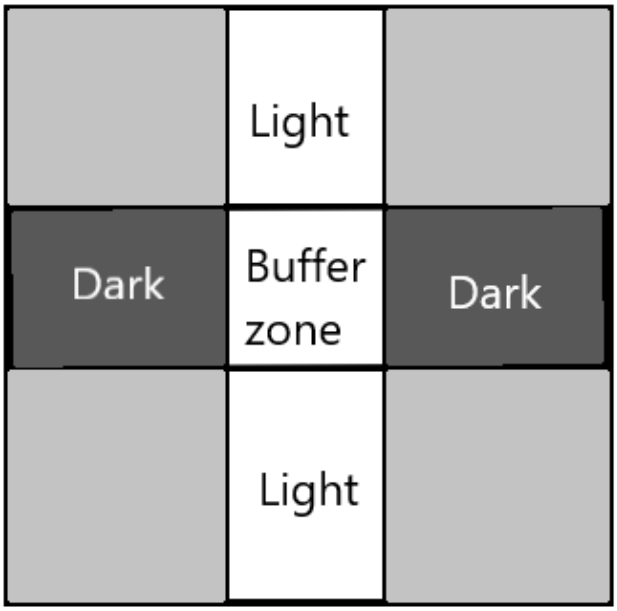


Figure 2.1: Plus-maze arena for behaviour investigation

2.3.3 Drug treatments

Serotonin creatinine sulphate monohydrate (5-HT), Dopamine hydrochloride (DA) and their blockers WAY-100635 maleate salt (WAY100635) as serotonin 5-HT1 receptor antagonist and methylergonovine (methergine) as non-specific DA receptor antagonist, were acquired from Sigma Aldrich (St. Louis, MO, USA) and dissolved in physiological saline (Van Harreveld, 1936) to obtain the desired concentration.

Experimental concentrations used by Ibuchi and Nagayama (Ibuchi and Nagayama, 2021) were used for drug injections (1µM and 10µM 5-HT, 1µM and 10µM DA, 50µM maleate salt and 10µM methergine).

2.3.4 Injection procedure

Physiological saline (control) and drugs dissolved in physiological saline were injected at a dose of 10 µg/g body weight (Berry and Breithaupt, 2010), into the pericardial sinus of crayfish without damaging the underlying heart. Injections were performed using a 250µL Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a 26-gauge needle (Hamilton Bonaduz, Bonaduz, Switzerland). Immediately after injection, the hole created by the injection needle was sealed using modelling clay to prevent haemolymph loss. Post injection, each animal was given a 15-minute adjustment time in its respective Tupperware box before introduction into the behaviour arena. All injections were performed in dim light conditions to minimize disturbance.

2.3.5 Behaviour testing

10-minutes after the injection, the animals were introduced to the plus maze arena. This process was also carried out under low-light conditions to keep disturbance to a minimum. Behaviour was recorded for 16 minutes from the point of introduction using a digital camera (Panasonic HC-V380, Panasonic, Japan) attached approximately 100cm above the arena.

2.3.6 Behaviour tracking

Lolitrack 5 (Loligo systems, Denmark) behavioural tracking software was used to analyse crayfish behaviour and time spent and active speed (Distance covered/Active time) in dark and light arms.

2.3.7 Statistical analysis

Statistical analysis was performed using RStudio programming software (R 4.3.0 version 1.4.1106, 2009-2021 RStudio, PBC, USA). One-way ANOVA followed by post hoc Tukey HSD was used where data were normally distributed and the Wilcoxon Rank sum test with continuity correction for non-normal distribution with Bonferroni corrected p values (Legendre and Legendre, 1998). $p < 0.05$ was considered significant.

2.3.8 Treatment groups

Table 2.3: Treatment groups and group sizes

Group	**Number of animals**
Control (saline-injected)	13
5-HT 1 µM	11
5-HT 10 µM	13
5-HT 10 µM + 5-HT blocker	10
5-HT 10 µM + DA blocker	10
DA 1 µM	14
DA 10 µM	14
DA 10 µM + DA blocker	10
DA 10 µM + 5-HT blocker	10
DA 1 µM + DA blocker	10
DA 1 µM + 5-HT blocker	10

2.4 Results

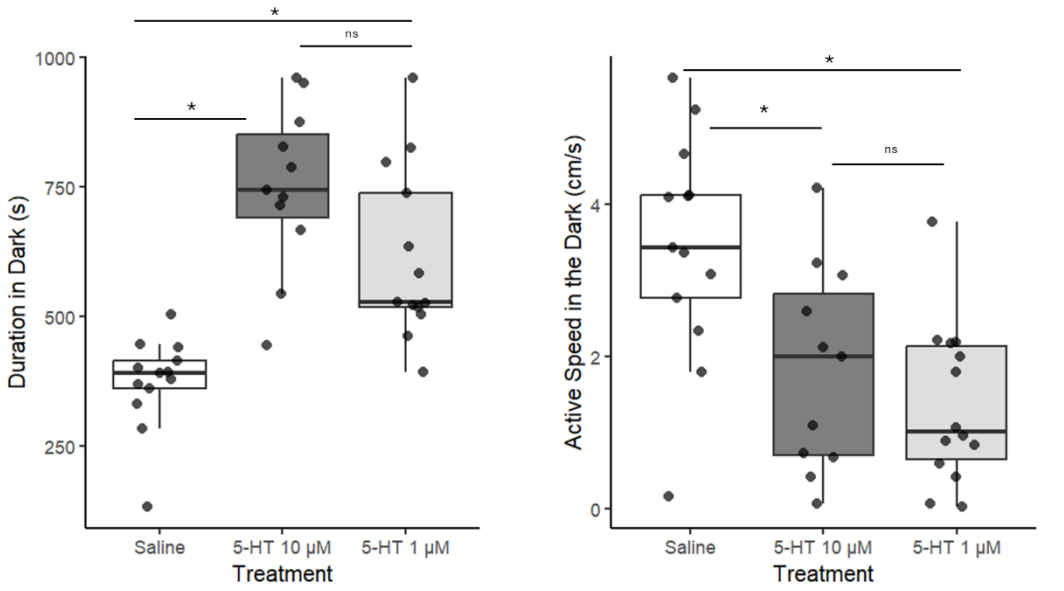
2.4.1 Effect of Serotonin (5-HT) injection on anxiety-like behaviour (ALB)

The results show a clear contrast in the behavioural responses between saline-injected (control) and 5-HT injected groups (5-HT 1µM and 10µM).

In contrast to the control group, exogenous serotonin at 10µM concentration resulted in the animals spending more time ($p < 0.05$, ANOVA, Tukey HSD, Figure 2.2a) with a slow active speed ($p < 0.05$, ANOVA, Tukey HSD, Figure 2.2b) in the dark zone.

Compared to the control group, animals injected with 5-HT 1µM, spent more time in the dark zone ($p < 0.05$, ANOVA, Tukey HSD, Figure 2.2a) and low active speeds ($p < 0.05$, ANOVA, Tukey HSD, Figure 2.2b).

5-HT led to the appearance of ALB at both tested concentrations and no differences were observed when comparing duration ($p = 0.07$, ANOVA, Tukey HSD, Figure 2.2a) and active speed ($p = 0.63$, ANOVA, Tukey HSD, Figure 2.2b) in the dark between 1µM and 10µM 5-HT.



a) b)

Figure 2.2: Duration and (a) Active speed (b) in the dark zone for saline (control), 1 µM 5-HT and 10 µM 5-HT injections. Boxplots display each treatment group's median, interquartile range (IQR), and data range. p-values from one-way ANOVA with Tukey HSD post-hoc comparisons are reported in the graphs. Significance is indicated by '*' for $p < 0.05$.

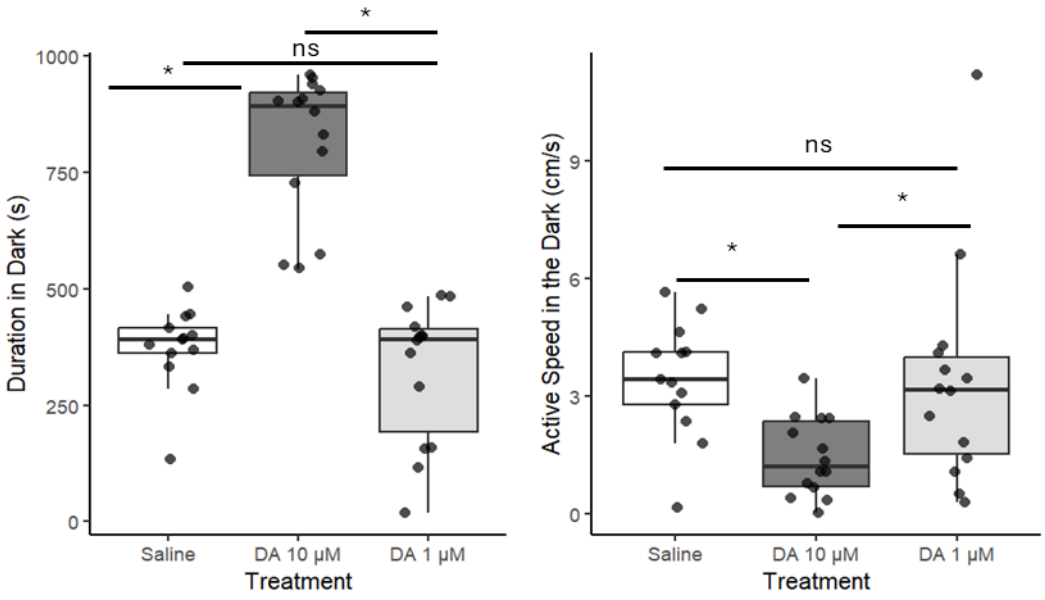
2.4.2 Effect of Dopamine injection on anxiety-like behaviour (ALB)

Like 5-HT treatment, I administered two doses of Dopamine (DA, 1µM and 10µM) to study its role in ALB and dose-dependent effects, if any. The results point towards a dose-dependent role of DA on anxiety responses in crayfish. Dopamine at a concentration of 10µM appears to have similar effects to 5-HT leading to ALB in crayfish.

Compared to the control group, animals injected with the 10µM dopamine dose spent significantly more time in the dark zone ($p < 0.05$, Wilcoxon rank-sum test, Bonferroni-corrected, see Figure 2.3a), and displayed significantly lower active speed ($p = 0.005$, Wilcoxon rank-sum test, Bonferroni-corrected, see Figure 3b).

The 1μM dopamine dose did not lead to any significant difference in either dark zone duration ($p = 1.0$, Wilcoxon rank-sum test, Bonferroni-corrected, see Figure 2.3a) or active speed ($p = 1.0$, Wilcoxon rank-sum test, Bonferroni-corrected, see Figure 3b) compared to the control group.

In contrast, animals injected with the 10μM dopamine dose spent significantly more time in the dark zone than those injected with the 1μM dopamine dose ($p = 0.021$, Wilcoxon rank-sum test, Bonferroni-corrected, see Figure 2.3a). For active speed, the 10μM dopamine dose showed a trend toward lower active speed compared to those injected with the 1μM dose ($p = 0.0570$, Wilcoxon rank-sum test, Bonferroni-corrected, see Figure 2.3b). However, this difference did not reach statistical significance after Bonferroni correction.



a) b)

Figure 2.3: Duration (a) and Active speed (b) in the dark zone for saline (control), 1 µM 5-HT and 10 µM DA injections. Boxplots display each treatment group's median, interquartile range (IQR), and data range. The graphs report the Wilcoxon rank sum test with Bonferroni adjusted p-value comparisons. Significance is indicated by '*' for $p < 0.05$.

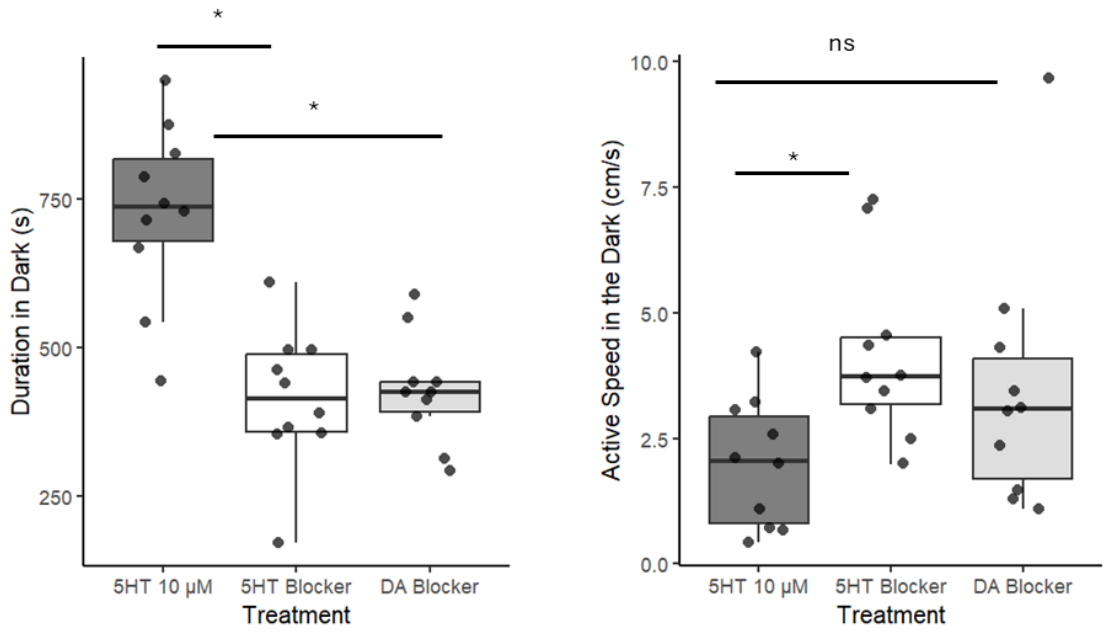
2.4.3 Effect of Serotonin (5-HT) and Serotonin blocker (maleate salt) co-injection

Since I did not find a statistically significant difference between the ALB response facilitated by 5-HT at the two varying concentrations (1µM and 10µM) and the effect appeared to be slightly more pronounced for the higher concentration of 5-HT, I decided to test the effect of blocker with 10µM 5-HT. Maleate salt when injected with 10µM 5-HT, was able to block ALB previously displayed when serotonin was administered alone. The duration in the dark was significantly shorter in the presence of combined 10µM 5HT and 5HT1 blocker injection compared to 10µM 5-HT injected animals ($p = 0.0000118$, Tukey HSD, ANOVA, Figure 2.4a). Similarly, active speed in the dark was significantly

increased in the presence of the blocker compared to sole serotonin injection (p = 0.024, Wilcoxon rank-sum test, Bonferroni corrected).

2.4.4 Effect of Serotonin 5-HT and Dopamine (DA) blocker (methergine) co-injection

There was a statistically significant difference with a drop in the duration and a rise in the active speed in dark zones when 10µM 5-HT was co-injected with 10µM methergine than when it was administered alone, p = 0.0000225, ANOVA, Tukey HSD (duration in dark, Figure 2.4a) and p = 0.3368, Wilcoxon rank-sum test, Bonferroni-corrected (active speed in dark, Figure 2.4b)) for 10µM 5-HT vs 10µM 5HT- maleate salt co-injection.



a) b)

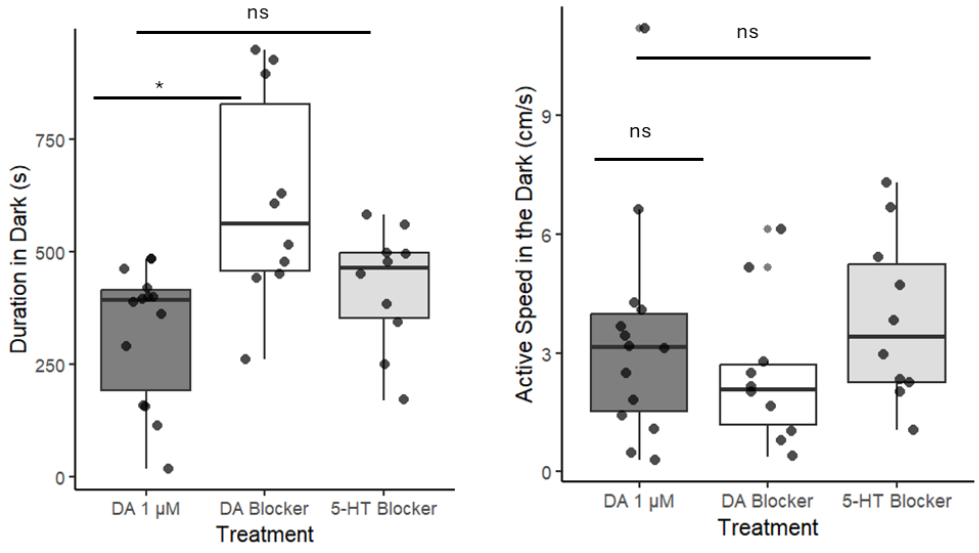
Figure 2.4: Duration (a) and Active speed (b) in the dark zone for 10 µM 5-HT injection vs 10 µM 5-HT- 5HT blocker and 10 µM 5-HT- DA blocker co-injections. Boxplots display each treatment group's median, interquartile range (IQR), and data range. p-values from one-way ANOVA with Tukey HSD posthoc and Wilcoxon rank sum test with Bonferroni adjusted p-value comparisons are reported in the graphs. Significance is indicated by '*' for $p < 0.05$.

2.4.5 Effect of Dopamine 1µM and Serotonin blocker (maleate salt) vs Dopamine blocker (methergine) co-injection

To understand the dose-dependent effect and crosstalk between DA and 5-HT receptors, we paired 1µM DA with DA blocker methergine and 1µM DA with 5-HT blocker

Co-injection of 1µM DA with maleate salt had no significant difference from when 1µM DA was administered alone ($p = 1.0$, Wilcoxon rank sum test, Bonferroni corrected (duration in dark, see Figure 2.5a) and $p= 1.0$ Wilcoxon rank sum test, Bonferroni adjusted (active speed in dark, see Figure 2.5b)), for 1µM DA vs 1µM DA- maleate co-injection.

Co-injection of 1µM DA with methergine led to ALB such that there was a statistically significant difference to the solo administration of 1µM DA vs when it was co-injected with methergine. Animals spent more time in the dark under 1µM DA and methergine combined injection ($p= 0.015$, Wilcoxon rank sum test, Bonferroni corrected (duration in dark, see Figure 2.5a) but the active speed did not differ $p= 0.176$ Wilcoxon rank sum test, Bonferroni corrected (active speed in dark, see Figure 2.5b)) from the group with 1µM DA injections.



a) b)

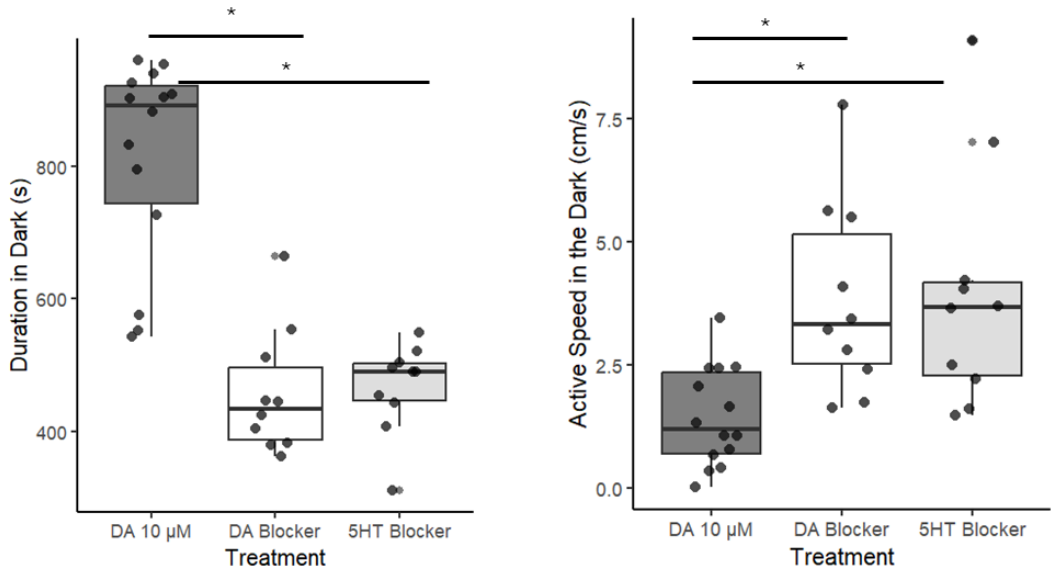
Figure 2.5: Duration (a) and Active speed (b) in the dark zone for 1 µM DA injection vs 1 µM DA- 5HT blocker and 1 µM DA- DA blocker co-injections. Boxplots display each treatment group's median, interquartile range (IQR), and data range. The graphs report the Wilcoxon rank sum test with Bonferroni-adjusted p-value comparisons. Significance is indicated by '*' for $p < 0.05$.

2.4.6 Effect of Dopamine 10µM and Serotonin blocker (maleate salt) vs Dopamine blocker (methergine) co-injection

Similar results were observed for 10µM DA vs 10µM DA- maleate salt co-injection, Welch two-sample t-test p = 0.0018, Wilcoxon rank-sum test, Bonferroni-corrected (duration in dark, Figure 2.6a) and p = 0.006, Wilcoxon rank-sum test, Bonferroni-corrected (speed in dark, Figure 2.6b)). At this concentration, the effect mimics the effect of DA blocker co-injection with 10µM 5-HT animals as reported earlier.

The ALB observed with the injection of 10µM DA was successfully blocked when administered together with methergine (p = 0.0005, Wilcoxon rank-sum test, Bonferroni-corrected (duration in dark, see Figure 2.6a) and p = 0.007, Wilcoxon rank-sum test,

Bonferroni-corrected (active speed in dark, see Figure 2.6b), for 10µM DA vs 10µM DA-methergine co-injection).



a) b)

Figure 2.6: Duration (a) and Active speed (b) in the dark zone for 10 µM DA injection vs 10 µM DA- 5HT blocker and 10 µM DA- DA blocker co-injections. Boxplots display each treatment group's median, interquartile range (IQR), and data range. The graphs report the Wilcoxon rank sum test with Bonferroni-adjusted p-value comparisons. Significance is indicated by '*' for $p < 0.05$.

2.5 Discussion

2.5.1 Serotonin (5-HT) and anxiety-like behaviour (ALB)

Anxiety is a prolonged state of alertness that persists even in the absence of any stressor (Fossat et al., 2014). It is a behavioural outcome of stress (Fossat et al., 2014). Serotonin (5-HT) has been characterized to play a role in driving stress-induced anxiety responses (Maximino et al., 2013, Fossat et al., 2014, Fossat et al., 2015). For example, Maximino et al. (2013) observed a positive relationship between 5-HT levels and anxiogenic response in zebrafish. Fossat et al. (2014) pointed out a positive relationship between stress and the intensity of anxiety-like behaviour (ALB). Stress responses are driven by 5-HT (Webster, 1996, Santos et al., 2001, Lorenzo et al., 2005, Aquiloni et al., 2012, Rajendran and Vasudevan, 2020). The latter results in an upregulation of the Crustacean Hyperglycemic Hormone (CHH), leading to a hyperglycemic effect and increased hemolymph glucose levels to support the fight-or-flight response (Webster, 1996, Santos et al., 2001, Lorenzo et al., 2005, Aquiloni et al., 2012, Rajendran and Vasudevan, 2020). As such, 5-HT can trigger ALB in crayfish (Fossat et al., 2015). Our results point towards a clear relationship between 5-HT and ALB. The data exhibit that animals displayed light avoidance and reduced active speeds for both concentrations of 5-HT injections (1 µM and 10 µM) compared to controls, which is a characteristic feature of ALB response (Fossat et al., 2014). Co-injection with a 5-HT1 blocker effectively reversed the anxiogenic response induced by 5-HT lone administration, further supporting the previous observations that 5-HT is involved in crayfish ALB.

2.5.2 Functional interplay between biogenic amines

We observed that the Dopamine (DA) blocker was able to block the effects of 5-HT when co-injected with 10 µM 5-HT. Additionally, 5-HT blocker co-injected with DA 10 µM was effective in reversing the anxiogenic response caused by DA. This indicates the possibility of cross-communication between biogenic amines in orchestrating behaviour among crayfish. It also points towards the possibility of parallel activation pathways

between 5-HT and DA with receptors being compatible with more than one biogenic amine.

Cross-communication between serotonergic and dopaminergic systems has been reported in both vertebrates as well as invertebrates (Sasaki-Adams, 2001, Niens et al., 2017, Scheunemann et al., 2018). For example, the enhanced motor activity in rats facilitated by intra-accumbent 5-HT was partially inhibited by DA blocker, pointing towards a potential 5-HT and DA interaction in regulating nervous processes (Sasaki-Adams, 2001). Niens et al. (2017) investigated the impact of altered DA signalling on the serotonergic circuit in the *Drosophila* brain and observed that DA is involved in the regulation of 5-HT innervation in brain circuitry. A study by Scheunemann et al. (2018) demonstrated that a pair of serotonin projection neurons were involved in the activation of long-term memory-gating dopaminergic neurons in the olfactory memory center, the mushroom body, in *Drosophila*. *In vivo* micro-dialysis in the anterior lateral striatum of the rat brain demonstrated that serotonin facilitates dopamine release, primarily mediated through 5-HT1 and 5-HT3 receptors (Benloucif et al., 1993). The study by Ibuchi and Nagayama on the agonistic behaviour of crayfish demonstrated that the facilitating effect of 10 µM DA on agonistic bouts was antagonised by 5-HT1 receptor antagonist, highlighting the potential cross-communication between dopaminergic and serotonergic systems in crayfish (Ibuchi and Nagayama, 2021).

In crustaceans, biogenic amines act as major modulators of behaviour and the crosstalk between these aminergic systems could be attributed to their distribution in the nervous system. Brain mapping of serotonergic and dopaminergic neurons in crayfish and lobsters has revealed that these groups of neurons occur in proximity which could explain the occurrence of functional interplay (Beltz and Kravitz, 1983, Tierney et al., 2003). Many of these neurons in crayfish and lobsters have anteriorly and posteriorly pointing axons in the central nervous system with their cytons placed in the suboesophageal ganglion

(Beltz and Kravitz, 1983, Tierney et al., 2003). As such, the suboesophageal ganglion could play a central role in facilitating cross-communication among biogenic amines (Beltz and Kravitz, 1983, Tierney et al., 2003, Ibuchi and Nagayama, 2021).

2.5.3 Dopamine (DA) and anxiety-like behaviour (ALB)

DA at 10 µM concentration had an anxiogenic effect on crayfish like 5-HT. Both 5-HT1 and DA blockers when co-administered with DA 10 µM, were effective in reversing the effects of DA 10 µM solo injection. This shows that at higher concentrations, DA behaves like 5-HT and leads to the appearance of ALB in crayfish. Interestingly, DA at 1 µM concentration did not affect behaviour and crayfish were comparable to saline-injected controls. Co-injection with 5-HT1 blocker did not appear to have any significant differences. However, when injected in combination with DA blocker, it led to the appearance of anxiety in crayfish. As such, crayfish subjected to a combination of 1 µM DA and DA blocker spent significantly higher time in dark zones of the plus-maze setup. This raises important questions about the role of DA in behavioural responses and needs to be further investigated.

Ibuchi and Nagayama's study on the role of dopamine in crayfish aggression suggested a potential dose-dependent receptor preference (Ibuchi and Nagayama, 2021). The study highlighted that the opposing effects of dopamine on crayfish fighting behaviour could be attributed to the different doses activating different receptors (Ibuchi and Nagayama, 2021). Among crustaceans, two types of dopamine receptors have been identified (Clark et al., 2008). Molecular characterization of DA receptors in the lobster nervous system reveals that D1 receptors lead to a rise in cAMP levels while D2 receptors via Gα mediated inhibitory effect on adenyl cyclase, can cause a net decrease in cAMP levels (Neve et al., 2004, Clark and Baro, 2006). An increase in cAMP levels is attributed to the loser effect and vice versa in crayfish aggression (Momohara et al., 2016). In Ibuchi and Nagayama's study, since 10 µM DA injection had an effect like 5-HT injection leading to

smaller crayfish winning agonistic bouts, it could indicate that at this concentration, D2 receptors are preferred. Notably, 5-HT has also been reported to cause a rise in cAMP levels in crayfish lateral giant (LG) interneurons to mediate escape behaviour (Araki et al., 2005). Such differential activity of different DA receptors has also been reported in marbled crayfish *Procambarus virginalis*, where the injection of a dopamine DA1 receptor antagonist inhibited reverse phototaxis, while dopamine DA2 receptor antagonists showed no effect (Shiratori et al., 2017). However, dose-dependent receptor binding preference still needs to be further investigated to confirm the observations and to understand the specific interactions between different biogenic amines.

Chapter 3 Effect of Fluoxetine on anxiety-like behaviour in crayfish *Pontastacus leptodactylus*

3.1 Abstract

The rising levels of pharmaceutically Active Compounds (PhACs) such as Selective Serotonin Reuptake Inhibitors (SSRIs) raise global concerns about their potential impact on the aquatic environment. SSRIs act by preventing the reuptake of Serotonin at synapses, thereby raising its levels in the haemolymph. Serotonin is a major modulator of behaviour among crustaceans. This study investigates the effects of Fluoxetine, an SSRI, on anxiety-like behaviour (ALB) in crayfish. Turkish crayfish (*Pontastacus leptodactylus*) were exposed to intermediate (0.56 ug/l), low (0.28 µg/l), and high (1.12 µg/l) concentrations of Fluoxetine. Crayfish exposed to Fluoxetine exhibited ALB, spending more time in dark zones of the experimental setup and reducing their speed when moving within dark zones compared to the control (0.00 µg/l). Haemolymph glucose levels were used as a physiological indicator of ALB and were found to be higher in crayfish exposed to Fluoxetine compared to the control group. The findings suggest an anxiogenic effect of Fluoxetine on crayfish, possibly due to increased serotonin levels in the haemolymph resulting from its nature as an SSRI. The behavioural changes are considered adaptive in the presence of a predator. However, an anxious response without a predatory event induces costs to the crayfish by reducing the exploration time and foraging efficiency. Since crayfish are keystone species such clear behavioural changes will also change the dynamics of the ecosystem. These findings imply that Fluoxetine exposure can induce ALB in crayfish, potentially mediated by serotonin.

3.2 Introduction

Pharmaceutically Active Compounds (PhAC) have become a cause for concern in the aquatic environment since their detection in freshwater systems around the mid-1970s (Tabak and Bunch, 1970). These compounds enter freshwater bodies mainly through human and animal excretion, medical, agricultural, and industrial runoff, and untreated or inefficiently treated wastewater (Fent et al., 2006, Santos et al., 2013). Among PhACs, the drugs that target neuro-endocrine systems are particularly concerning as they can disrupt the normal physiology and behaviour of aquatic animals exposed to them.

Selective Serotonin Reuptake Inhibitors (SSRIs) are a category of PhACs that are very frequently administered against depressive tendencies and various compulsive disorders in humans. (Hossain et al., 2019b). The neurotransmitter serotonin (5-Hydroxytryptamine, 5-HT) plays a vital role in the behavioural orchestration among aquatic animals such as fish, molluscs and crustaceans (Huber et al., 1997, Fossat et al., 2014, Lee et al., 2023). SSRIs bind to pre-synaptic reuptake transport proteins and prevent the reabsorption of 5-HT into synaptic clefts, consequently raising the level of this neurotransmitter in haemolymph (Kreke and Dietrich, 2008, Ford and Fong, 2016, Venkatachalam et al., 2023).

5-HT has been found to influence anxiety responses in both vertebrates and invertebrates (Bonhomme and Esposito, 1999, Lillesaar 2011, Fossat et al., 2014, Tu et al., 2020). Anxiety is a prolonged state of alertness which persists even in the absence of a stressor (Fossat et al., 2014). Crayfish subjected to a high-intensity electric field were found to have elevated brain 5-HT levels, and exogenous 5-HT injection into crayfish led to anxiety-like behaviour (ALB) (Fossat et al., 2015). The anxiolytic drug, chlordiazepoxide, reversed the effect (Fossat et al., 2014). In crayfish and other decapod crustaceans, 5-HT mediates anxiety responses by upregulating the crustacean

hyperglycaemic hormone (CHH), leading to heightened levels of haemolymph glucose (Webster, 1996, Santos et al., 2001, Lorenzo et al., 2005, Aquiloni et al., 2012, Rajendran and Vasudevan, 2020). Considering the significant role of crayfish in the aquatic ecosystem, even minor shifts in their behaviour patterns because of pollutants like SSRIs could have significant ecological consequences, potentially impacting nutrient cycling, decomposition of leaf litter, and population dynamics (Evans-White et al., 2003, Creed and Reed, 2004, Alp et al., 2016, Reisinger et al., 2021).

Fluoxetine, commercially known as Prozac® is a commonly prescribed anti-depressant belonging to the category of SSRIs (Henry et al., 2004, Hamilton et al., 2016, Kubec et al., 2019, Hossain et al., 2019b). Like other SSRIs, Fluoxetine leads to an elevated 5-HT level in the haemolymph (Hossain et al., 2019). In conditions of chronic exposure, it can also impact 5-HT regulation in the system thereby interfering with 5-HT release, impacting the expression of the 5-HT receptor gene, and the functioning of other neurotransmitters (Theodoridi et al., 2017, Cunha et al., 2018, Yamindago et al., 2021). Due to its widespread use, it is one of the most prevalent SSRI drugs in the aquatic environment. It has been detected in an average concentration ranging between 0.012 to1.4 μg/L in aquatic systems (Webb, 1999, Christensen et al., 2009).

Fluoxetine has been shown to impact behavioural tendencies among crustaceans (Huber et al., 1997, Santos, et al., 2001, Tierney et al., 2015, Hossain et al., 2019b). For example, ambient exposure to Fluoxetine was found to affect anxiety-like behaviour, foraging and anti-predatory behaviour among crabs (Hamilton et al., 2016, Peters et al., 2017). It was also observed to influence behaviour and growth in crayfish O*rconectes rusticus* (Tierney et al., 2015). Serotonin injected in combination with Fluoxetine reduced aggressive tendency in crayfish compared to injecting serotonin alone (Huber et al., 1997). Exposure to high level of Fluoxetine prolonged the fight duration and the time taken to reach peak fight intensity in crayfish *Faxonius virilis* (Hossain et al., 2019a). Since Fluoxetine works

by causing a rise in 5-HT levels, the exposure was observed to cause an upregulation of CHH, leading to a rise in haemolymph glucose levels in two species of decapod crustaceans, *Chasmagnatus granulate* and *Orconectes limosus* (Santos et al., 2001).

This study aims to investigate if exposure to Fluoxetine affects ALB in crayfish. Hence, I will expose crayfish to Fluoxetine in their Tupperware boxes for one week. I also want to test the effect at different concentrations. For this, I will use three concentrations of Fluoxetine: low (0.28 μg/l), intermediate (0.56 μg/l), and high (1.12 μg/l) dose, and compare the effect against a control (0.00 μg/l) group. Crayfish will be subjected to behavioural examinations in the plus-maze behaviour arena to test ALB. I also want to test if the behavioural effects of Fluoxetine exposure are translated into physiology by measuring haemolymph glucose levels under different exposure doses.

3.3 Materials and Methods

3.3.1 Experimental animals

110 male Turkish crayfish (*Pontastacus leptodactylus*) naïve individuals were selected from the aquarium culture and used in the study. The animals were sourced from South Norwood Lake, London, England (Crayaway crayfish removals, Bob Ring). 36 crayfish that moulted during the study were removed from the experimental trials. The animals used in the study had mean body weight and postorbital carapace length respectively being 50.35 ± 10 g and 41.9 ± 8.52 mm. There was no statistically significant difference in the weight (see Table 3.1) and carapace length of the animals under different treatment categories (see Table 3.2)

Table 3.1: Summary of weight across different groups

Group	**Weight ± sd**	**Comparison**	**Tukey HSD p adj.**
1.12 µg/L	62.30 ± 11.33	1.12 µg/L – 0.28 µg/L	0.53
0.56 µg/L	67.28 ± 8.35	0.56 µg/L – 1.12 µg/L	0.99
0.28 µg/L	62.16 ± 14.29	Control – 1.12 µg/L	0.82
Control	65.50 ± 8.26	0.56 µg/L – 0.28 µg/L	0.49
		Control – 0.28 µg/L	0.96
		Control – 0.56 µg/L	0.79

Table 3.2: Summary of carapace length across different groups

Group	**Length ± sd**	**Comparison**	**Tukey HSD p adj.**
1.12 µg/L	44.61 ± 8.58	1.12 µg/L – 0.28 µg/L	0.86
0.56 µg/L	42.31 ± 10.57	0.56 µg/L – 1.12 µg/L	0.92
0.28 µg/L	42.83 ± 6.79	Control – 1.12 µg/L	0.97
Control	45.92 ± 8.07	0.56 µg/L – 0.28 µg/L	0.99
		Control – 0.28 µg/L	0.59
		Control – 0.56 µg/L	0.70

3.3.2 Experimental design

Male Turkish crayfish (n= 110) were exposed to Fluoxetine (Sigma Aldrich, USA) in the ambient environment at concentrations of Control, 0.28 μg/L (low), 0.56 μg/L (intermediate) and high (1.12 μg/L) dose. The study involved behaviour testing (n= 66) and haemolymph glucose measurements (n= 44). Animals that moulted during isolation, were removed from the experiment.

Before the testing, crayfish were isolated for 7 days at 14°C in individual 1.5L Tupperware boxes. During this period of isolation, they were constantly exposed to ambient fluoxetine at the above-mentioned concentrations. Each such box was fitted with a siphoning system to eliminate any manual interferences during water changes with treatment water, which were performed every 24 hours. The animals were fed cooked prawns (1 medium-sized prawn per animal) a day before isolation and no feeding was done during the isolation period. An opaque cotton sheet (4mx4m) was used to visually separate the shelf where the crayfish were isolated from the main room to avoid any disturbance by the experimenter.

3.3.3 Behaviour testing

Post isolation, animals were introduced to the plus-maze behaviour arena (Refer to Figure 2.1, Chapter 2). This consisted of four arms (23 cm length, 12 cm width) and a central buffer zone (12 x 12 cm; see Figure 1). Two arms are lined with power-driven white LED strip lights to give an illumination of 70 lux in the light zone and the other two arms have power-operated red LEDs fixed vertically at the ends to produce 10 lux illumination in the dark zone (Figure 1). Light intensity was checked before each trial using a portable lux meter (Hanna instruments HI 97500). The maze was filled with 12 litres of demineralised water at pH8 maintained uniformly at 14°C throughout the trial. Two-thirds of the water in the maze was replaced by fresh stock water after each trial.

The animals were gently introduced to the arena in their respective Tupperware boxes with lids kept on. The process was carried out under low light conditions to cause minimum disturbance before trials. Behaviour was recorded for 16 minutes from the point of introduction into the plus-maze using a digital camera (Panasonic HC-V380, Panasonic, Japan) attached approximately 100 cm above the arena. Lolitrack 5 (Loligo systems, Denmark) behaviour software was used to track the animals in the arena and to record the time spent and active speed (distance covered/active time) in dark and light arms.

After behaviour recording, the carapace length of each crayfish was recorded to the closest 0.1 mm by Vernier callipers. The weight of each animal was measured on a digital weighing balance to the nearest 0.1 g. Animals used in the study had no missing appendages and were generally healthy and active as observed during the aquarium maintenance checks and feeding before the start of the study.

3.3.4 Haemolymph extraction and glucose analysis

Haemolymph extraction was done immediately after the exposure period for each treatment, control, low, intermediate and high doses of Fluoxetine. The process was always performed between 10 am to 1 pm to prevent circadian rhythm from influencing glucose results. A 100 µl of haemolymph sample was drawn from under the carapace using a sterile 0.8x40mm needle (BD Microlance, USA) in a 1ml syringe insert (BD Plastipak, USA). The sample was added to a centrifuge tube containing 200ul of 0.9M perchloric acid to prevent coagulation. The samples were used for analysis within 2 hours of collection.

Each sample was subjected to centrifugation. at 12000 rpm for 2 minutes. 100 µl of supernatant was placed in 200ul of Glucose assay reagent (GAGO20, Sigma Aldrich, USA) followed by a 30-minute incubation at 37°C in an incubator (Incubator Shaker, New Brunswick Scientific, USA). 400 µl of 3M H_2SO_4 was added to the tubes post-

incubation to stop the reaction. The optical density was recorded using a digital spectrophotometer (Helios α, Thermo Fisher Scientific, USA) at 540nm according to the procedures modified to adjust to 100 µl sample volume from the glucose assay kit. Glucose levels were calculated using the following formula and two standards (60mg/dl and 40mg/dl glucose) were prepared to compare glucose levels:

mg Glucose = ((ΔA540 of Test) * (mg Glucose in Standard)) / ΔA540 of Standard, where ΔA540 represents absorbance at 540nm (GAGO20, Sigma Aldrich, USA).

3.3.5 Chemicals and stock solutions

A solution of Fluoxetine hydrochloride (Sigma-Aldrich, USA) was prepared at a concentration of 1.12 µg/L and later diluted to obtain the required concentrations. Glucose Assay kit (GAGO20, Sigma-Aldrich, USA) was used for the assessment of haemolymph glucose levels in crayfish. The kit components (glucose oxidase/peroxidase reagent and *o*-danisidine reagents) were prepared in deionized water (peroxidase reagent dissolved in 39.2 mL and *o*-danisidine reagent in 0.8 mL of deionized water respectively). 0.8 mL of *o*-danisidine was added to 39.2 mL of peroxidase reagent stored in an amber container, to form the assay reagent for glucose analysis. It was stored at 4°C and used within four weeks.

3.3.6 Statistical calculations

Statistical analysis was performed using RStudio programming software (R 4.3.0 version 1.4.1106, 2009-2021 RStudio, PBC, USA). Time spent and active speed in the dark zone and the light zone of the plus-maze under different treatment conditions and differences in haemolymph glucose levels were compared using the Welch two-sample t-test (where data were normally distributed) or Wilcoxon rank sum test with continuity correction for non-normal data.

3.3.7 Treatment groups

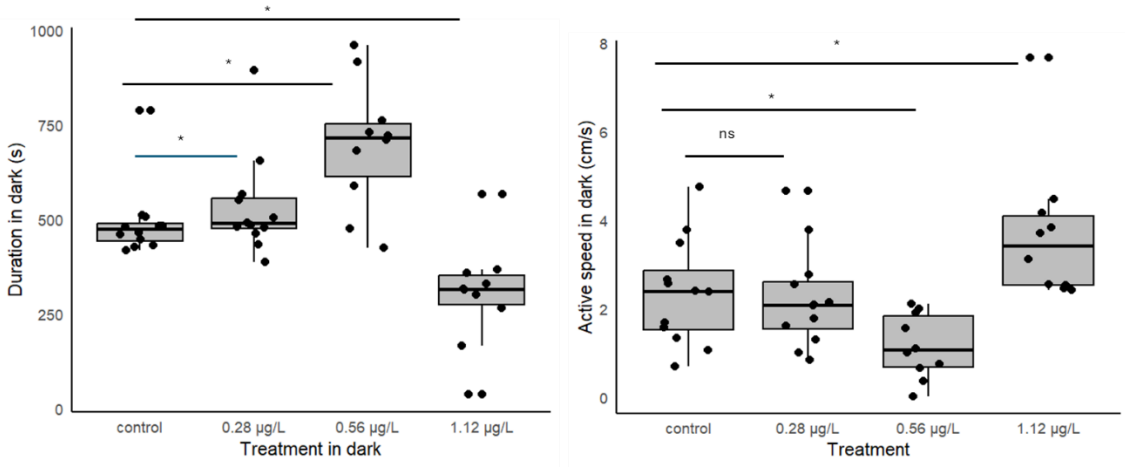
Table 3.3: Treatment groups and group sizes

Group	N for behaviour	N for glucose analysis
Control	11	7
0.28 µg/L Fluoxetine	12	7
0.56 µg/L Fluoxetine	11	7
1.12 µg/L Fluoxetine	10	7
Total	44	28

3.4 Results

3.4.1 Behaviour patterns

The tendency of light avoidance and dark preference among crayfish appeared to increase with the rise in the concentration of Fluoxetine. The average active speed (active speed) also exhibited a similar pattern. The duration of time spent in the dark at 0.28 µg/L Fluoxetine ($p < 0.05$, $t = -3.46$, $df = 11$), 0.56 µg/L Fluoxetine ($p < 0.05$, $t = -4.05$, $df = 21$; Welch two-sample t-test) and 1.12 µg/L Fluoxetine ($p < 0.05$, $W = 107$, $df = 17.47$, Wilcoxon rank-sum test with continuity correction) was higher when compared to the control group, see Figure 3.1A. Similarly, compared to the control crayfish had a reduced active speed in the dark zone at 0.56 µg/L Fluoxetine ($p < 0.05$, $t = -2.73$, $df = 18.75$, Welch two-sample t-test) and 1.12 µg/L Fluoxetine ($p < 0.05$, $t = 4.77$, $df = 14.52$, Wilcoxon rank-sum test with continuity correction) but not at 0.28 µg/L Fluoxetine ($p > 0.05$, $t = -1.91$, $df = 18.33$, Welch two-sample t-test), see Figure 3.1B.



b) a)

Figure 3.1: Duration (A) and Active speed (B) in dark zones for different concentrations of Fluoxetine. Boxplots display each treatment group's median, interquartile range (IQR), and data range. p-value from Welch's two-sample t-test or Wilcoxon rank sum test are reported in the graphs. Significance codes: '*' for $p < 0.05$.

3.4.2 Physiological patterns

Physiological measurements of haemolymph glucose levels indicated that Fluoxetine led to a rise in haemolymph glucose levels at all concentrations tested. (Figure 3.2). Statistics are reported from the Wilcoxon rank sum test with continuity correction. Haemolymph glucose levels were higher for 0.28 µg/L ($p < 0.05$, W= 7, df= 14), 0.56 µg/L ($p < 0.05$, W= 8, df= 14), 1.12 µg/L ($p < 0.05$, W= 3, df= 14) Fluoxetine when compared to the control group.

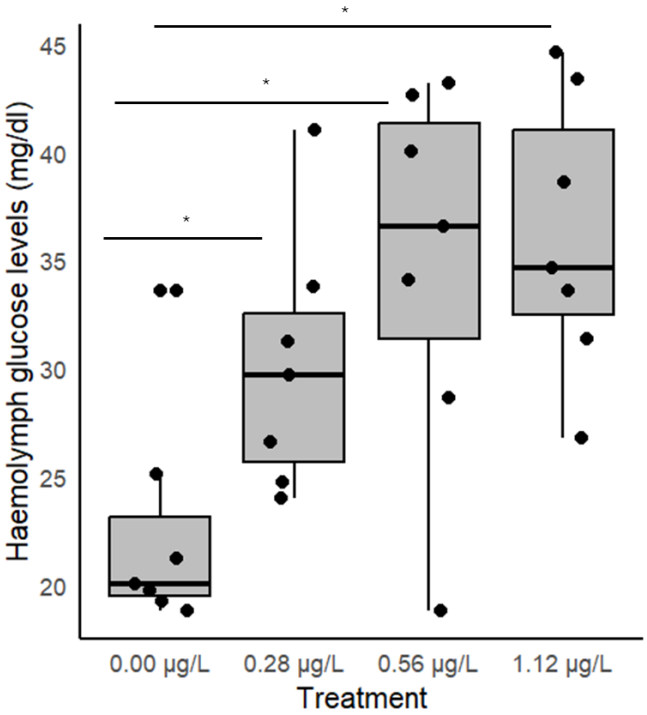


Figure 3.2: Haemolymph glucose levels for different concentrations of Fluoxetine. Boxplots display each treatment group's median, interquartile range (IQR), and data range. P-value Wilcoxon rank sum tests are reported in the graphs. Significance codes: '*' for $p < 0.05$.

3.5 Discussion

In the current study, I investigate the effect of Fluoxetine on anxiety-like behaviour (ALB) in the Turkish crayfish, *Pontastacus leptodactylus*. Three concentrations of Fluoxetine—0.28 µg/L (low), 0.56 µg/L (intermediate), and 1.12 µg/L (high)—were tested to evaluate behavioural and physiological responses. My findings indicate that exposure to Fluoxetine caused animals to spend more time in dark areas and have slower active speeds in comparison to the control group. Animals exposed to Fluoxetine also had an increased glucose level indicating higher stress levels compared to control animals. This behavioural pattern is consistent with anxiety-like responses where animals show dark preference to escape potential dangers (Fossat et al., 2014). Furthermore, physiological assessments revealed elevated haemolymph glucose levels in crayfish exposed to Fluoxetine at all concentrations when compared to the control group, further supporting the presence of anxiety-like behaviour (ALB). This could be attributed to the action of Fluoxetine as an SSRI leading to a rise in Serotonin (5-HT) levels in the brain, which upregulate the Crustacean Hyperglycaemic Hormone (CHH) hormone (Santos et al., 2001, Fossat et al., 2014). The latter raises glucose levels (Santos et al., 2001, Fossat et al., 2014) by rapidly mobilizing carbohydrate reserves (Fossat et al., 2014). The effect appeared even at the lowest concentration (0.28 µg/L) thereby raising concerns about the rising levels of SSRIs like Fluoxetine and other psychoactive drugs like Oxazepam (Lebreton et al., 2021), Citalopram (Holmberg et al., 2011), and Diazepam (Qiu et al., 2023) as well as their metabolites in aquatic water bodies.

Pharmaceutically active compounds (PhACs) are prevalent in aquatic bodies due to their high consumption and inadequate removal during wastewater treatment (Brodin et al., 2014). A broad range of pharmaceuticals are found in freshwater bodies and most of these are excreted out of the system in their pharmacologically active states (Brodin et al., 2014). Among PhACs, drugs that act on neurotransmitters such as Selective Serotonin Reuptake

Inhibitors (SSRIs), have raised a lot of concern due to their potential adverse impacts on the behaviour of aquatic animals (Kostich et al., 2008, Hossain et al., 2019a). SSRIs act by preventing the reabsorption of Serotonin at the pre-synaptic nerve cleft, leading to a rise of 5-HT levels in the haemolymph. 5-HT regulates major behavioural tendencies in aquatic animals, including stress, aggression and social behaviour (Huber et al., 1997, Lillesaar 2011, Fossat et al. 2014, Antunes et al., 2023).

Fluoxetine, an active compound in the commonly prescribed psychoactive drug Prozac, is a common SSRI found in freshwaters. It has been detected in a concentration ranging between 0.012 µg/L and 1.4 µg/L in a global average (Christensen et al., 2009, Kolpin et al., 2002, Weinberger and Klaper, 2014). Ambient exposure to Fluoxetine has been shown to affect behaviour among exposed aquatic organisms at concentrations detected in the environment for example, it can affect anti-predatory, foraging and reproductive behaviour in *Pimephales promelas* (Weinberger and Klaper, 2014). It has also been shown to influence aggression in toadfish (McDonald et al., 2011), cichlids (Stettler et al., 2021), and Betta fish (Kohlert et al., 2012), disrupt feeding in cichlid (Dorelle et al., 2020), and reduce swimming speed in response to predatory alarm in Arabian killifish (Barry, 2012) across concentrations detected in freshwater. The effect is also extended to invertebrates where Fluoxetine exposure was shown to affect ALB in crabs, attachment and righting behaviour in *Lymnea stagnalis* (Ford et al., 2018) and cause behavioural alterations among crayfish (Tierney et al., 2016, Hossain et al., 2019a).

Crayfish are considered to be keystone species in aquatic environments (Weinländer and Füreder, 2016). They are major consumers of leaf litter, biofilms and small benthic invertebrates (Evans-White et al., 2003, Reisinger et al., 2021). Even minor behaviour changes ensuing because of exposure to SSRIs like Fluoxetine, can affect essential aquatic processes such as the breakdown of leaf litter, trophic maintenance, and recycling

of nutrients (Evans-White et al., 2003, Creed and Reed, 2004, Alp et al., 2016, Reisinger et al., 2021). My findings indicate that the anxiogenic effect induced by the presence of Fluoxetine caused crayfish to exhibit dark preference and lower their activity levels even in the absence of an alarm stimulus. This kind of sustained alertness even in the absence of a predator is called anxiety (Fossat et al., 2014).

Such an effect when translated to the aquatic environment can negatively affect species fitness and survival as stress responses often entail high metabolic costs (Musil et al., 2023). Crayfish naturally tend to explore their surroundings (Fossat et al., 2014). Exploratory drive and enhanced activity are linked to fitness as they facilitate habitat expansion (Galib et al., 2022) and are necessary for foraging (Atkinson et al., 2023, O'hea Miller et al., 2024) and inter- and intra-specific interactions (Galib et al., 2022). Hence, it is crucial to understand the long-term effects of SSRI pollution in aquatic ecosystems and how the resulting behavioural alterations may impact community dynamics and ecological balance in the future.

Chapter 4 Effect of pH change on anti-predatory behaviour of crayfish *Pontastacus leptodactylus*

4.1 Abstract

In aquatic ecosystems, olfactory-mediated behaviour is a major facilitator of many survival activities including foraging, mating, and anti-predatory behaviour. Anthropogenic acidification of freshwater bodies can interfere with the detection or composition of olfactory chemical cues, affecting population dynamics and ecological structures. This study investigates the impact of acidification on crayfish anti-predatory response. In this study, I tested the response to alarm cues by crayfish upon exposure to low pH (pH6). At pH8 (control), the animals responded strongly to alarm odour. They exhibited a strong stress response by spending significantly more time with slow active speed in the dark zones of the plus-maze arena, compared to those at pH6. The behaviour results are supported by physiological results (changes in haemolymph glucose concentration). The pH8 group of animals had significantly higher glucose levels than the pH6 group for the alarm odour stimulus. It also highlights glucose as an indicator of stress in crayfish. To test if short-term acidification could affect non-chemically mediated responses in the central nervous processes, manual handling was used as a control stressor. It mimics transient capture by a predator and does not stimulate a chemical response such as olfaction. I did not observe any significant differences in the response to manual handling at pH8 and pH6 and the animals were equally stressed. This study suggests that water acidification affects the peripheral sensory detection of olfactory stimuli but not the higher-order central processing of stress perception such as those involved in handling by a predator.

4.2 Introduction

Predation is one of the most potent selection pressures in nature. Failure to escape predation can lead to injury of death (Acquistapace et al., 2004; Preisser et al., 2005). Anti-predatory behaviour is a crucial factor in determining the fitness and survivability of an organism (Lima and Dill 1990; Atkins et al., 2016). Successful predator avoidance depends on a prey's ability to detect predators and assess the risk involved (Lukas et al., 2021).

Crayfish respond in various ways to predators. Research on juvenile and adult signal crayfish, *Pacifastacus leptodactylus*, and crayfish *Orconectes sp.*, showed that predatory exposure reduced locomotion. (Blake and Hart, 1993; Hazlett and Schoolmaster, 1998; Stebbing et al., 2010). Increased shelter use is also one of the anti-predatory strategies among crayfish (Blake and Hart, 1993; Stein and Magnuson, 1977; Stein, 1976). An increased display of chelae is another defense mechanism, observed in *Paranephrops zealandicus* when exposed to skin mucus from predatory eels (Shave et al., 1994; Breithaupt et al. 2016). Finally, crayfish also employ the unique "tail-flip" escape response directed away from predators (Herberholz 2022).

Haemolymph from freshly injured conspecifics, also called alarm odour is a predatory cue. It is used by many crustaceans as a chemosensory cue that indicates a potential risk of predation (Hazlett 1994; Hazlett and McLay 2005; Briones Fourzán et al., 2008). When animals detect the presence of an alarm odour, they respond by employing defensive anti-predatory strategies to ensure their safety. Changes in water chemistry can affect the transmission and detection of alarm odour, as it is a chemical signal that travels through the water (Woelcke and Brown, 1969; Brown et al., 2000; Tierney and Atema, 1988). This can potentially affect anti-predatory response thereby increasing the risk of predation.

Previous studies have demonstrated that under acidic conditions, the ability of crayfish to detect chemical cues could be compromised. For instance, a significant loss of

responsiveness to basic food stimuli was observed under low pH conditions in crayfish *Faxonius virilis*, *Procambarus acutus* (Tierney and Atema, 1986; Tierney and Atema, 1987) and *Cambarus bartoni* (Allison et al., 1992). pH-mediated disruption of predatory information transfer in aquatic environments was also found to interfere with anti-predatory responses in freshwater snails (Cothran et al., 2021). These observations highlight the pH sensitivity of chemosensory responses among crayfish and other aquatic invertebrates, where even minor changes can lead to significant effects.

Ionic changes in water due to pH shift may affect the molecular structure of alarm odour, leading to a loss of chemosensory ability. For instance, earlier research in ostariophysan fish indicated Hypoxanthine-3(N)-oxide, a purine derivative, as the active ingredient of epidermal alarm cells (Pfeiffer et al., 1985). The compound is confirmed to induce alarm response (Pfeiffer et al., 1985; Brown et al., 2000). Information from analytical chemistry research suggests that under acidic conditions, this compound undergoes a molecular change and is converted to 6,8-dioxypurine with a loss of the 3-*N*-oxide functional group (Woelcke and Brown, 1969; Kawashima and Kumashiro, 2006; Leduc et al., 2013). This irreversible change in the chemical constitution of the active compound at low pH could render the alarm odour ineffective or non-functional (Brown et al., 2000).

Additionally, pH changes may result in physiological changes in odour receptors. Research on fish demonstrates that pH changes alter the state of ionisation of protein receptor sites (Tierney and Atema, 1988; Moore, 1994) This can affect the binding affinity of the receptor sites and render them incapable of binding to the alarm molecules (Tierney and Atema, 1988). This would diminish the anti-predatory response due to neurological change in stimulus-receptor binding (Tierney and Atema, 1988; Moore 1994)

Acidification can also impact central nervous processing and affect chemosensory abilities. The mechanism accounting for pH-dependent chemosensory impairment in freshwater and marine fish has previously been linked to the GABA-A signalling (Nilsson

et al., 2012). Acidification can disrupt normal Cl^- and/or HCO_3^- gradients over neuronal membranes, leading to depolarisation of some GABA-A receptors (Nilsson et al., 2012). This ionic imbalance during low pH exposure causes putative changes in the brain leading to dramatic shifts in chemosensory abilities (Nilsson et al., 2012). Disruption of GABAergic neurotransmission when exposed to near-future CO_2 levels has been accounted for sensory impairment in both freshwater fish like three-spined sticklebacks (Lai et al., 2016) as well as marine fish such as larval coral reef fish (Nilsson et.al 2012). It was also linked to an increased anxiety response in Rockfish (Hamilton et al., 2014).

Developing effective management strategies for the conservation of aquatic organisms requires an understanding of the impact of water acidification on aquatic ecosystems. Anthropogenic CO_2 accumulation is well-documented in marine systems, leading to ocean acidification, which disrupts marine ecosystems by altering food webs, nutrient cycles, and biodiversity (Weiss et al., 2018). The ocean absorbs large amounts of atmospheric CO_2, causing a decrease in pH with widespread biological consequences. In contrast, freshwater systems have received less attention despite their complex biogeochemistry. Long-term reservoir data indicate rising pCO_2 levels and decreasing pH, suggesting that CO_2 accumulation also affects inland waters. Experiments on *Daphnia* show that high pCO_2 levels impair predator detection, independent of pH changes, which could disrupt freshwater trophic interactions (Weiss et al., 2018). Unlike marine systems, where acidification is primarily studied in calcifying organisms, freshwater impacts appear to involve disruptions in chemical communication, potentially affecting species interactions across trophic levels.

Lowered freshwater pH can affect important behavioural tendencies mediated via chemosensory signaling including anti-predatory behaviour (Leduc et al., 2013). This can potentially interfere with life history traits, trophic maintenance, population structure, and ecological roles of species, thereby raising ecological concerns. An ineffective response

to predatory cues can cause a significant rise in prey mortality rates thereby potentially disturbing population dynamics. For instance, in staged encounters, the rate of predation of trout by largemouth bass was significantly higher when trout alarm odour was introduced at low pH (pH6) than at neutral pH (Leduc et al., 2009). In future scenarios, acidification can lead to naïve prey due to a lack of conditioning with alarm odour and hence lead to a greater predation cost to prey populations (Leduc et al., 2013).

Once a potential threat is detected, it can induce a stress response. In the current paper, we use time spent in dark and active speed in the dark zones of the plus maze arena, as behavioural indicators of stress (Fossat et al., 2015). Haemolymph glucose levels account for a physiological stress indicator (Webster, 1996; Jusilla et al., 1998; Lorenzon et.al 2004; Fossat et al., 2015; Soares et al., 2020). The glucose levels are mediated via crustacean hyperglycemic hormone (CHH) (Webster, 1996; Lorenzon et al., 2005; Fanjul-Moles, 2006; Fossat et al.; 2014; Rajendran and Vasudevan, 2020) and the latter has been linked to respond to changes in serotonin levels (Santos et al., 2001; Lorenzon et al., 2005; Aquiloni et al., 2012; Fossat et al., 2015).

I compared the stress response among crayfish in response to chemical (blood from conspecifics that acts as alarm odour), and physical (manual handling) stimuli. I introduced the stimuli under two different pH (pH6 and pH8). These different stimuli were selected to determine the nervous areas impacted. The introduction of alarm odour as a chemical stimulus will elicit a receptor-based peripherical response associated with the detection and transmission of chemical signals. On the other hand, manual handling mimics transient capture by a predator. It is a tactile stimulus and does not involve olfactory sensing. Response to tactile stimuli is mediated via the lateral giant (LG) neuron circuitry (Herberholz 2022).

The main aim of this study is to investigate whether exposure to low pH can lead to changes in the anti-predatory behaviour of crayfish. Hence, I exposed crayfish to alarm odour at two different pH. I also wanted to see if any pH-related changes in response are due to changes in the reception process or due to pH-induced changes in central processing of the predation stimulus. For this, I employed an olfactory stimulus (alarm odour) and a mechanosensory tactile stimulus (manual handling). Lastly, I wanted to investigate the physiological consequence of these changes. For this, I measured haemolymph glucose as an indicator of predation-borne stress.

4.3 Materials and methods:

4.3.1 Experimental animals

146 naïve male Turkish crayfish (Pontastacus leptodactylus) individuals were selected from the aquarium culture and used in the study. The animals were sourced from South Norwood Lake, London, England (Crayaway crayfish removals, Bob Ring). 10 animals were used as haemolymph donors for alarm odour preparation. 25 crayfish that moulted during the study were removed from the experimental trials. The animals used in the study had mean body weight and postorbital carapace length respectively being 40.3 ± 9.17 g and 43.86 ± 8.84 mm for pH8 and 38.8 ± 8.48 g and 39.75 ± 8.71 mm for pH6 group. There was no statistically significant difference in the weight and carapace length of the animals in different experimental groups (see Table 4.1)

4.3.2 Experimental design

Male Turkish crayfish (n= 120) were exposed to a set of treatments at two different pH (pH6 and pH8). The aquarium facility naturally houses animals at pH8 and for pH6 preparation, a small aquarium CO_2 bubbling machine (pH monitoring CO_2 systems premium, Guemmer, Germany) was used. The study was conducted in two parts involving behaviour testing (n= 60) and glucose level measurements (n= 42). 15 crayfish individuals were used as haemolymph donors for alarm odour exposure. The donors were re-used for haemolymph extraction after at least 10 days of recovery period.

Behavioural responses to two types of predatory stimuli namely haemolymph from conspecifics (alarm odour) and transient capture by the predator (manual handling) were assessed. 15 crayfish were exposed to alarm odour and 15 were subjected to manual handling for each pH. The experiments were performed using a paired design for treatment and control in alarm odour treatments. Different sets of animals were used for each pH condition such that the animals subjected to one pH were not exposed to another pH.

Glucose levels were measured in response to the above-described predatory stimuli. 7 animals each were tested for the following categories (repeated measure design was not employed here):

i. Control pH8(no alarm odour and no manual handling)
ii. Alarm odour exposure at pH8
iii. Manual handling at pH8
iv. Control pH6 (no alarm odour and no manual handling)
v. Alarm odour exposure at pH6
vi. Manual handling at pH6

Before behaviour testing as well as glucose level measurements, crayfish were isolated for 7 days at 14°C in individual 1.5L Tupperware boxes. Each such box was fitted with a flow-through siphoning system to eliminate any manual interferences during water changes which were performed every 24 hours. The animals were fed cooked prawns (1medium-sized prawn per animal) a day before isolation and no feeding was done during the isolation period. An opaque cotton sheet (4mx4m) was hung on the frame of the isolation set-up to avoid any disturbance from the outside.

4.3.3 Pre-behaviour trial/pre-glucose measurement preparations and treatments

a) Alarm odour collection: 0.lml of haemolymph samples were collected from below from the base of the carapace using a sterile 0.8x40mm needle (BD Microlance, USA) in 1ml syringe insert (BD Plastipak, USA). The collected haemolymph was added to 9.9ml of 0.1mM L-ascorbic acid to prevent coagulation. The prepared alarm odour samples were used in the experiments the same day.

b) pH changes: The inflow tubing of the siphoning Tupperware box for the animal to be tested was fitted with a funnel through which water at pH6 was introduced with a steady inflow (initialization period). Testing done before the study established that it takes 4

minutes for pH of the water to change in the Tupperware box. The animals were kept at pH6 for 4 minutes (exposure period) post which pH8 water was introduced through the inflow to restore pH to 8 in another 4 minutes (washing period). Changes to pH were made in situ in the isolation boxes of the animals. For pH8 treatment, the inflow valve was kept open for 4 minutes followed by a 4-minute exposure period and a 4-minute washing period as above.

c) Treatments: The animals were subjected to the following treatments for both behaviour trials as well as glucose measurements:

i. **Control:** The controls were the same for alarm odour testing and manual handling experiments. There was a repeated pairing between treatment and control for alarm treatment exposure only. Controls in the two pH categories were not exposed to alarm odour or manual handling during the 4-minute exposure period.

ii. **Alarm odour exposure:** The animals were exposed to alarm odour introduced through the inflow tubing at the start of the exposure period. Inflow and outflow were stopped during the exposure period and restarted during the washing period. Thus, the animals were in contact with a stagnant alarm substance for 4 minutes and gradually diminishing concentration during the washing period when the flow was started.

iii. **Manual handling:** After the initialization, exposure and washing periods spanning 12 minutes as described above, the animals were subjected to a 3-minute extensive manual handling. This involved manually lifting the crayfish from the Tupperware box and moving it between two hands for one and a half minutes. Next, crayfish were placed into a dry 1.5L Tupperware box and exposed to restrained horizontal vibration which caused the animal to slide around in the box. This process was also one and a half minutes in duration.

4.3.4 Behaviour testing and data collection

After the completion of 12-minute pre-behavioural treatments, crayfish were introduced to the behaviour arena. The latter comprises a plus-shaped matrix, a 63.5x63.5 square tank with arms of 23 cm length and 12 cm width and central area(buffer zone) of 12 cm length and 12 cm width (Refer to Figure 2.1 in Chapter 2). Its two opposite arms are lined with power-driven white LED strip lights to give the illumination of 70lx in the light zone. Power-operated red LEDs are fixed vertically at the ends of two opposite arms (dark zone) and the light strips have been wrapped in a single layer with a diffusing sheet to produce 10lx illumination in the dark zone. Light intensity was monitored before each trial using a portable luxmeter (Hanna instruments HI 97500). The maze was filled with 12 litres of demineralised water at pH8 maintained uniformly at 14°C throughout the trial. Two-thirds of the water in the maze was replaced by fresh stock after each trial.

The animals were introduced to the arena in their respective Tupperware boxes with lids on. The process was carried out under low light conditions to cause minimum disturbance before trials. Behaviour was recorded for 16 minutes from the point of introduction into the plus-maze using a digital camera (Panasonic HC-V380, Panasonic, Japan) attached approximately 100 cm above the arena. Lolitrack 5 (Loligo systems, Denmark) behaviour software was used to track the animals in the arena and to record the time spent and active speed (distance covered/active time) in dark and light arms.

At the end of the recording period, the carapace length of each crayfish was measured to the nearest 10^{-1}mm using Vernier callipers and each animal was weighed on a digital weighing balance to the nearest 10^{-1}g. Animals used in the study had no missing appendages and were generally healthy and active as observed during the aquarium maintenance checks and feeding before the start of the study. There was no significant difference in the body measurements of the animals used in different parts of the study (Table 2).

In the alarm odour trials, there was a randomised choice of the first treatment the animals were exposed to (control or alarm odour). After the first treatment, the animals were housed in the communal tanks for a week followed by a week of isolation in the siphoning Tupperware boxes before the next treatment. Animals which underwent moulting after the first treatment were not used in the second treatment.

4.3.5 Haemolymph extraction and glucose analysis

The animals underwent 1-week isolation in a siphoning system and were subjected to the treatments as described in sections 2.2 and 2.3 above. Haemolymph extraction was done immediately after exposure to a specific treatment (control, alarm odour, or manual handling). The process was always performed between 10 am to 1 pm to avoid any influence of circadian rhythm on haemolymph glucose levels. A 100ul of haemolymph sample was drawn from under the carapace using a sterile 0.8x40mm needle (BD Microlance, USA) in 1ml syringe insert (BD Plastipak, USA). The sample was added to a centrifuge tube containing 200ul of 0.9M perchloric acid to prevent coagulation. The samples were used for analysis within 2 hours of collection. Each sample was centrifuged at 12000 rpm for 2 minutes. 100ul of supernatant was placed in 200ul of assay reagent (GAGO20, Glucose (GO) Assay Kit, Sigma Aldrich, USA) and incubated for 30 minutes at 37°C in an incubator (Incubator Shaker, New Brunswick Scientific, USA). 400ul of 3M H_2SO_4 was added to the tubes post-incubation to stop the reaction. The optical density was recorded using a digital spectrophotometer (Helios α, Thermo Fisher Scientific, USA) at 540nm according to the procedures modified to cater to 100ul sample size from the glucose assay kit. Glucose levels were calculated using the following formula and two standards (60mg/dl and 40mg/dl glucose) were prepared to compare glucose levels:

mg Glucose = ((ΔA540 of Test)*(mg Glucose in Standard)) / ΔA540 of Standard, where ΔA540 represents absorbance at 540nm.

4.3.6 Chemicals and stock solution

Individual stock solutions of L-ascorbic acid (Sigma-Aldrich, USA), perchloric acid (Sigma Aldrich, USA) and sulfuric acid were prepared in deionized water at respective concentrations of 10 mM, 0.9M and 3M respectively. The stock solutions were stored at 14°C maintained consistently in a temperature-controlled room. Glucose Assay kit GAGO20 (Sigma-Aldrich, USA) was used for the assessment of haemolymph glucose levels in crayfish. The kit components (glucose oxidase/peroxidase reagent and o-danisidine reagents) were prepared in deionized water (peroxidase reagent dissolved in 39.2 mL and o-danisidine reagent in 0.8 mL of deionized water respectively). 0.8 mL of o-danisidine reagent was added to the amber bottle containing 39.2 mL of peroxidase reagent to form the assay reagent for glucose analysis. It was stored at 4°C and used within one month.

4.3.7 Statistical calculations

Statistical analysis was performed using RStudio programming software (R 4.3.0 version 1.4.1106, 2009-2021 RStudio, PBC, USA). Normality was established using Shapiro-Wilks test and one way ANOVA with TUKEY HSD posthoc test was used to compare the different groups. The null hypothesis was rejected at $p<0.05$.

4.3.8 Treatments

Table 4.1: Treatment groups and group sizes

Group	**Number of animals**
Behaviour experiments	
Control pH8/ Alarm pH8	17
Manual Handling pH8	14
Control pH6/ Alarm pH6	13
Manual Handling pH8	13
Glucose experiments	
Control pH8	8
Alarm odour pH8	6
Manual Handling pH8	8
Control pH6	8
Alarm odour pH6	6
Manual Handling pH6	8
Total	101

4.4 Results

4.4.1 Behaviour patterns

a) Behavioural response at pH8

Crayfish exposed to the alarm odour at pH8 spent more time and had significantly lower active speeds in the dark zones of the plus maze arena, $p < 0.05$, ANOVA, Tukey HSD duration in the dark, and $p < 0.05$, ANOVA, Tukey HSD, for active speed in the dark than the control group.

Animals subjected to manual handling treatment pH8 spent significantly more time and had less active speed in the dark zones when compared to controls, $p < 0.05$, ANOVA, Tukey HSD, duration in the dark, and $p < 0.05$, ANOVA, Tukey HSD, for active speed in the dark.

b) Behavioural response at pH6

Crayfish exposed to alarm odour at pH6 had no significant difference in the duration and active speed in the dark zones compared to controls ($p > 0.05$, ANOVA, Tukey HSD, duration in the dark, and $p > 0.05$, ANOVA, Tukey HSD, for active speed in the dark).

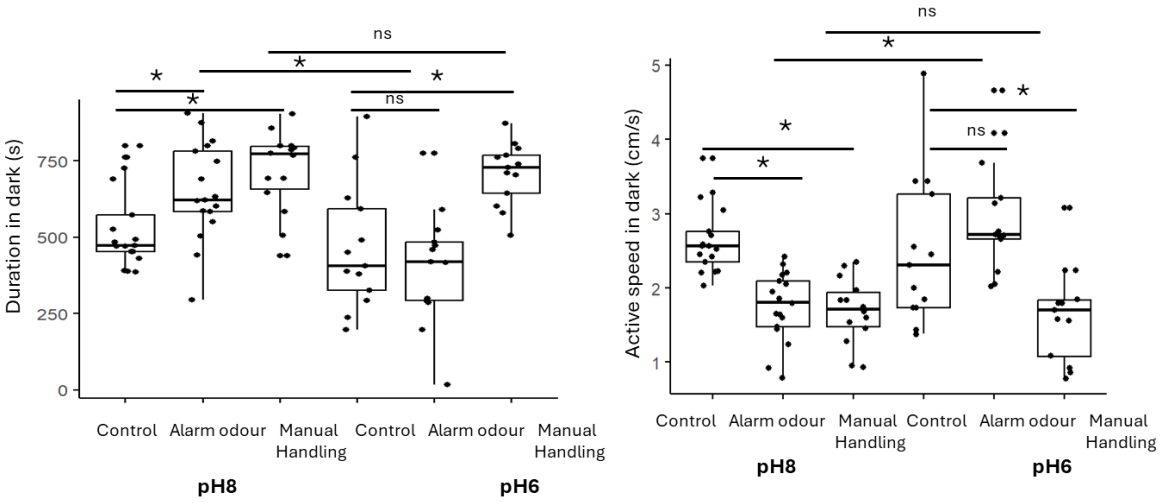
In the case of manual handling treatment at pH6, the animals spent significantly more time and had less active speed in the dark zones when compared to controls, $p < 0.05$, ANOVA, Tukey HSD, duration in the dark, and $p < 0.05$, ANOVA, Tukey HSD, for active speed in the dark.

c) Behavioural response pH6 vs pH8

There was a marked difference in behavioural response to alarm odour between pH6 and pH8. Crayfish exposed to alarm odour at pH6 were much bolder and hence spent less time in dark zones with a higher active speed in the dark when compared to those at pH8 ($p < 0.05$, ANOVA, Tukey HSD, duration in the dark), and $p < 0.05$, ANOVA, Tukey HSD for active speed in the dark).

However, there was no significant difference in the behavioural response to manual handling treatment between pH6 and pH8 ($p > 0.05$, ANOVA, Tukey HSD, duration in the dark, and $p > 0.05$, ANOVA, Tukey HSD, for active speed in the dark).

Note: Figure 4.1 a) and b) summarize the statistical output



a) **b)**

Figure 4.1: Duration and (a) Duration (b) Active speed in the dark zone for control, alarm odour and manual handling groups at pH8 and pH6. Boxplots display each treatment group's median, interquartile range (IQR), and data range. p-values from one-way ANOVA with Tukey HSD post-hoc comparisons are reported in the graphs. Significance is indicated by '*' for $p < 0.05$.

4.4.2 Physiological patterns

a) Haemolymph glucose levels at pH8

Haemolymph glucose levels of crayfish exposed to alarm odour at pH8 were significantly higher than controls ($p = 0.008$, ANOVA, Tukey HSD, Figure 4.2).

Similarly, animals subjected to manual handling treatment at pH8 also had significantly higher haemolymph glucose levels when compared to controls ($p = 0.0074$, ANOVA, Tukey HSD, Figure 4.2)

b) Haemolymph glucose levels at pH6

Haemolymph glucose levels of crayfish exposed to alarm odour at pH6 were comparable to controls. As such there was no significant elevation in haemolymph glucose compared to controls ($p = 0.7$, ANOVA, Tukey HSD, Figure 4.2)

In contrast, the animals subjected to manual handling treatment at pH6 had significantly higher haemolymph glucose levels when compared to controls ($p = 0.01$, ANOVA, Tukey HSD, Figure 4.2).

c) Haemolymph glucose levels pH6 vs pH8

There was a marked difference in haemolymph glucose levels between pH6 and pH8 for alarm odour exposure. Crayfish exposed to alarm odour at pH8 had significantly higher glucose levels compared to pH6 ($p = 0.0012$, ANOVA, Tukey HSD, Figure 4.2).

There was no significant difference in glucose levels for manual handling treatment between pH6 and pH8 ($p = 0.99$, ANOVA, Tukey HSD, Figure 4.2).

Note: Figure 4.2 summarizes the statistical results

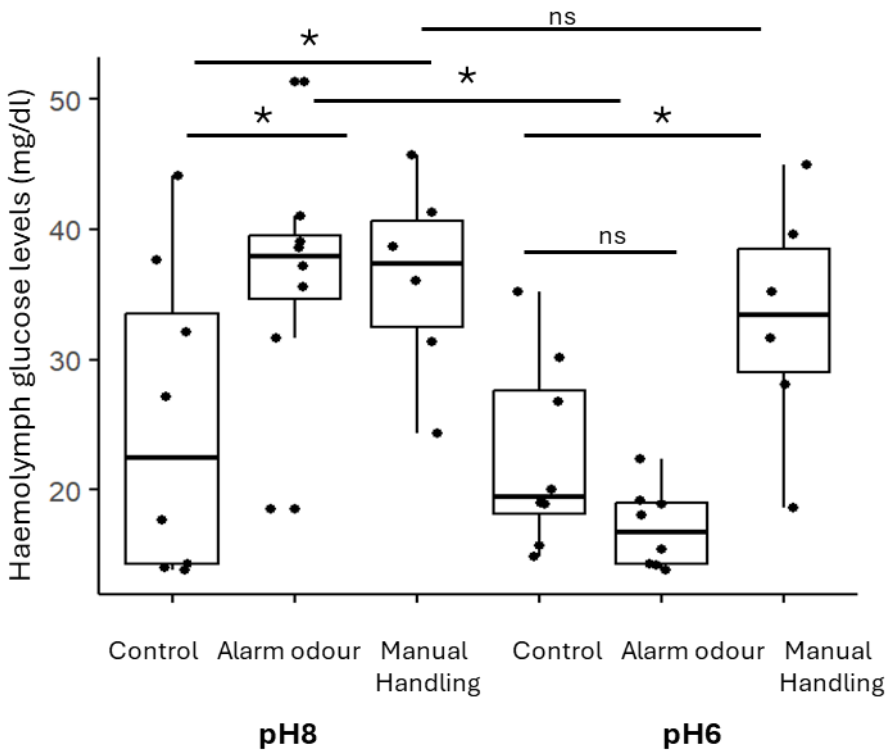


Figure 4.2: Haemolymph glucose levels (mg/dl) for control, alarm odour and manual handling groups at pH8 and pH6. Boxplots display each treatment group's median, interquartile range (IQR), and data range. p-values from one-way ANOVA with Tukey HSD post-hoc comparisons are reported in the graphs. Significance is indicated by '*' for $p < 0.05$.

4.4.3 Life history traits

The crayfish used in pH6 as well as pH8 trials did not differ statistically in weight and carapace length (Table 4.1). Also, no statistically significant difference was observed in moult frequency amongst animals exposed to pH6 and pH8 ($X^2 = 0$, $P = 1$) (Table 3). Crayfish that moulted within one week of exposure in behaviour trials were considered for treatment-related moulting instances in statistical calculations (Table 4.2).

Table 4.2: Weight and Carapace length of Turkish crayfish *Pontastacus leptodactylus* specimens in pH6 and pH8 groups. Data are presented as mean ± standard deviation.

Group	**Weight(g) ± sd**	***t*-test**	***P***	**Carapace(mm) ± sd**	***t*-test**	***P***
pH6	38.8 ± 8.48	1.45	0.15	39.75 ± 8.71	0.37	0.71
pH8	40.3 ± 9.17			43.86 ± 8.84		

Table 4.3: Moulting in Turkish crayfish *Pontastacus leptodactylus* specimens in pH6 and pH8 groups. Data are presented as mean ± standard deviation.

Group	**Moults in alarm odour/control category**	**Moults in manual handling category**	X^2 test	***P***
pH6	9	12	0	1
pH8	8	8		

4.5 Discussion

In the current study, I examine the impacts of low pH on anti-predatory behaviour in Turkish crayfish *Pontastacus leptodactylus*. Two types of stimuli were used to assess behavioural responses, alarm odour and manual handling. The results suggest that exposure to low pH may affect chemosensory processes, such as those involved in the sensing of alarm cues. The exposure to alarm odour at pH8 led to a stress response. The animals spent more time in the dark and had slower active speeds in the dark zones compared to the control. On the other hand, crayfish exposed to alarm odour at pH6 spent similar time in the dark zones of the plus-maze arena and had similar active speeds in the dark as controls. The diminished response to alarm odour at pH6 shows that low pH can interfere with the detection of alarm cues. A successful detection of alarm odour triggers adaptive anti-predator behaviour, but this was absent at low pH. However, the response to manual handling was similar at both pH levels. Hence, manual handling may not stimulate chemical sensing, so pH changes may not affect the behavioural response. The animals exposed to alarm odour at pH8 had higher haemolymph glucose levels than controls unlike those exposed at pH6 where the levels were comparable with the control group. The glucose results indicate that predatory threat leads to fear-borne stress response. The levels were higher for animals subjected to manual handling when compared to the controls in both pH groups. Glucose levels are sensitive to changes in brain serotonin levels (Santos et al., 2001; Lorenzon et al., 2005; Aquiloni et al., 2012) and were suggested to stimulate anxiety-like stress responses in crayfish (Fossat et al., 2015).

Peripheral chemosensory receptor-based response to alarm odour

The introduction of alarm odour is a chemical stimulus that elicits a peripheral response based on receptor detection and transmission of chemical signals. It is likely detected by aethestac chemoreceptors on the first antenna (antennules) of crayfish (Skog and Hallberg,

2011). These chemoreceptors are sensitive to chemosensory cues and have organised receptive fields called receptor hair bushes, on claws of ambulatory feet (AF), antennas, antennules, and other mobile appendages (Fedotov 2009). Crayfish have unimodal interneurons in the antennular neuropil of the supra-pharyngeal ganglion to detect chemical cues (Derby and Blaustein 1988, Wiersma and Roach 2011). Chemosensory interneurons in the first thoracic ganglion show an excitatory response when the exteroceptors of the first pair of walking legs, chelipeds, and maxillipeds are stimulated (Fedotov, 2009).

Tactile receptor-based central response to manual handling

Manual handling mimics transient capture by a predator. Tactile pressure applied around the tail and abdominal regions stimulates direction-sensitive hair mechanoreceptors (Wiese et.al 1976) in addition to proprioceptors (Anouma et al., 1999, Newland et al., 2000). These afferents make direct electrical synapses with the LG neuron and chemical synapses with many interneurons (Herberholz 2022). Thus, the response to manual handling stimulus is likely a central nervous system response mediated via the LG neuron circuitry. The response is generated via a tactile stimulus which does not involve chemical sensing and hence, is not affected by pH change.

Effect of pH change on chemical communication

pH changes in water can have detrimental consequences on chemical communication (Psenner 1994, Dangles and Guerold 1999, Guerold et al., 2000, Tix et al., 2017, Ninokawa and Ries 2022). Previous studies have demonstrated that chemosensory abilities of crustaceans can be affected in freshwater acidification scenario. For example, research has shown that under low pH conditions, crayfish such as *Faxonius virilis*, *Procambarus acutus* (Tierney and Atema 1986, Tierney and Atema 1987), and *Cambrus bartoni* (Allison et al., 1992) were less responsive to food cues, while freshwater snails

showed decreased responsiveness to predatory cues (Cothran et al., 2021). When considering anti-predatory behaviour, failure to respond to alarm cues could significantly lead to an increase in prey mortality rates. This can potentially impact population dynamics and species interactions.

In this research, I observed that the crayfish did not respond effectively to the alarm odor in in the low pH (pH6) group. The demonstrated disruption of response to predatory cues under low pH conditions could be a result of the chemosensory receptors being unable to recognize alarm odour, changes in the molecular structure of alarm chemical components or a combination of the two. Thus, under an acidified environment, the alarm cues can be subjected to molecular changes rendering them unrecognisable by chemosensory receptors or the concentration of the active component in alarm odour might no longer be able to meet the minimum threshold needed to elicit the required response (Leduc et al., 2013). For example, analytical chemistry applications have suggested that one of the active components of the alarm odour for freshwater *Ostariophysan* fish, hypoxanthine-3-*N*-oxide changes to 6,8-dioxypurine under low pH conditions. This happens due to the loss of 3-*N*-oxide functional group thereby rendering the alarm cue unrecognisable and hence ineffective in eliciting a behavioural response (Brown et al., 2002). Another possible way in which low pH may affect olfaction and chemosensitivity in aquatic settings is the physiological disruption of the chemosensory apparatus which can hamper the ability to detect alarm cues by impairment of receptor-ligand binding and hence negatively impact anti-predatory responses (Klaprat et al.,1988, Tierney and Atema 1988, Moore 1994, Tembo 2009, Leduc et al., 2013).

Central processing of stress in crayfish

In crustaceans, stress responses are processed centrally via the neuroendocrine centre known as the X organ and sinus gland of the eyestalk (Knigge et al., 2021). This is homologous to the hypothalamus-pituitary axis in the vertebrate brain (Knigge et al.,

2021). Stress involves a rapid mobilization of neuroendocrine centres. The crustacean hyperglycemic hormone (CHH) works similarly to the cortisol-cortisone stress signalling in vertebrate stress responses (Knigge et al., 2021). It is synthesized, stored and released from brain and eyestalk organs among decapod crustaceans (Lorendo-Ranjel et al., 2017). The neuropeptide for CHH is produced in the medulla terminalis ganglion in the X-organ and released in the sinus gland. It drives the stress response by mobilizing glucose, leading to an elevated level of glucose in the haemolymph (Lorenzon et al 2005, Fanjul-Moles 2006, Fossat et al 2014, Knigge et al., 2021).

Synthesis and Conclusion

The findings suggest that low pH affects crayfish chemosensory abilities, particularly in detecting alarm cues. At pH8, exposure to alarm odour triggered a stress response, with crayfish spending more time in dark zones and moving more slowly compared to controls. However, at pH6, their behaviour was similar to the control group, indicating a diminished response to alarm cues in acidic conditions.

Responses to manual handling were consistent across both pH levels, suggesting that this stimulus does not rely on chemical sensing and is therefore unaffected by pH changes. Physiological data supported these behavioural results—crayfish exposed to alarm odour at pH8 had elevated haemolymph glucose levels, while those at pH6 showed no significant increase. Manual handling, however, led to higher glucose levels at both pH levels, implying a stress response independent of chemosensory input.

These results highlight the potential ecological consequences of acidifying aquatic environments. The inability to detect alarm odours at low pH suggests that crayfish may struggle to recognize and respond to predatory threats, increasing their vulnerability (Briones Fourzán et al., 2008). Since alarm odour detection is crucial for adaptive anti-predator behaviour, this impairment could disrupt survival strategies, ultimately affecting

population dynamics. Additionally, the lack of an effect on manual handling responses suggests that different stressors engage distinct physiological pathways. Haemolymph glucose, known to be influenced by serotonin levels (Fossat et al., 2014), provides further insight into stress responses in crayfish. Future research should focus on the neural mechanisms underlying these changes and explore whether prolonged exposure to low pH results in long-term behavioural and physiological adaptations. Understanding these impacts is essential, as freshwater acidification continues to pose a growing challenge for aquatic ecosystems.

Chapter 5 Discussion and Scope for Further Research

This thesis explored the complex role of serotonin (5-HT) in modulating crayfish behaviour, with a particular emphasis on anxiety-like behaviour (ALB). It investigated the cross-communication between serotonergic and dopaminergic pathways, and how they can orchestrate anxiety responses in crayfish. Furthermore, the study highlights the vulnerability of this behaviour to the emerging issue of psychotropic drug pollution in freshwater ecosystems. These pharmacologically active compounds can interfere biogenic amine pathways, thereby influencing behavioural tendencies in non-target aquatic species like crayfish.

In addition, the research addresses the global issue of freshwater acidification and its detrimental impact on anti-predatory behaviour—a survival-critical trait in crayfish. By integrating findings from neurochemical and environmental manipulations, this study offers a multidimensional perspective on the influence of biogenic amines on crustacean behaviour and how external stressors may interfere with these processes.

5.1 The Role of Serotonin and Dopamine in Crayfish Behaviour

The results of this study emphasize the critical role of 5-HT in modulating ALB in crayfish, aligning with its well-established function as a neuromodulator in both vertebrates and invertebrates (Bonhomme and Esposito, 1999, Lillesaar 2011, Fossat et al., 2014, Fossat et al., 2015, Tu et al., 2020). Exogenous administration of 5-HT induced ALB, as evidenced by increased time spent in the dark and by slower walking speeds in the darker areas. The reversibility of these effects with 5-HT1 blockers indicates that specific serotonergic receptors mediate this behaviour. Interestingly, DA was also capable of inducing ALB at higher concentrations. The fact that DA blockers reversed the anxiogenic effects induced by 5-HT and vice-versa, suggests a possible interaction or overlap in the roles of 5-HT and DA in anxiety-like behaviour. Another interesting

finding was that the co-introduction of a blocker with a lower concentration of DA but not the sole injection of a low concentration of DA-led to the appearance of anxiety. This dose-dependent role of DA has previously been observed by Ibuchi and Nagayama (2021) in their study of crayfish aggression. These findings warrant further investigation, as they point towards a complex interplay of biogenic amines in crayfish neural circuits.

While the primary focus of this study was on 5-HT, the crosstalk between serotonergic and dopaminergic systems may reflect a broader neuromodulatory network governing behavioural responses in crustaceans. In vertebrates, such interactions between 5-HT and DA are well-documented, often influencing emotional states and decision-making processes (Sasaki-Adams, 2001, Fischer and Ullsperger, 2017, De Deurwaerdère et al., 2021). This study provides preliminary evidence for similar mechanisms in invertebrates, suggesting a potential evolutionary conservation of biogenic amine functions. Further research should aim to map the molecular pathways underlying this cross-communication and assess their broader behavioural implications.

5.2 Environmental Stressors and Behavioural Modulation

The thesis demonstrates how environmental factors, such as acidification and SSRI exposure, influence crayfish behaviour by interfering with chemosensory signalling (Leduc et al., 2013) and potentially disrupting biogenic amine pathways (Theodoridi et al., 2017; Cunha et al., 2018) respectively. The anxiogenic effects of Fluoxetine, an SSRI, underscore the ecological risks associated with pharmaceutical contamination. Since Fluoxetine works by causing a rise in 5-HT, it causes an increase in hemolymph glucose levels via the Crustacean Hyperglycaemic Hormone (CHH) (Santos et al., 2001)), promoting ALB in crayfish, potentially compromising their tendency to perform other essential behaviours such as foraging or courtship. These behavioural shifts in a keystone species such as crayfish may have cascading effects on aquatic ecosystems, affecting processes such as nutrient cycling and detritus breakdown (Evans-White et al., 2003,

Creed and Reed). Future studies should address the chronic impacts of SSRIs and their potential interactions with other environmental stressors, such as temperature fluctuations and hypoxia, to develop a comprehensive understanding of their ecological consequences.

The study further demonstrates how environmental factors, such as acidification and SSRI exposure, influence crayfish behaviour by potentially disrupting olfactory responses (Leduc et al., 2013). Exposure to low pH impaired crayfish responses to alarm odour, most likely due to a loss of chemosensory abilities necessary for the detection of alarm odour. This finding is consistent with previous studies indicating that acidification can disrupt olfactory perception in aquatic organisms (Brown et al., 2002, Leduc et al., 2003). The unaffected response to mechanical stimuli suggests that the central nervous system processes remain intact under acidic conditions, while only the peripheral chemosensory mechanisms are affected. This highlights the need to investigate how acidification alters the molecular structure and functionality of chemical cues and receptor sites in crayfish and other aquatic species.

The measurement of haemolymph glucose levels in stressed animals (animals exposed to manual handling, alarm odour and Fluoxetine) were consistently higher when compared to the unstressed control animals in both acidification as well as pharmacological studies, reinforcing that glucose can serve as a reliable indicator of stress in crustaceans (Fossat et al., 2014).

5.3 Comparative Insights and Evolutionary Implications

The findings of this thesis contribute to a broader understanding of the role of biogenic amines in invertebrate behaviour. Comparative studies across species could provide valuable insights into the evolutionary conservation of these pathways and their functional adaptations to different ecological contexts. For instance, do other invertebrates exhibit similar serotonin-mediated ALB responses, and how might these vary across taxa? What role does dopamine play in shaping these behavioural responses,

and could it interact with serotonin in complex ways? Furthermore, how does ALB relate to the complexity of the central nervous system in different invertebrates, and what does this reveal about the evolution of behavioural plasticity?

5.4 Limitations and Future Directions

While this thesis offers valuable insights, it is not without limitations. First, the experiments primarily relied on exogenous administration of biogenic amines. Future research should employ techniques such as genetic manipulation or in vivo monitoring of neuromodulator levels to better understand their natural roles. Second, the short-term nature of environmental stressors exposure limits our understanding of their long-term effects. Whether animals are capable of adapting to the changed condition when exposed to scenarios such as acidification when exposed for longer periods, would be interesting to study further to provide a more comprehensive picture of the ecological impact.

Additionally, the observed crosstalk between 5-HT and DA systems raises intriguing questions about their underlying mechanisms. Electrophysiological and molecular studies are needed to explore the receptor-level interactions and neural circuitry involved. Expanding the scope of research to include other behaviours, such as aggression and social interactions, could further elucidate the roles of biogenic amines in crayfish and other invertebrates.

5.5 Conclusion

Overall, this thesis demonstrates the multifaceted role of serotonin (5-HT) in crayfish behaviour and highlights their vulnerability to pharmacologically active compounds in the aquatic environment that target biogenic amine pathways. Additionally, it addresses the dose-dependent role of dopamine (DA) in crayfish anxiety and cross-communication among biogenic amines. Lastly, it explores the wider implications of acidification of aquatic ecosystems and their potential to affect anti-predatory behaviour by potentially disrupting olfactory sensing. By bridging the fields of neurobiology, biochemistry and

environmental science, it provides a foundation for future research aimed at conserving aquatic biodiversity and understanding the broader ecological implications of behavioural modulation in keystone species.

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Appendix

Raw data

Chapter 2: Role of biogenic amines Serotonin and Dopamine in anxiety-like behaviour of crayfish *Pontastacus leptodactylus*

duration	event	treatment	ac speed	carapce length mm	weight g
949.61	dark	ser10mM	0.67	52.02	66.329
827.66	dark	ser10mM	0.72	43.82	50.649
714.59	dark	ser10mM	1.09	42.12	52.074
743.61	dark	ser10mM	3.22	43.62	51.249
444.76	dark	ser10mM	4.21	24.82	34.388
667.59	dark	ser10mM	2.12	41.92	55.529
787.22	dark	ser10mM	0.42	43.62	57.929
729.1	dark	ser10mM	1.99	41.42	50.185
875.31	dark	ser10mM	3.06	45.32	53.069
543.36	dark	ser10mM	2.59	43.82	50.835
959.09	dark	ser10mM	0.07	30.22	37.98
366.32	dark	ser10mMserB	4.55	30.92	44.63
389.45	dark	ser10mMserB	2.49	49.52	63.798
496.91	dark	ser10mMserB	3.76	33.42	49.309
354.91	dark	ser10mMserB	7.25	37.62	53.53
356.54	dark	ser10mMserB	3.09	45.82	54.708
462.55	dark	ser10mMserB	4.35	42.02	50.941
440.03	dark	ser10mMserB	1.99	44.22	58.653
170.67	dark	ser10mMserB	7.09	24.12	35.609
496.41	dark	ser10mMserB	3.45	28.52	43.535
609.88	dark	ser10mMserB	3.71	34.02	52.309
293.38	dark	10serDAblocker	9.67	32.92	49.869
426.01	dark	10serDAblocker	1.29	43.42	59.249
412.63	dark	10serDAblocker	3.05	32.02	50.395
550.13	dark	10serDAblocker	3.44	45.62	65.612
313.1	dark	10serDAblocker	4.3	35.72	51.995
383.71	dark	10serDAblocker	2.37	60.12	75.9
425.67	dark	10serDAblocker	5.09	34.82	43.142
441.49	dark	10serDAblocker	1.46	34.12	49.538
441.46	dark	10serDAblocker	1.09	30.82	52.768
589.54	dark	10serDAblocker	3.12	38.62	52.43
332.78	dark	control	2.34	59.12	77.99
504.42	dark	control	4.09	38.92	53.388
446.03	dark	control	4.65	32.02	50.711
400.78	dark	control	3.07	44.32	57.649
392.15	dark	control	4.11	36.62	50.613
285.28	dark	control	1.79	21.42	22.262
133.36	dark	control	5.65	46.82	50.395

415.47	dark	control	4.12	44.02	51.99
380.16	dark	control	3.36	41.12	51.368
393.65	dark	control	2.77	50.12	57.888
361.63	dark	control	0.16	42.62	51.129
440.76	dark	control	5.24	20.92	29.052
369.29	dark	control	3.42	46.82	51.855
392.68	dark	ser1mM	0.59	29.32	31.34
504.42	dark	ser1mM	1.06	58.42	72.118
523.07	dark	ser1mM	2.21	42.02	50.331
518.94	dark	ser1mM	3.76	51.82	72.53
796.67	dark	ser1mM	0.06	43.62	50.755
738.44	dark	ser1mM	0.83	38.72	52.108
526.04	dark	ser1mM	2	45.42	50.641
824.6	dark	ser1mM	0.42	45.12	53.23
463.42	dark	ser1mM	1.79	44.12	51.663
528.58	dark	ser1mM	2.19	21.72	27.262
635.08	dark	ser1mM	0.89	42.42	51.311
584.2	dark	ser1mM	2.18	41.82	50.39
959	dark	ser1mM	0.02	18.92	20.652
419.4	dark	dop1mM	3.45	41.12	50.313
290.35	dark	dop1mM	4.29	45.82	54.39
398.89	dark	dop1mM	1.82	42.92	50.655
388.84	dark	dop1mM	0.49	40.12	50.524
399.89	dark	dop1mM	3.67	41.82	50.798
395.41	dark	dop1mM	4.1	40.12	50.69
361.63	dark	dop1mM	2.5	43.92	52.088
18.32	dark	dop1mM	11.2	44.52	52.319
114.95	dark	dop1mM	1.42	43.02	50.885
159.25	dark	dop1mM	3.17	45.82	52.548
155.98	dark	dop1mM	6.62	52.02	63.155
484.9	dark	dop1mM	1.07	34.72	50.434
461.74	dark	dop1mM	0.29	40.92	49.889
484.9	dark	dop1mM	3.14	43.62	54.31
901.7	dark	dop10mM	0.67	42.52	50.469
904.11	dark	dop10mM	2.44	37.92	50.799
794.67	dark	dop10mM	1.65	46.32	55.589
881.38	dark	dop10mM	0.34	42.62	50.743
575.09	dark	dop10mM	2.06	42.02	51.141
908.21	dark	dop10mM	1.07	24.02	32.37
552.56	dark	dop10mM	0.78	22.02	35.874
831.6	dark	dop10mM	2.44	58.02	78.368
727.11	dark	dop10mM	3.45	43.62	50.666
952.65	dark	dop10mM	1.07	45.42	51.284
924.99	dark	dop10mM	0.01	43.82	53.708
959.09	dark	dop10mM	0.41	40.32	50.07
939.11	dark	dop10mM	1.33	42.42	51.998
543.36	dark	dop10mM	2.46	43.92	50.849
424.07	dark	10DopDAblocker	1.62	46.42	53.632

362.75	dark	10DopDAblocker	2.42	40.92	50.358
382.13	dark	10DopDAblocker	4.09	42.42	52.129
512.09	dark	10DopDAblocker	5.51	42.82	53.148
446.47	dark	10DopDAblocker	3.44	45.02	53.282
554.26	dark	10DopDAblocker	1.74	43.62	51.101
664.54	dark	10DopDAblocker	3.21	19.62	23.363
379.2	dark	10DopDAblocker	2.79	46.12	51.786
444.27	dark	10DopDAblocker	5.63	18.02	24.309
405.29	dark	10DopDAblocker	7.79	43.92	51.18
521.2	dark	10Dopserblocker	2.49	31.92	50.406
496.54	dark	10Dopserblocker	3.66	23.02	35.029
490.71	dark	10Dopserblocker	7.02	36.92	50.775
489.64	dark	10Dopserblocker	1.6	42.32	50.101
310.5	dark	10Dopserblocker	2.21	42.62	50.628
549.3	dark	10Dopserblocker	9.09	43.42	52.309
442.73	dark	10Dopserblocker	1.48	30.02	43.695
453.91	dark	10Dopserblocker	4.21	41.72	51.008
503.62	dark	10Dopserblocker	3.69	44.12	52.09
407.33	dark	10Dopserblocker	4.05	43.82	50.69
171.27	dark	1DopSerblocker	1.07	43.02	50.159
343.39	dark	1DopSerblocker	2.25	45.42	55.749
383.87	dark	1DopSerblocker	5.43	42.02	50.919
560.1	dark	1DopSerblocker	2.97	45.32	50.611
494.78	dark	1DopSerblocker	6.67	58.62	74.048
582.8	dark	1DopSerblocker	2.34	42.82	50.695
250.64	dark	1DopSerblocker	3.83	44.12	51.39
498.25	dark	1DopSerblocker	7.32	43.52	51.029
450.6	dark	1DopSerblocker	4.71	42.02	50.508
477.8	dark	1DopSerblocker	2.03	46.42	50.63
946.35	dark	1DopDopblocker	0.81	30.22	38.96
261.92	dark	1DopDopblocker	2.17	43.62	50.848
924.46	dark	1DopDopblocker	1.04	25.82	32.292
607.29	dark	1DopDopblocker	2.01	43.12	50.215
628.97	dark	1DopDopblocker	0.39	44.02	50.874
441.83	dark	1DopDopblocker	5.17	45.82	52.476
450.6	dark	1DopDopblocker	6.14	42.12	51.656
477.8	dark	1DopDopblocker	2.78	41.82	50.7
894.03	dark	1DopDopblocker	1.65	21.02	22.413
516.53	dark	1DopDopblocker	2.49	42.92	50.88

Chapter 3: Effect of Fluoxetine on anxiety-like behaviour in crayfish *Pontastacus leptodactylus*

Behaviour data

duration	speed	treatment	zone	weight g	carapace length mm
35.97	7.67	control	dark	80.518	55.6
328.82	2.46	control	dark	64.838	47.4
312.6	3.71	control	dark	67.258	45.7
366.49	2.56	control	dark	65.024	47.2
264.46	4.18	control	dark	58.169	28.4
315.24	2.43	control	dark	58.819	45.5
165.9	4.49	control	dark	77.987	47.2
299.32	3.85	control	dark	63.498	45
358.42	3.12	control	dark	64.584	48.9
565.75	2.54	control	dark	79.801	47.4
461.51	2.78	control	dark	66.184	33.8
549.7	1.779	0.56 µg/L	dark	78.089	34.5
433.62	2.56	0.56 µg/L	dark	57.331	53.1
485	3.79	0.56 µg/L	dark	63.727	37
490.64	4.67	0.56 µg/L	dark	66.957	41.2
480.23	1.01	0.56 µg/L	dark	66.619	49.4
654.2	1.63	0.56 µg/L	dark	92.179	45.6
478.26	2.07	0.56 µg/L	dark	36.451	47.8
892.89	0.862	0.56 µg/L	dark	64.584	27.7
503.92	1.31	0.56 µg/L	dark	66.179	32.1
386.17	2.15	0.56 µg/L	dark	65.557	37.6
565.75	2.09	0.56 µg/L	dark	72.077	36.5
786.46	0.71	0.56 µg/L	dark	65.318	47
446.76	2.67	0.28 µg/L	dark	59.241	35.6
481.06	1.7	0.28 µg/L	dark	66.044	49.2
506.86	2.57	0.28 µg/L	dark	49.529	39.3
425.71	1.34	0.28 µg/L	dark	86.307	63.7
418.5	3.78	0.28 µg/L	dark	64.52	38.4
459.18	3.49	0.28 µg/L	dark	86.719	37.7
464.35	4.76	0.28 µg/L	dark	64.944	34.4
431.18	1.59	0.28 µg/L	dark	66.297	42.2
510.39	1.07	0.28 µg/L	dark	64.83	62.7
482.67	2.39	0.28 µg/L	dark	67.419	42.5
480.36	2.41	0.28 µg/L	dark	65.852	35.6
680.22	1.02	1.12 µg/L	dark	41.451	47.9
476.03	2.11	1.12 µg/L	dark	65.5	40.2
720.06	0.67	1.12 µg/L	dark	64.579	25

710.08	0.76	1.12 µg/L	dark	44.841	50.4
729.67	1.92	1.12 µg/L	dark	64.502	47.6
423.07	1.58	1.12 µg/L	dark	68.579	44.7
914.98	0.02	1.12 µg/L	dark	77.344	53.7
959.09	0.38	1.12 µg/L	dark	64.623	46.2
760.44	1.11	1.12 µg/L	dark	64.078	24.5
588.53	2.01	1.12 µg/L	dark	68.499	50.4

Glucose data

Treatment	**Glucose mg/dl**	**weight g**	**carapace length mm**
0.00 µg/L	25.1	64.658	32.9
0.00 µg/L	19.2	64.988	62
0.00 µg/L	33.6	69.778	45.6
0.00 µg/L	18.8	64.932	55.4
0.00 µg/L	19.7	65.33	47.2
0.00 µg/L	21.22	46.559	42.3
0.00 µg/L	20.01	56.063	49
0.28 µg/L	31.27	72.557	48.7
0.28 µg/L	29.67	64.855	47.7
0.28 µg/L	24.73	65.473	25.3
0.28 µg/L	41.06	66.318	46
0.28 µg/L	26.63	67.337	45.4
0.28 µg/L	33.78	67.471	22.5
0.28 µg/L	24.01	65.29	44.7
0.56 µg/L	42.64	27.552	49.4
0.56 µg/L	36.59	65.975	46.5
0.56 µg/L	40.07	43.498	43.7
0.56 µg/L	28.65	65.369	45.4
0.56 µg/L	18.78	64.595	43.7
0.56 µg/L	43.2	54.218	47.5
0.56 µg/L	34.11	64.8	48.1
1.12 µg/L	34.67	78.237	46.6
1.12 µg/L	31.39	66.665	49.4
1.12 µg/L	26.78	65.845	55.6
1.12 µg/L	44.61	64.889	38.3
1.12 µg/L	43.39	36.602	44.5
1.12 µg/L	38.64	65.069	47.2
1.12 µg/L	33.58	57.791	46.1

Chapter 4: Acidification and Its Effects on Anti-Predatory Behaviour

Behaviour data

duration (s)	event	treatment	pH	speed (cm/s)	weight g	carapce length mm
471.37	dark	Control	eight	2.52	52.8	55.6
725.77	dark	Control	eight	3.225	44.6	47.4
798.24	dark	Control	eight	2.56	42.9	45.7
469.22	dark	Control	eight	3.05	44.4	47.2
451.47	dark	Control	eight	2.71	25.6	28.4
492.54	dark	Control	eight	2.23	42.7	45.5
524.86	dark	Control	eight	3.74	44.4	47.2
473.06	dark	Control	eight	2.42	42.2	45
386.38	dark	Control	eight	2.76	46.1	48.9
431.36	dark	Control	eight	2.35	44.6	47.4
571.84	dark	Control	eight	2.21	31	33.8
390.81	dark	Control	eight	2.22	31.7	34.5
761.24	dark	Control	eight	2.59	50.3	53.1
689.96	dark	Control	eight	2.03	34.2	37
387.94	dark	Control	eight	3.28	38.4	41.2
452.47	dark	Control	eight	2.56	46.6	49.4
482.7	dark	Control	eight	2.45	42.8	45.6
904.67	dark	Alarm odour	eight	0.925	same as control	same as control
293.8	dark	Alarm odour	eight	1.8	same as control	same as control
799.4	dark	Alarm odour	eight	2.42	same as control	same as control
584.03	dark	Alarm odour	eight	2.32	same as control	same as control
781.36	dark	Alarm odour	eight	2.175	same as control	same as control
618.6	dark	Alarm odour	eight	1.6	same as control	same as control
440.89	dark	Alarm odour	eight	1.45	same as control	same as control
585.3	dark	Alarm odour	eight	1.64	same as control	same as control
633.32	dark	Alarm odour	eight	2.05	same as control	same as control
814.45	dark	Alarm odour	eight	2.2	same as control	same as control
874.61	dark	Alarm odour	eight	1.24	same as control	same as control
748.85	dark	Alarm odour	eight	1.95	same as control	same as control

621.13	dark	Alarm odour	eight	1.48	same as control	same as control
503.75	dark	Alarm odour	eight	1.86	same as control	same as control
550.6	dark	Alarm odour	eight	2.09	same as control	same as control
689.96	dark	Alarm odour	eight	0.79	same as control	same as control
600.72	dark	Alarm odour	eight	1.65	same as control	same as control
583.16	dark	Handling	eight	1.74	45.1	47.9
774.95	dark	Handling	eight	0.93	37.4	40.2
438.5	dark	Handling	eight	2.3	22.2	25
692.27	dark	Handling	eight	1.54	47.6	50.4
855.85	dark	Handling	eight	1.84	44.8	47.6
693.07	dark	Handling	eight	1.6	41.9	44.7
791.93	dark	Handling	eight	1.46	50.9	53.7
903.77	dark	Handling	eight	1.68	43.4	46.2
798.49	dark	Handling	eight	2.16	21.7	24.5
504.95	dark	Handling	eight	2.35	47.6	50.4
646.39	dark	Handling	eight	1.84	30.1	32.9
768.12	dark	Handling	eight	0.95	59.2	62
798.37	dark	Handling	eight	1.28	42.8	45.6
786.15	dark	Handling	eight	1.97	52.6	55.4
235.99	dark	Control	six	2.56	42.6	21.7
894.69	dark	Control	six	3.44	22.5	42.4
196.66	dark	Control	six	3.44	26.9	41.8
761.46	dark	Control	six	1.73	32.4	18.9
489.62	dark	Control	six	1.73	31.3	41.1
387.12	dark	Control	six	3.26	41.8	45.8
592.39	dark	Control	six	1.85	30.4	42.9
406.04	dark	Control	six	4.88	44	40.1
627.27	dark	Control	six	2.45	34.1	41.8
378.84	dark	Control	six	1.38	58.5	40.1
450.3	dark	Control	six	2.31	33.2	43.9
325.47	dark	Control	six	1.44	42	44.5
292.02	dark	Control	six	2	37.1	43
484.03	dark	Alarm odour	six	2.66	same as control	same as control
590.37	dark	Alarm odour	six	3.68	same as control	same as control
196.66	dark	Alarm odour	six	2.68	same as control	same as control
522.55	dark	Alarm odour	six	2.72	same as control	same as control
285.91	dark	Alarm odour	six	3.21	same as control	same as control

299.59	dark	Alarm odour	six	4.08	same as control	same as control
473.37	dark	Alarm odour	six	2.02	same as control	same as control
458.45	dark	Alarm odour	six	2.05	same as control	same as control
419.15	dark	Alarm odour	six	2.22	same as control	same as control
415.9	dark	Alarm odour	six	2.76	same as control	same as control
774.95	dark	Alarm odour	six	3.14	same as control	same as control
17.08	dark	Alarm odour	six	4.66	same as control	same as control
292.92	dark	Alarm odour	six	2.71	same as control	same as control
789.36	dark	Handling	six	1.84	same as control	same as control
644.12	dark	Handling	six	1.56	43.8	45.8
710.65	dark	Handling	six	1.58	43.5	52
601.37	dark	Handling	six	2.24	42.5	34.7
703.64	dark	Handling	six	0.78	20.1	40.9
579.38	dark	Handling	six	2.24	40.8	43.6
760.69	dark	Handling	six	0.92	40.2	42.5
727.23	dark	Handling	six	1.8	17.3	37.9
738.65	dark	Handling	six	1.08	39.5	46.3
768.15	dark	Handling	six	1.7	44.2	42.6
873.41	dark	Handling	six	3.08	41.3	42
805.88	dark	Handling	six	0.86	42.3	24
505.67	dark	Handling	six	1.8	42.9	22

Glucose data

Treatment	pH	Glucose (mg/dl)	Weight g	Carapace length mm
Control	eight	27.14	43.2	47.8
Control	eight	14.36	43.6	27.7
Control	eight	44.13	45.8	32.1
Control	eight	17.71	44.4	37.6
Control	eight	13.85	20.4	36.5
Control	eight	14.04	46.9	47
Control	eight	15.77	18.8	35.6
Control	eight	18.96	44.7	49.2

Alarm odour	eight	18.5	32.7	39.3
Alarm odour	eight	35.6	23.8	63.7
Alarm odour	eight	38.59	37.7	38.4
Alarm odour	eight	51.28	43.1	37.7
Alarm odour	eight	41.02	43.4	34.4
Alarm odour	eight	37.19	44.2	42.2
Handling	eight	45.68	34.9	62.7
Handling	eight	24.33	31.6	42.5
Handling	eight	41.32	39.4	35.6
Handling	eight	31.35	59.9	47.2
Handling	eight	38.64	39.7	42.3
Handling	eight	36.07	32.8	49
Handling	eight	32.14	33.6	48.7
Handling	eight	37.61	37.9	47.7
Control	six	14.85	41.4	58
Control	six	18.92	44.2	43.6
Control	six	30.11	50.4	45.4
Control	six	35.25	33.1	43.8
Control	six	20	39.3	40.3
Control	six	26.8	42	42.4
Control	six	19.16	40.9	43.9
Control	six	18.04	36.3	46.4
Alarm odour	six	22.42	44.7	40.9
Alarm odour	six	18.91	41	42.4
Alarm odour	six	15.4	40.4	42.8
Alarm odour	six	14.2	22.4	45
Alarm odour	six	13.86	20.4	43.6
Alarm odour	six	14.37	56.4	19.6
Handling	six	28.11	42	46.1
Handling	six	35.2	43.8	18
Handling	six	18.6	42.2	43.9
Handling	six	39.6	38.7	31.9
Handling	six	44.97	40.8	23

Handling	six	31.62	42.3	36.9
Handling	six	39.01	44.8	42.3
Handling	six	31.61	39.3	42.6