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Effects of weathered polyethylene microplastic ingestion on sexual maturation, fecundity and egg quality in maturing broodstock Atlantic cod *Gadus morhua*☆

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ABSTRACT

Microplastics (MPs) have become a global issue as they are omnipresent in the ocean. Fish ingesting MPs through feed could be affected in their physiological function, e.g., disrupted enzyme production and function, reduction of feeding and reproductive failure. This study assessed the effects of feed containing naturally weathered MPs from the Oslofjord (Norway) on the reproductive physiology of Atlantic cod (*Gadus morhua*). Farmed cod broodstock were fed either control (C-diet) or feeds containing 1% microplastic (MP-diet) starting nine months prior to spawning, from June until May. No major differences were found between diet groups in overall biometrics or gonad histology. Sex steroid levels (testosterone, 11-ketotestosterone and 17β-estradiol) resulted in expected profiles increasing over time without any significant differences between treatments. Gene expression levels of the steroidogenic enzyme 20β-hydroxysteroid dehydrogenase (*20β-hsd*) and vitellogenin1 (*vtg1*) showed significant differences between dietary treatments with lower expression in the control group. This can be a direct effect of MPs, but endocrine disrupting effects of potentially leachable plastic additives cannot be completely ruled out. Thus, these enzymes could be indicators of exposure to contaminants that disrupt sexual maturation by affecting the production of primarily maturation-inducing steroid. Although the concentration of MPs employed in this study may not be high enough to elicit any observable short-term biological effects, the observed gene expression suggests that long-term consequences should be considered caused by an expected increase of MPs in marine environments.

1. Introduction

The increase in production of plastics over the last few decades has made its litter, arising from the inappropriate disposal of plastic products, a major worldwide problem (MacLeod et al., 2021). For instance, plastics comprise the main litter source (80%–90%) collected from beaches (Sundt et al., 2014). Over time, plastic litter breaks down to fragments, with those smaller than 5 mm called microplastics (MPs) (GESAMP, 2015). Besides this breakdown of larger plastic particles (secondary), MPs also occur in the environment due to the release of manufactured MPs (primary) from products such as personal care products, medicines or with laundry wastewater (Wright et al., 2013). MPs can be found throughout the aquatic environment, and concentrate in harbours, industrial coastal areas and ocean gyres (Goldstein et al., 2013; Bronzo et al., 2021). In recent years, the ingestion of MPs has been described in various aquatic systems (Vázquez-Rowe et al., 2021) and many animal taxa, including zooplankton (Cole et al., 2013), fish (Foekema et al., 2013; Kibria et al., 2022), birds (Wang et al., 2021) and marine mammals (Zantis et al., 2020). Much ongoing research focuses on the effects of MPs entering and channelling up to several trophic levels through aquatic food chains (Prinz and Korez, 2020; Bronzo et al., 2021; Kibria et al., 2022), but it has also been a field of increased

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attention to identify the short and/or long-term effects on marine bioresources (Wang et al., 2019; De Vries et al., 2020; Solomando et al., 2020; Kim et al., 2021; Rios-Fuster et al., 2021; Mkuye et al., 2022).

Microplastics consisting of e.g., polyethylene, polypropylene and polystyrene, are reported to be ingested by fish, but their effects on fish health and possible routes of transport upon gut absorption are still unclear (Roch et al., 2020; Kim et al., 2021; Rios-Fuster et al., 2021). MP exposure was reported to have several effects on fish. For instance, MPs induced liver toxicity, hepatic stress and the disruption of endocrine function in Japanese medaka, *Oryzias latipes* (Rochman et al., 2013, 2014), while in zebrafish (*Danio rerio*), MP exposure led to inflammatory responses of the innate immune system (Brun et al., 2018) and the disruption of metabolic processes (Lu et al., 2016). MPs may also cause disturbance in reproductive physiology affecting fecundity and offspring performance due to decreased oocyte diameter and sperm mobility, thus reducing the fertilization rate (Sharifinia et al., 2020; Wang et al., 2019). The question of interactions with chemical contaminants has been discussed, including MPs operating as a passive sampler of previously ingested chemicals (Mohamed Nor and Koelmans, 2019). However, MP ingestion did not cause a measurable enhancement of pollutants depuration in rainbow trout (*Onchorhynchus mykiss*) (Rummel et al., 2016). These studies point out the possibility of different effects of MPs, presumably influenced by upper and lower size range, polymer type, concentration, sorption capacity, and the species involved.

Furthermore, plastics contain many chemicals as additives, ensuring certain functionalities of the plastic material, and have also been shown to adsorb hydrophobic persistent organic pollutants (POPs) from the environment, favoured by their high surface area to volume ratio (Goldstein et al., 2013; Fauser et al., 2022). POPs include chemical contaminants such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). These chemicals can be desorbed from MPs faster in the gut of organisms than in natural water due to processes in the digestion and concentration driven transfer of chemicals (Bakir et al., 2014). In theory, the ingestion of MPs could lead to increased toxic effects from POPs and plastic additives some of which are known to be carcinogenic and endocrine disruptors (Oehlmann et al., 2009; Fauser et al., 2022). The concentration of adsorbed POPs in weathered MPs are generally low, but assumed to be higher close to point sources and cities.

Atlantic cod (*Gadus morhua*) is an important commercial finfish in the North Atlantic. In Norway, its habitat ranges from the continental shelf edge to the inner parts of fjords where it is exposed to anthropogenic litter (Foekema et al., 2013). Available studies of MP ingestion by Atlantic cod have shown that 3% of the studied individuals on the Norwegian coast had MPs in their stomach (Bråte et al., 2016), which is lower than in other areas such as the North Pacific (35%; Boerger et al., 2010) or the English Channel (37%; Lusher et al., 2013). However, studies on how naturally weathered MP particles affect the digestion, reproductive physiology and egg production in Atlantic cod are still limited.

The aim of this study was to explore the effects of 1% MPs, retrieved after natural weathering in the sea and added to feed, on digestion, gonadal development, and reproductive endocrinology performance (i.e., egg production and fertilization success) of Atlantic cod, from early maturation until past spawning. Such knowledge is essential to understand potential consequences of the anticipated increase of MPs in marine environments and to direct further research and monitoring in this field.

2. Material and methods

All animal procedures and handling described in this study were carried out according to Norwegian Animal welfare laws and were approved by the Norwegian Animal Research Authority (Forsøksdyrutvalget; FOTS ID 11236).

2.1. Feed composition

2.1.1. Sorption of contaminants to microplastics

The MPs used in this study originated from an industrial linear low density polyethylene powder (ICORENE®3545, LyondellBasell, Åstorp, Sweden) purchased from Plastinvent AS (Radøy, Norway) that use it for rotational moulding. The powder were sieved at the Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima AS) to a fraction between 0.3 and 0.6 mm. Five-hundred grams of this powder were packed in water permeable filter bags (pore size 0.2 mm, NMO20012X18D, Midwest Filter LLC, West Chicago, USA) and deployed in the inner areas of the Oslofjord (59.88° N, 10.68° E; Oslo County) at 2–3 m depth for 4 months to study the sorption of POPs. After weathering, no biofilm on the MPs were observed only outside the permeable bags. Detailed information about the weathering process and POP concentration before and after weathering of MPs, silicone and polyoxymethylene at the Oslofjord and seven other locations along the coast of Norway will be published elsewhere (Vorkamp et al., 2019). Briefly, the polymers were analysed for congeners of PCB, PBDE, hexabromocyclododecane (HBCD), toxaphene (CHB) and chlordane-related pesticides. Weathered MPs sampled from the Oslofjord showed from the sum of congeners a 44-fold increase in PCB, 5-fold increase in PBDE, 8-fold increase in HBCD, 2-fold increase in CHB and 17-fold increase of chlordane-related pesticides compared to clean MP in its original form.

2.1.2. Microplastics in experimental feeds

Control (C-diet) and microplastic (MP-diet) feed were produced from the same ingredient mix at the Nofima Feed Technology Centre in Bergen (Suppl. Table 1; Suppl. Table 2) with the only difference that 1% weathered MPs (low density PE at 0.3–0.6 mm size) was added to the experimental diet (MP-diet) prior to extrusion. The contribution of accumulated contaminants (PCBs and PBDEs) from MPs in the fish feed was low compared to the levels in the feed ingredients (Suppl. Table 3). Any effects on the cod broodstock fed the experimental diet were assumed to be the result of MPs inclusion in the diet, because the concentrations of POPs in the control and the experimental feed were similar (Bogevik et al., in preparation).

2.2. Broodstock maintenance

In June 2017, four hundred farmed Atlantic cod broodstock (four years old; 5.5 ± 1.6 kg average weight) of third generation (F3) were transferred from sea cages (Centre for Marine Aquaculture – CMA sea cage facility, Røsnes, Norway) to six 25 m^3 tanks located at a CMA land-based facility (Kvaløya, Tromsø). Three tanks (29–37 fish per tank) were established as the control group (C1–C3) and three other tanks conformed the experimental group (MP1-MP3). Fish were fed diets *ad libitum* and according to their appetite, previously estimated to regulate the optimal food intake, i.e. three times a week from early maturation (June 2017; 5.5 kg) until spawning (March–April 2018; 7.1 kg). The light and temperature regimes followed the natural rhythm of Tromsø, Norway (69.6492° N, 18.9553° E).

2.3. Broodstock sampling

Based on the general model of cod oocyte maturation by Kjesbu et al. (2010), fish were sampled at 5 time points: June 2017 (initial sampling after the spawning and post-spawning recovery), September 2017 (prior to the start of vitellogenesis), December 2017 (mid/late vitellogenesis), late February 2018 (gonadal maturation) and May 2018 (post spawning, only biometric measurements). At the initial sampling, 16 fish (8 males and 8 females) were sampled from the common pool (without treatment separation), while in subsequent samplings 16 fish per tank were sampled targeting equal number of samples from both sexes. Sex identification was difficult, especially at early maturation due to the lack of external signs, resulting in uneven sex ratio among samples (Suppl.

Table 1
Names, abbreviations, primer sets, annealing temperature (Tm, °C), efficiency (Eff., %) and correlation index (R2) of the analysed genes and target tissues (B, Brain; P, Pituitary; G, Gonad and L, Liver).

Gene	Code	5′-Forward primer-3′	5′-Reverse primer-3′	Tm	Tissue	Eff	R2	Source
Target genes								
Luteinizing Hormone Subunit Beta	*lhb*	GTGGAGAAGAAGGGCTGTCC	GGACGGGTCCATGGTG	60	P	112.73	0.993	Hodne et al., 2010/ Von Krogh et al., 2017
Luteinizing hormone receptor	*lhr*	GCCACTGTTGTGCCTTCCA	GAGCCTTGTGAGGTTCTTTAATGC	62	G	114.91	0.965	Mittelholzer et al., 2009/Breton et al. (2012)
Follicle Stimulating Hormone Subunit Beta	*fshb*	GAACCGAGTCCATCAACACC	GGTCCATCGGGTCCTCCT	60	P	107.39	0.997	Hodne et al., 2010/ Von Krogh et al., 2017
Follicle Stimulating Hormone Receptor	*fshr*	CACGCCAACCTCACCTATCAC	TGAACAGATGGAGTCCCACTTG	62	G	100.11	0.973	Mittelholzer et al., 2009/Breton et al. (2012)
Estrogen receptor	*esr1*	CGCTTTCGGATGCTCCAG	ACGAGAAGGCCCCAGAGTTG	60	B, P, G, L	103.64	0.991	Yadetie et al. (2018)
Vitellogenin 1	*vtg1*	AGACTGGCCTGGTCGTCAAA	GCGAGGATAGAGGCAGGGAT	62	L	84.46	0.993	Yadetie et al. (2018)
20β-hydroxysteroid dehydrogenase	*20β-hsd*	ATCACTGTGCAGTGTGTGG	GCAGCCGTTGGTGTAGTT	60	G	104.27	0.984	Dale et al. (2019)
Gonadotropin-Releasing Hormone 3	*gnrh3*	AGAAGTGTGGGAGAGCTGGA	CCTCGTCGTTGACCAGACT	60	B	104.94	0.986	Hildahl et al. (2011)
Gonadotropin Releasing Hormone Receptor 2a	*gnrhr2a*	TTCACCTTCTGCTGCCTCTT	TCCGTGGAGGAAAGATTGTC	60	P	115.41	0.977	von Krogh et al., 2017
Gonadotropin-Releasing Hormone 2	*gnrh2*	CACTGGTCTCATGGCTGGTA	GGCCAGGACATCCATAAAGA	62	B	90.03	0.956	Hildahl et al. (2011)
Reference genes								
Translation elongation factor 1alpha	*ef1α*	CGGTATCCTCAAGCCCAACA	GTCAGAGACTCGTGGTGCATCT	60	B, P, G, L	108.32	0.992	Dale et al. (2019)
β-Actin	*actb*	CGACGGGCAGGTCATCACCATCG	CCACGTCGCACTTCATGATGCTGT	60	B, P, G, L	101.68	0.991	Yadetie et al. (2018)

Table 2
Spawning biometrics of cod broodstock fed with control (C-diet) and microplastics added feeds (MP-diet).

Treatment	Tank no.	Female biomass (kg)	Egg volume (mL)	Egg output (mL kg^{-1})
MP-1	5	50	44	893
MP-2	6	60	64	1073
C-1	7	87	53	614
C-2	5	39	28	702
P-value		0.764	0.489	0.083

Statistics by one-way ANOVA mean effect of diet ($P < 0.05$).

Table 3
Quality parameters of spontaneously and manually stripped eggs of cod broodstock fed control (C-diet) and microplastics added feeds (MP-diet).

Quality parameter	Spontaneous spawning		Manual stripping		*P*-value	
	C-diet	MP-diet	C-diet	MP-diet	Method	Diet
Fertilization success (%)	20.4 ± 8.4[a]	18.6 ± 3.7[a]	50.9 ± 1.8[b]	54.6 ± 1.5[b]	0.001	0.762
Normal cell division (%)	36.8 ± 3.4[a]	38.3 ± 2.3[a]	45.1 ± 2.3[a]	48.0 ± 4.2[a]	0.016	0.382

Mean ± st.dev., n = 2 tanks. Statistics by two-way ANOVA mean effect of egg collection method and diet ($P < 0.05$). Superscripts with different letters indicate significant differences ($P < 0.05$) between bars as determined by Tukey's post hoc test.

Table 4). In September 2017, 16 fish were sampled from each tank (C1, C2, MP1 and MP2) and fish from C3 and MP3 were randomly divided and transferred to C1, C2 and MP1, MP2, respectively. This was done to account for the intense sampling design (5 sampling points including initial control), cost to run six tanks per year and limitation of number of adult fish that can be stocked in one tank at an optimal density to preserve fish welfare (Fig. 1).

Sampling involved random selection of fish that were netted and euthanized in an overdose Finquel (60 mg L^{-1}; Scan Aqua AS, Årnes, Norway). Total body length and weight were measured and blood was collected from the caudal vein using heparinised tubes (BD Vacutainer LH 68 I.U.), centrifuged at 3750 *g* for 10 min to extract blood plasma, and stored at −25 °C for later sex steroid analysis. Gonad, liver, pituitary and brain samples were preserved in RNAlater and stored at −80 °C for quantitative real-time PCR (qPCR) analysis. Total weight of gonad and liver were also recorded. Ovary and testis samples were fixed in 10% buffered formalin for histology analysis. At the sampling point in February 2018, the content of distal gut was collected in glass breakers, pooled per sex in each tank, frozen on dry ice and stored at −20 °C for digestibility analysis. The apparent digestibility for crude protein (and similar for dry matter and total fat) was calculated as follows: $100–100 \times (\frac{\text{Feed I x Faeces CP}}{\text{Feed CP x Faeces I}})$ where I is concentration of digestibility indicator yttrium (III) oxide and CP is crude protein (Nx6.25) content.

2.4. Egg quality measurements

Prior to the anticipated spawning, egg collectors were placed inside the tank and checked daily. When spawning commenced, eggs were collected daily and egg volume registered, in addition to fertilization success and proportion of eggs with normal cell cleavage recorded from a subsample of the egg collected per tank from March 8th to May 22nd' 2018 (204 batches in total during the trial). The fertilization success was calculated as percentage of eggs with symmetrical cell division among 50 cleaved eggs (Hansen et al., 2013). Besides the collection of eggs from the tanks, eggs and sperm were also hand stripped from selected broodstock at mid-spawning season (9 parents per dietary group), cross-fertilized and incubated until hatching. For the stripped eggs, conventional egg quality indicators, such as fertilization success, embryonic development and hatching rate, were recorded and determined based on previous studies (Pickova et al., 1997; Hansen and Puvanendran, 2010).

2.5. Histology

From the late February sampling (gonad maturation), ovary and

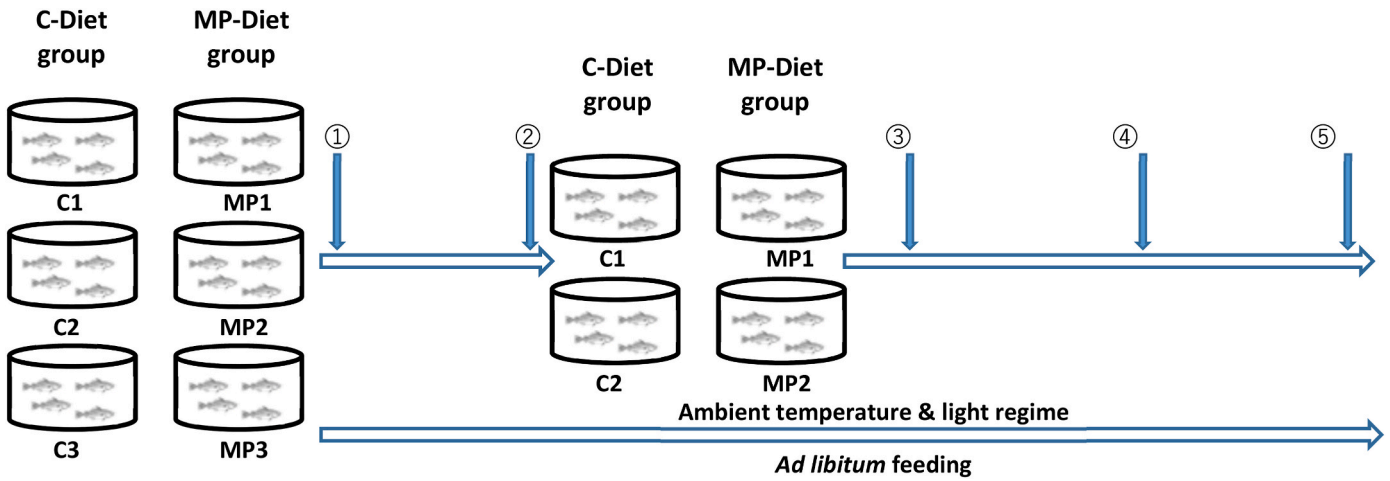

Fig. 1. Schematic diagram the experimental set-up. ① - ⑤ denote the sampling points in June, September, December 2017, February and May 2018, respectively. Tank numbers were reduced from six to four in September 2017 after the second sampling.

testis samples (n = 8–10 per group) were embedded, sectioned and stained at the Norwegian Veterinary Institute (Harstad, Norway). Briefly, prior to embedding in paraffin, samples were dehydrated following an ethanol series and Histo-clear (National Diagnostics). Sections of 4 μm thickness were obtained and were stained with haematoxylin-eosin (H-E). Pictures of the slides sections were captured using a Nikon DS-Fi2 camera coupled to a Nikon Eclipse Ci microscope (Nikon) using the NIH elements program for visualisation. Oogonia were staged following Hall et al. (2004) and differentiated between pre-vitellogenesis, early vitellogenesis, mid-vitellogenesis, late vitellogenesis and spawning (Suppl. Figure 1). To determine the maturation status, oocyte stage percentages were calculated from three different sections per slide by the number of oocytes in a stage divided by the total amount of oocytes. Similarly, testis was staged following Nagasawa et al. (2014) and classified as spermatogonia (SPG), spermatocytes (SPC), spermatids (SPD) and spermatozoa (SPZ) (Suppl. Figure 2). The maturation status was determined based on the percentage of the area obtained per stage, calculated from three different sections per slide.

2.6. Plasma steroid measurements

To investigate possible endocrine disrupting effects of MPs, levels of testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (E2) in blood plasma were analysed using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, USA). Plasma from six fish per dietary treatment and sex sampled September, December and February were selected (72 in total). Prior to the analysis, steroids were extracted using diethyl ether (Sigma-Aldrich) following previously established protocols (Tveiten et al., 2010). Briefly, in a borosilicate glass tube, 200 μL plasma was mixed thoroughly on a plate shaker in 4 ml diethyl ether, and thereafter allowed to separate into an aqueous and organic phase. The aqueous phase was frozen on dry ice and the diethyl ether containing the steroids was transferred into a new borosilicate glass tube. The diethyl ether was evaporated for ca. 30 min using a water bath at 45 °C. The dry extracts were resuspended in 600 μL ELISA buffer for ca. 10 min using a water bath at 45 °C and stored at −20 °C until analysis.

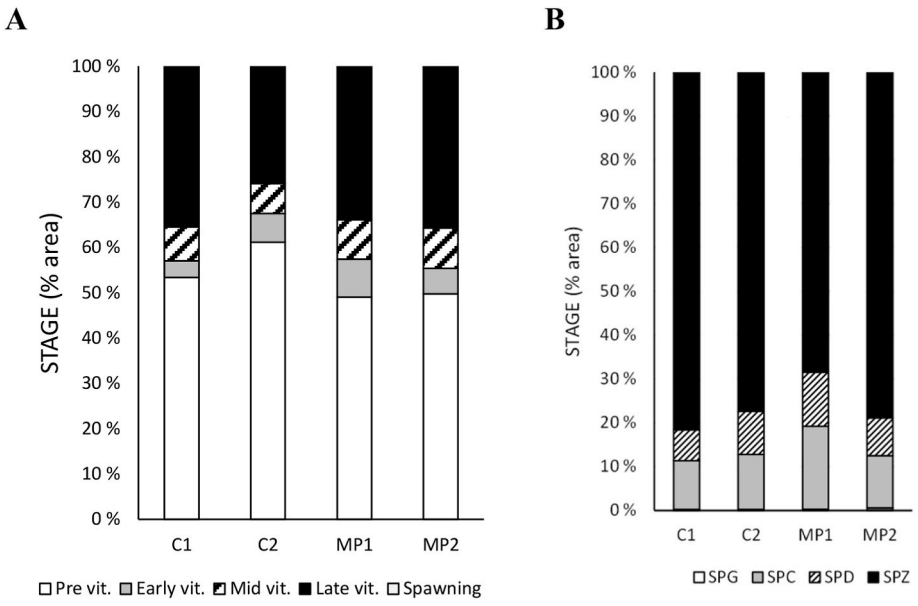

Fig. 2. Gonadal histology in females (**A**) and males (**B**) sampled February 2018. Abbreviations; vit = vitellogenic; SPG = spermatogonia; SPC = spermatocytes; SPD = spermatids; SPZ = spermatozoa.

2.7. Gene expression analysis

The relative expression of genes involved in reproduction was investigated in brain, pituitary, gonad and liver tissues using quantitative real-time PCR (qPCR) analysis. Five males and three females sampled June 2017, and six fish per sex and dietary treatments were selected for RNA analysis from tissue sampled September, December, and February (80 in total). The RNA was extracted from 30 mg of tissue using the total RNA Isolation kit (NZYTech, Lisbon, Portugal) and following the manufacturer's instructions. Since all tissues but pituitary contained high lipid concentration, a final step was added to prevent DNA contamination, i.e., samples were incubated with 5 U of DNase I (Thermo Fisher) at 37 °C for 15 min, followed by enzyme inactivation with chloroform-isoamilic alcohol. RNA integrity and quantity were assessed with the Thermo Scientific NanoDrop Lite Spectrophotometer and visualised by electrophoresis in 2% agarose gels. Reverse transcription was performed from 500 ng of total RNA using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) in a reaction volume of 20 μL using a thermocycler GeneAmpPCRSystem 9700 (Thermo Fisher Scientific).

The amplification efficiency was determined for each primer set assayed using a five-fold standard curve of cDNA. Ten genes were selected for qPCR reactions (Table 1) as seven with optimal efficiency (90–110%). The qPCR was run in duplicate using an ABI 7900HT Real-Time PCR System (Applied Biosystems). Each reaction contained 5 μL NZYSpeedy qPCR Green Master Mix (2×) ROX plus (NZYtech) with SYBR Green Reagent, 0.4 μL 10 μM per primer, 2 μL diluted cDNA and 2.2 μL nuclease-free water to a final reaction volume of 10 μL. The profile of the qPCR consisted of a 2 min activation and denaturing step at 95 °C, followed by 40 cycles of 95 °C for 5s, 30 s for annealing, extension at 72 °C for 30 s and ended by a melting curve stage. The annealing temperature (Tm) ranged between 58 and 62 °C and aimed to achieve standard curve correlation values of 0.99 and efficiency of 100–105%. The SDS 2.3 software (Applied Biosystems) was used for data collection. The C_T mean values of the reference genes, i.e. translation elongation factor 1alpha (ef1α) and β-actin (actb) (Table 1) were used to calculate the relative expression of the target genes by applying the comparative CT method (Xia et al., 2010). This study used the initial sampling ('Start'; June 2017) as calibrator (reference sample) for it was a common factor across tanks before treatments.

2.8. Data analysis

Statistical analyses were conducted using SPSS v26.0 (IBM Corporation, NY, USA). Fish total body weight (BW, kg), HSI (Hepatosomatic index (%) = (liver weight/BW) x 100), GSI (Gonadosomatic index (%) = (gonad weight/BW) x 100) and Fulton's condition factor (K= (W/L3) × 100), where W represents body weight in grams and L fish length in cm) were rank transformed to meet the requirements of normality. Statistical analyses were performed with a three-way ANOVA with sampling date, treatment (MP-diet and control diet) and sex as variables. Significant effects in any variable were followed by Tukey post hoc test. Spawning data (fecundity, fertilization success and normal cleavage) were analysed with one-way ANOVA. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Broodstock biometrics – growth and reproductive indices

In June 2017 the sampled males and females had an average body weight (BW) of 5.0 ± 2.0 kg and 4.3 ± 1.0 kg, respectively (Suppl. Figure 3A). The BW increased during maturation and dropped after spawning; however, no significant differences were observed either between sexes or between diets. The condition factor (CI) increased from 1.0 to 1.5 in the experimental period and peaked prior to spawning

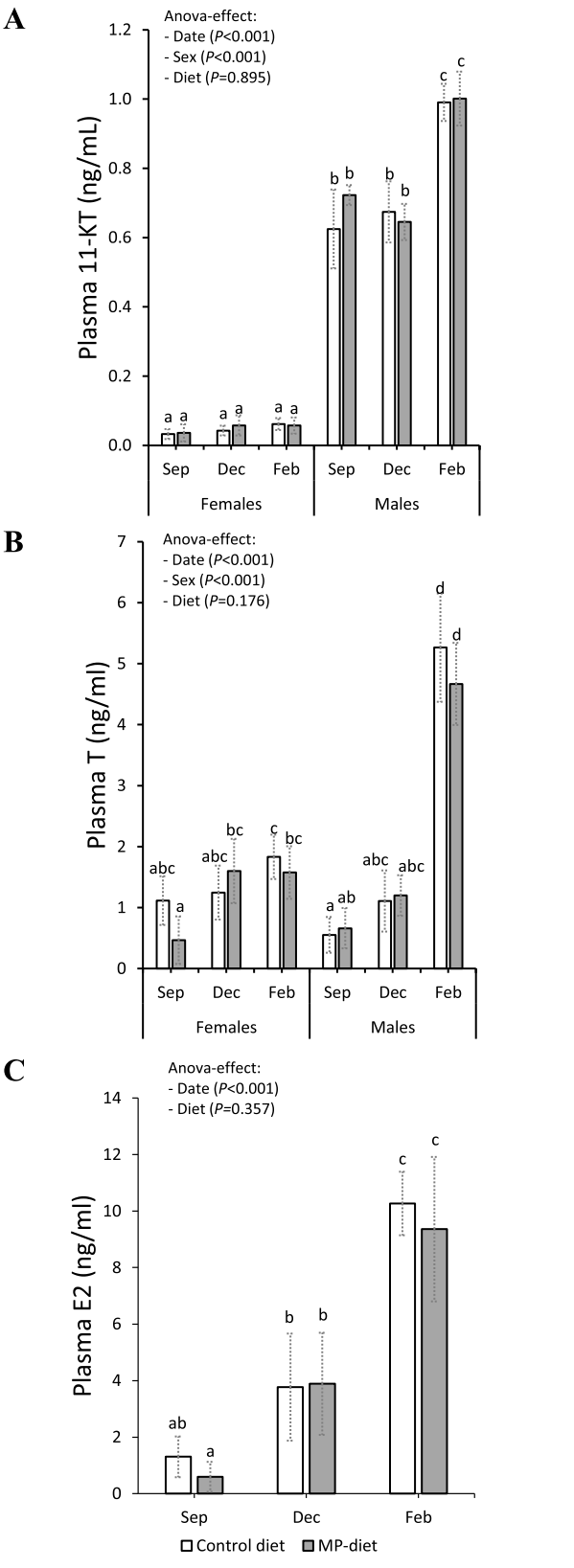

Fig. 3. Plasma levels of **A**) 11-keto testosterone (ng/mL) and **B**) testosterone (ng/mL) in males and females, and **C**) 17β-estradiol (ng/ml) in females. Mean ± SE; n = 6. ANOVA, mean effect of sampling dates, diet and sex. Superscripts with different letters indicate significant differences (P < 0.05) between bars as determined by Tukey's post hoc test.

(Suppl. Figure 3B).

Overall, females showed significantly higher hepatosomatic index (HSI) than males, but there were no effects between diets or among samplings (Suppl. Figure 4A). Similarly, females had larger gonads than males but there were no significant differences in gonad weight between diets. In both sexes, the gonadosomatic index (GSI) gradually increased between September 2017 and February 2018, from 5% to 1%–20% and 15%, respectively (Suppl. Figure 4B).

3.2. Feed intake and digestibility

There were no differences in feed intake between diets. During the first period of the experiment (June–September 2017), the daily feed intake was low (<0.2% of fish weight) and increased during the maturation process (September–December 2017) up to 0.4–0.5%. Feed intake diminished during final maturation and spawning (Suppl. Figure 5A). Pooled content from distal intestine (February 2018; Suppl. Figure 5B) showed no significant differences in apparent digestibility coefficient (ADC) of dry matter, protein and lipid, either between diets or between sexes.

3.3. Histology

The gonadal maturation status showed small differences between treatments, e.g., more pre-vitellogenic oocytes in tank C2 and spermatozoa in tank MP1. Overall, there were no significant differences between diets (Fig. 2). Sampled fish were entering different stages of maturation as females had most of the oocytes at the previtellogenic stage (50–60%; Fig. 2A), while spermatozoa dominated in males (70–80%; Fig. 2B). Gut histology was inconclusive and showed no signs of inflammation or unexpected observations.

3.4. Fecundity and egg quality

No significant differences between diets were found in fecundity, fertilization success and normal cleavage ($P = 0.083$, 0.762 and 0.382, respectively; Tukey post hoc test). One of the tanks fed on the MP-diet tended to produce more eggs (1073 ml egg per kg female) compared to both tank MP1 (893 ml/kg) and control tanks C1 and C2 (614 and 702 ml/kg) (Table 2). The spawning lasted eleven weeks and peaked between weeks 5–7 (Suppl. Figure 6A). Manually stripped cod produced egg of a significantly higher quality than eggs collected after spontaneous spawning, i.e., the average fertilization success was 50–55% vs. 20% and the ratio of normal cell division was 44–48% vs. 37–38%, respectively (Table 3). Eggs from spontaneous spawning also showed a poor quality throughout the spawning season, and their fertilization success showed no tendency to peak at mid spawning (Suppl. Figure 6B).

3.5. Plasma steroids

The plasma content of the sex steroids 11-KT, T and E2 increased during maturation from September 2017 to February 2018. No significant differences were observed between diets. Males had high level of 11-KT and a peak level of T in February 2018, while females had a barely detectable level of 11-KT and a peak level of E2 in February 2018 (Fig. 3).

3.6. Gene expression

The relative expression levels of some genes involved in sexual maturation were analysed in four tissues, i.e., brain, pituitary, gonad and liver, in order to investigate the potential effect of MPs consumption on the reproductive pathways.

3.6.1. Brain tissue

Gonadotropin-releasing hormone 2 (*gnrh2*) expression levels appeared to increase from June 2017 (start) to September 2017 and thereafter decreased toward spawning (February 2018). This was particularly pronounced in females (Fig. 4A). Gonadotropin-releasing hormone 3 (*gnrh3*) expression showed no significant differences among diets, sexes or samplings (Fig. 4B). The estrogen receptor (*esr1*) showed a downregulation towards spawning in females and males. A higher expression level was observed in the MP-diet as compared to the C-diet ($P = 0.05$); however, no significant differences were identified between diets at specific sampling points (Fig. 4C).

3.6.2. Pituitary tissue

In both sexes, the relative expression levels of gonadotropin releasing hormone receptor 2a (*gnrhr2a*) increased over time from June 2017 to February 2018 (Fig. 4D). In females, the expression levels of the luteinizing hormone subunit beta (*lhb*) showed the same trend as *gnrhr2a*. However, upregulation appeared earlier in females compared to males (Fig. 4E). Follicle stimulating hormone subunit beta (*fshb*) exhibited an overall increased expression in the pituitary of all groups after June, except September males fed the C-diet showing a comparable expression to males in June (Fig. 4F). Expression of the estrogen receptor (*esr1*) in the pituitary samples of both sexes showed an overall reduction in expression since the beginning of the trial (Fig. 4G). This reduction was similar in both the MP-diet and the C-diet.

3.6.3. Gonad tissue

Between June 2017 and February 2018, the luteinizing hormone receptor (*lhr*) showed an increase in relative expression levels in both sexes and with only minor differences among MP-diet and C-diet (Fig. 5A). Follicle stimulating hormone receptor (*fshr*) showed overall differences among samplings and sexes. However, the main differences being that males (except MP-diet fish sampled in December 2017) appeared to have similar expression throughout the experiment, while females showed an upregulation in the expression levels of *fshr* in December and February as compared to June and September samples (Fig. 5B). Estrogen receptor (*esr1*) showed similar profiles as *fshr*, upregulation for females in December and February compared to June and September (Fig. 5C). The expression levels of 20β-hydroxysteroid dehydrogenase (*20β-hsd*) exhibited an upregulation over time, higher levels in males compared to females as well as in the MP-diet as compared to the control. Females showed an overall increased expression in time, while this was not observed in males. In addition, there appeared to be differences between diets, not significantly different at the same sampling points, but with a higher expression of *20β-hsd* in the MP-diet in December and February compared to the C-diet. The only significant dietary differences observed were in females with a higher expression in the MP-diet sampled in December compared to females sampled from the C-diet in February ($P = 0.035$; Fig. 5D).

3.6.4. Liver tissues

The relative expression levels of estrogen receptor vitellogenin 1 (*vtg1*) increased in females over time, being on average higher in the MP-diet compared with the C-diet at all time points from September 2017–February 2018 (Fig. 5E). In MP-diet males, *vtg1* expression levels showed a respectively 10 and 80-fold increase in September and December, relative to the start values, which was followed by a decrease in February. On the other hand, the C-diet males showed first a non-significant downregulation between June and December, followed by a significant upregulation between December and February. There was an overall effect of the dietary treatments with a marginal significant higher expression level in cod fed the MP-diet compared to C-diet ($P = 0.056$). The expression levels of estrogen receptor (*esr1*) in females increased in September compared to the initial sampling and a faster regulation appeared to take place in the MP-diet. In males, expression was similar among diets and sampling months (Fig. 5F).

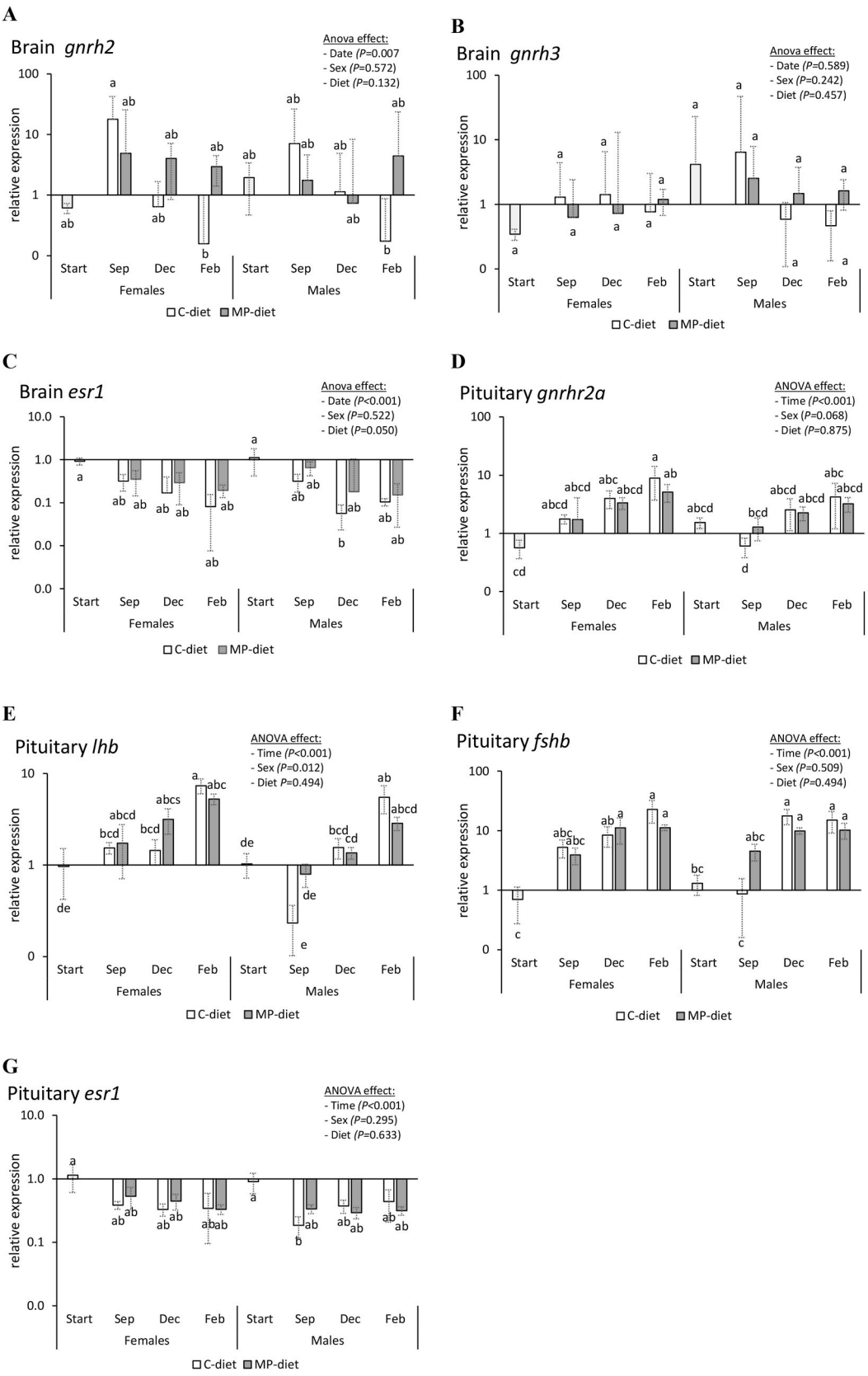

Fig. 4. Variation of relative expression of seven target genes in female and male cod among microplastic (MP-diet) and control (C-diet) feeds from September to February. **Brain:** **A**) *gnrh3*, gonadotropin-releasing hormone 3; **B**) *gnrh2*, gonadotropin-releasing hormone 2; **C**) *esr1*, estrogen receptor. **Pituitary**: **D**) *gnrhr2a*, gonadotropin releasing hormone receptor 2a; **E**) *lhb*, luteinizing hormone subunit beta; **F**) *fshb*, follicle stimulating hormone subunit beta; **G**) *esr1*, estrogen receptor. 'Start' was used as a calibrator sample. Mean ± SE; n = 6. ANOVA, mean effect of sampling dates, diet and sex. Superscripts with different letters indicate significant differences ($P < 0.05$) between bars as determined by Tukey's post hoc test.

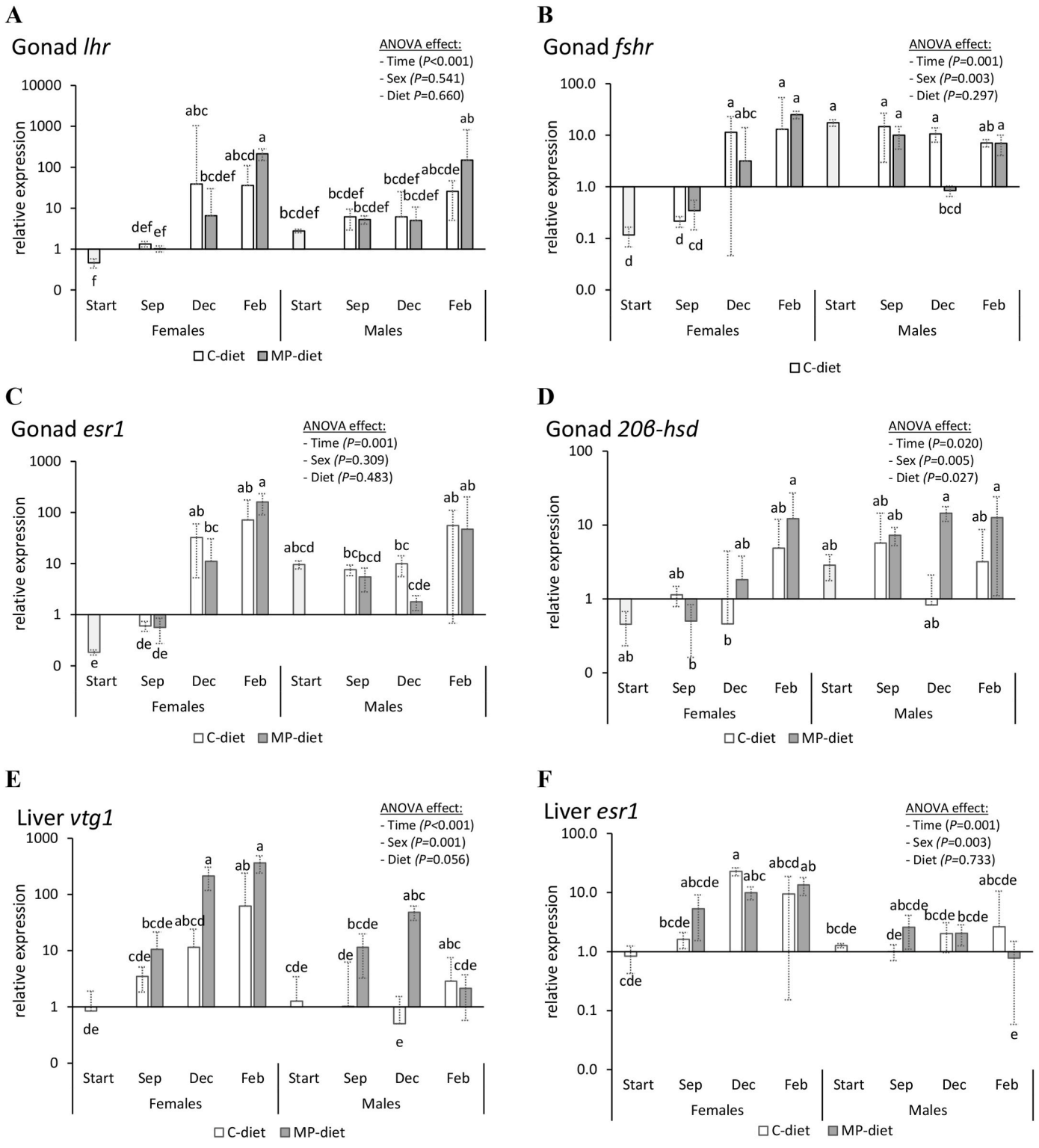

Fig. 5. Variation of relative expression of seven target genes in female and male cod among microplastic (MP-diet) and control (C-diet) feeds from September to February. **Gonad: A**) *lhr*, luteinizing hormone receptor; **B**) *fshr*, follicle stimulating hormone receptor; **C**) *esr1*, estrogen receptor; **D**) *20β-hsd*, 20β-hydroxysteroid dehydrogenase. **Liver: E**) *vtg1*, estrogen receptor vitellogenin 1; **F**) *esr1*, estrogen receptor. 'Start' was used as a calibrator sample. Mean ± SE; n = 6. ANOVA, mean effect of sampling dates, diet and sex. Superscripts with different letters indicate significant differences ($P < 0.05$) between bars as determined by Tukey's post hoc test.

4. Discussion

The present study evaluates the potential effects of naturally weathered MPs on the digestion, gonadal development, endocrine and reproductive performance in Atlantic cod. Plastics can accumulate chemicals from the environment, that, if released in the gut, can exhibit toxic effects (Oehlmann et al., 2009; Goldstein et al., 2013). Here, Atlantic cod was fed a diet including fish meal and oil, with or without

1% MPs. The chemical analysis of the weathered MPs revealed that the concentration of POPs that may have sorbed during the weathering process was low compared to the natural background in fish feed ingredients. Any differences observed between the two types of diets were, therefore, assumed to be related to the presence of MPs in the diets. However, it cannot be completely ruled out that other chemicals not analysed in the present study and/or inherited chemicals in MPs, i.e., chemical additives, could be released in the gut and affect fish health (Schrank et al., 2019).

The most common consequences of the MPs ingestion are the blockage of the digestive tract, feeding reduction followed by false satiation of the fish (Mallik et al., 2021) and a decrease in caloric intake (Galloway et al., 2017), which could be also induced by nonedible natural particles of similar size and shape at high concentrations (Ogonowski et al., 2018). The effects of these non-palatable materials are tightly related to their small size as they can easily be phagocytized and transported into tissues where they may cause inflammatory responses, oxidative stress and the expression of stress-related genes (Brun et al., 2018). However, unrealistically high concentrations are often administered to obtain measurable effects in studies (Lenz et al., 2016), where high exposure levels can provoke effects that are transient under natural conditions (Ogonowski et al., 2018). In the context of this study, 1% naturally weathered MPs added to extruded fish feed showed no significant differences in feed intake, nutrient digestibility and biometrics (e.g., body weight, HSI and GSI) of maturing Atlantic cod. This result is in agreement with previous studies using naturally occurring concentrations of MPs (Ašmonaité et al., 2018). The absence of MP effects on feed intake, nutrient digestibility and biometric measures could be explained by the fact that fish are used to ingesting indigestible particles and their gut is adjusted to deal with such undesired compounds provided their low amount and exposure time (Mallik et al., 2021; De Vries et al., 2020). Nonetheless, these particles can be absorbed and interfere with signal pathways in the gut-brain axis or desorb inherited chemicals (e.g., additives) that may affect physiological functions in the fish (Wang et al., 2019; Zhu et al., 2020).

The posterior movement of particles to the gut or other organs through the circulatory system is mainly governed by particle size, concentration and time of exposure (Bhagat et al., 2020). This size-dependent accumulation is based on the fact that smaller particles (<0.1 mm) could be incorporated in the gut and transported to the liver or other peripheral organ, while larger particles are confined to gill and the digestive tract (Qiao et al., 2019; Bhagat et al., 2020). Smaller MP particles, in particular at the nanoscale, are also likely to cause more pronounced adverse effects (Kögel et al., 2020). However, studies in this size range are still challenged by methodological limitations. Besides MP size, concentrations and exposure time (Kögel et al., 2020), shape has also been reported to influence MPs toxicity as fibers tend to embed easily into the tissue as compared to fragments and have longer gut passage time than spherical shape beads (Bhagat et al., 2020). In this study polyethylene powder at 0.3–0.6 mm were used in the feeds. These particles were most likely transported through the gastrointestinal tract (GI), but it is unknown if the MPs had an impact on gastrointestinal functions, although no effect on feed intake and nutrient digestibility were observed. This is in agreement with Ašmonaité et al. (2018) who observed no measurable effects on fish intestinal permeability, active transport or electrophysiology in rainbow trout (*Oncorhynchus mykiss*) after 4 weeks of 0.1–0.4 mm polystyrene exposure.

Sexual maturation in fish is controlled by the brain–pituitary–gonad (BPG) axis. In the brain gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary, which in turn stimulate gonadal steroidogenesis and gametogenesis for the production of sex steroids and gametes, respectively (Kagawa et al., 1982; Weltzien et al., 2004; Rocha et al., 2008; Von Krogh et al., 2017). Most of the impacts on reproductive systems resulting from MP exposure tend to be induced indirectly by oxidative stress, apoptosis of germ cells, or energy shift to consumption for growth instead of reproduction (Qiang and Cheng, 2021). However, no differences in histological maturation status, fecundity or egg quality measures were observed between treatments in the present study. This result may suggest that the exposure concentration of MPs should be higher, the particle size should be smaller or the exposure time should be longer in order to observe the changes reported by Qiang and Cheng (2021).

The presence of MPs and the associated chemicals have also been described as potential endocrine disruptors affecting estrogenic activity and reproduction (Wang et al., 2019; Zhu et al., 2020), e.g., decreasing sex hormone levels of female fish and subsequently their fecundity, while in males it led to increased testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (E2) levels (Brander et al., 2016; Wang et al., 2019). Therefore, it is evident that the influence of MPs on the sex steroids of fish differs with sex as previously reported by Mallik et al. (2021), but those differences are not significant in cod under the characteristics of our study (i.e., MPs amount and time of exposure).

Gonadotropin releasing hormone (GnRH) is a key regulator of sexual development and reproduction in vertebrates. Several isoforms of GnRH receptors (GnRHr) have been identified (Hildahl et al., 2011). All of them are expressed in the brain and pituitary, but *gnrhr2* and *gnrhr3* showed lower expression in pituitaries and ovaries. The expression of *gnrhr2a* is well correlated to gonadal maturation, stimulating both gonadotropins Fsh and Lh, while *gnrhr3* expression levels are reported to increase in the pituitary during puberty (Hildahl et al., 2011). Our results showed that relative expression levels of *gnrhr2a* in the pituitary increased over time following gonadal maturation. This profile is also obtained for luteinizing hormone subunit beta (*lhb*) and follicle stimulating hormone subunit beta (*fshb*), thus corroborating the connection between the hormone receptor activation and the gonadotropin release. Endocrine disruption described by MPs to affect steroid production (Brander et al., 2016; Wang et al., 2019) is closely related to the expression of the genes in the BPG-axis. During oocyte maturation, a shift in steroidogenesis occurs in ovarian follicles, mediated by an increased gene expression of the steroidogenic enzymes (Mittelholzer et al., 2007). In agreement with this, ovarian *20β-hsd* showed increased expression during maturation in the present study. Also, the significant higher expression of *20β-hsd* in females exposed to dietary MP, indicates early sign of endocrine disruption, although no dietary effects on other BPG-genes, steroid levels, fecundity, or egg quality measures were observed. MP size and concentration may matter for a more disruptive effect of MP as observed by Brander et al. (2016) and Wang et al. (2019), and should be studied in depth to have a better understanding of how MP can affect ovarian steroid production, fecundity and egg quality.

Although analysis of genes in BPG-tissues showed minor differences between dietary treatments, the main effect of MPs appears to be in the liver in the present study. In agreement with previous studies showing effects on liver histology and endocrine disruption (Bhagat et al., 2020), we observed an upregulation of vitellogenin 1 (*vtg1*) in the MP group. This is a yolk precursor protein synthesised by the liver under stimulation of E2 during sexual maturation. It is a useful biomarker of hormonal control in relation to oogenesis and it can act as an indicator of exposure to estrogenic or anti-estrogenic substances in aquatic environments (Arukwe and Goksøyr, 2003). In general, male fish will not express *vtg1*, as the level of endogenous E2 is too low to induce its synthesis; however, they do possess the genes for *vtg*, and a *de novo* synthesis can be induced by exposure to E2 (Arukwe and Goksøyr, 2003). In our analysis, compared with males of the C-diet, males of the MP group had higher levels of *vtg1* expression in liver samples from September and December 2017, while no differences were observed in February 2018. The increase of *vtg*1 expression levels observed in males may be a result of external exposure to E2 in a shared tank by maturing females to synchronise spawning (Henry et al., 2009). Another plausible explanation is the potential endocrine disrupting effect caused by plastic additives known to be endocrine disruptors (Rochman et al., 2014). Further, E2 up-regulates the expression of its receptor (ESR) in brain, pituitary and

ovaries and induces the synthesis of Vtg (Arukwe and Goksøyr, 2003; Dale et al., 2019). The expression of *esr1* decreased towards spawning in brain and pituitary for both sexes in the present study. In contrast, the expression of *esr1* increased during maturation in the gonad samples concomitant with an increased expression of *vtg1* in the liver. These results are in accordance with Morini et al. (2017), where *esr1* expression levels decreased in pituitary until testis development was complete and only increased in some brain areas (mes-/diencephalon) of the European eel. The upregulation of *vtg1* liver samples exposed to MPs agrees with the proposed disruption of oogenesis process observed by Mak et al. (2019), with no effect in reproductive success in zebrafish. These changes in *vtg1* regulation may lead to variation in fish fecundity (Zhang et al., 2008), although not clearly observed in the present study. Plasma vitellogenin analysis and sperm quality measure were not included in the present study.

5. Conclusions

The inclusion of 1% polyethylene MPs at 0.3–0.6 mm had no effect on feed intake, nutrient digestibility or biometric measures (including gonadal development) during maturation of Atlantic cod, nor on fecundity or egg quality measures during its spawning. Measure of gene expression through the BPG-axis and plasma steroid analysis revealed normal development through maturation, with minor differences between sexes and diets, but a significant effect on gonadal *20β-hsd* and liver *vtg1* expression. It will be relevant to study further whether these effects are likely related to MP exposure or to a leaching of endocrine disrupting chemicals present in the plastic polymer. In addition, given the expected increase of MP amounts in the oceans, more knowledge is needed of the putative effects of different polymers, particle sizes and concentrations of MPs on physiology and fecundity of fish broodstock.

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Author contributions statement

All the authors analysed the data, discussed results and implications, commented on the manuscript at all stages and approved the final article for submission. More specific tasks were the responsibility of M.F-M., V. P., H.T., and A.B. (conceptualization and sampling), K.V., Ø.J.H., G.J. (biometrics, histology and plasma sampling), M.F-M., P.P. and E.B. (acquisition of molecular data), M. F-M., E.B, V.P. and A.B. (data analysis and manuscript drafting).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

Arukwe, A., Goksøyr, A., 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp. Hepatol. 2 (1), 1–21. https://doi.org/10.1186/1476-5926-2-4.

Ašmonaité, 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 (24), 14392–14401. https://doi.org/10.1021/acs.est.8b04848.

Bakir, A., Rowland, S.J., Thompson, R.C., 2014. Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. Environ. Pollut. 185, 16–23. https://doi.org/10.1016/j.envpol.2013.10.007.

Bhagat, J., Zang, L., Nishimura, N., Shimada, Y., 2020. Zebrafish: an emerging model to study microplastic and nanoplastic toxicity. Sci. Total Environ. 728, 138707 https://doi.org/10.1016/j.scitotenv.2020.138707.

Boerger, C.M., Lattin, G.L., Moore, S.L., Moore, C.J., 2010. Plastic ingestion by planktivorous fishes in the North Pacific central gyre. Mar. Pollut. Bull. 60 (12), 2275–2278. https://doi.org/10.1016/j.marpolbul.2010.08.007.

Bogevik, A.S., Von Krogh, K., Bjørndal, G.T., Nourizadeh-Lillabadi, R., Hodne, K., Ropstad, E., Haug, T.M., Weltzien, F.A., 2017. Sex steroids differentially regulate fshb, lhb and gnrhr expression in Atlantic cod (*Gadus morhua*). Reproduction 154 (5), 581–594. https://doi.org/10.1530/REP-17-0208.

Brander, S.M., Jeffries, K.M., Cole, B.J., DeCourten, B.M., White, J.W., Hasenbein, S., Fangue, N.A., Connon, R.E., 2016. Transcriptomic changes underlie altered egg protein production and reduced fecundity in an estuarine model fish exposed to bifenthrin. Aquat. Toxicol. 174, 247–260. https://doi.org/10.1016/j.aquatox.2016.02.014.

Bråte, I.L.N., Eidsvoll, D.P., Steindal, C.C., Thomas, K.V., 2016. Plastic ingestion by Atlantic cod (*Gadus morhua*) from the Norwegian coast. Mar. Pollut. Bull. 112 (1–2), 105–110. https://doi.org/10.1016/j.marpolbul.2016.08.034.

Breton, T.S., Anderson, J.L., Goetz, F.W., Berlinsky, D.L., 2012. Identification of ovarian gene expression patterns during vitellogenesis in Atlantic cod (*Gadus morhua*). Gen. Comp. Endocrinol. 179 (2), 296–304. https://doi.org/10.1016/j.ygcen.2012.09.003.

Bronzo, L., Lusher, A.L., Schøyen, M., Morigi, C., 2021. Accumulation and distribution of microplastics in coastal sediments from the inner Oslofjord, Norway. Mar. Pollut. Bull. 173, 113076 https://doi.org/10.1016/j.marpolbul.2021.113076.

Brun, N.R., Koch, B.E., Varela, M., Peijnenburg, W.J., Spaink, H.P., Vijver, M.G., 2018. Nanoparticles induce dermal and intestinal innate immune system responses in zebrafish embryos. Environ. Sci. Nano 5 (4), 904–916. https://doi.org/10.1039/C8EN00002F.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T. S., 2013. Microplastic ingestion by zooplankton. Environ. Sci. Technol. 47 (12), 6646–6655. https://doi.org/10.1021/es400663f.

Dale, K., et al., 2019. Contaminant accumulation and biological responses in Atlantic cod (*Gadus morhua*) caged at a capped waste disposal site in Kollevåg, Western Norway. Mar. Environ. Res. 145, 39–51. https://doi.org/10.1016/j.marenvres.2019.02.003.

De Vries, A.N., Govoni, D., Árnason, S.H., Carlsson, P., 2020. Microplastic ingestion by fish: body size, condition factor and gut fullness are not related to the amount of plastics consumed. Mar. Pollut. Bull. 151, 110827 https://doi.org/10.1016/j.marpolbul.2019.110827.

Fauser, P., Vorkamp, K., Strand, J., 2022. Residual additives in marine microplastics and their risk assessment–A critical review. Mar. Pollut. Bull. 177, 113467 https://doi.org/10.1016/j.marpolbul.2022.113467.

Foekema, E.M., De Gruijter, C., Mergia, M.T., van Franeker, J.A., Murk, A.J., Koelmans, A.A., 2013. Plastic in North sea fish. Environ. Sci. Technol. 47 (15), 8818–8824. https://doi.org/10.1021/es400931b.

Galloway, T.S., Cole, M., Lewis, C., 2017. Interactions of microplastic debris throughout the marine ecosystem. Nat. Ecol. Evol. 1 (5), 1–8. https://doi.org/10.1038/s41559-017-0116.

GESAMP, 2015. Sources, Fate and Effects of Microplastics in the Marine Environment: A Global Assessment.

Goldstein, M.C., Titmus, A.J., Ford, M., 2013. Scales of spatial heterogeneity of plastic marine debris in the northeast Pacific Ocean. PLoS One 8 (11), e80020. https://doi.org/10.1371/journal.pone.0080020.

Hall, T.E., Smith, P., Johnston, I.A., 2004. Stages of embryonic development in the Atlantic cod *Gadus morhua*. J. Morphol. 259 (3), 255–270. https://doi.org/10.1002/jmor.10222.

Hansen, Ø.J., Puvanendran, V., 2010. Fertilization success and blastomere morphology as predictors of egg and juvenile quality for domesticated Atlantic cod, *Gadus morhua*, broodstock. Aquacult. Res. 41 (12), 1791–1798. https://doi.org/10.1111/j.1365-2109.2010.02506.x.

Hansen, Ø.J., Puvanendran, V., Mortensen, A., 2013. Importance of broodstock holding temperature on fecundity and egg quality in three groups of photo-manipulated Atlantic cod broodstock. Aquacult. Res. 44 (1), 140–150. https://doi.org/10.1111/j.1365-2109.2011.03018.x.

Henry, T.B., McPherson, J.T., Rogers, E.D., Heah, T.P., Hawkins, S.A., Layton, A.C., Sayler, G.S., 2009. Changes in the relative expression pattern of multiple vitellogenin genes in adult male and larval zebrafish exposed to exogenous estrogens. Comparative biochemistry and physiology. Comp. Biochem. Physiol., A 154 (1), 119–126. https://doi.org/10.1016/j.cbpa.2009.05.009.

Hildahl, J., Sandvik, G.K., Edvardsen, R.B., Norberg, B., Haug, T.M., Weltzien, F.A., 2011. Four gonadotropin releasing hormone receptor genes in Atlantic cod are differentially expressed in the brain and pituitary during puberty. Gen. Comp. Endocrinol. 173 (2), 333–345. https://doi.org/10.1016/j.ygcen.2011.06.002.

Hodne, K., Haug, T.M., Weltzien, F.A., 2010. Single-cell qPCR on dispersed primary pituitary cells-an optimized protocol. BMC Mol. Biol. 11 (1), 1–15. https://doi.org/10.1186/1471-2199-11-82.

Kagawa, H., Young, G., Adachi, S., Nagahama, Y., 1982. Estradiol-17β production in amago salmon (*Oncorhynchus rhodurus*) ovarian follicles: role of the thecal and granulosa cells. Gen. Comp. Endocrinol. 47 (4), 440–448. https://doi.org/10.1016/0016-6480(82)90122-8.

Kibria, G., Nugegoda, D., Haroon, A.K.Y., 2022. Microplastic pollution and contamination of seafood (including fish, sharks, mussels, oysters, shrimps and seaweeds): a global overview. In: Hashmi, M.Z. (Ed.), Microplastic Pollution. Emerging Contaminants and Associated Treatment Technologies. Springer, Cham., Denmark, pp. 277–322. https://doi.org/10.1007/978-3-030-89220-3_14.

Kim, J.H., Yu, Y.B., Choi, J.H., 2021. Toxic effects on bioaccumulation, hematological parameters, oxidative stress, immune responses and neurotoxicity in fish exposed to microplastics: a review. J. Hazard Mater. 125423 https://doi.org/10.1016/j.jhazmat.2021.125423.

Kjesbu, O.S., Righton, D., Krüger-Johnsen, M., Thorsen, A., Michalsen, K., Fonn, M., Witthames, P.R., 2010. Thermal dynamics of ovarian maturation in Atlantic cod (*Gadus morhua*). Can. J. Fish. Aquat. Sci. 67 (4), 605–625. https://doi.org/10.1139/F10-011.

Kögel, T., Bjorøy, Ø., Toto, B., Bienfait, A.M., Sanden, M., 2020. Micro-and nanoplastic toxicity on aquatic life: determining factors. Sci. Total Environ. 709, 136050 https://doi.org/10.1016/j.scitotenv.2019.136050.

Lenz, R., Enders, K., Nielsen, T.G., 2016. Microplastic exposure studies should be environmentally realistic. Proc. Natl. Acad. Sci. USA 113 (29), E4121–E4122. https://doi.org/10.1073/pnas.1606615113.

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 (7), 4054–4060. https://doi.org/10.1021/acs.est.6b00183.

Lusher, A.L., Mchugh, M., Thompson, R.C., 2013. Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. Mar. Pollut. Bull. 67 (1–2), 94–99. https://doi.org/10.1016/j.marpolbul.2012.11.028.

MacLeod, M., Arp, H.P.H., Tekman, M.B., Jahnke, A., 2021. The global threat from plastic pollution. Science 373 (6550), 61–65. https://doi.org/10.1126/science.abg5433.

Mak, C.W., Yeung, K.C.F., Chan, K.M., 2019. Acute toxic effects of polyethylene microplastic on adult zebrafish. Ecotoxicol. Environ. Saf. 182, 109442 https://doi.org/10.1016/j.ecoenv.2019.109442.

Mallik, A., Xavier, K.M., Naidu, B.C., Nayak, B.B., 2021. Ecotoxicological and physiological risks of microplastics on fish and their possible mitigation measures. Sci. Total Environ. 779, 146433 https://doi.org/10.1016/j.scitotenv.2021.146433.

Mittelholzer, C., Andersson, E., Consten, D., Hirai, T., Nagahama, Y., Norberg, B., 2007. 20b-hydroxysteroid dehydrogenase and CYP19A1 are differentially expressed during maturation in Atlantic cod (*Gadus morhua*). J. Mol. Endocrinol. 39 (4), 319–328. https://doi.org/10.1677/JME-07-0070.

Mittelholzer, C., Andersson, E., Taranger, G.L., Consten, D., Hirai, T., Senthilkumaran, B., Nagahama, Y., Norberg, B., 2009. Molecular characterization and quantification of the gonadotropin receptors FSH-R and LH-R from Atlantic cod (*Gadus morhua*). Gen. Comp. Endocrinol. 160 (1), 47–58. https://doi.org/10.1016/j.ygcen.2008.10.015.

Mkuye, R., Gong, S., Zhao, L., Masanja, F., Ndandala, C., Bubelwa, E., Yang, C., Deng, Y., 2022. Effects of microplastics on physiological performance of marine bivalves, potential impacts, and enlightening the future based on a comparative study. Sci. Total Environ. 155933 https://doi.org/10.1016/j.scitotenv.2022.155933.

Mohamed Nor, N.H., Koelmans, A.A., 2019. Transfer of PCBs from microplastics under simulated gut fluid conditions is biphasic and reversible. Environ. Sci. Technol. 53 (4), 1874–1883. https://doi.org/10.1021/acs.est.8b05143.

Morini, M., Peñaranda, D.S., Vílchez, M.C., Tveiten, H., Lafont, A.G., Dufour, S., Pérez, L., Asturiano, J.F., 2017. The expression of nuclear and membrane estrogen receptors in the European eel throughout spermatogenesis. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 203, 91–99. https://doi.org/10.1016/j.cbpa.2016.08.020.

Nagasawa, K., Presslauer, C., Kirtiklis, L., Babiak, I., Fernandes, J.M., 2014. Sexually dimorphic transcription of estrogen receptors in cod gonads throughout a reproductive cycle. J. Mol. Endocrinol. 52 (3), 357–371. https://doi.org/10.1530/JME-13-0187.

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., Van Look, K.J., Tyler, C.R., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philos. Trans. R. Soc. B Biol. Sci. 364 (1526), 2047–2062. https://doi.org/10.1098/rstb.2008.0242.

Ogonowski, M., Gerdes, Z., Gorokhova, E., 2018. What we know and what we think we know about microplastic effects–A critical perspective. Curr. Opin. Environ. Sci. Health. 1, 41–46. https://doi.org/10.1016/j.coesh.2017.09.001.

Pickova, J., Dutta, P.C., Larsson, P.O., Kiessling, A., 1997. Early embryonic cleavage pattern, hatching success, and egg-lipid fatty acid composition: comparison between two cod (*Gadus morhua*) stocks. Can. J. Fish. Aquat. Sci. 54 (10), 2410–2416. https://doi.org/10.1139/f97-148.

Prinz, N., Korez, Š., 2020. Understanding how microplastics affect marine biota on the cellular level is important for assessing ecosystem function: a review. YOUMARES 9-The Oceans: our Research. Our Future 101–120. https://doi.org/10.1007/978-3-030-20389-4.

Qiang, L., Cheng, J., 2021. Exposure to polystyrene microplastics impairs gonads of zebrafish (*Danio rerio*). Chemosphere 263, 128161. https://doi.org/10.1016/j.chemosphere.2020.128161.

Qiao, R., Lu, K., Deng, Y., Ren, H., Zhang, Y., 2019. Combined effects of polystyrene microplastics and natural organic matter on the accumulation and toxicity of copper in zebrafish. Sci. Total Environ. 682, 128–137. https://doi.org/10.1016/j.scitotenv.2019.05.163.

Rios-Fuster, B., Arechavala-Lopez, P., García-Marcos, K., Alomar, C., Compa, M., Álvarez, E., Julià, M.M., Martí, A.S., Sureda, A., Deudero, S., 2021. Experimental evidence of physiological and behavioral effects of microplastic ingestion in *Sparus aurata*. Aquat. Toxicol. 231, 105737 https://doi.org/10.1016/j.aquatox.2020.105737.

Roch, S., Friedrich, C., Brinker, A., 2020. Uptake routes of microplastics in fishes: practical and theoretical approaches to test existing theories. Sci. Rep. 10 (1), 1–12. https://doi.org/10.1038/s41598-020-60630-1.

Rocha, M.J., Arukwe, A., Kapoor, B.G., 2008. Fish reproduction. In: Enfield (Ed.), New Hampshire. Science Publishers, Oxford, p. 632.

Rochman, C.M., Hoh, E., Kurobe, T., Teh, S.J., 2013. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. Sci. Rep. 3, 3263. https://doi.org/10.1038/srep03263.

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.

Rummel, C.D., Adolfsson-Erici, M., Jahnke, A., MacLeod, M., 2016. No measurable "cleaning" of polychlorinated biphenyls from Rainbow Trout in a 9 week depuration study with dietary exposure to 40% polyethylene microspheres. Environ. Sci.: Process. Impacts 18 (7), 788–795. https://doi.org/10.1039/C6EM00234J.

Schrank, I., Trotter, B., Dummert, J., Scholz-Böttcher, B.M., Löder, M.G., Laforsch, C., 2019. Effects of microplastic particles and leaching additive on the life history and morphology of *Daphnia magna*. Environ. Pollut. 255, 113233 https://doi.org/10.1016/j.envpol.2019.113233.

Sharifinia, M., Bahmanbeigloo, Z.A., Keshavarzifard, M., Khanjani, M.H., Lyons, B.P., 2020. Microplastic pollution as a grand challenge in marine research: a closer look at their adverse impacts on the immune and reproductive systems. Ecotoxicol. Environ. Saf. 204, 111109 https://doi.org/10.1016/j.ecoenv.2020.111109.

Solomando, A., Capó, X., Alomar, C., Álvarez, E., Compa, M., Valencia, J.M., Pinya, S., Deuderoc, S., Sureda, A., 2020. Long-term exposure to microplastics induces oxidative stress and a pro-inflammatory response in the gut of *Sparus aurata* Linnaeus, 1758. Environ. Pollut. 266, 115295 https://doi.org/10.1016/j.envpol.2020.115295.

Sundt, P., Schulze, P.E., Syversen, F., 2014. Sources of microplastic-pollution to the marine environment Project report. Norweg. Environ. Agency. M-321/2015., 86pp.

Tveiten, H., Frantzen, M., Scott, A.M., Scott, A.P., 2010. Synthesis of 17, 20β, 21-trihydroxypregn-4-en-3-one by ovaries of reproductively mature Atlantic cod *Gadus morhua*. J. Fish. Biol. 77 (1), 33–53. https://doi.org/10.1111/j.1095-8649.2010.02655.x.

Vázquez-Rowe, I., Ita-Nagy, D., Kahhat, R., 2021. Microplastics in fisheries and aquaculture: implications to food sustainability and safety. Curr. Opin. Green Sustain. Chem. 100464 https://doi.org/10.1016/j.cogsc.2021.100464.

Vorkamp, K., Karlsson-Drangsholt, A., Burgerhout, E., Puvanendran, V., Tveiten, H., Diepens, N.J., Koelmans, A., Rønnestad, I., Bogevik, A.S., 2019. Microplastics and Contaminants: Field Experiments to Assess the Role of Microplastics as a Vector for Contaminants, p. 102. Abstract book SETAC 3-7 Nov 2019, Toronto, Canada. ISSN 1087-8939.

Wang, J., Li, Y., Lu, L., Zheng, M., Zhang, X., Tian, H., Wang, W., Ru, S., 2019. Polystyrene microplastics cause tissue damages, sex-specific reproductive disruption and transgenerational effects in marine medaka (*Oryzias melastigma*). Environ. Pollut. 254, 113024 https://doi.org/10.1016/j.envpol.2019.113024.

Wang, L., Nabi, G., Yin, L., et al., 2021. Birds and plastic pollution: recent advances. Avian Res 12, 59. https://doi.org/10.1186/s40657-021-00293-2.

Weltzien, F.A., Andersson, E., Andersen, Ø., Shalchian-Tabrizi, K., Norberg, B., 2004. The brain–pituitary–gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 137 (3), 447–477. https://doi.org/10.1016/j.cbpb.2003.11.007Get.

Wright, S.L., Thompson, R.C., Galloway, T.S., 2013. The physical impacts of microplastics on marine organisms: a review. Environ. Pollut. 178, 483–492. https://doi.org/10.1016/j.envpol.2013.02.031.

Xia, M., Sherlock, J., Hegerich, P., You, X., Lee, K., Walworth, C., Spier, E., 2010. DataAssist–data analysis software for TaqMan real-time PCR data. IMECS 1, 210–212. http://www.iaeng.org/publication/IMECS2010/IMECS2010_pp210-212.pdf.

Yadetie, F., et al., 2018. RNA-Seq analysis of transcriptome responses in Atlantic cod (*Gadus morhua*) precision-cut liver slices exposed to benzo [a] pyrene and 17α-ethynylestradiol. Aquat. Toxicol. 201, 174–186. https://doi.org/10.1016/j.aquatox.2018.06.003.

Zantis, L., Carroll, E.L., Nelms, S.E., Bosker, T., 2020. Marine mammals and microplastics: a systematic review and call for standardisation. Environ. Pollut. 116142 https://doi.org/10.1016/j.envpol.2020.116142.

Zhang, X., Zha, J., Li, W., Yang, L., Wang, Z., 2008. Effects of 2, 4-dichlorophenol on the expression of vitellogenin and estrogen receptor genes and physiology impairments in Chinese rare minnow (*Gobiocypris rarus*). Environ. Toxicol. Int. J. 23 (6), 694–701. https://doi.org/10.1002/tox.20375.

Zhu, M., Chernick, M., Rittschof, D., Hinton, D.E., 2020. Chronic dietary exposure to polystyrene microplastics in maturing Japanese medaka (*Oryzias latipes*). Aquat. Toxicol. 220, 105396 https://doi.org/10.1016/j.aquatox.2019.105396.