An examination of the short- and long-term effects of microplastics in mammals using a mouse model

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Abstract

The United Nations has recognized plastic pollution as the second most concerning environmental problem after climate change. Photo-oxidation and other natural processes can fragment plastics into smaller particles, giving rise to another problem: microplastics. Despite many studies showing that humans are exposed to microplastics, their toxic effects are uncertain. The aim of this study was to contribute to the understanding of microplastic health impacts on mammals, using a mouse model. For the manuscript 1 study, we exposed female mice to two different shapes of microplastics (microbeads, microfibers) for up to four weeks. Although microbead and microfiber-treated groups showed a slightly altered food consumption, body weight was not affected in both groups. Microbeads, but not microfibers, were able to translocate from gut lumen to the liver, however no histopathological changes were observed. The expression levels of genes associated with inflammation (*Apcs, Crp, Cfb*) and tissue injury repair (*Ccn1*) did not show a statistically significant difference after exposure to microbeads and microfibers. For the manuscript 2 study, we exposed female and male mice to microbeads for up to sixteen weeks, followed by a four-week recovery period. Although microbeads translocated to deeper organs in both female and male mice, only females showed significant changes in gene expression. Overall, longer exposure caused more aggravated effects than short exposure; some of these effects were reversed after the recovery period, while others showed a delayed manifestation. Biological response to microplastic exposure has proven complex, depending on time of exposure and showing remarkable sex differences.

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C-reactive protein (CRP)

Damage-associated molecular patterns (DAMPs)

Dextran sodium sulfate (DSS)

Extracellular matrix (ECM)

Glyceraldehyde-3-phosphate dehydrogenase (GADPH)

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Inflammatory bowel disease (IBD)

Interferon (IFN)

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Membrane attack complex (MAC)

Microbeads (MB)

Microfibers (MF)

Nasal associated lymphoid tissue (NALT)

Pathogen-associated molecular pattern (PAMPs)

Pattern recognition receptors (PRRs)

Peripheral blood mononuclear cells (PBMCs)

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Polycarbonate (PC)

Polyethylene (PE)

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Polymethylmethacrylate (PMMC)

Polypropylene (PP)

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Polyurethane (PU)

Polyvinyl alcohol (PVA)

Polyvinylchloride (PVC)

Reactive oxygen species (ROS)

Serum amyloid P-component (SAP)

Transcriptional co-activator with PDZ-binding motifs (TAZ)

Tumor necrosis factor (TNF)

Yes-associated protein (YAP)

1- Introduction

1.1 Plastic pollution and microplastics

Plastic pollution has become a global environmental issue and a growing concern since the rise of the plastic industry in the mid-1950s. The long durability of plastics in the environment and deficient recycling policies contribute to environmental pollution, especially in aquatic ecosystems. Once in nature, plastics are exposed to a variety of physical and chemical processes, such as photolysis, thermal-oxidation, and hydrolysis, and can be fragmented into smaller pieces known as microplastics (Thompson, 2015). Microplastics are defined as plastic particles smaller than 5mm and are categorized as primary or secondary according to their source. Primary microplastics are originally manufactured to be that size, while secondary microplastics result from the breakdown of larger plastic items. Examples of primary microplastics include pellets/powders and engineered plastic microbeads used in cosmetic formulations, cleaning products and industrial abrasives (EFSA, 2016; GESAMP, 2016).

In the environment, microplastics differ in shape, size, source and chemical composition. The most common microplastic composition are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), polyamide (PA), polyethylene terephthalate (PET), polycarbonate (PC), polyvinyl alcohol (PVA), polyurethane (PU), polymethylmethacrylate (PMMC) and plastic additives such as stabilizers, flame retardants, plasticizers, fillers and pigments (Hirt and Body-Malapel, 2020). There is still some debate over the most appropriate upper and lower size limit, but usually researchers classify microplastics as particles <5mm and nanoplastics as particles ≤0.1µm. Microplastics can be

subcategorized according to their shape and includes beads, nurdles, fibers, foam, fragments, pellets, films, filaments, cylindrical, disk, flat, ovoid, spheruloid, elongated, rounded and irregular (Hirt and Body-Malapel, 2020).

Microplastics have been reported on a global scale. Many studies have reported microplastics in marine environments (Eriksen et al., 2014), freshwater ecosystems (Dris et al., 2015; Imhof et al., 2013), soil (Rillig, 2012), ground water (Mintenig et al., 2018), sewage and the atmosphere (Dris et al., 2015) and even in remote areas such as in the Arctic Sea ice (Obbard et al., 2014), Mount Everest (Napper et al., 2020) and Swiss Alps (Bergmann et al., 2019). Microplastics have also been found in bottled mineral water (Schymanski et al., 2018), tap water (Kosuth et al., 2018), beer (Kosuth et al., 2018; Liebezeit and Liebezeit, 2014), tea (Hernandez et al., 2019), cow milk (Kutralam-Muniasamy et al., 2020), table salt (Yang et al., 2015; Kosuth et al., 2018), honey (Liebezeit and Liebezeit, 2015, 2013), sugar (Liebezeit and Liebezeit, 2013), fruits and vegetables (Conti et al., 2020) and seafood (Mathalon and Hill, 2014; Murray and Cowie, 2011; Smith et al., 2018).

A growing body of evidence suggests that humans are exposed to microplastics. Recently microplastics were found in eight human stool samples (on average 20 particles/10g of feces, size range: 50 - 500µm) with PP and PET microparticles being the most abundant (Schwabl et al., 2019). Similarly, microplastics were found in eleven human colectomy samples (on average 331 particles/individual or 28.1 particles/g of colon tissue, size range: 0.8 - 1.6mm) of which 90% were PC, 50% PA and 40% PP (Ibrahim et al., 2021). In addition, pigmented microplastics were observed in four out of six human placenta samples (12 particles, size range: 5-10µm) where most of them were PP microplastics (Ragusa et al., 2021). Washes from hand, face, hair and mouth showed a remarkable inter and intra-individual variability of microplastic count within a 24-hour and 7-day period. This heterogeneity suggests that

human exposure to microplastics depends on the individual habits and environment, such as household furnishings, clothing, cleaning frequency, use of cosmetics and urbanization (Abbasi and Turner, 2021).

1.2 Microplastic exposure and potential health risks

1.2.1 Exposure routes and translocation

The routes of human exposure might be oral, inhalation and dermal, although the latter seems to have little effect on an intact skin and is only relevant for nanoparticles (Revel et al., 2018). Synthetic textiles, the erosion of rubber tires and city dust are thought to be the most important source of airborne microplastics (Hirt and Body-Malapel, 2020). Dermal contact occurs through the use of cosmetics and hygiene products that contain microbeads, such as facial cleansers, toothpaste, shampoos and scrubs (Hirt and Body-Malapel, 2020). The estimated annual consumption of microplastics for a North American diet ranges from 39,000 to 52,000 depending on age and sex. Inhalation contributes up to 35,000 - 62,000 additional particles and these numbers escalate even more if the source of water is exclusively plastic bottled water (Cox et al., 2019).

Microplastics can cross the intestinal and pulmonary epithelial barrier, causing systemic exposure. Transcytosis via M-cells in the Peyer's patch seems to be the main entry pathway of nano/microplastics across the intestinal wall, followed by endocytosis, paracellular transport and persorption (Powell et al., 2010; Stock et al., 2019). Approximately 10% of the PS nanoplastic dose administered to rats was recovered from the gastrointestinal tract, and 60% of the particle uptake occurred through the Peyer's patch (Hillery et al., 1994). In rats,

translocation of PS nanoparticles from intestinal lumen to the lymph occurred within minutes after exposure (Seifert et al., 1996) and these particles were observed in the stomach, intestines, kidneys, heart, spleen, lungs, and testes within 6 hours of oral administration (Walczak et al., 2015). Inhaled microplastics might escape mechanical and mucociliary clearance in the upper airways and advance to deeper respiratory tract reaching the alveoli (Dong et al., 2020; Pauly et al., 1998). After intranasal application, PS microplastics were found in the mouse nasal associated lymphoid tissue (NALT) and bronchopulmonary deposits were observed within 15 minutes. These particles were found in the spleen and liver 10 days after exposure (Eyles et al., 2001). In rats, PS nanoplastics accumulated in maternal lungs, heart and spleen and translocated to fetal liver, lungs, heart, kidney and brain 24 hours after intratracheal instillation (Fournier et al., 2020).

1.2.2 Microplastic toxicity studies

Many studies using *in vivo* analyses showed that microplastic exposure causes harmful effects, such as genotoxicity, neurotoxicity, oxidative stress, hepatotoxicity, metabolic disorder, disturbed immune response, endocrine system dysfunction, microbiome dysbiosis, intestinal barrier dysfunction, reproductive impairment, reduced feeding activity and growth, altered behaviour and reduced survival rate. These adverse effects have been shown in algae, amphipods (Au et al., 2015), *Daphnia* (Besseling et al., 2014; Ogonowski et al., 2016), waterfleas (Ziajahromi et al., 2017), copepods (Cole et al., 2015), mussels (Avio et al., 2015; von Moos et al., 2012), crabs (Watts et al., 2015), lugworms (Browne et al., 2013), *Caenorhabditis elegans* (Dong et al., 2018; Lei et al., 2018b, 2018a), fish (Ding et al., 2018; Jin et al., 2018b; Lu et al., 2016; Mattsson et al., 2017, 2015; Rochman et al., 2014), tadpoles (Araújo et al., 2020b, 2020a), rats (Amereh et al., 2020; Brown et al., 2001; Donaldson et al.,

2000; Fournier et al., 2020; J. Hou et al., 2021; Porter et al., 1999; Xu et al., 2004) and mice (Araújo and Malafaia, 2021; Deng et al., 2021, 2020, 2018, 2017; B. Hou et al., 2021; Jin et al., 2021, 2018a; Li et al., 2020; Lu et al., 2018; Luo et al., 2019a, 2019b; Park et al., 2020; Xie et al., 2020; Zheng et al., 2021). Other studies have found that microplastics might pose a minimum health risk.

Some results suggest that the exposure to microplastics at environmental concentrations does not bear a remarkable potential to cause toxic effects (Stock et al., 2019). After a short depuration period, microplastics were quickly egested by *Xenopus* tadpole (Hu et al., 2016), sea bass larvae (Mazurais et al., 2015) and *Daphnia* (Rosenkranz et al., 2009) and were almost completely eliminated after 6 days, 2 days and 4 hours, respectively. Zebrafish larvae exposed to PE fragments showed no histopathological signs, no oxidative stress, and no significant difference in the expression of genes related to neurotransmission and apoptosis (Karami et al., 2017). Similarly, PS microplastic exposure did not cause intestinal dysfunction and immune response in rainbow trout (Ašmonaite et al., 2018). Mice exposed to PS microparticles three times per week for 28 days showed no histological alterations and only a few particles were detected in the intestinal wall, while no particles were found in the liver, spleen, and kidney. In addition, there was no activation of the heme oxygenase 1 promoter, which is associated with oxidative stress and the inflammatory response (Stock et al., 2019). The variation among studies could be explained by the different microplastic characteristics (concentration, size, and chemistry), administration method, exposure time, and the use of different animal models, strains, or different stages of their life cycle.

Exposure to microplastics affects gene expression by downregulating or upregulating a variety of genes. Whole animal transcriptomics showed that larval zebrafish exposed to PE microplastics for 48 hours presented a transient and extensive change in up to 1,734 genes,

most of them were downregulated and are associated with the central and peripheral nervous systems (LeMoine et al., 2018). Zebrafish embryo and larvae injected with PS microplastics showed 26 and 51 differently expressed genes, respectively, mostly related to lipid metabolism, oxidative stress and complement pathway (Veneman et al., 2017). Hepatic transcriptome of mice exposed to PS microplastics showed that 200 genes were downregulated and 218 upregulated, most of them related to locomotor rhythm, insulin-activated receptor activity, negative regulation of insulin secretion, heat generation and energy metabolism, such as lipid metabolism and gluconeogenesis (Luo et al., 2019a). The gut transcriptome in mice showed that di- (2-ethylhexyl) phthalate (DEHP)-contaminated PE microplastics had a stronger effect than exposure to virgin PE microplastics or DEHP alone. Most of the 703 differentially expressed genes are related to oxidative response, hormone and lipid metabolism and immune response (Deng et al., 2020).

Microplastics can cause reproductive impairments and the adverse effects might persist across generations. Blood-testis barrier disruption, abscission, disordered arrangement of spermatogenic cells, multinucleated gonocytes, reduced testosterone serum levels along with lower sperm cell count and increase in sperm cell abnormalities were observed in male mice after PS microplastic exposure (B. Hou et al., 2021; Jin et al., 2021; Xie et al., 2020). Moreover, the expression of the anti-apoptotic protein Bcl2 was decreased while the pro-apoptotic protein Bax increased, which explains the increased number of apoptotic cells in the mouse testes (B. Hou et al., 2021). Exposure to PS microplastics induced oxidative stress and apoptosis in ovarian granulosa cells, leading to reduced number of growing follicles and reproductive toxicity in female rats (J. Hou et al., 2021). Mice dams exposed to PS microparticles during gestation and lactation showed metabolism disorder that persisted for the F1 and F2 generations (Luo et al., 2019a, 2019b). In addition, exposure to PE microplastics affected the number of live births, sex ratio and body weight as well as spleen

lymphocyte count of the offspring (Park et al., 2020). After intratracheal instillation of PS nanoparticles in pregnant rats, particles were observed in the fetal lungs, liver, heart, kidney, and brain 24 hours after the exposure, suggesting translocation across the maternal pulmonary barrier to placental and fetal tissues (Fournier et al., 2020). Recently a pilot study reported the presence of microplastics in four human placentas, drawing attention to the risks microplastics might pose to pregnancy and fetal development (Ragusa et al., 2021).

Microplastics represent a new substrate for microbial colonization and transportation. Genome sequence data showed that the microbial community living on plastics from the North Atlantic Subtropical Gyre was distinct from the microorganisms in the surrounding seawater and included opportunistic pathogens (Zettler et al., 2013). Likewise, potentially pathogenic *Vibrio* spp. were found on microplastics collected in the North and Baltic Sea (Kirstein et al., 2016). Interestingly, the biofilms on the microplastic surface are structurally different compared to those on natural substrates and might favor the propagation of antibiotic resistant microorganisms. The complex microbial consortia in microplastic biofilms may promote horizontal gene transfer between phylogenetically distinct microbes more rapidly than in free-living microorganisms (Wu et al., 2019). Therefore, microplastics may function as a vector for dispersion of invasive species and pathogenic organisms.

1.2.3 Effects of plastic additives

The adverse effects on organisms exposed to microplastics are categorized by physical or chemical effects (Campanale et al., 2020). Physical damage is associated with microplastic shape, size, and concentration while chemical damage is caused by leaching of environmental pollutants adsorbed on their surfaces and the release of plastic additives into the tissues

(Bouwmeester et al., 2015; Browne et al., 2013; Campanale et al., 2020). As microplastics have a high surface area to volume ratio, the concentration of these adsorbed chemicals can be orders of magnitude greater than those in the surrounding environment. These pollutants include heavy metals and persistent organic pollutants, such as polychlorinated biphenyls, organo-halogenated pesticides, nonylphenol, polycyclic aromatic hydrocarbons and dioxins (Endo et al., 2005; Heskett et al., 2012; Hirai et al., 2011; Ogata et al., 2009). More than a pathway to transfer pollutants into tissues, microplastics might augment their toxic effects, synergizing with the adverse effects of the toxics that they contain or have adsorbed (Deng et al., 2021, 2020, 2018; Luís et al., 2015). A possible explanation is that microplastics augment the exposure and accumulation of these chemicals and slowly release them into the tissues, increasing their bioavailability (Deng et al., 2018).

Plastic additives account on average for 4% of the plastic composition (EFSA, 2016) and usually are not chemically bound to the plastic polymer, being easily released into the tissues (Campanale et al., 2020). Plasticizers, such as phthalates and bisphenol A, are used to confer flexibility to plastic products and are well known endocrine disrupting chemicals. These chemicals interfere with receptor binding and hormone signaling, disrupting endocrine functions and leading to reproductive abnormalities (Rattan et al., 2017). Phthalates can affect ovarian follicles at different stages of folliculogenesis, either activating apoptosis (atresia), inducing follicle arrest or accelerating their development (Hannon and Flaws, 2015). Each of these outcomes can affect the production of ovarian hormones, leading to reproductive and non-reproductive detrimental effects (Hannon and Flaws, 2015). A significant reduction of ovarian weight was observed in mice after bisphenol A treatment, likely due to the inhibition of follicular growth during puberty (Nah et al., 2011).

Microplastics were able to adsorb, transport and accumulate phthalate esters into mouse tissues, indicating that microplastics are a route of exposure to plasticizers (Deng et al., 2021, 2020). Moreover, the accumulation of phthalates in the gut and in the liver was higher when transported by PE microplastics compared with phthalates alone (Deng et al., 2021, 2020). Microplastics enhanced the reproductive toxicity caused by phthalates in male mice, as demonstrated by spermatogenesis disorder, testicular oxidative stress, reduced sperm cell quality and altered testicular transcriptome (Deng et al., 2021). Microplastic and phthalate co-exposure also caused aggravated adverse effects on mouse gut, leading to microbiota dysbiosis, intestinal inflammation and altered transcriptome (Deng et al., 2020). Similarly, organophosphorus flame retardants in the presence of PE and PS microplastics led to worse harmful effects in mice, such as oxidative stress, neurotoxicity and metabolism disorder (Deng et al., 2018). Due to their toxic effects, some additives have been replaced by alternatives. However, as they have similar structure, the alternatives are thought to cause equivalent toxicological effects (Rattan et al., 2017).

1.3 Microplastics and the immune system

1.3.1 Microplastics and inflammation

It is reasonable to expect the immune system to be affected by microplastic exposure, as its main function is to provide protection against foreign particles. Inflammation is an immune response against damaged cells, irritants and pathogens that is highly coordinated by genes expressed in immune cells, particularly neutrophils. Inflammation can be acute (short-term) followed by healing or chronic (long-term) and characterized by persistent and increased

expression of inflammatory cytokines (e.g. interleukin [IL]-1, IL-6, and tumor necrosis factor [TNF]-α). Physical damage to tissue induces the secretion of inflammatory cytokines and other mediators typical of the innate immune response, leading to chronic inflammation if the damage is not resolved (Owen et al., 2018). Therefore, the microplastic potential to trigger an acute or chronic inflammation will depend upon the cell damage caused by microplastics, if they are eliminated/neutralized by the organism or if they persist in damaging cells.

Many *in vitro* studies have shown the nano/microplastic potential to induce an inflammatory response. Exposure to PS and PC nanoparticles induced phagocytosis of particle aggregates and individual particles by neutrophils. In addition, these particles induced degranulation of primary granules of neutrophils, respiratory burst and extracellular trap release (Greven et al., 2016). Exposure to PP microparticles caused hemolysis of red blood cells (RBCs) and increased reactive oxygen species (ROS), IL-6, TNF-α and histamine levels in dermal fibroblasts, peripheral blood mononuclear cells (PBMCs), macrophages and mast cells, while no effect was observed in IL-2, IL-10 and IL-1β secretion levels (Hwang et al., 2019). Human lung epithelial cells exposed to PS microparticles showed oxidative stress, loss of epithelial barrier integrity, and increased secretion of proinflammatory cytokines IL-6 and IL-8 (Dong et al., 2020). Mouse testicular cells (germ cell-1, Leydig and Sertoli cells) showed increased levels of TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP-1) and chemokine (C-X-C motif) ligand 10 (CXCL10) after PS exposure, indicating a strong testicular inflammation (Jin et al., 2021).

Microplastics have been reported to cause an inflammatory response in both invertebrate and vertebrate species. Mussels showed a sharp increase in hyalinocytes (precursor of granulocytes), decreased lysosomal membrane stability and granulocytoma formation after exposure to microplastics (Avio et al., 2015; von Moos et al., 2012). Gut inflammatory cell

infiltration was observed in mice after oral exposure to PE microplastics (Deng et al., 2020) as well as in adult zebrafish exposed to PS microparticles (Qiao et al., 2019b). After an intraperitoneal injection of PS microplastics, mice showed a strong inflammatory response, characterized by the influx of monocytes, macrophages and neutrophils into the particle-infiltrated area. Macrophages rapidly engulfed small and medium sized particles (1.2 - 6.2µm), whereas larger particles (12µm) were surrounded by macrophages that apparently fused to each other to create giant cells (Tomazic-Jezic et al., 2001). After oral PE exposure, mice showed a decreased number of leukocytes in the blood with an elevated proportion of neutrophils, increased helper T cell to cytotoxic T cell ratio and decreased proportion of mature dendritic cells in the spleen; some of these effects were also observed in the offspring (Park et al., 2020). Inhaled microplastics that have escaped clearance mechanisms in the upper airways interact with lung cells and lung fluid triggering an inflammatory response, usually mediated by macrophages (Dong et al., 2020; Pauly et al., 1998).

Adult zebrafish exposed to PS microplastics presented increased mRNA levels of the pro-inflammatory cytokines IL-1α, IL-1β, IL-8 and interferon (IFN) in the gut (Jin et al., 2018b). Mice exposed to PE microplastics showed altered serum concentration of the cytokines IL-1α, granulocyte colony-stimulating factor (G-CSF), IL-2, IL-5, IL-6, IL-9, interferon gamma-induced protein 10 (IP-10), and RANTES (regulated on activation, normal T cell expressed and secreted, also known as CCL5) (Li et al., 2020) and higher levels of immunoglobulin (Ig) A, while no change was observed for IgM, IgE and IgG (Park et al., 2020). Caspase-3, NF-κB, TNF-α, IL-1β and IL-6 levels were increased while the anti-inflammatory molecules Nrf2 and HO-1 were decreased in the testicular tissue of mice exposed to PS microplastics (B. Hou et al., 2021; Xie et al., 2020). These effects were reversed by the use of an antioxidant agent and a p38 MAPK inhibitor, suggesting that PS microplastic toxicity involves oxidative stress and activation of the MAPK signaling pathway (Xie et al., 2020).

Moreover, microplastics can exacerbate inflammation in a pre-existing inflammatory condition, as demonstrated in dextran sodium sulfate (DSS)-induced colitis mice exposed to PS microplastics (Zheng et al., 2021). This study showed that microplastics can have a stronger adverse effect on people with chronic inflammation, such as inflammatory bowel disease (IBD) patients (Zheng et al., 2021).

Microplastic exposure seems to activate the complement system. The complement system includes 50-plus serum proteins that cooperate with both innate and adaptive immune systems to eliminate pathogens, dying cells and immune complexes (Owen et al., 2018). Once in biological fluids, nanoparticles can bind proteins and form protein coronas within 30 seconds of exposure; most of these corona proteins are complement components (Tenzer et al., 2013). The complement system can be initiated through three different ways: classical, lectin and alternative pathways. The classical and lectin pathways rely on the binding of pattern recognition receptors (PRRs) to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) while the alternative pathway is initiated by the spontaneous hydrolysis of the complement protein C3 (Thorgersen et al., 2019; Owen et al., 2018). Larval zebrafish injected with PS microparticles showed altered transcriptome profile, including the upregulation of genes of the complement pathway (*cfb, cfh, c3a.2, c3a.3, c3a.6* and *c9*), indicating that microplastics can activate the complement system, mainly through the alternative pathway (Veneman et al., 2017). All three initiation pathways converge in the generation of a C5 convertase, which cleaves C5 molecule into C5a and C5b and ultimately leads to the formation of the membrane attack complex (MAC) and cell death (Owen et al., 2018). It is important to notice that although the MAC formation is the final product of the complement cascade, the molecules that are generated in the process also play a major role on the immune response, such as opsonins (C3b, C4b) and anaphylatoxins (C3a, C5a) that mediate inflammation.

1.3.2 Acute-phase reactants

Acute-phase reactants (APR) are serum proteins that rapidly increase or decrease in response to infection, tissue damage or inflammation. These proteins are commonly recognized inflammation markers and are extensively used in diagnostics and prognostics in a variety of clinical conditions. C-reactive protein (CRP) is a major APR in humans. During an inflammatory response, blood levels of CRP can rapidly increase up to 1,000-fold and decrease just as rapidly when the stimuli end. Studies have shown that CRP opsonize pathogens and apoptotic cells and activate the classical complement pathway (Sproston and Ashworth, 2018). The name CRP arose because it was first identified as a substance that reacted with the C-polysaccharide of pneumococcal cell wall in patients with acute pneumonia. Similarly, CRP binds to exposed or denatured chromatin and phosphatidylcholine present on damaged cell membranes, promoting clearance by macrophages and neutrophils (Du Clos, 2000). By contrast, CRP in mice is less inducible by inflammatory stimuli and is deemed a minor APR. Serum amyloid P-component (SAP), however, is the major inflammation marker in mice (Pepys et al., 1979).

Similar to CRP, SAP is a pattern recognition molecule secreted by the liver and modulates the innate immune response (Cox et al., 2014). Its concentration in the serum increased up to 50-fold in mice within 24-48 hours of injection of lipopolysaccharides (LPS) and returned to the basal level after 32 days (Pepys et al., 1979). The main roles of SAP are the regulation of neutrophil migration and spreading, inhibition of monocyte differentiation into fibrocyte, opsonization of cell debris, regulation of macrophage polarization and activation of the classical complement cascade (Cox et al., 2014). Studies have shown that SAP binds to a variety of microbial polysaccharides and toxins as well as chromatin and DNA in circulation, enhancing the clearance of autoantigens to prevent autoimmunity (Mold et al., 2001).

1.3.3 The Hippo Pathway and tissue injury repair

As studies have reported that microplastics can potentially cause tissue damage, it is expected that injury repair mechanisms to be engaged following microplastic exposure. The Hippo Signaling Pathway is a protein kinase cascade that modulates cell proliferation, differentiation, growth and death (Yu and Guan, 2013). Cell contact, mechanical stress, cytoskeletal tension, cell polarity, and growth factor receptor signaling are known upstream regulators of this pathway. Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motifs (TAZ) are the major downstream effectors of the Hippo pathway in mammals. When the Hippo pathway is activated, YAP/TAZ is phosphorylated and unable to enter the nucleus, resulting in inhibition of target gene transcription. On the other hand, inactive upstream kinases result in nonphosphorylated YAP/TAZ that translocate into the nucleus and interact with DNA-binding transcription factors, promoting target gene transcription (Yu and Guan, 2013). Abnormal YAP/TAZ overexpression leads to uncontrolled cell growth and cancer development (Wang et al., 2013). This pathway regulates the transcription of a diverse range of growth-promoting and apoptosis-inhibiting genes, including cellular communication network factor 1 (CCN1), previously named cysteine-rich 61 (Cyr61) (Lee et al., 2019).

Cellular communication network proteins are conserved matricellular proteins that regulate cell adhesion, migration, differentiation, proliferation, and connective tissue remodeling (Jun and Lau, 2013; Lee et al., 2019). These proteins modulate extracellular signal molecules, such as growth factors, chemokines, cytokines and enzymes, or directly bind to integrin receptors to activate pathways in a cell-type dependent manner (Jun and Lau, 2013). While in adults CCN1 expression has been associated with inflammation and tissue injury repair, during embryogenesis it is essential for cardiovascular and skeletal development (Jun and

Lau, 2013; Kim et al., 2018). Wound healing and injury repair consist of three temporarily overlapping but biologically distinct phases - inflammation, proliferation and maturation – and CCN1 has been shown to play an important role in each of these phases (Kim et al., 2018). Immune cells, particularly neutrophils, infiltrate the damaged area during inflammation. However, neutrophils are short-lived and rapidly undergo apoptosis. The clearance of apoptotic neutrophils is facilitated by CCN1, that functions as a bridging molecule between the phosphatidylserine molecules of apoptotic cells and integrins in macrophages (Jun et al., 2015). During the proliferation phase, myofibroblasts are activated to produce extracellular matrix (ECM) components to promote tissue integrity. Treatment with CCN1 induced cell proliferation and differentiation after bile duct injury (Kim et al., 2015) and DSS-induced colitis (Choi et al., 2015) leading to biliary repair and intestinal mucosal healing, respectively. During the maturation phase, CCN1 triggers myofibroblast senescence, ceasing ECM production in order to avoid fibrosis while upregulating ECM degradation enzymes to promote matrix remodeling (Kim et al., 2018).

This research tested the ability of microplastics to affect the health of mammals using a mouse model. We assessed the microplastic effects on food consumption, body and organ weights, the effects on the expression levels of genes related to inflammation (complement factor B - *Cfb*, C-reactive protein - *Crp*, serum amyloid P-component - *Apcs*) and tissue injury repair (cellular communication network factor 1 - *Ccn1*) and microplastic tissue distribution, accumulation and associated histopathology. Manuscript 1 summarizes the pilot project results, where two different shapes of microplastics were used (microbeads and microfibers). Manuscript 2 discusses the follow-up project, where the exposure time was increased and sex-based differences were observed.

2- (In prep) Short-term exposure to microbeads and microfibers pose minimal risk of inflammation and tissue injury in mice

2.1 Chapter summary

Microplastics are ubiquitous pollutants, traveling in the atmosphere and depositing all over the world, whether at sea or land. Recent studies have found microplastics in human stools, colectomy samples and placenta. Despite considerable evidence suggesting that microplastics cause negative outcomes in model organisms and wild species, the risks to human health are unknown. Moreover, most researches use manufactured single-shaped microplastics while in reality humans are exposed to a variety of shapes and sizes of microplastics. Here we investigated the biological effects of two different shapes and sizes of microplastics (microbeads and microfibers) using a mouse model. Female mice were fed microplastic-containing food for two and four weeks, followed by a four-week recovery period. Microplastic exposure did not affect body weight and only minor changes were observed in food consumption for both microbead and microfiber-treated groups. Microfibers were not able to translocate from the gut lumen into tissues, while microbeads were observed in the liver and small intestinal wall. Although microbeads remained embedded in the liver after the recovery period, no histopathological signs were observed. Similarly, microplastic exposure did not affect the expression levels of genes related to inflammation (*Apcs, Crp, Cfb*) and tissue repair (*Ccn1*). Although we did not find any significant change for the biomarkers used in the present study, other researches using different endpoints have reported a broad range of adverse effects. Further research is needed to investigate the fate and effects of different types of microplastics and the impact of long-term exposure on biological systems.

2.2 Introduction

The distribution and abundance of plastics into the world are so extensive that they have earned a historical epoch: The Plasticene (Campanale et al., 2020). Microplastics are plastic particles smaller than 5mm that are purposefully manufactured in this size (primary microplastics) or derived from the fragmentation of larger pieces of plastic (secondary microplastics) due to natural processes, such as photo-oxidation, hydrolysis, abrasion, and biodegradation. Microplastics are ubiquitous, have been reported in aquatic systems (Dris et al., 2015; Eriksen et al., 2014; Imhof et al., 2013), soil (Rillig, 2012), ground water (Mintenig et al., 2018), sewage and the atmosphere (Dris et al., 2015). In addition, studies have found microplastics in drinking water (Kosuth et al., 2018) and food destined for human consumption (Conti et al., 2020; Kosuth et al., 2018; Smith et al., 2018; Yang et al., 2015; Liebezeit and Liebezeit, 2015; 2013; Mathalon and Hill, 2014; Murray and Cowie, 2011).

Emerging evidence suggests that humans are exposed to microplastics mainly through diet and inhalation. Recently, microplastics were found in human stools (Schwabl et al., 2019), colectomy samples (Ibrahim et al., 2021) and placenta (Ragusa et al., 2021). However, microplastic exposure risk to humans is unresolved. A remarkable inter and intra-individual variability of microplastic abundance in washes from hand, face, hair and mouth suggests that human exposure to microplastics depends on the individual habits and environment (Abbasi and Turner, 2021). A recent study found that the dietary exposure to microplastics depends on sex and age, with an annual consumption ranging from 39,000 to 52,000 particles (Cox et al., 2019).

Mouse models have been used to elucidate the microplastic potential risk to mammalian health. Studies have shown that microplastics can translocate across the gut barrier into different organs, being found in the mouse gut, liver and kidney (Araújo and Malafaia, 2021;

Deng et al., 2018, 2017; Jin et al., 2018a). Some studies have shown that microplastics can lead to intestinal homeostasis disturbance, as revealed by gut inflammation, oxidative stress, reduced mucus secretion, increased intestinal permeability and microbiota dysbiosis (Jin et al., 2018a; Li et al., 2020; Lu et al., 2018; Luo et al., 2019a). Neurotoxic effects have also been observed in mice exposed to microplastics, as indicated by inhibition of acetylcholinesterase activity (Deng et al., 2017) and behavioural disorders (Araújo and Malafaia, 2021). Microplastic exposure caused changes in serum and hepatic metabolite levels, suggesting lipid, amino acid and energy metabolism disorders (Deng et al., 2017; Jin et al., 2018a; Lu et al., 2018); some of these adverse effects persisted across generations (Luo et al., 2019b, 2019a). Additionally, microplastics caused reproductive impairment in male mice, as shown by testis inflammation, reduced serum testosterone and decrease in sperm cell quality (B. Hou et al., 2021; Jin et al., 2021; Xie et al., 2020).

In the environment, microplastics differ in shape, size and composition; this is not always reflected in laboratory studies. Microplastics can be subcategorized according to their shape and includes beads, nurdles, fibers, foam, fragments, pellets, films, filaments, cylindrical, disk, flat, ovoid, spheruloid, elongated, rounded, and irregular. (Hirt and Body-Malapel, 2020). While spherical particles (microbeads) are most commonly used in laboratory studies, fibers and fragments are the most common types detected in field samples (de Sá et al., 2018). Filaments or fibers accounted for 96.1% of microplastics found in human colon samples (Ibrahim et al., 2021) while fragments and films were the most abundant in stool samples (Schwabl et al., 2019). Spherical and irregular shapes were predominant in human placenta samples (Ragusa et al., 2021).

Although in the natural environment organisms are exposed to different types of microplastics, their toxicity is more dependent on their size and shape than their chemical

composition (Lei et al., 2018b), with fibers showing greater adverse effects in comparison to beads. Waterflea (*Ceriodaphnia dubia*) exposed to PET microfibers showed a more pronounced reduction in growth and brood size along with body deformations compared with PE microbead exposure (Ziajahromi et al., 2017). Similarly, PP microfiber exposure caused higher mortality, slower egestion time and reduced growth compared with PE microbead exposure in amphipod (*Hyalella azteca*) (Au et al., 2015). Secondary irregular shaped microplastics caused elevated mortality, decreased reproduction and longer egestion time compared with microbeads in *Daphnia magna* (Ogonowski et al., 2016). It should be noted that the microfiber adverse impacts reported in small zooplankton might be related to the physical stress promoted by entanglement rather than ingestion (Ziajahromi et al., 2017). In zebrafish, however, PP microfibers had higher tissue accumulation and caused more intestinal damage than PS microbeads and PS fragments. Additionally, microfibers caused body weight loss and gut dysbiosis, along with stronger oxidative stress and higher IL-1α level compared with microbeads and fragments (Qiao et al., 2019a). Rounded shapes might be transported through the gut more easily while non-spherical particles have a longer gut passage and may be easily embedded in the tissues (Au et al., 2015; Qiao et al., 2019a).

Considering the knowledge gap concerning the impact of different shapes of microplastics on vertebrate species, particularly mammals, the aim of the present study was to evaluate the health risks posed by microbeads (MB) and microfibers (MF) using a mouse model. BALB/c female mice were exposed to MB or MF via diet and sacrificed at three different time points: after two weeks of exposure, after four weeks of exposure and after four weeks of exposure followed by a four-week recovery period. We monitored food consumption, body weight, gene expression and tissue distribution, accumulation, and associated histopathology. Our hypothesis is that microplastics will cross the intestinal barrier and reach other organs triggering an immune response. The expression levels of genes related to inflammation (*Apcs*,

Cfb, Crp) and tissue repair (*Ccn1*) are expected to be high after microplastic exposure and return to normal levels after the recovery period. If there is a difference between MB and MF, we hypothesize that MF will cause more aggravated outcomes.

2.3 Methods

Care of animals

Five to six-week old female BALB/c mice (n=27) were ordered from Charles River Laboratories (Pointe-Claire, Quebec, Canada). The mice weighed approximately 20 grams. The animals were housed in the Animal Care Facility, Brandon University, Brandon, Canada, on a 12-hour light/dark period with food and water provided *ad libitum*. Each cage housed three mice. Temperature and relative humidity were maintained between 20°C - 25°C and 50% - 70%, respectively. The cages used were clear polycarbonate with stainless steel lids. The composition of the bedding used for nesting was corn cob and natural paper. Paper rolls were used for environmental enrichment. Upon arrival, the mice were acclimated for two weeks before beginning the experiment to ensure there were no signs of stress or illness. The animals were randomly divided into three groups: nine were assigned to the control group, nine were assigned to the microbead (MB)-treated group, and nine were assigned to the microfiber (MF)-treated group. The cages were cleaned once a week, when the feces were collected and the mice were weighed. Mice were anesthetized with 5% isoflurane until unconscious and then immediately euthanized with CO_2 at a flow rate of 20% chamber air displacement per minute. The animal care protocol was approved by the Brandon University Animal Care Committee (BUACC) in accordance with guidelines of the Canadian Council on Animal Care (BUACC Certificate #2019R04-1).

Microplastic exposure and sampling

Fluorescent red polyethylene microbeads (PE MB) were purchased from Cospheric (UVPMS-BR-0.995, size range: 10 - 45 µm, mean diameter: 36.4µm, density: 0.995g/cc,

excitation: 300-550 nm, peak emission of 607 nm when excited at 575nm). The source of microfibers (MF) was a red felt fabric composed by 15% acrylic and 85% of a polyester fiber made from recycled plastic bottles (Ecofi®, size range: 36.20 - 2953.33µm, density: 0.213g/cc). The fabric was cut into 2 x 2 cm pieces and rinsed with deionized (DI) water and 96% ethanol to remove possible surface contamination. The material was dried and cut into smaller pieces and then placed in a Petri dish with DI water to allow the fibers to spread. The coarse parts were removed from the dish and the remaining floating MF were filtered, dried and stored until use. MF were counted and measured using Nikon Eclipse T*i* inverted microscope with a Nikon DS-Ri1 camera and NIS-Elements Advanced Research Software (version 4.13.00). The MF mean length was 609.06± 413.98 µm ranging from 36.20µm to 2953.33µm (Figure 2-1).

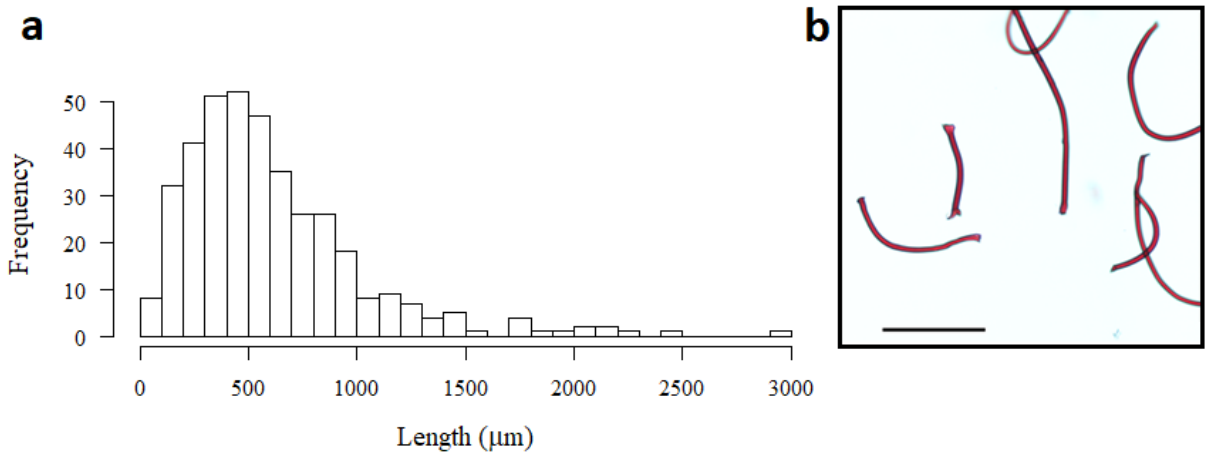


Figure 2-1: Microfiber length.
Felt fabric was manually cut, washed and dried. The MF mean length was 609.06 ± 413.98µm and size range 36.20 - 2953.33µm. **a)** MF length histogram. **b)** MF photomicrograph, scale bar = 250µm.

The mice were exposed to microplastics via food intake. Commercial mouse feed (LabDiet Rodent 5001) was ground and mixed with microplastics at a concentration of 100µg/Kg, except for the control food that was microplastic-free. It is worth mentioning that we chose to

use the same mass/mass concentration for both microplastic exposures (100µg/Kg), however the number of microplastic particles differed between MB and MF as they have different sizes and densities (approximately 3,960 MB particles/Kg and 150 MF particles/Kg). After mixing the microplastics with the ground feed, distilled water was added until the dough became smooth and uniform (approximately 1L of water to 1Kg of food). The dough was cut into a square shape and dried for 48 hours at 45°C.

The experimental procedure was conducted as outlined in the Figure 2-2. The animals were randomly divided into three groups: nine were assigned to the control group, nine were assigned to the MB-treated group and nine were assigned to the MF-treated group. Upon arrival, the mice were fed microplastic-free food and the experiment began after two weeks of acclimation. After two weeks of exposure, three mice from each group were sacrificed and samples collected. To sacrifice the animals, they were first sedated and anaesthetized with isoflurane gas, followed by carbon dioxide euthanasia. Kidney, liver, spleen, small and large intestines were harvested. Another three mice from each group were sacrificed after four weeks of exposure. At this point, all the remaining mice were fed microplastic-free food and were sacrificed after four weeks (recovery period).

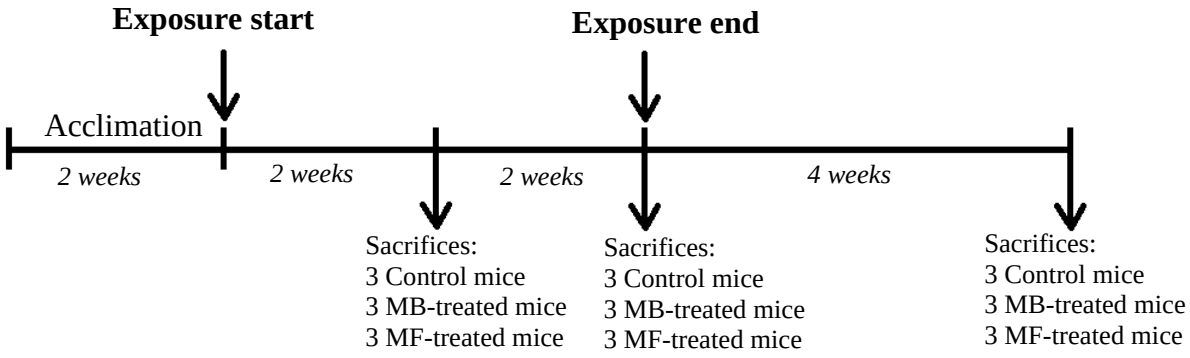


Figure 2-2: Experimental design.
Microplastic exposure began after two weeks of acclimation. The sacrifices occurred after two and four weeks of exposure. The exposure was terminated and all remaining mice were fed microplastic-free food and sacrificed after the four-week recovery period.

Microplastic intake and egestion

The microplastic intake and egestion were estimated by the amount of ingested food and the number of microplastics present in the feces, respectively. Feces were collected from each cage once a week and separated from the bedding using test sieves. Feces were weighed and reconstituted in DI water (0.3g of feces in 150mL of DI water). The homogenate was filtered through a Whatman® qualitative filter paper (grade 595, pore size: 4 -7µm) using vacuum filtration (Millipore® vacuum pump). The filter papers were placed in a Petri dish and all MB or MF particles were counted with the use of an Olympus MVX10 stereomicroscope with X-Cite® 120 fluorescence mercury lamp and U-MRFPHQ/XL mirror unit (excitation: 545 +/- 10nm, emission: 597.5 +/- 27.5nm, dichroic mirror: 565nm). All samples were analyzed in 3 replicates.

Food consumption and body weight

Food and water were provided *at libitum* and the amount of food in the cages was measured daily. When the amount of food in each cage was low (around 30 grams), more food was added to completely fill the feed hopper (approximately 75 grams). Food was added to all cages at the same time to prevent differences in food consumption that could be caused by the better palatability of fresh food. Daily food consumption was calculated by the difference in the amount of food left in the cage from the previous day, e.g. food consumed on Day 1 = amount of food left in the cage on Day 1 – amount of food left in the cage on Day 2 (all measurements were taken around 8:00am). The individual mice were weighed weekly.

Histological analysis and tissue accumulation

Samples from liver, kidney, spleen, small and large intestines were collected from control and experimental groups, fixed in 10% formalin, embedded in paraffin wax, sectioned at 5µm thickness, and stained with hematoxylin and eosin (H&E) for microscopic observation (Appendix 1). The "Swiss roll" technique as described by Moolenbeek and Ruitenberg (1981) was performed for the small and large intestines.

As a section is a two-dimensional image taken from a three-dimensional object (Altunkaynak et al., 2012), the sections were chosen in a way that different depths of the tissues were represented. The analyzed sections were cut 10-60µm apart (depending on the thickness of the whole tissue) until the whole embedded organ had been sampled. At least 50 sections were analyzed per tissue of each mouse.

The histological sections were analyzed using a Nikon Eclipse T*i* inverted microscope and the photomicrographs were captured using a Nikon DS-Ri1 camera and NIS-Elements Advanced Research Software. In order to detect the MB fluorescence, the sections from MB-treated group were analyzed using an Olympus BX51 microscope with X-Cite® 120 fluorescence mercury lamp and TRITC filter cube (excitation: 543 +/- 11nm, emission: 591.5 +/- 21.5nm, dichroic mirror: 562nm). The MB photomicrographs were captured using an Olympus SC100 camera and cellSens Imaging software.

Gene expression analysis

- RNA extraction

Liver samples were harvested and immediately placed in 2mL cryotubes containing RNA*later*™ RNA Stabilization Solution (Invitrogen, Thermo Fisher Scientific, Lithuania). Samples were incubated overnight at 2-8°C and stored at -80°C until RNA extraction. Approximately 100mg of tissue was used for total RNA extraction with TRIzol® Reagent (Ambion, Life Technologies, USA) according to the manufacturer's protocol (Appendix 2). The extracted total RNA was eluted with nuclease-free water and the concentration and quality were assessed with a NP80 NanoPhotometer (Implen, Munich, Germany) using nuclease-free water as a blank. Samples with optical density ratios of 260/280nm close to 2.0 and concentrations above 500ng/µl were considered acceptable for downstream application (Matlock, 2015). The extracted total RNA samples were aliquoted and stored at -80°C until reverse transcription (cDNA synthesis).

- Reverse Transcription

Following nuclease digest treatment with DNase I, total RNA (2,000 ng) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, Vilnius, Lithuania) (Appendix 3). Quantity and quality of transcribed cDNA samples were measured using a NP80 NanoPhotometer (Implen, Munich, Germany) using a solution of RT buffer and nuclease-free water as a blank. Samples with 260/280 ratio between 1.6 - 1.8 and concentration above 500ng/µl were considered acceptable for downstream application. Transcribed cDNA was aliquoted and stored at -20°C until qPCR.

- Quantitative Real-Time PCR (qPCR)

Gene expression analysis was performed using commercially available gene-specific TaqMan probes (Applied Biosystems, Pleasanton, CA, USA).

Table 2-1: TaqMan probe details

Gene name	Gene assay	Amplicon length	PCR setting	Expression sites
Complement Factor B (*Cfb*)	Mm00433909_m1	85	Hold: 95°C 10min Cycle: (x50) 95°C 15sec 58-60°C 60sec	Mainly in the liver, followed by placenta, kidney, large intestine, mammary glands, small intestine, bladder, spleen, lung, ovary, etc.
Cellular Communication Network Factor 1 (*Ccn1*)	Mm00487498_m1	112		Broad expression including lung, limb, bladder, placenta, ovary, heart, spleen, stomach, intestines, CNS, liver, thymus, etc.
C-Reactive Protein (*Crp*)	Mm00432689_g1	134		Liver
Serum Amyloid P-Component (*Apcs*)	Mm00488099_g1	114		Liver
18S ribosomal RNA (*18s*)	Mm04277571_s1	115		Ubiquitous
Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)	Mm99999915_g1	109		Ubiquitous

For qPCR, cDNA samples were diluted in nuclease-free water (1:80). The qPCR was performed according to the manufacturer's instructions with a reaction final volume of 20µl (forward and reverse primer concentration: 900nM, probe concentration: 250nM, and 4µL of cDNA) (Appendix 4). The amplification efficiency was determined by constructing standard curves for each TaqMan assay using a 5-point 2-fold dilution series of pooled cDNA templates. Efficiencies between 90-105% and R^2 above 0.985 were considered acceptable. All samples and non-template control (NTC) were run in duplicate and 3 independent runs were performed for each assay. Replicates from the same run were less than 0.5 cycle apart and replicates from different runs were less than 1.0 cycle apart.

The cycle threshold (Ct) values were calculated using the Corbett Rotor-Gene 6000 (Qiagen) software using the corresponding standard curve following manufacturer's instructions.

Relative gene expression ratio was calculated according to the ΔΔCt method (Livak and Schmittgen, 2001) using the geometric mean of two housekeeping genes (*18s* and *Gapdh*) as normalizers.

Statistical analysis

Food consumption and body weight data were tested using two-way repeated measures ANOVA (one factor repetition) and multiple comparisons versus control group (Holm-Sidak method) was used to isolate the group that differed from the others. Gene expression data were tested using one-way ANOVA. All tests were performed using SigmaPlot 11.0 at a significance level of $p \leq 0.05$ after passing normality and equal variance tests.

2.4 Results

Food consumption and body weight

Food consumption was measured by cage daily - each cage housed three mice. The results of the two-way repeated measures ANOVA revealed that there was no significant main effect of microplastic exposure on food consumption (df=2, p=0.089). However, there was a significant interaction between treatment (control, MB, MF) and time (days 1 to 56) (df=110, p<0.001), i.e. the effect of MB or MF microplastic exposure on food consumption depends on the day. Multiple comparisons versus control group (Holm-Sidak method) showed significant difference on days 13, 20, 45 and 51 for MB-treated mice, on day 7 for MF-treated mice and on days 4, 5, 8, 31, and 40 for both MB and MF-treated mice compared to the control group (marked with * in the graph – see Figure 2-3a). There was no consistency if the consumption was increased or decreased compared with the control group.

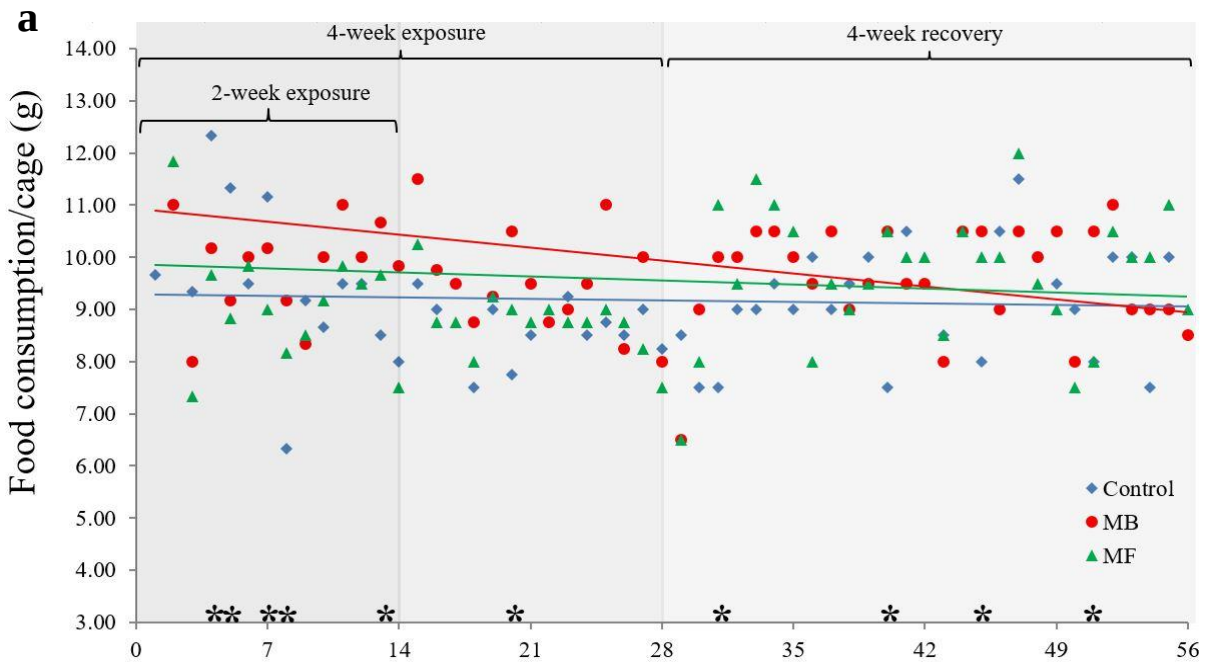
Individual mice were weighed weekly. Two-way repeated measures ANOVA analysis revealed that there was a statistically significant effect of time on body weight (df=7, p<0.001), however it was due to the mouse expected growth during puberty phase. There was no statistically significant effect of microplastic exposure on body weight (df=2, p=0.143) and there was no statistically significant interaction between treatment (control, MB, MF) and time (weeks 1 to 8) (df=14, p=0.062) (Figure 2-3b).

Microplastic intake and egestion

Food consumption was measured by cage daily. On average, MB and MF-treated cages consumed 10.25g/day and 9.46g/day, respectively. As each cage housed three mice, each MB-treated mouse consumed 3.42g/day and each MF-treated mouse consumed 3.15g/day,

assuming equal food consumption. As the microplastic concentration in the mouse food was 3,960MB/Kg or 150MF/Kg, each mouse was exposed to approximately 13.53 MB/day or 0.47 MF/day.

Microplastic egestion was estimated counting the number of MB or MF present in the feces. Both MB and MF egested in the feces did not show any sign of deterioration (Figure 2-4). Both MB and MF-treated mice were able to egest microplastics within the first week of exposure. There was a significant decrease in the amount of microplastics in feces after the fourth week, when the exposure was terminated for both MB and MF-treated cages ($df=7$, $p=0.003$). MB-treated mice continued to eliminate MB until the eighth week, while no MF was visualized in MF-treated mouse feces in week 3 and in the recovery period (Figure 2-3c).


a
4-week exposure
4-week recovery
2-week exposure
Food consumption/cage (g)
14.00
13.00
12.00
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
0
7
14
21
28
35
42
49
56
Control
MB
MF
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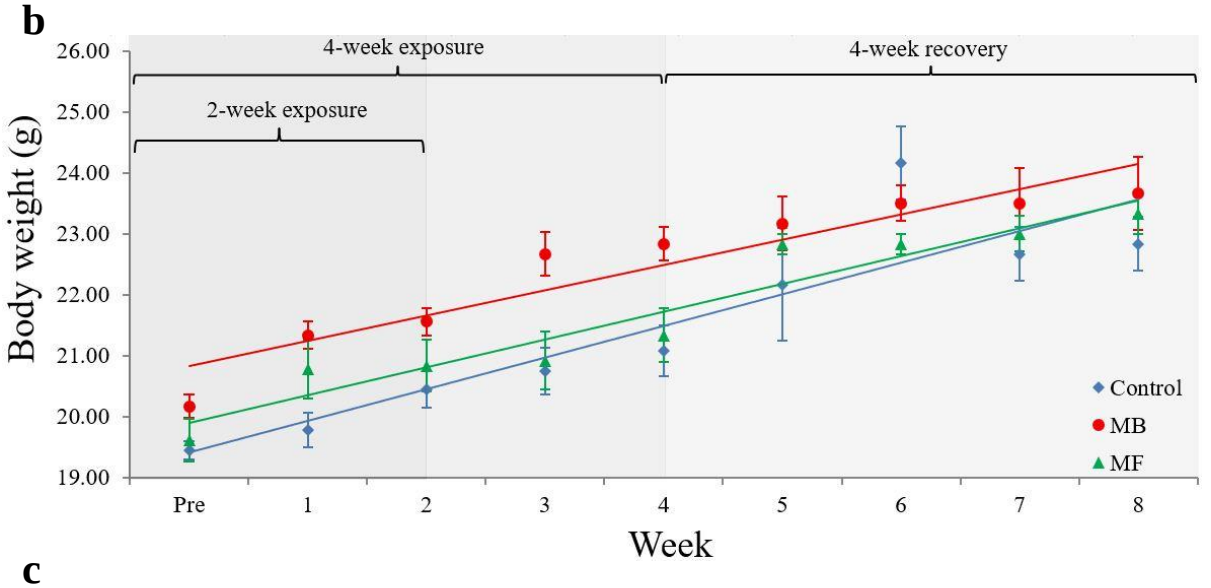

b
4-week exposure
4-week recovery
2-week exposure
Body weight (g)
26.00
25.00
24.00
23.00
22.00
21.00
20.00
19.00
Pre
1
2
3
4
5
6
7
8
Week
Control
MB
MF

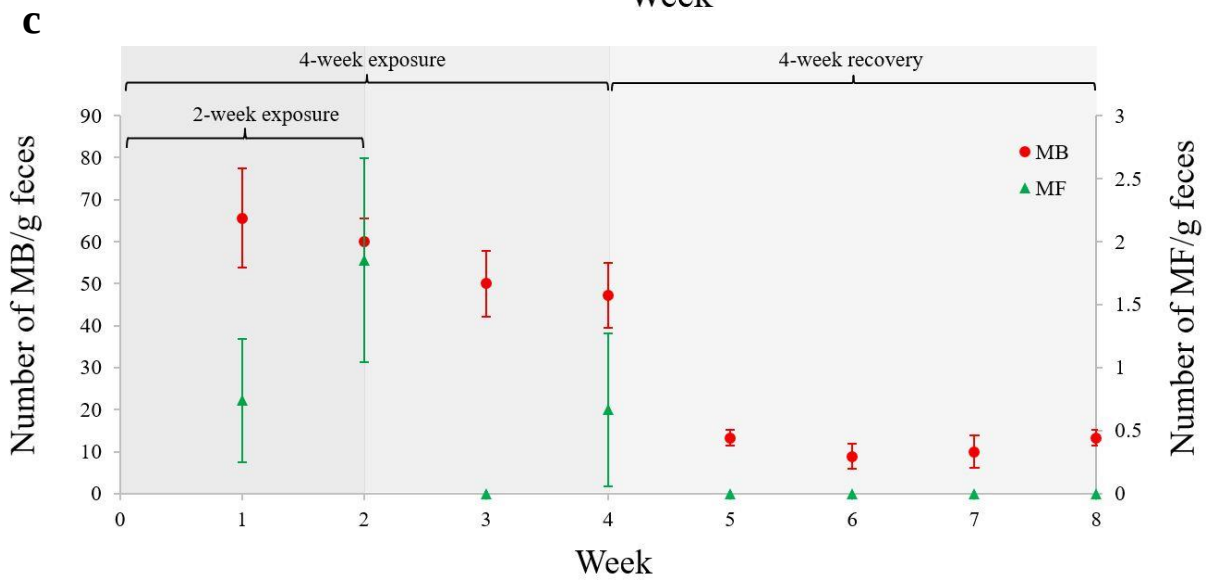

c
4-week exposure
4-week recovery
2-week exposure
Number of MB/g feces
90
80
70
60
50
40
30
20
10
0
Number of MF/g feces
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
8
Week
MB
MF

Figure 2-3: Food consumption, body weight and microplastic egestion.
a) The presented values are the average food consumption per cage. Statistically significant difference was observed on days 13, 20, 45 and 51 for MB-treated group, on day 7 for MF-treated group and on days 4, 5, 8, 31 and 40 for both MB and MF-treated groups compared to the control group (marked with *). Linear regression for control group: $y = -0.0041x + 9.281$ $R^2 = 0.0034$, MB group: $y = -0.0355x + 10.933$ $R^2 = 0.0565$, and MF group: $y = -0.0107x + 9.8547$ $R^2 = 0.0064$.
b) Individual mouse weight. The presented values are the mean±SEM. No statistically significant difference was observed between control and microplastic-treated groups. Linear regression for control group: $y = 0.5181x + 18.891$ $R^2 = 0.816$, MB group: $y = 0.4148x + 20.414$ $R^2 = 0.8869$, and MF group: $y = 0.4579x + 19.43$ $R^2 = 0.9224$. "Pre" refers to the body weight before exposure.
c) Microplastic egestion. MB and MF in feces were counted using a stereo microscope. The presented values are the mean±SEM amount of microplastics found in 1g of feces (each sample was run in triplicate). There was a sharp drop after the fourth week, when the exposure was terminated.

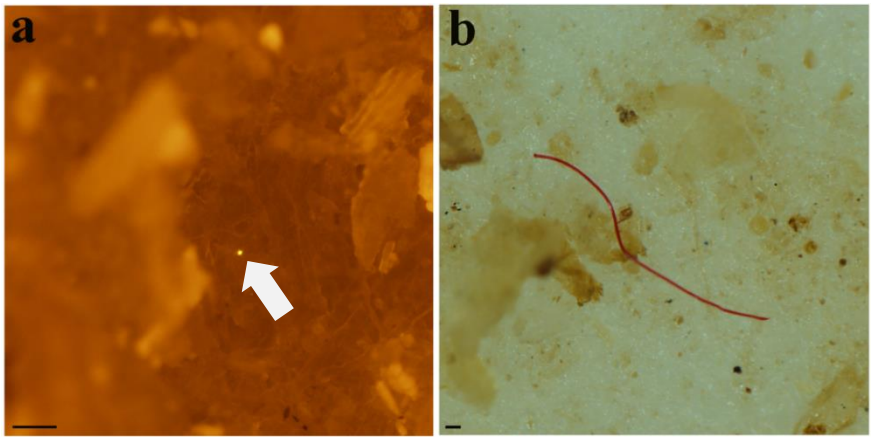


Figure 2-4: Microplastics in feces.
Feces were reconstituted in DI water and filtered through a filter paper with pore size of 4-7µm (Whatman®). The filters were analyzed using a stereo microscope. **a)** Fluorescent red PE MB and **b)** red polyester MF found in the feces. Scale bar = 100µm.

Histological analysis and tissue accumulation

The kidney, liver, spleen and small and large intestine were harvested for histological analysis. The H&E staining showed normal cellular morphology with no signs of inflammation in all tissues analyzed after two and four weeks of exposure and recovery period. There was no evidence of necrosis or immune cell infiltration in control mice as well as microplastic-treated mice (Figure 2-5).

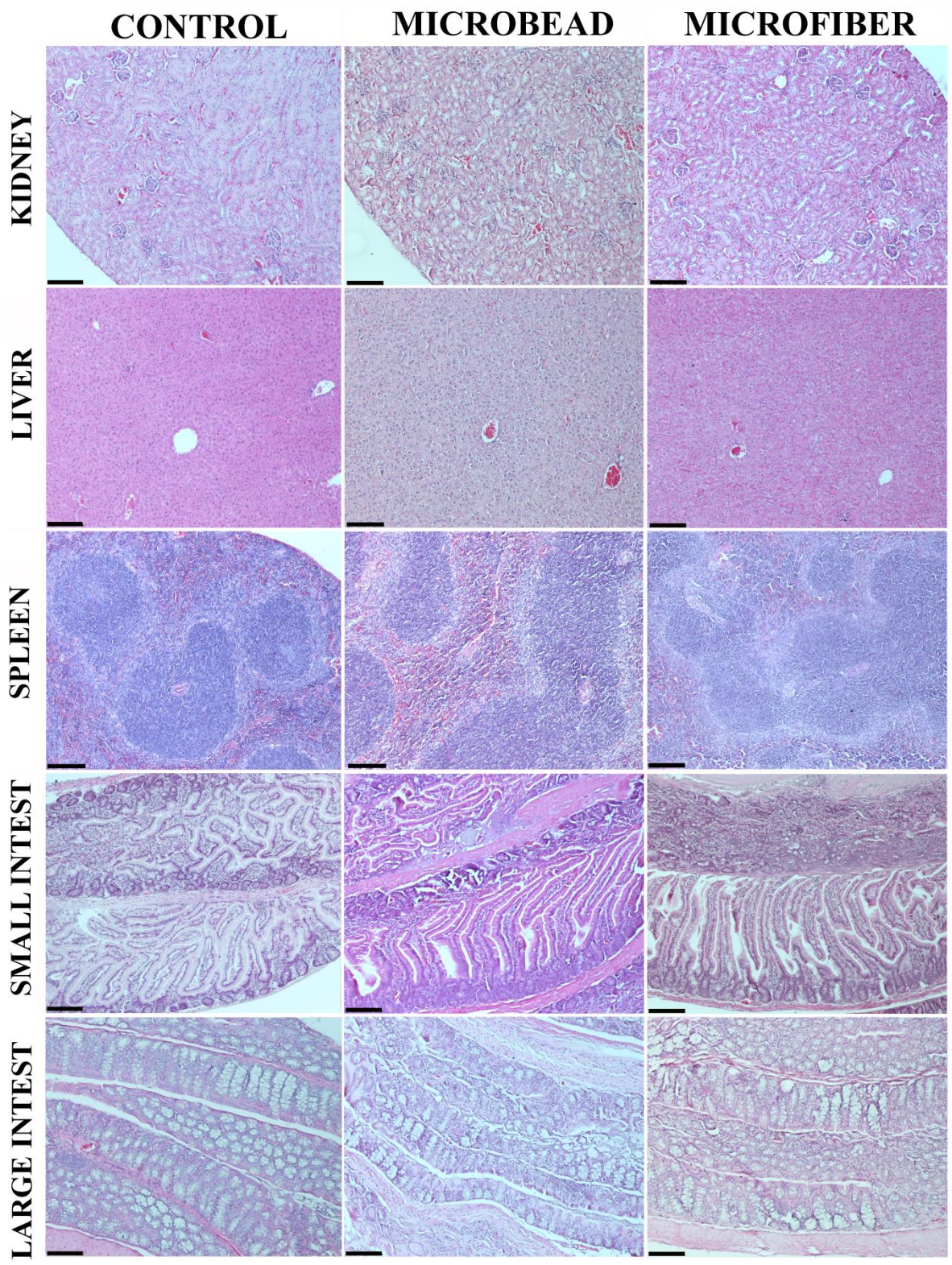


Figure 2-5: Histological analysis after microplastic exposure.
Representative photomicrographs of H&E stained kidney, liver, spleen, small and large intestines of control, MB and MF-treated mice. There are no histological alterations. Scale bar = 100µm.

No MF was found in MF-treated mouse tissues, while two MB particles were found in the small intestine after four weeks of exposure and one MB particle was found in the liver after the four-week recovery period (Figure 2-6).

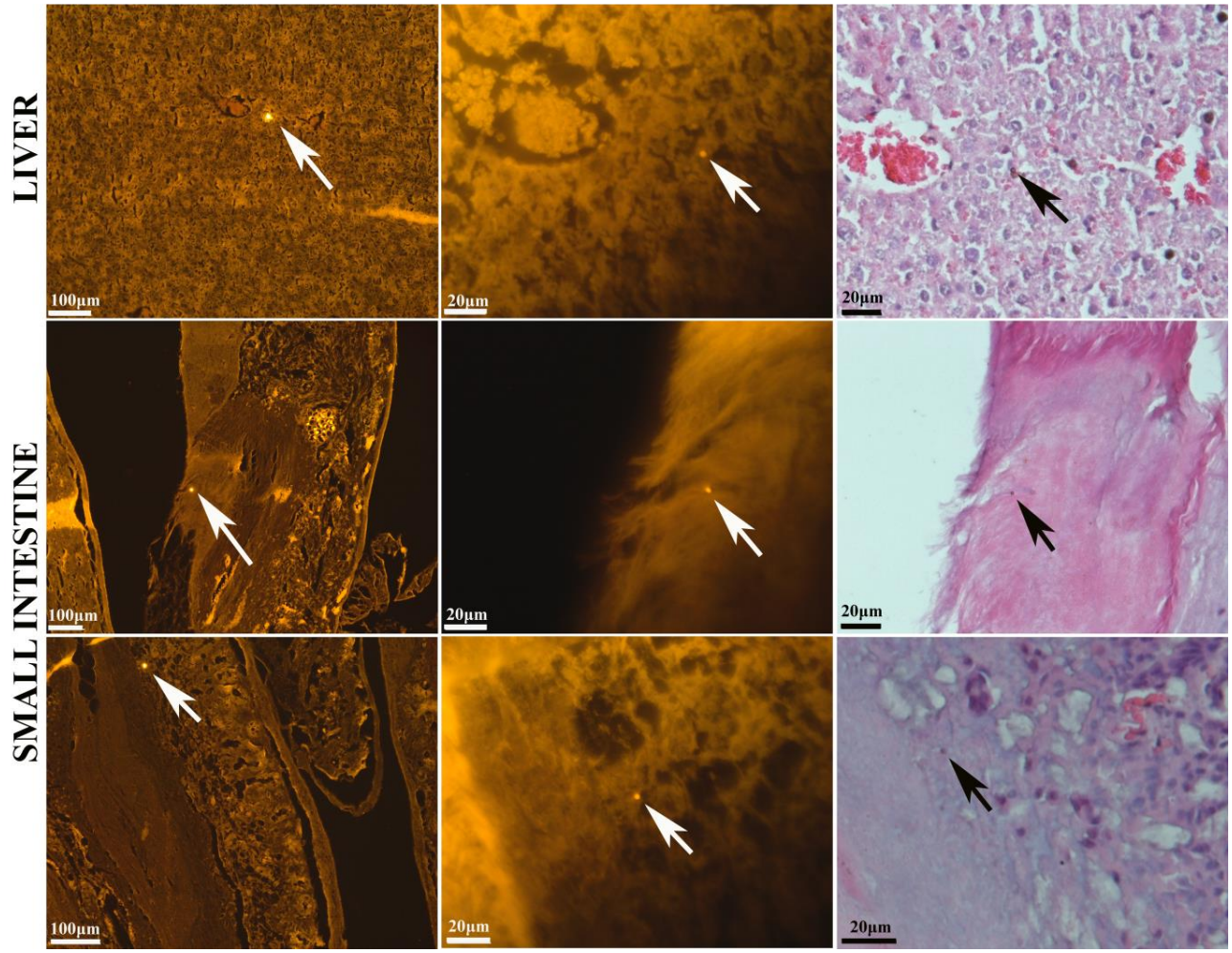
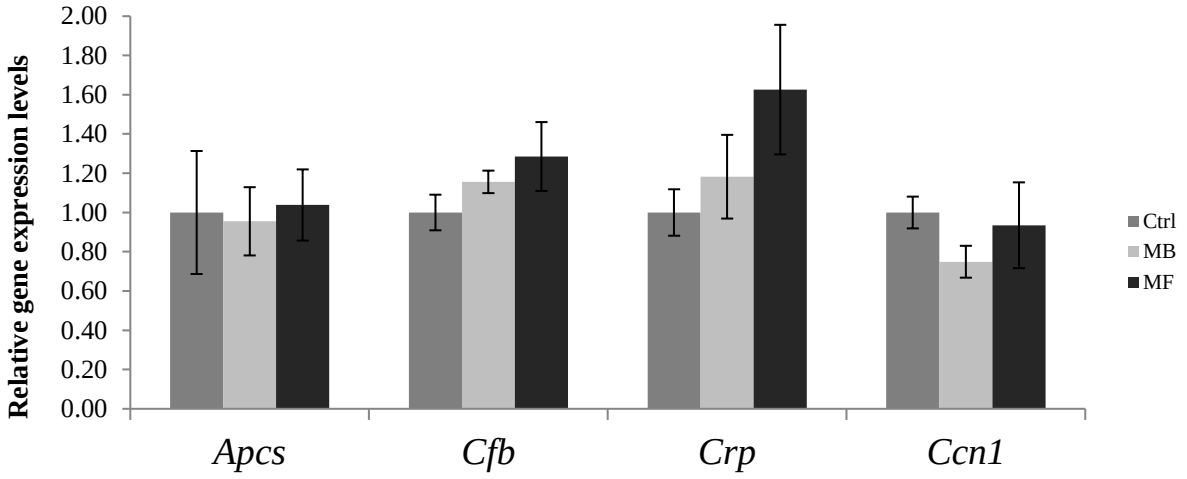


Figure 2-6: MB found in mouse tissues.
Fluorescent red PE MB in the liver (recovery period) and small intestine (4-week exposure) of MB-treated mice. Left column: 20x objective (scale bar = 100µm), middle and right columns: 40x objective (scale bar = 20µm).

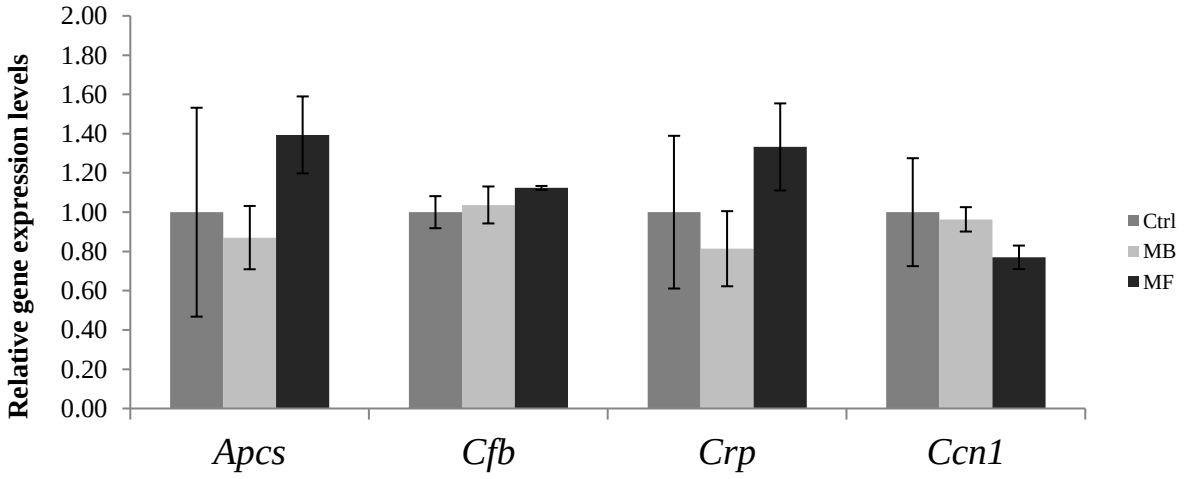
Gene expression analysis

The expression level of genes related to inflammation (*Apcs, Cfb, Crp*) and tissue injury repair (*Ccn1*) was measured in the liver. Although the expression pattern of microplastic-treated groups differed from the control group, the difference was not statistically significant ($p>0.05$) (Figure 2-7).

a



b



c

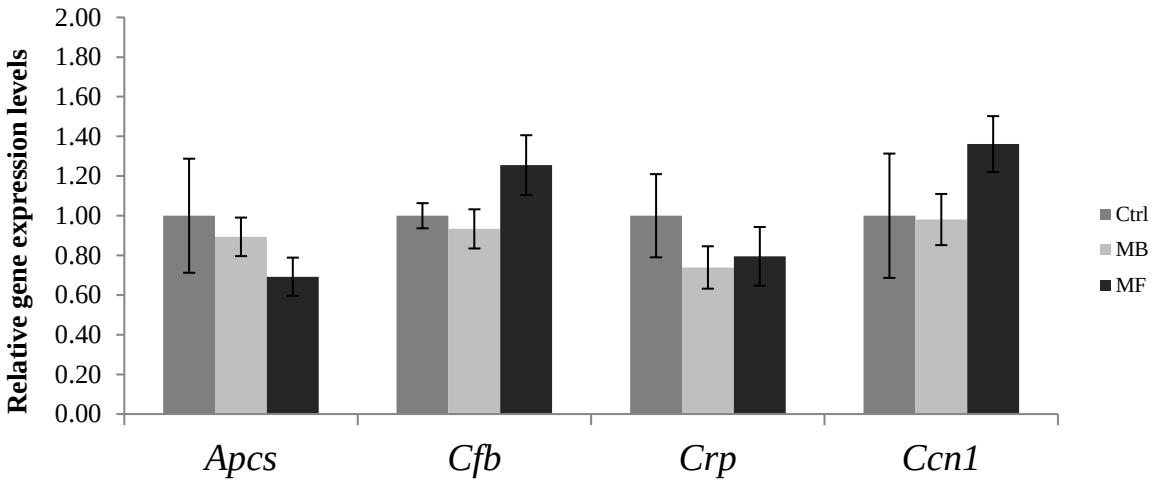


Figure 2-7: Gene expression in the liver.
Apcs, *Cfb*, *Crp* and *Ccn1* gene expression profiles of control (Ctrl), MB and MF-treated groups after **a)** two weeks of exposure, **b)** four weeks of exposure, and **c)** recovery period. *18s* and *Gapdh* were used as normalizer genes. Mean ± SEM (n=3). No statistically significant differences were observed.

2.5 Discussion

Microplastics are a pollutant of emerging concern, being reported in the atmosphere, drinking water and food destined for human consumption (Wright and Kelly, 2017). Although recent evidence suggests that humans are exposed to microplastics, their fate and effects on the human body are still controversial and uncertain (Campanale et al., 2020). Studies have shown that particles smaller than 150µm might translocate from the gut into the lymph and circulatory system, causing systemic exposure (Barboza et al., 2018). The adverse effects on organisms exposed to microplastics might be caused by the physical or chemical microplastic properties. Physical damage is associated with microplastic shape, size, and concentration while chemical damage is caused by microplastic monomers and additives as well as the environmental chemicals they adsorb on their surface (Campanale et al., 2020).

The microplastic effects on feeding activity and growth depend on the biology, physiology and behaviour of organisms. Species with lower energy demand might be less vulnerable to low-calorie diet and species able to modulate food intake can avoid plastic-contaminated food (Watts et al., 2015). Microplastics have been thought to cause false satiation, damage and blockage of the intestinal tract that could lead to reduced feeding activity and growth. On the other hand, organisms might show a compensatory response to non-nutritious plastic content in the food and temporarily increase feeding activity (Watts et al., 2015). Crabs fed PP microfibers showed reduced food consumption and a significant reduction in energy available for growth (Watts et al., 2015). Similarly, lugworms showed reduced feeding activity and depleted energy reserves after exposure to PVC microparticles (Wright et al., 2013).

In the present study, change in food consumption was observed on days 13, 20, 45 and 51 for MB-treated mice, on day 7 for MF-treated mice and on days 4, 5, 8, 31, and 40 for both MB and MF-treated mice compared with the control group. However, microplastic exposure did

not affect body weight. In contrast, a significant loss of body weight was observed in male mice after PS (Jin et al., 2021; Lu et al., 2018; Xie et al., 2020) and PE microplastic exposure (Park et al., 2020) with or without changes in food/water consumption. Other researchers have reported no effect on body weight and food consumption in male rats (Rafiee et al., 2018) and male mice (Deng et al., 2017; Stock et al., 2019) after PS exposure. These contradictory results could be explained by differences in experimental design, such as microplastic characteristics and route of administration. Most of the studies that reported a significant reduction in body weight used PS microplastics and an extremely high dose administered by oral gavage.

Both MB and MF-treated mice were able to egest microplastics in feces within the first week of exposure. MF were found in MF-treated mouse feces during the exposure phase, except for week 3, and no MF were found during the recovery period. On the other hand, MB were found in MB-treated mouse feces throughout the experiment although in a lower concentration during recovery period. This finding suggests that MB might be retained in the gastrointestinal tract for a longer time than MF, which could be explained by the higher MB concentration exposure and/or by the MB properties, such as smaller size and spherical shape. In contrast, microplastic beads were egested more rapidly than microfibers and irregular shaped microplastics in amphipods and *Daphnia*, respectively (Au et al., 2015; Ogonowski et al., 2016). Plastic microspheres were quickly egested by *Xenopus* tadpoles (Hu et al., 2016), sea bass larvae (Mazurais et al., 2015) and *Daphnia* (Rosenkranz et al., 2009) and were almost completely eliminated after 6 days, 2 days and 4 hours of depuration, respectively.

Under visual inspection, the egested microplastics showed no signs of deterioration or fragmentation, which confirms microplastic bioresistance, i.e. refractory to digestion or

dissolution. PP microfibers were significantly altered by the passage through crab foregut, being excreted with a smaller size and length (Watts et al., 2015). The different result may be due to the crab foregut complex gastric mill used for grinding carapace shells and animal and plant tissues (Watts et al., 2015). In humans, digestive enzymes and bile salts are thought to affect the color of microplastics, as the majority of the microfilaments found in human colectomy samples was transparent (Ibrahim et al., 2021) whereas all microplastics found in human placenta were pigmented (Ragusa et al., 2021).

Histological analysis revealed normal tissue morphology without noticeable histopathological signs and inflammatory response in the liver, kidney, spleen, and intestines in control, MB and MF-treated groups throughout the experiment (2-week and 4-week exposure and 4-week recovery period). Similarly mice exposed to PS microplastics had no histopathological changes in the above mentioned tissues (Stock et al., 2019) and rats exposed to PS nanoparticles showed no histological alterations in the liver, kidney, spleen, lung, brain, testes, stomach, small and large intestines (Walczak et al., 2015). Zebrafish showed no histological changes in the gills, liver, brain, kidney and intestines after exposure to PE micro fragments (Karami et al., 2017) and PS microbeads (Lei et al., 2018b). Conversely, zebrafish showed intestinal injury, such as epithelial damage, thinning of the gut wall and congestive inflammation (Qiao et al., 2019b) as well as cracking of villi and splitting of enterocytes (Lei et al., 2018b) after exposure to different types of microplastics. In mice, histological alterations have been reported in the gut, liver and testes. PE microplastic exposure caused intestinal damage and inflammation, as indicated by edema, loose glands, and infiltration of lymphocytes and plasma cells into the lamina propria (Li et al., 2020). Accumulation of lipid droplets and ballooning degeneration of hepatocytes were observed in mouse liver after PS microplastic exposure (Deng et al., 2017; Luo et al., 2019a). Additionally, PS microplastic exposure caused histopathological changes in mouse testicular tissue, as shown by disordered

arrangement of spermatid cells, pyknosis, nuclear rupture, cell detachment, and reduced spermatogenic cell number (B. Hou et al., 2021; Xie et al., 2020).

Researches have shown that microplastics can cross the intestinal barrier and accumulate in different organs (Araújo and Malafaia, 2021; Deng et al., 2018, 2017; Jin et al., 2021). Recent studies have found that different sizes of microplastics (<40µm) can accumulate in mouse gut wall, liver, kidney and testes (Araújo and Malafaia, 2021; Deng et al., 2017; Jin et al., 2021, 2018a; Stock et al., 2019). In the present study, histological sections were analyzed to assess microplastic tissue distribution and accumulation. Only two MB particles were found in the small intestine after four weeks of exposure and one MB particle was observed in the liver after the recovery period. No MF was observed in the MF-treated mouse tissues. Studies have confirmed that only particles smaller than 150µm can translocate across the intestinal epithelial barrier and only a small fraction is expected to penetrate deeply into organs (Barboza et al., 2018). This could explain the absence of MF and the small number of MB found in the tissues, as the MF average length used in this study exceeds the size limit. The estimate bioavailability of PS nanoplastics (50nm) in rats ranged from 0.2 – 1.7% of the administered dose, being found mostly in the stomach and intestines, followed by kidney, spleen, testes, heart and lungs (Walczak et al., 2015).

It is noteworthy that in the present study MB persisted in the liver after four weeks of recovery, confirming the plastic biopersistence, i.e. ability to escape clearance mechanisms and reside in the tissues indefinitely. It is also important to note that although MF were not able to cross the gut barrier, they might have local effects on the gut lumen. MF could be retained in the mucous layer lining the gastrointestinal tract and interfere with the gut homeostasis, leading to microbiota dysbiosis, epithelial disruption, oxidative stress and local inflammatory response (Hirt and Body-Malapel, 2020). In adult zebrafish, microplastic fibers

caused more severe intestinal toxicity than microplastic beads and fragments, as demonstrated by increased histological damage, stronger oxidative stress and more pronounced decline in mucus secretion in the gut (Qiao et al., 2019a). In addition, cellulosic and plastic fibers greater than 250μm have been found in human lung specimens and have been associated with acute and chronic inflammation that can cause a range of respiratory diseases (Pauly et al., 1998).

In the present study, the expression of genes related to inflammation and tissue injury repair were measured in the liver. The alternative pathway of complement activation is independent of antibody-antigen interaction. Complement factor B (CFB) is unique to the alternative complement pathway and is part of the C3 and C5 convertase complex, which generates potent opsonins and anaphylatoxins, such as C3b and C5a (Owen et al., 2018). CRP and SAP are pattern recognition molecules produced by the liver and secreted into the blood to interact with pathogens and cell debris and promote their clearance by phagocytic cells (Mold et al., 2001). In addition, SAP can activate the classical complement cascade and interact with monocytes, neutrophils and macrophages to modulate the immune response and wound healing (Cox et al., 2014; Pilling and Gomer, 2018). CCN1 is a secreted matricellular protein that is involved in all tissue repair phases: inflammation, proliferation and maturation. CCN1 binds to specific integrin receptors on distinct cell types to promote clearance of apoptotic neutrophils by macrophages, induce cell proliferation and differentiation, promote angiogenesis and matrix remodeling (Kim et al., 2018). In the present study, gene expression of *Cfb, Crp, Apcs,* and *Ccn1* were measured in the liver. Although the expression pattern in microplastic-treated groups differed from the control group throughout the experiment, there was no statistically significant difference.

2.6 Conclusions

Microplastics have emerged as one of the most concerning environmental problems in recent years. A growing body of evidence indicates that humans are exposed mainly through diet and inhalation. Although a broad range of microplastic types is found in the environment, most studies have used manufactured single-sized microbeads to assess microplastic health risks. Moreover, researchers have used an extremely high microplastic concentration in an attempt to induce more aggravated outcomes. Although several studies have shown the harmful effects caused by microplastics, research comparing the effects of different shapes of microplastics is still lacking. In the present study, we used two different shapes of microplastics (beads and fibers) in a wide range of sizes and a realistic environmental concentration. We also included a recovery period in our experimental design in order to evaluate the reversibility of the adverse effects.

Microplastic exposure affected mouse food consumption however did not influence the body weight. Microbeads were able to cross the gut barrier and only a few particles accumulated in the liver and small intestine wall. Although microfibers were not able to cross the gut barrier due to their larger size, it is worth mentioning the potential local effects on the intestinal lumen. Microfibers could be retained in the mucous layer lining the gastrointestinal tract and interfere with the gut homeostasis, causing microbiota dysbiosis, oxidative damage and local inflammatory response. Microbeads remained in the liver even after the recovery period; however, the biological markers used in this study failed to indicate inflammation or tissue injury. Other researches using different biomarkers, however, have shown that microplastics affect the innate and adaptive immune systems, and cause cellular and tissue damage. Nevertheless, the toxicity mechanisms and defense against microplastics are still unclear.

Human exposure to microplastics is much more complex than the scenario simulated herein. Further research using different experimental designs are needed to better understand the microplastic effects on human health, such as long-term exposure, different microplastic shapes, sizes and chemical composition, and presence of contaminants or microorganisms adsorbed on the microplastic surface. Therefore, the study described in manuscript 2 compared the health effects on female and male mice exposed to microplastics for a longer period of time.

3- (In prep) Sex-based and time-dependent effects of microplastic exposure in mice

3.1 Chapter summary

Plastic pollution has been identified by the United Nations as the second most ominous threat to the global environment after climate change. Once in nature, plastics can be fragmented into smaller pieces giving rise to another problem: microplastics. Despite substantial evidence suggesting that humans are exposed to microplastics mainly through diet and inhalation, little is known about the fate and effects of these particles on mammals. Here we investigated the effects of polyethylene microplastics on mammalian health, using a mouse model. To do this, female and male mice were fed food containing microplastics for four and sixteen weeks and a third group was allowed an additional four-week recovery period to assess the reversibility of the potential effects. Microplastics were able to translocate from the intestinal lumen into different organs of both female and male mice and remained embedded in the tissues even after the recovery period, confirming plastic biopersistence. Although no histopathological signs were observed in the tissues, female mice showed altered mRNA levels of genes related to inflammation and tissue injury repair. Overall, longer exposure to microplastics caused more pronounced effects than short exposure, and some of these effects were reversed after the recovery period. The present study shed light on the often-overlooked sex differences in the response to microplastic exposure. Further research is warranted to better understand the effects of exposure times, particularly long-term impacts, and the use of female and male models is imperative to translate experimental findings to human populations.

3.2 Introduction

Plastics are ubiquitous in both terrestrial and aquatic ecosystems, with an estimated 250 million metric tons entering the oceans by 2025 (Wright and Kelly, 2017). Microplastics come from a variety of sources, including manufactured small plastic particles designed for commercial use (primary microplastics) and larger plastic debris that degrades into smaller pieces due to natural processes (secondary microplastics). Although a modern wastewater treatment plant can remove up to 98% of the microplastics in the effluent, it is estimated that 65 million microplastic particles are still released into the receiving water every day (Murphy et al., 2016). In addition, microplastics are concentrated in the sludge subsequently used as fertilizer thus contaminating the soil (Hirt and Body-Malapel, 2020).

Emerging evidence suggests that human exposure to microplastics occurs mainly through diet and inhalation. Although the effects on human health are uncertain, recent studies have found microplastics in human tissues (Ibrahim et al., 2021; Ragusa et al., 2021; Schwabl et al., 2019). Mouse models have been used to elucidate the microplastic potential risk to mammalian health. Researches have shown that microplastics can translocate across the gut barrier into different organs (Araújo and Malafaia, 2021; Deng et al., 2018, 2017; Jin et al., 2018a) and cause intestinal homeostasis disturbance (Jin et al., 2018a; Li et al., 2020; Lu et al., 2018; Luo et al., 2019a), neurotoxic effects (Deng et al., 2017), metabolism disorder (Deng et al., 2017; Jin et al., 2018a; Lu et al., 2018) and reproductive impairment (B. Hou et al., 2021; Jin et al., 2021; Xie et al., 2020).

Recent researches have highlighted the possible adverse effects of microplastic exposure on immune responses. It is thought that microplastics greater than 150μm are not able to cross the intestinal barrier, however they remain in the mucus layer and might lead to local intestinal inflammation and microbiota disturbance (Hirt and Body-Malapel, 2020). Particles

smaller than 150μm may translocate from the gut cavity to the lymph and circulatory system, causing systemic exposure (Barboza et al., 2018). Microplastic oral exposure caused gut inflammatory cell infiltration (Deng et al., 2020; Qiao et al., 2019b), altered immune cell counts in the blood and spleen (Park et al., 2020), and elevated levels of immunoglobulins and pro-inflammatory cytokines (B. Hou et al., 2021; Jin et al., 2018b; Li et al., 2020; Park et al., 2020; Xie et al., 2020). Moreover, microplastics can exacerbate inflammation in a pre-existing inflammatory condition, as demonstrated in dextran sodium sulfate (DSS)-induced colitis mice exposed to PS microplastics (Zheng et al., 2021). However, these studies have not considered the sex-based differences in immune response against microplastics.

Immune responses against both foreign and self-antigens depend on sex. Generally, females have stronger innate and adaptive immune responses compared with males in a variety of species, including human and mouse (Klein and Flanagan, 2016). The sex differences in immune responses are caused by genetic, hormonal and environmental factors (Costa et al., 2018; Klein and Flanagan, 2016). Many signaling proteins are encoded on the X chromosome, such as pattern recognition receptors (toll-like receptor (TLR) 7 and TRL8), cytokine receptors (IL2RG and IL13RA2) and transcriptional regulators (FOXP3) (Klein and Flanagan, 2016). Males are more vulnerable to X-linked diseases as they have a single X chromosome, while the cellular mosaicism associated with X inactivation in females provides a more extensive repertoire of proteins (Fish, 2008). Additionally, these X genes are found in higher copy number in females, as approximately 15% of X genes in humans and 3% in mice escape X inactivation (Klein and Flanagan, 2016). Moreover, some immune cells, such as natural killer cells, macrophages, dendritic cells, neutrophils, B- and T-cells, have sex-steroid hormone receptors and respond directly to the absence, presence or change of hormone levels. Environmental factors, such as microbiome and diet, also have differential effects on the immune response. Nutritional supplementation caused different epigenetic changes of

immune-related genes in female and male fetuses. Similarly, vitamin supplementation differently affected immune cell numbers and cytokine production in female and male infants (Klein and Flanagan, 2016).

Complement pathway activation and complement levels in the serum differ between females and males in both humans (Costa et al., 2018) and mice (Kotimaa et al., 2016). Although female and male mice show similar complement functional activity at the level of C3 activation in all three initiating pathways, females have lower terminal functionality, i.e. C6 – C9 activation (Kotimaa et al., 2016). Generally, testosterone and progesterone have a suppressive effect on immune function, while estradiol can be pro-inflammatory at low concentrations or anti-inflammatory at high concentrations (Klein and Flanagan, 2016). Estrogens and progesterone tend to favor immune responses towards T-helper (T_H) 2 while supressing T_H1 and T_H17 differentiation. Testosterone supresses T_H2 and T_H17-induced adaptive immune responses while the effect on T_H1 differentiation is unclear (Roved et al., 2017). Estrogens increase the size of regulatory T (T_{REG})-cell population and decrease the negative selection of naïve B-cells, which leads to survival of autoreactive B-cells and higher antibody production (Fish, 2008). Testosterone decreases B- and T-cell proliferation, antibody and cytokine production and supresses TLR4 expression in monocytes and macrophages (Fish, 2008).

The immunocompetence-handicap hypothesis is a model that explains the interactions between endocrine system, secondary sexual traits, immune system and parasite burden (Folstad and Karter, 1992). Briefly, testosterone is required for the development of many secondary sexual traits and dominance in males, therefore relating to reproductive success. However, testosterone supresses the immune system and increases the susceptibility and pathogenicity of parasitic infections. Hormonal self-regulation and plasticity of sexual trait

expression play an important role balancing the costs and benefits of each component (Folstad and Karter, 1992).

Sex-based differences in immune response play a major role in disease incidence in females and males. In general, men are more susceptible to viral, bacterial, fungal and parasitic infections while the prevalence of autoimmune diseases is higher in women (Fish, 2008). Sex-dependent factors influence susceptibility and progression of diseases, as well as efficacy and side-effects of drugs. These findings made the National Institutes of Health (NIH) require that all NIH-funded clinical trials include women as subjects. Similarly, the Natural Sciences and Engineer Research Council of Canada (NSERC) asks the grant applicants to consider sex and gender in the project's research design.

The present study aimed to determine the microplastic effects on mammalian health using a mouse model. We analyzed microplastic tissue distribution and accumulation, histopathology and expression levels of genes related to inflammation (complement factor B - *Cfb*, C-reactive protein - *Crp*, serum amyloid P-component - *Apcs*) and tissue injury repair (cellular communication network factor 1 - *Ccn1*). We compared sex-specific responses to microplastics as well as the effects of different exposure times.

3.3 Methods

Care of animals

Five to six-week old BALB/c mice (n=18 females, n=18 males) were ordered from Charles River Laboratories (Pointe-Claire, Quebec, Canada). The mice weighed approximately 20 grams. The animals were housed in the Animal Care Facility, Brandon University, Brandon, Canada, on a 12-hour light/dark period with food and water provided *ad libitum*. Each cage housed three mice. Temperature and relative humidity were maintained between 20°C - 25°C and 50% - 70%, respectively. The cages used were clear polycarbonate with stainless steel lids. The composition of the bedding used for nesting was corncob and natural paper. Paper rolls and apple tree sticks were used for environmental enrichment. Upon arrival, the mice were acclimated for two weeks before beginning the experiment to ensure there were no signs of stress or illness. The male and female mice were randomly assigned to one control group (n=9) and one microplastic-treated group (n=9). The cages were cleaned once a week, when the feces were collected and the mice were weighed. Mice were anesthetized with 5% isoflurane until unconscious and then immediately euthanized with CO_2 at a flow rate of 20% chamber air displacement per minute. The animal care protocol was approved by the Brandon University Animal Care Committee (BUACC) in accordance with guidelines of the Canadian Council on Animal Care (BUACC Certificate #2019R04-1).

Microplastic exposure and sampling

MB-containing food was prepared as described in manuscript 1. Please refer to page 21.

The experimental procedure was conducted as outlined in the Figure 3-1. The animals were randomly divided into two groups: nine were assigned to the control group and nine were

assigned to the MB-treated group. Upon arrival, the mice were fed microplastic-free food and the experiment began after two weeks of acclimation. After four weeks of exposure, three mice from each group were sacrificed and samples collected. To sacrifice the animals, they were first sedated and anaesthetized with isoflurane gas, followed by carbon dioxide euthanasia. Kidney, liver, spleen, ovaries, testes, small and large intestines were harvested and weighed. Another three mice from each group were sacrificed after sixteen weeks of exposure. At this point, all the remaining mice were fed microplastic-free food and were sacrificed after four weeks (recovery period).

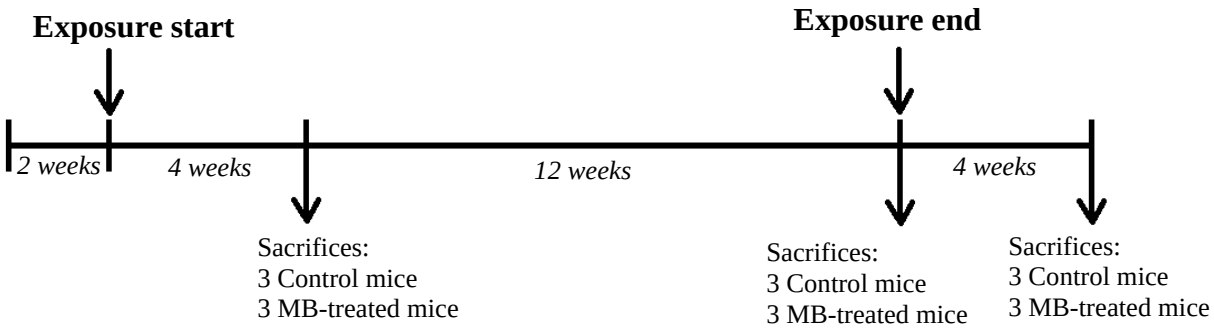


Figure 3-1: Experimental design
Microplastic exposure began after two weeks of acclimation. The sacrifices occurred after four weeks and sixteen weeks of exposure. The exposure was then terminated and all remaining mice were fed microplastic-free food and were sacrificed after four weeks.

Microplastic intake and egestion

Microplastic intake and egestion analyses were performed as described in manuscript 1. Please refer to page 24.

Food consumption, body weight and relative organ weight

Food consumption and body weight were measured as described in manuscript 1. Please refer to page 24. Liver, spleen, kidneys, ovaries, testes, small and large intestines were harvested and weighed. Small and large intestines were opened longitudinally, and the intestinal content

was carefully removed with forceps before weighing the tissues. To calculate the relative organ weight the organ weight was divided by the body weight measured immediately after euthanasia.

Histological analysis and tissue accumulation

Histological analysis was performed as described in manuscript 1. Please refer to page 25.

Gene expression analysis

Liver, spleen and large intestine were harvested for gene expression analysis. The protocol was described in manuscript 1. Please refer to page 26.

Statistical analysis

Food consumption and body weight data were tested using two-way repeated measures ANOVA (one factor repetition) and multiple comparisons versus control group (Holm-Sidak method) was used to isolate the group that differs from the others. Relative organ weight and gene expression data were tested using unpaired t-Test. All tests were performed using SigmaPlot 11.0 at a significance level of $p \leq 0.05$ after passing normality and equal variance tests.

3.4 Results

Food consumption and body weight

Food consumption was measured by cage daily – each cage housed three mice. The results of the two-way repeated measures ANOVA revealed that there was no significant main effect of microplastic exposure on food consumption of female mice (df=1, p=0.169). However, there was a significant interaction between treatment (control, microplastic) and time (days 1 to 140) (df=139, p=0.041), i.e. the effect of microplastic exposure on food consumption depends on the day. Multiple comparisons versus control group (Holm-Sidak method) showed that significant difference was observed on days 6, 7, 70, 83, 96, 99, 109, 111, 113 and 123, with decreased food consumption on all these days, except for day 7 when the consumption was increased (marked with * - see Figure 3-2a). For male mice, however, statistical analysis showed no statistically significant effect of microplastic exposure on food consumption (df=1, p=0.846) and no statistically significant interaction between treatment and time (df=139, p=0.281) (Figure 3-3a).

Individual mice were weighed every week. The results of the two-way repeated measures ANOVA revealed that there was no statistically significant effect of microplastic exposure on body weight of female and male mice (Figure 3-2b, Figure 3-3b). There was a statistically significant effect of time on body weight of female (df=19, p<0.001) and male mice (df=19, p<0.001) however it was due to the mouse expected growth during puberty phase. There was no statistically significant effect of microplastic exposure on body weight of female (df=1, p=0.802) and male mice (df=1, p=0.477) and there was no statistically significant interaction between treatment (control, microplastic) and time (weeks 1 to 20) in female (df=19, p=0.429) and male mice (df=19, p=0.978).

Microplastic intake and egestion

Food consumption was measured by cage daily - each cage housed three mice. On average, PE microplastic-treated cages consumed 9.03g/day, i.e. each mouse consumed 3g/day, assuming equal food consumption. As the microplastic concentration in the mouse food was approximately 3,960particles/Kg, each mouse was exposed to 11.88 PE particles/day for up to sixteen weeks followed by a four-week recovery period.

Microplastic egestion was estimated counting the number of PE fluorescent microplastics present in the feces. There was a significant decrease in the amount of microplastics in the feces after the sixteenth week, when the exposure was terminated for both female and male mice ($df=19$, $p<0.001$) (Figure 3-2c, Figure 3-3c).

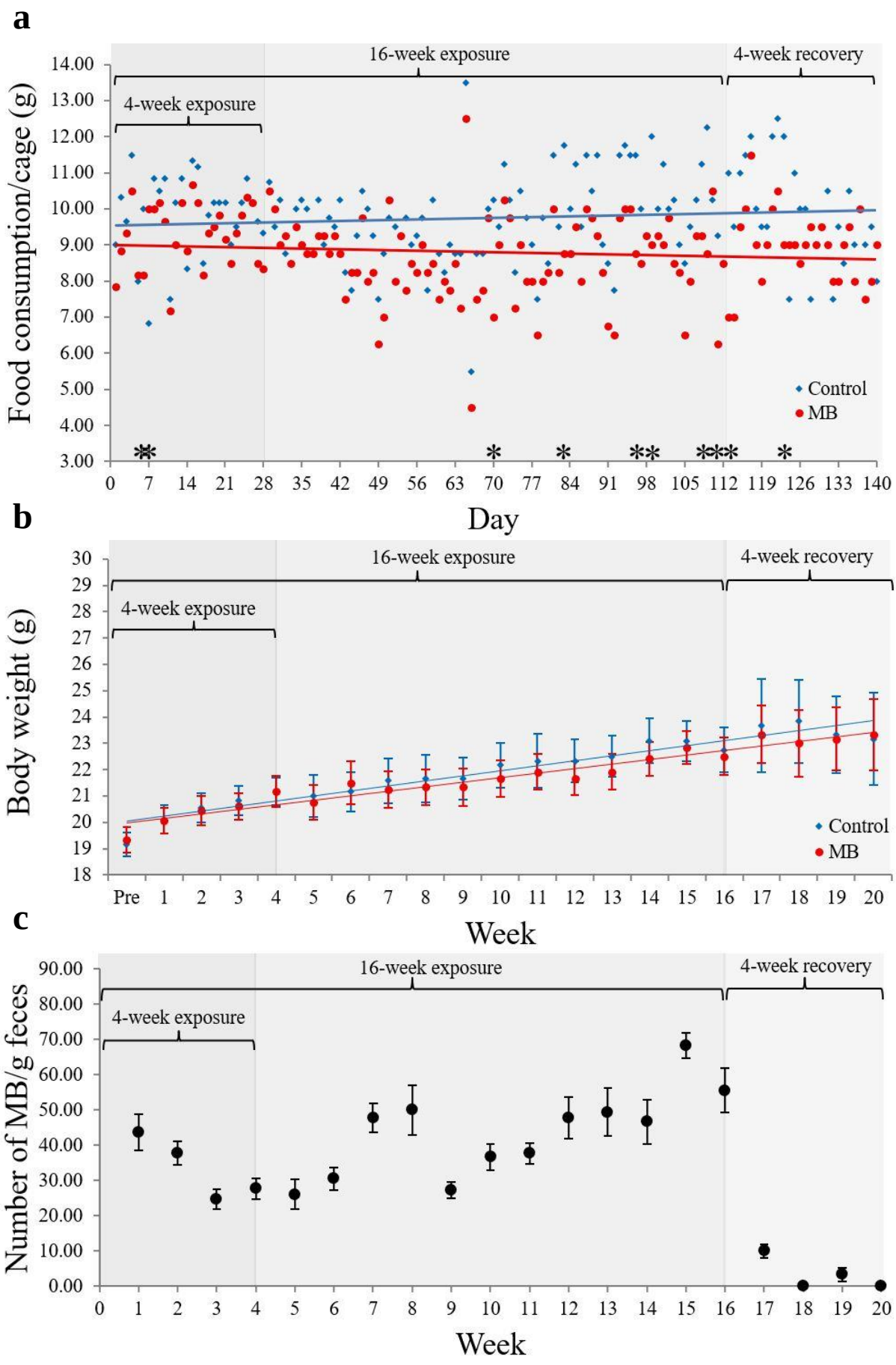

a
16-week exposure
4-week recovery
4-week exposure
Food consumption/cage (g)
Control
MB
Day
b
16-week exposure
4-week recovery
4-week exposure
Body weight (g)
Control
MB
Week
c
16-week exposure
4-week recovery
4-week exposure
Number of MB/g feces
Week

Figure 3-2: Female food consumption, body weight and microplastic egestion.
a) The presented values are the average food consumption per cage. Statistically significant difference was observed only on days 6, 7, 70, 83, 96, 99, 109, 111, 113 and 123 (marked with *). Linear regression for female control group: $y = 0.003x + 9.5519$ $R^2 = 0.0092$, and female MB group: $y = -0.0027x + 8.9928$ $R^2 = 0.0098$.
b) Individual body weight. The presented values are the mean±SEM. No statistically significant difference was observed between control and MB-treated groups. Linear regression for female control group: $y = 0.1914x + 19.855$ $R^2 = 0.9255$, and female MB group: $y = 0.1725x + 19.794$ $R^2 = 0.9363$.
c) Microplastic egestion. Feces were reconstituted in DI water, filtrated and analyzed under a fluorescence stereomicroscope. The presented values are the mean±SEM of the amount of microplastics found in 1g of feces. There was a sharp drop after the sixteenth week, when the exposure was terminated.

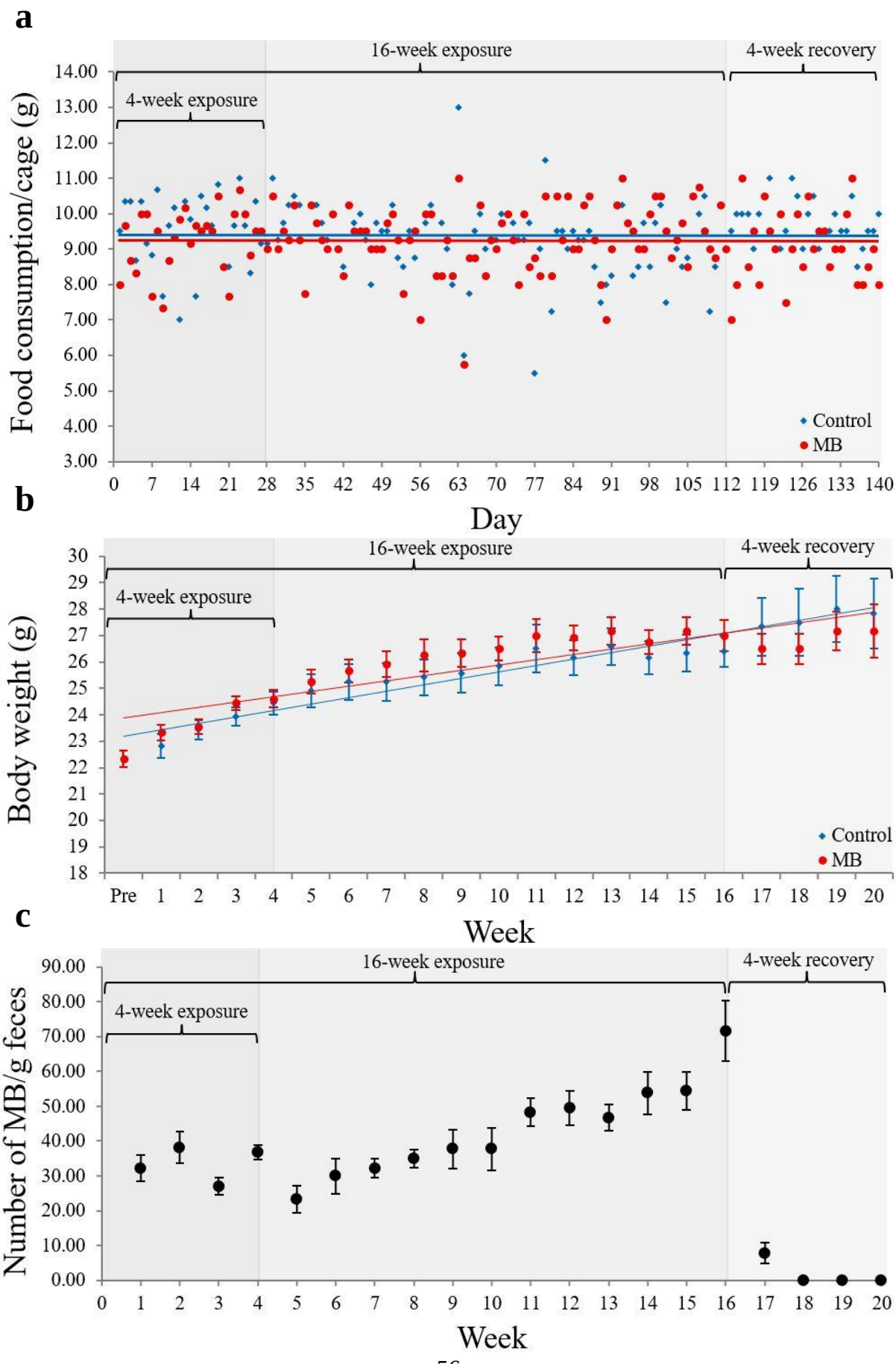
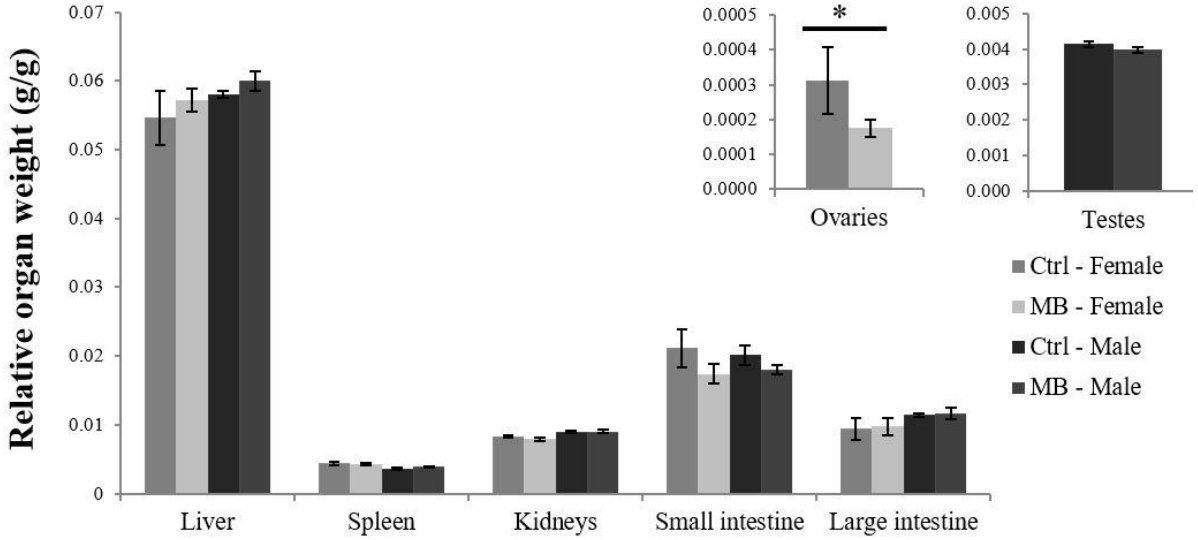

a
16-week exposure
4-week recovery
4-week exposure
Food consumption/cage (g)
Control
MB
Day
b
16-week exposure
4-week recovery
4-week exposure
Body weight (g)
Control
MB
Pre
Week
c
16-week exposure
4-week recovery
4-week exposure
Number of MB/g feces
Week

Figure 3-3: Male food consumption, body weight and microplastic egestion.
a) The presented values are the average food consumption per cage. No statistically significant difference was observed. Linear regression for male control group: $y = -0.0002x + 9.3986$ $R^2 = 4E\text{-}05$, and male MB group: $y = -0.0003x + 9.2572$ $R^2 = 0.0002$.
b) Individual body weight. The presented values are the mean±SEM. No statistically significant difference was observed between control and MB-treated groups. Linear regression for male control group: $y = 0.2424x + 22.956$ $R^2 = 0.9269$, and male MB group: $y = 0.201x + 23.671$ $R^2 = 0.7581$.
c) Microplastic egestion. Feces were reconstituted in DI water, filtrated and analyzed under a fluorescence stereomicroscope. The presented values are the mean±SEM of the amount of microplastics found in 1g of feces. There was a sharp drop after the sixteenth week, when the exposure was terminated.

Relative organ weight

Liver, spleen, kidneys, ovaries, testes, small and large intestines were harvested and weighed immediately after euthanasia. The relative organ weight was calculated by dividing the organ weight by body weight (g/g). Ovaries from microplastic-treated mice showed a decreased relative weight compared to the control after four weeks ($p \leq 0.05$) and sixteen weeks of exposure ($p \leq 0.001$). A significant decrease was also observed for female liver after the recovery period ($p \leq 0.05$). Spleen, kidneys, testes, small and large intestines showed no significant change in relative weight throughout the experiment (Figure 3-4).

a


Relative organ weight (g/g)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Liver
Spleen
Kidneys
Small intestine
Large intestine
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0.0005
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Testes
Ctrl - Female
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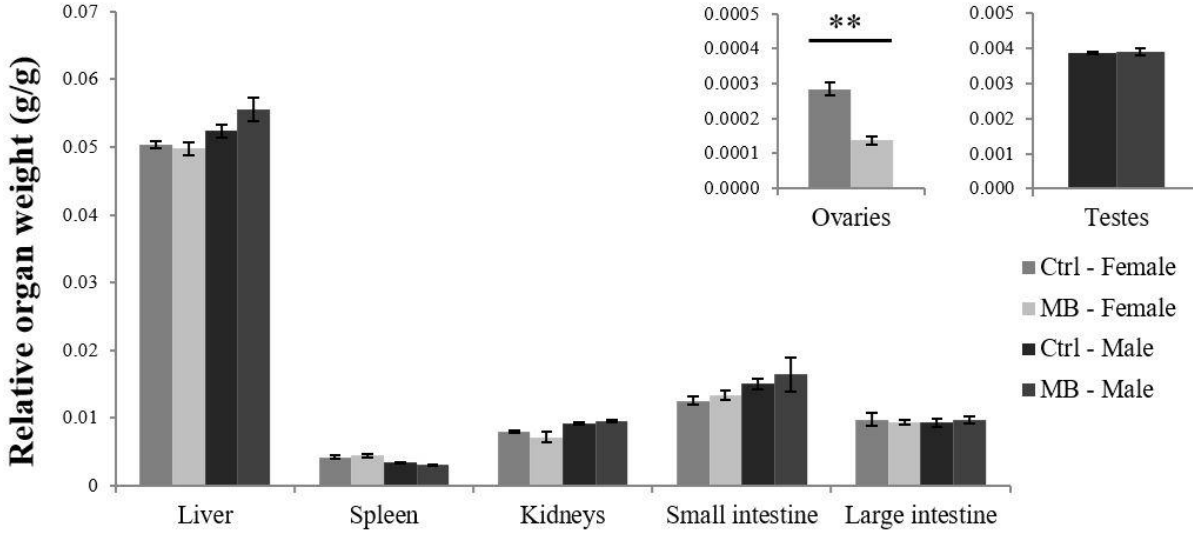
b


Relative organ weight (g/g)
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Liver
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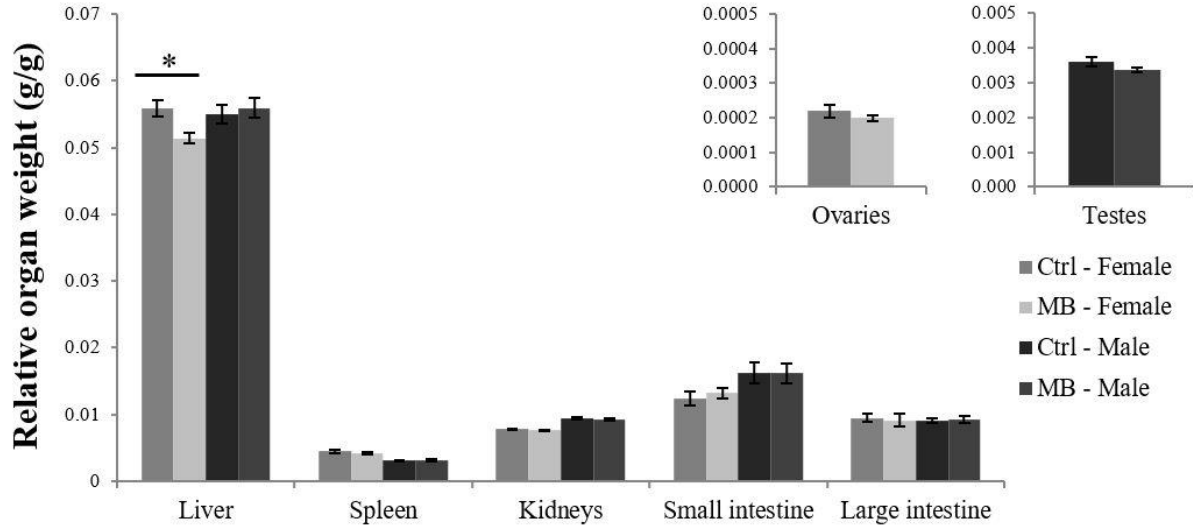
Relative organ weight (g/g)
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Testes
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MB - Female
Ctrl - Male
MB - Male

Figure 3-4: Relative organ weight.
Liver, spleen, kidneys, ovaries, testes, small and large intestines were harvested and weighed. Control (Ctrl) and MB-treated groups after **a)** four weeks of exposure, **b)** sixteen weeks of exposure and **c)** recovery period. The relative organ weight was calculated dividing the organ weight by the body weight (g/g). The presented values are the mean±SEM. Statistically significant differences are marked with * ($p \leq 0.05$) and ** ($p \leq 0.001$).

Histological analysis and tissue accumulation

Liver, spleen, kidney, ovary, small and large intestines were used for histopathological analysis. The H&E staining showed normal cellular morphology with no signs of inflammation in all tissues analyzed after four and sixteen weeks of exposure and four week recovery period. There was no evidence of necrosis or neutrophil infiltration in control mice as well as microplastic-treated mice (Figure 3-5).

No PE microplastic was found in the kidney and ovary, while 1-3 particles were found in the liver and intestines of both female and male mice and in the spleen of female mice after four and sixteen weeks of exposure. Some PE microplastics were observed in the liver and intestines after the recovery period (Table 3-1 and Figure 3-6).

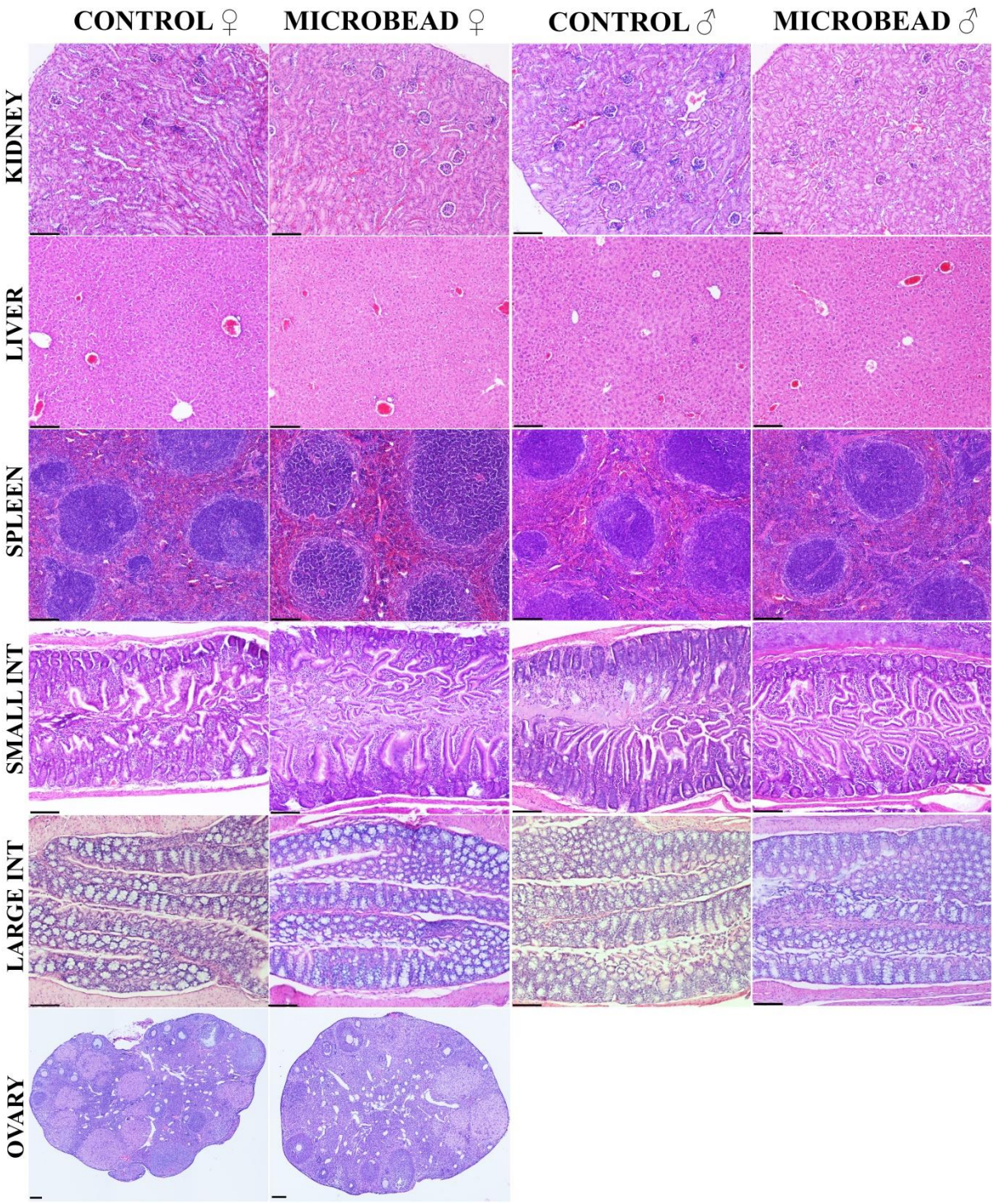

Figure 3-5: Histological analysis after microplastic exposure.
Representative photomicrographs of H&E stained liver, spleen, kidney, small and large intestines and ovary of control and MB-treated mice. Scale bar = 100µm.

Table 3-1: MB accumulation in different tissues.
MB particles were observed in female and male mouse tissues after four and sixteen weeks of exposure and recovery period.

	Female	Liver	Spleen	Kidney	Ovary	Small intest.	Large intest.
4-week exposure	I B1					1	2
	I B2	2	2			1	
	I B3						1
16-week exposure	II B1	1					
	II B2					1	1
	II B3		1			2	
4-week recovery	III B1	2					
	III B2						
	III B3					1	

	Male	Liver	Spleen	Kidney	Small intest.	Large intest.
4-week exposure	I B1	2			2	1
	I B2					
	I B3					
16-week exposure	II B1				3	2
	II B2	2			2	1
	II B3					
4-week recovery	III B1	1			1	1
	III B2					
	III B3					

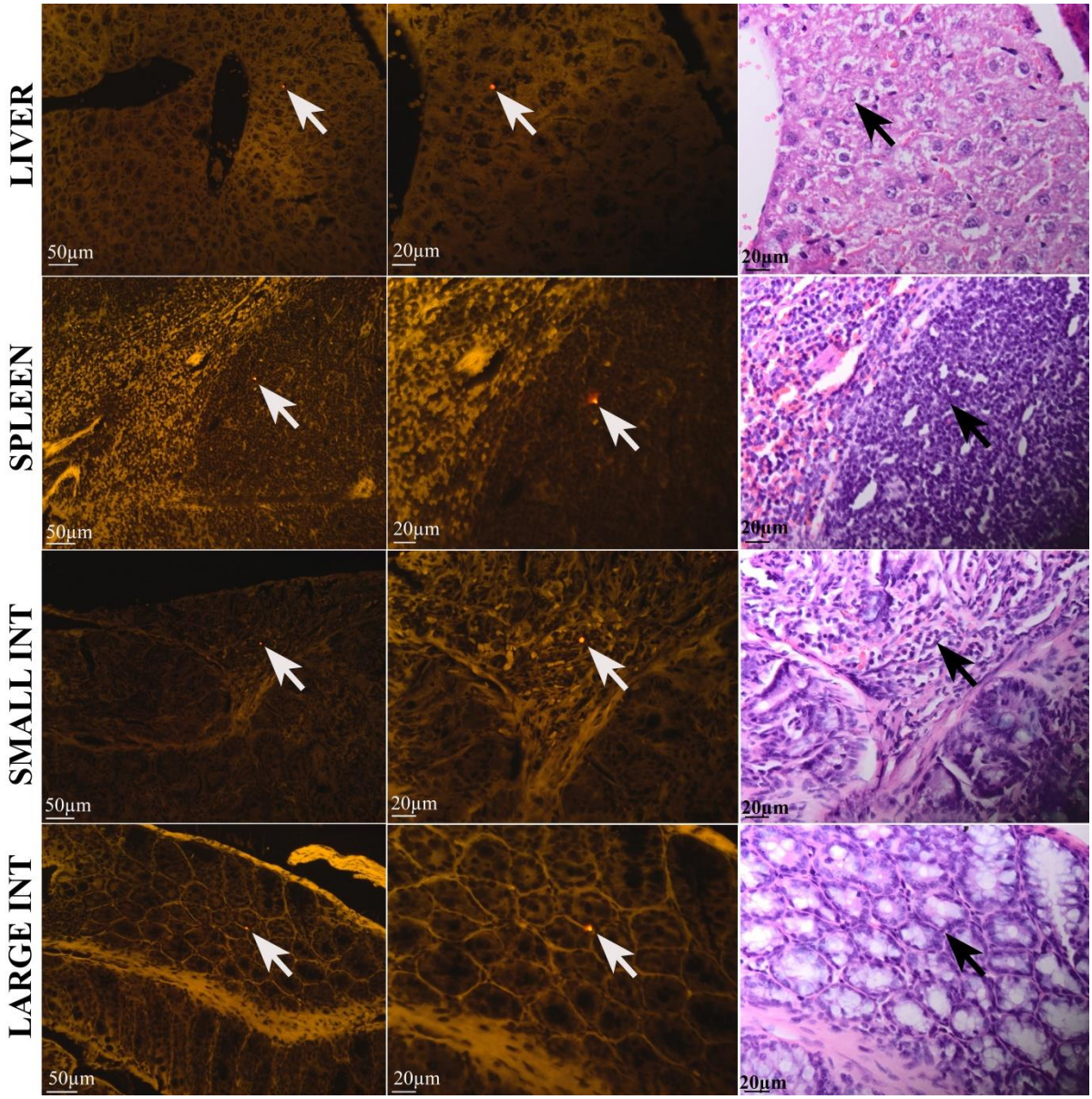

Figure 3-6: MB found in mouse tissues.
Fluorescent red PE MB (arrows) in the liver, spleen, small and large intestines. Right column: 20x objective (scale bar = 50 µm), middle and right columns: 40x objective (scale bar = 20 µm).

Gene expression analysis

The expression levels of serum amyloid P-component (*Apcs*), complement factor B (*Cfb*), C-reactive protein (*Crp*) and cellular communication network factor 1 (*Ccn1*) were measured in the liver (Figure 3-7). *Cfb* and *Ccn1* gene expressions were also measured in the large intestine (Figure 3-8) and spleen (Figure 3-9). Small intestine gene expression analysis was not included in this study as the qPCR data did not show consistency and reproducibility.

In general, female and male mice showed different gene expression patterns throughout the experiment. Microplastic-treated mice appeared to show upregulation or downregulation of some genes compared with the control mice, however this was only statistically significant ($p \leq 0.05$) in females for *Cfb* and *Ccn1* after sixteen weeks of exposure and *Apcs* after the recovery period in the liver, and *Ccn1* after sixteen weeks of exposure in the large intestine.

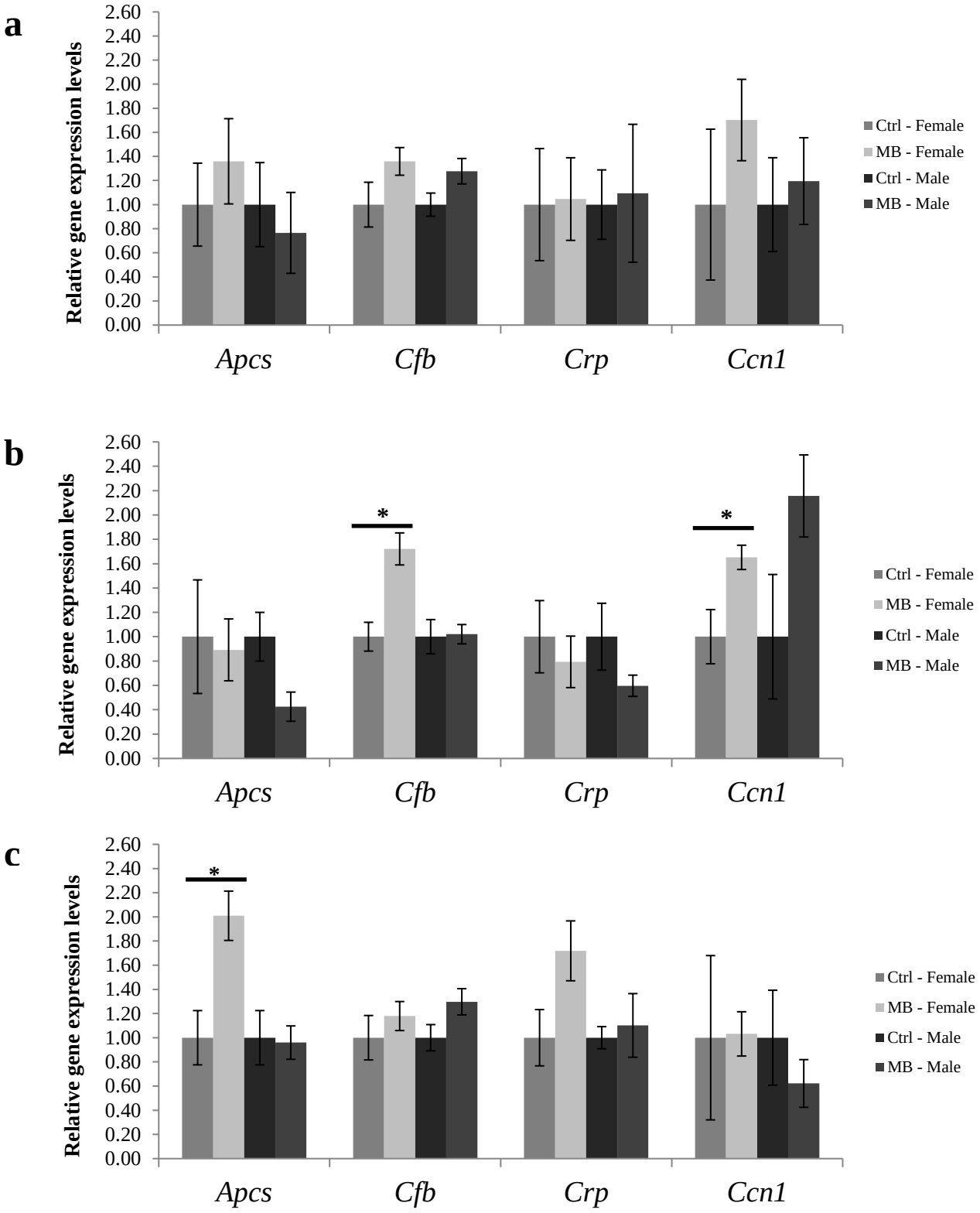

Figure 3-7: Gene expression in the liver.
Apcs, *Cfb*, *Crp* and *Ccn1* gene expression profiles of control (Ctrl) and MB-treated female and male mice after **a)** four weeks of exposure, **b)** sixteen weeks of exposure, and **c)** recovery period. *18s* and *Gapdh* were used as normalizer genes. Mean ± SEM (n=3). Statistically significant differences are marked with * ($p \leq 0.05$).

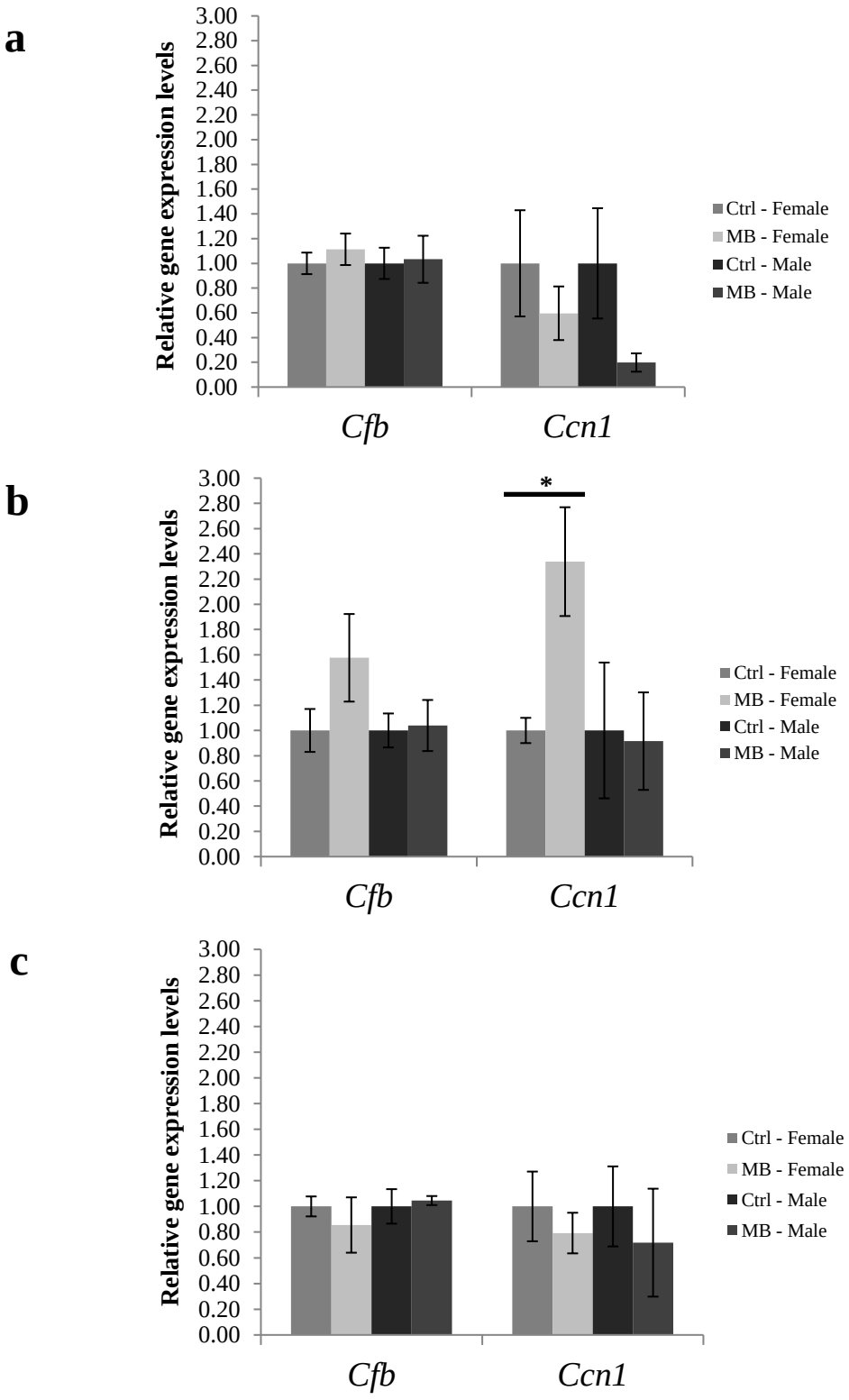

Figure 3-8: Gene expression in the large intestine.
Cfb and *Crp* gene expression of control (Ctrl) and MB-treated female and male mice after **a)** four weeks of exposure, **b)** sixteen weeks of exposure, and **c)** recovery period. *18s* and *Gapdh* were used as normalizer genes. Mean ± SEM (n=3). Statistically significant differences are marked with * ($p \leq 0.05$).

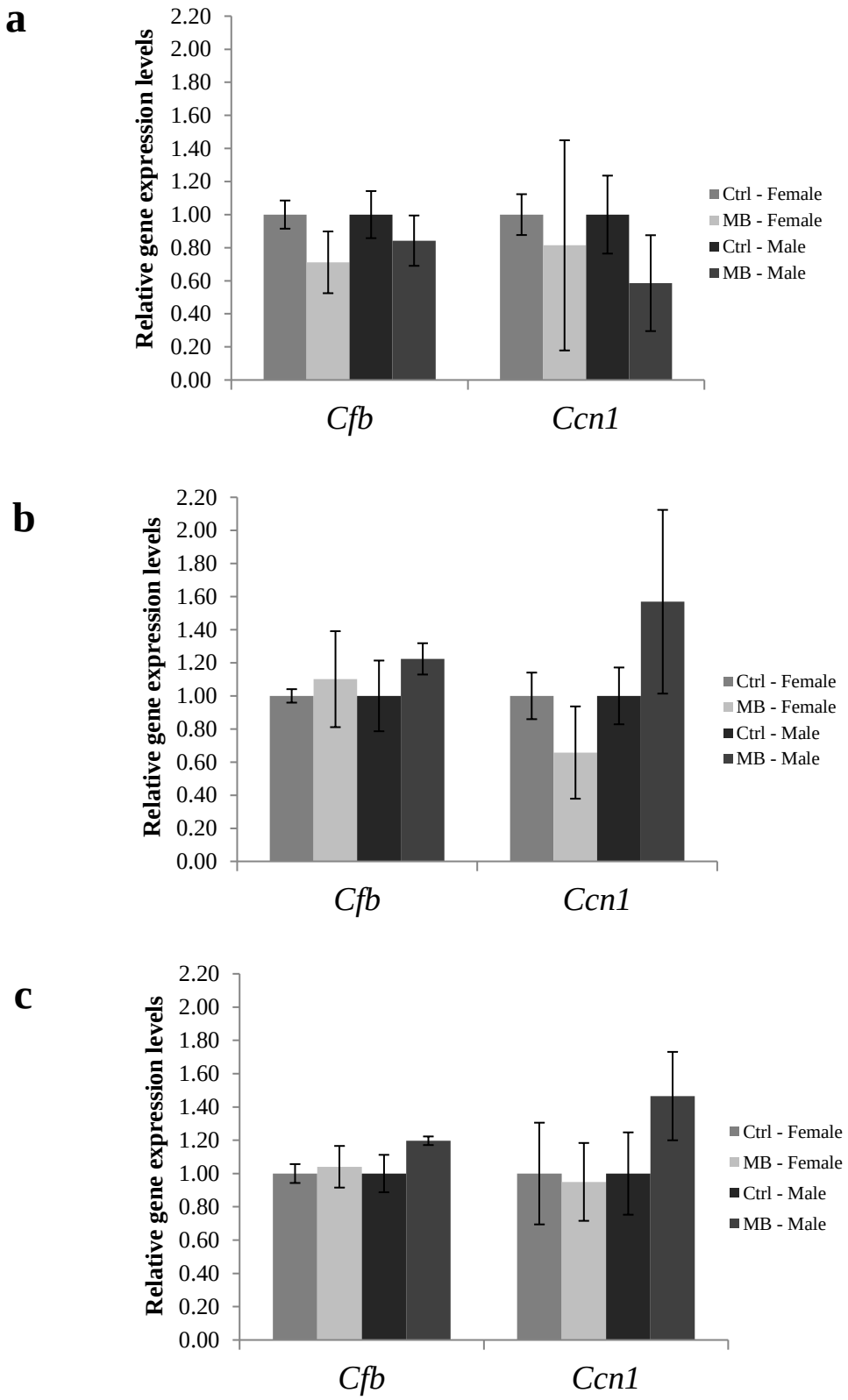

Figure 3-9: Gene expression in the spleen.
Cfb and *Crp* gene expression of control (Ctrl) and MB-treated female and male mice after **a)** four weeks of exposure, **b)** sixteen weeks of exposure, and **c)** recovery period. *18s* and *Gapdh* were used as normalizer genes. Mean ± SEM (n=3).

3.5 Discussion

Microplastics have been reported worldwide and a growing body of evidence suggests that humans are exposed to these environmental pollutants. Although the effects on human health are unknown, microplastic potential to cause toxic effects has been documented in both invertebrate and vertebrate species (Lei et al., 2018b), and in *in vitro* as well as in *in vivo* studies (Brown et al., 2001). The estimated daily consumption of microplastics for a North American diet ranges from 106 to 142 particles depending on age and sex. Inhalation contributes an additional 97 to 170 particles and these numbers escalate even more if the source of water is exclusively bottled water (Cox et al., 2019). In the present study, mice were exposed to approximately 11.88 PE microplastic particles/day for up to sixteen weeks followed by a four-week recovery period. According to Cox et al. (2019) this value represents a realistic, but low concentration that humans might be exposed to via diet.

In the present study, PE microplastic exposure did not affect food consumption of male mice while only minor changes were observed for female mice. No significant difference was observed in body weight of female and male mice after four weeks and sixteen weeks of exposure and recovery period. In contrast, a significant loss of body weight was observed in male mice after PS and PE microplastic exposure as early as three weeks of oral exposure (Jin et al., 2021; Lu et al., 2018; Park et al., 2020; Xie et al., 2020). Other researchers have reported no effect on body weight and food consumption after microplastic exposure in male rats (Rafiee et al., 2018; Xu et al., 2004) and male mice (Araújo and Malafaia, 2021; Deng et al., 2017; B. Hou et al., 2021; Stock et al., 2019). These contradictory results could be explained by differences in experimental design, such as microplastic characteristics and administration method. Most of the studies that reported a significant reduction in body weight used PS microplastics and an extremely high dose administered by oral gavage.

Both female and male mice were able to egest microplastics in feces within the first week of exposure. A drastic reduction was observed after the sixteenth week, when the exposure was terminated. Nonetheless, a small number of microplastics was observed in the recovery period. This finding suggests that PE microbeads might be retained in the gastrointestinal tract; this could be explained by the microbead properties, such as small size and spherical shape. Similarly, plastic microspheres were quickly egested by *Xenopus* tadpoles (Hu et al., 2016), sea bass larvae (Mazurais et al., 2015) and *Daphnia* (Rosenkranz et al., 2009) and were almost completely eliminated after 6 days, 2 days and 4 hours, respectively. Under visual inspection, the microplastics showed no signs of deterioration or fragmentation, which confirms that microplastics are bioresistant, i.e. refractory to digestion and dissolution.

In the present study, no significant change was observed in the relative weight of kidney, spleen, testes and small and large intestines of female and male mice throughout the experiment. Likewise, no significant change was reported in relative weight of kidney, spleen, liver, heart, lung, and testes after oral exposure to PS microparticles (Stock et al., 2019). Although no difference was observed in the liver weight of mouse dams exposed to PS microplastics during gestation and lactation, their offspring showed an increased liver weight along with hepatic lipid accumulation and altered metabolite levels, suggesting a transgenerational effect (Luo et al., 2019a).

The liver's main function includes the production of blood plasma proteins and bile, endocytosis-based filtering of the blood and detoxification of substances. Approximately 25% of the incoming blood supply comes from the aorta via the hepatic artery, while 75% comes from the lower gastrointestinal tract through the portal vein. The arterial and venous blood flows into the sinusoids, passing along and over the basolateral membrane of rows of hepatocytes and drains into the hepatic vein and ultimately the inferior vena cava. This

organization ensures that hepatocytes are the first cells exposed to everything ingested and absorbed, either nutrients or toxic substances. Hepatocytes excrete lipids, salts and degraded proteins from their apical membrane into small channels (canaliculi) that feed bile contents through the intrahepatic biliary tree. Bile is drained from the liver into the gall bladder for storage and released into the intestinal lumen during feeding to promote emulsification, digestion and adsorption of dietary fats as well as the removal of foreign substances and endogenous waste products (Schulze et al., 2019). The liver also produces a variety of proteins that play an important role in the innate immunity, such as complement proteins, opsonins, chemokines and fibrinogen. As some of these proteins rapidly increase in the blood upon inflammatory stimulation they are called acute-phase reactants (APR) (Zhou et al., 2016).

As would be expected for a highly vascularized organ that receives blood directly from the gut and has a pivotal role in the immune system, the liver is thought to be affected by microplastic exposure. In the present study, 1-2 microplastic particles were found in the liver of female and male mice after four and sixteen weeks of exposure, as well as after the recovery period. Interestingly, a significant relative weight decrease was observed in female mouse liver after the recovery period, whereas no difference was found during the microplastic exposure period. Increased liver weight in diabetic mice has been associated with increased triglyceride accumulation and influx of fatty acid into the liver (Zafar and Naqvi, 2010). However, mice exposed to PS microplastics showed hepatic lipid droplets and decreased liver relative weight (Deng et al., 2017). Similarly, a significant loss of liver and epididymal fat relative weight has been reported in mice after PS microplastic exposure (Lu et al., 2018). Exposure to microplastics has been reported to cause hepatic metabolism disorder, as indicated by altered metabolomic profile and biomarkers related to amino acid, bile acid, glucose and lipid metabolism (Deng et al., 2017; Jin et al., 2018a; Lu et al., 2018;

Luo et al., 2019a, 2019b; Zheng et al., 2021). In addition, PE microplastic exposure caused hepatotoxicity in tadpoles, promoting immune cell infiltration, blood vessel dilation, sinus congestion, hydropic degeneration, steatosis, hypertrophy and hyperplasia along with hepatocyte nuclear abnormalities (Araújo et al., 2020b). In the present study, however, no histological difference was observed between control and PE microplastic treated group throughout the experiment.

Contrary to expectations, the reduction of liver relative weight as well as upregulation of *Apcs* gene expression in the liver occurred in female mice only after the recovery period; no significant difference was observed after four and sixteen weeks of exposure. Although the mice were allowed a four-week period without exposure, microplastics remained embedded in the liver and probably exerted a delayed toxic effect. The reason why these effects were not observed during the exposure period is unknown and intriguing, since SAP is a major acute-phase reactant in mice. CRP is a major acute-phase reactant in humans, while in mice it is a trace plasma component and only a minor acute-phase reactant. In contrast, SAP is highly inducible in mice but only slightly in humans (Roy et al., 2017), showing a 50-fold increase in mouse serum within 24-48 hours of injection of LPS and returning to the basal level after 32 days (Pepys et al., 1979).

CRP and SAP belong to the pentraxin superfamily, characterized by a cyclic pentameric structure, sequence homology and calcium-dependent interactions with the ligands (Mold et al., 2001). CRP and SAP are pattern recognition molecules produced by the liver and secreted into the blood to interact with pathogens and cell debris and promote their clearance by binding to IgG receptors on phagocytic cells (Mold et al., 2001). In addition, SAP can activate the classical complement cascade and interact with monocytes, neutrophils and macrophages to modulate the immune response and wound healing (Cox et al., 2014; Pilling

and Gomer, 2018). SAP influences the migration and spreading of neutrophils, inhibits monocyte differentiation into fibrocytes and promotes macrophage polarization toward an anti-inflammatory/anti-fibrotic phenotype (Cox et al., 2014). SAP has been demonstrated to bind to a variety of microbial polysaccharides and toxins as well as chromatin and DNA in circulation, enhancing the clearance of autoantigens to prevent autoimmunity (Mold et al., 2001).

Hepatocytes constitutively produce most of the complement proteins and further elevate their production after inflammatory stimuli (Zhou et al., 2016). Transcriptome analysis showed that up to 51 genes were differentially expressed in larval zebrafish injected with PS microplastics, most notably the alternative complement pathway genes (eg.: *cfhl3, cfhl4, cfb* and *c9*), suggesting that microplastics are integrated in immunological recognition processes (Veneman et al., 2017). In the present study, female mice showed an increased *Cfb* gene expression after sixteen weeks of exposure to PE microplastics. Although microplastics do not have any corresponding receptors, they need to be tagged by complement proteins and cleared from the body by phagocytic cells. The alternative complement pathway seems likely to be affected by microplastic exposure since its activation does not depend on the presence of antibodies (Veneman et al., 2017). CFB is unique to the alternative complement pathway and undergoes cleavage when bound to spontaneously occurring C3 (H_2O), forming the C3 convertase (C3bBb). During infection, C3b on the microbial surface recruits more CFB to amplify the concentration of C3 convertase and generate large numbers of C3b. Regardless of the initiating pathway, up to 90% of the C3b molecules are generated via activation of the alternative complement pathway (Owen et al., 2018).

Considering that microplastics can potentially cause tissue damage, it is expected that injury repair mechanisms to be engaged after microplastic exposure. CCN1 has been related to

inflammation, injury repair and wound healing (Choi et al., 2015; Kim et al., 2018). CCN1 is a secreted matricellular protein that is involved in all tissue repair phases: inflammation, proliferation and maturation. CCN1 binds to specific integrin receptors on distinct cell types to promote clearance of apoptotic neutrophils by macrophages, induce cell proliferation and differentiation through Jag1/Notch signaling, and promote angiogenesis and matrix remodeling. CCN1 is essential to prevent fibrosis during wound healing, as it induces fibrogenic myofibroblast senescence (Kim et al., 2018). In the present study, the expression level of *Ccn1* gene was measured. After sixteen weeks of exposure to PE microplastics, female mice showed upregulation of *Ccn1*in the liver and in the large intestine.

As the intestinal epithelium is constantly exposed to microbes and ingested toxins, the intestinal cells are prone to damage (Kim et al., 2018). CCN1 plays an essential role in intestinal tissue repair by promoting proliferation of colonic epithelial cells. Administration of CCN1 accelerated mucosal restitution of DSS-induced colitis in mice mostly due to macrophage and fibroblast IL-6 secretion (Choi et al., 2015). In the present study, however, histological analysis did not reveal any signs of intestinal damage in PE microplastic-treated female and male mice despite the presence of 1-3 particles in the small and large intestine after four and sixteen weeks of exposure and recovery period. A reasonable explanation is that early stages of tissue damage may not be detected in histological analysis.

The spleen is the largest secondary lymphoid organ in the body and is responsible for filtering the blood of foreign materials and old or damaged erythrocytes and for initiating an immune response against blood-borne pathogens (Cesta, 2006). The spleen is functionally and morphologically divided into red pulp and white pulp. The white pulp is essentially made of lymphatic tissue while the red pulp consists of venous sinuses and splenic cords that filter the blood (Cesta, 2006). Particles larger than 100-200nm are incapable of crossing the

endothelial slit of splenic sinuses and are retained in the red pulp, where they are internalized by macrophages and slowly destroyed (Cataldi et al., 2017).

In the present study, 1-2 microplastic particles were found in the splenic red pulp and white pulp of female mice after four weeks and sixteen weeks of exposure, while no particle was observed after the recovery period. In a recent study, abnormal nuclear and mitochondrial morphology was observed in female mouse spleens after PE microplastic oral exposure, along with inhibited dendritic cell maturation and increased helper T cell to cytotoxic T cell ($CD4^+/CD8^+$) ratio (Park et al., 2020). After intraperitoneal injection of PS microplastics in mice, no microplastics were found in the spleen, nonetheless, the red pulp and the marginal zone of the white pulp seemed to be activated (Tomazic-Jezic et al., 2001). In the present study, however, no histopathological changes were observed in female and male mouse spleen after exposure to PE microplastics.

Histological analysis revealed normal tissue morphology without noticeable pathological signs in the liver, kidney, spleen, ovary and intestines of female and male mice after four and sixteen weeks of exposure to PE microplastics and after the recovery period. Similarly exposure to PS nano/microplastics showed no histological alteration in kidney, liver, spleen, lung, testis and intestine of mice (Stock et al., 2019) and rats (Walczak et al., 2015). No histological changes were observed in zebrafish gill, liver, brain, kidney and intestines after exposure to PE micro fragments (Karami et al., 2017) and PS microbeads (Lei et al., 2018b). On the other hand, histopathological changes have been reported in other studies. Zebrafish showed intestinal damage, such as villi and epithelial damage, thinning of the gut wall and congestive inflammation (Qiao et al., 2019b) as well as cracking of villi and splitting of enterocytes (Lei et al., 2018b). Mice exposed to PS microplastics showed hepatic ballooning degeneration (Luo et al., 2019a) and lipid droplets and inflammation in the liver (Deng et al.,

2017). Edema, loose glands, small vessels proliferation and inflammatory cell infiltration has been reported in mouse intestine after PE microplastic exposure (Li et al., 2020). Histopathological changes were also reported in mouse testes after exposure to PS microplastics, as shown by disordered spermatogenic cells, abscission and multinucleated gonocytes (B. Hou et al., 2021; Jin et al., 2021; Xie et al., 2020).

Histological sections were used to investigate microplastic distribution and accumulation in the liver, spleen, kidney, ovary and small and large intestines. No PE microplastic was found in the kidney or ovary, while 1-3 particles were found in the liver and intestines of both female and male mice and in the spleen of female mice after four and sixteen weeks of exposure. Studies have shown that particles smaller than 150µm may translocate from the gut to the lymph and circulatory system, however the absorption of these particles is limited (<0.3%) and only particles smaller than 20µm is expected to penetrate deeply into organs (Barboza et al., 2018). In the present study, some PE microplastics remained in the liver and intestines even after the recovery period, confirming plastic bioresistance and biopersistence. A recent study reported only a few particles in the gut wall and no particles in the liver, kidney and spleen of mice exposed to PS microplastics (Stock et al., 2019), whereas other studies showed microplastic accumulation in the mouse gut, liver, kidney and testes (Araújo and Malafaia, 2021; Deng et al., 2018, 2017; Jin et al., 2021, 2018a).

Studies have shown that microplastic adverse effects might be caused by plastic additives, as they are not chemically bound to the polymeric chain and can be easily released into the tissues (Campanale et al., 2020). Plasticizers are well known endocrine disrupting chemicals and mainly affect female fertility in a range of species, including humans (Rattan et al., 2017). Phthalates and bisphenol A are used to confer flexibility to plastic products but due to their toxic effects, they have been replaced by alternative plasticizers. However, due to their

similar structure, the alternatives are thought to cause equivalent toxicological effects (Rattan et al., 2017). In the present study, a significant decrease in ovarian relative weight was observed after four and sixteen weeks of exposure to PE microplastics. Due to the supplier's trade secret, it was not possible to associate this result with microplastic chemical composition; however it is reasonable to speculate that some plasticizer might have contributed to ovarian weight decrease. It is worth mentioning that the inherent toxic effects of microplastics might augment the toxicity of phthalates, as indicated by the aggravated toxic effects on mice co-exposed to DEHP and PE microplastics compared with exposure to DEHP or microplastics alone (Deng et al., 2020). Recently, microplastic reproductive toxicology has been reported in male mice, as indicated by testicular histopathological changes, inflammation and oxidative stress, low-quality sperm cells and decreased serum testosterone levels (B. Hou et al., 2021; Jin et al., 2021; Xie et al., 2020).

As might be expected, no significant difference was observed in ovarian weight between PE microplastic-treated mice and control mice after the recovery period, suggesting that the effect was reversed. The reversibility of the toxic effects depends on which follicle stage was targeted. For example, targeting of primordial follicles for an extended period of time leads to irreversible toxic effects, as the follicular reserve is non-renewable. By contrast, the effects on more mature follicles, such as antral follicles, can be reversed if the toxicant is removed, as the early stages of folliculogenesis are unaffected (Hannon and Flaws, 2015). However, in the present study no difference was observed in ovarian histology between control and treatment groups after exposure to microplastics. In addition, no PE microplastics were found in the ovaries, suggesting that the toxic effect is not related to the physical damage caused by microplastics. Further research is warranted to investigate how microplastics affect ovarian function and the potential to cause reproductive disorders in females.

It is especially important to notice the sex-based differences in the response to microplastic exposure. Females and males have distinct innate and adaptive immune responses as well as different immunological responses to self and foreign antigens (Klein and Flanagan, 2016). Research has shown that female and male mice have similar complement activity until the C3 activation level, while females have lower terminal pathway activation (C6-C9) (Kotimaa et al., 2016). In the present study, both male and female mice accumulated PE microplastics in the tissues, but only females appeared to have triggered immune response and tissue repair mechanisms, as demonstrated by the upregulation of *Cfb*, *Apcs* and *Ccn1* genes. These results suggest that the alternative complement pathway and the pattern recognition molecule SAP are likely to be involved in the immune response against microplastics in females, while it was not possible to determine the defense mechanisms induced in males.

3.6 Conclusions

Emerging evidence shows that humans are exposed to microplastics, yet the effects on human health have not been clarified. The present study aimed to contribute to the understanding of the microplastic effects on mammalian health using an original approach. Our experimental design included a four-week recovery period to assess the reversibility of the adverse effects and we used both female and male mouse models to compare sex-based differences in the response to microplastic exposure.

Microplastics were able to translocate across the intestinal epithelial barrier and accumulated in the liver, spleen and intestinal wall and remained in the tissues even after the recovery period, confirming microplastic biopersistence and bioresistance. Although tissue accumulation was reported in both female and male mice, females seemed to be more affected. Females showed increased expression levels of genes related to inflammation (*Cfb*, *Apcs*) and tissue injury repair (*Ccn1*) as well as reduction in ovarian weight. Some of these effects appeared to be reversible, as the ovarian weight and *Cfb* and *Ccn1* gene expression returned to normal levels after the recovery period, while the effect on *Apcs* gene expression seemed to be delayed and noticeable only in the recovery period.

Overall, longer exposure to microplastics caused more pronounced effects than short exposure, and some of these effects were reversed after the depuration period. Microplastics affected the expression of genes that coordinate a broad range of biological functions, such as immune system activation, wound healing and tissue repair. These results suggest that microplastics might have a systemic impact and the severity of effects depends on the exposure time. Further research is warranted to better understand the potential long-term health hazards and the sex-based differences in the biological response against microplastics.

4- Conclusions

Microplastics have become one of the most concerning environmental problems in recent years. Although a growing body of evidence suggest that humans are exposed, the fate and effects of microplastics on human body are controversial and unclear. Many studies have used invertebrate and vertebrate models to evaluate the potential adverse effects on wildlife and humans.

The present research project confirmed that only a small fraction of microplastics cross the gut barrier and accumulate in the tissues; the majority of them are more likely to be eliminated via feces. Either microbeads or microfibers had a minor effect on food consumption and no effect on body weight. At environmental concentrations, microplastics do not seem to pose a serious health risk during short-term exposure, however longer exposure seems to be more harmful. Microplastics affected more females than males, and the effects were time-dependent. Some effects worsened with longer exposure (e.g. ovarian weight), others reversed after a depuration period (e.g. *Cfb* and *Ccn1* gene expression, ovarian weight) and others have a delayed manifestation (e.g. *Apcs* gene expression, liver weight). Only a few microplastics were found in the tissues, nevertheless they remained there even after the recovery period, confirming plastic bioresistance and biopersistence. The genes analyzed in this study are involved in a variety of biological functions, such as immune system activation, wound healing and tissue repair, which suggests that microplastics might have a systemic impact.

The microplastic problem has just started; even if plastic production were completely stopped, the existing plastic in the landfills and environment would continue to degrade into microplastics and nanoplastics and human exposure to these particles would keep increasing. We acknowledge that the mouse exposure to microplastics performed herein does not

completely represent the human exposure, as it did not take into consideration the nano/microplastic diversity and the cumulative effects of a lifetime of exposure. The present study, however, described for the first time the sex-based differences in the mouse response to microplastics, showing that females are more affected than males. Furthermore, exposure time plays an important role on the outcome, as some effects were time-dependent, with longer exposure causing worse effects, others reversed after a period without exposure, and others showed a delayed manifestation.

References

Abbasi, S., Turner, A., 2021. Human exposure to microplastics: A study in Iran. J. Hazard. Mater. 403, 123799. https://doi.org/10.1016/j.jhazmat.2020.123799

Altunkaynak, B.Z., Önger, M.E., Altunkaynak, M.E., Ayranci, E., Canan, S., 2012. A brief introduction to stereology and sampling strategies: Basic concepts of stereology. NeuroQuantology 10, 31–43. https://doi.org/10.14704/nq.2012.10.1.427

Amereh, F., Babaei, M., Eslami, A., Fazelipour, S., Rafiee, M., 2020. The emerging risk of exposure to nano(micro)plastics on endocrine disturbance and reproductive toxicity: From a hypothetical scenario to a global public health challenge. Environ. Pollut. 261. https://doi.org/10.1016/j.envpol.2020.114158

Araújo, A.P. da C., de Melo, N.F.S., de Oliveira Junior, A.G., Rodrigues, F.P., Fernandes, T., de Andrade Vieira, J.E., Rocha, T.L., Malafaia, G., 2020a. How much are microplastics harmful to the health of amphibians? A study with pristine polyethylene microplastics and Physalaemus cuvieri. J. Hazard. Mater. 382, 121066. https://doi.org/10.1016/j.jhazmat.2019.121066

Araújo, A.P. da C., Gomes, A.R., Malafaia, G., 2020b. Hepatotoxicity of pristine polyethylene microplastics in neotropical physalaemus cuvieri tadpoles (Fitzinger, 1826). J. Hazard. Mater. 386. https://doi.org/10.1016/j.jhazmat.2019.121992

Araújo, A.P. da C., Malafaia, G., 2021. Microplastic ingestion induces behavioral disorders in mice: A preliminary study on the trophic transfer effects via tadpoles and fish. J. Hazard. Mater. 401.

Ašmonaite, G., Sundh, H., Asker, N., Carney Almroth, B., 2018. Rainbow Trout Maintain Intestinal Transport and Barrier Functions Following Exposure to Polystyrene Microplastics. Environ. Sci. Technol. 52, 14392–14401. https://doi.org/10.1021/acs.est.8b04848

Au, S.Y., Bruce, T.F., Bridges, W.C., Klaine, S.J., 2015. Responses of Hyalella azteca to acute and chronic microplastic exposures. Environ. Toxicol. Chem. 34, 2564–2572. https://doi.org/10.1002/etc.3093

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., D'Errico, G., Pauletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environ. Pollut. 198, 211–222. https://doi.org/10.1016/j.envpol.2014.12.021

Barboza, L.G.A., Dick Vethaak, A., Lavorante, B.R.B.O., Lundebye, A.K., Guilhermino, L., 2018. Marine microplastic debris: An emerging issue for food security, food safety and human health. Mar. Pollut. Bull. 133, 336–348. https://doi.org/10.1016/j.marpolbul.2018.05.047

Bergmann, M., Mützel, S., Primpke, S., Tekman, M.B., Trachsel, J., Gerdts, G., 2019. White and wonderful? Microplastics prevail in snow from the Alps to the Arctic. Sci. Adv. 5, 1–11. https://doi.org/10.1126/sciadv.aax1157

Besseling, E., Wang, B., Lürling, M., Koelmans, A.A., 2014. Nanoplastic affects growth of S. obliquus and reproduction of D. magna. Environ. Sci. Technol. 48, 12336–12343. https://doi.org/10.1021/es503001d

Bouwmeester, H., Hollman, P.C.H., Peters, R.J.B., 2015. Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology. Environ. Sci. Technol. 49, 8932–8947. https://doi.org/10.1021/acs.est.5b01090

Brown, D.M., Wilson, M.R., MacNee, W., Stone, V., Donaldson, K., 2001. Size-dependent proinflammatory effects of ultrafine polystyrene particles: A role for surface area and

oxidative stress in the enhanced activity of ultrafines. Toxicol. Appl. Pharmacol. 175, 191–199. https://doi.org/10.1006/taap.2001.9240
Browne, M.A., Niven, S.J., Galloway, T.S., Rowland, S.J., Thompson, R.C., 2013. Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. Curr. Biol. 23, 2388–2392. https://doi.org/10.1016/j.cub.2013.10.012
Campanale, C., Massarelli, C., Savino, I., Locaputo, V., Uricchio, V.F., 2020. A detailed review study on potential effects of microplastics and additives of concern on human health. Int. J. Environ. Res. Public Health 17. https://doi.org/10.3390/ijerph17041212
Cataldi, M., Vigliotti, C., Mosca, T., Cammarota, M.R., Capone, D., 2017. Emerging role of the spleen in the pharmacokinetics of monoclonal antibodies, nanoparticles and exosomes. Int. J. Mol. Sci. 18. https://doi.org/10.3390/ijms18061249
Cesta, M.F., 2006. Normal Structure, Function, and Histology of the Spleen. Toxicol. Pathol. 34, 455–465. https://doi.org/10.1080/01926230600867743
Choi, J.S., Kim, K.-H., Lau, L.F., 2015. The Matricellular Protein CCN1 Promotes Mucosal Healing in Murine Colitis through IL-6. Physiol. Behav. 8, 1285–1296. https://doi.org/10.1038/mi.2015.19.The
Cole, M., Lindeque, P., Fileman, E., Halsband, C., Galloway, T.S., 2015. The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. Environ. Sci. Technol. 49, 1130–1137. https://doi.org/10.1021/es504525u
Conti, G.O., Ferrante, M., Banni, M., Favara, C., Nicolosi, I., Cristaldi, A., Fiore, M., Zuccarello, P., 2020. Micro- and nano-plastics in edible fruit and vegetables. The first diet risks assessment for the general population. Environ. Res. 187, 109677. https://doi.org/10.1016/j.envres.2020.109677
Costa, M.G., Poppelaars, F., van Kooten, C., Mollnes, T.E., Tedesco, F., Wurzner, R., Trouw, L.A., Truedsson, L., Daha, M.R., Roos, A., Seelen, M.A., 2018. Age and Sex-Associated Changes of Complement Activity and Complement Levels in a Healthy Caucasian Population. Front. Immunol. 9.
Cox, K.D., Covernton, G.A., Davies, H.L., Dower, J.F., Juanes, F., Dudas, S.E., 2019. Human Consumption of Microplastics. Environ. Sci. Technol. 53, 7068–7074. https://doi.org/10.1021/acs.est.9b01517
Cox, N., Pilling, D., Gomer, R.H., 2014. Serum amyloid P: a systemic regulator of the innate immune response. J. Leukoc. Biol. 96, 739–743. https://doi.org/10.1189/jlb.1mr0114-068r
de Sá, L.C., Oliveira, M., Ribeiro, F., Rocha, T.L., Futter, M.N., 2018. Studies of the effects of microplastics on aquatic organisms: What do we know and where should we focus our efforts in the future? Sci. Total Environ. 645, 1029–1039. https://doi.org/10.1016/j.scitotenv.2018.07.207
Deng, Y., Yan, Z., Shen, R., Huang, Y., Ren, H., Zhang, Y., 2021. Enhanced reproductive toxicities induced by phthalates contaminated microplastics in male mice (Mus musculus). J. Hazard. Mater. 406, 124644. https://doi.org/10.1016/j.jhazmat.2020.124644
Deng, Y., Yan, Z., Shen, R., Wang, M., Huang, Y., Ren, H., Zhang, Y., Lemos, B., 2020. Microplastics release phthalate esters and cause aggravated adverse effects in the mouse gut. Environ. Int. 143, 105916. https://doi.org/10.1016/j.envint.2020.105916
Deng, Y., Zhang, Y., Lemos, B., Ren, H., 2017. Tissue accumulation of microplastics in mice and biomarker responses suggest widespread health risks of exposure. Sci. Rep. 7, 1–10. https://doi.org/10.1038/srep46687

Deng, Y., Zhang, Y., Qiao, R., Bonilla, M.M., Yang, X., Ren, H., Lemos, B., 2018. Evidence that microplastics aggravate the toxicity of organophosphorus flame retardants in mice (Mus musculus). J. Hazard. Mater. 357, 348–354. https://doi.org/10.1016/j.jhazmat.2018.06.017

Ding, J., Zhang, S., Razanajatovo, R.M., Zou, H., Zhu, W., 2018. Accumulation, tissue distribution, and biochemical effects of polystyrene microplastics in the freshwater fish red tilapia (Oreochromis niloticus). Environ. Pollut. 238, 1–9. https://doi.org/10.1016/j.envpol.2018.03.001

Donaldson, K., Stone, V., Gilmour, P.S., Brown, D.M., Macnee, W., 2000. Ultrafine particles: Mechanisms of lung injury. Philos. Trans. R. Soc. A Math. Phys. Eng. Sci. 358, 2741–2749. https://doi.org/10.1098/rsta.2000.0681

Dong, C. Di, Chen, C.W., Chen, Y.C., Chen, H.H., Lee, J.S., Lin, C.H., 2020. Polystyrene microplastic particles: In vitro pulmonary toxicity assessment. J. Hazard. Mater. 385. https://doi.org/10.1016/j.jhazmat.2019.121575

Dong, S., Qu, M., Rui, Q., Wang, D., 2018. Combinational effect of titanium dioxide nanoparticles and nanopolystyrene particles at environmentally relevant concentrations on nematode Caenorhabditis elegans. Ecotoxicol. Environ. Saf. 161, 444–450. https://doi.org/10.1016/j.ecoenv.2018.06.021

Dris, R., Gasperi, J., Rocher, V., Saad, M., Renault, N., Tassin, B., 2015. Microplastic contamination in an urban area: A case study in Greater Paris. Environ. Chem. 12, 592–599. https://doi.org/10.1071/EN14167

Du Clos, T.W., 2000. Function of C-reactive protein. Ann. Med. 32, 274–278. https://doi.org/10.3109/07853890009011772

EFSA, (EFSA Panel on Contaminants in the Food Chain), 2016. Statement on the presence of microplastics and nanoplastics in food, with particular focus on seafood. EFSA J. 14, 4501. https://doi.org/10.2903/j.efsa.2016.4501

Endo, S., Takizawa, R., Okuda, K., Takada, H., Chiba, K., Kanehiro, H., Ogi, H., Yamashita, R., Date, T., 2005. Concentration of polychlorinated biphenyls (PCBs) in beached resin pellets: Variability among individual particles and regional differences. Mar. Pollut. Bull. 1103–1114. https://doi.org/10.1016/j.marpolbul.2005.04.030

Eriksen, M., Lebreton, L.C., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F., Ryan, P.G., Reisser, J., 2014. Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. PLoS One 9.

Eyles, J.E., Bramwell, V.W., Williamson, E.D., Alpar, H.O., 2001. Microsphere translocation and immunopotentiation in systemic tissues following intranasal administration. Vaccine 19, 4732–4742. https://doi.org/10.1016/S0264-410X(01)00220-1

Fish, E.N., 2008. The X-files in immunity: sex-based differences predispose immune responses. Nat. Rev. Immunol. 8, 737–744.

Folstad, I., Karter, A.J., 1992. Parasites, bright males, and the immunocompetence handicap. Am. Nat. 139, 603–622. https://doi.org/10.1086/285346

Fournier, S., D'Errico, J., Adler, D., Kollontzi, S., Goedken, M., Fabris, L., Yurkow, E., Stapleton, P., 2020. Nanopolystyrene Translocation and Fetal Deposition After Acute Lung Exposure During Late-Stage Pregnancy 9, 1–11. https://doi.org/10.21203/rs.3.rs-39676/v1

GESAMP, 2016. Sources, fate and effects of microplastics in the marine environment: part 2 of a global assessment.

Greven, A.C., Merk, T., Karagöz, F., Mohr, K., Klapper, M., Jovanović, B., Palić, D., 2016. Polycarbonate and polystyrene nanoplastic particles act as stressors to the innate immune system of fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 35,

3093–3100. https://doi.org/10.1002/etc.3501
Hannon, P.R., Flaws, J.A., 2015. The effects of phthalates on the ovary. Front. Endocrinol. (Lausanne). 6, 1–19. https://doi.org/10.3389/fendo.2015.00008
Hernandez, L.M., Xu, E.G., Larsson, H.C.E., Tahara, R., Maisuria, V.B., Tufenkji, N., 2019. Plastic Teabags Release Billions of Microparticles and Nanoparticles into Tea. Environ. Sci. Technol. 53, 12300–12310. https://doi.org/10.1021/acs.est.9b02540
Heskett, M., Takada, H., Yamashita, R., Yuyama, M., Ito, M., Geok, Y.B., Ogata, Y., Kwan, C., Heckhausen, A., Taylor, H., Powell, T., Morishige, C., Young, D., Patterson, H., Robertson, B., Bailey, E., Mermoz, J., 2012. Measurement of persistent organic pollutants (POPs) in plastic resin pellets from remote islands: Toward establishment of background concentrations for International Pellet Watch. Mar. Pollut. Bull. 64, 445–448. https://doi.org/10.1016/j.marpolbul.2011.11.004
Hillery, A.M., Jan, P.U., Florence, A.T., 1994. Comparative, quantitative study of lymphoid and non-lymphoid uptake of 60 nm polystyrene particles. J. Drug Target. 2, 151–156. https://doi.org/10.3109/10611869409015904
Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., Kwan, C., Moore, C., Gray, H., Laursen, D., Zettler, E.R., Farrington, J.W., Reddy, C.M., Peacock, E.E., Ward, M.W., 2011. Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. Mar. Pollut. Bull. 62, 1683–1692. https://doi.org/10.1016/j.marpolbul.2011.06.004
Hirt, N., Body-Malapel, M., 2020. Immunotoxicity and intestinal effects of nano- and microplastics: a review of the literature. Part. Fibre Toxicol. 17, 1–22. https://doi.org/10.1186/s12989-020-00387-7
Hou, B., Wang, F., Liu, T., Wang, Z., 2021. Reproductive toxicity of polystyrene microplastics: In vivo experimental study on testicular toxicity in mice. J. Hazard. Mater. 405.
Hou, J., Lei, Z., Cui, L., Hou, Y., Yang, L., An, R., Wang, Q., Li, S., Zhang, H., Zhang, L., 2021. Polystyrene microplastics lead to pyroptosis and apoptosis of ovarian granulosa cells via NLRP3/Caspase-1 signaling pathway in rats. Ecotoxicol. Environ. Saf. 212. https://doi.org/10.1016/j.ecoenv.2021.112012
Hu, L., Su, L., Xue, Y., Mu, J., Zhu, J., Xu, J., Shi, H., 2016. Uptake, accumulation and elimination of polystyrene microspheres in tadpoles of Xenopus tropicalis. Chemosphere 164, 611–617. https://doi.org/10.1016/j.chemosphere.2016.09.002
Hwang, J., Choi, D., Han, S., Choi, J., Hong, J., 2019. An assessment of the toxicity of polypropylene microplastics in human derived cells. Sci. Total Environ. 684, 657–669.
Ibrahim, Y.S., Tuan Anuar, S., Azmi, A.A., Wan Mohd Khalik, W.M.A., Lehata, S., Hamzah, S.R., Ismail, D., Ma, Z.F., Dzulkarnaen, A., Zakaria, Z., Mustaffa, N., Tuan Sharif, S.E., Lee, Y.Y., 2021. Detection of microplastics in human colectomy specimens. JGH Open 5, 116–121. https://doi.org/10.1002/jgh3.12457
Imhof, H.K., Ivleva, N.P., Schmid, J., Niessner, R., Laforsch, C., 2013. Contamination of beach sediments of a subalpine lake with microplastic particles. Curr. Biol. 23, R867–R868. https://doi.org/10.1016/j.cub.2013.09.001
Jin, H., Ma, T., Sha, X., Liu, Z., Zhou, Y., Meng, X., Chen, Y., Han, X., Ding, J., 2021. Polystyrene microplastics induced male reproductive toxicity in mice. J. Hazard. Mater. 401, 123430. https://doi.org/10.1016/j.jhazmat.2020.123430
Jin, Y., Lu, L., Tu, W., Luo, T., Fu, Z., 2018a. Impacts of polystyrene microplastic on the gut barrier, microbiota and metabolism of mice. Sci. Total Environ. 649, 308–317. https://doi.org/10.1016/j.scitotenv.2018.08.353
Jin, Y., Xia, J., Pan, Z., Yang, J., Wang, W., Fu, Z., 2018b. Polystyrene microplastics induce

microbiota dysbiosis and inflammation in the gut of adult zebrafish. Environ. Pollut. 235, 322–329. https://doi.org/10.1016/j.envpol.2017.12.088
Jun, J. Il, Kim, K.H., Lau, L.F., 2015. The matricellular protein CCN1 mediates neutrophil efferocytosis in cutaneous wound healing. Nat. Commun. 6. https://doi.org/10.1038/ncomms8386
Jun, J.-I., Lau, L.F., 2013. Taking Aim at the Extracellular Matrix: CCN Proteins as Emerging Therapeutic Targets. Nat Rev Drug Discov. 10, 945–963. https://doi.org/10.1038/nrd3599.Taking
Karami, A., Groman, D.B., Wilson, S.P., Ismail, P., Neela, V.K., 2017. Biomarker responses in zebrafish (Danio rerio) larvae exposed to pristine low-density polyethylene fragments. Environ. Pollut. 223, 466–475. https://doi.org/10.1016/j.envpol.2017.01.047
Kim, K.H., Chen, C.C., Alpini, G., Lau, L.F., 2015. CCN1 induces hepatic ductular reaction through integrin αvβ5-mediated activation of NF-κB. J. Clin. Invest. 125, 1886–1900. https://doi.org/10.1172/JCI79327
Kim, K.H., Won, J.H., Cheng, N., Lau, L.F., 2018. The matricellular protein CCN1 in tissue injury repair. J. Cell Commun. Signal. 12, 273–279. https://doi.org/10.1007/s12079-018-0450-x
Kirstein, I. V., Kirmizi, S., Wichels, A., Garin-Fernandez, A., Erler, R., Löder, M., Gerdts, G., 2016. Dangerous hitchhikers? Evidence for potentially pathogenic Vibrio spp. on microplastic particles. Mar. Environ. Res. 120, 1–8. https://doi.org/10.1016/j.marenvres.2016.07.004
Klein, S.L., Flanagan, K.L., 2016. Sex differences in immune responses. Nat. Rev. Immunol. 16, 626–638. https://doi.org/10.1038/nri.2016.90
Kosuth, M., Mason, S.A., Wattenberg, E. V, 2018. Anthropogenic Contamination of Tap Water, Beer, and Sea Salt. PLoS One 13.
Kotimaa, J., Klar-Mohammad, N., Gueler, F., Schilders, G., Jansen, A., Rutjes, H., Daha, M.R., van Kooten, C., 2016. Sex matters: Systemic complement activity of female C57BL/6J and BALB/cJ mice is limited by serum terminal pathway components. Mol. Immunol. 76, 13–21. https://doi.org/10.1016/j.molimm.2016.06.004
Kutralam-Muniasamy, G., Pérez-Guevara, F., Elizalde-Martínez, I., Shruti, V.C., 2020. Branded milks – Are they immune from microplastics contamination? Sci. Total Environ. 714, 136823. https://doi.org/10.1016/j.scitotenv.2020.136823
Lee, S., Ahad, A., Luu, M., Moon, S., Caesar, J., Cardoso, W. V, Grant, M.B., 2019. CCN1–Yes-Associated Protein Feedback Loop Regulates Physiological and Pathological Angiogenesis Sangmi. Mol. Cell. Biol. 39.
Lei, L., Liu, M., Song, Y., Lu, S., Hu, J., Cao, C., Xie, B., Shi, H., He, D., 2018a. Polystyrene (nano)microplastics cause size-dependent neurotoxicity, oxidative damage and other adverse effects in Caenorhabditis elegans. Environ. Sci. Nano 5, 2009–2020. https://doi.org/10.1039/c8en00412a
Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K.M., He, D., 2018b. Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. Sci. Total Environ. 619–620, 1–8. https://doi.org/10.1016/j.scitotenv.2017.11.103
LeMoine, C.M.R., Kelleher, B.M., Lagarde, R., Northam, C., Elebute, O.O., Cassone, B.J., 2018. Transcriptional effects of polyethylene microplastics ingestion in developing zebrafish (Danio rerio). Environ. Pollut. 243, 591–600. https://doi.org/10.1016/j.envpol.2018.08.084
Li, B., Ding, Y., Cheng, X., Sheng, D., Xu, Z., Rong, Q., Wu, Y., Zhao, H., Ji, X., Zhang, Y., 2020. Polyethylene microplastics affect the distribution of gut microbiota and

inflammation development in mice. Chemosphere 244, 125492. https://doi.org/10.1016/j.chemosphere.2019.125492
Liebezeit, G., Liebezeit, E., 2015. Origin of synthetic particles in honeys. Polish J. Food Nutr. Sci. 65, 143–147. https://doi.org/10.1515/pjfns-2015-0025
Liebezeit, G., Liebezeit, E., 2014. Synthetic particles as contaminants in German beers. Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 31, 1574–1578. https://doi.org/10.1080/19440049.2014.945099
Liebezeit, G., Liebezeit, E., 2013. Non-pollen particulates in honey and sugar. Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 30, 2136–2140. https://doi.org/10.1080/19440049.2013.843025
Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCt Method. Methods 25, 402–408.
Lu, L., Wan, Z., Luo, T., Fu, Z., Jin, Y., 2018. Polystyrene microplastics induce gut microbiota dysbiosis and hepatic lipid metabolism disorder in mice. Sci. Total Environ. 631–632, 449–458. https://doi.org/10.1016/j.scitotenv.2018.03.051
Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., Ding, L., Ren, H., 2016. Uptake and Accumulation of Polystyrene Microplastics in Zebrafish (Danio rerio) and Toxic Effects in Liver. Environ. Sci. Technol. 50, 4054–4060. https://doi.org/10.1021/acs.est.6b00183
Luís, L.G., Ferreira, P., Fonte, E., Oliveira, M., Guilhermino, L., 2015. Does the presence of microplastics influence the acute toxicity of chromium(VI) to early juveniles of the common goby (Pomatoschistus microps)? A study with juveniles from two wild estuarine populations. Aquat. Toxicol. 164, 163–174. https://doi.org/10.1016/j.aquatox.2015.04.018
Luo, T., Wang, C., Pan, Z., Jin, C., Fu, Z., Jin, Y., 2019a. Maternal Polystyrene Microplastic Exposure during Gestation and Lactation Altered Metabolic Homeostasis in the Dams and Their F1 and F2 Offspring. Environ. Sci. Technol. 53, 10978–10992. https://doi.org/10.1021/acs.est.9b03191
Luo, T., Zhang, Y., Wang, C., Wang, X., Zhou, J., Shen, M., Zhao, Y., Fu, Z., Jin, Y., 2019b. Maternal exposure to different sizes of polystyrene microplastics during gestation causes metabolic disorders in their offspring. Environ. Pollut. 255, 113122. https://doi.org/10.1016/j.envpol.2019.113122
Mathalon, A., Hill, P., 2014. Microplastic fibers in the intertidal ecosystem surrounding Halifax Harbor, Nova Scotia. Mar. Pollut. Bull. 81, 69–79.
Matlock, B., 2015. Assessment of Nucleic Acid Purity. Tech. Bull. NanoDrop Spectrophotometers 1–2.
Mattsson, K., Ekvall, M.T., Hansson, L.A., Linse, S., Malmendal, A., Cedervall, T., 2015. Altered behavior, physiology, and metabolism in fish exposed to polystyrene nanoparticles. Environ. Sci. Technol. 49, 553–561. https://doi.org/10.1021/es5053655
Mattsson, K., Johnson, E. V., Malmendal, A., Linse, S., Hansson, L.A., Cedervall, T., 2017. Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain. Sci. Rep. 7, 1–7. https://doi.org/10.1038/s41598-017-10813-0
Mazurais, D., Ernande, B., Quazuguel, P., Severe, A., Huelvan, C., Madec, L., Mouchel, O., Soudant, P., Robbens, J., Huvet, A., Zambonino-Infante, J., 2015. Evaluation of the impact of polyethylene microbeads ingestion in European sea bass (Dicentrarchus labrax) larvae. Mar. Environ. Res. 112, 78–85. https://doi.org/10.1016/j.marenvres.2015.09.009
Mintenig, S.M., Löder, M.G.J., Primpke, S., Gerdts, G., 2018. Low numbers of microplastics

detected in drinking water from ground water sources. Sci. Total Environ. 648, 631–635. https://doi.org/10.1016/j.scitotenv.2018.08.178
Mold, C., Gresham, H.D., Du Clos, T.W., 2001. Serum Amyloid P Component and C-Reactive Protein Mediate Phagocytosis Through Murine FcγRs. J. Immunol. 166, 1200–1205. https://doi.org/10.4049/jimmunol.166.2.1200
Moolenbeek, C., Ruitenberg, E.J., 1981. The "Swiss roll": A simple technique for histological studies of the rodent intestine. Lab. Anim. 15, 57–59.
Murphy, F., Ewins, C., Carbonnier, F., Quinn, B., 2016. Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment. Environ. Sci. Technol. 50, 5800–5808. https://doi.org/10.1021/acs.est.5b05416
Murray, F., Cowie, P.R., 2011. Plastic contamination in the decapod crustacean Nephrops norvegicus (Linnaeus, 1758). Mar. Pollut. Bull. 62, 1207–1217. https://doi.org/10.1016/j.marpolbul.2011.03.032
Nah, W.H., Park, M.J., Gye, M.C., 2011. Effects of early prepubertal exposure to bisphenol A on the onset of puberty, ovarian weights, and estrous cycle in female mice. Clin. Exp. Reprod. Med. 38, 75–81. https://doi.org/10.5653/cerm.2011.38.2.75
Napper, I.E., Davies, B.F.R., Clifford, H., Elvin, S., Koldewey, H.J., Mayewski, P.A., Miner, K.R., Potocki, M., Elmore, A.C., Gajurel, A.P., Thompson, R.C., 2020. Reaching New Heights in Plastic Pollution—Preliminary Findings of Microplastics on Mount Everest. One Earth 3, 621–630. https://doi.org/10.1016/j.oneear.2020.10.020
Obbard, R.W., Sadri, S., Wong, Y.Q., Khitun, A.A., Baker, I., Thompson, R.C., 2014. Global warming releases microplastic legacy frozen in Arctic Sea ice. Earth's Futur. 2, 315–320. https://doi.org/10.1002/2014EF000240
Ogata, Y., Takada, H., Mizukawa, K., Hirai, H., Iwasa, S., Endo, S., Mato, Y., Saha, M., Okuda, K., Nakashima, A., Murakami, M., Zurcher, N., Booyatumanondo, R., Zakaria, M.P., Dung, L.Q., Gordon, M., Miguez, C., Suzuki, S., Moore, C., Karapanagioti, H.K., Weerts, S., Mcclurg, T., Burres, E., Smith, W., Van Velkenburg, M., Lang, J.S., Lang, R.C., Laursen, D., Danner, B., Stewardson, N., Thompson, R.C., 2009. International Pellet Watch: Global monitoring of persistent organic pollutants (POPs) in coastal waters. 1. Initial phase data on PCBs, DDTs, and HCHs. Mar. Pollut. Bull. 58, 1437–1446. https://doi.org/10.1016/j.marpolbul.2009.06.014
Ogonowski, M., Schür, C., Jarsén, Å., Gorokhova, E., 2016. The effects of natural and anthropogenic microparticles on individual fitness in daphnia magna. PLoS One 11, 1–20. https://doi.org/10.1371/journal.pone.0155063
Park, Eun-Jung, Han, J.-S., Park, Eun-Jun, Seong, E., Lee, G.H., Wan Kim, D., Son, H.-Y., Han, H.-Y., Lee, B.-S., 2020. Repeated-oral dose toxicity of polyethylene microplastics and the possible implications on reproduction and development of the next generation. Toxicol. Lett. https://doi.org/10.1016/j.toxlet.2020.01.008
Pauly, J.L., Stegmeier, S.J., Allaart, H.A., Cheney, R.T., Zhang, P.J., Mayer, A.G., Streck, R.J., 1998. Inhaled cellulosic and plastic fibers found in human lung tissue. Cancer Epidemiol. Biomarkers Prev. 7, 419–428.
Pepys, M.B., Baltz, M., Gomer, K., Davies, A.J.S., Doenhoff, M., 1979. Serum amyloid P-component is an acute-phase reactant in the mouse [18]. Nature. https://doi.org/10.1038/278259a0
Pilling, D., Gomer, R.H., 2018. The development of serum amyloid p as a possible therapeutic. Front. Immunol. 9, 1–10. https://doi.org/10.3389/fimmu.2018.02328
Porter, D.W., Castranova, V., Robinson, V.A., Hubbs, A.F., Mercer, R.R., Scabilloni, J., Goldsmith, T., Schwegler-Berry, D., Battelli, L., Washko, R., Burkhart, J., Piacitelli, C., Whitmer, M., Jones, W., 1999. Acute inflammatory reaction in rats after intratracheal

instillation of material collected from a nylon flocking plant. J. Toxicol. Environ. Heal. - Part A 57, 25–45. https://doi.org/10.1080/009841099157845
Powell, J.J., Faria, N., Thomas-McKay, E., Pele, L.C., 2010. Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. J. Autoimmun. 34, J226–J233. https://doi.org/10.1016/j.jaut.2009.11.006
Qiao, R., Deng, Y., Zhang, S., Wolosker, M.B., Zhu, Q., Ren, H., Zhang, Y., 2019a. Accumulation of different shapes of microplastics initiates intestinal injury and gut microbiota dysbiosis in the gut of zebrafish. Chemosphere 236.
Qiao, R., Sheng, C., Lu, Y., Zhang, Y., Ren, H., Lemos, B., 2019b. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. Sci. Total Environ. 662, 246–253. https://doi.org/10.1016/j.scitotenv.2019.01.245
Rafiee, M., Dargahi, L., Eslami, A., Beirami, E., Jahangiri-rad, M., Sabour, S., Amereh, F., 2018. Neurobehavioral assessment of rats exposed to pristine polystyrene nanoplastics upon oral exposure. Chemosphere 193, 745–753. https://doi.org/10.1016/j.chemosphere.2017.11.076
Ragusa, A., Svelato, A., Santacroce, C., Catalano, P., Notarstefano, V., Carnevali, O., Papa, F., Rongioletti, M.C.A., Baiocco, F., Draghi, S., D'Amore, E., Rinaldo, D., Matta, M., Giorgini, E., 2021. Plasticenta: First evidence of microplastics in human placenta. Environ. Int. 146, 106274. https://doi.org/10.1016/j.envint.2020.106274
Rattan, S., Zhou, C., Chiang, C., Mahalingam, S., Brehm, E., Flaws, J.A., 2017. Exposure to endocrine disruptors during adulthood: Consequences for female fertility. J. Endocrinol. 233, R109–R129. https://doi.org/10.1530/JOE-17-0023
Revel, M., Châtel, A., Mouneyrac, C., 2018. Micro(nano)plastics: A threat to human health? Curr. Opin. Environ. Sci. Heal. 1, 17–23. https://doi.org/10.1016/j.coesh.2017.10.003
Rillig, M.C., 2012. Microplastic in terrestrial ecosystems and the soil? Environ. Sci. Technol. 46, 6453–6454. https://doi.org/10.1021/es302011r
Rochman, C.M., Kurobe, T., Flores, I., Teh, S.J., 2014. Early warning signs of endocrine disruption in adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from the marine environment. Sci. Total Environ. 493, 656–661. https://doi.org/10.1016/j.scitotenv.2014.06.051
Rosenkranz, P., Chaudhry, Q., Stone, V., Fernandes, T.F., 2009. A Comparison of Nanoparticle and Fine Particle Uptake by Daphnia magna. Environ. Toxicol. Chem. 28, 2142–2149.
Roved, J., Westerdahl, H., Hasselquist, D., 2017. Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. Horm. Behav. 88, 95–105. https://doi.org/10.1016/j.yhbeh.2016.11.017
Roy, N., Ohtani, K., Hidaka, Y., Amano, Y., Matsuda, Y., Mori, K., Hwang, I., Inoue, N., Wakamiya, N., 2017. Three pentraxins C-reactive protein, serum amyloid p component and pentraxin 3 mediate complement activation using Collectin CL-P1. Biochim. Biophys. Acta - Gen. Subj. 1861, 1–14. https://doi.org/10.1016/j.bbagen.2016.11.023
Schulze, R.J., Schott, M.B., Casey, C.A., Tuma, P.L., Mcniven, M.A., 2019. Beyond the Cell The cell biology of the hepatocyte: A membrane trafficking machine. https://doi.org/10.1083/jcb.201903090
Schwabl, P., Koppel, S., Konigshofer, P., Bucsics, T., Trauner, M., Reiberger, T., Liebmann, B., 2019. Detection of various microplastics in human stool: A prospective case series. Ann. Intern. Med. 171, 453–457. https://doi.org/10.7326/M19-0618
Schymanski, D., Goldbeck, C., Humpf, H.U., Fürst, P., 2018. Analysis of microplastics in water by micro-Raman spectroscopy: Release of plastic particles from different

packaging into mineral water. Water Res. 129, 154–162. https://doi.org/10.1016/j.watres.2017.11.011
Seifert, J., Haraszti, B., Sass, W., 1996. The influence of age and particle number on absorption of polystyrene particles from the rat gut. J. Anat. 189, 483–486. https://doi.org/10.1007/s004490050709
Smith, M., Love, D.C., Rochman, C.M., Neff, R.A., 2018. Microplastics in Seafood and the Implications for Human Health. Curr. Environ. Heal. reports 5, 375–386. https://doi.org/10.1007/s40572-018-0206-z
Sproston, N.R., Ashworth, J.J., 2018. Role of C-reactive protein at sites of inflammation and infection. Front. Immunol. 9, 1–11. https://doi.org/10.3389/fimmu.2018.00754
Stock, V., Böhmert, L., Lisicki, E., Block, R., Cara-Carmona, J., Pack, L.K., Selb, R., Lichtenstein, D., Voss, L., Henderson, C.J., Zabinsky, E., Sieg, H., Braeuning, A., Lampen, A., 2019. Uptake and effects of orally ingested polystyrene microplastic particles in vitro and in vivo. Arch. Toxicol. 93, 1817–1833. https://doi.org/10.1007/s00204-019-02478-7
Tenzer, S., Docter, D., Kuharev, J., Musyanovych, A., Fetz, V., Hecht, R., Schlenk, F., Fischer, D., Kiouptsi, K., Reinhardt, C., Landfester, K., Schild, H., Maskos, M., Knauer, S.K., Stauber, R.H., 2013. Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. Nat. Nanotechnol. 8, 772–781. https://doi.org/10.1038/nnano.2013.181
Thompson, R.C., 2015. Marine Anthropogenic Litter. Springer. https://doi.org/10.1007/978-3-319-16510-3
Thorgersen, E.B., Barratt-Due, A., Haugaa, H., Harboe, M., Pischke, S.E., Nilsson, P.H., Mollnes, T.E., 2019. The Role of Complement in Liver Injury, Regeneration, and Transplantation. Hepatology 70, 725–736. https://doi.org/10.1002/hep.30508
Tomazic-Jezic, V.J., Merritt, K., Umbreit, T.H., 2001. Significance of the type and the size of biomaterial particles on phagocytosis and tissue distribution. J. Biomed. Mater. Res. 55, 523–529. https://doi.org/10.1002/1097-4636(20010615)55:4<523::AID-JBM1045>3.0.CO;2-G
Veneman, W.J., Spaink, H.P., Brun, N.R., Bosker, T., Vijver, M.G., 2017. Pathway analysis of systemic transcriptome responses to injected polystyrene particles in zebrafish larvae. Aquat. Toxicol. 190, 112–120. https://doi.org/10.1016/j.aquatox.2017.06.014
von Moos, N., Burkhardt-Holm, P., Köhler, A., 2012. Uptake and effects of microplastics on cells and tissue of the blue mussel Mytilus edulis L. after an experimental exposure. Environ. Sci. Technol. 46, 11327–11335. https://doi.org/10.1021/es302332w
Walczak, A.P., Hendriksen, P.J.M., Woutersen, R.A., van der Zande, M., Undas, A.K., Helsdingen, R., van den Berg, H.H.J., Rietjens, I.M.C.M., Bouwmeester, H., 2015. Bioavailability and biodistribution of differently charged polystyrene nanoparticles upon oral exposure in rats. J. Nanoparticle Res. 17. https://doi.org/10.1007/s11051-015-3029-y
Wang, L., Shi, S., Guo, Z., Zhang, X., Han, S., Yang, A., Wen, W., Zhu, Q., 2013. Overexpression of YAP and TAZ Is an Independent Predictor of Prognosis in Colorectal Cancer and Related to the Proliferation and Metastasis of Colon Cancer Cells. PLoS One 8. https://doi.org/10.1371/journal.pone.0065539
Watts, A.J.R., Urbina, M.A., Corr, S., Lewis, C., Galloway, T.S., 2015. Ingestion of Plastic Microfibers by the Crab Carcinus maenas and Its Effect on Food Consumption and Energy Balance. Environ. Sci. Technol. 49, 14597–14604. https://doi.org/10.1021/acs.est.5b04026
Wright, S.L., Kelly, F.J., 2017. Plastic and Human Health: A Micro Issue? Environ. Sci.

Technol. 51, 6634–6647. https://doi.org/10.1021/acs.est.7b00423
Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013. Microplastic ingestion decreases energy reserves in marine worms. Curr. Biol. 23, 1–3. https://doi.org/10.1016/j.cub.2013.10.068
Wu, X., Pan, J., Li, M., Li, Y., Bartlam, M., Wang, Y., 2019. Selective enrichment of bacterial pathogens by microplastic biofilm. Water Res. 165, 114979. https://doi.org/10.1016/j.watres.2019.114979
Xie, X., Deng, T., Duan, J., Xie, J., Yuan, J., Chen, M., 2020. Exposure to polystyrene microplastics causes reproductive toxicity through oxidative stress and activation of the p38 MAPK signaling pathway. Ecotoxicol. Environ. Saf. 190, 110133. https://doi.org/10.1016/j.ecoenv.2019.110133
Xu, H., Verbeken, E., Vanhooren, H.M., Nemery, B., Hoet, P.H.M., 2004. Pulmonary toxicity of polyvinyl chloride particles after a single intratracheal instillation in rats. Time course and comparison with silica. Toxicol. Appl. Pharmacol. 194, 111–121. https://doi.org/10.1016/j.taap.2003.09.018
Yang, D., Shi, H., Li, L., Li, J., Jabeen, K., Kolandhasamy, P., 2015. Microplastic Pollution in Table Salts from China. Environ. Sci. Technol. 49, 13622–13627. https://doi.org/10.1021/acs.est.5b03163
Yu, F.X., Guan, K.L., 2013. The Hippo pathway: Regulators and regulations. Genes Dev. 27, 355–371. https://doi.org/10.1101/gad.210773.112
Zafar, M., Naqvi, S.N.-H., 2010. Effects of STZ-Induced Diabetes on the Relative Weights of Kidney, Liver and Pancreas in Albino Rats: A Comparative Study. Int. J. Morphol. 28. https://doi.org/10.4067/s0717-95022010000100019
Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the “plastisphere”: Microbial communities on plastic marine debris. Environ. Sci. Technol. 47, 7137–7146. https://doi.org/10.1021/es401288x
Zheng, H., Wang, J., Wei, X., Chang, L., Liu, S., 2021. Proinflammatory properties and lipid disturbance of polystyrene microplastics in the livers of mice with acute colitis. Sci. Total Environ. 750.
Zhou, Z., Xu, M.J., Gao, B., 2016. Hepatocytes: A key cell type for innate immunity. Cell. Mol. Immunol. 13, 301–315. https://doi.org/10.1038/cmi.2015.97
Ziajahromi, S., Kumar, A., Neale, P.A., Leusch, F.D.L., 2017. Impact of Microplastic Beads and Fibers on Waterflea (Ceriodaphnia dubia) Survival, Growth, and Reproduction: Implications of Single and Mixture Exposures. Environ. Sci. Technol. 51, 13397–13406. https://doi.org/10.1021/acs.est.7b03574

Appendices

Appendix 1: Histological preparations

1. Tissues were fixed in 10% neutral buffered formalin for at least 24 hours at room temperature.

Reagent	**Quantity**
Formaldehyde 37-40%	100mL
Distilled water	900mL
Sodium phosphate monobasic	4g
Sodium phosphate dibasic	6.5g
Adjust the pH to 6.8, if necessary.	

2. After trimming, tissues were dehydrated as follows:

70% ethanol: 1 hour

80% ethanol: 1 hour

95% ethanol: 1 hour

100% ethanol I: 1 hour

100% ethanol II: 1 hour

Xylene I: 1 hour

Xylene II: 1 hour

3. Tissues were infiltrated with paraffin wax (Fisher Scientific TissuePrep®) in stainless steel base molds on a hot plate at 95°C. The tissues were infiltrated with paraffin twice, for 1 hour each. After infiltration, samples were embedded in paraffin and immediately placed on ice to for fast cooling. Embedded samples were kept at room temperature until sectioning.

4. Samples were sectioned at 5µm thickness using a Shandon AS325 microtome. Sections were 10-60µm apart in order to sample different depths of the tissue as follows:

Tissue	**µm apart**
Spleen	10
Ovary	10
Liver	50
Kidney	60
Small intestine	50
Large intestine	25

5. The sections were floated in waterbath at 42°C (Fisher TissuePrep^{TM} flotation bath, model 134) and placed on glass slides.

6. The slides were air dried and placed on a hot plate for 30 minutes at 60°C to allow the sections to adhere to the slide.

7. Slides were dewaxed and hydrated as follows:

Xylene I: 10 minutes

Xylene II: 10 minutes

100% Ethanol I: 5 minutes

100% Ethanol II: 5 minutes

95% Ethanol: 2 minutes

70% Ethanol: 2 minutes

Washing in running tap water: 2 minutes

8. Slides were stained as follows:

Harris hematoxylin: 2 minutes

Washing in running tap water: 3 minutes

Acid-alcohol solution: 10 dips

Washing in running tap water: 1 minute

95% ethanol: 30 seconds

Eosin Y: 30 seconds

95% ethanol: 2 minutes

100% ethanol I: 1 minute

100% ethanol II: 1 minute

Xylene: 5 minutes

Cover slipping using Permount® mounting media.

Appendix 2: Total RNA extraction protocol (TRIzol method)

The protocol was obtained from Invitrogen User Guide.

1. 1 mL of TRIzol® Reagent (Ambion, Life Technologies, USA) was added per 50–100 mg of tissue and the sample was homogenized using a tissue homogenizer (Tissue-Tearor, Biospec Products). The sample volume should not exceed 10% of the volume of TRIzol® Reagent used for lysis.

2. The homogenized sample was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoproteins complex.

3. 0.2 mL of chloroform (Sigma-Aldrich) was added per 1 mL of TRIzol® Reagent used for lysis and sample was incubated for 2-3 minutes at room temperature.

4. Sample was centrifuged for 15 minutes at 12,000 x g at 4°C (Eppendorf refrigerated centrifuge 5417R). The mixture separated into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase.

5. The aqueous phase containing the RNA was transferred to a new tube by angling the tube at 45° and pipetting the solution out carefully to avoid transferring any of the interphase or organic layer into the pipette.

6. 0.5 mL of isopropanol (Fisher Scientific) was added to the aqueous phase, per 1 mL of TRIzol® Reagent used for lysis and sample was incubated for 10 minutes at room temperature.

7. Sample was centrifuged for 10 minutes at 12,000 x g at 4°C.

8. Total RNA precipitate formed a white gel-like pellet at the bottom of the tube. The supernatant was discarded.

9. The pellet was resuspended in 1 mL of 75% ethanol (BDH®) per 1 mL of TRIzol® Reagent used for lysis. The sample was vortexed briefly.

10. Sample was centrifuged for 5 minutes at 7500 x g at 4°C and the supernatant was discarded.

11. The RNA pellet was air dried for 5–10 minutes at room temperature.

12. The pellet was resuspended in 20 - 100 µL of nuclease-free water (Invitrogen) by pipetting up and down.

13. Sample was incubated in a water bath at 55–60°C for 10–15 minutes.

14. The RNA yield was measured using a nanophotometer (NP80, Implen) using nuclease-free water as a blank. Samples with a 260/280 ratio close to 2.0 and concentration above 500ng/µL were considered acceptable for downstream applications.

RNA samples were stored at -80°C until reverse transcription.

Appendix 3: Reverse transcription (cDNA synthesis)

The reverse transcription protocol was obtained from Thermo Fisher Scientific user guide with addition of a pre-treatment with nuclease digest. All reagents were thawed on ice and homogenized through vortexing and spinning. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, Lithuania).

1. RNA samples were pre-treated with nuclease digest as follows:

Reagent	**Volume (µL)**
RNase free water	5.0*
RNA sample (2µg AKA 2000ng)	3.0*
10x DNase I buffer	1.0
DNase I	1.0
Total volume	**10**

*Note: The volume of RNA sample was calculated to correspond to 2µg depending on the extraction concentration. Water was added to complete the total volume (10µL).

2. Samples were incubated for 15 minutes at room temperature.

3. 1µL of 25mM EDTA was added to each sample.

4. After homogenization, samples were incubated for 10 minutes at 65°C using a thermal cycler (Techne Progene).

5. The 2X Reverse Transcription master mix was prepared as follows:

Reagent	Volume (µL)
10x RT buffer	2.0
25x dNTP mix	0.8
10x random primer	2.0
Multiscribe reverse transcriptase	1.0
RNase inhibitor	1.0
Nuclease-free water	3.2
Total	**10.0**

6. 10 µL of 2X RT master mix was pipetted into each 0.2mL PCR tube.

7. 10 µL of RNA sample from the nuclease digest was added to each tube, pipetting up and down two times to mix.

8. Samples were vortexed and centrifuged to spin down the contents and to eliminate any air bubbles.

9. Samples were loaded into the thermal cycler programmed for the following conditions:

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	hold

10. The cDNA yield was measured using a NP80 NanoPhotometer (Implen, Munich, Germany) using a solution of nuclease-free water (15µL) and 10x RT buffer (2µL) as a blank. Samples with a 260/280 ratio between 1.6-1.8 and concentration above 500ng/µL were considered acceptable for downstream application.

11. cDNA samples were diluted (1:80), aliquoted and stored at -20°C until qPCR.

Appendix 4: TaqMan RT-PCR

This protocol was adapted from Thermo Fisher Scientific and Corbett Rotor-Gene 6000 user guides. All test samples (expression assay), no template control (NTC), and normalizer genes (18S ribosomal RNA and GADPH) were run in duplicate. Statistical analysis was performed using the average from 3 independent runs.

1. The efficiency of the reaction was determined for both target and reference genes before running the test samples. cDNA from treated and control samples were pooled together to make a 2-fold serial dilution across 5 points. This serial dilution was used to stablish a standard curve (concentration plotted against the Ct values). The efficiency was calculated as follows:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

Efficiency between 90 – 105% and $R^2 > 0.985$ were considered acceptable.

2. All reagents were thawed on ice. The reagents were resuspended by vortexing then briefly centrifuged. The reaction mix was prepared as follows:

Reagent	**Volume (µL)**
20x Taqman Expression Assay	1.0
2x Taqman Gene Expression Master Mix	10.0
cDNA template*	4.0
RNase free water	5.0
Total volume	**20.0**

*Note: The volume of cDNA template was calculated to correspond to approximately 100ng, not exceeding 20% of the PCR reaction mix. Water was added to complete the total volume (20µL).

3. Reactions were prepared in 0.1mL strip tubes placed into a precooled loading block.

4. Corbett Rotor-Gene 6000 was set as follows:

Stage	Temperature	Time
Hold	95°C	10 minutes
Cycle (x50)	95°C	15 seconds
	58 - 60°C	60 seconds

Channel set up:
Name = green
Source = 470nm
Detection = 510nm
Gain = 10

The "Perform Optimization Before 1st Acquisition" function was selected.

5. The Ct values were calculated by importing the appropriate standard curve and following slope correction by the Rotor-Gene software.

6. Relative quantification of mRNA levels was performed according to $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). Briefly, $\triangle$Ct was calculated by subtracting the geometric mean Ct of the normalizer genes (*18s* and *Gapdh*) from the target gene Ct. Then, $\triangle\triangle$Ct was calculated by subtracting the control group average $\triangle$Ct from each individual $\triangle$Ct. The fold change was calculated by the formula $2^{-\triangle\triangle Ct}$ for each mouse.