Oyster reproduction is affected by exposure to polystyrene microplastics

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Plastics are persistent synthetic polymers that accumulate as waste in the marine environment. Microplastic (MP) particles are derived from the breakdown of larger debris or can enter the environment as microscopic fragments. Because filter-feeder organisms ingest MP while feeding, they are likely to be impacted by MP pollution. To assess the impact of polystyrene microspheres (micro-PS) on the physiology of the Pacific oyster, adult oysters were experimentally exposed to virgin micro-PS (2 and 6 μm in diameter; 0.023 mg·L−1) for 2 mo during a reproductive cycle. Effects were investigated on ecophysiological parameters; cellular, transcriptomic, and proteomic responses; fecundity; and offspring development. Oysters preferentially ingested the 6-μm micro-PS over the 2-μm-diameter particles. Consumption of microalgae and absorption efficiency were significantly higher in exposed oysters, suggesting compensatory and physical effects on both digestive parameters. After 2 mo, exposed oysters had significant decreases in oocyte number (−38%), diameter (−5%), and sperm velocity (−23%). The D-larval yield and larval development of offspring derived from exposed parents decreased by 41% and 18%, respectively, compared with control offspring. Dynamic energy budget modeling, supported by transcriptomic profiles, suggested a significant shift of energy allocation from reproduction to structural growth, and elevated maintenance costs in exposed oysters, which is thought to be caused by interference with energy uptake. Molecular signatures of endocrine disruption were also revealed, but no endocrine disruptors were found in the biological samples. This study provides evidence that micro-PS cause feeding modifications and reproductive disruption in oysters, with significant impacts on offspring.

Significance

Plastics are a contaminant of emerging concern accumulating in marine ecosystems. Plastics tend to break down into small particles, called microplastics, which also enter the marine environment as fragments from a variety of sources, including cosmetics, clothing, and industrial processes. Given their ubiquitous nature and small dimensions, the ingestion and impact of microplastics on marine life are a cause for concern, notably for filter feeders. Oysters were exposed to polystyrene microparticles, which were shown to interfere with energy uptake and allocation, reproduction, and offspring performance. A drop in energy allocation played a major role in this reproductive impairment. This study provides ground-breaking data on microplastic impacts in an invertebrate model, helping to predict ecological impact in marine ecosystems.


The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE71845).

See Commentary on page 2331.

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Total numbers of oocytes were 2.3 ± 0.9 μm for exposed females and 32.2 ± 1.1 μm for the control females. As an oocyte quality proxy, D-larval yield was estimated after making crosses by mixing oocytes collected from exposed and control females with control spermatzoa. A significant reduction in D-larval yield was observed in exposed females (29.6 ± 0.3%) compared with control females (49.8 ± 1.6%).

For males, significantly lower sperm velocity (−23%, P < 0.05) was observed in exposed individuals (59.5 ± 14.5 μm/s, P < 0.05) compared with controls (77.5 ± 9.3 μm/s). The percentage of motile sperm was similar between the two treatments, 40 ± 16% and 51 ± 11% for exposed and control males, respectively.

Finally, the larval growth was significantly slower (P < 0.001) (Fig. 2) in progeny issued from exposed genitors than in progeny issued from control genitors. A mean reduction in size of 18.6% was observed at 17 d postfertilization: mean shell length was 279.8 ± 12.5 μm for control progeny and 227.5 ± 8.5 μm for progeny issued from exposed genitors, for which a 6-d lag in time to metamorphosis was observed.

Transcriptomic and Proteomic Analyses. In the digestive gland, 76 transcripts were differentially expressed between exposed and control oysters (P < 1.10^-5, false-discovery rate (FDR) < 5%) (Dataset S1) and 1,266 transcripts were differentially expressed between sampling times T1 and T3 (P < 0.01, FDR < 5%). Two clusters of transcripts with similar expression patterns, down-regulated (cluster 1, n = 51) and up-regulated (cluster 2, n = 25), were revealed in exposed digestive glands compared with controls (Fig. S2). Response to glucocorticoid stimulus, fatty acid catabolic processes, respiratory burst, and cellular response to mechanical stimulus were the main significantly enriched Gene Ontology (GO) biological processes.

In gonads, 46 transcripts were differentially expressed between exposed and control oysters (P < 0.01, FDR < 5%) (Dataset S2), and 8,136 between the sampling time T1 and T3 (P < 1.10^-5, FDR < 5%). Two distinct clusters with similar expression patterns were found, with transcripts down-regulated (cluster 1, n = 31) and up-regulated (cluster 2, n = 15) in exposed gonads compared with controls (Fig. S2). Glutamine biosynthetic processes, positive regulation of insulin secretion, positive regulation of epithelial cell proliferation, and ovarian follicle cell–cell adhesion were among the significantly enriched GO biological processes.

In oocytes, 81 transcripts were differentially expressed between the two treatments (P < 0.01, FDR < 5%) (Dataset S3); 41 transcripts appeared to be down-regulated (cluster 1, n = 41) and 40 up-regulated (cluster 2, n = 40) in oocytes collected from exposed females compared with controls (Fig. S2). Proteolysis, embryo development, and ion binding were some of the enriched GO biological processes. Finally, the proteome of oocytes revealed two abundant protein spots that showed a marked difference in the oxidative burst, and cellular response to mechanical stimulus were the main significantly enriched Gene Ontology (GO) biological processes.

Reproduction, Gamete Quality, and Larval Development. Histological examination at T3 revealed that all control and exposed oysters were in stage 3, corresponding to ripeness.

For females, the total number of oocytes collected by stripping and oocyte diameter were significantly lower in exposed females than controls (−38%, P < 0.01 and −5%, P < 0.05, respectively). Total numbers of oocytes were 2.3 × 10^5 ± 0.6 for the exposed females and 3.8 × 10^5 ± 0.9 for controls. Oocyte diameter was 30.6 ± 0.9 μm for exposed females and 32.2 ± 1.1 μm for the control females. As an oocyte quality proxy, D-larval yield was estimated after making crosses by mixing oocytes collected from exposed and control females with control spermatzoa. A significant reduction in D-larval yield was observed in exposed females (29.6 ± 0.3%) compared with control females (49.8 ± 1.6%).

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between exposed and control samples. These two spots were identified as arginine kinase, characterized by a lower amount in oocytes collected from exposed females, and the protein seervin, which was present in a higher amount in oocytes collected from exposed females than in oocytes collected from controls.

**Dynamic Energy Budget Model Simulations.** Control oysters were simulated with standard dynamic energy budget (DEB)-model parameters (action of energy used for growth plus somatic maintenance, $\kappa = 0.45$, and volume-specific cost of maintenance $[p_M] = 44 \text{ J cm}^{-3} \text{ d}^{-1}$) and with the absorption efficiency measured in the control (Fig. 3, "control"). Exposed oysters were simulated with standard DEB model parameters and the absorption efficiency measured for this condition (Fig. 3, "micro-PS.std"). Simulated relative differences in final dry flesh mass (DFM) and oocyte production were overestimated compared with values observed at T3. To make the model parameters fit with observed DFM and oocyte production, numerous simulations were performed with a set of parameter values ($\kappa$ from 0 to 1 and $[p_M]$ from 0 to 200 J cm$^{-3}$ d$^{-1}$). The best fit between observations and simulations (Fig. 3, “micro-PS.cal”) was reached with a single set of the two parameters $\kappa = 0.77$ and $[p_M] = 84 \text{ J cm}^{-3} \text{ d}^{-1}$, which corresponds to increases of 71% and 90% beyond standard values, respectively.

**Chemical Analysis.** Following methods described in the Supporting Information, analyses on extracted micro-PS particles detected bibenzyl and 1(2H)naphthalenone,3,4-dihydro4phenyl with >90% correspondences (Fig. S3). Analyses in the aequous phase or digestive styles did not show any molecules leaching from micro-PS particles compared with the controls, with a detection limit at 0.1 ng L$^{-1}$ for compounds with a log $K_{ow}$ less than 3.

**Discussion**

**Ingestion and Fate of Micro-PS in Oyster.** Micro-PS were efficiently ingested by filtration in oysters, presumably because of their similarity in size and shape to phytoplankton. Oysters preferentially ingested the 6-μm micro-PS over the 2-μm-diameter particles. This result may be explained by the oyster particle selection mechanism, which is 100% efficient for 5- to 6-μm particles (27). Ingested micro-PS particles were visually observed in feces (under microscope) and no accumulation in the gut was observed on histological slides, suggesting a high potential of egestion of micro-PS. However, smooth and spherical micro-PS beads differ greatly from plastic debris, such as the fibers and fragments of varying form and roughness present in the marine environment. Therefore, caution must be taken when extrapolating the rapid egestion rate observed here (28). Despite evidence of MP translocation in bivalves from some other studies (14, 15, 17), here no evidence of micro-PS transfer from the digestive tract to the circulatory system and other tissues was detected on the histological slides. Future studies on marine bivalves should address translocation processes by testing nonspherical fragments down to nano-sized particles, the size class most prone to this phenomenon via transcellular uptake in the gastrointestinal epithelium in mammals (29).

**Impacts of Micro-PS on Energy Uptake and Allocation.** Consumption of microalgae and absorption efficiency appeared significantly higher in exposed oysters, suggesting a compensatory effect on food intake and absorption efficiency and an enhancement of mechanical digestion. Indeed, an improvement of mechanical disruption in the stomach of mussels was demonstrated in response to moderate silt ingestion, which enhances clearance rate and absorption efficiency (30). Nevertheless, increased food consumption can be viewed as compensation to adjust energy intake in response to digestive interference caused by micro-PS in the gut. The variations in mRNA levels of lipid-related proteins, such as enzymes involved in fatty acid oxidation, also suggest impairment of fatty acid metabolism and reduced energy intake from food (31). In any case, this compensation is insufficient to counterbalance the energy-flow disruption induced by micro-PS uptake as demonstrated by DEB modeling. Energy flows seem to shift toward organism maintenance and structural growth at the expense of reproduction. A recent study on mussels revealed increased energy consumption measured by respiration in MP-exposed animals, suggesting increased stress and energy demand to maintain homeostasis (16). Furthermore, in our data, there are signs of disturbance of homeostasis reflected by changes in hemocyte size and oxidative activity (32), and enrichment of transcripts involved in the response to glucocorticoid stimulus GO process. Glucocorticoids are hormonal corticosteroids involved in stress response, able to inhibit the expression of enzymes involved in fatty acid oxidation (33, 34).

**Micro-PS Impaired Gametogenesis, Gamete Quality, and Fecundity.** Strong negative effects were observed on reproductive health indices, which significantly impacted fecundity and offspring performance during larval stages. The 23% reduction in sperm velocity in exposed oysters may lower their ability to fertilize oocytes. Indeed, in sea urchin a decrease in sperm motility was linked to an increase in the number of sperm required for fertilization success (35). Oyster oocyte number and size in micro-PS–exposed oysters were

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**Fig. 3.** DEB modeling. DEB model simulations for the DFM and oocyte number. Simulations named “control” represent simulations with standard parameters (i.e., fraction of energy allocated to soma, $\kappa = 0.45$, and volume-specific cost for maintenance, $[p_M] = 44 \text{ J cm}^{-3} \text{ d}^{-1}$) and with absorption efficiency measured in controls. Simulations named “micro-PS.std” represent simulations with standard parameters and with absorption efficiency measured for oysters exposed to micropolyethylene. Simulations named “micro-PS.cal” represent simulations with calibrated parameters (i.e., $\kappa = 0.77$ and $[p_M] = 84 \text{ J cm}^{-3} \text{ d}^{-1}$) and with absorption efficiency measured for exposed oysters. Initial and final dry flesh mass and oocyte production observed are plotted.
also significantly reduced over the same period (−38% and −5%, respectively). As oocyte quality predictors, mean oocyte diameter has been identified as a direct consequence of nutrition (36), supporting the hypothesis of energetic disruption in exposed oysters. Moreover, egg size and shape were reflected in morphological traits directly related to larval survival and growth in subsequent progeny (37). In oocytes, maternally inherited mRNAs can have multiple functions, from regulation of cell-cycle progression and cellular metabolism, to regulation of developmental processes, such as fertilization, activation of zygotic transcription, and formation of body axes (38). Ion binding was greatly affected by MP exposure: 10 transcripts coding for proteins involved in this function were differentially expressed. Transcripts coding for proteins involved in Ca2+ binding may have affected the Ca2+ signaling pathway in exposed oocytes, thus affecting oocyte maturation (39). Severin is a Ca2+-dependent actin-binding protein regulating the completion of cell division (40); its up-regulation may reflect a deleterious effect of micro-PS on cytoskeletal dynamics, which are essential during oocyte maturation, fertilization, and subsequent embryo development (41). Other candidates, down-regulated in exposed oocytes, also indicate potential impairment of embryo development: transcripts in the categories of embryogenesis, cell differentiation, and proliferation, and the arginine kinase protein, responsible in invertebrates for ATP buffering on phosphagens, which are essential for metabolism, to regulation of developmental processes, such as stress or endocrine-disrupting chemicals. A disturbance in fecundity, estimated through D-larval yield, offspring growth, and settlement, was observed for larvae produced from gametes collected from micro-PS exposed oysters. Negative effects of MP had already been observed on fecundity in copepods using similar micro-PS (20), and in Daphnia exposed to nano-PS, where numbers and body size of neonates fell and malformation rates rose (21). The parental effect of micro-PS on subsequent offspring growth may potentially affect recruitment of wild and farmed populations of Pacific oysters, with consequences for both ecology and aquaculture.

It is hypothesized that the energetic fraction allocated to reproduction seemed to shift toward structural growth and high maintenance costs. Disruption of energy balance may result from the down-regulation of several transcripts coding for proteins involved in the insulin pathway, with GO terms corresponding to cell proliferation and differentiation processes, in both digestive gland and gonads. The insulin pathway plays a crucial role in mobilizing reserves during gametogenesis, and has an essential role in germlinal cell proliferation and maturation (43). We thus hypothesize that micro-PS exposure negatively impacts cell proliferation and differentiation processes in gonads through the down-regulation of several transcriptomic protein-coupled receptor transcript, also down-regulated in digestive gland, has a key role in the reproductive function, binding the kisspeptin hormone responsible for the gonadotropic axis in vertebrates (44). The differential expression of hormone receptors or transcripts involved in different hormonal pathways in micro-PS–exposed animals suggests endocrine disruption. Endocrine system function can be affected by factors such as stress or endocrine-disrupting chemicals. A disturbance in individual energetics revealed by DEB modeling suggested that micro-PS particles have threatened the physiological integrity of oysters and consequently increased the maintenance costs, as described in response to various stresses and species (45–47). Micro-PS particles may potentially act as endocrine disruptors. The chemical analyses of virgin micro-PS only revealed bibenzyl and 1(2H)naphthalenone, 3,4-dihydroxyphenyl in destructive conditions after dichloromethane extraction. Bibenzyl-diol core molecules may have endocrine disruption properties, as established in mammalian cells, because they are structural analogs of estrogens (48). No substances were found in seawater or in the digestive style extracts used to mimic oyster digestive conditions in vitro. However, it is now known that endocrine disrupters are often present below the detection limits and that bio-assays are sometimes more powerful than chemical quantification methods to detect their presence and effect (49). Although we cannot establish an impact of these molecules in our experiment, reprotoxic effects induced by virgin MP have recently been revealed in Daphnia (21) and in fish (50), suggesting that they could be a concern for endocrine disruption, induced by MP alone or in combination with other persistent pollutants.

**Implications.** The micro-PS concentration tested in the present study was below the one estimated in Besseling et al. (21) that may occur at the sediment–water interface, where wild oysters live (Table S1). The exposed mass concentration (0.023 mg L−1) was also in the range of the highest estimated field concentration >33 μm, from manta trawl sampling (Table S1), based on the assumption of a steady fragmentation of plastic debris (9, 51). It should, nonetheless, be noted that there is a lack of consistent field evaluations of the presence of microplastics as an exposure used in the present study. This is mainly because of methodological limitations: current methods exclude the possibility of quantifying small size domains (reviewed by Filella (51)). Moreover, assuming no waste management infrastructure improvements, the cumulative quantity of plastic waste available to enter the marine environment from land is predicted to increase by an order-of-magnitude by 2025 (2), especially in estuaries and coastal waters where oysters live and where waters are greatly influenced by increased human expansion. Therefore, our study also contributes to an early warning system and provides stakeholders with the necessary data to limit the impact of the microplastic legacy in decades to come.

To conclude, this study highlighted microplastic impacts on energy uptake and allocation and on reproductive health indices (i.e., quantity and quality of gametes produced), when oysters were exposed to micro-PS during gametogenesis. Strong negative effects were shown on broodstock fecundity and offspring growth at larval stages. The two explanatory hypotheses discussed in the present paper, a fall in energy allocated to reproduction via interference in digestive processes and endocrine disruption, are not mutually exclusive. We believe that, considering the strength of the impact on reproductive health indices, both forms of disruption may have occurred. However, the absence of endocrine disruptor detection in biological samples prevents us from drawing stronger conclusions about this second hypothesis. Transcriptomic profiles support this hypothesis, notably highlighting an alteration in glucocorticoid response, insulin pathway, and fatty-acid metabolism in oysters in response to micro-PS exposition. Further investigations are now necessary: first, to provide full environmental data on small microplastics <10 μm, requiring fundamental analytical developments (51) and, second, to compare our experimental results with in situ and experimental studies that closely mimic in situ conditions, in particular by using different shapes and forms of MP representative of those found in the field.

**Methods**

**Experimental Exposure of Adult Oysters to Micro-PS.** The experimental procedures comply with French law and with institutional guidelines. Adult oysters purchased from a commercial hatchery (18 mo, 16.9 ± 3.3 g) were transferred to Institut Français de Recherche pour l’Exploitation de la Mer’s experimental facilities in March 2013. Histological visual inspection showed they were at reproductive stage 0 to early stage 1, corresponding to an undifferentiated state or developing early active gametogenesis (52). After acclimatization, the oysters were conditioned for 2 mo under suitable conditions for germ cell maturation (52). The oysters were placed in six experimental 50-L tanks (40 oysters per tank) supplied with filtered (1 μm), UV-treated running seawater (12.5 L h−1) at 17.1 ± 0.5 °C and 34 PSU, and fed continuously on a mixed diet of Tisochrysis and microalgal (Tisochrysis for T. iso; formerly Isochrysis sp., Tahitian strain: T. iso; CCAP 927/14, and Chaetoceros gracilis, UTEX LB2658) at a daily ratio equal to 8% dry weight algae/dry weight oyster. Control and micro-PS exposed treatments were set up with three tanks per condition. For each treatment, a fourth tank was deployed without oysters to evaluate algal and micro-PS sinking or sticking to the tank walls. To prevent micro-PS sinking, the water inflow was pressurized to create the regulating flow and the tank bottoms, and micro-PS particles were supplied to tanks with Tween-20 at a final concentration of 0.0002%. The same concentration of Tween-20 was supplied to the control tanks.
The purchased micro-PS were yellow-green fluorescent polystyrene beads (2 and 6 μm; Polysciences). These were supplied continuously to the tanks by peristaltic pumps from a concentrated micro-PS solution, maintained in a glass flask on a magnetic stirrer. Micro-PS concentrations were daily counted using an EasyCyte Plus flow cytometer (Guava-Merck-Millipore) giving an inflow concentration of 2,062 ± 170 and 118 ± 15 beads per mL for 2- and 6-μm particles, respectively (namely a mass concentration of 0.023 mg L⁻¹) corresponding to an inflow daily ratio of 9.6-mg micro-PS d⁻¹. The mass concentration in the surrounding water was of 0.01 mg L⁻¹ (i.e., 1,816 ± 76 and 21 ± 6 beads per mL for 2- and 6-μm particles, respectively), which is far lower than most to which marine invertebrates have been exposed (from 0.8 to 2,500 mg L⁻¹) (10, 15, 17, 21) (Table S1). Microplastic concentration corresponded to 0.21% of the volume (μm³) of algae supplied.

Ecophysiological Measurements. Once a day, inflow and outflow seawater was sampled from each tank. Phytoplankton counts were made using an electronic particle counter (MultiSizer 3 equipped with a 100-μm aperture tube) to provide 50 d of continuous data. Algal consumption (C) was expressed in algal cell volume per oyster per day (μm³ oyster⁻¹ d⁻¹), as in Savina and Pouvreau (53). Polystyrene particle ingestion (I) was estimated in percentage micro-PS ingested: I = [(I_i-I_c)/I_i] × 100, I_i being number of beads at the inlet, I_c number of beads at the outlet, I_c number of beads remaining in the tank without oysters by subtracting inlet from outlet. Once a week, feces were collected from the tanks to calculate absorption efficiency (percent) of organic matter from ingested food (53).

Sampling. At the beginning and the end of the experiment, 12 oysters per condition were killed to measure biometric parameters (total, shell, and dry weight). Condition index was calculated as: dry weight/total weight-shell weight. At 2, 5, and 8 wk after the beginning of exposure (corresponding to T1, T2, and T3, respectively) eight animals per tank were sampled for histological analysis (hemolymph [taken as described by Haberkorn et al. (54)], and a transversal section of the gonadic area for histological examination. The remainder of the gonad and digestive gland were immediately frozen in liquid nitrogen for subsequent analyses. Oocytes were collected from five females per treatment, filtered in a 40-μm sieve, counted, and transferred into 1.5 mL extract-all reagent (Eurobio) (20,000 oocytes) and 5 mL lysis buffer (55) (200,000 oocytes) for RNA and protein analyses, respectively. For gamete quality measurements, oocytes were collected from five females per treatment, fixed in 0.1% formaldehyde-seawater solution until image analysis for size measurement. Oocytes were processed and analyzed using the language R/BioConductor (60) as in Sussarellu et al. (59).

Preprocessing and Microarray Data Analysis. Microarray data were processed using the R-based BioConductor framework (60). Normalized hybridization values were deposited in the Gene Expression Omnibus (GEO) repository with the accession number GSE71845. Statistical analyses to identify the differentially expressed transcripts in digestive glands and gonads were carried out by ANOVA. The fixed factors for the two-way ANOVA were treatment (IM exposure vs. control) and sampling time (T1 or T3). For oocytes, differentially expressed transcripts were detected by t-test. The FDR associated with the selected transcripts was determined by a total number of analyzed transcripts (31,918) × P value/number of differentially expressed transcripts × 100; the FDR cut-off value was 5%. Hierarchical clustering was performed using the Ward method, and 1-correlation as similarity matrix. Putative annotations of transcripts were identified using ngKlaster software (KL Korilg Bioinformatics Solutions) against a protein database (E-value 1.0 × 10⁻5) obtained from the C. gigas sequenced genome available on GenBank. Differentially expressed transcripts were tested against the Swissprot database (E-value 1.0 × 10⁻5). GO term enrichment analysis was performed using the Fisher’s Exact test on Blast2Go (62).

Dynamic Energy Budget Design. The DEB model simulations were performed as in Bernard et al. (63) to evaluate how physiological changes induced by micro-PS exposure affect energy fluxes and could explain observed phenotypic changes. The DEB model describes dynamics of four state variables: (i) the energy stored in reserves, E; (ii) energy allocated to structural growth, EV; (iii) energy allocated to development and reproduction, ER; and (iv) energy used in the construction of gametes, E_G (see ref. 63 for a full description). Initial state was obtained from the initial biometrics measurements and maturity observations. Oocyte production was calculated according to an energy content of 9.3 ± 4 J (spermatozoa) and 1.5 ± 0.2 J (ova) (56). Two parameters, namely the allocation fraction to structural growth and structural maintenance from reserves (the remainder being allocated to development/reproduction and maturity maintenance, x) and the volume specific cost for maintenance rate (p_m), are free fitted to evaluate the disturbance level in terms of micro-PS exposure that would lead to the observed growth and reproductive traits.

Statistical Analysis. All analysis data were processed and analyzed using the language RBioConductor (60), R Development Core Team (2008) by ANOVA (fixed factors were condition and sampling date) or t tests. Normality was screened on residuals and further tested using the Shapiro-Wilk test. When necessary, data were log-transformed, and angular transformation was used for percentage data. Homogeneity of variance matrices was assessed with a Fligner test. Least-significant difference post hoc tests were performed to discriminate groups. Data are expressed as mean ± SD or SEM. Analyses of microarray data are detailed above in the microarray data analysis section.

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