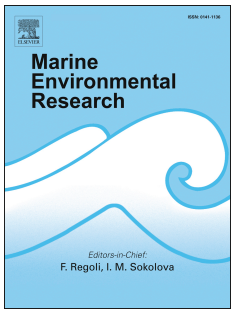

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Author contributions

AM, AC and LP originally formulated the idea. AM, GB and LP conceived and designed the experiments; AM, TC, GB and MLG developed and constructed experimental setup; AM, TC, GB and MLG conducted fieldwork and performed the experiments; AM, CV and LP performed statistical analyses; AM wrote the manuscript. All the authors contributed with the interpretation of results and provided editorial advice.

Adverse Effects of Plastic Ingestion on the Mediterranean Small-Spotted Catshark (*Scyliorhinus canicula*).

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Keywords

Microplastics, macroplastics, μ-Raman spectroscopy, small-spotted catshark, immunity

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Abstract

Plastics are widely diffused in the oceans and their ingestion by marine organisms is raising concern for potentially adverse effects. The risk of harmful interactions with marine plastic pollution depends on the biology of the species as well as the distribution and abundance of the different plastic types.

The aim of this study was to assess the occurrence of plastic ingestion by the small-spotted catshark (*Scyliorhinus canicula*), one of the most abundant elasmobranchs in the Mediterranean Sea. The expression levels of genes indicative of total immune system function were analyzed to gather preliminary data for further investigation of any potential correlations between plastic presence and immune activation.

One hundred catsharks were collected during the Spring 2018 in two geographic locations in the southern region of the central Mediterranean Sea: 1) near Mazara del Vallo, SW Sicily and 2) near Lampedusa island, Italy's southernmost. Standard measurements were recorded for each specimen and its organs and sex was determined. The gastrointestinal tract (GIT) was preserved for plastic detection and identification. Where present, plastics (macro- and micro-) were characterized in terms of size, shape and polymer typology through microscopy and μ-Raman spectroscopy. Spleen from a subset of thirty samples was preserved for RNA extraction, then used to quantify by real time PCR the transcripts of T cell receptor beta (TCRB), T cell receptor delta (TCRD) and IgM genes.

The results indicated that ingestion of plastic is widespread, with microplastics (MP, from 1 μm to <1 mm) abundantly present in nearly all samples and macroplasticplastic (MaP, > 1 cm) in approximately 18% of the specimens collected. A significant increase in the expression of TCRB, TCRD and IgM was observed in the spleen of MaP+ specimens from Mazara del Vallo waters, in parallel with 67% increase in liver weight.

43 While the presence of MP alone is not enough to induce a strong activation of the immunity, some type
44 of plastics falling into the MaP category may be more toxic than others and crucial in the activation of
45 the immune response.
46 The results of this study represent a first evidence that plastic pollution represents an emerging threat to
47 *S. canicula*, the Mediterranean food web and human consumers.
48

1. Introduction.

The abundance of plastic debris floating in Mediterranean waters was first reported in 1980 (Morris RJ 1980). Later studies confirmed the distribution, abundance and characteristics of plastic debris in the basin thanks to visual counts supported by surface nets tows allowing detection and quantification of microplastics, (MP, from 1 μm to <1 mm) (Aliani et al., 2003; Suaria et al., 2014; Faure et al., 2015; Ruiz-Orejón et al., 2016; Zeri et al., 2018). Plastic particles are in fact typically grouped into categories from macro (MaP >1 cm) to nano (from 1 to <1000 nm) depending on their size (as measured by their diameter or by considering the larger dimension as classifier for irregular of fiber debris) according to the classification proposed by Hartmann et al., 2019.

In the Mediterranean Sea, MP concentrations is ranging from tens to hundreds of thousands of items per square kilometer; this abundant presence of buoyant plastic debris is likely related to the high human pressure and the hydrodynamics of this semi-enclosed basin (Eriksen et al., 2014; Cozar et al., 2015) which is also one of world's busiest shipping routes, receiving waters from densely populated river shorelines (e.g., Nile, Ebro, Rhone and Po) while being connected to the Atlantic Ocean only by the Strait of Gibraltar. For its characteristics, the Mediterranean basin has a water residence time as long as a century (Lacombe et al., 1981) and its shores house10% of the global coastal population (ca. 100 million people within the 10-km coastal strip (CIESIN, 2012)).

MP are divided in two types: 1) primary, found in most commonly utilized products (e.g. cosmetic and personal care products, insect repellents, sunscreens, products for children), and 2) secondary, originating from the fragmentation of larger plastic debris through biological degradation, photo-degradation, chemical deposition and physical fragmentation (Auta et al., 2017). Both primary and secondary MP are present in sea water where the most represented synthetic polymers are polypropylene (PP), polyethylene (PE), polystyrene (PS), polyvinylchloride (PVC)and polyethylene terephthalate (PET) (Rocha-Santos and Duarte, 2015).

MP in the marine environment are dispersed via oceanic currents and wind patterns throughout the water column (Lebreton et al., 2012), in a variety of colors, shapes, sizes and densities (Reisser et al., 2014). Their persistence, availability and the biomagnification of the associated harmful chemicals, represent a potential hazard to marine life throughout the food web also if there is very few studies in field (Lusher et al., 2017; Romeo et al., 2015). MP interaction with marine organisms has been described for zooplankton (Botterell et al., 2019), invertebrates (Avio et al., 2015; Digka et al., 2018), fish (Digka et al., 2018; Compa et al., 2018; Renzi et al., 2019), turtles (Domenech et al., 2019), birds (Wilcox et al., 2015) and mammals (Fossi et al., 2016), including endangered species (Deudero and Alomar, 2016).

Fish may accidentally ingest particulate while they are feeding on their prey or ingest plastic debris because of their resemblance to prey: the first report of MP ingestion in fish was in 1972 (Carpenter et al., 1972). Depending on plastic size and species, particles may be expelled or accumulate in the gastrointestinal tract (GIT), where could cause physical damage (e.g. block of feeding appendages or filters, and obstruction of GIT), and in some cases inflammation leading to death (Li et al., 2016; Werner et al., 2016).

In the last few years, the presence of plastics debris in fish has been described in species captured in the oceans, seas and freshwater raising concerns on their potential negative effects (e.g Ory et al., 2018; Bessa et al., 2018; Pellini et al., 2018; Silva-Cavalcanti et al., 2017). In addition, several authors have studied in controlled conditions the effects of virgin MP intake or as vehicles of other toxic compounds. Potential damage is related to the physical properties, regarding the interaction of the particles with the organism tissues and to the chemical properties, concerning the transfer of contaminants or leaching of plastic additives (Rochman et al., 2013; Pedà et al., 2016; Limonta et al., in review). Indeed, plastics, due to their lipophilic nature, have the potential to adsorb many hydrophobic persistent organic pollutants which may increase their harmful effect on biota. Up to now, it has not

been completely elucidated the contribution of chemicals to plastic toxicity. Recent studies suggest that the two main routes of uptake in fish are represented by the ingestion and inhalation, since MP were found to accumulate and cause tissue damage in GIT and gills (Li et al., 2016, Pedà et al., 2016). Histological observations on exposed fish confirmed that MP were able to induce a strong inflammatory response in the target tissues (Limonta et al., 2019). The transcriptomic profiling of zebrafish larvae exposed to PS also suggested the activation of immune response, with the up-regulation of genes related to the complement system (Veneman et al., 2017; Pitt et al., 2018). Lu et al. (2016) reported how the accumulation of PE in gills, gut and liver of zebrafish causes oxidative stress, inflammation in fish liver and a disturbed lipid and energy metabolism (Lu et al., 2016).

Besides inflammation and metabolic disorders, MP absorption and distribution in different tissues and cells can result in several types of effects, such as behavior alterations, predatory performance reduction, neurotoxicity, decreased growth (Rochman et al., 2013; Rochman et al., 2014; Ferreira et al., 2016; Pedà et al., 2016; Barboza et al., 2018a; Barboza et al., 2018b). Moreover, given the chemical properties, the MP uptake by aquatic organisms with other contaminants is a route to harmful chemicals including styrene, metals, phthalates, bisphenol A, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) or polybrominated diphenyl ethers (PBDEs) (Koelmans et al., 2014; Barboza et al., 2018c). For example, the japanese medaka (*Oryzias lapites*), exposed for short-time to both virgin and marine PE fragments, showed bioaccumulation of PAHs, PCBs and PBDEs, with signs of liver stress and early tumor formation (Rochman et al., 2013; Rochman et al., 2014).

The small-spotted catshark (*Scyliorhinus canicula*, Linnaeus, 1758, SC) is a species of the family Scyliorhinidae. It is one of the most abundant cartilaginous fish in the central Mediterranean Sea (Ragonese et al., 2013) and inhabits the continental shelves of off the coasts of Norway and the British Isles up to Senegal. It is a small, shallow-water shark inhabiting waters of depths ranging from a few

meters (mt) down to 400 mt (Geraci et al., 2017; Rodriguez-Cabello et al. 2007). SC feeds opportunistically on a wide range of macrobenthic fauna, with Crustacea, Mollusca, Annelida, and Echinodermata as preferred prey. Feeding preference may depend on SC age and feeding intensity is highest during the summer due to the higher availability of living preys (Rodriguez-Cabello et al. 2007).

Given their low commercial value and abundance in the Mediterranean Sea (this species is currently listed as "Least Concern" on the IUCN Red List of Threatened Species), SC has been chosen as model in this study, as representative sample of the potential hazard fish in the southern waters of Italy are subject.

Here we present the description and analysis of effects of plastics (MP and MaP) in SC from two different geographic locations in the southern region of the central Mediterranean Sea, near: 1) Mazara del Vallo (MDV), SW Sicily and 2) Lampedusa (LMP), Italy's southernmost island. Plastics have been isolated from the GIT of SC, quantified and analyzed with Raman spectroscopy to identify the polymer category. *S. canicula* was further investigated through the analysis of spleen transcripts of key genes involved in adaptive and innate immunity to evaluate the potential of future research hypothesis linking plastic presence to health status.

2. Materials and Methods.

2.1 Samples collection.

One hundred specimens of SC were collected on March 16, 2018 near Mazara del Vallo (N=48, N=25 females, F, and N=23 males, M), SW Sicily, Italy and on May 7th, 2018, near Lampedusa island (N=52, N=14 females, F, and N=38 males, M), Italy's southernmost island, in the FAO General Fisheries Commission for the Mediterranean (GFCM) areas marked as Geographical Sub-Area (GSA) 16 and GSA13 in Figure 1, respectively (Figure 1; Supplementary Table 1).

2.2 Morphometric indices.

Fish total length (TL), body weight (BW), spleen weight (SPL W), liver weight (LIV W) and GIT weight (GIT W), gender and maturity stage were recorded. Visceral weight (VW) was calculated subtracting the carcass weight (CW) to the BW. Fish were weighed after sampling, then spleen, liver and GIT were removed by dissection and weighted after evisceration. All the weights were measured using Sartorius balance (model: MSEE6202P-000-D0) to an accuracy of 0.01 grams.

Sexual maturity was defined by 6 stages of gonadic development according to the Medits (International Bottom Trawl Survey in the Mediterranean) scale (Anon., 2016).

Condition factor (CF) was calculated as follows: (BW*100)/TL^3. The hepato-somatic index (HSI), the spleno-somatic index (SSI) and the GIT somatic index (GSI) were calculated as follows: HSI = liver weight (g) × 100/body weight (g), SSI = spleen weight (g) × 100/body weight (g), GSI=GIT weight (g) × 100/body weight (g) (Supplementary Table 1).

2.3 Chemistry: plastic isolation and identification in the GIT.

After sampling, fish were quickly frozen and stored at -20°C. Successively, they were processed in the laboratory, where they were washed with MilliQ water, sectioned and the entire GIT (esophagus to

163 vent), liver and spleen were removed. Liver and spleen were fixed in RNAlater® and stored in
164 separated closed container at -20°C for subsequent isolation of RNA, whereas GIT was wrapped in
165 aluminum foil and then frozen at −20°C in a closed container.
166 GIT samples were digested at 60°C for 24 h in 10% KOH (Merck) in MilliQ® water, filtered with 0.45
167 μm nitrocellulose filter before use, according to the protocol published by Dehaut A et al. (2016).
168 Following digestion, samples were filtered on 8 μm cellulose nitrate filters (Whatman). In the case of
169 the presence of debris in the digestate, a density-based separation step using a ipersaline solution of
170 NaCl (Sigma Aldrich) was performed. The solution was added to the digestate (2:1, v/v) and stirred for
171 10 min before being left to settle for 1 h. The supernatant, containing the floating plastic particles was
172 collected and filtered, as previously described. Plastic debris of big size that could not be digested nor
173 filtered were isolated and analyzed as undigested MaP. The filters that by visual inspection contained
174 plastic debris contaminated with undigested organic residues or minerals were subject to an additional
175 basic digestion step according to the procedure reported in Roch et al. (2017) and washed by a dilute
176 acid aqueous solution (HCl 0.1M); it was observed that even after this treatment plastic debris were not
177 broken into smaller fragments. Blank samples were prepared and analyzed in parallel as controls to
178 account for MP contamination from the digestion and filtering processes (i.e., 'digestion control'). In
179 detail, digestion solutions were placed into a clean beaker, heated at 60 °C for 24 h, and vacuum
180 filtered onto a nitrocellulose filter 0.45 μm, (the procedure was repeated ten times; n = 10). MP was
181 quantified and characterized for fish samples (see below). The blank samples contamination (mean No.
182 filter−1) consisted only of fibers with average values of 4.2 (± 0.5).
183 Measure to avoid contamination were adopted during all the extraction procedure, cotton lab coat and
184 nitrile gloves were used and glassware equipment were thoroughly washed and rinsed with MilliQ
185 water before use, all materials were covered between use with aluminum foils, and filters were stored
186 in glass petri dishes.

Filters were first examined and sorted by visual inspection with a stereo microscope following protocol (Dehaut A et al., 2016). Microscopic analysis of the filters was performed with a Nikon SMZ745T stereomicroscope, equipped with a Nikon Digital Sight DS-F12 camera.

All the MaP, fragments and filaments, were analyzed with Raman spectroscopy. The size of the analyzed subset should insure a representative view of the particle size distribution and chemical nature as suggested by Kedzierski M et al. 2019. Spectra were generated with a LabRam HR800 micro-Raman instrument from Horiba Scientific equipped with an air-cooled CCD detector at −70 °C, an Olympus BXFM microscope, a 600 groove/mm grating and a 50× objective were used to collect the Raman scattering signals. The excitation source was a He-Ne laser (632.8 nm line) with a maximum laser power of 20 mW. A minimal spectral accumulation of 10 times 1 s was used; if a high background was recorded the accumulations were increased to a maximum of 100 times 1 s to improve the signal-to-noise ratio.

2.4 Gene expression analysis.

RNA Extraction. Total RNA from spleen samples of thirty selected samples (Table 1) was extracted using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Tissue (about 30 mg) lysis and homogenization was performed with T 10 basic ULTRA-TURRAX® (IKA, Staufen, Germany). Genomic DNA was removed through an in-column DNase I digestion (Qiagen). The RNA concentration and the quality of the extractions were assessed with a BioSpec-nano UV–Vis spectrophotometer (Shimadzu Italia S.r.l., Milan, Italy). The RNA samples that did not meet the absorbance ratio cutoff (1-8-2.0 for the 280/260; 2.0-2.2 for the 260/230) were extracted more than once.

Quantitative real time PCR (q-PCR). A panel of immunity genes [*T cell receptor beta* (*TCRB*), *T cell receptor delta* (*TCRD*) and *immunoglobulin M* (*IgM*)], and housekeeping genes [*ribosomal protein 13* (*RPL13*), *actin beta 1* (*ACTb*) and *ribosomal protein L29* (*RPL29*)] was selected from the literature. Primers for the selected genes were obtained from literature or designed with Primer 3 on different exons to exclude any genomic DNA co-amplification: ncbi accession numbers of the sequences used were KY434203 (TCRB); KY434205 (TCRD); JX555996 (µ heavy chain IgM) (Crouch et al., 2013; Li et al., 2015; Pettinello et al., 2017) (Table 2). Reverse transcription was performed using iScript™ cDNA synthesis kit (Bio-Rad, California, USA) according to the manufacturer's instructions using 1 µg total RNA. The qPCR reaction was performed in triplicates in 96 wells plates, using the EvaGreen Dye Master mix (Bio-Rad) on CFX Connect Real-Time Detection system (Bio-Rad). qPCR efficiencies were calculated using the equation from Dhar et al. (2009). The amplification efficiency of each primer couple was checked through the creation of a five points standard curve with serially diluted 1:5 cDNA from 5 samples (MDV: SC7, SC9, SC11 and LMP: SC56, SC57) (Table 2). cDNA was reverse transcribed from 1 µg of total RNA using SsoFastTM EvaGreen® Supermix (Bio-Rad) in a total volume of 10 µl of a reaction mix containing 10 ng cDNA, 0.3 µM of each primer, 2× Evagreen enzyme and DNase-free sterile water. qPCR reactions were run as follows: 1 cycle of 98 °C for 30 min, 49 cycles of 95 °C for 5 s, 60 °C for 10 s; melting curve 65 °C–95 °C: increment 0.5 °C every 5 min. Each reaction was run in triplicate, together with a tri- plicate of no-template controls. The average Ct values were normalized to the values of the housekeeping genes RPL13. RPL13 was the most stable house-keeping gene amongst those tested (e.g RPL13, ACTb, RPL29). Comparative Ct method of analysis ($2^{-\Delta\Delta ct}$) was used to determined changes of expression between control and treated samples on CFX connect manager software 3.1 (Bio-Rad). Fold differences were calculated accounting for differences in primer efficiencies using the Pfaffl method (Pfaffl, 2001).

233 **2.5 Statistical analysis.**

234 Data were analyzed within and between locations. Morphometric data were analyzed within MDV and
235 LMP, related to gender (male and females) and/or presence or absence of ~~MP and~~ MaP in the GIT.
236 Student t-test and ANOVA were used in comparison to detect significant differences between groups
237 compared, for morphometric data and for gene expression analysis.

238

239 # 3. Results and Discussion.

240 ## 3.1 Morphometric data analysis.

241 All the morphometric data for the 100 samples were analyzed to examine if significant differences exist
242 between the samples from the two locations studied. Variation in TL and BW did not account for a
243 difference in CF between MDV and LMP while the gonadal maturity and liver size had interesting
244 dissimilarities (Table 3, Supplementary Table 2).
245 In detail: MDV, both females and males SC have smaller (< 34%) index of gonadal maturity when
246 compared to samples from LMP. Moreover, MDV males have bigger liver (HSI > 20%), while females
247 have smaller liver (HSI < 24%) compared to LMP specimens.
248 The smaller gonadal maturity in MDV specimens may be linked to the great difference in the existing
249 anthropogenic activities in the two locations of sampling. MDV sampling site is close to a port with the
250 largest fishing fleet in Italy, while LMP sampling site is in a pristine area, near Lampedusa, the Italian
251 southernmost island inhabited by only 6.000 people in an area of 20.2 square kilometers. The
252 difference in human activity, contamination and consequently greater stress conditions in MDV, may
253 have an effect on the appropriate development of the MDV fish and may correlate to the overall
254 differences we observed amongst the two shark's populations. The translation of stress to the
255 organismal/population level and higher is not straightforward. In teleosts, acute and chronic stressors
256 can stimulate physiological changes at the organismal level, impacting growth rate, reproductive output
257 or investments, and disease resistance (Davis, 2002; Iwama et al., 2006; Ramsay et al., 2009). In
258 contrast, very little is known about the physiological changes linked to stress in elasmobranchs. Indeed,
259 the extent to which elasmobranchs are affected by pervasive anthropogenic threats, such as habitat
260 degradation, climate change and pollution and its transient or prolonged impacts on health and fitness
261 (e.g., somatic growth and reproduction) remains poorly understood (Skomal and Mandelman, 2012).

262 We could assume that they respond to threats in a similar way teleosts do, but the nature and magnitude
263 of the response could be highly species-specific and related to ecological factors and the type and
264 duration of the stressors. Plastic contamination and the ingestion by aquatic organisms, including
265 species of commercial importance for fisheries, are well documented in the Mediterranean Sea (Lusher
266 et al., 2017) and we can reasonably assume that it will continue to increase in the foreseeable future;
267 filling the knowledge gaps on the occurrence and adverse effects caused by (of the) polymers actually
268 present in the environment is necessary to assess / understand the impact of these contaminants on biota
269 (degree of the biological impact).
270
271 **3.2 Effects and composition of plastics in the GIT of *S. canicula*.**
272 The GIT of 50 selected specimens was analyzed for plastic presence, quantity and type. Plastic were
273 either grouped as fibers, MP fragments or MaP (Figure 2 and Figure 3) following the classification
274 proposed by Hartmann et al., 2019. The occurrence of plastic was considered by the number of
275 ingested plastics as well as the frequency of ingestion (Avio et al., 2020).
276 We found debris in the shape of fibers (filaments and lines) and fragments in about 80% of the samples
277 analyzed (86.3 % of the MDV samples, and 75.7 % from LMP samples), both colored and clear (Figure
278 3; Table 4). Artificially dyed debris and filaments ~~and debris~~ identified by Raman spectroscopy where
279 referred as plastic particles. Fibers in most cases were characterized by a not regular diameter along the
280 particle, with diameter values lower than 10 μm. A total number of 138 particles were counted by
281 visual inspection: 115 fibers shape (83.3%; which were classified based on the diameter size in 68
282 fibers and 59 filaments) and fragments (16.7%). According to size classes, the plastic debris were
283 divided up as follows: 17 were in the range 1-10 μm, 72 were in the range 20 μm e 100 μm, 38 the
284 range 20 μm e 100 μm and 11 were in the range 1cm – 5cm (MaP). Considering the color, 88 debris
285 were dark colored (63.8%), 30 were light colored (21.7%) and 20 were transparent (14.5%).

All fragments and filaments were analyzed by Raman microspectroscopy: fibers identification resulted in many cases problematic possibly due to additive/pigments contained in the fibers or to adhesion of organic residues. The uncertainty in identification of fibers constituents and therefore the bias induced by a partial identification of the samples, combined with the fact that fibers can also derive from contamination during the sample preparation procedure suggest us to limit the data only to MaP, fragments and filaments.

In addition, in the present work we aimed to investigate possible correlation of MaP presence and variation of immune-related gene expression in *S. canicula*.

MP filaments and fragments accounted for a similar number in the two location with an ingested average by individual of 1.32 items in MDV vs 1.04 items in LMP and a frequency of ingestion of 71% in MDV vs 62% in LMP (Table 4).

Differently, MaP were detected in 18% of the samples analyzed. Specifically, in 20% of the MDV samples, and 16% from LMP samples (Table 4). All specimens with MaP from MDV were females, while those with MaP from LMP were 50% males and 50% females (Supplementary Table 3).

MaP were composed of the polymers polypropylene, polyethylene and polyethylenterephtalate whereas MP were mainly identified as polyester, acrylic and nylon 6~~, and colorant~~ (Figure 4). Average values of MP composition were not significantly different in the two sites.

The analysis of morphological data associated to the presence of MaP in samples from both locations showed no correlation to CF and VW. However, a significant increase in liver weight (49%, $p<0.05$) and HIS (26%, $p<0.01$) was observed (Supplementary Table 4A). In the correlation to MaP, MDV specimens showed an increase in the weight of all organs, although only the liver and HSI returned a significant increase of 67% and 34%, respectively of the total weight ($p<0.005$) (Figure 5, Supplementary Table 4B). In LMP, the MaP specimens showed an increase of VW but the variation

309 was non-significant; nevertheless, the liver was still subject to the highest increase (Figure 5,
310 Supplementary Table 4C).
311 In general, females from both location have higher VW probably due to higher structural and functional
312 demands linked to vitellogenesis and maternal immunity (LIV W is 25% and 44% higher in females
313 than males in MDV and LMP, respectively) (Supplementary Table 4D and 4E).
314 The analysis of morphometric data of specimens sampled at MDV and LMP related to both MaP
315 detection and gender, resulted in non-significant differences although it is evident that MDV females
316 have an increase of BW that is only in part related to the increase in VW (Supplementary Table 5A). In
317 LMP the increase of VW is more evident in females than males (Supplementary Table 5B). The
318 females have VW that is 31% ($p<0.05$) than males (Supplementary Table 5C). BW and VW were
319 higher in samples with MaP females and lower in males (Supplementary Table 5D and 5E). The
320 correlation analysis is clearly showing that females in MDV have smaller liver activity than those in
321 LMP, while the males in MDV have higher liver activity, almost as the males in MDV are stimulated to
322 invest more in liver functions, which is usually a female feature (e.g. vitellogenesis) rather than in male
323 characters (in males the body weight should be higher because is correlated to bones and muscles, for
324 examples). The feminization-like evidence and the smaller gonadal maturity overall observed could
325 severely impact the reproduction rate and fitness of the SC population in MDV. It has been previously
326 observed that the exposure to endocrine disrupting chemicals (additives of plastics) can strongly
327 influence the course of sex differentiation and unbalance the sex ratio in zebrafish populations; in other
328 freshwater species vitellogenin concentration in male fish have been described in correlation to the
329 exposure to estrogenic contaminants (Von Hippel et al., 2017; Santos et al., 2017). Decreased growth
330 rate, decreased fecundity and negative impacts on subsequent generations have also been linked to
331 plastic exposure in both marine and terrestrial species (Huerta-Lwanga et al., 2016; Sussarellu et al.,
332 2016).

333

334 **3.3 Gene Expression.**

335 In order to move beyond simply studying the presence and type of plastics, the effects on the
336 underlying physiological and biochemical mechanisms should be investigated. The effects can be many
337 and diverse, and may involve a stress response implicating several systems, such as the immune, the
338 endocrine or reproductive one. A first step to identify changes that may be associated to the presence of
339 plastic described was to evaluate changes in the expression of genes known linked to the immune
340 system**.** There are two main layers of immune responses: innate immune responses and adaptive
341 immune responses. The innate immune system creates a fast, non-specific reaction to the pathogen
342 infecting the host organism. If the pathogen persists despite innate defenses, then the adaptive immune
343 system will engage the microbe with specificity and memory. The adaptive (or acquired) immune
344 system mounts to a discriminating long lasting immune response directed by two types of lymphocytes,
345 T cells (cell-mediated immunity) and B cells producing immunoglobulins (Ig) (humoral immune
346 response) (Rauta et al., 2012). Cartilaginous fish and elasmobranchs (sharks, skates and rays), in
347 particular, are the first jawed vertebrate group to emerge in evolution and are the oldest group relative
348 to mammals having an immune system grounded upon Ig, T cell receptors (TCR), the major
349 histocompatibility complex (MHC), as well as RAG-mediated rearrangement, somatic hypermutation
350 and the presence of primary and secondary lymphoid tissues (Flajnik MF, 2002). Immunoglobulins
351 (IgM) were discovered in sharks almost 40 years ago and while some features of the immune system
352 are simple and primordial, other features, including the Ig system, can be quite complex (e.g. the
353 presence of two non-IgM isotypes, IgW and IgNAR) (Dooley and Flanjk, 2006; Flajnik MF, 2002). It
354 is highly probable that each of these isotypes evolved to mediate a particular type of defense
355 mechanism, although there are no functional data as yet for the non-IgM isotypes. The genes that were

analyzed in this study were those related to canonical immune response pathways, *IgM*, *TCRB* and *TCRD* (Pettinello et al., 2017; Crouch et al., 2017).

RNA extraction from spleen of the 30 specimens was successful and all samples were retrotranscribed and used as template in the real time qPCR of *TCRB* and *IgM*, specifically related to adaptive immunity activity and *TCRD*, linked to innate immunity activity. Samples analyzed within location group (MDV and LMP separately), considering the presence (MaP +) or absence (MaP -) of plastics, showed differences in the expression of the genes (immune-related) tested.

In MDV (5 MaP + and 8 MaP -) specimens, MaP + spleens showed a significant increase in the expression of all immune-related genes: fold increases were: 1.2 for *TCRB* ($p<0.02$), 2.1 for *TCRD* ($p<0.01$) and 3.1 for IgM ($p<0.01$) (Figure 6). When the analysis was restricted to only females (5 MaP + and MaP -), fold increases were: 3.5 for *TCRB* (not significant), 6.3 for *TCRD* ($p<0.02$), and 16.4 for *IgM* ($p<0.02$) (Figure 6).

In LMP specimens (4 MaP + and 11 MaP -), MaP + spleens showed minimal and not significant variations of immune-related gene transcripts: fold increases were 0.13 (*TCRB*), 0.23 (TCRD) and 0.18 (*IgM).* Data analyzed separating males and females gave the same outcome (data not shown).

Results from spleen gene expression were somewhat correlated with what we observed in the previous analyses. The changes observed in the expression of the three immune-related genes in spleen were greater in MDV samples than in LMP samples, consistent with the hypothesis that the adverse effects observed may be correlated to the highest degree of MDV anthropogenic pollution, in which MaP, co-present with MP (detected in on nearly all samples) are most likely additive for chemical co-contaminants.

4. Conclusion.

379 The present study reports high frequencies of microplastic consumption as well as the presence of
380 macroplastic ingestion in the small spotted shark, *S. canicula*, sampled in two different locations of the
381 Mediterranean Sea. From a first examination, it may be hypothesized that sharks could be less
382 susceptible to microplastic ingestion than macroplastics, given the potential correlation of macroplastic
383 presence to changes in expression of immune-related genes. But the link between plastics and the
384 unavoidable absorbed chemicals, differently distributed in the locations examined, needs to be
385 specifically addressed, given the estrogenic effects hereby reported on maturity and gender
386 development that could be caused, for example, by endocrine disruptors present in the most
387 contaminated site. Correspondingly, immunology data describing the full functionality of T- and B-
388 cells needs to be specifically gathered and integrated.
389 To our knowledge, this is the first study to explore the influence that plastic ingestion by a shark
390 species in the Mediterranean Sea. The occurrence and high frequency of ingested plastic debris hereby
391 reported highlights the ubiquitous nature of this pollutant throughout the Mediterranean Sea and the
392 importance of targeting plastics and their co-contaminants in future pollution control efforts.
393

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397

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694 ## Figure legends.

695 ***Figure 1. Sampling sites, located within FAO GFCM GSAs.***

696 Dots represent the 2 locations of sampling, south of Mazara del Vallo, GSA 16 and south of
697 Lampedusa, GSA 13. Lampedusa samples were collected in deep water. GFCM, General Fisheries
698 Commission for the Mediterranean; GSA, Geographical Sub-Area.

699

700 ***Figure 2. Images of undigested plastics MaP from 3 different samples.***

701 ***A,*** Digested GIT tissue with undigested plastic; same specimen is shown in ***B. C- D*** MaP found in the
702 GIT of two other specimens. MaP were identified by Raman spectroscopy: ***B*** (polypropylene), ***C***
703 (Polyethylene terephthalate), ***D*** (polyethylene).

704

705 ***Figure 3. Microscope images of MP debris found on filters in 2 different samples.***

706 ***A, B.*** Filters after digestion of SC6 and SC7. ***C, D.*** Microscope image of plastic debris on filters.

707

708 ***Figure 4. Plastic debris composition.***

709 ***A, B.*** MaP and MP composition: PE polyethylene, PP polypropylene, PET Polyethylene terephthalate,
710 PA Polyamide, PAC Polyacrylate, PAN Polyacrylonitrile. The numbers refer to percentage values.

711

712 ***Figure 5. Morphometric changes correlated to the presence of MaP in GIT samples from both***
713 ***locations.***

714 ***A,*** Condition factor, CF. ***B,*** Viscera weight. ***C,*** liver weight, LIV W. ***D,*** Hepato-somatic index, HSI.
715 (+), MaP isolated; (–), no MaP detected. * $p < 0.05$, ** $p < 0.01$. *30.* Samples/individuals: MDV, N =
716 15; LMP, N = 15. MDV: N = 10 MaP (+), N = 5 MaP (-); LMP: N = 4 MaP (+), N = 11 MaP (-).

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718 ***Figure 6. Expression of immune-related genes in S. canicula spleen samples from MDV.***

719 ***A, D,*** TCRB; ***B, E,*** TCRD; ***C, F,*** IgM. Normalized fold expression relative to HK gene (RPL13) in
720 spleen of specimens witht macroplastic MaP (+) vs specimens without MaP (-) in the digested GIT of
721 all MDV samples (A-C) or only female MDV samples (D-F). Statistically different comparison are
722 represented by asteriscs: *, $p<0.02$; **, $p<0.01$. MDV, 1, Mazara del Vallo.

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Table 1. Sample subset of *S. canicula* used in the chemistry and gene expression analyses.

Sample ID	Gender	MAT	TL	BW	CW	VW	CF	SPL W	SSI	LIV W	HSI	GIT W	GSI (g)	Date 2018	LS
SC1	M	1	42,00	245,10	200,62	44,48	0,33	0,66	0,27	17,17	7,01	16,78	6,85	16-mar	MDV
SC2	M	2	39,00	186,11	157,77	28,34	0,31	0,75	0,40	10,34	5,56	11,01	5,92	16-mar	MDV
SC3	F	4	45,00	294,11	230,23	63,88	0,32	0,79	0,27	14,39	4,89	20,60	7,00	16-mar	MDV
SC4	F	5	43,00	254,05	198,29	55,76	0,32	0,98	0,39	26,66	10,49	19,43	7,65	16-mar	MDV
SC5	M	2	39,50	182,92	155,00	27,92	0,30	0,66	0,36	10,58	5,78	10,50	5,74	16-mar	MDV
SC6	M	1	33,50	104,68	91,01	13,67	0,28	0,37	0,35	4,76	4,55	7,72	7,37	16-mar	MDV
SC7	F	3	40,50	245,02	187,62	57,40	0,37	0,88	0,36	24,58	10,03	14,43	5,89	16-mar	MDV
SC8	F	2	33,50	118,53	99,08	19,45	0,32	0,78	0,66	6,08	5,13	11,09	9,36	16-mar	MDV
SC9	M	4	45,00	303,99	248,30	55,69	0,33	0,67	0,22	14,10	4,64	13,57	4,46	16-mar	MDV
SC10	M	1	39,50	209,30	166,67	42,63	0,34	0,79	0,38	13,16	6,29	15,38	7,35	16-mar	MDV
SC11	F	3	41,00	260,30	205,87	54,43	0,38	0,78	0,30	21,25	8,16	17,17	6,60	16-mar	MDV
SC12	F	1	34,50	127,30	110,60	16,70	0,31	0,51	0,40	6,03	4,74	8,87	6,97	16-mar	MDV
SC13	F	1	35,50	137,50	118,32	19,18	0,31	0,57	0,41	10,01	7,28	8,23	5,99	16-mar	MDV
SC14	M	4	40,50	197,58	159,56	38,02	0,30	0,60	0,30	10,95	5,54	10,36	5,24	16-mar	MDV
SC15	F	4	41,00	267,70	186,99	80,71	0,39	0,43	0,16	14,07	5,26	24,32	9,08	16-mar	MDV
SC49	M	4	36,50	151,80	127,41	24,39	0,31	0,36	0,24	5,14	3,39	7,97	5,25	7-mag	LMP
SC50	F	3	35,00	151,82	118,48	33,34	0,35	0,54	0,36	15,59	10,27	10,66	7,02	7-mag	LMP
SC51	F	2	32,00	121,80	98,75	23,05	0,37	0,45	0,37	10,85	8,91	8,87	7,28	7-mag	LMP
SC52	M	2	36,00	155,08	130,58	24,50	0,33	0,68	0,44	7,03	4,53	9,13	5,89	7-mag	LMP
SC53	F	2	37,00	157,90	131,39	26,51	0,31	0,67	0,42	10,87	6,88	11,62	7,36	7-mag	LMP
SC54	M	2	38,00	173,59	138,99	34,60	0,32	0,51	0,29	9,66	5,56	11,92	6,87	7-mag	LMP
SC55	M	4	35,50	129,70	105,39	24,31	0,29	0,29	0,22	6,76	5,21	9,87	7,61	7-mag	LMP
SC56	M	2	36,00	144,72	115,63	29,09	0,31	0,57	0,39	9,42	6,51	13,71	9,47	7-mag	LMP
SC57	F	4	36,50	161,30	122,43	38,87	0,33	0,56	0,35	13,03	8,08	8,64	5,36	7-mag	LMP
SC58	M	2	35,50	147,88	126,27	21,61	0,33	0,40	0,27	7,39	5,00	6,83	4,62	7-mag	LMP
SC59	M	5	38,50	206,30	167,04	39,26	0,36	0,62	0,30	10,38	5,03	12,78	6,19	7-mag	LMP
SC60	F	5	35,50	155,94	123,01	32,93	0,35	0,63	0,40	15,95	10,23	10,13	6,50	7-mag	LMP
SC61	F	3	36,50	174,00	131,86	42,14	0,36	0,53	0,30	20,13	11,57	8,77	5,04	7-mag	LMP
SC62	M	2	35,50	120,46	102,45	18,01	0,27	0,62	0,51	6,21	5,16	6,62	5,50	7-mag	LMP
SC63	M	4	38,50	171,80	145,30	26,50	0,30	0,55	0,32	10,25	5,97	6,59	3,84	7-mag	LMP

SC, *S. canicula*. Gender: M, male sample, F, female sample. MAT, stage of gonadal maturity (1-5). L, total length; W, total weight. CW, carcass weight. VW, viscera weight. SPL W, spleen weight. LIV W, liver weight. GIT W, gastro-intestinal tract weight. SSI, spleno-somatic index index. HIS, hepato-somatic index. GSI, GIT somatic index. Length is measured in centimeters (cm); weight is measured in grams (g). LS, location of sampling. MDV, Mazara del Vallo; LMP, Lampedusa.

Table 2. Sequences and amplification efficiency of primers used in q-PCR analysis.

Label	Gene Description	Sequence (5' -> 3')	Acc. n.	Slope	R^2	Eff	Amp
RPL13	Large Ribosomal Subunit Protein 13	F: GCTCCAAGTTAATCATCTTCCCA R: GCCTTGAAATTCTTCTCATCCTC	AY130423	-3,2	0,91	104	2,0
ACTb	Actin beta	F: CGAGACCTTCAATACCCCTGC R: CATAACCTTCGTAGATGGGCACAG	AJ312004	-3,1	0,95	110	2,1
RPS29	Large Ribosomal Subunit Protein 29	F: CATCAGCAGCTTTACTGGTCTCATC R: GAAGCCGATGTCTTTAGCGTATTG	n/a	-3,0	0,95	114	2,1
TCRB	T cell receptor beta, B (TCRbB)	F: CGTCAATGGCGAAGAAATGC R: TGTCATGTTGCGTGCTCTTGG	KY434203	-3,1	0,9	110	2,1
IgM	Immunoglobulin M heavy chain	F: CGATGGACTACTCCCCAGAA R: CACAGCTGATTTTGCTGCAT	JX555996	-3,1	0,97	110	2,1
TCRD	T cell receptor delta (TCRD)	F: TGCTTGGCATCAGACTTCTACCC R: TTACCCAGGTGAGATTTTCGG	KY434205	-3,2	0,98	100	2,0

Slopes (-3.1, -3.6), R^2, efficiencies (Eff, 90-110) and amplification (Amp) for each primer couple was checked through the creation of a five points standard curve with serially diluted 1:5 cDNA from 5 samples (MDV: SC7, SC9, SC11 and LMP: SC56, SC57). Amplification and efficiency were calculated using the equation from Dhar et al. (2009).

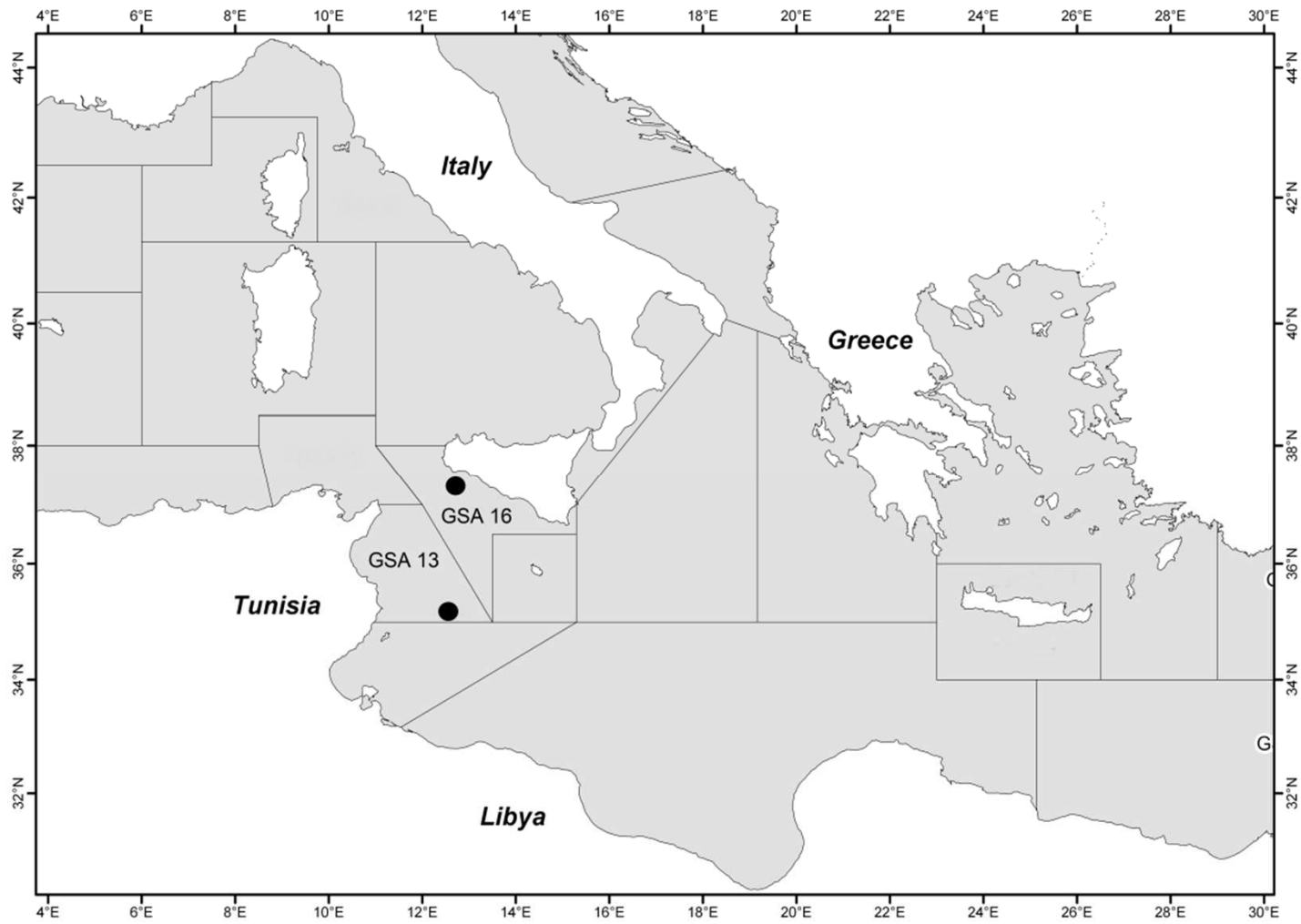
Table 3. Percentage of change in MDV samples compared to LMP samples.

Md / Diff	MAT	CF	HSI
F	- 34%	0%	- 24%
M	- 34%	- 3%	+ 20%

Md, Morphometric data. Diff, % of change in MDV vs LMP. F, females; M, males. MAT, gonadal maturity. CF, condition factor. HIS, hepato-somatic index.

Table 4. Microplastic (MP) and Macroplastic (MaP) total number, particles, fibers, average ingested by individual fish and frequency of ingestion.

Catch area	N. specimens	Total number MP	Filaments	Fragments	Average / fish	Frequency of ingestion
GSA 16 MDV	25	33	24	9	1.32	71%
GSA 13 LMP	25	26	19	7	1.04	62%
Catch area	**N. specimens**	**Total number MaP**	**Filaments**	**Fragments**	**Average / fish**	**Frequency of ingestion**
GSA 16 MDV	25	6	-	6	0.24	20%
GSA 13 LMP	25	5	-	5	0.2	16%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6°E
8°E
10°E
12°E
14°E
16°E
18°E
20°E
22°E
24°E
26°E
28°E
30°E
44°N
42°N
40°N
38°N
36°N
34°N
32°N
Italy
Greece
GSA 16
GSA 13
Tunisia
Libya

Figure 1.

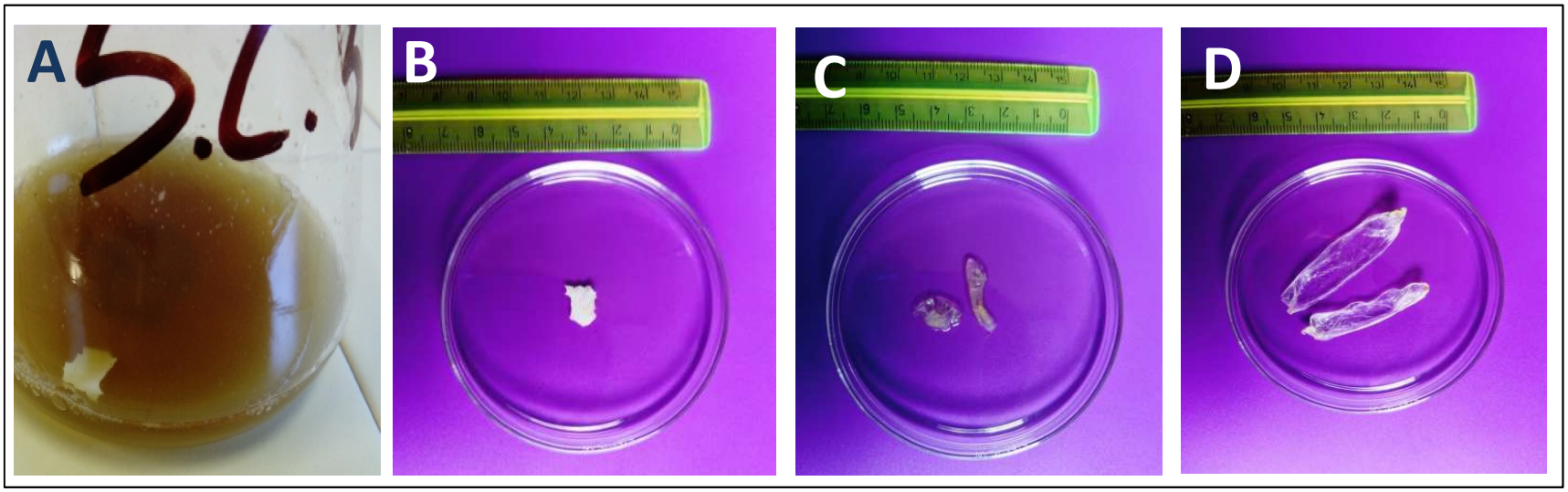


Figure 2.

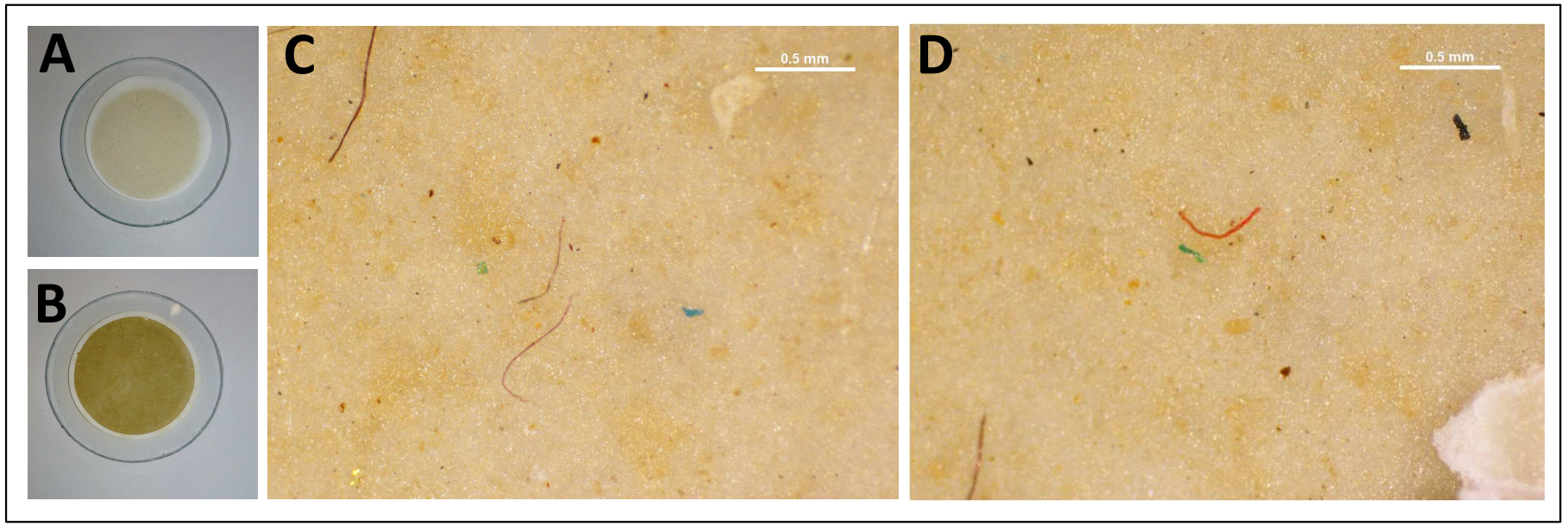


Figure 3.

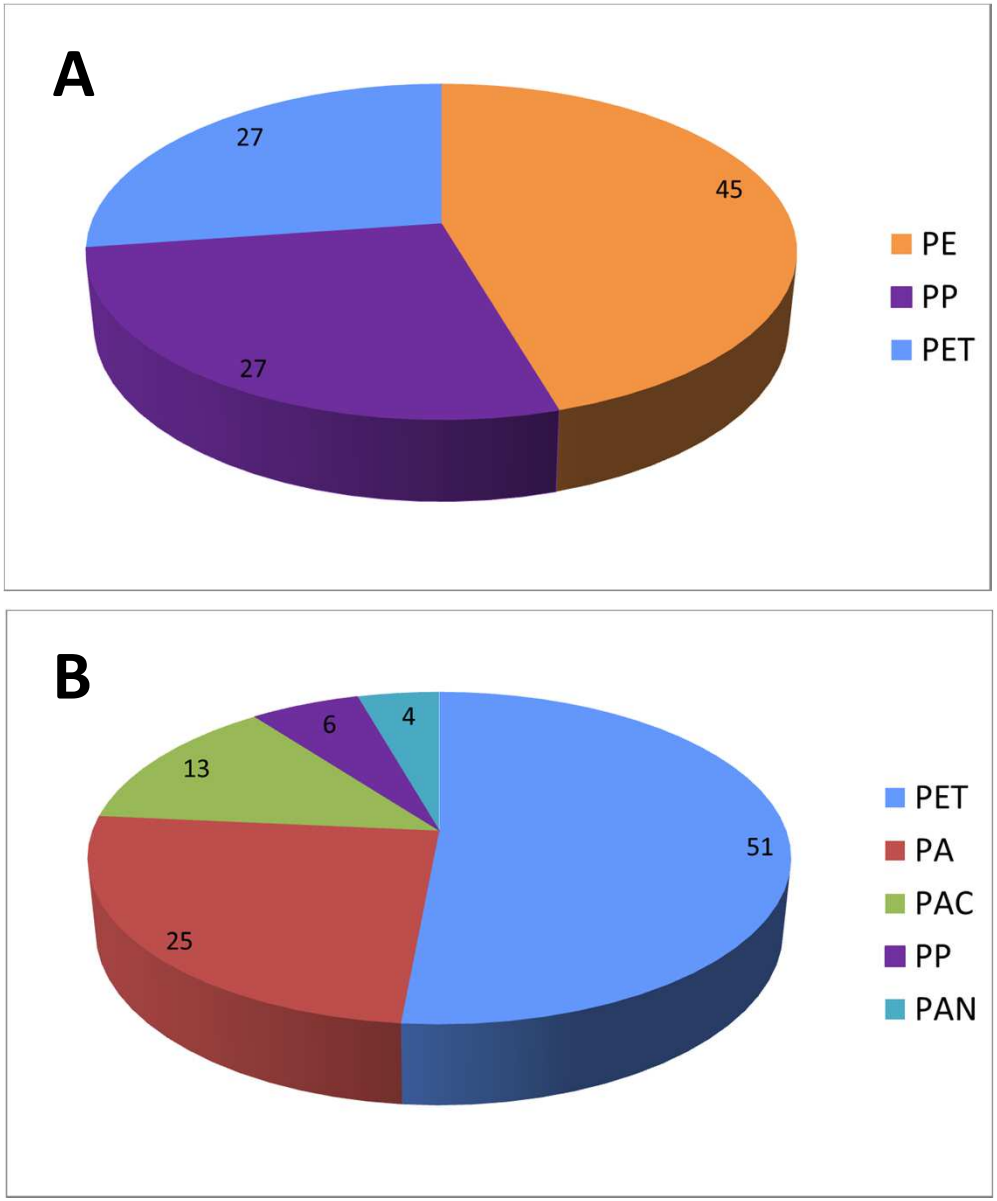
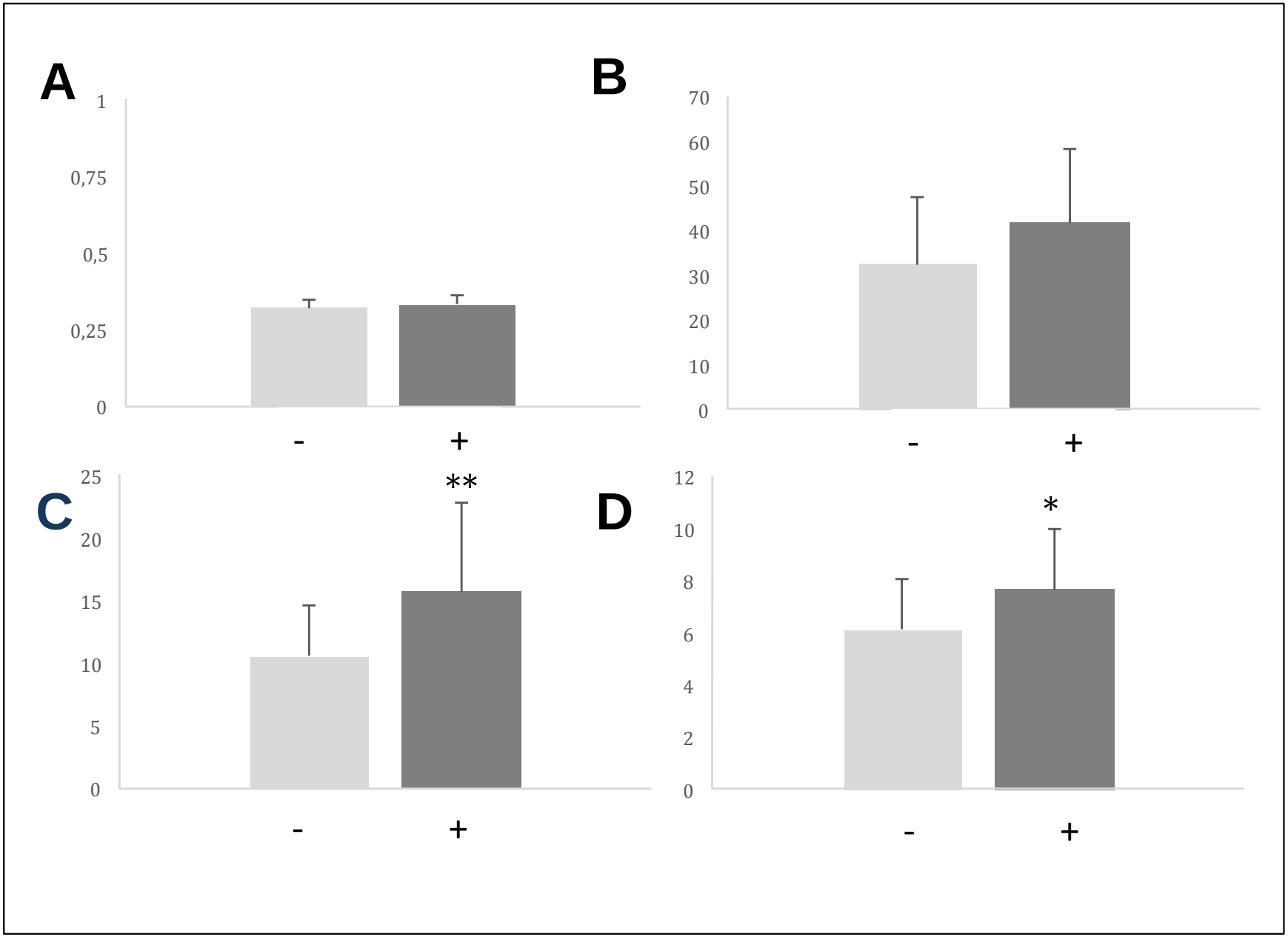


Figure 4.



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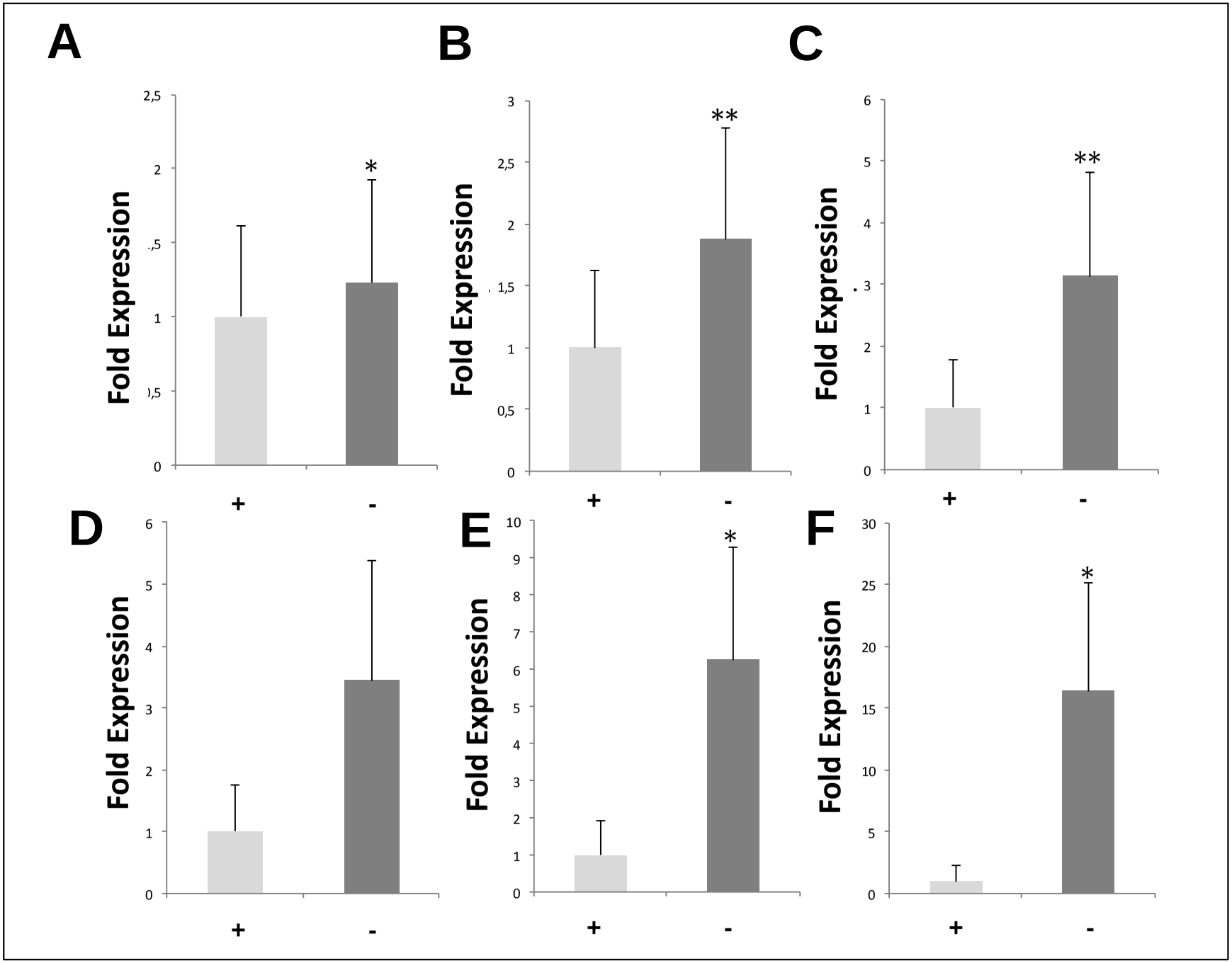

Figure 6.

- We describe microplastics and macroplastics in small spotted sharks (S. canicula) sampled in two geographic locations in the southern region of the central Mediterranean Sea;
- We characterized the plastics in the shark's gastrointestinal tract (GIT) using microscopy and μ-Raman spectroscopy;
- We analyzed morphometric data in correlation of geographical location and plastic load;
- We measured the expression of immune-related genes for potential correlation to location and plastic detection.