How does the presence of macroplastic impact the reproductive behaviour and fecundity of Zebrafish (*Danio rerio*)?

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Submitted to Swansea University in fulfilment of the requirements for the Degree of MRes Biosciences

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Abstract

Plastic pollution is emerging as a growing environmental problem worldwide, with plastic waste entering our seas and rivers at an unprecedented rate. However, most plastic waste is stored in river systems, with an estimated influx of 0.8 million tonnes entering 84% of rivers worldwide, a number that is expected to triple by 2060. The current research into the impacts of macroplastics on freshwater species mainly looks at threats such as ingestion and entanglement, however this study is one of the first to look at the impacts on reproductive behaviours and fecundity. A change in reproductive behaviours can impact spawning success, thus with plastic being deposited on the bottom sediment where most species forage and spawn, this can alter the distribution of species. A consistent change in behaviours over a prolonged period of time can be inherited by offspring as personality traits. This suggests that as the plastic problem grows, future generations will have adapted to the changing environment by inheriting the most resilient personality traits through behavioural selection. Here we show that zebrafish (*Danio rerio*) exhibited changes in their reproductive behaviours in the presence of macroplastic, with benthic plastic having the most negative impact on behaviour and egg survival rates. Individuals presenting bold behavioural syndromes reproduced more successfully and had a higher egg survival rate compared to individuals displaying timid behaviours, which implicates future natural selection that favours bolder fish. This research suggests that freshwater ecosystems with a higher amount of macroplastic pollution will see detrimental impacts to population structure of the species inhabiting these environments.

Lay Summary

Zebrafish are a widely studied species that inhabit freshwater ecosystems such as rivers, streams, and ponds. Plastic pollution of these systems is a growing problem, as approximately 0.8 million tonnes of plastic is entering 84% of rivers worldwide, and this number is expected to triple by 2060. Freshwater systems act as a transport to the oceans, including pollution and the dangers that come with it. This impacts all species inhabiting these environments, not just because plastic can be ingested and cause entanglement, but also because it can alter the behaviour of species over time. Here we looked at zebrafish that showed both timid and bold behaviours and how these behaviours changed when two types of plastic were present, benthic (sunken) and floating plastic. Benthic plastic had the most negative effect on their reproductive behaviours. These changes in behaviours impacted all stages of their courtship and reproduction, which lead to a decrease in egg survival. We found that zebrafish displaying bold behaviours reproduced more successfully than zebrafish displaying timid behaviours, and they also had a higher rate of egg survival, yet this was still a lot lower than zebrafish that were not exposed to the plastic. As bolder fish reproduce more successfully in the presence of plastic, this means that zebrafish inhabiting freshwater systems that are polluted by plastic are the most likely to pass on these behaviours to future offspring. This highlights the need for a raised awareness of the plastic pollution problem, as impacts to one species will impact all species in the food web, leading to catastrophic outcomes for our planet's waterways.

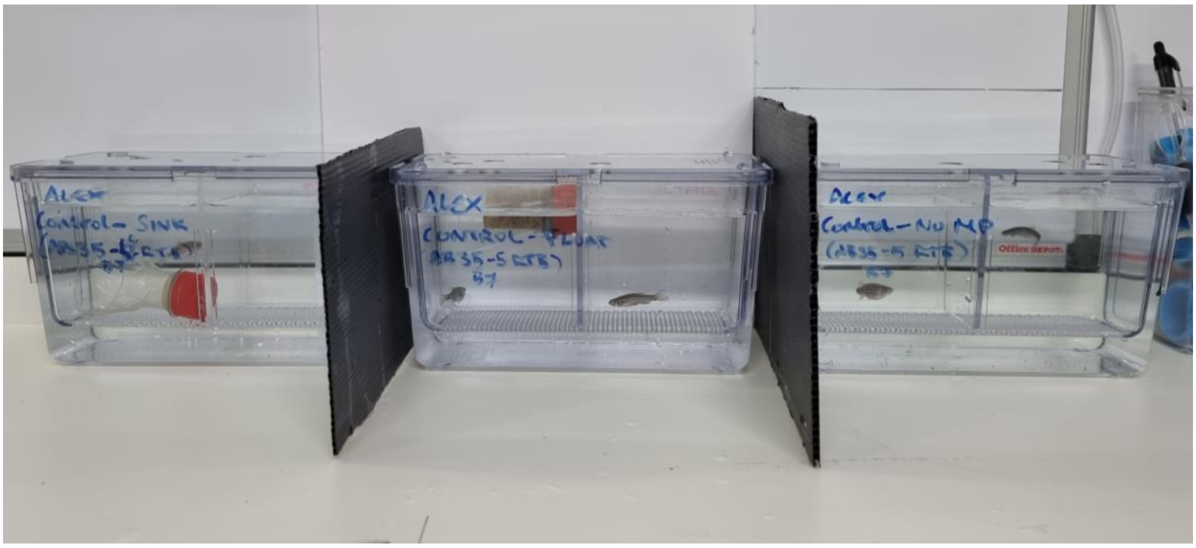

Figure 1. Example of the experimental set up of a male and female from the control behavioural selection line with benthic, floating and control plastic treatments.

Declarations

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed......[redacted]

Date.....12/06/2023..

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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I hereby give consent for my thesis, if accepted, to be available for electronic sharing

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The University's ethical procedures have been followed and, where appropriate, that ethical approval has been granted.

Signed....[redacted]

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Statement of Expenditure

Student Name: Alexandra Chand

Student Number: [redacted]

Project Title: How does the presence of macroplastic impact the reproductive behaviour and fecundity of zebrafish (*Danio rerio*)?

Category	Item	Description	Cost
Facilities	CSAR Lab Use	22 days of facility use including tanks, fish and equipment (clicker counter, net, bleach solution).	£100/day (approx.)
Software	GraphPad Prism 9	One year subscription to data analysis software.	£102.31
Total			£2,302.31

I hereby certify that the above information is true and correct to the best of my knowledge.

Signed,

[redacted] [redacted]

Signature (supervisor) Signature (student)

Statement of Contributions

Contributor role	Persons involved
Conceptualisation	FJH
Data Curation	EP
Formal Analysis	EP
Funding Aquisition	NE
Investigation	FJH
Methodology	FJH
Project Administration	FJH
Resources	FJH, RS, CH
Software	EP
Supervision	FJH, NE, EP
Validation	NE
Visualisation	NE, EP
Writing - Original draft preparation	NE
Writing – Review & editing	NE, EP

CH – Craig House

EP - Dr Ed Pope

FJH - Dr Fraser Januchowski-Hartley

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Ethics Approval

Project Ethics Assessment Confirmation|Cadarnhad o Asesiad Moeseg Prosiect

Retention: Exchange Retention Policy

C

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Wed 09/06/2021 16:16

This is an automated confirmation email for the following project. The Ethics Assessment status of this project is: APPROVED

Applicant Name: Alexandra Chand
Project Title: The effects of macro-plastic on reproductive potential in Zebrafish
Project Start Date: 01/04/2021
Project Duration: 6 Months
Approval No: SU-Ethics-Student-090621/3774

NOTE: This notice of ethical approval does not cover aspects relating to Health and Safety. Please complete any relevant risk assessments prior to commencing with your project.

Ethics:AWERB Review Form|Moeseg: Ffurflen Adolygiad AWERB

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To: Hartley F.A.
Cc: [redacted]

Wed 09/06/2021 16:16

Your application: Ethics:AWERB Review Form has been reviewed by College Ethics Committee/AWERB Group and it's status indicates as Approved Proposal.

Please ensure to use the link below to view 'AWERB APPROVAL NOTIFICATION AND IP REFERENCE NUMBER'.

Comments:
The AWERB committee approve this application on the condition that the plastics used are not soft enough for the fish to ingest and that all hard plastic items are checked for rough or sharp edges before being used (09/06/21).

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Acknowledgements

Thank you to Dr Nicole Esteban for being my guiding light and pushing me to be the best that I can be. Thank you to Dr Ed Pope for being my statistical rabbi and keeping me inspired (and caffeinated). Also, a big thanks to the wonderful staff at CSAR for allowing me to use their brilliant facilities and providing some comic relief when I needed it.

To my best friends in the entire world, Jasmine Knight and Molly-Jane Eley, for being my emotional support emergency responders and to my partner Jose Daniel Gallardo for his unwavering belief in me, I cannot thank you enough. Most of all, a massive thankyou to my mum Joy for supporting me through my university journey, all of the ups and downs that came with it, and for reminding me to never give up. I wouldn't be where I am now without all of you, and I am eternally grateful.

"If you trust in yourself… and believe in your dreams… and follow your star… you'll still get beaten by people who spent their time working hard and learning things and weren't so lazy"
– Sir Terry Pratchett

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CSAR – Centre for Sustainable Aquatic Research

PET – Polyethylene terephthalate

B[a]P – Benzo[a]pyrene

1.0 Introduction

Plastic pollution is emerging as a growing environmental problem worldwide, with plastic waste being disposed of and deposited in our seas and rivers both directly and indirectly over several decades (Faure *et al*., 2015; Lechthaler *et al*., 2020). A 2023 study estimated that in 2015 an annual influx of 0.8 million tonnes of plastic was entering 84% of rivers worldwide, with this amount expected to triple by 2060 (Nyberg *et al*., 2023). River systems act as a transport network to the oceans, with an estimated amount between 0.41 and 8 million tonnes of plastic waste being deposited annually (Nyberg *et al*., 2023). However, most plastic waste is stored in river systems (van Emmerick *et al*., 2022), with under 2% of global plastic waste being deposited into marine environments (Meijer *et al*., 2021). Between 1961 and 2017, 25.3 million tonnes of macroplastic entered the oceans via rivers and the fisheries industry, yet this is only 4.7% of today's global plastic waste (Isobe & Iwasaki, 2022).

Macroplastics have been recently defined as having a diameter greater than or equal to five mm (Lechthaler *et al*., 2020; Karpova *et al*., 2022). The obvious hazard of macroplastics being present in freshwater systems is a physical one, as large items of plastic can lead to accidental harm by ingestion, entanglement or entrapment (Rochman, 2013). A recent study conducted across three cities around the Paraná river in Argentina evaluated 90 separate cases of interactions between freshwater species and macroplastic (Blettler & Mitchell, 2021). 33% of the 44 species documented were cases of entanglement/entrapment, 60% of which were fatal. 72.2% of these interactions were birds, whilst 8.9% were fish species, with remaining encounters being between mammals, reptilians, and invertebrates (4.4%, 5.6% and 8.9%, respectively) (Blettler & Mitchell, 2021).

Macroplastic can remain in a freshwater system for up to four decades in a reoccurring cycle of entering the system, being deposited on the sediment and then remobilising (Lechthaler *et al*., 2020). The entry of plastic into the freshwater environment is only the beginning of the plastic life cycle, progressing to the breakdown of large debris into smaller particles that are more easily ingested, after which bioaccumulation can occur, as well as the leaching of harmful chemicals (Rochman, 2013). This study focusses on the effects of early life plastic, as there is a noticeable gap in the knowledge between macro and microplastic. Microplastics are defined as having a diameter between one and five mm (Bhatt & Chuahan, 2023).

1.1 Macroplastic pollution

It has been shown that freshwater ecosystems are declining in resilience due to the growing problem of plastic pollution, as its presence is impacting the food web and therefore creating a cascade of unprecedented environmental issues, such as eutrophication (Karpova *et al*., 2022). Eutrophication happens when an influx of nutrients such as phosphorous and nitrogen enter a freshwater system, resulting in the acceleration of phytoplankton production and subsequent decrease in oxygen levels and water quality, impacting all species inhabiting that ecosystem (Geletu, 2023). Through the process of plastic degradation, plastic particles become suspended in the water column and enter the bottom sediments, with research showing that increased plastic deposits are causing resuspension of nutrients in the bottom sediments, which can accelerate eutrophication (Zhang *et al*., 2019). A 2019 study on the effect of eutrophication on adult carp (*Cyprinus carpio*) simulated the stages of eutrophication in a tank designed to mimic their natural environment (Huang *et al*., 2019). Throughout the study, the carp exhibited short term avoidance, swam slower due to onset hypoxia and stopped responding to external stimuli before they suspended movement completely, sank to the bottom and died (Huang *et al*., 2019).

However, research into behavioural effects in the presence of macroplastic is lacking. Research into the effects of ingested microplastic on male guppies (*Poecilia reticulata*) found that when a small percentage of microplastics were mixed with their dry food, there was a substantial impact to their reproductive behaviour and survival (Rahman *et al*., 2022). Courting displays were significantly reduced, as well as producing significantly lower amounts of sperm and ultimately a decreased survival rate (Rahman *et al*., 2022).

A change in reproductive behaviours can impact spawning success and cause later stage problems with fecundity (Blettler *et al*., 2018), thus with plastic being deposited on the bottom sediment where most species forage and spawn (Bukola *et al*., 2015), this can alter the distribution of species. A study conducted in Vietnam along the Mekong river (Karpova *et al*., 2022) found that macroplastic deposited on the bottom sediment had a negative impact on the abundance and behaviour of benthic families such as decapods and drums (family *Sciaenidae*). Plastic pieces containing anthropogenic food particles attracted species such as anchovy (family *Engraulidae*), changing typical feeding behaviours and affecting grazing processes (Karpova *et al*., 2022). A change in behaviours over a prolonged period of time can be inherited by offspring as personality traits/phenotypes, meaning that as the plastic problem grows, by means of behavioural selection, future generations will have adapted to the changing environment by inheriting the most resilient personality traits (Sbragaglia *et al*., 2019).

Investigation into plastic pollution in oceans is becoming a much more widespread research effort, whereas the focus on effects plastic waste is having on freshwater systems is fairly diminished by comparison (Blettler *et al*., 2017).

1.2 Behavioural Response

A behavioural response is typically the first way an animal reacts to a change in its environment, as it can increase the chances of survival and successful reproduction (Wong & Candolin, 2015). Behavioural responses that lead to successful reproduction or predator avoidance for example, are more likely to be passed on to offspring and facilitate adaption to novel environments (Wong & Candolin, 2015). Animals can adapt to natural changes in the environment through genetic change over many generations, however the rapid anthropogenic changes we are experiencing require phenotypic plasticity, as survival may depend on an immediate change in behaviour (Hendry *et al*., 2008). The 2008 study found that phenotypic changes in response to an anthropogenically altered environment were greater than those in response to naturally varying environment (Hendry *et al*., 2008). A later study on the impact of polyethylene plastics in common carp juveniles found that the behaviour of the test subjects changed in the presence of plastics, including macro, micro and nano plastics. The fish showed abnormal swimming patterns, such as vertical swimming due to loss of movement coordination, as well as higher respiration rates (Hamed *et al*., 2022).

1.3 Zebrafish

Zebrafish typically inhabit slow-moving bodies of water such as rivers, streams, ponds and rice fields across South Asia (Aleström *et al*., 2020), mostly located around the Brahmaptura and Ganges river basins (Spence *et al*., 2008). *Danio rerio* make an excellent model species due to their small size (average 3.5 ± 0.2 cm) which makes them easier to house, short life cycle, which makes developmental stages easier to identify, and high fecundity which allows for efficient reproductive studies (Bhagat *et al*., 2020). They show robust responses to stress and are highly adaptable animals which minimises adverse reactions during behavioural studies (Egan *et al*., 2009).

They spawn frequently and produce large quantities of eggs, with females being able to lay over 700 eggs in one spawning event. The eggs are also relatively large, with a diameter of around 0.7mm when fertilised. Embryos are external and transparent, meaning that studying their viability is feasible without the need for a microscope, reducing inflicted stress as well as rendering them perfect subjects for determining factors that impact reproductive behaviours and fecundity (Bhagat *et al*., 2020, Spence *et al*., 2008). Zebrafish are a perfect model vertebrate, making them a commonly studied species used in environmental effect studies due to their short lifecycle, the ease of assessing developmental stages, and being small, robust animals (Egan *et al.*, 2009).

1.4 Reproductive Behaviour

In the wild, zebrafish spawning behaviours tend to commence at dawn (Nasiadka & Clark, 2012). This has also been seen in laboratory settings in the early hours of an artificial circadian light cycle, with zebrafish achieving most effective reproduction when the females have been separated from the male until the lights are turned on at sunrise (Tsang *et al.*, 2017). In a laboratory setting zebrafish tend to reach sexual maturity during the third month post-hatch, with males reaching the peak of their average sperm cell count at around 10 months old. However, spawning has been observed in fish as young as 2.5 months (Tsang *et al.*, 2017). In older individuals, eggs deteriorate in quality and fewer eggs are released during spawning when they reach approximately 1.5 years old, which is why the optimal time for observing reproductive behaviours is between 6 months and 1 year for natural, uninduced mating (Nasiadka & Clark, 2012).

There are different stages when it comes to reproductive behaviour in zebrafish. The first stage, termed initiation, involves a period where the male will chase and follow the female; biting, nudging or tail-nose touching, where the male will touch the female's tail or side with his nose/head; receptive/rejective stage where the female will either allow the male to court her by remaining at his side, or not follow and show avoidance behaviour; spawning stage, where if the male has been successful during courtship he will oscillate his tail against the side of the female to trigger oviposition and simultaneous sperm release (Darrow & Harris, 2004; Spence *et al.*, 2008; Ribas & Piferrer, 2014). Changing environmental conditions can impact the different stages of zebrafish reproductive behaviour, for example elevated CO_2 levels can be detrimental to behavioural responses such as following and biting/nudging as well as egg production (Thomas & Kumar, 2023). These courtship behaviours are sensitive to other changes in the environment such as turbidity after heavy rainfall and hypoxia due to eutrophication (Thomas & Kumar, 2023).

Current research primarily focusses on marine environments and microplastics (Blettler *et al*., 2018). Microplastic may influence survival, growth and reproductive traits of fish species. For example, microplastic ingestion by another model fish species, guppy (Poecilia reticulata) caused increased mortality, shorter standard length, smaller body, lower number of sperm bundles and sigmoid displays and decreased sexual interest (Rahman *et al*. 2022). Deterioration in reproductive function occurred in zebrafish exposed to nanoplastics as evidenced by nanoplastics accumulation in gonads and pronounced behaviour alterations (e.g., aggressiveness, shoal formation, predatory avoidance behaviour) (Sarasamma *et al*. 2020). The effects that the presence of macroplastic has on the reproductive behaviour of fish are relatively unknown, as there is little research into this specific topic (Blettler *et al*., 2018).

1.5 Personality Phenotypes

When an individual exhibits consistent and distinctive behaviours over time, due to either environmental factors or genetics or a combination of both, these are then classed as personality traits (Ariyomo *et al*., 2013; Vargas *et al*., 2018). These traits influence the fitness of the individual, and how they adapt to changing environmental conditions and stressors such as predation, which in turn impacts reproductive success through natural selection (Vargas *et al*., 2018; Khan & Echevarria, 2017). Personalities can then be passed down to offspring, and it has been shown that the mother's phenotype is of importance as it can influence the phenotype of her offspring and how they react to their environment, which differs from their inherited genetics (Ariyomo *et al*., 2013). A 2018 study on the effects of personality on the reproductive success of zebrafish found that the offspring of individuals that had bolder personalities, such as a willingness to explore novel environments and take higher risks, had a high growth rate and longer survival due to having increased access to resources (Vargas *et al*., 2018). Boldness also influences social rank, as this personality type tends to have greater success in competing for resources and acquiring mates, thus leading to a higher egg fertilisation rate than that of zebrafish exhibiting shy personalities (Khan & Echevarria, 2017).

1.6 Objectives of the study

This study aims to provide increased understanding of the impact of macroplastic debris on zebrafish behaviour with a focus on courtship behaviours and reproductive success, and how it may interact with behavioural selection. Advancing our understanding of which types of plastic are important, and in what ways they may influence reproductive success (e.g., by scavenging eggs from the water column once laid or by barring access to mates) will fill current knowledge gaps on the influence of plastics in the environment. By testing different personality phenotypes, we expect to provide insights into how other species of fish that undergo fisheries behavioural selection pressures (as has recently been shown in northern pike, *Esox Lucius* (Monk *et al*., 2021)) may respond to plastics in their environment.

The aims of the experiment were to examine the impacts of macroplastic on zebrafish reproductive behaviours and fecundity, including behavioural phenotypes (timid and bold). Understanding of a possible effect on behaviour caused by the presence of macroplastic could give insight as to whether present and future population structures of zebrafish will be changed by plastic pollution. These factors influencing personality phenotype will also provide an insight into zebrafish psychology, and how boldness may impact fecundity and reproductive rates in wild zebrafish populations. The hypotheses of this study are therefore that stress caused by the presence of macroplastic differentially inhibits reproductive behaviours and fecundity causing (a) reduced courtship behaviour, (b) reduced egg production and (c) lower egg survival rates, and that (d) reproductive behaviour shifts may be personality dependent.

2.0 Materials and Methods

An AB wild strain of zebrafish (*Danio rerio*) housed in the Centre for Sustainable Aquatic Research at Swansea University in Wales, (here after written as CSAR) were previously tested and categorised into selection lines according to their presented behavioural syndromes; bold and timid. Three generations of behavioural selection were completed, thus a bold-shy spectrum across the population had already been established. Group AB32 displayed bold behaviours, AB34 timid behaviours and AB35 were categorised as the control group as they had not undergone testing for behavioural syndromes.

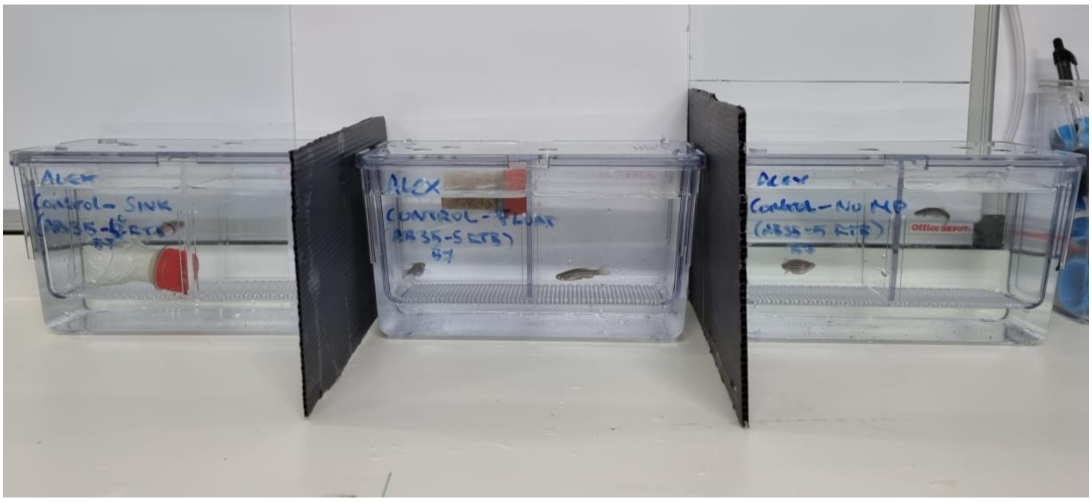

Figure 1. Example of the experimental set up of a male and female from the control behavioural selection line with benthic, floating and control plastic treatments.

In August 2021, three identical containers (top: length 22cm by 10cm width, bottom: length 20cm by 9cm width, volume: 1.95 litres) were filled with dechlorinated water from the recirculating system in CSAR to test two treatments; (1) floating plastic, (2) benthic plastic. A third container was a control with zero plastic. The plastic segments used in both treatments were cut to identical sizes and shapes (7.5 cm by 5.5 cm) from small Coca Cola™ bottles. All cut edges were sanded down to avoid causing any injuries to the fish. A cork was added to one piece to enable it to float for treatment 1. All plastic pieces and corks were boiled before experimentation commenced, and then further sterilised by dipping in a 5% bleach solution and rinsing prior to and in between uses.

Fish that were chosen for the trials were hatched in November 2020 so that they were 9 months old, as most successful spawning is achieved in the first 12 months of the lifecycle (Nasiadka & Clark, 2012). From each selection line, one male and one female were sexed and removed from their tanks and placed in heterosexual breeding pairs into the testing containers with the appropriate treatment before sundown. The individuals selected were those that were healthy and swimming around the tank. Selected females had slightly swollen abdomens, showing presence of eggs in attempt to reduce study limitations. The containers were fitted with a mesh insert to allow any eggs produced to settle at the bottom of the tank undisturbed, as well as a clear plastic divider in the middle of the container that allowed the male and female to see, smell and sense each other without being able to physically interact. After being placed in the trial containers, the behaviour of the individuals was monitored for the first five minutes after being placed in the container with the appropriate treatment, to ensure that they were not distressed or harmed by the plastic.

The morning after the introducing the fish to the experimental containers, the clear separation plates were removed to allow the individuals to interact. Behavioural activity of all fish in the container was recorded using a camera for ten minutes so that after the experiment I could reassess behaviours if needed. The number of bites/nudges (hereafter referred to as bites/biting behaviour) during the initiation stage of courtship was recorded manually with a clicker counter and re-counted after the experiment by reviewing the video footage to minimise counting errors. When each trial was completed, the test subjects were retrieved from the spawning containers and placed back in their original tanks. Any eggs produced during spawning were extracted from the bottom of the container using a pipette and placed onto a petri dish to be counted and separated into viable and non-viable categories; if the eggs were cloudy rather than clear they were classed as non-viable. Viable eggs were then housed in petri dishes inside an incubator at 28 ˚C, as research has shown that temperatures between 22˚C and 32˚C increase survival rates and hatching success (Schnurr *et al*., 2014). Every 24 hours the survivors were checked and counted, at 96 hours post-fertilization embryos were discarded before hatching in line with ethical procedure, as hatching occurs between 72 and 96 hours post fertilisation in lab reared zebrafish (Singleman & Holtzman, 2014).

Nine trials were conducted for each behavioural selection line for each macroplastic treatment, totalling 81 trials (figure 2). One male and one female were used in each trial, 54 individuals were used from each of the three selection lines (n = 162) as previous studies (e.g. Ariyomo & Watt, 2012) indicate that this number of fish provide appropriate statistical power.

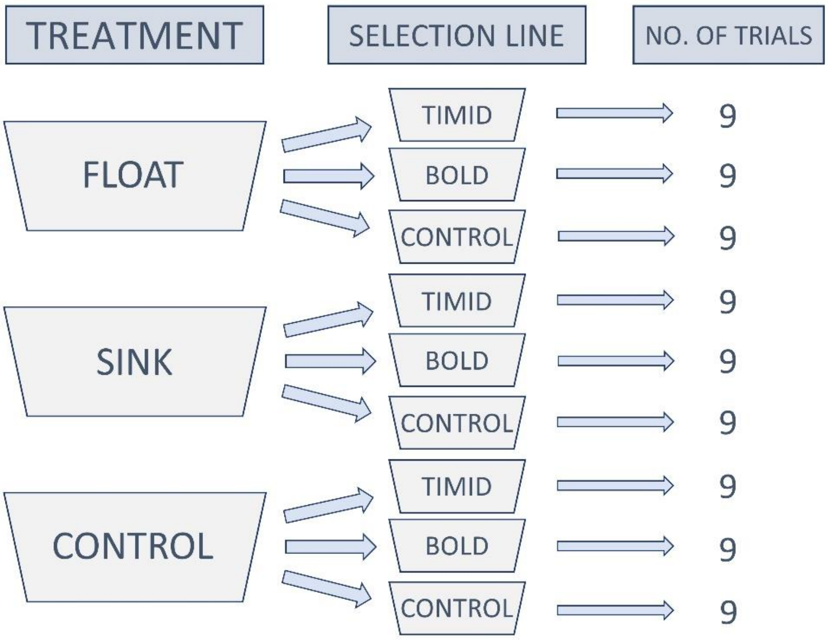

Figure 2. A visualisation of experimental design.

2.1 Statistical Analysis

All data analyses were conducted using the GraphPad Prism statistical package version 9.5.0 (GraphPad Prism version 9.5.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). A two-way ANOVA was performed to analyse the relationship between both the treatment and time to first bite, as well as the treatment and the number of bites pre-spawn within each selection line. Tukey's multiple comparisons test was also used during the analysis of the number of bites in relation to treatment and selection line. When looking at the relationship between spawning and the number of bites, a two-way ANOVA was performed for each selection line with a 95% confidence interval. The percentage of egg survival in each selection line was also analysed using a two-way ANOVA. A non-parametric (due to a high number of zero values) correlation was used to identify the relationship between the number of eggs produced and the number of bites recorded pre spawning. A spearman's rank correlation was used to assess the relationship between the number of eggs produced and the number of eggs that survived.

3.0 Results

A total of 162 zebrafish were successfully assessed and timid and bold behaviours quantified in August and September 2021. No fish were injured during the trial and 100% were returned to their original main tanks.

3.1 Assessment of biting courtship behaviour

The presence of plastic in tanks significantly affected the number of bites, with benthic (sink) plastic affecting all three behavioural selection lines, timid being the most negatively impacted (Fig. 3, B). There were no significant relationships between treatment or selection line on how long it took the male to initiate biting (Fig. 3, A) (interaction: $f\ (4, 72) = 0.057$, $p = 0.994$, treatment: $f\ (2, 72) = 0.356$, $p = 0.702$, selection line: $f\ (2, 72) = 0.332$, $p = 0.719$).

The number of bites (Fig. 3, B) showed a significant relationship between treatment and the number of bites ($f\ (2, 71) = 6.077$, $p= 0.004$), however interaction and selection line were both insignificant ($f\ (4, 71) = 0.2998$, $p= 0.877$ and $f\ (2, 71) = 1.687$, $p = 0.192$, respectively).

The relationship between the plastic treatment, selection line and biting behaviour (figure 3) found that there were no significant relationships between any of the factors in terms of how long it took the male to initiate biting (figure 3, A), however the number of bites (figure 3, B) had a significant relationship with the treatment ($p= 0.004$) but not with selection line. Tukey's multiple comparisons test was also conducted on the number of bites trial (figure 3, B), which found no interaction between treatment and selection line but showed that benthic plastic was the significant source of variation (Control vs Sink: $p = 0.004$, Sink vs Float: $p = 0.029$). The post hoc test further clarified that the treatment effect shows benthic plastic to have the largest impact, but there were no significant differences between selection lines.

When evaluating the relationship between biting and spawning (figure 4), for the timid selection line the spawning factor was significant ($f(1, 21) = 15.86$, $p = 0.001$), with a higher number of bites leading to successful spawning. The interaction was not significant ($f(2, 21) = 1.180$, $p = 0.326$) and neither was treatment ($f(2, 21) = 0.2079$, $p = 0.814$). This shows a threshold at around 200 bites pre-spawn in order to produce eggs. The bold selection line showed a significant spawning relationship ($f(1, 21) = 6.738$, $p= 0.017$). There was no significant interaction ($f(2, 21) = 0.058$, $p = 0.944$) and there was no significant relationship with the treatment ($f(2, 21) = 0.909$, $p = 0.418$). The control selection line also had a significant spawning relationship ($f(1, 21) = 26.77$, $p <0.0001$), however interaction was not significant ($f(2, 21) = 0.227$, $p = 0.799$) and treatment had no significant effect on spawning ($f(2, 21) = 2.157$, $p = 0.141$) (figure 4). In total, the timid selection line had seven spawning events when no plastic was present, five times with floating plastic, and two times with benthic plastic. The bold selection line had six spawning events with no plastic present, eight times with floating plastic present, and five times with benthic plastic present.

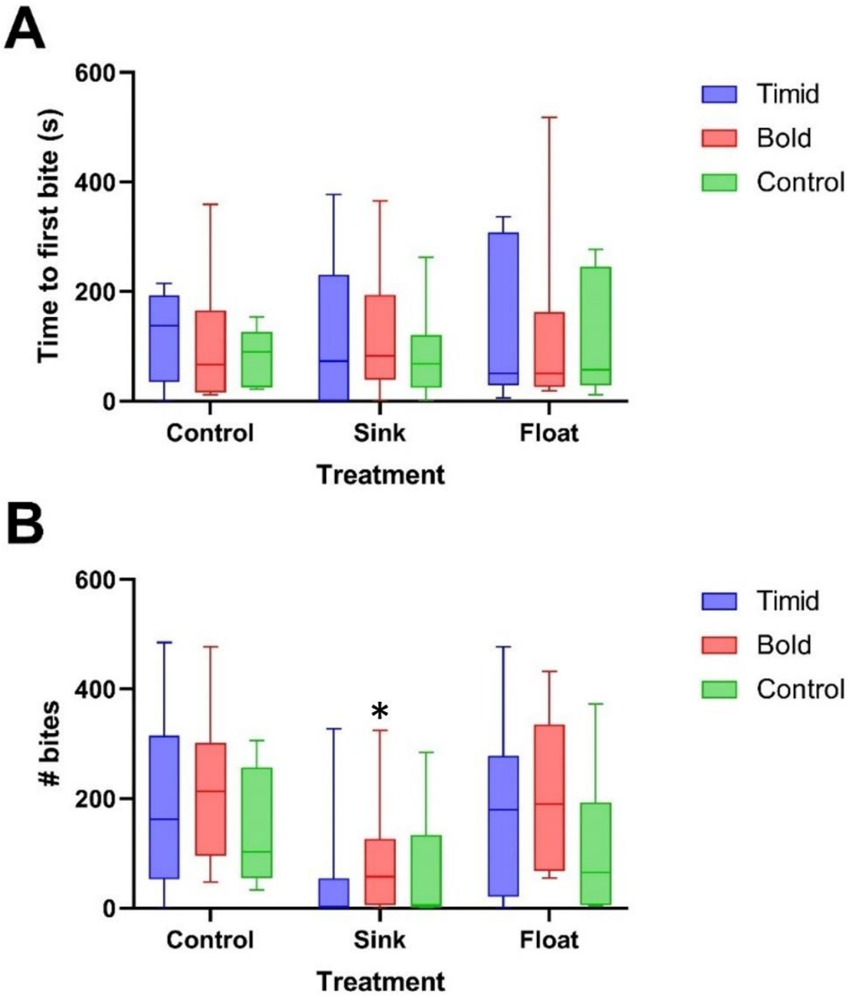

Figure 3. Biting behaviour responses to benthic and floating plastic are shown by (A) the time to first bite and (B) the number of bites by each selection line. (A) Neither treatment or selection line had a significant impact on the time taken to initiate biting (p = 0.7015, p = 0.7187, respectively). (B) Treatment had a significant relationship (*) with the number of bites (p= 0.004) but selection line did not (p = 0.192).

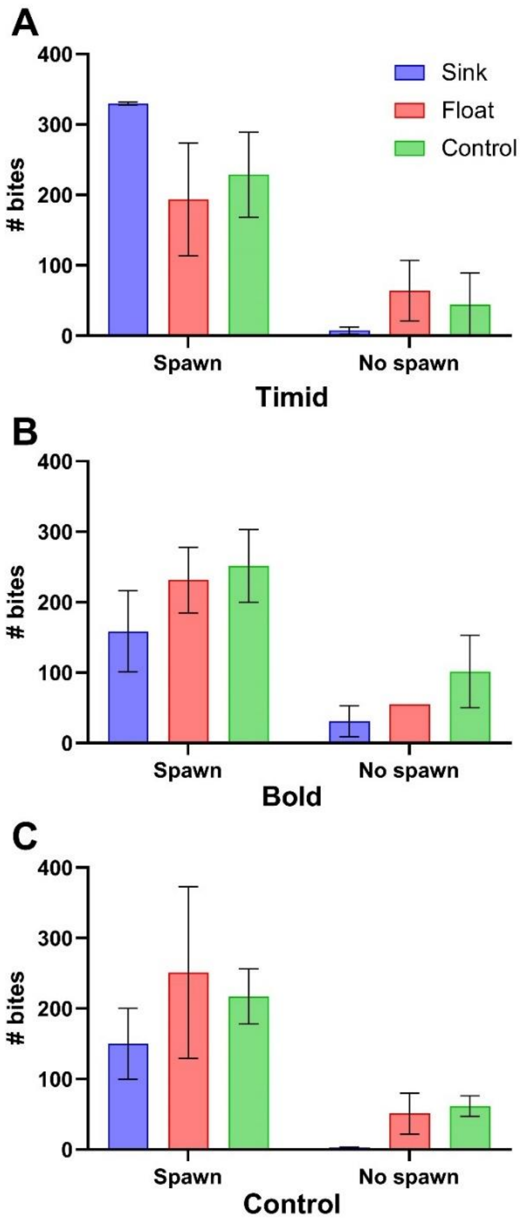

Figure 4. Spawning in response to the number of bites in the presence of benthic and floating plastic showed that a higher number of bites lead to successful spawning, with a threshold of approximately 200 bites to achieve spawning. The spawning factor was significant (p= 0.001), whereas treatment and selection line were not (p = 0.814 and p = 0.327, respectively).

3.2 Estimating Egg Production

There was a significant relationship between the number of eggs produced dependent on the number of bites prior to spawning (figure 5) (timid: $r = 0.660$, $p = 0.0002$; bold: $r = 0.522$, $p = 0.005$; control: $r = 0.647$, $p = 0.0003$), meaning that this relationship has a positive correlation for each selection line.

This relationship was also tested for in the three treatment types (figure 6). The control tank with no plastic treatment showed a significant correlation between the number of bites before spawning and the number of eggs produced consequently ($r = 0.687$, $p < 0.0001$). The tank with the floating plastic also showed a significant correlation ($r = 0.582$, $p = 0.002$). The tank with the benthic plastic again showed a significant correlation ($r = 0.645$, $p = 0.0003$).

Overall, the benthic plastic had the most negative effect on the number of eggs produced dependent on the number of bites prior to spawning, and the timid selection line had the highest number of bites which lead to the highest number of eggs produced.

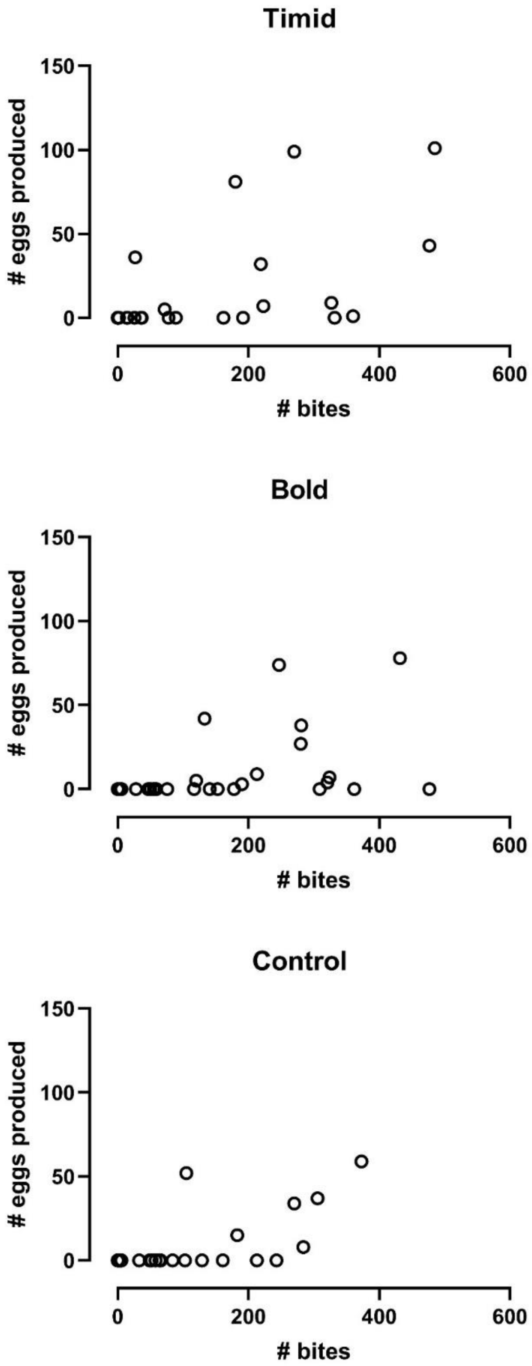

Figure 5. The number of eggs produced in response to the number of bites prior to spawning showed a positive correlation for all three selection lines (timid: $p = 0.0002$, bold: $p = 0.005$, control: $p = 0.0003$).

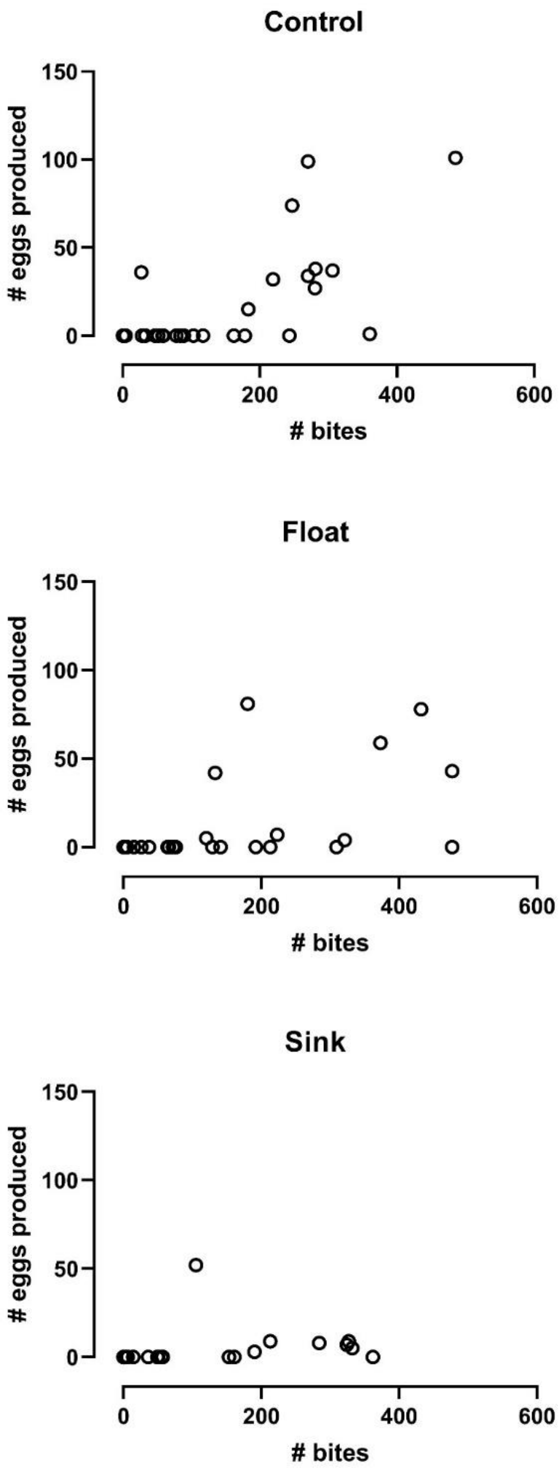

Figure 6. The number of eggs produced in response to the number of bites prior to spawning decreased in the presence of macroplastic. The benthic plastic treatment had the lowest number of bites and eggs produced. All three treatments showed a significant correlation (control $p < 0.0001$, float $p = 0.002$, sink (benthic) $p = 0.0003$).

3.3 Estimating Egg Survival Rates

A significant relationship was found for each behavioural selection line between the number of eggs produced and the number of eggs that survived (Figure 7). The timid selection line correlation was significant ($r = 0.928$, $p < 0.0001$). The bold selection line correlation was also significant ($r = 0.901$, $p < 0.0001$). The control selection line correlation was also significant ($r = 0.788$, $p < 0.0001$) (figure 7).

The same test was performed for each of the macroplastic treatment types (figure 8). The control plastic treatment showed a significant correlation ($r = 0.958$, $p < 0.0001$). The floating plastic treatment also showed a significant correlation ($r = 0.9963$, $p < 0.0001$). The benthic plastic treatment showed a less significant correlation ($r = 0.4849$, $p = 0.010$).

The percentage of eggs that survived for each selection line in relation to the three macroplastic treatments (figure 9) showed no statistically significant results, however there is a visible pattern that shows benthic plastic to have the most negative impact on egg survival, and the bold selection line to be the most resilient to both types of plastic treatment. Selection line had an insignificant result ($f(2, 4) = 4.589$, $p = 0.092$) as did treatment type ($f(2, 4) = 4.259$, $p = 0.102$).

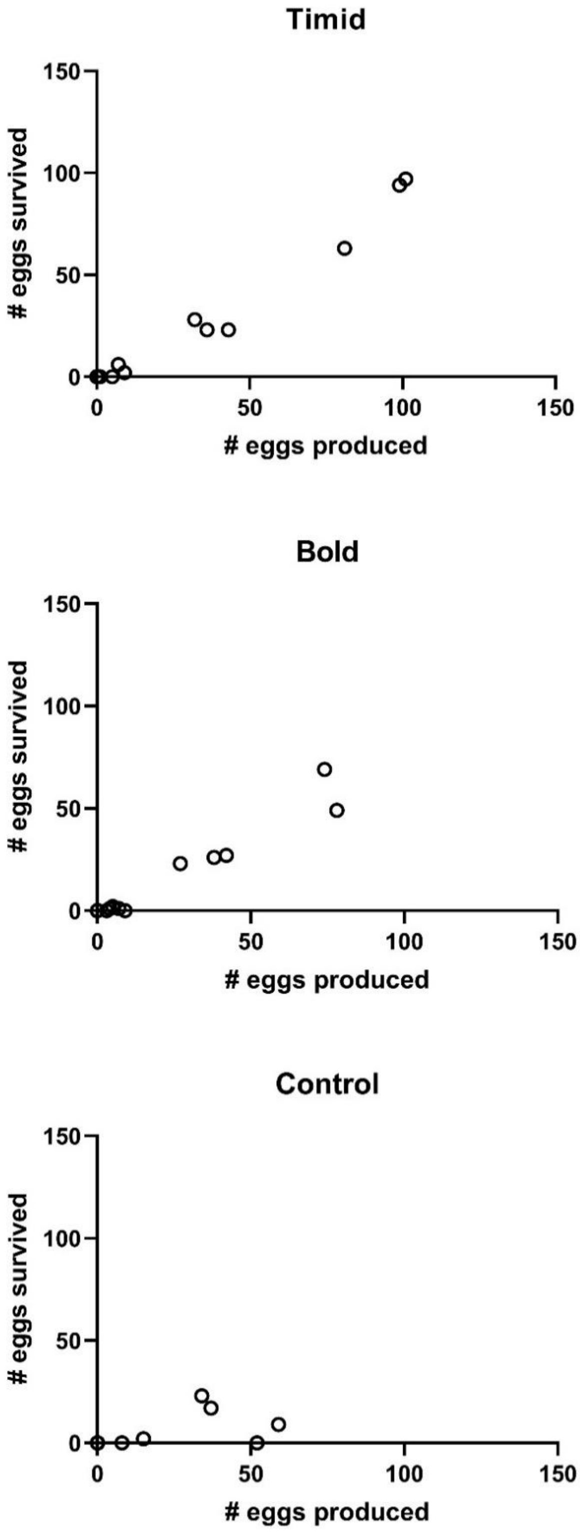

Figure 7. Zebrafish egg survival is associated with egg production across all three selection lines. There was a strong, positive correlation between egg production and survival ($p<0.0001$ for timid, bold and control).

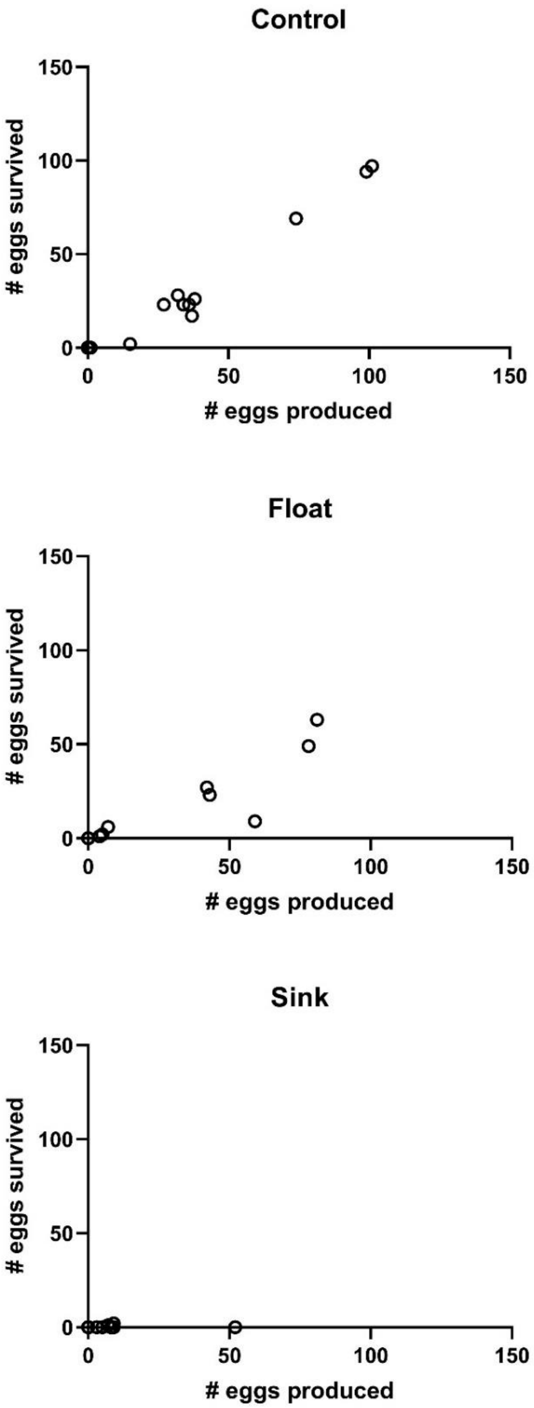

Figure 8. The presence of macroplastic in the tank has a negative impact on both egg production and egg survival, benthic macroplastic having the most detrimental impact. There was a strong correlation between all three treatments (control $p < 0.0001$, float $p < 0.0001$, sink $p = 0.010$).

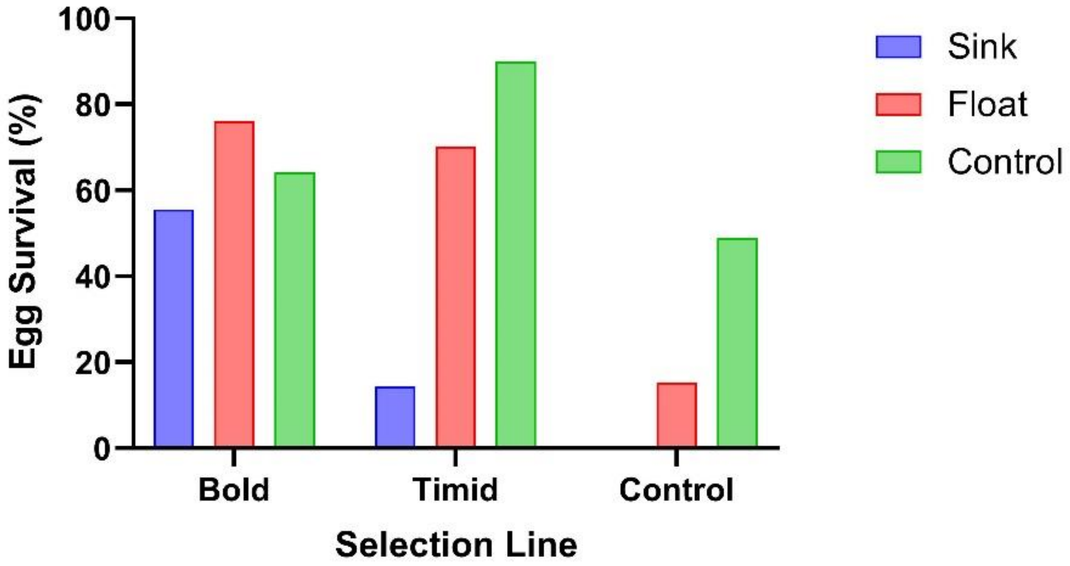

Figure 9. Benthic plastic (sink) had the most negative impact on egg survival rate. The bold selection line had the highest egg survival rate in the presence of both benthic and floating macroplastic (Selection line p =0.092, Plastic treatment p =0.102).

3.4 Effect of selection line on reproductive behaviour

In summary, the bold selection line took the least amount of time to initiate the biting stage of courtship behaviour and had a higher number of bites when subjected to both plastic treatments. When counting the number of spawning events, the bold selection line spawned more in the presence of both plastic treatments and had the highest egg survival rate in the presence of both macroplastic treatments.

4.0 Discussion

This is the first study to investigate the effects of macroplastic on reproductive behaviours of zebrafish. The key findings were that benthic plastic has the most detrimental impact to each stage of zebrafish reproduction for both timid and bold behavioural selection lines, however individuals that exhibit bold behaviours were most resilient in the presence of macroplastic.

A possible side effect of testing behaviour in the presence of macroplastic was that over time, leaching of chemicals may create another variable that could impact their behaviour, as the physiological effects of long-term exposure to chemicals that leach from plastics into aquatic and marine systems requires further investigation. Chemicals that leach from plastics used in 3D printing, for example, can cause changes to neurological pathways in the brain of zebrafish, effecting behaviour. They can also trigger oxidative stress, as well as extreme adverse reactions such as apoptosis (Walpitagama *et al.*, 2019). A 2009 study on mudsnails (*Potamopyrgus antipodarum*) found that polyethylene terephthalate (PET), a toxin released during plastic water bottle degradation, disrupted endocrine function (Wagner & Oehlmann, 2009).

In this study, this was not a variable that I could measure, as research into the time taken for toxins to be released from macroplastics like mineral water bottles is still ongoing. However, if leaching had occurred there would not have been a statistical difference between the results of the floating and benthic plastic treatments, as it would have impacted them equally. There are currently no published studies into the time taken for chemicals to leach from plastic bottles into the aqueous environment, however there is research pertaining to leaching into water contained within plastic bottles, but this is still highly dependent on various factors such as temperature (Filella, 2020, Xu *et al.*, 2020).

4.1 Effect of macroplastic on biting as courtship behaviour

This study investigated the reproductive behaviour of three behavioural selection lines of wild strain *Danio rerio* after treatment with macroplastics. Three treatments were used; floating (float), benthic (sink) and no plastic (control). The plastic had no significant effect on the time taken to initiate biting (figure 2; A) across all three selection lines, as there was no significant difference between the control treatment and the plastic treatments. These results show that the time taken to initiate biting as a proxy for measuring the effects of plastic on reproductive behaviour requires further investigation.

The number of bites counted prior to spawning (figure 2; B) showed a significant effect between treatments but not selection line, with the sink plastic having the lowest number of bites across all three selection lines. This implies that the presence of sink plastic impacts this aspect of reproductive behaviour in zebrafish regardless of behavioural syndromes.

A similar study examined the behavioural effects of polycyclic aromatic hydrocarbons (which can be found in plastic) on *Danio rerio*, by exposing them to acute levels of benzo[a]pyrene (B[a]P). Results showed that short term exposure to this chemical increased bold behaviours such as aggression and exploration whilst decreasing levels of anxiety, which has the potential to influence courtship and disrupt population dynamics (Hamilton *et al*, 2021).

4.2 Effect of macroplastic on spawning and egg production

When evaluating the relationship between the number of bites and spawning (figure 3), the results showed that the number of bites (figure 3, B) had a significant effect on whether the subjects spawned. A higher number of bites lead to a higher likelihood of a spawning event, however, neither treatment or selection line had a significant effect on whether they spawned or not. This concurs with previous research showing that biting is an important component of reproductive behaviour in *Danio rerio* (Darrow & Harris, 2004). Referring back to figure 1, as sink plastic is shown to have a significant effect on the number of bites prior to spawning, this then leads to a lower chance of a spawning event in the presence of benthic plastic.

Each selection line showed a significant positive correlation between the number of bites counted prior to spawning and the number of eggs produced (figure 4). This again concurs with previous research that a higher number of bites leads to a higher chance of spawning and thus a larger amount of eggs. When observing this in the presence of macroplastic (figure 5), the results showed that the number of eggs produced decreased slightly when exposed to floating plastic, and then even further with the benthic plastic. This follows on from the hypothesis that the plastic acts as a stressor rather than enrichment and negatively impacts reproductive behaviour in *Danio rerio* regardless of behavioural syndromes.

4.3 Effect of macroplastic on egg survival rates

Benthic plastic had the most negative effect on egg survival when compared with floating plastic and control, though this was not statistically significant. It appeared that egg survival (to 72 h) was highest within the bold selection line, suggesting that being bold is a more successful behavioural type than being timid, and these fish would adapt easier to the growing presence of plastic in their natural habitat. Similar patterns of better response to environmental stressors by bolder fish have been reported in the three-spined stickleback (*Gasterosteus aculeatus*) in Anglesey, north Wales where the relationship between boldness and exploratory behaviours of higher risk, novel environments was stronger in individuals inhabiting environments where predators were more prevalent (Dingemanse *et al*., 2007).

4.4 Effect of personality on reproductive behaviour

The key result of this study is that individuals presenting a bold personality had higher reproductive success when exposed to both plastic treatment types.

My findings are supported by a number of studies that reported that bolder fishes would be impacted less by plastic, as individuals possessing these behavioural traits thrive in "high-risk, high-reward" scenarios (Daniel & Bhat, 2020). This supports the theory that a higher level of activity and exploratory behaviours allow fish to adapt to new environments faster (Daniel & Bhat, 2020), so therefore may not be as inhibited by macroplastic as individuals that exhibit timid behaviours.

4.5 Effect of enrichment on reproduction

An alternative hypothesis could have been that macroplastic provides cognitive enrichment, shelter or substrate for egg laying, and causes positive impacts on reproductive behaviour and fecundity.

Zebrafish produced a higher number of eggs when spawning in the presence of plastic grass than with no environmental enrichment (Wafer *et al*., 2016). Providing structures can reduce anxiety behaviours, as zebrafish can use objects as shelter from aggressive interactions with other individuals and as an escape from stress stimuli, improving welfare (Stevens, Reed & Hawkins, 2021). Females in particular prefer to stay near to structures such as plants rather than barren areas (Delaney *et al*., 2002, Stevens, Reed & Hawkins, 2021). This supports the hypothesis that the presence of macroplastic in the tank may provide shelter and reduce stress, thus benefiting reproduction by minimising anxiety responses.

However, my results have not supported this alternative hypothesis, as both selection lines would have shown greater reproductive success when plastic was present.

5.0 Recommendations for future research

In attempting to use different individuals for each trial, the plastic remained a novel object which may have induced stress responses. Future research could investigate the long-term exposure to macroplastic to address whether over time individuals become habituated to these objects and begin to use these structures as shelter, meaning that timid and bold individuals may begin to succeed equally.

In the wild zebrafish spawn in groups, however, to closely observe their behaviour they had to be separated into pairs. Future research could compare the impacts of macroplastic on the behaviour of a group, as the safety of being with other individuals during testing may produce a different outcome. As they were assessed in pairs, behavioural differences between males and females were not studied. Female zebrafish have been shown to have higher fecundity in the presence of structures/shelter, so this may also be an avenue worth exploring.

Overall, this area of study needs further research, as current literature is sparse when it comes to the impacts of macroplastic on animal behaviour. Ingestion and entanglement have been studied extensively, with microplastics and nano plastics currently at the forefront of research on the impacts of plastic in the environment. This study has been one of the first of its kind, and whilst the problem of micro and nano plastics are of high importance, of the few other studies currently looking at the topic it is obvious that the impacts of macroplastics on animal behaviour have been undervalued. This particular area should be researched further as changes in behavioural response are a precursor to the survival of future generations within the growing problem of plastic pollution.

6.0 Conclusions and implications of research

In conclusion, benthic plastic has the most significantly negative impact on reproductive behaviour and egg survival rates, suggesting that freshwater ecosystems with a higher amount of plastic deposited on the sediment will see the most detrimental impacts to zebrafish populations. This in turn impacts all other aspects of the food web, with a potential for causing a trophic cascade due to the suppression of this species.

As prolonged and consistent behaviours become classed as personality, these personality traits are inherited just as morphological traits are (Sbragaglia *et al*., 2019). This suggests that if bolder fish reproduce more successfully and have a higher egg survival rate in the presence of macroplastic, then the growing problem of plastic pollution may directly select for bolder personalities, meaning individuals with more timid phenotypic traits will not reproduce as successfully, which may evolve the behaviour of future generations of zebrafish in the wild and change population dynamics as a whole. This change in behaviours may lead to a decline in not just zebrafish numbers, but also other species that exhibit altered behaviours in the presence of plastic.

Appendix

Raw data for group AB32 Bold

Date	Tank No.	Test	First Bite (seconds)	No. Bites	Spawn	No. Eggs	No. Eggs Survived	% eggs survived
05/08/2021	AB32 1	Control	359	47	Yes	0	0	
		Sink	148	4	No	0	0	
		Float	120	178	Yes	0	0	
08/08/2021	AB32 2	Control	160	281	Yes	38	26	68.42
		Sink	239	28	No	0	0	
		Float	518	59	Yes	0	0	
12/08/2021	AB32 3	Control	17	280	Yes	27	23	85.19
		Sink	76	117	No	0	0	
		Float	21	247	Yes	74	69	93.24
16/08/2021	AB32 2	Control	16	321	Yes	4	1	25
		Sink	n/a	0	No	0	0	
		Float	18	309	Yes	0	0	
19/08/2021	AB32 1	Control	11	477	Yes	0	0	
		Sink	33	120	Yes	5	2	40
		Float	45	432	Yes	78	49	62.82
27/08/2021	AB32 4	Control	170	141	Yes	0	0	
		Sink	120	133	Yes	42	27	57.45
		Float	205	76	Yes	0	0	
29/08/2021	AB32 1	Control	66	213	Yes	9	0	0
		Sink	45	324	Yes	7	1	14.29
		Float	31	190	Yes	3	0	0
04/09/2021	AB32 4	Control	15	153	No	0	0	
	AB32 3	Sink	365	6	No	0	0	
		Float	50	55	No	0	0	
07/09/2021	AB32 1	Control	106	50	No	0	0	
		Sink	82	57	Yes	0	0	
		Float	85	362	Yes	0	0	
					total	287	198	68.989

Raw data for group AB34 Timid

Date	Tank No.	Test	First bite (seconds)		No. Bites	Spawn	No. Eggs	No. Eggs Survived	%eggs survived
08/08/2021	AB34 3	Control		198	162	Yes	0	0	
		Sink	n/a		0	No	0	0	
		Float		124	180	Yes	81	63	77.78
10/08/2021	AB34 1	Control		46	89	No	0	0	
		Sink	n/a		0	No	0	0	
		Float		22	192	No	0	0	
16/08/2021	AB34 2	Control		215	270	Yes	99	94	94.95
		Sink		377	2	No	0	0	
		Float		298	15	Yes	0	0	
18/08/2021	AB34 4	Control		187	78	Yes	0	0	
		Sink		73	36	No	0	0	
		Float		5	1	No	0	0	
20/08/2021	AB34 4	Control	n/a		0	No	0	0	
		Sink		334	14	No	0	0	
		Float		336	37	No	0	0	
26/08/2021	AB34 3	Control		33	360	Yes	1	0	0
		Float		126	72	yes	0	0	
		Sink		46	332	Yes	5	0	0
28/08/2021	AB34 2	Control		37	485	Yes	101	97	96.04
		Sink	n/a		0	No	0	0	
		Float		50	26	No	0	0	
30/08/2021	AB34 2	Control		137	27	Yes	36	23	63.89
		Sink	n/a		0	No	0	0	
		Float		318	223	Yes	7	6	85.71
04/09/2021	AB34 3	Control		174	219	Yes	32	28	87.5
		Sink		93	327	Yes	9	2	22.23
		Float		36	477	Yes	43	23	53.49
						total	414	336	81.159

Raw data for group AB35 Control

Date	Tank No.	Test	First Bite (seconds)	No. Bites	Spawn	No. Eggs	No. Eggs Survived	% eggs survived
03/08/2021	AB35 2	Control	115	243	Yes	0	0	
		Sink	128	3	No	0	0	
		Float	11	5	No	0	0	
06/08/2021	AB35 3	Control	136	33	No	0	0	
		Sink	n/a	0	No	0	0	
		Float	n/a	0	No	0	0	
11/08/2021	AB35 3	Control	27	270	yes	34	23	67.65
	AB35 5	Sink	262	105	yes	52	0	0
	AB35 5	Float	42	129	Yes	0	0	
13/08/2021	AB35 5	Control	69	58	No	0	0	
		Sink	n/a	0	No	0	0	
		Float	130	64	No	0	0	
17/08/2021	AB35 3	Control	90	84	Yes	0	0	
		Sink	68	49	Yes	0	0	
		Float	28	213	No	0	0	
20/08/2021	AB35 2	Control	109	183	Yes	15	2	13.33
		Sink	67	284	Yes	8	0	0
		Float	271	4	No	0	0	
28/08/2021	AB35 1	Control	23	52	No	0	0	
		Sink	110	6	No	0	0	
		Float	277	5	No	0	0	
01/09/2021	AB35 1	Control	22	306	Yes	37	17	45.95
		Sink	113	3	No	0	0	
		Float	57	66	No	0	0	
02/09/2021	AB35 2	Control	153	103	No	0	0	
		Sink	48	161	Yes	0	0	
		Float	30	373	Yes	59	9	15
					total	205	51	24.878

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