The effect of plastic pollution on the reproductive success of the cryptobenthic fish *Forsterygion capito*.

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Abstract

Marine microplastic pollution is of growing concern, thus research to understand the effects of microplastic ingestion has been studied in a variety of organisms. Previous research on the effects of both microplastic ingestion and exposure to plastic associated chemicals (e.g., plasticisers) have occurred in isolation of one another. Therefore, the aim of the present work was to assess the effect of exposure to the plasticiser di-(2-ethylhexyl)-phthalate (DEHP) via ingestion of dosed microplastics on the reproductive output, behaviour, and biochemistry of the mottled triplefin *Forsterygion capito*. Here, 60 fish (30 breeding pairs) were split into three treatment groups, control, virgin polystyrene (PS) and DEHP-dosed polystyrene (DEHP) and were exposed to a given treatment over a five-week period. In Chapter Two, five-weeks exposure had no impact on the reproductive output, gonad condition and vitellogenin mRNA expression. Despite this, in Chapter Four, all markers of both antioxidant defence (enzyme activity) and oxidative damage (protein carbonyls and lipid peroxides) were significantly increased in the white muscle tissue of fish from Chapter Two. DEHP exposure therefore may incur an energetic cost, the consequences of which could manifest over time. In addition to this experiment, reproductive behaviour of male *F. capito* was assessed following the exposure of 90 fish (30 per group) to one of the same treatments (control, PS, and DEHP) over a ten-week period (Chapter Three). Though no change to aggressive or attractive behaviours was observed, ten weeks exposure did cause significant increased dopamine concentrations as well as decreased serotonin concentrations in the brain. This experiment also saw the ability to display nuptial colouration was decreased in DEHP-exposed fish compared to that of the control. This could indicate a change in the reproductive condition of exposed fish. DEHP exposure via microplastic ingestion did not have the same impacts as those seen in studies of constant aqueous exposure, this highlights the importance of environmentally relevant protocols as the delivery method will impact the degree of change observed.

Overall, it was found that, in terms of reproductive effects, the variable exposure resulting from the present treatment method led to results vastly different from those observed in studies of constant exposure to aqueous DEHP. Though this exposure method did not cause reproductive dysfunction or change to reproductive behaviours, it was clear that the fish

were responding to the chemical as a result of ingesting dosed microplastics. This is evidenced by the increased oxidative stress markers as well as changed neurotransmitter levels and presentation of reproductive signals. The work in the present study therefore highlights the importance of exposure vector on observed results as well as the importance of environmental relevance of experiments. A comprehensive understanding as to the effects of microplastics and their additives on marine taxa is crucial in our assessment of the impact anthropogenic activity has on the marine environment. As such, research conducted must be environmentally relevant to allow the clearest picture possible.

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Abbreviations

Abbreviation	Full term
DEHP	Di-(2-ethylhexyl)-phthalate
PVC	Polyvinyl Chloride
PS	Polystyrene
PC	Protein Carbonyl
SOD	Superoxide dismutase
CAT	Catalase
GPOX	Glutathione peroxidase
GST	Glutathione S-transferase
ROS	Reactive Oxygen Species
ELISA	Enzyme-linked immunosorbent assays
LP	Lipid peroxides
DA	Dopamine
5-HT	Serotonin
VTG	Vitellogenin
PML	Portobello Marine Laboratory

Chapter 1 – General Introduction

1.1 Plastic pollution

Plastics are synthetic organic polymers made with the intention of tailoring materials to specific needs (Mulder and Knot 2001). The first plastic was produced in 1862, with Bakelite being the first commercially successful plastic, patented in 1907 (Mulder & Knot, 2001). Resource shortages during World War II lead to the mass production of plastics in the 1940's (Cole et al. 2011; Freinkel 2011). Since then, global consumption of plastic has grown exponentially across multiple industries such as food retailing, construction, transportation and medicine (e.g. PPE) (Dauvergne 2018). Now rivalling metals in their breadth of application, plastics and their associated additives (such as plasticisers) are mass produced in the millions of metric tonnes each year (Geyer, Jambeck, and Law 2017; Lehtiniemi et al. 2018; Singh and Sharma 2008).

Disposal of plastic occurs via recycling, incineration or discard into landfills (Ritchie 2018). Recycling is the process of sorting plastics by type, grinding, cleaning and clumping them together after which they can be granulated and reused (Santos et al. 2005). Only polyethylene terephthalate and high-density polyethylene are widely recyclable (Ritchie 2018). These plastics are two of eight plastic types made globally (Geyer et al., 2017). Plastics that cannot be recycled, are often taken to landfills or dumps, buried in or piled on top of earth and left to break down naturally (Geyer et al. 2017; Jambeck et al. 2015). However, degradation can take anywhere from 0.25 to 600 years to breakdown completely (Ritchie 2018). Once exposed to both terrestrial and marine environments, macroplastics (> 5 mm diameter) are broken down by processes such as photodegradation, hydrolysis, and biodegradation (Gewert, Plassmann, and MacLeod 2015). Photodegradation occurs when UV radiation from sunlight breaks the bonds of a polymer, allowing it to be oxidised and in doing so causing disintegration (Singh and Sharma 2008). Hydrolysis occurs as a plastic is oxidised in the presence of water, this again leading to the breakdown of the structure of the polymer (Chamas et al. 2020). Biodegradation occurs when plastic fragments are

colonised by microorganisms which break polymers using enzymatic action (Bahl et al. 2021; Gewert et al. 2015). All degradation processes weaken plastics causing them to fragment in to smaller pieces known as microplastics (< 5 mm diameter) (Cole et al. 2011; Law 2017). In addition to secondary microplastics, which result from the breakdown of macroplastics, there are also primary microplastics such as nurdles and microbeads which were purpose made at small sizes (fig. 1) (Lehtiniemi et al. 2018). These primary particles are used for the transport of raw material in industry as well as in cosmetics and personal care products (Hammer, Kraak, and Parsons 2012; van Wezel, Caris, and Kools 2016).

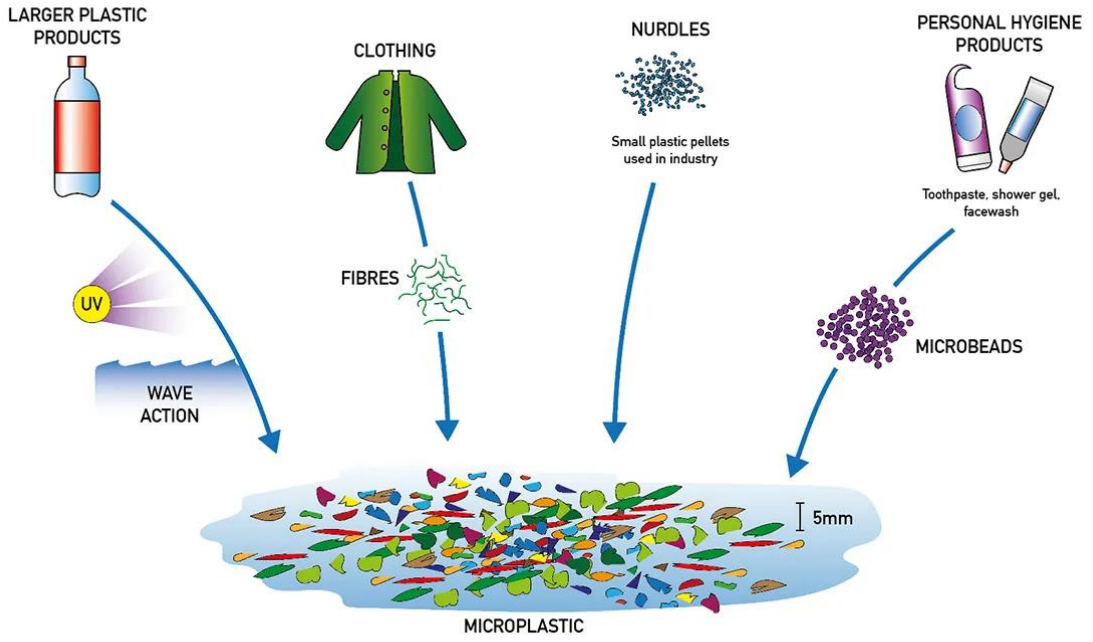

Figure 1. Diagram of microplastic origins, secondary microplastics on the left and primary microplastics on the right (Encounter Edu n.d.).

The problematic disposal of plastics comes from the lack of proper governance and accountability (Dauvergne 2018). Corporations are not held accountable for their plastic products and for too long the 'disposal' of plastics has relied on shifting the problem to other countries by export of plastic waste (Dell 2019). This also breeds the misconception that a few countries are responsible for the bulk of plastic pollution when all countries

contribute to the issue. The mismanagement of plastic waste leads to a large proportion of used plastics ending up in the environment, in particular the marine environment owing to the proximity of disposal sites to the coast (Geyer et al. 2017). In addition, rivers serve as a means of transport for plastics from land to the sea, for example the Amazon and Yangtze rivers are estimated to transport 38,900 and 333,000 tonnes of plastic into the Northern Atlantic and South China Sea respectively (Lebreton et al. 2017; Meijer et al. 2021). Addressing ocean plastic pollution is complicated as plastic pollution is land derived so ocean-based approaches will not properly address the problem (Haward 2018).

Microplastic pollution in the top 200m of the Atlantic Ocean was estimated be 11.6-21.1 million tonnes (Pabortsava and Lampitt 2020). Pabortsava and Lampitt (2020) highlight that the true extent of plastic pollution may have previously been underestimated as contamination occurs throughout the water column, not just at the surface or in the sediment. Other papers have documented the ubiquity of plastic pollution throughout the marine environment and have demonstrated that plastic pollution occurs in the marine environment from the Arctic to the Antarctic and in the depths of the Mariana Trench (Chiba et al. 2018; Cózar et al. 2017; Lacerda et al. 2019). Extensive contamination of the marine environment by plastics means that marine organisms are likely to interact with the pollutant.

From an ecological context, plastics are a growing concern owing to the potential risk they exert on marine organisms should they accidentally ingest or become entangled (Kiessling et al., 2015; Gregory, 2009). Globally, interactions between marine wildlife (e.g. whales, fish, turtles and birds) and plastic are well documented (Li, Tse, and Fok 2016). For example, in the Greek Seas, it has been estimated that half of all cetacean species have ingested macroplastics (Alexiadou, Foskolos, and Frantzis 2019). Off the Californian coast, over two decades, there were 914 recordings of pinnipeds entangled in plastic waste (Hanni and Pyle 2000). There have also been observations of megafauna such as whales foraging in the Great Pacific Garbage Patch (Gibbs et al. 2019; Kahane-Rapport et al. 2022). Entanglement in and ingestion of macroplastics has been associated with reduced reproductive ability,

predator avoidance and increased mortality for a variety of organisms from seals to sea birds, fish, and turtles (for review see Li et al., 2016).

In Aotearoa, New Zealand, marine plastic interactions with wildlife are well documented For example, the entanglement of New Zealand sea lions is well studied with 185 entanglement events recorded in a 10 year period in Kaikōura alone (Boren et al. 2006). On Ohinau, an island on the north-eastern coast of Aotearoa, a positive linear relationship was found between plastic fragment density and burrow density of flesh-footed shearwater (Buxton et al. 2013). The plastic density on this island was likely the result of the adult birds having ingested plastic debris (Buxton et al. 2013). These papers, and others, highlight that Aotearoa is not immune from ocean plastic pollution (Clere et al. 2022). Studies on plastics in the environment have grown in number in recent years, with 200 articles written, worldwide, in 2017 (Dauvergne 2018). As a result, studies on the interaction of wildlife with marine macroplastics are being undertaken globally. Table 1 below shows a selection of data for the interactions between macroplastics and marine life, highlighting that these interactions occur on a global scale.

Table 1 A selection of studies on the effect of macroplastics on marine fauna

Location	Species	Plastic Type	Main finding	Reference
Eastern North Pacific	Seabirds	Various	29% of ingested plastic was industrial pellets, 71% was discarded products.	(Blight and Burger 1997)
California, USA	Red phalarope	Unspecified	Six of the seven sampled birds had plastic in their gut.	(Connors and Smith 1982)
Bering Sea	Short-tailed Shearwaters	Various	Type of plastic did change from industrial to consumer sourced plastics.	(Vlietstra and Parga 2002)
Hawaii	Laysan Albatross and Wedge-tailed Shearwaters	Various	90% of chicks surveyed in 1982 and 1983 had plastic fragments in their gut.	(Fry, Fefer, and Sileo 1987)
Macquarie Island, Tasmania	Seabirds	Various	The ingestion of plastics by seabirds, has increased since the late 1970's.	(Slip, Green, and Woehler 1990).
Germany	Gannets	Nets	313 observations of gannets entangled in nets were made between 1984 and 1985.	(Schrey and Vauk 1987)
Bouvetoya Island	Antarctic fur seals	Various	119 entanglement sightings were made over 301 days.	(Hofmeyr et al. 2006)
South Georgia	Antarctic fur seals	Various	1033 entanglement events were recorded between 1989 and 2013.	(Waluda and Staniland 2013)
Hawaii	Hawaiian Monk Seal	Fishing nets	10.8 metric tonnes of abandoned fishing gear was recovered from reef habitats around Hawaii	(Boland and Donohue 2003)
Pribilof Island, Bering Sea	Northern fur seals	Fishing net	Juvenile fur seals were likely to become entangled.	(Feldkamp, Costa, and DeKrey 1988)
Farallon Island, California	Pinnipeds	Fishing gear	A total of 914 pinnipeds were observed entangled in plastics between 1976 and 1998.	(Hanni and Pyle 2000)
Tasmania	Australian fur seals	Various	Entanglement of fur seals in plastics led to permanent collars being observed for 75 seals over two seasons.	(Pemberton, Brothers, and Kirkwood 1992)
Adriatic Sea	Loggerhead sea turtles	Various	35.2% of 54 turtles had ingested plastic. Ingested plastic included rope, polystyrene and fishing line.	(Lazar and Gračan 2011)
Australia	Sea turtles	Various	96.8% of reviewed papers reported plastic ingestion in sea turtles.	(Schuyler et al. 2014)
Brazil	Sea turtles	Unspecified	Anthropogenic debris had been ingested in 60.5% of sea turtles (n =38).	(Bugoni, Krause, and Virgínia Petry 2001)
Nova Scotia, Canada	Harbour porpoise	Unspecified	Plastic was found blocking the oesophagus of a juvenile harbour porpoise which was found dead.	(Baird and Hooker 2000)
Florida, USA	Manatees	Various	Manatees ingest plastic Entanglement killed 11 manatees from 1974 to 1985	(Beck and Barros 1991)
Pacific Ocean	Various cetaceans	Various	14 individual cetaceans, were observed within the Great Pacific Garbage Patch.	(Gibbs et al. 2019)
Western North Pacific	Various	Fishing net	A recovered, lost driftnet in 1978 had caused the mortality of 99 seabirds, 2 sharks, 1 ragfish and over 200 salmon.	(Degange and Newby 1980)

As well as macroplastics, microplastics are a concern for marine organisms. First detected in the ocean in the 1970's, microplastics are now a ubiquitous environmental pollutant (Costa and Barletta 2015). Ingestion of microplastics occurs readily throughout the food web, as their small size allows both intentional and accidental ingestion (Germanov et al. 2018; Sun et al. 2017). Ingestion of microplastics has been recorded in a range of marine taxa from zooplankton to humpback whales (Germanov et al. 2018; Sun et al. 2017). Furthermore, the microbial communities associated with microplastics have been found to increase microplastic ingestion rates (Rummel et al. 2017). This is likely due to the microplastic now appearing to be food organisms that zooplankton naturally graze on (Vroom et al. 2017). For example, the soaking of polystyrene beads in local seawater for three weeks prior to experiments allowing settlement of a microbial community on the plastics resulted in higher ingestion rates in zooplankton compared to the clean control particles (Vroom et al. 2017). This means that estimates of microplastic ingestion based on laboratory experiments of virgin microplastic exposure are likely underestimating the rate at which organisms will ingest plastics under field conditions (Vroom et al., 2017). This may have consequences for our understanding of the trophic transfer of microplastics.

Once ingested, microplastics can accumulate in the gut, where they may have long gut residence times (Welden and Cowie 2016). These characteristics can lead to false satiation signals in exposed organisms with direct implications on fitness (Cole et al. 2015; Welden and Cowie 2016; Wright et al. 2013). For example, the consumption of polyvinyl chloride (PVC) microplastics was found to deplete energy reserves in lugworms (*Arenicola marina*) as they fed less often due to the long gut residence time of the microplastic particles (Wright et al. 2013). Similarly, exposure to polystyrene microplastics caused reduced feeding rates in copepods (*Calanus helgolandicus*) which lead to depleted energy reserves through time (Cole et al. 2015). In addition to causing energy depletion, the ingestion of microplastics can cause gross, morphological impairment of the gastrointestinal (GI) tract. For example, ingestion of PVC microplastics caused morphological changes in the GI tract of the European sea bass (*Dicentrarchus labrax*) (Pedà et al. 2016). Following a 30-day exposure to PVC microplastics, the structure of the GI tract was modified as evidenced by an increase in the density of goblet cells. Goblet cells, which produce protective mucus, likely increased in

density owing to the mechanical stress associated with ingestion of plastic particles (Pedà et al. 2016). Similarly, in zebrafish a 21 day exposure to water suspended PS microplastics lead to intestinal damage shown as an increase in cilia defects and inflammation (Qiao, Deng, et al. 2019; Qiao, Sheng, et al. 2019). The ubiquity and ease of ingestion of microplastics has led to extensive research as to which species are ingesting particles in the wild as well as the effects of ingestion in experimental protocols. Table 2 below shows a subset of previous literature, highlighting the breadth of taxa which are ingesting plastic in the environment as well as the negative effects microplastic consumption has been found to have on experimental animals.

Table 2. A selection of studies of the ability of organisms to ingest microplastics.

Species	Plastic type	Main Finding	Reference
Zooplankton	Various	Multiple species of zooplankton ingested plastic	(Sun et al. 2017)
Sea Urchin	Polyethylene	Ingestion of virgin pellets indicates that particles could act as vectors for pollutant transfer.	(Nobre et al. 2015)
Isopod	Various	All plastic types were ingested and excreted.	(Hämer et al. 2014)
Barnacles	Various	33.5% of 385 barnacles had ingested plastics. 99% were degraded fragments.	(Goldstein and Goodwin 2013)
Shore crab	Polystyrene	Microplastic particles were taken up by the gills of the crabs.	(Watts et al. 2016)
Mud crabs	Various	The size of the plastic particle influenced the gut residence time.	(Torn 2020)
Mussels and lugworms	Polystyrene	Mussels were able to excrete ingested microplastics after 20 minutes of exposure.	(Gonçalves et al. 2019)
Mussels and lugworms	Various	Ingestion of microplastics was confirmed for all wild-caught animals.	(Van Cauwenberghe et al. 2015)
Mussels and lugworms	Polystyrene	Microplastic ingestion by mussels lead to an alteration of physiological processes.	(Paul-Pont et al. 2016)
European sea bass	Polyethylene	Ingestion of microplastics by larvae was confirmed for two of three treatment groups.	(Mazurais et al. 2015)
Sardines and anchovies	Various	96% of sardines and 91% of anchovies had ingested microplastics.	(Renzi et al. 2019)
Kreffts frillgoby	Polyethylene	Ingestion of microplastic exposed sand hoppers by gobies caused no significant behaviour changes.	(Tosetto, Williamson, and Brown 2017)
Zebrafish	Polyethylene	Exposure of embryonic and larval fish showed plastic accumulation in the gills of the fish.	(LeMoine et al. 2018)
Zebrafish	Polystyrene	Exposure of embryos to water borne microplastics caused malformation and mortality.	(Tang, Zhang, and He 2019)
Northern Fur Seal	Various	All 44 scat samples collected contained plastic fragments or fibres.	(Donohue et al. 2019)
Various	Unspecified	A review of the ubiquity of microplastics and potential for transfer up the food chain.	(Santillo, Miller, and Johnston 2017)
Various	Various	A review of the presence of microplastics in the marine environment.	(Galloway, Cole, and Lewis 2017)
Various	Various	9% of 2233 fish had ingested plastic. The average number ingested was 1.06.	(Vendel et al. 2017)
Various	Polystyrene	All experiment taxa were found to ingest plastic.	(Setälä, Fleming-Lehtinen, and Lehtiniemi 2014)
Various	Various	36.5% of 504 fish consisting of 10 species had ingested plastic.	(Lusher, McHugh, and Thompson 2013)

1.2 Plasticisers – DEHP

Microplastics are not benign substances. They are often associated with hazardous chemicals, derived from either the plastic manufacturing process or are adsorbed to the plastic once in the environment (Bakir, Rowland, and Thompson 2012; Rodrigues et al. 2019; Sarkar et al. 2021). Plastics can therefore be associated with pollutants such as heavy metals, like copper, or persistent organic pollutants (POPs) like DDT (dichlorodiphenyltrichloroethane) (Verla et al. 2019). Plasticisers such as bisphenols and phthalates are added to plastics during manufacture to add qualities such as strength and flexibility (Carnevali et al. 2010; Cole et al. 2011; Kim, Kim, and Lee 2002; Tyler et al. 2018; Ye et al. 2014). Di-(2-ethylhexyl)-phthalate (DEHP) is an extremely common phthalate plasticiser of which 1.8 million metric tons are produced annually (Rowdhwal and Chen 2018). DEHP is primarily used in the formation of polyvinyl chloride (PVC). As a result, PVC can consist of up to 50% DEHP and other phthalates (Tyler et al. 2018). One of the most produced and used plastics today, PVC has a wide range of applications from construction products, medical devices and equipment to clothing and children's toys (Chikae et al. 2004; Mulder and Knot 2001; Tyler et al. 2018). Plasticisers are not covalently bonded to the plastic molecules, as they are simply additives; this results in the ability of plasticisers to leach readily from the plastics after production (Guerranti et al. 2019; Magdouli et al. 2013). Due to this leaching and the use of DEHP in everyday life, and a lack of regulation for plasticisers in general, DEHP is now considered a "ubiquitous environmental pollutant" which is now detectable throughout the environment (Chikae et al. 2004; Liu et al. 2017; Magdouli et al. 2013). DEHP has been found to occur in soil, freshwater and sediment, marine water and sediment, and the atmosphere (Guerranti et al. 2019; Peijnenburg and Struijs 2006; Sirivithayapakorn and Thuyviang 2010).

In addition to being found throughout the environment, DEHP is also a known 'endocrine disruptor' (Erkekoglu et al. 2012). Endocrine disruptors are defined by the European Commission as 'an exogenous substance or mixture, that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub)population' (Waring and Harris 2005). Endocrine disruptors in the body mimic

hormones such as testosterone or estrogen and in doing so can disrupt the balance of hormones needed for essential biological processes such as reproduction (Carnevali et al. 2018). Furthermore, they can induce oxidative stress on exposed organisms (Cui et al. 2020). Stress in fish has been shown to inhibit reproduction (Pankhurst and Munday 2011). Under stress, hormones such as cortisol are increased whilst reproductive hormones such as estrogen and testosterone are supressed (Pankhurst and Munday 2011). As reproduction is key to the maintenance of populations and biodiversity, alterations to the reproductive capability of a population can have significant impacts on the trophic structure of an ecosystem by removal of affected species. The removal or suppression of one trophic level can cause trophic cascades in which the functioning and structure of an ecosystem is altered (Myers et al. 2007). Hence understanding potential changes to the reproductive output of an organism is important not only from a population context but also from an entire ecosystem perspective. This is especially true if the organism plays an important ecosystem role either by being part of a functional assemblage or by being numerically abundant, and thus an important prey item (Brandl et al., 2019).

1.3 Biomarkers

The effect of an environmental stressor on reproduction can be demonstrated in several different ways. Histology allows visual observation of cellular change in response to environmental stressors and can reveal changes in reproductive health, stage and identification of abnormalities (Blazer 2002). Histopathology has been acknowledged as an effective tool in the assessment of endocrine disrupting molecules on the reproductive health of taxa (Blazer 2002). Additionally, histology allows the staging of gametes which can indicate whether gametogenesis has been disrupted (Blazer, 2002). The quantification of the egg yolk protein vitellogenin (VTG), especially of that in males, allows insight into whether and how the reproductive processes within an animal has been altered by an exogenous stressor (Costa et al. 2010; Rose et al. 2002) – quantification of VTG production is extremely useful in this instance as the pathway for VTG production is extremely sensitive to the presence of estrogen (Sullivan and Yilmaz 2018). For this reason, VTG can be used as an indicator of whether estrogenic endocrine disruption has occurred (Sullivan & Yilmaz, 2018).

Using a combination of gonad histology and VTG analysis can give a clear understanding of the reproductive condition of an animal (Blazer, 2002).

There are many mechanisms by which a stressor may be impacting the reproductive capability of an organism. Biochemical change such as the production of reactive oxygen species (ROS) can also lead to a reduced reproductive output (Aitken et al. 2016; Kasahara et al. 2002). Antioxidants such as superoxide dismutase (SOD) and catalase (CAT) are produced to remove ROS which are formed during normal cellular function, or due to exposure to environmental stressors (Lister, Lamare, and Burritt 2015). However, if ROS production outpaces their removal by antioxidants, then oxidative damage occurs. This can occur when an organism is exposed to exogenous substances such as plasticisers and can result in altered reproductive success (Kasahara et al. 2002). Oxidation to integral structures such as proteins, lipids and DNA can impede cellular function resulting in changes to the fitness of an organism (Aitken et al. 2016; Junaid et al. 2018; Kasahara et al. 2002). Oxidative biomarkers have been used in the past to show the effects of direct waterborne exposure to DEHP and microplastics on the reproductive ability and physiological changes of a variety of organisms (see table 5).

Neurotransmitters have a wide variety of functions throughout the body and in the central nervous system. The neurotransmitters serotonin, 5-hydroxytryptamine (5H-T), and dopamine (DA) are found throughout the central nervous system and are involved in motor control, locomotion, and aggression (Weis et al., 2001; Winberg & Thörnqvist, 2016). As these neurotransmitters are linked to fish behaviour and reproduction (Ciranna, 2006; Klein et al., 2019; Sears & Hewett, 2021), changes in neurotransmitter concentrations may prevent or exaggerate the expression of adaptive or maladaptive behaviours. In addition to the potential for changed behaviour, changed levels of neurotransmitters can occur in response to stress (de Abreu et al. 2020; Thörnqvist et al. 2019). Increased DA has been identified by previous research as a common stress response in teleosts (de Abreu et al. 2020; Chabbi and Ganesh 2015; Prasad, Ogawa, and Parhar 2015). In contrast, stress in teleosts causes a decrease in 5H-T levels (de Abreu et al. 2020). As a result, analysing

neurotransmitters as well as behaviour allows a comprehensive understanding of how fish are responding to a stressor.

1.31 Behaviour as an endpoint for exposure experiments

Quantifying whether a treatment causes changes to behaviour can serve as an indicator for decreased fitness (Bjerselius et al., 2001; Brian et al., 2006). Previous studies have shown that reproductive, and territoriality-associated behaviours can be altered by exposure to estrogen mimics and serve as an indicator for altered reproductive success (Bell, 2001; Bjerselius et al., 2001; Brian et al., 2006). Because many fish have mating systems in which males display fitness through courtship behaviours and defence of territories against male conspecifics, this is important to understand (Bell, 2001; Bjerselius et al., 2001; Brian et al., 2006; Goatley and Brandl, 2017).

Assessing reproductive behaviours is extremely relevant for cryptobenthic fish. Cryptobenthic fish is a group made up by a variety of species, primarily those of the Orders Gobiiformes and Blenniiformes (Goatley and Brandl 2017). These fish are small, often less than 5cm in length and camouflage well in their environment (Goatley and Brandl 2017). These fish often have polygamous mating systems which requires the establishment and guarding of a territory and nest site against male conspecifics (Hastings & Peterson, 2010). Therefore, for the triplefin *Forsterygion capito*, member of Suborder Blennioidei, understanding impacts on reproductive behaviour will allow a broader scope for understanding the effects of DEHP on the fitness of these fish.

Table 3. Assays used to assess reproductive and physiological change.

Assay	Species	Contaminant	Finding	Reference
Histology	Pacific Oyster	PS microplastics	Decreased oocyte density and size due to exposure.	(Sussarellu et al. 2016)
Histology	Marine Medaka	Aqueous DEHP	Increased number of atretic follicles observed in ovaries. Number of spermatozoa decreased.	(Ye et al. 2014)
Histology	Chinese Rare Minnow	Aqueous DEHP	Significant increases in the number of immature gametes in both testes and ovaries.	(Guo et al. 2015)
Histology	Zebrafish	DEHP injection	Proportion of spermatozoa was significantly lower in DEHP treated fish.	(Uren-Webster et al. 2010)
Vitellogenin	Chinese Rare Minnow	Aqueous DEHP	Increased transcription of VTG was observed in both male and female fish.	(Guo et al. 2015)
Oxidative stress	Wistar rat	Oral administration of DEHP	Increased production of ROS in the testes of male rats as well as the antioxidants SOD and CAT.	(Kasahara et al. 2002)
Oxidative stress	Zebrafish	PS microplastics	Significant increases in SOD and CAT occurred due to microplastic exposure.	(Lu et al. 2016)
Behaviour	Goldfish	17β-estradiol	Decreased courtship behaviours in male goldfish.	(Bjerselius, Lundstedt-Enkel, and Dimberg 2001)
Behaviour	Three-spined stickleback	Aqueous ethinyl oestradiol	Exposure caused reduced male territorial behaviour.	(Bell 2001)
Behaviour	Guppy	Aqueous 4-tert-octylphenol and 17β-estradiol	Treatment groups exhibited decreased courtship behaviours.	(Bayley, Nielsen, and Baatrup 1999)
Neurotransmitters	Zebrafish	PS nanoplastics	Decreased dopamine and serotonin expression	(Sarasamma et al. 2020)

1.4 Aims of this research.

Despite the rapid increase in marine plastic pollution, and the potential impacts of exposure to endocrine disrupting plasticisers having been acknowledged (see Table 3), whether or not plasticisers leaching out of consumed microplastics cause similar effects seen in cases of direct exposure remains largely understudied. The resulting knowledge gap means that the extent of the impact that microplastics have on wildlife and ecosystems once they enter the environment, is not completely understood. Therefore, the aim of this study is to identify whether DEHP leaching from consumed microplastics impacts the reproductive and physiological processes of the cryptobenthic fish *Forsterygion capito.*

The hypotheses of the proposed research are:

1) Exposure to DEHP-dosed microplastics will negatively affect fecundity in *F. capito*. (Chapter Two)
2) Exposure to DEHP-dosed microplastics will negatively affect reproductive behaviours in *F. capito*. (Chapter Three)
3) Exposure to DEHP-dosed microplastics will negatively impact the levels of neurotransmitters in the brains of *F. capito*. (Chapter Three)
4) Exposure to DEHP-dosed microplastics will exert a physiological stress on exposed *F. capito* as evidenced by an increase in oxidative stress biomarkers. (Chapter 4)

In addition to the importance of understanding the impact of leaching plasticisers, particularly on reproduction, this study focusses on the reproductive capability of a cryptobenthic fish. Cryptobenthic fishes are an integral part of their associated ecosystems owing to their numerical abundance, and high larval outputs (Brandl et al. 2019; Kohn and Clements 2011). These characteristics of cryptobenthic fishes, though they have remained largely unstudied, are now being acknowledged. This is because recent research has found that these fish serve as major sources of prey within ecosystems as both adults and larvae (Brandl et al. 2019; Goatley and Brandl 2017). Therefore, changes to the reproductive output of these fishes, due to DEHP exposure via ingested microplastics, could have serious implications for the associated marine food web of this species.

Chapter 2 – Exposure to microplastics containing di(2-ethylhexyl) phthalate does not affect reproductive traits in *Fosterygion capito*

2.1 Introduction

The rapid uptake and consumption of plastic products has outpaced waste management solutions, leading to accumulation of plastic waste in landfills (Geyer et al. 2017; Jambeck et al. 2015; Ritchie 2018). This frequent mismanagement of plastics, in combination with their mass production, has led to these products being a prominent pollutant in the marine environment (Eriksen et al. 2014; Geyer et al. 2017). As such, plastics have contaminated every marine ecosystem from the Artic Circle to the bottom of the Mariana Trench (Chiba et al. 2018; Cózar et al. 2014; Lacerda et al. 2019). Microplastics, particles less than 5 mm in diameter, are of particular concern due to the ease with which they are ingested by a range of animal taxa from copepods to whales (Germanov et al. 2018; Sun et al. 2017). Microplastic ingestion poses a threat to animals via a suite of impacts including damage or blockage of the gastrointestinal (GI) tract (Cole et al., 2015; Welden & Cowie, 2016; Wright et al., 2013). In addition, the particles themselves are not inert and can often have persistent organic pollutants (POPs) or heavy metals adsorbed to the surface (Bakir et al. 2012; Rodrigues et al. 2019; Sarkar et al. 2021) or may contain chemicals such as plasticisers (Pedà et al. 2016).

Plasticisers are added during manufacture to add qualities such as UV resistance and flexibility (Carnevali et al. 2010; Kim et al. 2002; Tyler et al. 2018). The phthalate DEHP, di (2-ethylhexyl)-phthalate, is one of the most commonly used plasticisers (Chikae et al., 2004; Mulder & Knot, 2001; Tyler et al., 2018), and is often found in products including clothing, construction materials, medical devices, and children's toys (Chikae et al. 2004; Mulder and Knot 2001; Tyler et al. 2018). The everyday use of plastics containing this plasticiser have led to DEHP being considered a ubiquitous environmental contaminant (Chikae et al. 2004; Liu et al. 2017; Magdouli et al. 2013). DEHP however, is also a known endocrine disrupting chemical (EDC) as it can mimic sex hormones, such as estrogen, once in the body of exposed

animals (Erkekoglu et al. 2012). Endocrine disruption resulting from exposure to DEHP has been found to cause reproductive dysfunction in various animal taxa, including fish (Guo et al. 2015; Uren-Webster et al. 2010; Ye et al. 2014).

Reproductive dysfunction can occur via altered hormone production as well as disrupted gametogenesis with implications for clutch output (Guo et al. 2015; Uren-Webster et al. 2010; Ye et al. 2014). Previous research on teleost's (e.g., zebrafish (*Danio rerio*) and marine medaka (*Oryzias melastigma*)) has demonstrated that aqueous exposure to DEHP can cause an increased abundance of immature germ cells and a decrease in that of mature gametes (Uren-Webster et al. 2010; Ye et al. 2014). In addition, other reproductive measures, such as gonadosomatic index (GSI) are used to measure the effect of environmental stress on fish. GSI assesses the reproductive status of an animal by expressing gonad weight as a percentage of the body weight of an organism (Brewer, Rabeni, and Papoulias 2008; Htun-Han 1978). Throughout a reproductive cycle GSI changes, being highest at the peak of reproduction and lowest outside of a breeding season (Htun-Han, 1978). GSI is therefore useful in determining whether the reproductive condition of an animal has changed due to an environmental stressor, such as endocrine disrupters (Louiz, Ben-Attia, and Ben-Hassine 2009). For example, exposure to aqueous DEHP concentrations of 13.3 μg L^{-1} and 40.8 μg L^{-1} resulted in 32.7% and 40.0% decreases in egg production respectively in the Chinese rare minnow (*Gobiocypris rarus*) reducing reproductive success (Guo et al., 2015). Similarly exposure of *D. rerio* to 0.02, 0.2, 2, 20 and 40 mg L^{-1} of aqueous DEHP reduced the percentage of embryos produced at all concentrations (Carnevali et al. 2010). In the case of the highest dosage, embryo production was 1% of that control fish (Guo et al., 2015). Decreased fertilisation success was noted after the exposure of *O. melastigma* to both DEHP and it's metabolite MEHP, mono(2-ethylhexyl)-phthalate, at aqueous concentrations of 0.1 and 0.5 mg L^{-1} (Ye et al. 2014). Reductions in clutch output can be caused by a variety of mechanisms such as altered production of proteins such as vitellogenin (VTG). The production of VTG, a protein that serves as the main nutritive component of egg yolks, is controlled by an estrogen-mediated pathway (Villalobos 2018). Because the pathway for VTG production is very sensitive to estrogens (Copeland et al., 1986), assessing whether the quantity of this protein has changed in response to a treatment is indicative of endocrine

disruption, particularly in male fish which generally have very low blood VTG concentrations (Copeland et al. 1986). Because of this, high levels of VTG production in male fish may be indicative of reproductive dysfunction.

Cryptobenthic fish are a group of small fishes primarily belonging to the Orders Blenniiformes and Gobiiformes (Brandl et al. 2019). Cryptobenthic fish are extremely important in their ecosystems as their numerical dominance and high reproductive output make adult and larval fish important prey sources (Brandl et al. 2019; Depczynski and Bellwood 2003). The role of a high reproductive output in the ecological functioning of these fish highlights the importance of understanding the impact of anthropogenic stressors, such as plasticisers, on their reproductive success. Changes in reproductive success can have the potential to alter ecosystem dynamics. Therefore, the aim of the present study was to explore how exposure to DEHP-dosed microplastics would affect the reproductive capability of a cryptobenthic fish. To understand the impacts of DEHP on cryptobenthic fish, breeding pairs of adult triplefins (*Forsterygion capito*) were fed DEHP-dosed polystyrene microplastics along with virgin polystyrene microplastics (PS) and control food over a five-week period during which the presence and absence of egg clutches was monitored. After the exposure period, fish were euthanised and liver and gonad tissue were removed and analysed for vitellogenin mRNA concentrations and changes to gonad structure, respectively. Predictions from previous research were:

(i) Exposure to DEHP, via ingested microplastics, would disrupt gametogenesis evidenced by changed gamete proportions upon histological analysis in male fish.
(ii) Exposure to DEHP, via ingested microplastics, would result in decreased clutch output of exposed fish.
(iii) Exposure to DEHP, via ingested microplastics, would increase the levels of vitellogenin mRNA in the liver.

The reproductive ability of an organism is crucial to population maintenance and more broadly, ecosystem functioning. Therefore, insight as to the influence of anthropogenic

stressors on the reproductive success of taxa is crucial to understanding how these stressors will impact ecosystems.

2.2 Methods

2.21 Ethics Statement

All work carried out in Ōtepoti (Dunedin), Aotearoa (New Zealand) was in accordance with the University of Otago Animal Ethics guidelines (Animal Ethics approvals AUP-19-70, MPI collection permit 644).

2.22 Study location and animal collection

A total of 34 male and 26 female *F. capito* were collected in May of 2019 on the eastern side of Muaūpoko (Otago Peninsula), Ōtepoti, Aotearoa (-45.828172, 170.640949) (fig. 2). The males were collected by locating nests and removing the guarding male (fig 3). Females were collected using minnow traps (fig. 3) baited with crushed green lip mussel (*Perna canaliculus*), which were set in the intertidal zone, left overnight, and cleared in the morning. All fish were immediately transferred to the Portobello Marine Lab (PML) where they were held in 70 L tanks with flow through seawater and supplied with an air stone and

Figure 2. Map showing the study location (black box) and fish collection site (red circle).

habitat (PVC pipe) in densities of 20 fish per tank. Males and females were housed separately until they were assigned into breeding pairs and treatment tanks.

Figure 3. Left) A nest-guarding male with a clutch of eggs. Right) Minnow trap used to collect female fish (image sourced from marine deals)

2.23 Aquaria conditions

Prior to introduction to the treatment tank, each fish was selected randomly from the holding tank and weighed. Weighing the fish pre- and post- experimental period, allowed assessment of whether there was a treatment effect on body weight (fig. 5). Breeding pairs were established by introducing an individual male to each treatment tank, allowing acclimation over a 48-hour period as per the methods of Wellenreuther and Clements (2007). Individual females were randomly selected and placed into a treatment tank containing a male fish. Individual breeding pairs were housed in one of thirty 20 L glass tanks which were separated by an opaque background removing the potential for interactions between breeding pairs which may confound the results (i.e. agonistic interactions between males). The tanks were distributed across two temperature control rooms with the order of the treatment tanks randomised to control for spatial differences. Each temperature control room had five tanks from each treatment. The flow rate of the

tanks was 600 mL per minute, with a complete water change occurring every 40 minutes. Water parameters (temperature, flow rate and aeration) were monitored daily and recorded. The tanks had constant aeration provided by an air stone and the temperature of the tanks was maintained at ambient winter conditions 8.6-10°C (Smith 2023). All tanks contained a halved terracotta pot to act as a spawning substrate. The behaviour of the fish was observed and recorded as was the presence or absence of an egg clutch. Finally, each tank was carefully syphoned daily to remove any leftover food. No aggression or abnormal behaviour was recorded during the treatment period; no mortality occurred during the five-week treatment period.

2.24 Plastic preparation

All plastics were produced at James Cook University, Townsville Australia. To produce microplastics of the desired size, 305 g of polystyrene was dissolved in 1 L of dichloromethane and left to stir overnight using a magnetic stirrer at room temperature in a 2 L bottle. Next, 68.4 mL of DEHP dissolved in 0.5 L of dichloromethane was added to this solution. This homogenous solution was then drop cast on watch glasses (50 mm in diameter) and allowed to evaporate in a fumehood over 60 hours to form a thick white membrane. The membranes were dried further in vacuo overnight to form brittle plates of polystyrene containing DEHP. The materials were ground into microplastic particles using a food processor (NutriBullet, 900 Series) and sieved in the range of 200 to 400 µm (Geo-Con). The size was confirmed using optical microscopy.

2.25 Plastic-dosed food treatments

There were three treatment groups, control, virgin polystyrene (PS) and PS dosed with DEHP (DEHP). These groups were each assigned ten pairs in ten replicate tanks. Male and female gonads consist of two separate lobes, one intact lobe from each fish was preserved in buffered formalin for histological analysis and the other lobe was snap frozen in liquid nitrogen. Prior to preservation, both gonad lobes were weighed for calculation of the GSI $\frac{Gonad\ weight}{Body\ weight} \times 100$. The liver was also weighed and split into at least two pieces and snap frozen in liquid nitrogen.

Fish in treatment groups were exposed to either DEHP-dosed PS or virgin PS particles for a five-week period. Microplastics were embedded in pulverized green lip mussels at a ratio of 2.5% plastic by food weight (5 g mussel block; 0.125 g PS microplastics). Mussel blocks were weighed down using a stainless-steel nut, simulating the benthic feeding of triplefins (Feary et al., 2009). To control for the temporal nature of environmental plastic exposure, fish were assigned diet treatments on alternate days. The degree of environmental microplastic pollution is currently unknown for the Otago region. Therefore, we used previously published data from Rochman et al., (2013) to set our plastic loading concentration. However, this value was deemed too high (10% plastic to food) and was reduced by 75% (2.5% plastic to food). At the end of the five-week exposure period, all fish were euthanised as per Otago animal ethics guidelines (AUP-19-70) using Aqui-S (6 mL per litre seawater).

2.26 Clutch output

Each day, the presence, or absence of an egg clutch was noted. The clutch was then removed from the spawning substrate and discarded. A fresh spawning substrate was then placed back in the tank.

2.27 Histological analysis

Male gonads were placed in labelled cassettes and then dehydrated in graded ethanol, and xylene. After dehydration, the samples were placed in 60°C paraffin wax which was changed twice. Samples were embedded in paraffin wax, as flat as possible so that a complete cross-section could be obtained. Sections 0.5 µm thick were then taken using a microtome, these were then placed on a slide and dried. The prepared slides were then stained. The presence of mature gametes and immature germ cells were quantified (using ImageJ, version 1.52a) by overlaying a grid and classifying the cells at each of the 108 intersections as either mature or immature. The proportion of mature and immature germ cells was then calculated for analysis. Mature was defined as spermatozoa, and everything which was not spermatozoa was deemed immature. A diagram of this is shown in fig. 4 below.

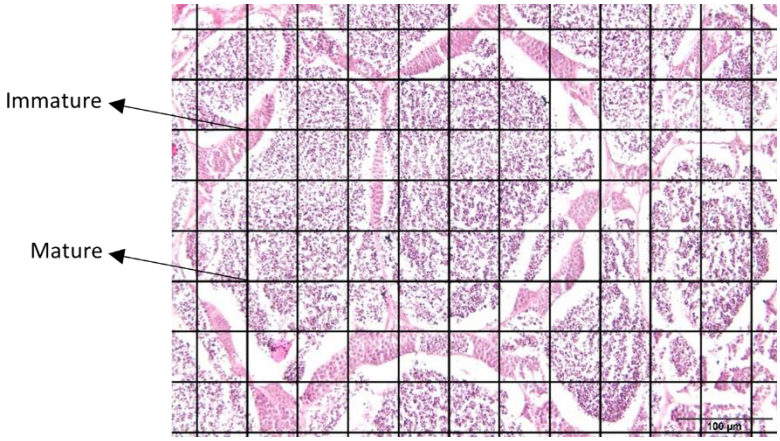

Figure 4. Shows the grid used in analysis and an example of staging for both mature gametes and immature germ cells.

2.28 Vitellogenin expression

2.28.1 RNA Extraction

Total RNA was extracted from this tissue by homogenisation in 1 mL of Trizol using a Next Advance bullet blender homogeniser. Lipids were removed by aspiration after centrifugation for 5 minutes at 12000 rpm at 4°C. After centrifugation, 0.2 mL of chloroform was then added, and each sample was shaken for 15 seconds and incubated at room temperature for 3 minutes. Samples were then centrifuged at 10000 rpm for 15 minutes. The supernatant was removed and placed in a new tube containing 0.5 mL of isopropanol. The samples were inverted to mix and incubated at room temperature for 10 minutes. After incubation, the samples were centrifuged for 10 minutes at 10000 rpm. The supernatant was removed, and the pellet was washed twice by addition of 1 mL of 75% ethanol and centrifugation at 7500 rpm for 5 minutes. After washing, the ethanol was discarded, and the samples were left to air dry for 5 minutes. Once dry, the RNA was reconstituted by addition of 20-50 µl of milli-Q water. To ensure the samples were dissolved, they were placed in a 55°C water bath for 5 minutes. RNA quality was assessed using fragment analysis, by an Agilent Bioanalyzer 2100 (Otago Genomics Facility, University of Otago).

2.28.2 DNAse treatment

After extraction, a DS-11 series spectrophotometer was used to estimate the concentration of RNA in each sample. Samples were then diluted 10-fold. The DNA-*free*™ kit (Life Technologies, USA) was then used to DNAse treat 5 µg of RNA per sample as per kit instructions (DNAase free). The RNA containing supernatant was then transferred to a new tube.

2.28.3 Reverse Transcription

After DNAase treatment the RNA samples were reverse transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) as per kit instructions (High-capacity cDNA reverse transcription).

2.28.4 Primer Design

To design species-specific qPCR primers, a PCR was run on cDNA from a wild-caught female fish (for PCR primers see Table 1). To do this 30 µL reactions were run, containing 6 µL 5x buffer, 1.2 µL of 50 mM $MgCl_2$, 2 µL of 8mM dNTPs, 1 µL of 20 ng/µL of cDNA, 1 µL of both the forward and reverse primers at 10 mM, 0.5 µL of Mangotaq (Meridian Bioscience, USA) and 17.3 µL of milli-Q water. PCR conditions were 95°C for 5 minutes, followed by 35 amplification cycles of (i) 95°C for 30 seconds, (ii) 45°C for 30 seconds (iii) 72°C for 1 minute, and finally 72oC for 1 minute. Samples were then placed in a 1% agarose gel at 100 V for 45 minutes. Bands of interest were extracted; the gel was eluted using NucleoSpin™ Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) as per kit instructions (NucleoSpin Gel and PCR Clean-up). The resulting product was sequenced using Sanger sequencing and BLASTn (Altschul et al. 1990) was used to confirm the identity of the sequence. Lastly, Primer3 (Kõressaar et al. 2018; Koressaar and Remm 2007; Untergasser et al. 2012) was used to design primers nested withing the Sanger-sequenced cDNA. It was predicted that the primers produced would amplify mRNA for both types of VTG.

2.28.5 qRT-PCR protocol

The specific primers (Table 4) were used to design a qRT-PCR protocol using standard dilution from a positive control (female liver tissue). To do this, the primers were first run on a temperature gradient so that the annealing temperatures used were 60, 61, 62 and 63°C. This was to determine which temperature had the best annealing success as shown by the quantification cycle (Cq). An annealing temp of 61°C was selected for both actin and vitellogenin. A standard curve was then obtained for both genes with different primer amounts of 0.5, 0.2 and 0.1 µL. PCR product was diluted 1:10 starting from a 1:100 dilution of the cDNA concentration.

Table 4. Primers used in the PCR and qRT-PCR protocols

				qRT-PCR			
Gene Name	Sequence 5' to 3'	Annealing Temperature	Product size (bp)	Gene name	Sequence 5' to 3'	Annealing Temperature (°C)	Product size (bp)
Actin	Forward: CATGGACTCTGGTGATGGTG Reverse: GATGCCAGGGTACATGGTG	45	472	Actin	Forward: TACAGCTTCACCACCACAGC Reverse: GCTCAAAGTCCAGTGCAACA	61	82
Vitellogenin (VTG)	Forward: TGTGGACTCTGTGGAAAGG Reverse: CTGCTGTTTCCCTCAGGT	45	355	Vitellogenin (VTG)	Forward: GAGTACACCGCACCCAACAC Reverse: AGCACGGGCTCAACAGAGTA	61	185

2.29 Statistical Analysis

Changes in body weight between treatment groups and over time were identified using a one-way repeated measures ANOVA male body weight (three levels, control, PS and DEHP). Whilst for gamete proportion (three levels), and female GSI (three levels) a one-way ANOVA was used. During analysis, four histological samples (one control and 3 PS) could not be photographed due to the inability to take a representative picture. The body weight of female fish (three levels) initially violated the assumption of normality so was log-transformed and subsequently analysed using a one-way ANOVA. The nature of any significant differences detected was then determined using Tukey's HSD post hoc test. Assumptions of ANOVA were tested using Levene's test for homogeneity of variance and a Shapiro Wilks test for normality of data as well as visual inspection of residual plots. Data which violated the assumptions of ANOVA were analysed using a Kruskal-Wallis nonparametric test (male GSI (three levels)). The nature of any observed significance was determined using the Dunn post hoc test. Unfortunately, due to the high fat content of the

livers, all but three samples of extracted RNA per group were degraded (Appendix A). The VTG mRNA levels in a gravid untreated female was therefore used both to ensure primer function but also to add context to the mRNA measured in the nine experimental fish. The mRNA data (three levels) was analysed using a one-way ANOVA. Clutch production data (two levels) was analysed using the Fisher test.

2.3 Results

2.31 Fish health

No mortality occurred during the experimental period. Upon dissection, some fish were not the intended sex resulting in seven pairs, one in the DEHP and six in the PS group being male-male rather than male-female pairings.

2.32 Body weight

Prior to treatment, the average weight of male fish in the control (6.89 g), PS (5.9 g) and DEHP (6.36 g) groups were not significantly different from each other ($F_{2,30}$= 1.011, p-value = 0.376). Though in the male control and PS groups there appeared to be a decrease in body weight post-treatment, The average body weights of each group, control (6.39 g), PS (5.6 g), and DEHP (6.47 g) was not significantly different from their starting weight ($F_{1,2}$= 1.664, p-value > 0.326) (fig. 5A). The same was true for females as prior to treatment weight was consistent among groups control (4.4 g), PS (4.47 g), and DEHP (3.8 g) ($F_{2,23}$= 0.551, p-value > 0.584). The post-treatment weights of females following treatment, control (4.3 g), PS (4.64 g) and DEHP (4.05 g), were also not significantly different from the original weights ($F_{1,2}$= 1.179, p-value > 0.391) (fig. 5B).

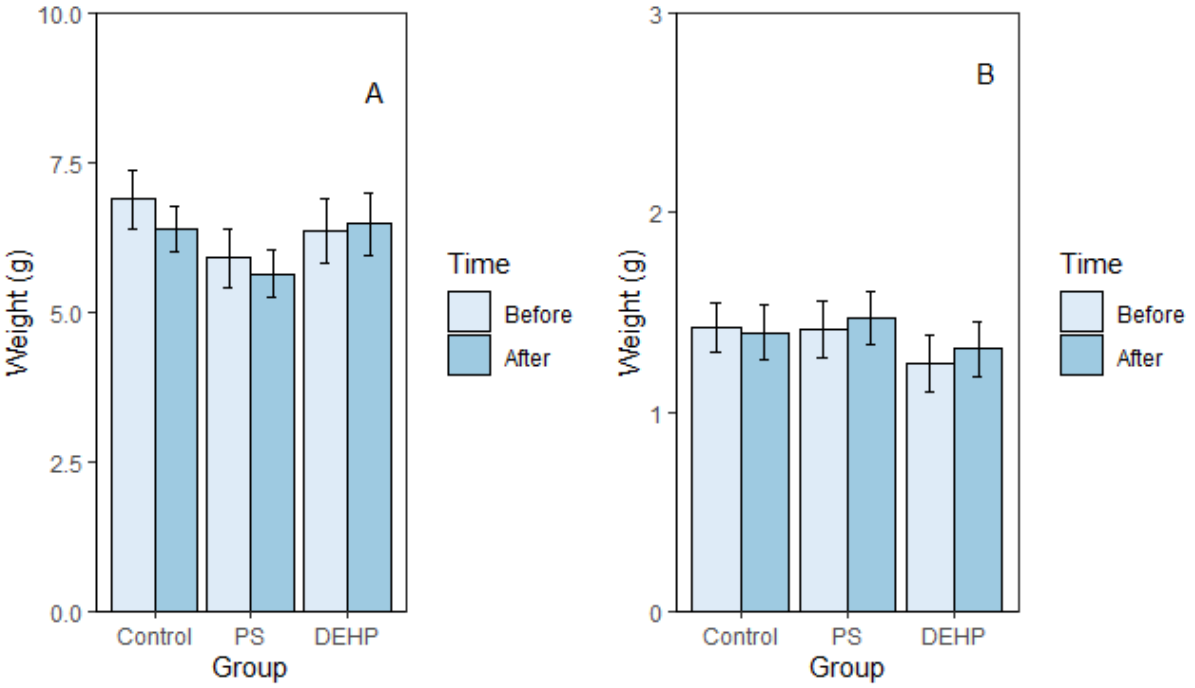

Figure 5. The mean body weight of *F. capito* ± standard error before and after treatment in A) males (control n=11, PS n=12, DEHP n=10) and B) females (control n=9, PS n=8, DEHP n=9) of each group.

2.33 Clutch output

Of the nine viable pairs in the DEHP group, three laid an egg clutch, whilst of the ten viable control pairs six laid a clutch. This meant that the reproductive output in the DEHP group was reduced by 50% compared to that of the control (33% and 60% respectively; see fig. 7A). But this change was not statistically significant (p-value= 0.369). Due to erroneous fish sexing in the PS group, resulting in only six of the ten pairs being male-female pairs, the clutch production of this group could not be compared to that of the other two groups.

2.34 Gonadosomatic index (GSI)

In both sexes it appeared as though the GSI was increased in fish exposed to virgin PS. However, in males the average GSI of PS-exposed fish (1.93 GSI%), was no different from that of control (1.49 GSI%) and DEHP-exposed fish (1.38 GSI%) (chi-squared=2.63, df=2, p-value>0.1) see fig. 7B. The same was observed in female fish as the average GSI value in the control (7.16 GSI%) and DEHP (7.19 GSI%) fish was no different to that of the PS group (8.86 GSI%) ($F_{2,23}$=0.414, p-value>0.1) see figure 7C.

2.35 Histological analysis

The average proportion of mature gametes in the control group of 0.64 was not significantly different ($F_{2,26}$=0.661, p-value >0.05) to that of the PS (0.68) or the DEHP (0.55) groups (fig. 7D). The same was true for immature germ cells ($F_{2,26}$=0.528, p-value >0.05), control (0.35), PS (0.31) and DEHP (0.44). Although trends suggested that exposure to DEHP-dosed microplastics led to an increased proportion of immature germ cells and a decreased proportion of mature gametes, this was not significant. For representative cross sections of each group see fig. 6.

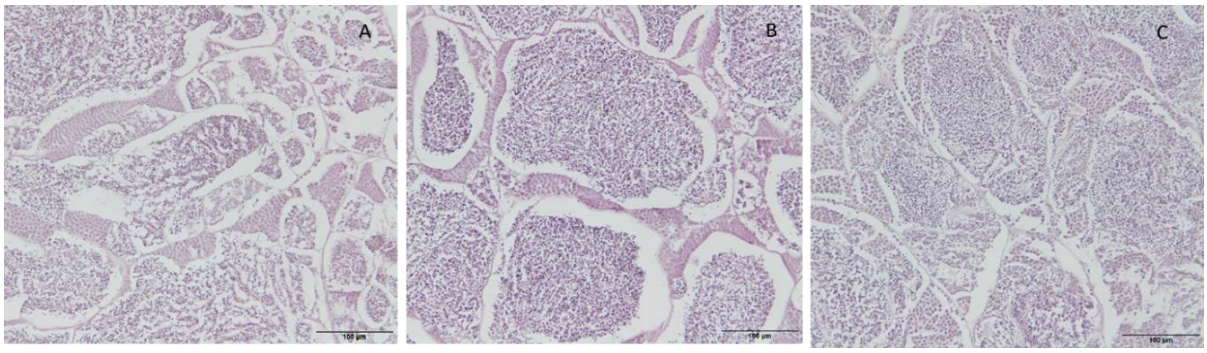

Figure 6. A representative cross section of a stained slide of male *F. capito* gonad, from the control (A), PS (B) and DEHP (C) group. Scale bar is equal to 100 µm.

2.36 VTG mRNA analysis

Analysis of the mRNA expression in the nine undegraded samples showed that VTG mRNA levels were not influenced by exposure to DEHP when compared to the control and PS groups ($F_{2,6}$=3.493, p-value>0.05). This is as the average VTG mRNA level of each group control (1.23), PS (0.49) and DEHP (0.82) were not significantly different from one another. Furthermore, the VTG mRNA levels in the experimental male fish were less than 10% of that in an untreated gravid female fish (11.4) (fig. 7E).

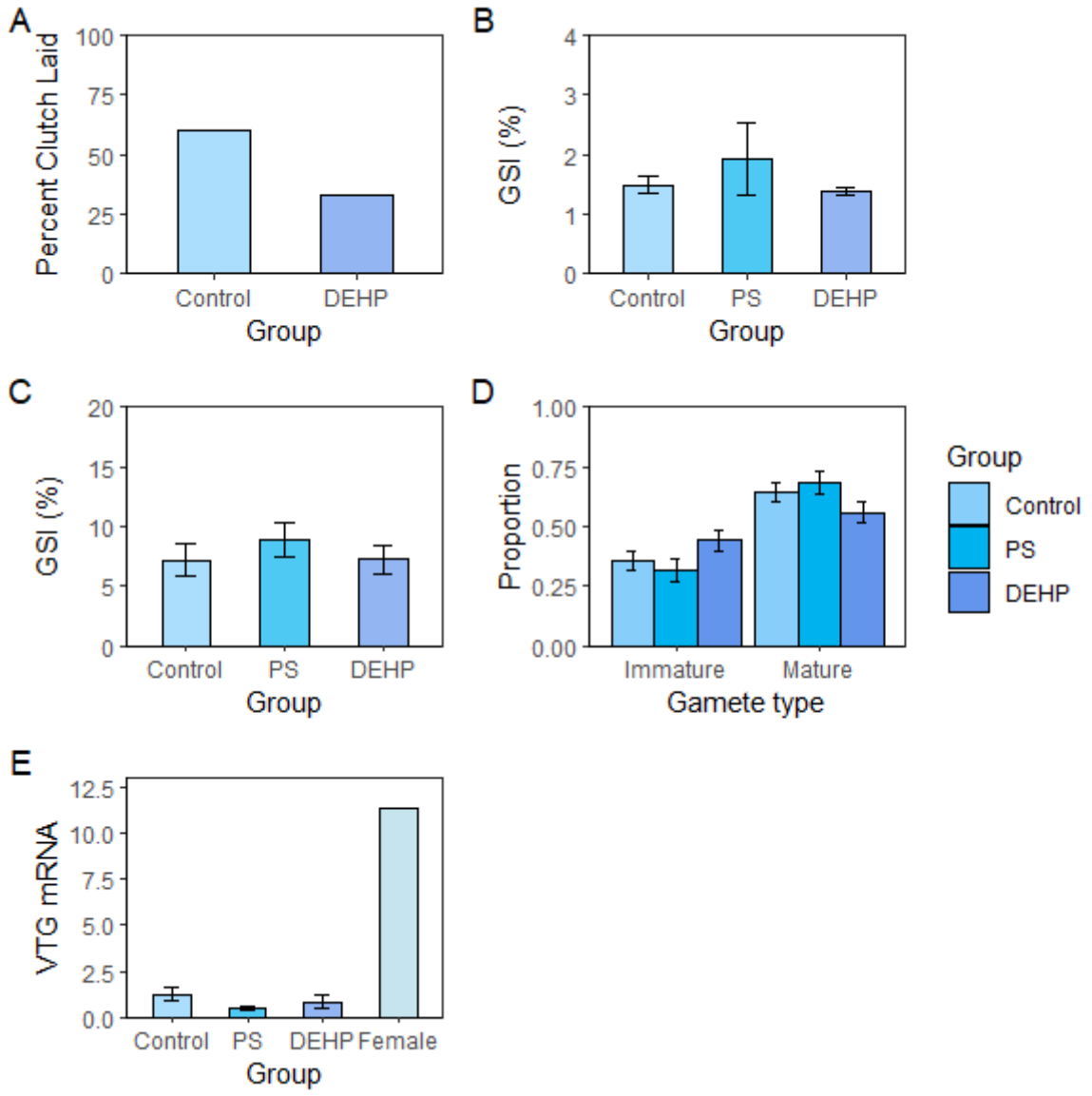

Figure 7. A) The percentage of pairs which produced an egg clutch in the control n = 10 and DEHP n= 9 groups. B) Mean GSI ± standard error per group in males, control n=11, PS n=12, DEHP n=10. C) Mean GSI ± standard error per group in females control n=9, PS n=8 DEHP n=9. D) Proportion ± standard error of immature and mature gametes for each treatment group, control (n=10), PS (n=9), DEHP (n=10). E) Vitellogenin mRNA expression in control, PS and DEHP treated male fish (n=3 per group) and a wild caught gravid female (n=1).

2.4 Discussion

The phthalate plasticiser DEHP is a ubiquitous environmental pollutant. To date, most research has focused on exposing marine and freshwater taxa to aqueous DEHP which has been shown to have consequences for the reproductive success in a range of taxa including fish (Chikae et al. 2004; Magdouli et al. 2013; Uren-Webster et al. 2010; Ye et al. 2014). However, despite its common use in plastics, it is poorly understood whether exposure to DEHP via ingestion of plastics yields similar effects. Here, it is demonstrated that ingestion of DEHP-dosed microplastics has little impact on the reproductive success of two cryptobenthic fish. For example, clutch output, GSI, gametogenesis and VTG mRNA expression were unchanged in exposed *F. capito*. This was unexpected as many past papers have observed decreased reproductive output, linked with altered gametogenesis and changes to hormone concentrations in response to aqueous DEHP exposure (Carnevali et al. 2010; Guo et al. 2015; Ye et al. 2014).

Histological analysis can be used to assess the condition of a given tissue. For example, it can be used to assess whether the production of gametes is occurring as expected given the reproductive cycle of the studied species (Blazer 2002). Previous research has found that exposure to aqueous DEHP causes the disruption of gametogenesis in both male and female fish, preventing the production of mature gametes (Ahmadivand et al. 2016; Corradetti et al. 2013; Uren-Webster et al. 2010). For instance, in zebrafish, aqueous DEHP exposure caused a significant increase in the number of immature germ cells and decreases in the number of mature gametes (Uren-Webster et al. 2010). Also in zebrafish, aqueous DEHP concentrations of 0.2 and 20 μg L^{-1} for 3 weeks led to an increased proportion of early-stage germ cells and a decreased proportion of intermediate germ cell stages (Corradetti et al., 2013). Additionally, male rainbow trout exposed to 50 mg kg^{-1} injections of DEHP had few mature gametes after 10 days of exposure (Ahmadivand et al., 2016). The accumulation of immature germ cells suggests that DEHP exposure disrupts gametogenesis, preventing the transition of immature germ cells to mature ones (Ahmadivand et al. 2016; Corradetti et al. 2013; Uren-Webster et al. 2010). Although it was not significant, we observed a similar trend in the DEHP exposed fish in *F. capito* (fig. 7D). In addition, GSI remained unchanged across treatment groups, suggesting that gonad condition was not altered by DEHP

exposure (see fig 7B). These findings contrast with previous studies that have shown that DEHP alters GSI, negatively impacting gonad condition, finding which often accompanies the disruption of gametogenesis (Corradetti et al., 2013; Uren-Webster et al., 2010). The present study differs from published literature in that exposure to DEHP occurred via ingestion of dosed microplastics rather than aqueous exposure. The exposure method in this study meant that the dose of DEHP fish were exposed to in the present study likely varied between feedings. The difference in exposure method, consistency and duration may explain the different observations between this and previous research.

VTG can be used as a proxy for changed estrogen levels as the pathway to produce this protein is regulated by estrogen, so increases in estrogen can cause increased VTG production (Copeland et al. 1986). In male fish, this serves as a clear indicator of endocrine disruption as naturally, male fish produce very low levels of VTG as it is not required in the production of sperm (Copeland et al. 1986). The present study failed to detect significant change to the VTG expression of exposed fish (fig. 7E), additionally the VTG mRNA detected in male fish was substantially lower than that of the female control. The female control in this case highlights that the primers and PCR protocol were successful in being able to produce VTG mRNA. The female control therefore confirms that the low VTG levels observed in the treated male fish were a true result. A caveat of this being the low sample size for each group (n=3), due to poor RNA quality. This poor quality likely being due to the high fat content of the livers, making extraction of quality RNA difficult. These high fat levels potentially owing to the fat content of food provided. However, as the VTG mRNA in all groups was less than 10% of that of the gravid female (fig. 7E), there is no reason to believe that DEHP influenced VTG mRNA expression. The effect of DEHP on VTG expression is varied as previous literature observed the upregulation of this protein as a result of exposure (Guo et al. 2015; Uren-Webster et al. 2010; Ye et al. 2014) whilst others did not observe any change to VTG levels (Golshan et al. 2015; Ye et al. 2016). The variation in findings around the influence of DEHP on VTG expression indicates that the concentration and length of exposure can influence whether DEHP will impact the endocrine system.

The results of the present study contradict those of published studies of aqueous DEHP exposure and are instead supported by published literature focussing on microplastic exposure. For example, 3 weeks exposure to waterborne 2 μm PS microplastic at concentrations of 44 μg L^{-1} did not alter the reproductive success of Japanese medaka (*Oryzias latipes*) (Assas et al. 2020). Similarly, in male and female zebrafish, GSI and the proportion of early and late-stage gametes was unchanged at all suspended PS concentrations (10,100,1000 μg L^{-1}) after 21 days of exposure (Qiang and Cheng 2021). However some studies have found significant effects; for instance 60 days exposure to suspended 10 μm PS microplastics significantly altered the GSI and fecundity of marine medaka at concentrations of 2, 20 and 200 μg L^{-1} (Wang et al. 2019). The variable nature of effects of microplastic exposure highlights the need for environmentally relevant studies to be undertaken in all systems, as studies of the same plastic type can have different outcomes. Furthermore, many of the previously mentioned studies used high and constant exposure conditions of very small microplastics which are not environmentally relevant.

The contrasting results of the present study and published DEHP literature is likely due to the method used to expose fish to DEHP. Past literature has focussed on aqueous exposure, the protocol for which animals are kept at a constant concentration of DEHP for a given period. Though the concentrations used in past literature have been largely based on DEHP levels detected in the environment, these studies do not account for spatiotemporal dynamics of environmental concentrations. DEHP in the environment does biodegrade, a process which can take up to one month, meaning that the DEHP concentrations in surface waters is likely to be highly variable (Magdouli et al. 2013). Furthermore, the movement of water by currents will cause varied concentrations across a surveyed site (Paluselli and Kim 2020). This means that though the aqueous concentrations of DEHP used in previous literature may be environmentally relevant, the conditions of exposure were not. The present exposure method led to variable exposure to DEHP, as fish were unlikely to ingest the same amount of plastic each feeding and were only given dosed food every second day. The present study has, as a result, shown that variable and low-dose exposure to DEHP is unable to elicit the effects observed seen in previous studies constant aqueous exposure. The findings of the present study therefore stress the importance of environmental

relevance in studies as to the effects of pollutants on organisms as only realistic study models can properly inform subsequent decision making.

Chapter 3 – Exposure to DEHP alters neurochemistry and reproductive signals in male *Forsterygion capito.*

3.1 Introduction

Globally, marine ecosystems are facing a barrage of threats ranging from climate change to increased pollution. Of concern are endocrine disrupting chemicals (EDCs). EDC's can alter hormone production and neurochemistry which, in turn, may impact behaviour of marine taxa following exposure (Carbone et al. 2019; Huang et al. 2022; Nilsson et al. 2012). One well studied group of EDCs are phthalate plasticisers. Plasticisers are chemicals added to plastics during manufacture to add qualities such as strength and flexibility (Carnevali et al. 2010; Cole et al. 2011; Kim et al. 2002; Tyler et al. 2018; Ye et al. 2014). Because plasticisers are not bound to the molecular structure of plastics, they are released by diffusion, abrasion and leeching thereby entering the environment (Tyler et al. 2018). As plasticisers leech readily from plastics, published literature acknowledges that microplastics can act as vehicles for exposure to plasticisers after the particles are ingested (Nobre et al. 2015; Rodrigues et al. 2019).

One of the most used phthalate plasticisers is di-(2-ethylhexyl)-phthalate (DEHP) which is used as an additive to polyvinyl chloride (PVC) plastic products (Tyler et al., 2018). The common use of PVC and its associated plasticiser DEHP has led to DEHP being a ubiquitous environmental pollutant (Chikae et al. 2004; Liu et al. 2017; Magdouli et al. 2013). DEHP is a known endocrine disruptor and has previously been found to have estrogenic influences on a variety of taxa such as goldfish (*Carassius auratus*), marine medaka (*Oryzias melastigma*) and zebrafish (*Danio rerio*), with negative implications for reproductive success (Cole et al. 2015; Erkekoglu et al. 2012; Golshan et al. 2015; Wang et al. 2019). DEHP exposure has also been found to cause altered neurotransmitter concentrations as both increases and decreases have been observed in a range of taxa (Carbone et al. 2019; Tran, Do, and Kim 2021) as well as changed behaviour in larval medaka fish (*Oryzias latipes*) (Yang et al. 2018).

There is currently no literature as to the effects of DEHP on the reproductive behaviour of teleosts. However, DEHP was found to alter the reproductive behaviour of male *Spodoptera littoralis* (a species of moth) (Avilès et al. 2020). Though research with a focus on reproductive behaviour is absent, previous studies have shown that DEHP exposure can impact other behaviours such as the activity levels of exposed organisms (Huang et al. 2022; Tran et al. 2021). Furthermore, exposure to other estrogenic EDCs has negatively impacted reproductive behaviour in fish. The estrogen mimic 17α-ethinylestradiol (EE2) caused decreased display behaviour when male *Betta splendens* (Siamese fighting fish) were exposed to 10 ng L^{-1} for two weeks (Dzieweczynski and Kane 2017). Exposure to EE2 in this case caused males to be less attractive to both exposed and unexposed female conspecifics (Dzieweczynski and Kane 2017). Similarly, aqueous exposure to the plasticiser triphenyl phosphate (TPhP) at concentrations of 1.6, 8, and 40 µg L^{-1} decreased chasing reproductive behaviours in male *O. latipes* (Li et al. 2018). As DEHP exposure has been demonstrated to impact the behaviour of exposed fish, and because EDCs with estrogenic influences cause disruption of reproductive behaviours, the present study aims to assess whether DEHP influences reproductive behaviour. Behaviour, in all its forms, is influenced by endogenous factors such as neurotransmitters. Therefore, changes in neurotransmitter levels can in turn negatively impact the energy budget of animals exposed to a stressor (Anacleto et al. 2018; Huang et al. 2022; Weis et al. 2001). Because of this it is also important to assess whether exposure to DEHP via dosed microplastics may impact behaviour or neurotransmitter concentrations.

The production of reproductive hormones and performance of behaviours are, in part, regulated by neurotransmitters (Chabbi and Ganesh 2015; Prasad et al. 2015). Neurotransmitters have a variety of functions throughout the body as well as in the central nervous system. The neurotransmitter serotonin, or 5-hydroxytryptamine, (5-HT) is distributed throughout the central nervous system and is involved in a large variety of physiological functions such as motor control, immune system function, food intake and is involved in the production of estrogen (Ciranna 2006; Prasad et al. 2015). Similarly, dopamine (DA) is used widely throughout the central nervous system and has been linked to motor control, cognitive function, and the reproductive behaviours of mammals (Klein et al.

2019). In fish, 5-HT and DA have been found to be involved in locomotion, aggression and feeding behaviour (Weis et al. 2001; Winberg and Thörnqvist 2016). Previous research has demonstrated that exposure to DEHP can influence the production and regulation of neurotransmitters. For example, exposure of zebrafish to aqueous DEHP caused disordered swimming behaviour likely due to changed expression of genes involved in DA production (Tran et al. 2021). Similarly, exposure of zebrafish to polystyrene nanoplastics suspended in the water column caused decreases in levels of DA and 5-HT (Sarasamma et al. 2020). Sarasamma et al., (2020) suggested that the observed changes in DA and 5-HT concentrations may have led to increases in locomotory and exploratory behaviours as well as a decrease in predator avoidance and aggression. DA and 5-HT have also been found to be involved in the response of animals to a stressor (de Abreu et al. 2020; Chabbi and Ganesh 2015; Prasad et al. 2015; Szabo et al. 1991; Thörnqvist et al. 2019). As these neurotransmitters are involved in both stress response and reproductive processes it is important to understand how exposure to DEHP will impact their global concentrations and whether changes to these may have negatively impact reproductive ability.

Cryptobenthic fish are a group of small fishes which primarily belong to the Orders Blenniiformes and Gobiiformes (Brandl et al. 2019). Due to their numerical dominance in both the adult and larval stages (Brandl et al. 2018), cryptobenthic fish are extremely important in their ecosystems as prey sources (Brandl et al. 2019; Depczynski and Bellwood 2003). Many cryptobenthic fish have a planktonic larval stage, where the mortality rates are uniformly high and can exceed 99% (Shima et al. 2021). This high mortality is compensated for by high quantities of offspring reaching the planktonic stage (Shima et al. 2021). Therefore, embryo survival is a crucial part of the reproductive fitness of these fish. Due the importance of embryo survival, for blennioid and many other cryptobenthic fish, parental care is undertaken by the male fish (Brandl et al. 2018). The males parental care tasks include guarding the embryos from conspecific and heterospecific predators, also fanning, cleaning and application of antibiotics which is secreted from the tips of fin rays of male fish (Hastings and Peterson 2010). These parental behaviours increase embryo survival and therefore benefit the reproductive fitness of the individual and the population (Hastings & Peterson, 2010).

For blennioid fish, the establishment of a nest site and associated territory serves as an honest signal to females of the males suitability as a mate (Pärssinen et al. 2019). Once established, males must recognise female conspecifics and display courtship behaviours, after which eggs are laid in the males' nest (Hastings and Peterson 2010; Tornquist 2020; Wellenreuther, Syms, and Clements 2008). Nesting territories require guarding, which results in territorial behaviours leading to aggression between male conspecifics (Bell 2001; Hastings and Peterson 2010). These behaviours are important for embryo survival and clutch success (Hastings & Peterson, 2010). Triplefins (Family Tripterygiidae), a common group of cryptobenthic fishes found in Aotearoa (New Zealand), exhibit these characteristic behaviours which involve the defence of a territory and guarding of embryos (Tornquist 2020; Wellenreuther et al. 2008). Courtship behaviours displayed by triplefins include lateral displays and habitat presentations whilst territorial behaviours include, biting, chasing, and lateral threat (Tornquist, 2020). Additionally, male fish assume a nuptial coloration during the breeding season to advertise their availability as a mate (Tornquist, 2020; Wellenreuther et al., 2008). Like other triplefins in Aotearoa, *Forsterygion capito* displays a nuptial colouration of a solid, whole body, jet black colour (Tornquist 2020; Wellenreuther et al. 2008). The expression of territorial and courtship behaviours can be influenced by environmental factors, such as habitat quality and the size of other male conspecifics (Thompson 1986; Tornquist 2020)**.** Because habitat quality and male size are determinants of female interest in a male's nest site (Thompson 1986; Tornquist 2020), this means that in both courtship and defence of habitat, emphasis is placed on displaying the size of the guarding fish, hence lateral displays and lateral threat occur as males attempt to look as large as possible (Butler and Maruska 2016).

For a large proportion of cryptobenthic species, territorial, courtship and parental behaviours underpin successful reproduction. However, these behaviours can be disrupted by exposure to EDC's (Dzieweczynski and Kane 2017; Li et al. 2018). Therefore, the aim of the present study was to explore, for the first time, how exposure to DEHP-dosed microplastics would affect the behaviour of a cryptobenthic fish and whether this could be correlated with changes in global neurochemistry. This study also assessed whether exposure to DEHP would alter the appearance of reproductive signals. Adult male *F. capito*

were fed DEHP-dosed polystyrene microplastics (DEHP) along with virgin polystyrene (PS) and controls over a nine-week period after which their courtship behaviour was monitored as well as agonistic interactions with another male fish. To analyse behaviours, the number of interactions the treated male had with an untreated female fish as well as interactions with an untreated male (interactions included lateral displays, bites and darts) were measured. To study the neurochemical mechanisms which may influence observed behavioural changes, neurotransmitter levels (e.g., 5-HT, and DA) in the treated male brains was quantified. Predictions from previous research were:

(i) Exposure to DEHP, via ingested microplastics, would decrease the frequency of courtship displays when treated fish are presented with a female conspecific.

(ii) Exposure to DEHP, via ingested microplastics, would decrease the frequency of territorial and defence behaviours when treated fish are exposed to a male conspecific.

(iii) Exposure to DEHP, via ingested microplastics, would increase global concentrations of neurotransmitters correlating with a change in behaviour.

3.2 Methods

3.21 Ethics Statement

See section 2.21

3.22 Study location

See section 2.22

3.23 Plastic preparation

See section 2.24

3.24 Animal Collection

Ninety male *F. capito* were collected by locating nests and removing the guarding fish. Collected fish were then transported to PML and placed in individual 3.5 L tanks in which they were treated for a nine-week period. Fish were size matched to 4.49 ± 0.03 g at the beginning of the treatment period. Towards the end of the treatment period nine female fish and an additional nine male fish were caught using minnow traps and the location of nests, respectively. These fish collected and designated as the stimulus fish, were individually housed in 3.5 L tanks, and were fed control food only. During the collection of stimulus fish, photos were taken of 31 nest-guarding males for colour analysis and comparison, these fish were not included in the experiment.

3.25 Plastic addition and treatments

For food preparation see section 2.25. The treatments here remained the same as those in Chapter Two (control, PS and DEHP) but fish in this experiment were fed one 1 g, instead of 5 g, food blocks daily and were given treated feeds every second day. This is as the 5 g food blocks in Chapter Two were never entirely consumed so the amount of food offered was decreased to reduce waste.

3.26 Reproductive behaviours

At the end of the nine-week treatment period, fish were transferred individually to a static 20 L glass tank fitted with an air stone. Each tank was covered on three sides with black plastic to ensure visual isolation from neighbouring tanks. A 4×4 cm grid was drawn in the front of the tank to allow the observer to record behavioural parameters. The PVC pipe shelter with each fish was also transferred over to allow familiarity within this new setup. Fish were left overnight to acclimate, and lights were switched on at 9am the following day and the fish left for another hour to allow them to settle with the change in light. After this hour, filming commenced. Each fish was filmed for 10 minutes pre-stimulus to establish baseline behaviour. A resealable plastic bag containing only sand was introduced and after a 10-minute acclimation period, the behaviour of the fish in response to the bag was filmed for 10 minutes. After this, the fish were left with an empty tank for 10 minutes, a plastic resealable bag containing sand and a female fish was then introduced to the tank and left for a 10-minute acclimation period. The response of each male to the introduction of the female was recorded for 30 minutes. The female fish was then removed, and the tank was left empty for 10 minutes, after which a resealable bag containing sand and a male fish, also between four and five grams was introduced, after a 10-minute acclimation period the response of the experimental males to the introduced males was recorded for 30 minutes.

The behaviours analysed in all videos were: the number of habitat entries as presentations, erection of the dorsal fins, number of bites, number of darts, activity level, with lines crossed as proxy. The number of lateral displays was also analysed as well as the colour of the fish in each video. A diagram of what the tank set up looked like is shown in fig. 8 below.

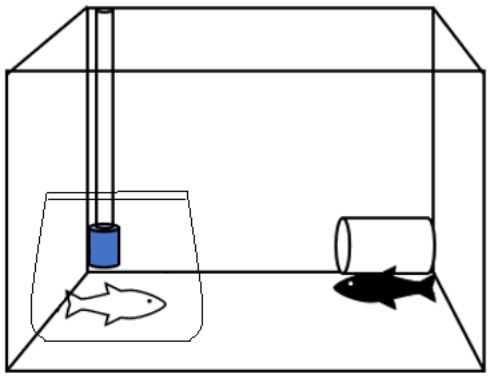

Figure 8. A schematic of the set up used to film reproductive behaviours. Treated fish in black on the right, the stimulus fish (white) in bag on the left, air stone (in blue) behind that.

3.26.1 Behaviour analysis

Collected count data for the measured behaviours were combined to produce two composite variables. These were display, a combination of fin erections, mouth gaping and lateral display and the other was aggression, a combination of number of darts and bites. These two variables as well as activity measured as number of lines crossed and shelter, the number of habitat entries were then analysed using a principal component analysis. Fish which remained inside the habitat for the duration of all four recording sessions were labelled non-responders and were removed from analysis. An example of a video analysis can be found here.

3.27 Colour analysis

To determine whether plastic exposure affected nuptial coloration, each fish was analysed using ImageJ (version 1.52a). A screen capture was taken of each treatment fish at a point in which the length of the body could be seen by the observer. Fish which did not present a whole side of the body long enough for a capture to be taken were excluded from analysis. The images were loaded into ImageJ and analysed by selecting an area, just behind the operculum, of equal size in each image. Using the histogram tool, a mean intensity value for the selected pixels was produced. The intensity values are between 0 and 255, 0 being black and 255 being white.

3.28 Neurotransmitters

After behaviour data had been collected, all fish were euthanised and the brains were removed, weighed and snap frozen in liquid nitrogen and stored at −80 °C. The concentration of neurotransmitters 5-HT and DA were measured using enzyme-linked immunosorbent assays (ELISA) kits (MyBioSource, USA). Samples were analysed using ELISA kits following the manufacturers protocol. Extractions of the neurotransmitters was completed by homogenisation of the brain tissue in a 10% weight: Phosphate-buffered saline (PBS) at 7.4 pH. Following this step, the homogenates were centrifuged at 3,000 rpm for 20 minutes at ambient temperature (25 °C) as per kit instructions. The supernatant was collected and used for analysis using the ELISA kit. Neurotransmitter levels were measured

using a SpectraMax M2 microplate reader (Molecular Devices, USA) with absorbance value at 450 nm. The 5-HT plate contained duplicate standards and five blank wells in a 96 well plate with 12 replicates of each of the control, PS, and DEHP groups. For the DA kit there were duplicate standards and two blank wells in a 96 well plate with 12 replicates for each of the control, PS, and DEHP groups. As there were not enough resources to have all 30 replicates from each group, 12 samples from each group were selected randomly for these assays.

3.29 Statistical Analysis

As analysis of individual behaviours was not very informative (Appendix B) a principle component analysis was used to analyse the composite variables of aggressive and attractive behaviours as well as shelter and activity. PC1 (three levels, control, PS and DEHP) violated the assumptions of ANOVA and so was analysed using a Kruskal-Wallis nonparametric test. The nature of any observed significance was determined using the Dunn post hoc test, these analyses were completed in PAST (4.10). Other data which violated the assumptions of ANOVA were also analysed using the Kruskal-Wallis nonparametric test these were, body weight prior to treatment (three levels) and 5-HT (three levels), this and all other analysis was completed in R (1.2.500). Condition post-treatment also violated the assumptions of ANOVA and so a paired Wilcoxon signed rank test was used to analyse these data. DA (three levels) and colour change (three levels) were analysed using ANOVA. Tukey's HSD test followed this to determine the nature of any significant differences.

3.3 Results

3.31 Fish health

Two mortalities were recorded during this period, the cause of death was not clear as the fish displayed no abnormalities prior to death. One fish was discovered to be female, this fish was removed from the experiment but could not be replaced as the treatment period had begun.

3.32 Body Weight

Prior to treatment, the average weight of fish did not differ significantly (chi-squared = 0.4941, df = 2, p-value = 0.781) between groups, control (4.4 g), PS (4.5 g), DEHP (4.4 g). The body weight of fish in all treatment groups were significantly increased post treatment control (5.2 g) (V = 404, p-value<0.001), PS (5.1 g) (V = 397.5, p-value<0.001) and DEHP (5.3 g) (V = 406, p-value<0.001) (fig. 9). This weight gain was consistent among groups and all fish appeared to have gained a similar amount of weight.

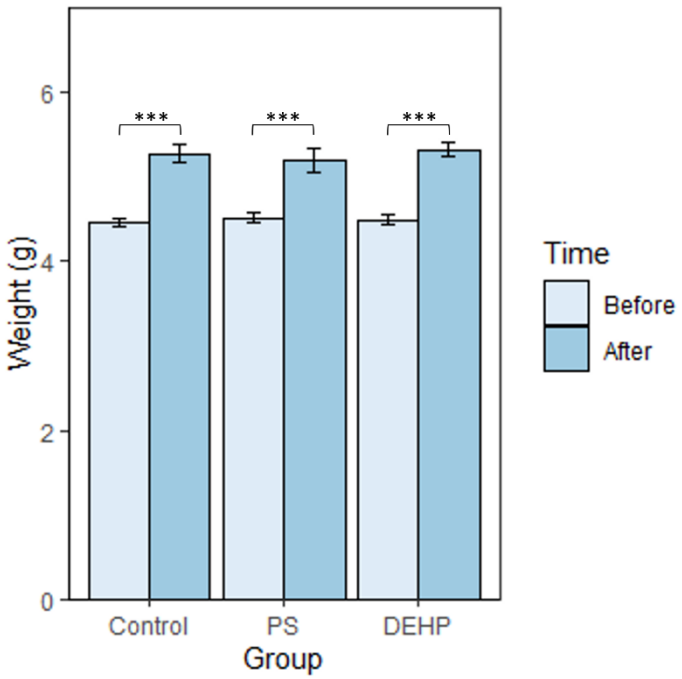

Figure 9. Mean body weight ± standard error before and after treatment of each group. Control n=29, PS n=29, DEHP n=29. *** =p-value<0.01

3.33 Behaviour

Mean activity (PC1) explained 85% of the variance when fish were responding to a male stimulus fish and 83% in response to a female stimulus fish. Though it appeared that PS treated fish were on average more active (5.28) in the presence of male stimulus fish than the other treatment groups, control (-2.03) and DEHP (-3.4), there was no significant difference (chi-squared=0.7135, df=1, p-value = 0.699) (fig. 10A). In response to female stimulus fish, DEHP-exposed fish seemed to be the least active (-0.94) compared to that of the control (0.3) and PS (0.5) groups, however, this was not statistically significant (chi-squared=0.022828, df=1, p-value = 0.988) (fig. 10B).

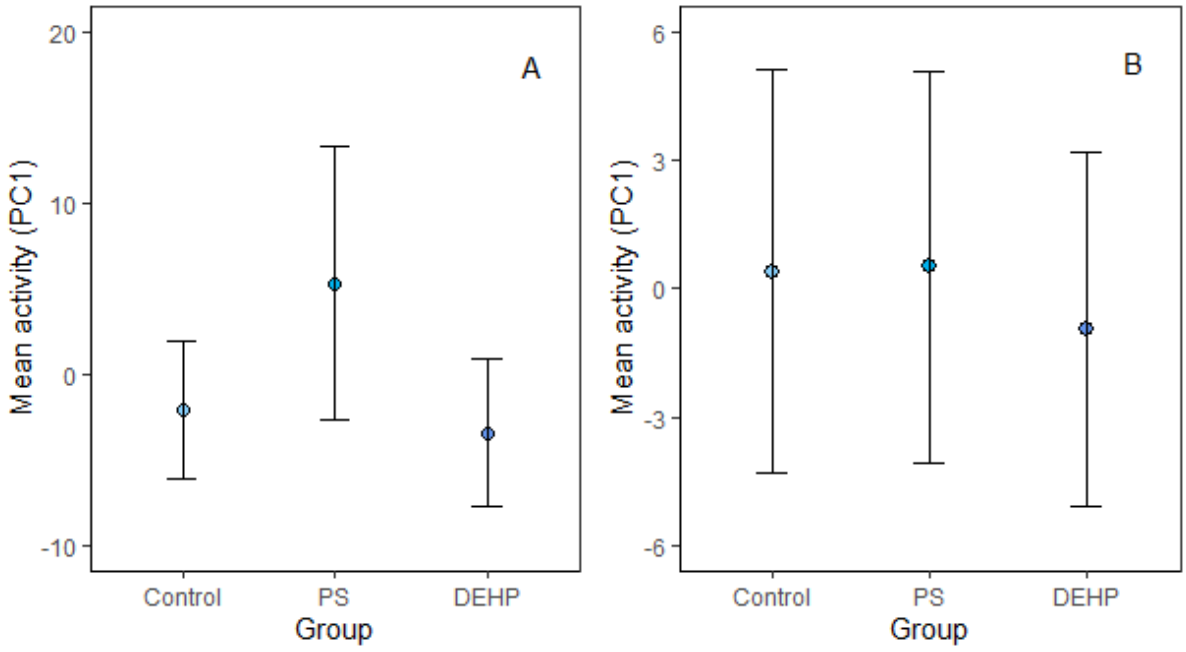

Figure 10. The mean PC1 value ± standard error of fish when exposed to a male (A) and female (B) stimulus fish for control (n=20), PS (n=20) and DEHP (n=19).

3.34 Colour change

Though in the presence of female stimulus fish both the PS (96.4) and DEHP (120.3) groups were lighter in colour than control (64.8) fish, this was only significant in DEHP-treated fish ($F_{2,26}$= 4.62, p-value = 0.0192) (fig. 11B). However, in the presence of a male stimulus fish both the PS (95.5) and DEHP groups (103.4) groups were significantly lighter in colour than the control (55.2) ($F_{2,26}$= 5.061, p-value = 0.0139) (fig. 11A). In comparison to wild nest-guarding fish (59.8), the colour of control fish was not significantly different in the presence of both sexes of stimulus fish, male ($F_{1,38}$=0.129, p-value = 0.721) and female ($F_{1,38}$=0.44, p-value = 0.511).

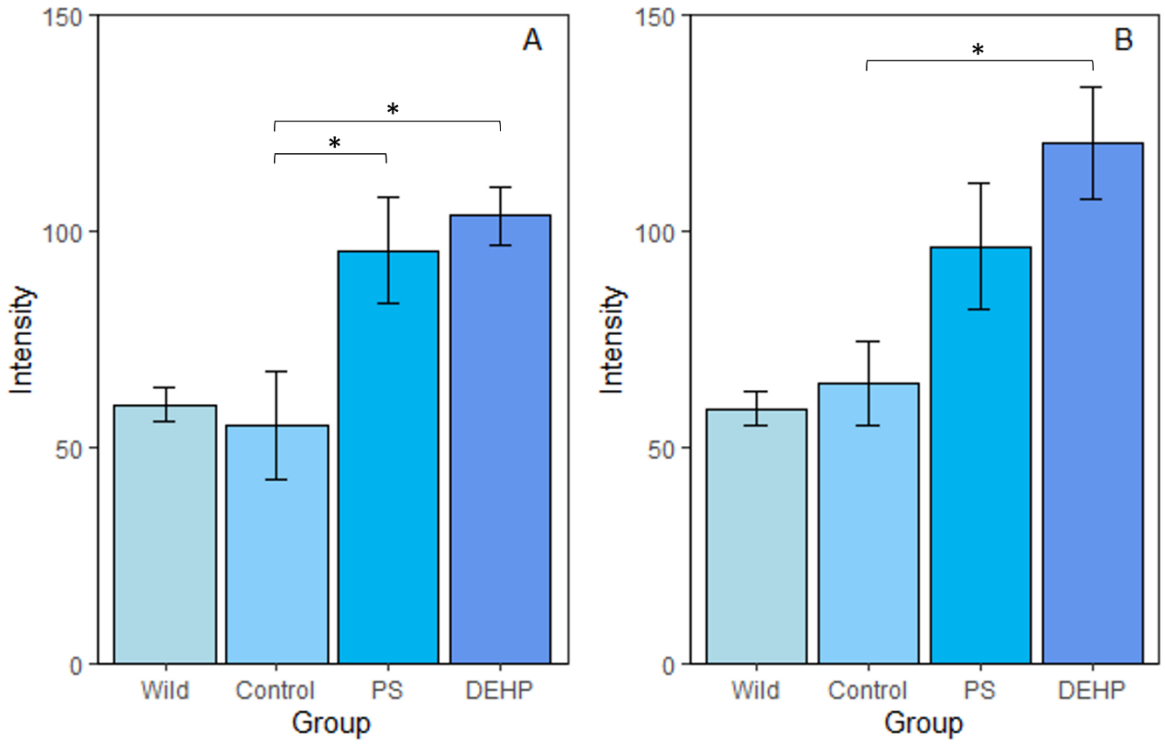

Figure 11. The mean colour intensity ± standard error of fish when exposed to a male stimulus fish (A) and a female stimulus fish (B). Wild guarding fish (n= 31) used as comparison to validate the colour presented by control treatment fish. Control (n=9), PS (n=10), DEHP (n=10). *= p-value<0.05

3.35 Neurotransmitters

Though trends showed that the mean DA concentration detected increased in a stepwise manner with the control group the lowest (75.5 ng mL^{-1}), the DEHP group the highest (129.7 ng/mL) and the PS-exposed fish in the middle (99.27 ng mL^{-1}). A statistically significant increase was only detected between the control and DEHP exposed group ($F_{2,32}$= 3.562, p-value = 0.0401) (fig. 12A). Additionally, a significant decrease in the amount of 5-HT was detected between the control (40.2 ng mL^{-1}) and both the PS (34.1 ng mL^{-1}) and DEHP (33.9 ng mL^{-1}) exposure groups (chi-squared= 9.3989, df=2, p-value = 0.0091) (fig. 12B). For DA concentrations the PS and DEHP exposed groups were not significantly different from one another.

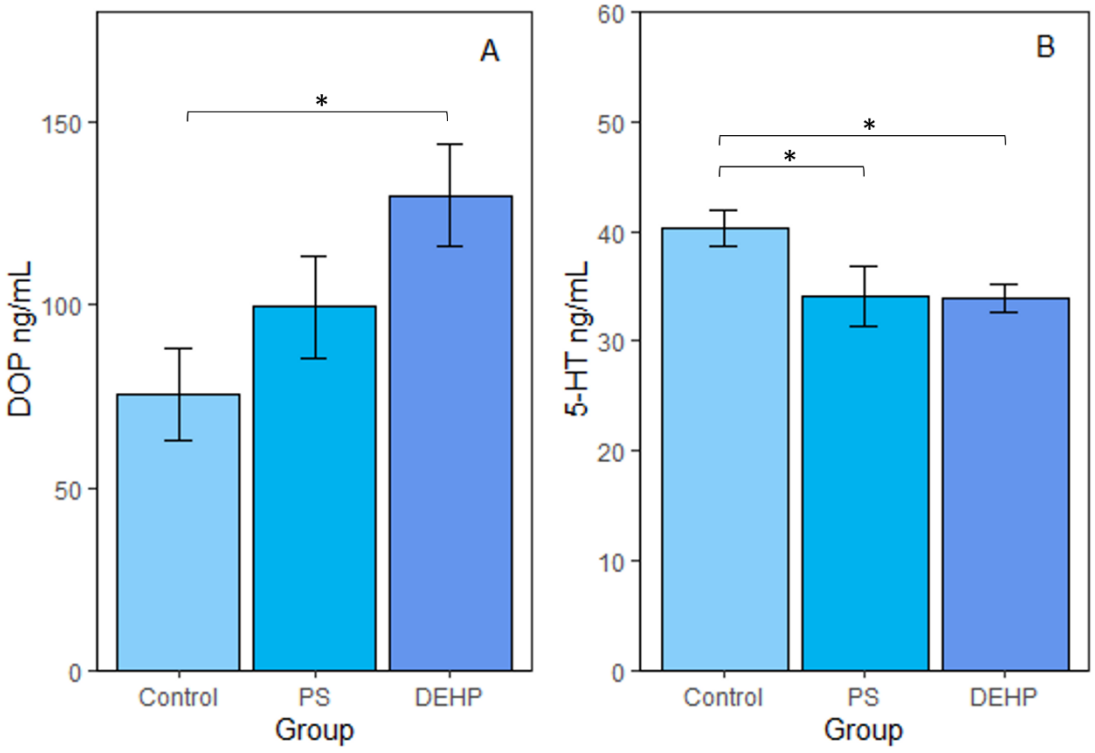

Figure 12. The mean concentration of A) DA ng mL^{-1} ± standard error and B) 5-HT ng mL^{-1} ± standard error in each group, n=12 for each group. *= p-value<0.05

3.4 Discussion

Microplastic pollution poses a threat to marine taxa via the dangers posed by the plastics themselves as well as the additives associated with them (Cole et al. 2011; Tyler et al. 2018; Ye et al. 2014). Published literature has demonstrated that microplastics and their additives can have negative effects on the behaviour of exposed organisms (Carbone et al. 2019; Chen et al. 2020; Li et al. 2017, 2018; McCormick et al. 2020). Additionally, both microplastics and plasticisers have been found to alter the neurochemistry of exposed organisms (Barboza et al. 2018; Carbone et al. 2019; Tran et al. 2021). Here, it is demonstrated that exposure to DEHP-dosed microplastics has varied effects on the behaviour and neurochemistry of a cryptobenthic fish (*F. capito*). Specifically, we found that whilst reproductive and agonistic behaviours were unchanged (see fig. 10), fish exposed to both virgin PS and DEHP-dosed microplastics failed to display nuptial colouration to the same degree as that exhibited by control fish (fig. 11). Levels of both measured neurotransmitters were impacted by exposure to DEHP-dosed microplastics. DA levels were significantly increased (fig. 12A) whilst 5-HT levels significantly decreased (fig. 12B).

During the breeding season, male *F. capito* take on a nuptial colouration (a darkening of the body) whilst guarding a nest site and therefore, the colour of fish post treatment can be indicative of whether a fish is signalling that it is in reproductive condition (Johnstone 1996; Price et al. 2008; Tornquist 2020; Wellenreuther and Clements 2007). The present study has found that DEHP-treated fish were lighter in colour than control fish when exposed to both male and female stimulus fish (fig. 11A and B). This could be due to these fish being less fit as reproductive individuals. Previous research using guppies (*Poecilia reticulata*) has found that exposure to EDCs can impact the presentation of reproductive colouring (Baatrup and Junge 2001; Toft and Baatrup 2003). For example, Baatrup and Junge (2003) found that after 30 days of exposure to food dosed with 1 μg mg^{-1} of the fungicide vinclozolin, both the area and intensity of orange colouration of male guppies was reduced. Similarly, in cases of exposure to 0.5 μg L^{-1} of E2 (17β-estradiol) as well as 200 μg L^{-1} of OP (4-tert-octylphenol) the area and intensity of orange colouration of male guppies was reduced after three months (Toft and Baatrup 2003). For the present study, this could mean that ten weeks

exposure to DEHP resulted in changes to the endocrine system, leading to failure to present proper nuptial colouration. Though sex hormones were not measured in the present study, DEHP is a well-known endocrine disruptor. Previous studies of aqueous exposure have shown that, dependent on exposure concentration and duration, DEHP can have both estrogenic and anti-androgenic effects (Golshan and Alavi 2019; Guo et al. 2015).

To date, few studies have investigated whether ingestion of DEHP-dosed microplastics affects neurochemistry. In the present study, it was found that exposure to DEHP-dosed microplastics resulted in significant increases in DA and significant decreases in 5-HT global brain concentrations (fig. 12A and B). Neurotransmitters are associated with a range of functions in the body of an organism (Chabbi and Ganesh 2015; Dufour et al. 2010), including reproductive performance. For example, increased DA can inhibit reproduction in teleosts, by preventing gamete development (Chabbi and Ganesh 2015; Dufour et al. 2010). Further, research has shown that DA is involved in stress-induced reproductive dysfunction (Chabbi & Ganesh, 2015). The exposure of the cichlid fish *Oreochromis mossambicus* to aquaculture-related stresses such as handling and chasing led to fewer mature oocytes which was linked to the observed increase in DA (Chabbi & Ganesh, 2015). Similarly, the administration of a DA blocker was able to restart ovarian function in female zebrafish that had previously ceased reproduction due to age (Fontaine et al. 2013). Furthermore, exposure of *Oncorhynchus mykiss* (Rainbow Trout) to sublethal concentrations of cyanide caused reproductive dysfunction in both male and female fish which was also linked to DA levels (Szabo et al. 1991). These studies indicate that under a given stressor DA levels increase, and this can have negative consequences for the reproductive success of effected individuals. They also suggest that, in the presence of toxicants, increased DA is a common response among teleosts. Likely as under conditions of stress survival, rather than reproduction, is prioritised. In contrast, 5-HT has been shown to have a stimulatory effect on gametogenesis (Prasad et al. 2015). Therefore, the observed trends for both neurotransmitters point to the potential for an altered reproductive output for exposed fish. The observed increase in DA and decrease in 5-HT in DEHP-exposed fish in the present study shows that the neurochemistry of these fish was altered. It is important to note that the present study measured global DA and 5-HT concentrations, therefore it cannot be

determined for what purpose the measured neurotransmitters were being used. Though the reproductive output of exposed fish was not assessed in the present study, it is possible that the observed change to colour expression indicates that the reproductive condition of exposed fish was compromised by treatment.

The present study observed no change to the performance of either display (attractive) or aggressive behaviours in the presence of either stimulus fish (fig 10A and B). Though little-to-no literature as to the effects of DEHP on the behaviour of adult fish exists, some work has been published as to the effects of other phthalate plasticisers. Exposure of *D. rerio* to aqueous concentrations up to 50 µg L^{-1} diheptyl phthalate (DHpP) and diisodecyl phthalate (DIDP), in separate experiments, caused abnormal swimming behaviour (Poopal et al. 2020). Similarly, exposure of three-spined sticklebacks (*Gasterosteus aculetaus*) to di-n-butyl phthalate caused a delay in nest building in male fish but not significantly so (Aoki et al. 2011). The difference between published literature and the current study being DEHP delivery method, as this study used dosed microplastics as a vector of transfer. A lack of behaviour change in the present study could be due to different phthalates having different toxicities. It is also possible that in consuming dosed microplastic particles exposure to the plasticiser itself is variable depending on the number of particles ingested during a feeding, therefore exposure may not have been consistent enough to elicit a behaviour change. The ability of DEHP to impact neurochemistry and to potentially change the suitability of an animal as a mate in the present study are noteworthy observations.

The role of cryptobenthic fishes in their associated ecosystems is to serve as a food source owing to their numerical dominance both as larvae and adults (Brandl et al. 2018; Depczynski and Bellwood 2003), decreased reproductive output in these species could therefore have flow on effects to population and ecosystem dynamics. Though in the present study, behaviour in response to both female and male conspecifics was unchanged, it could be that the consequences of altered neurochemistry had not had time to manifest. Future research could focus on mate selection trials to assess whether the observed change in colour presentation would cause female fish to preferentially select control males over DEHP or PS microplastic exposed ones.

Chapter 4 – Exposure to DEHP via microplastic ingestion causes oxidative stress in *Forsterygion capito*

4.1 Introduction

The mass disposal of plastic products has caused the widespread occurrence of plastic pollution in the ocean (Cole et al. 2011; Freinkel 2011; Ostle et al. 2019). Microplastics, plastics under 5 mm in diameter, were first recorded in the marine environment in the 1970's (Costa and Barletta 2015). Microplastics are easily ingested by animal taxa with ingestion confirmed in taxa from humpback whales to copepods (Germanov et al. 2018; Sun et al. 2017). Microplastics however, are often associated with chemicals derived from manufacture or adsorption from the environment. Chemicals added during manufacture, known as plasticisers, are added to plastics to give the polymers favourable properties such as flexibility and UV resistance (Cole et al. 2011; Kim et al. 2002). Di(2-ethylhexyl)phthalate (DEHP), one of the most commonly used plasticisers, is a known endocrine disruptor as well as a ubiquitous environmental pollutant (Chikae et al. 2004; Liu et al. 2017). Previous literature has outlined that exposure to DEHP can cause negative biochemical change in the form of oxidative stress (Huang et al. 2011; Kasahara et al. 2002; Luo et al. 2019).

Oxidative stress occurs when the production of antioxidant defence is outpaced by the production of reactive oxygen species (ROS) which can damage proteins, lipids and DNA, inhibiting their ability to function (Aitken et al. 2016; Birnie-Gauvin et al. 2017; Choi and Oris 2000). Measuring markers of this condition is useful for quantifying the body's response to a stressor. ROS are produced in aerobic organisms as a component of routine cellular function. In response, defensive enzymes such as superoxide dismutase (SOD) and catalase (CAT) are produced to convert oxidising species like the superoxide anion ($^{-}O_2$) into harmless molecules such as H_2O (Carney Almroth et al. 2008; Ighodaro and Akinloye 2018). This occurs as SOD converts the $^{-}O_2$ to hydrogen peroxide (H_2O_2) whilst CAT reduces H_2O_2 to water (Ighodaro and Akinloye 2018). Another important enzyme is glutathione peroxidase (GPOX) (Carney Almroth et al. 2008). GPOX can reduce both H_2O_2 and lipid peroxides (LPs) to

prevent them doing oxidative damage (Carney Almroth et al. 2008). The enzyme glutathione S-transferase (GST) is important in aiding the excretion of xenobiotics (Carney Almroth et al., 2008). These enzymes work in tandem to prevent oxidative damage caused by ROS.

This system however can be overwhelmed, leading to oxidative damage in tissues which may in turn have consequences for fitness of the individual and reproductive capability (Aitken et al. 2016; Zhang et al. 2016). Altered reproductive capability due to oxidative stress can occur via oxidative damage to gametes, preventing their function (Aitken et al., 2016), or by the trade-off between gamete production or mate attraction with oxidative defence (Birnie-Gauvin et al. 2017). In addition to defensive enzymes, protein carbonyls (PCs) and LPs can be used as indicators for oxidative stress (Choi and Oris 2000; Fedorova, Bollineni, and Hoffmann 2014; Lister et al. 2015). PCs are formed when the oxidation of proteins causes irreversible damage; PCs are therefore indicative of potential tissue damage (Fedorova et al. 2014). LPs are formed when the lipids of cell membranes are oxidised by ROS and therefore no longer function within the membrane (Choi and Oris 2000). The accumulation of PCs and LPs can therefore be used as evidence of oxidative damage. Oxidative stress occurs when the rate of ROS production exceeds the capacity of an organism's antioxidant mechanisms and can ultimately result in cell death, behavioural impairments and mortality (Ankley et al. 2010; Choi and Oris 2000).

Published literature has shown that exposure to microplastics can induce oxidative stress (Kim, Yu, and Choi 2021; Prokić et al. 2019). In roundworm (*Caenorhabditis elegans*) exposure to PS microplastics of various densities increased production of the detoxifying enzyme GST-4, indicating increased antioxidant defence as a result of plastic exposure (Lei et al. 2018). Similarly, exposure of zebrafish (*Danio rerio*) to waterborne PS microplastics of 20, 200, or 2000 μg L^{-1} of 5 μm particles caused significant increases in activity of both CAT and SOD (Lu et al. 2016). In another study, exposure of *D. rerio* to 50 μg L^{-1} and 500 μg L^{-1} of pure and polluted waterborne polystyrene microplastics showed increased antioxidant defence as SOD and CAT activities were again increased (Qiao, Sheng, et al. 2019). Additionally, the increase in enzyme activity was higher for fish exposed to polluted microplastic than the pure plastics groups (Qiao, Sheng, et al. 2019), this indicates that the

leaching of chemicals from ingested microplastics may add to the stress caused by the plastics alone.

Similarly, direct exposure to the plasticiser DEHP has also been found to cause oxidative stress in a range of exposed organisms (Brassea-Pérez et al. 2022). Oral administration of 1 g kg^{-1} of DEHP to male rats (*Rattus norvegicus*) caused an increase in the antioxidant enzymes GPOX and CAT in the testes (Kasahara et al. 2002). Similarly, exposure of quails (*Coturnix japonica*) to DEHP via oral administration was found to cause increased lipid peroxidation products in the brain (Luo et al. 2019). The kidneys of rats have also been found to show increased oxidative stress resulting from DEHP exposure (Erkekoglu et al. 2012). As it is well established that direct DEHP exposure causes oxidative stress, this begs the question whether DEHP exposure via leaching from ingested microplastics would have similar effects.

The aim of the present work is to assess the impact of DEHP exposure via ingested microplastics on the antioxidant system in the cryptobenthic fish *F. capito*. Analysis of the oxidative state of these fish was done by extraction of protein and lipids from white muscle tissue. Predictions based on previous research was that, exposure to DEHP, via ingested microplastics, would cause increased oxidative stress evidenced by increased antioxidant enzyme activity and markers of oxidative damage.

4.2 Materials and methods

4.21 Tissue collection

Analyses in the present chapter were conducted on white muscle tissue harvested from fish following the treatment period in Chapter Two. White muscle was collected from each fish, snap frozen in liquid nitrogen, and stored at -80°C until analysis.

4.22 Oxidative stress in muscles

One muscle sample per fish was prepared for oxidative stress analysis by extracting total protein from the tissue. Total protein was extracted by homogenising the sample in 750 µL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM Na_2 EDTA (ethylenediaminetetraacetic Acid), 1% PVP-44 (polyvinylpyrrolidone), 1 mM PMSF (phenylmethylsulfonyl fluoride) and 0.5% v/v TritonX-100 using six zirconia beads and five 15 second cycles in a BioSpec 3110BX Mini-BeadBeater. Once homogenised, samples were centrifuged at 4°C for 15 minutes at 14000 rpm. The resulting supernatant was then removed for ultrafiltration and the pellet was stored at -80°C prior to lipid analysis (section 4.23). Ultrafiltration was undertaken to produce semi-purified protein extracts, to remove any small molecules that could interfere with subsequent assays. For the ultrafiltration step, 500 µL of the supernatant was placed in an ultrafilter (Amicon^{TM}, 10 kD molecular weight cut-off (MWCO), maximum capacity 500 µL) and spun for 15 minutes at 10000 rpm at 4°C. The semi-purified protein extract was then washed by addition of 50 mM (pH 7.0) 500 µL phosphate (PO_4) buffer to the ultrafilter and another centrifugation step for 20 minutes at 10000 rpm at 4°C. The remaining protein samples were then reconstituted by addition of 500 µL of PO_4 buffer and separated into four 100 µl aliquots which were stored at -80°C. PC levels were determined in semi-purified protein extract using the 2.4-dinitrophenylhydrazine (DNPH) method (see Lister et al., 2015). The activities of the enzymes SOD, CAT, GPOX and GST were also analysed using microplate assays (Lister et al. 2015).

4.23 Lipid extractions

Tissue pellet remaining from protein extraction was prepared for a lipid peroxidase assay. This was done by homogenizing the tissue (~100 mg) in 0.6 mL of methanol: chloroform (2:1 v/v) and vortexed for at least 30 seconds. Next, 0.4 mL of chloroform was added, samples were then vortexed again for 30 seconds and left for one minute. Lastly, 0.4 mL of deionised water was added, and samples were centrifuged for 30 seconds at 12000 rpm. After this, 200 µL of extract from the lipid phase was transferred in a new tube, these were then stored at -80°C until analysis. Samples were analysed as per Lister et al. (2015).

4.24 Statistical analysis

All statistical analyses were undertaken in R-studio version 1.2.5001. All data which met the assumptions of normality and homogeneity of variance were analysed using a one-way ANOVA. The nature of any significant differences detected was then determined using Tukey's HSD post hoc test. Assumptions of ANOVA were tested using Levene's test for homogeneity of variance and a Shapiro Wilks test for normality of data as well as visual inspection of residual plots. If data which violated the ANOVA assumption of normality could be corrected by log transformation then an ANOVA was carried out on the log transformed data. This was the case for the GPOX and GST data of female fish (three levels). However, if data violated the assumption of homogeneity of variance or could not be corrected by log transformation then the Kruskal-Wallis nonparametric test was used. This was true for male SOD, GPOX and GST data as well as the PC and LP data for both *F.capito* sexes (three levels in all cases). The nature of any observed significance was then determined using the Dunn post hoc test.

4.3 Results

4.31 *F. capito* muscle oxidative stress

SOD activity in males in the control group averaged 142.28 ± 5 units/mg protein. This value was significantly lower (chi-squared =27.457, df =2, p-value <0.001) than that of both the PS (212.7 ± 10 units/mg protein) and the DEHP (363.03 ± 21 unit/mg protein) (fig. 13). The same occurred for female fish as the control (140.6 ± 6 units/mg protein) was significantly lower ($F_{2,23}$ = 173.3, p-value <0.001) than that of the PS (218.7 ± 7 units/mg protein) and DEHP (341.4 ± 9 units/mg protein) (fig. 13). No differences were detected between sexes within any group.

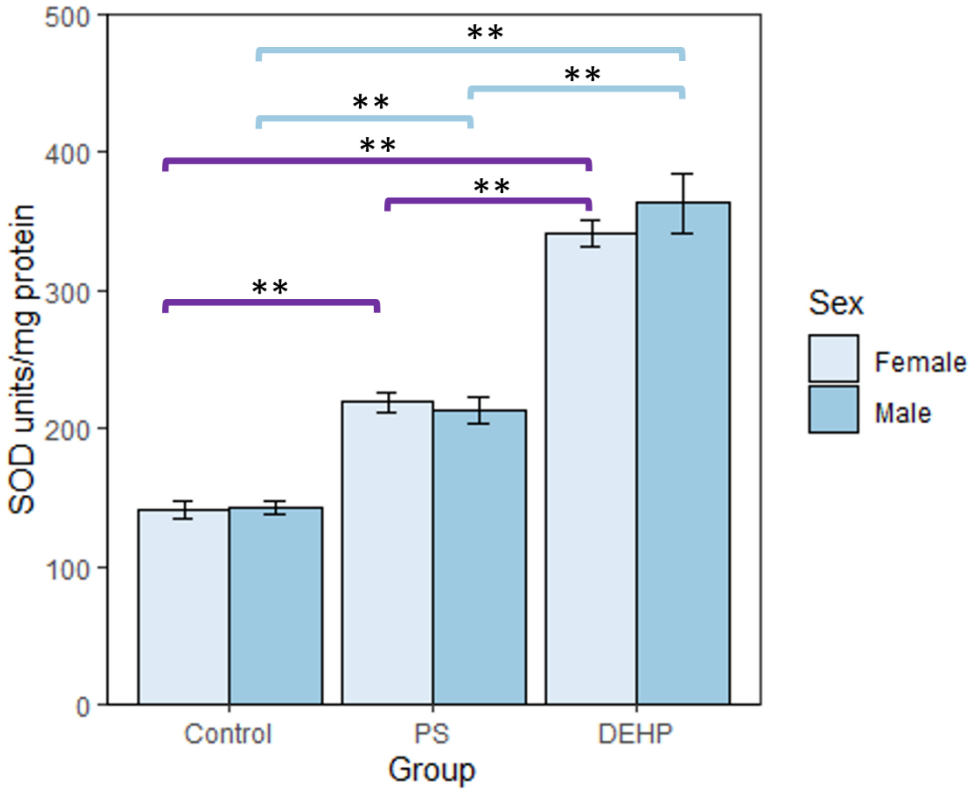

Figure 13. Mean concentration of superoxide dismutase SOD units/mg protein ± standard error in male and female white muscle. Male control n= 10, female control n=10, male PS n=12, female PS n=8, male DEHP n=11, female DEHP n=8. Blue markers indicate significance between female fish whilst purple markers indicate that of male fish. **p-value<0.01

CAT activity in males in the control group averaged 11.08 ± 0.47 µmol/min/mg protein. This value was significantly lower ($F_{2,30}$ =144.2, p-value <0.001) than that of both the PS (18.7 ± 0.62 µmol/min/mg protein) and the DEHP (31.4 ± 1 µmol/min/mg protein) (fig. 14). The same was true for female fish as the control (12.04 ± 0.5 µmol/min/mg protein) was significantly lower ($F_{2,23}$ =49.41, p-value <0.001) than that of the PS (17.07 ± 0.7 units/mg protein) and DEHP (23.7 ± 1 µmol/min/mg protein) (fig. 14). Additionally, the average male CAT activity of 31.4 ± 1 µmol/min/mg protein was significantly ($F_{1,17}$ =18.88, p-value <0.001) higher than that of female fish (in the DEHP treatment groups (23.7 ± 1 µmol/min/mg protein). There was no difference between sexes in the control or PS groups.

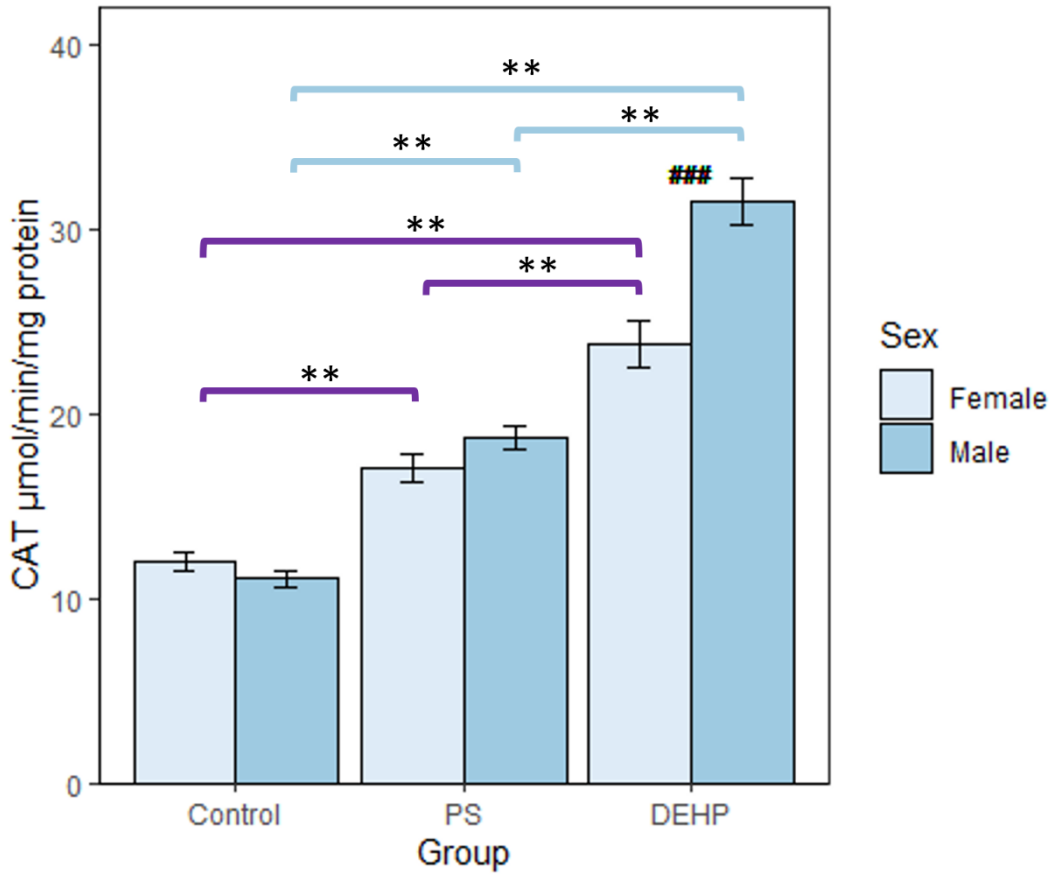

Figure 14. Mean concentration of CAT µmol/min/mg protein ± standard error in male and females white muscle. Male control n= 10, female control n=10, male PS n=12, female PS n=8, male DEHP n=11, female DEHP n=8. Blue markers indicate significance between groups of female fish whilst purple markers indicate those of male fish. # denotes significant difference between sexes, ### p-value<0.0001. **p-value<0.01.

GPOX activity in males in the control group averaged 32.8 ± 1.2 µmol/min/mg protein. This value was significantly lower (chi-squared = 28.412, df = 2, p-value<0.001) than that of both the PS (47.5 ± 1.4 µmol/min/mg protein) and the DEHP (93.3 ± 5.2 µmol/min/mg protein) (fig. 15). The same was true for female fish as the control (33.4 ± 1.3 µmol/min/mg protein) was significantly lower ($F_{2,23}$ =88.98, p-value <0.001) than that of the PS (47.6 ± 2 µmol/min/mg protein) and DEHP (72.1 ± 2.5 µmol/min/mg protein) (fig. 15). Furthermore, the average activity of GPOX enzymes in DEHP-exposed male fish was significantly higher than that of DEHP-exposed female fish ($F_{1,17}$ =10.54, p-value =0.00475). There were no statistical differences between the sexes within the control and PS groups.

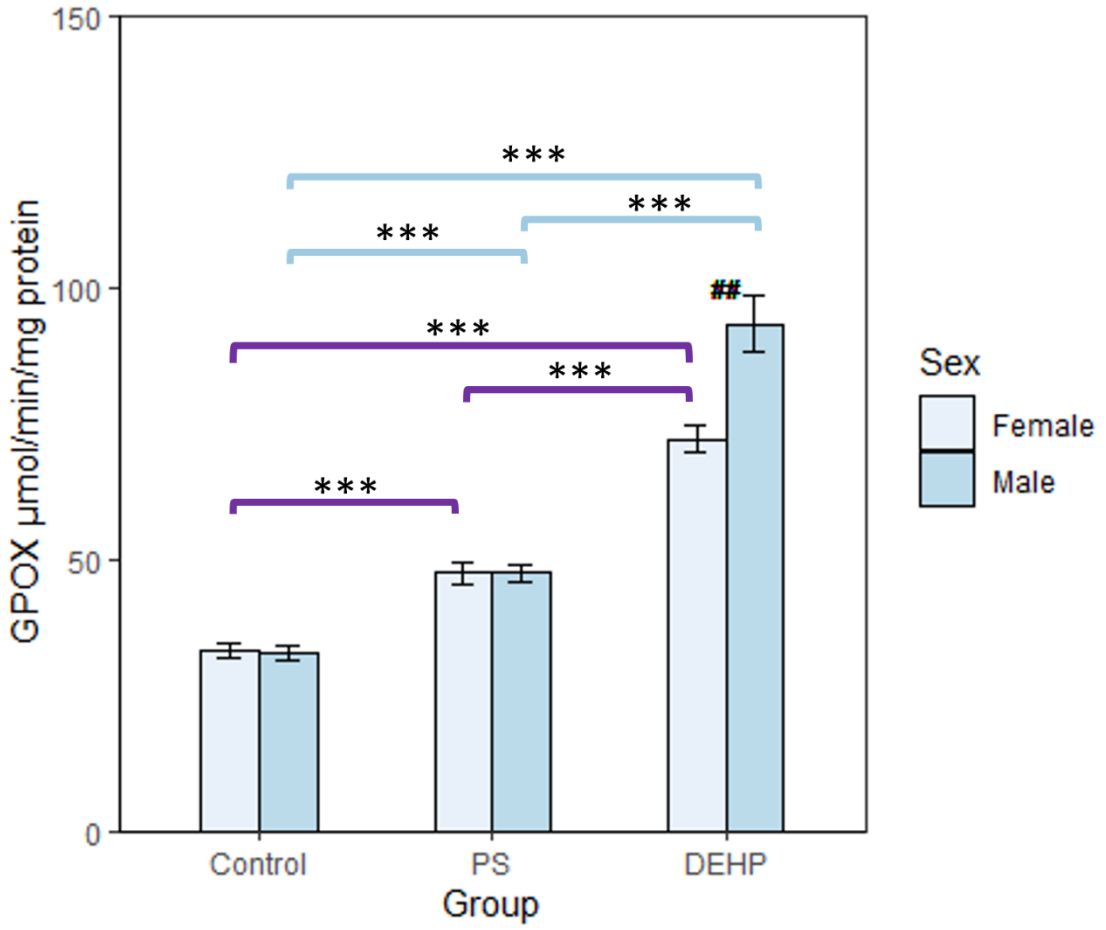

Figure 15. A) Mean concentration of GPOX µmol/min/mg protein ± standard error in male and female white muscle. Male control n= 10, female control n=10, male PS n=12, female PS n=8, male DEHP n=11, female DEHP n=8. Blue markers indicate significance between groups of female fish whilst purple markers indicate those of male fish. # denotes significant difference between sexes, ## p-value<0.01. ***p-value<0.0001

GST activity in males in the control group averaged 43.4 ± 1.43 nmol/min/mg protein. This value was significantly lower (chi-squared =28.178, df =2, p-value <0.001) than that of both the PS (64.8 ± 2.5 nmol/min/mg protein) and the DEHP (119.2 ± 6.7 nmol/min/mg protein) (fig. 16). The same was true for female fish as the control (43.8 ± 1.8 nmol/min/mg protein) was significantly lower ($F_{2,23}$ =110.8, p-value <0.001) than that of the PS (69.3 ± 2.8 nmol/min/mg protein) and DEHP (103.4 ± 2.5 nmol/min/mg protein) (fig. 16). No statistical significance was detected between sexes within any group.

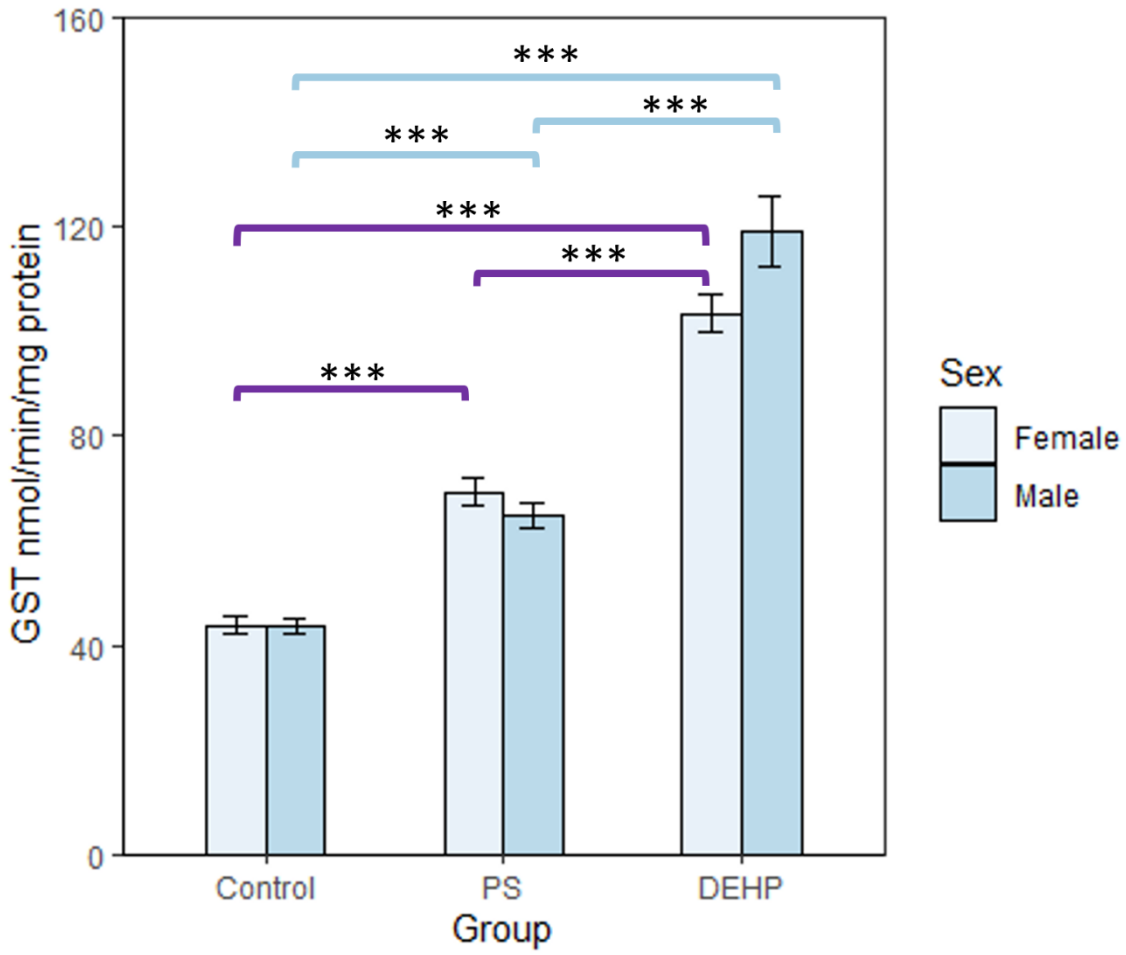

Figure 16. Mean concentration of GST nmol/min/mg pro ± standard error in male and white muscle in each group. Blue markers indicate significance between groups of female fish whilst purple markers indicate those of male fish. Male control n= 10, female control n=10, male PS n=12, female PS n=8, male DEHP n=11, female DEHP n=8. ***p-value<0.0001

LP levels in males in the control group averaged 20.2 ± 0.84 nmol/mg protein. This value was significantly lower (chi-squared = 28.168, df = 2, p-value <0.001) than that of both the PS (31.3 ± 1.3 nmol/mg protein) and the DEHP (109.1 ± 5.3 nmol/mg protein) (fig. 17). The same was true for female fish as the control (25.6 ± 1.4 nmol/mg protein) was significantly lower (chi-squared = 20.886, df = 2, p-value < 0.001) than that of the PS (36.5 ± 2.2 nmol/mg protein) and DEHP (143.4 ± 5.3 nmol/mg protein) (fig. 17). Additionally, LP levels were significantly higher in female fish compared to that of male fish within both the control (chi-squared = 5.915, df = 1, p-value = 0.0150) and DEHP (chi-squared = 10.14, df = 1, p-value = 0.00145) groups. No difference was observed between the sexes within the PS group.

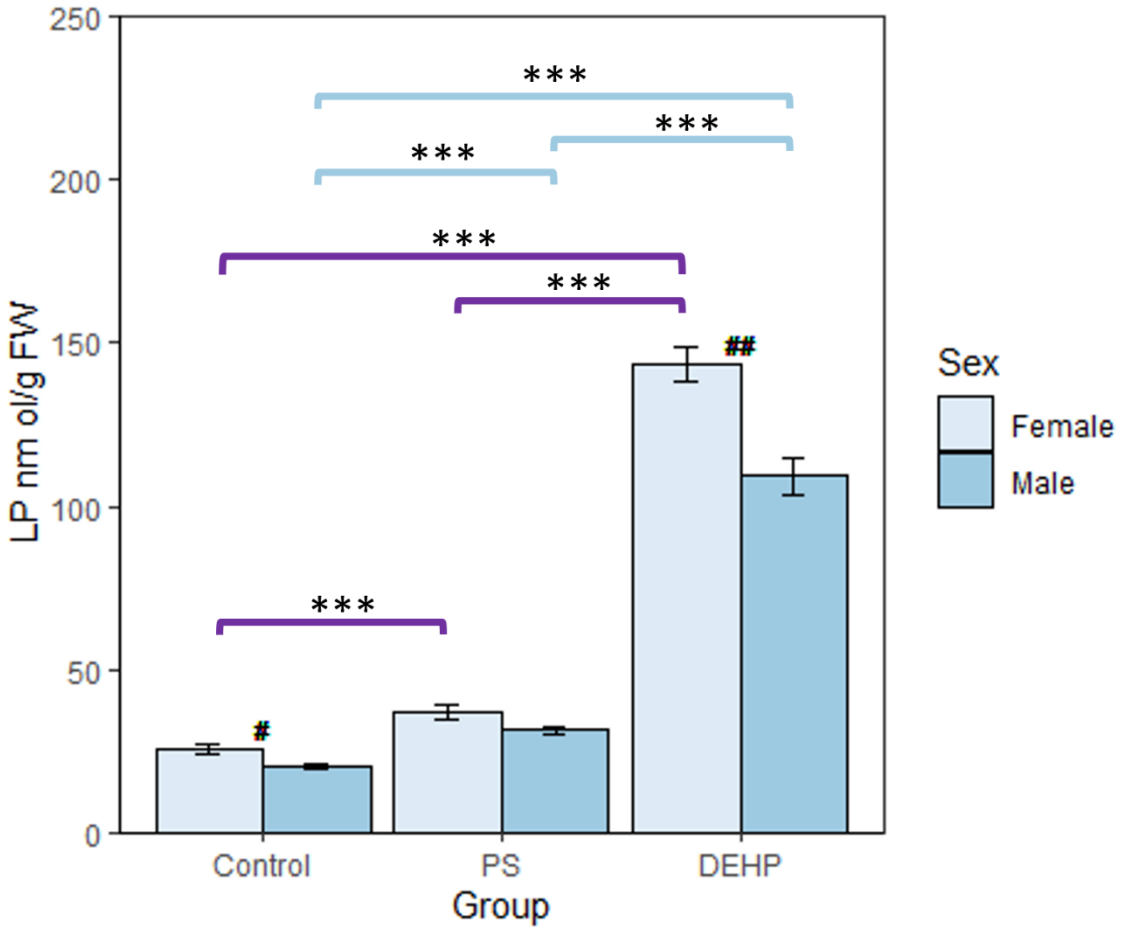

Figure 17. A) Mean concentration of lipid peroxide (LP) nmol/g FW ± standard error in male and female white muscle in each group. Blue markers indicate significance between groups of female fish whilst purple markers indicate those of male fish. Male control n= 11, female control n=9, male PS n=12, female PS n=8, male DEHP n=10, female DEHP n=9. # denotes significant difference between sexes, # p-value<0.05, ## p-value<0.01. *p-value<0.05, ***p-value<0.001

As well as enzyme activity, PC levels in males in the control group averaged 2.5 ± 0.1 nmol/mg protein. This value was significantly lower (chi-squared = 28.412, df = 2, p-value<0.001) than that of both the PS (3.6 ± 0.15 nmol/mg protein) and the DEHP (12.8 ± 0.6 nmol/mg protein) (fig. 18). The same was true for female fish as the control (3 ± 0.1 nmol/mg protein) was significantly lower (chi-squared = 20.992, df = 2, p-value<0.001) than that of the PS (4.1± 0.1 nmol/mg protein) and DEHP (17 ± 1 nmol/mg protein) (fig. 18). In each group, female PC levels were significantly higher than that of males, control (chi-squared = 6.0448, df_1, p-value = 0.0139), PS (chi-squared = 4.5049, df = 1, p-value =0.0338), and DEHP (chi-squared = 6.5523, df = 1, p-value = 0.01048).

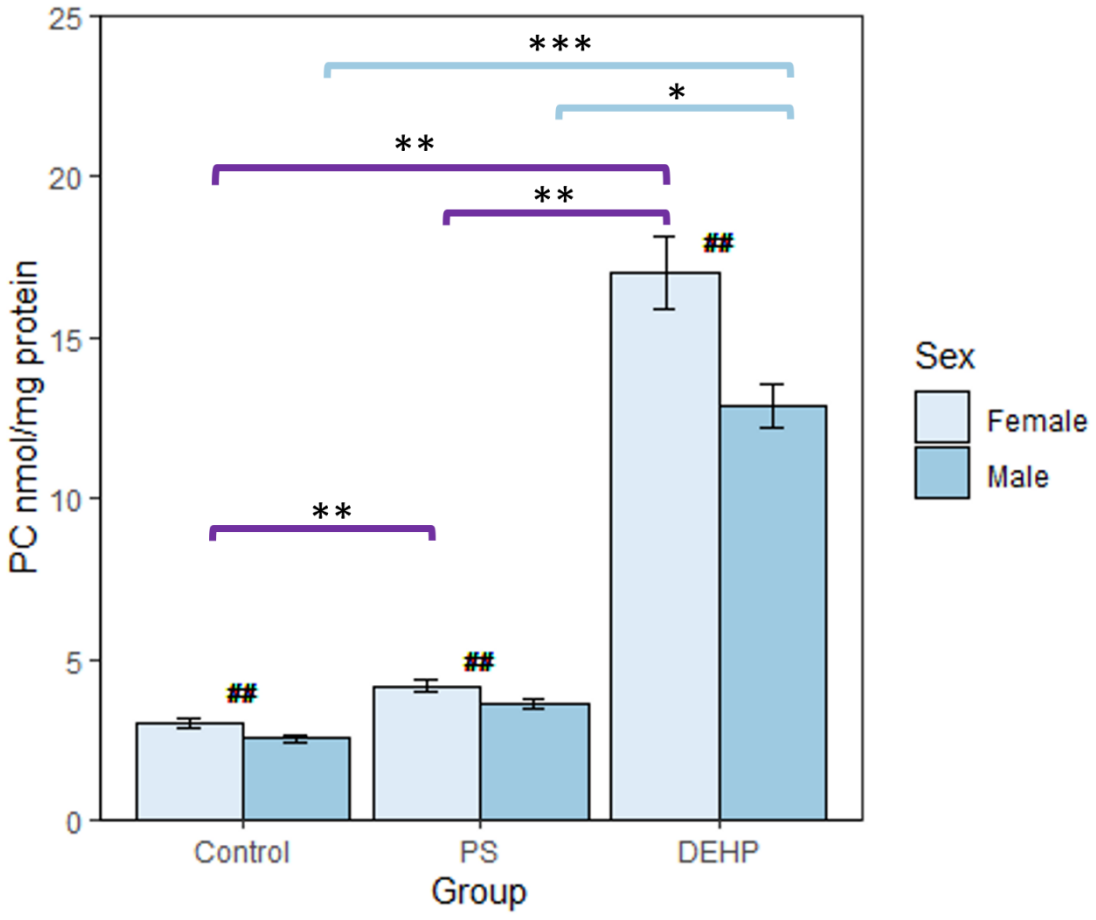

Figure 18. A) Mean concentration of PC nmol/mg pro ± standard error in male and female white muscle in each group. Blue markers indicate significance between groups of female fish whilst purple markers indicate those of male fish. Male control n= 10, female control n=10, male PS n=12, female PS n=8, male DEHP n=11, female DEHP n=8. # denotes significant difference between sexes, ## p-value<0.01. *p-value<0.05, **p-value<0.01.

4.4 Discussion

The ubiquity of plastic pollution in the marine environment has led to a range of taxa having records of microplastic ingestion (Germanov et al. 2018; Sun et al. 2017). In addition to the threat posed by microplastics themselves, microplastics are often associated with plasticisers, chemicals added to plastics during their manufacture (Tyler et al. 2018). One of the most used plasticisers is DEHP, which itself is detected throughout the marine environment (Chikae et al. 2004; Liu et al. 2017). However, despite the frequent use of DEHP-containing plastics, research as to the effects of plasticiser exposure following microplastic ingestion is scarce. This study is one of the first to assess the effects of DEHP exposure via consumption of dosed microplastics on the physiological condition of a cryptobenthic fish. In *F. capito* white muscle, the oxidative biomarkers PCs, LPs, and the activity of enzymes SOD, CAT, GPOX and GST were all significantly increased due to DEHP exposure. This study also observed significant differences in activities of enzymes and the production of both LPs and PCs between the sexes.

Increased PC and LP production serves as an indicator of failing oxidative defence mechanisms of an organism, as their presence indicates the oxidation of structural proteins and lipids by ROS. The defensive enzymes SOD, CAT, GPOX and GST all work together to prevent harm done by ROS (Ighodaro and Akinloye 2018). Therefore, when increased ROS production occurs, enzyme production will also increase to prevent oxidative damage. The present study not only observed increased enzyme activities in DEHP exposed fish compared to control and PS groups (figs. 13-16) but also detected significant increases in both LPs and PCs (figs. 17 and 18 respectively). These findings indicate that DEHP exposure caused ROS production at a rate which overwhelmed defensive systems as though more enzymes were active, damage to both proteins and lipids of the white muscle occurred at an increased rate compared to that of the control and PS groups. The findings of the present study are supported by that of published literature. For example, exposure of *D. rerio* embryos to DEHP for 96h caused increased production of LP's but did not affect SOD or CAT levels (Mankidy et al. 2013). It appears to be a somewhat consistent finding among studies of direct DEHP exposure to observe increased PC and LP levels but no change or suppression of

antioxidant enzyme activity levels (Mo et al. 2019; Revathy and Chitra 2018; Xiang et al. 2017). The difference between past literature and the present study being the exposure method as constant aqueous exposure is the most common but here, dosed microplastics were vectors for DEHP exposure. Perhaps in conditions of constant exposure, enzyme activity is supressed whereas in low dose conditions, such as those of the present study, enzyme activity is increased but still outpaced by ROS production. In both instances, oxidative stress and damage to tissue and cellular structures occurs. The outpacing of defensive systems in white muscle tissue is an important finding as oxidation of proteins, DNA and lipids by ROS may prevent them from functioning correctly (Aitken et al. 2016; Mo et al. 2019; Revathy and Chitra 2018).

In addition to the above findings, in *F. capito* a difference was observed between sexes in oxidative response to DEHP exposure. For example, for the enzymes CAT and GPOX males had higher enzyme activity than females (figs. 14 and 15 respectively). Different antioxidant response between the sexes have been observed in past literature (Adeogun et al. 2020; Costantini 2018). This literature suggests that this difference is due to the high energetic cost of egg production preventing female fish mounting antioxidant defence equal to that of male fish (Adeogun et al. 2020; Costantini 2018). In the present study, this reduced antioxidant defence was associated with a higher level of both PCs and LPs being produced in female fish (figs. 17 and 18 respectively). As past literature has noted that females are most vulnerable to oxidative stress during their breeding season (Costantini 2018), this means that environmental pollutants such as DEHP could have the most significant impact on an organism's health during this part of their lifecycle. Furthermore, past literature has found that animals which undertake parental care are more susceptible to the effects of ROS's (Costantini 2018). This is important to note as *F. capito* is a species which performs parental care as the male guards and cares for embryos until hatching like other intertidal triplefins in Aotearoa (Thompson 1986; Tornquist 2020). Understanding how animals of reproductive age respond to a given stressor is extremely useful as a larger energetic cost during the breeding season may impact the survival of adults between seasons reducing the number of mature, breeding individuals season to season.

The findings of the present study indicate that DEHP exposure causes the production of ROS at a rate which overwhelms defensive pathways leading to oxidative stress. This study also found that the effect of DEHP on the antioxidant defence between sexes was not equal. This is significant to note as with the potential to impact both gamete quality as well as the health of adult fish, the accumulation of DEHP in marine ecosystems could impact ecosystem dynamics. The present findings are also significant as exposure to DEHP was variable, this is as the fish were unlikely to ingest the same number of plastic particles each feeding and were only offered treated feeds every second day. This is an important detail to note as exposure in previous research has focussed on the effects of direct DEHP, largely via aqueous exposure. And as the maintenance of a given concentration of DEHP for an extended period is not environmentally relevant, tailoring exposure experiments to a given species is crucial to obtaining as clear a picture as possible to the impacts of a given stressor. In summary the present study observed that even low doses of DEHP can induce oxidative stress in the body of exposed fish. This is noteworthy as the inability of oxidative defence systems to cope with DEHP exposure may have energetic costs over time.

Chapter 5 – General discussion

5.1 Overview

Mass production of plastics since the 1940s and their prominent role in everyday life has led to both macro and microplastics being ubiquitous throughout the marine environment (Cole et al. 2011; Freinkel 2011). As a result, many studies have observed reproductive, energetic and oxidative costs of microplastic ingestion on marine fauna (Dauvergne 2018). Microplastics have a large surface area which allows for environmental toxins to be adsorbed from or leached out into the environment (Guerranti et al. 2019; Magdouli et al. 2013; Sarkar et al. 2021). Plasticisers, such as DEHP, are chemicals added to plastics during manufacture (Chikae et al. 2004; Magdouli et al. 2013; Tyler et al. 2018). They are often endocrine disruptors as well as ubiquitous environmental pollutants (Chikae et al. 2004; Magdouli et al. 2013; Tyler et al. 2018). Endocrine disruptors can change the reproductive capacity of exposed organisms as they can influence the levels of sex hormones in the body which are required in pathways such as gamete production and the expression of reproductive behaviours (Carnevali et al. 2018). Reproduction is crucial to the maintenance of populations so its disruption can have negative effects for biodiversity and ecosystem dynamics. Both microplastics and plasticisers have been found to cause reproductive dysfunction, but these stressors have largely been studied in isolation of one another. Therefore, it was the aim of this study to assess how the ingestion of DEHP-dosed microplastics impacted the reproductive output, behaviour and biochemistry of *F. capito*. This was done via two experiments, one to assess reproductive output and biochemistry (Chapters Two and Four) and the other to assess behaviour and neurochemistry (Chapter Three).

5.2 Exposure to microplastics containing di(2-ethylhexyl) phthalate does not affect reproductive traits in *Forsterygion capito*.

The present study found that DEHP exposure via ingestion of dosed microplastics did not change the reproductive fitness of *F. capito* after five weeks of exposure. All measures analysed were unaffected, showing no significant differences between groups (fig. 7). The

findings of this chapter contradict those of published literature which have shown that constant exposure to aqueous DEHP does cause reproductive dysfunction teleosts (e.g., zebrafish and medaka) (Guo et al. 2015; Uren-Webster et al. 2010; Ye et al. 2014). The findings of the present study thereby highlight the importance of environmentally relevant experiments as a different exposure method led to the opposite finding.

There are numerous published studies in which constant exposure to aqueous DEHP led to negatively changed reproductive fitness and outputs (Ahmadivand et al. 2016; Carnevali et al. 2010; Corradetti et al. 2013; Uren-Webster et al. 2010; Ye et al. 2014). Though many studies used environmentally relevant concentrations of DEHP, the constant maintenance of exposure concentrations is not. DEHP concentrations in the marine environment are unlikely to be constant due to processes such as currents and ocean mixing by wave action. It is therefore important to understand how variable exposure to pollutants such as DEHP will impact marine species.

5.3 Exposure to DEHP alters neurochemistry and reproductive signals in male *Forsterygion capito.*

The ingestion of DEHP containing microplastics did not alter the reproductive behaviours of male triplefins. It did however impact both the presentation of nuptial colouration and levels of the measured neurotransmitters of exposed fish. Fish exposed to virgin and DEHP-dosed microplastics were unable to maintain nuptial colouration (full body jet black colour) to the same extent as the control fish after ten weeks of exposure (fig. 11). This could be a result of the endocrine-based mechanisms which regulate colour expression being altered (Baatrup and Junge 2001; Toft and Baatrup 2003). Also in this chapter, DA levels were significantly increased whilst those of 5-HT were significantly decreased following DEHP exposure (fig. 12A and B). Though this finding did not correlate with changed behaviour as predicted, it does suggest that in conditions of variable exposure, longer exposure times may be needed to induce endocrine disruption. The inability of males to maintain nuptial colouration after ten weeks of DEHP exposure could also indicate that after longer exposure periods, variable exposure to DEHP may have more pronounced effects.

5.4 Exposure to DEHP via microplastic ingestion causes oxidative stress in *Forsterygion capito*.

In present study, DEHP exposure induced oxidative stress. All measured markers of antioxidant defence and oxidative stress significantly increased. This suggests that an increased antioxidant defence was mounted in response to DEHP exposure, but ROS production outpaced enzyme activity leading to the oxidation of proteins and lipids. Damage to proteins and lipids in this manner can negatively impact cellular function. The present study observed lower enzyme activity in female fish compared to that of males. Previous studies suggest this difference between sexes results from the high energetic cost of egg production compromising the ability of females to mount a defence equal to that of male fish (Adeogun et al. 2020; Costantini 2018).

5.6 Limitations

In Chapter Two, we were unable to assess the effects of DEHP exposure on the female gonad as the tissue could not be successfully fixed on a slide. Additionally, the VTG data was limited to a sample size of three fish per group instead of the intended ten as the RNA extracted from the other 27 samples were too degraded for analysis. This degradation was likely a result of the high fat content of the livers. The low replication of the VTG mRNA data means that these analyses are limited in their ability to support the findings of the present research. Also, due to the erroneous sexing of fish, the PS group consisted of primarily same-sex pairs preventing the clutch output of this group being to be included in the analysis alongside that of the control and DEHP groups.

5.6 Summary and future directions

In looking at the results of both Chapters Two and Four, the fish were responding to the DEHP exposure as evidenced by the increase in all measured oxidative markers (figs. 13 - 18). But it did not cause reproductive dysfunction as both clutch output and gametogenesis were unaffected (figs. 7A and B). It may be that as the breeding of *F. capito* occurs between July and October (Wellenreuther and Clements 2007), investment in gamete production had already been largely completed when fish begun treatment in late June so reproductive

output went unaffected. To assess whether timing may determine whether DEHP exposure impacts reproduction it would be of value for future studies to expose fish to DEHP earlier in the year. Additionally, considering the results of both Chapter Two and Chapter Three, it appears that in conditions of variable exposure, DEHP may only compromise reproductive success after longer exposure periods. This is as although DEHP had no reproductive effects after five weeks of exposure, the colour presentation of males was impacted after ten weeks. This change in colour presentation could be indicative of compromised health or reproductive ability. As the present work could not elucidate which is true, future work could aim to seek the true cause. A repeat experiment to assess the reproductive ability of fish after ten weeks exposure could help answer this question. Also, to follow on from the work undertaken in Chapter Three, future research could assess whether the observed change in nuptial colouring will impact mate selection of female fish. By measuring whether females would preferentially select control over exposed males, this will give another angle from which to understand the effects of DEHP on fish reproduction. Investigating the impacts that parental DEHP exposure may have on subsequent offspring would also be extremely useful in the assessment of threat posed by DEHP. Previous literature has focussed on the effect of pollutants on juvenile fish likely due to the potential heightened susceptibility as development is ongoing (Leeuwen et al. 1985; Ory et al. 2018). Therefore, the effect of DEHP via ingested microplastics on juvenile fish could be assessed in future studies, whether this is via direct or parental exposure. Lastly, as the marine environment is currently facing a barrage of human-derived threats it would be useful for future studies to investigate the impacts of DEHP exposure in combination with stressors such as climate change or ocean acidification.

5.7 Conclusions

Plastic polymers have become an integral part of everyday life. The ubiquity of these particles and their associated chemicals in the environment are a cause for concern regarding the health of wildlife and ecosystems. The present work has shown that even conditions of variable exposure the plasticiser DEHP can still have a suite of effects on the condition of an animal. The effects observed having the potential to illicit negative consequences on organisms' health. Findings of this study emphasise the importance of environmentally relevant experiments to allow us the best understanding of the true

impacts of plastics. A comprehensive understanding of the threat posed by plastics and plasticisers is required for appropriate management strategies to take place, environmental relevance should be a key consideration as to how studies are carried out.

6 – Appendix

6.1 Appendix A – Bioanalyser results.

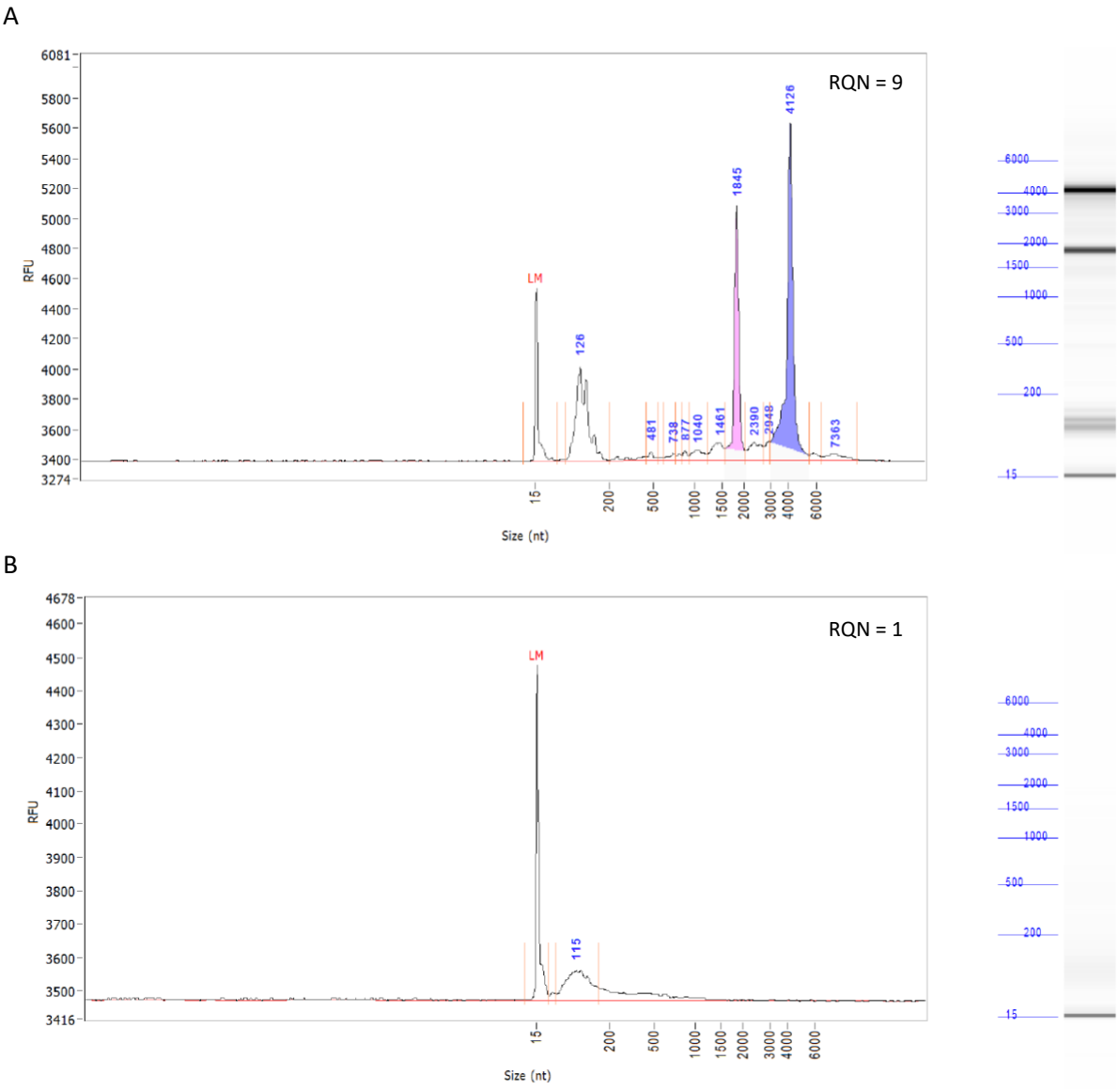

Figure 19. Bioanalyser results for extracted RNA A) Undegraded RNA with an RQN value of 9. B) A degraded RNA sample with an RQN value of 1.

6.2 Appendix B – Raw behaviour data

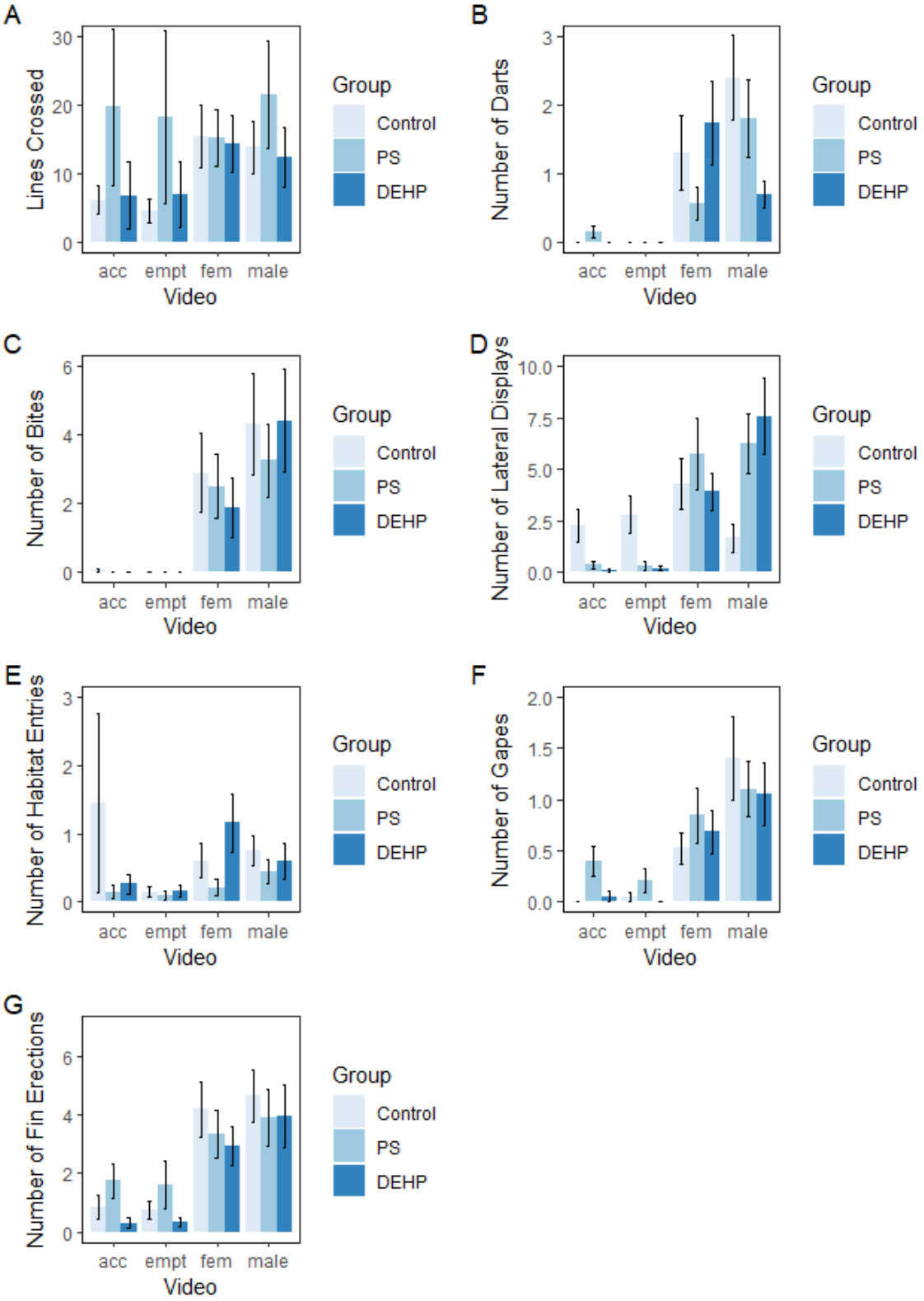

Figure 20. Behaviour data prior to PCA A) Number of lines crossed ± standard error B) Number of darts ± standard error C) Number of bites ± standard error D) Number of lateral displays ± standard error E) Number of habitat entries ± standard error F) Number of gapes ± standard error G) Number of fin erections ± standard error in each video. Acc = no bag, Empt = empty bag, fem = female stimuls fish in bag and male = male stimulus fish in bag for each group. Control (n=20), PS (n=20) and DEHP (n=19) for all data.

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