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Proteomic analysis of seminal plasma and nanoplastics effects on sperm cells in Sparus aurata

Hayam Djafar

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Proteomic analysis of seminal plasma and nanoplastics effects on sperm cells in *Sparus aurata*

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Contents

Acknowledgements 5
Summary 7

Introduction 9

Ecotoxicological effects of nanoplastics in aquatic environments 11
Effects of nanoplastics on fish reproduction 13
Effects of nanoplastics in Gilthead Seabream (*Sparus aurata*) 16
References 18

Objectives 24

Proteomic profile of gilthead seabream (*Sparus aurata*) seminal plasma 25

Abstract 27
Introduction 28
Materials and Methods 29
Results 31
Discussion 39
References 44

Short-term exposure to polystyrene nanoplastics alters sperm viability and motility, and seminal plasma composition in the gilthead seabream (*Sparus aurata*) 53

Abstract 55
Introduction 56
Materials and Methods 57
Results 62
Discussion 71
Conclusions 75
References 76

The impact of nanoplastics on the quality of fish sperm: a review 87

Abstract 89
Introduction 90
Materials and Methods 91
Effects of NPs on the quality of sperm in fish 96
Knowledge gaps 102
Conclusions 105
References 106

General discussion and conclusions 113

Minimum common set of protein functionalities in the seminal plasma of fish 116
The impact of nanoplastics on sperm performance and metabolite composition in the seminal plasma 118
Conclusions 121
References 122

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Summary

In species with external fertilization, such as fish, exposure to nanoplastics may lead to changes in both sperm motility and viability and, ultimately, affect fertilization, proper development and survival. This is particularly important in commercially valuable species such as the gilthead seabream (*Sparus aurata*), as it may affect both wild and cultured populations due to the widespread presence of contaminants such as nanoplastics not only in aquatic environments, but also in the plastic tools and equipment used in aquaculture practices.

Research on the effects of nanoplastics on fish reproductive physiology remains limited to a handful of species, mostly laboratory models and some commercially important species. However, studies focusing on the impact of nanoplastic exposure on male reproductive function in gilthead seabream are still scarce. Therefore, this thesis addresses the issue from two complementary perspectives. First, the proteomic profile of gilthead seabream seminal plasma was characterized to obtain a reliable representation of its protein composition. This characterization provides a reference framework and establishes a common baseline for future analyses of proteomic deviations under pathological or stressful conditions. Second, the performance of *S. aurata* spermatozoa exposed to increasing doses of nanoplastics was evaluated to determine their effects on sperm motility and viability. In addition, metabolites affected by exposure to polystyrene nanoplastics (PS-NPs) in seminal plasma were also assessed. Our results indicate that the proteomic characterization of gilthead seabream seminal plasma identified key biological processes represented within the seminal plasma proteome, namely innate immune responses, coagulation processes, and proteolytic activity. The complement system and other acute-phase proteins appear to orchestrate the immune response in *S. aurata* seminal plasma, as suggested by the abundance and composition of the identified proteins. Exposure to polystyrene nanoplastics (PS-NPs) altered sperm motility and viability and may consequently affect fertilization success. PS-NPs reshaped sperm motility patterns in a dose-dependent manner. Exposure to high concentrations of PS-NPs (50 μg/mL) induced erratic sperm movements, whereas low concentrations (1 μg/mL) enhanced sperm velocity and increased levels of metabolites associated with motility support. Exposure to high concentrations of PS-NPs also increased the levels of lipid-related markers, including cholesterol and triglycerides, in the seminal plasma of *S. aurata*. Under PS-NP exposure, betaine and glycine

emerged as key protective metabolites in gilthead seabream seminal plasma. The thesis is further complemented by an analysis of recent experimental evidence in the form of a review of the current state of the art regarding the general effects of nanoplastics on fish reproductive function, helping to contextualize and support the arguments developed throughout the work. Overall, the characterization of the effects of nanoplastics on the male fish reproductive system remains incomplete and appears to be strongly species-specific.

1

Introduction

1. Ecotoxicological effects of nanoplastics in aquatic environments

Aquatic ecosystems are increasingly exposed to a wide range of emerging contaminants, including pharmaceuticals, personal care products, heavy metals, and plastics. Among these, plastic pollution has become a major global concern. Plastics degrade over time into smaller particles, forming microplastics (MPs) (<5 mm) and nanoplastics (NPs) (<100 nm) (Amereh et al., 2020; Koelmans et al., 2015). NPs originate from the degradation of larger plastic debris, industrial nanomaterials, cosmetic and pharmaceutical products **(Figure 1)**.

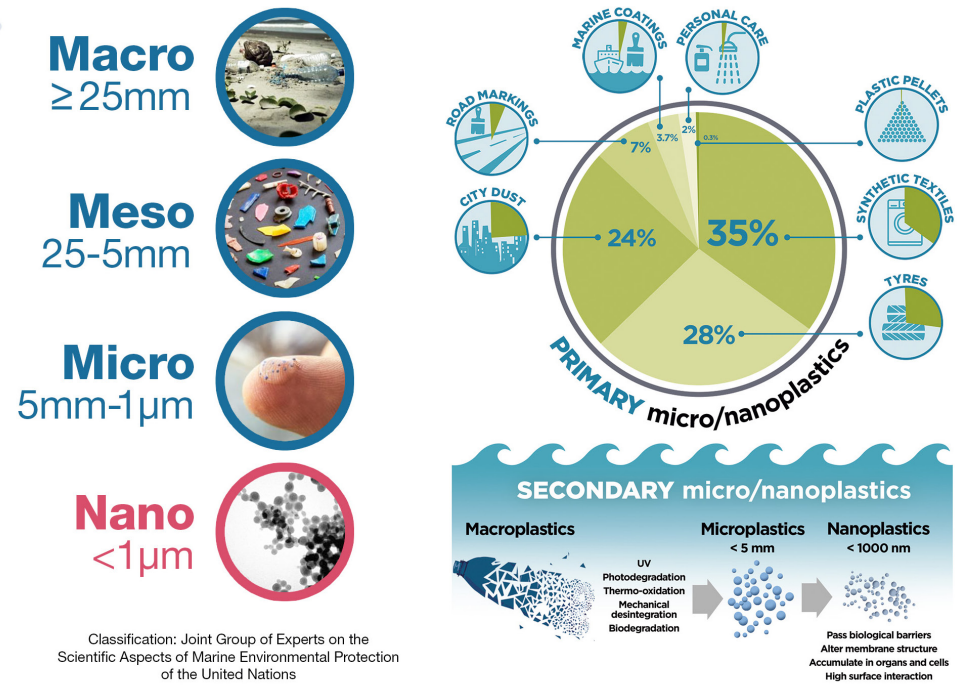


Figure 1. Sources of primary and secondary MPs and NPs found in marine waters. Adapted from Boucher and Friot (2017), IUCN.

Once released into aquatic environments, nanoplastics can aggregate, or remain suspended, bind to other pollutants or be ingested by aquatic organisms. Various types of MNPs (<50,00,000 nm), such as polystyrene (PS), polyvinyl chloride (PVC), polyethylene (PE), polyethylene terephthalate (PET), polymethyl methacrylate (PMMA), polyoxymethylene, and

polypropylene (PPP), have been identified across multiple environmental compartments (De Sa et al., 2018). Because of their extremely small size and large surface area, NPs are particularly effective at both physically and chemically adsorbing a wide range of environmental contaminants (Hartmann et al., 2017; Lee et al., 2019; Trevisan et al., 2019; Bhagat et al., 2020; Bhagat et al., 2021). In addition, they can be readily transferred along the food chain (Chae et al., 2018).

Their small size enables them to cross biological barriers, including cell membranes, and potentially even nuclear membranes, leading to intracellular accumulation, and potentially disrupting physiological processes (Alimi et al., 2018). The European Food Safety Authority (EFSA) has reported that particles smaller than 150 μm (150,000 nm) are capable of crossing the intestinal mucosal barrier, whereas those below 1.5 μm (1,500 nm) may penetrate further into deeper tissues, including essential organs (Horvatits et al., 2022). Moreover, these particles have also been detected in the brain and reproductive organs (Lehner et al., 2019).

NPs exert toxic effects through several interconnected mechanisms. Briefly, NPs can induce oxidative stress, mitochondrial dysfunction, inflammation and cellular damage, and metabolic disruption. Thus, NPs can induce the production of reactive oxygen species (ROS), leading to oxidative damage in lipids, proteins, and DNA (Panizzolo et al., 2023), interfere with mitochondrial function, reducing ATP production and impairing cellular energy balance (Liang et al., 2022). Likewise, exposure to NPs can trigger inflammatory responses and alter cell signalling pathways, contributing to tissue damage, and affect key metabolic pathways, including glycolysis, lipid metabolism, and amino acid metabolism. These disruptions can impair cellular homeostasis and energy production (Zhao et al., 2023). Numerous studies have investigated the ecotoxic effects of NPs on aquatic organisms, showing that NPs can readily cross cell membranes, accumulate in tissues, and transfer through food chains (Bouwmeester et al., 2015; Chae et al., 2018; Lee et al., 2019., Toussaint et al., 2019).

These particles have been reported to negatively affect behaviour, feeding, growth, reproduction, and survival (Chen et al., 2017). Contaminants present in aquatic environments can also directly interact with sperm cells, consequently affecting their structure, function and fertilising ability (Amereh et al., 2020). Reproductive endpoints such as sperm motility, viability, and metabolic activity are widely used as sensitive biomarkers of environmental stress (Gallego et al., 2018).

1.2 Effects of nanoplastics on fish reproduction

Fish represent the most diverse group of vertebrates, with more than 34,000 described species inhabiting a wide range of aquatic environments, from freshwater to marine ecosystems. The aquatic environment is central to the biology and survival of marine fish, serving not only as their living habitat but also as the medium in which critical life processes, including reproduction, occur (Trindade-Santos et al., 2015). Freshwater ecosystems host a greater number of fish species (~15,200) than marine ecosystems (~14,800), even though oceans cover about 70% of the Earth's surface and account for roughly 97% of all water. In marine systems, fish diversity is largely concentrated in shallow coastal areas (less than 200 m deep), which make up under 1% of the global ocean surface (May, 1994; Carrete Vega & Wiens, 2012).

This diversity is reflected in their reproductive strategies, which include both internal and external fertilisation, hermaphroditism, and complex life cycles (Alavi et al., 2021). Among marine teleosts, external fertilisation is the dominant reproductive mode. In this process, gametes are released into the surrounding water, where fertilisation occurs. This reproductive strategy requires precise synchronisation between male and female gamete release, as well as optimal environmental conditions (Dzyuba and Cosson, 2014). Due to this exposure, fish gametes are highly vulnerable to environmental stressors, including chemical contaminants. Therefore, fish are widely used as bioindicators in ecotoxicology and environmental monitoring studies (Wang et al., 2022)m due to (1) direct exposure of gametes to waterborne pollutants, (2) high sensitivity of reproductive tissues, and (3) ecological relevance in aquatic food webs. Furthermore, because fish have numerous biomarkers of contamination comparable to those in mammalian models and hold significant ecological and economic importance, they are considered one of the most sensitive bioindicators of environmental change and play a crucial role in ecotoxicological assessments aimed at protecting ecosystems and preventing contamination (Brooks, 2019; Ribeiro et al., 2020). However, in fish, studies are mostly restricted to model organisms that are easy to obtain and manipulate, such as zebrafish (Oliveira et al., 2019). The primary advantage of zebrafish as a model organism lies in their ease of rearing, handling, observation, and analysis. Research findings using zebrafish provide significant reference values, making them an ideal model for studies in developmental biology, genetics, and molecular biology. They

are widely used in applications such as drug screening, disease modelling, and environmental assessment (MacRae et al., 2015; Bambino et al., 2017).

NPs can negatively affect reproductive physiology in both aquatic and terrestrial organisms **(Figure 2)**. Exposure to 100-nm diameter polystyrene nanoplastic particles (PS-NPs) reduces hatching and survival rates of zebrafish embryos while inhibiting heart rate and body length by activating oxidative stress and base excision repair pathways in zebrafish (Feng et al., 2022). Increasing doses of 100-nm diameter PS-NPs exacerbated oxidative stress in oocytes, leading to oocyte apoptosis and impaired reproductive function in zebrafish (Chatterjee et al., 2022). NPs also inhibit spermatogenesis and oogenesis in medaka and delay gonadal maturation (Zhou et al., 2023). Exposure to 50 nm (PS-NPs) at concentrations ranging from 1 to 10 ppm significantly reduced the survival of embryonic and larval freshwater medaka (*Oryzias latipes*) (Manabe et al., 2011). Moreover, exposure to NPs may worsen the effects of other endocrine-disrupting chemicals. For instance, adult zebrafish exposed to bisphenol A for 21 days displayed marked structural and ultrastructural changes in the gonads, including ovarian atresia and degeneration, as well as degeneration of testicular cells and disruption of normal cellular organisation.

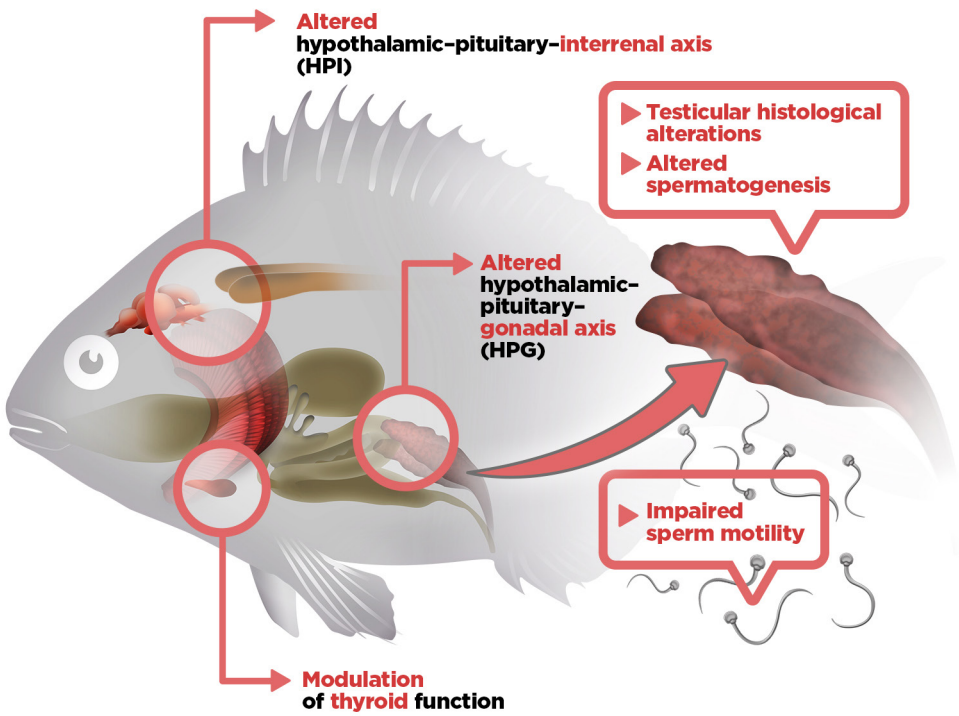


Figure 2. **Effects of NPs in fish hormonal reproductive axis.**

These pathological alterations may ultimately impair reproductive capacity in both females and males (Reis et al.2022). Chronic exposure of female zebrafish to PS-NPs has been reported to disrupt reproductive regulation at both endocrine and molecular levels. After 42 days of exposure, a significant decrease in gonadal 17β-estradiol (E2) levels was observed, combined with an increase in testosterone (T) concentrations, indicating an imbalance in sex hormone regulation (Zhang et al., 2024; Wu et al., 2025). Moreover, Zhang et al. (2024) observed that prolonged exposure to PS-NPs can modify the expression of genes involved in sex differentiation, suggesting possible consequences for reproductive development and function.

Adverse reproductive effects have also been documented in male zebrafish. Exposure to NPs at a concentration of 1000 μg/L resulted in a significant increase in apoptotic cells in the testes, which was linked to activation of the p53-mediated apoptotic signalling pathway (Qiang et al,2021). Such cellular damage may interfere with spermatogenesis and negatively affect male fertility. In male fish, sperm quality is a key determinant of reproductive success in fish species with external fertilisation. It is commonly evaluated using several parameters, such as motility, viability, morphology and metabolic activity. Motility is especially critical, as fertilisation must occur within a short time window after sperm activation. Fish sperm rely primarily on ATP for movement, and this energy is rapidly depleted during motility (Gallego et al., 2018). Any disruption in ATP production, mitochondrial function, or membrane integrity can significantly reduce sperm performance and fertilisation success.

Alterations in germ cell quality and hormonal homeostasis in exposed parents may also provoke consequences for the next generation. Teng et al. (2022) demonstrated that PS-NPs can be transmitted from exposed adults to their offspring, where the particles accumulate in the gastrointestinal tract of F1 larvae. Parental exposure was associated with disturbances in early embryonic development. As early as 2 hours post-fertilisation, embryos showed changes in spontaneous motility, lower hatching rates, and reduced body length (Zhang et al., 2024). In addition, maternal exposure to PS-NPs significantly decreased both E2 and T concentrations in F1 zebrafish, providing further evidence of transgenerational endocrine disruption (Zhang et al,2024). Most studies focus on general reproductive toxicity. However, fewer studies specifically investigate sperm-level effects. Sperm cells are highly sensitive to environmental stress due to their high metabolic demands, their limited repair mechanisms, and their dependence on membrane integrity.

1.3 Effects of nanoplastics in gilthead seabream (*Sparus aurata*)

Beyond classical laboratory organisms, commercially important marine fish species are increasingly investigated to better understand the ecological significance and aquaculture implications of (NPs) contamination. Among these, the gilthead seabream, *Sparus aurata*, stands out as a particularly relevant marine teleost model due to its high economic value and central role in Mediterranean aquaculture production (Brandts et al., 2020; Okon et al., 2025). Its well-characterised physiology, wide distribution, and sensitivity to environmental stressors make it a suitable organism for linking mechanistic toxicological responses with applied aquaculture outcomes.

Short-term waterborne exposure to polymethylmethacrylate nanoplastics (PMMA-NPs) in *S. aurata* has been shown to induce marked molecular and biochemical alterations (Brandts et al., 2020). Specifically, there is up-regulation of genes involved in lipid metabolism and antioxidant defence pathways, accompanied by increases in plasma cholesterol and triglyceride concentrations. In parallel, total antioxidant capacity rises, suggesting activation of compensatory redox mechanisms. However, erythrocytic nuclear abnormalities have also been reported, indicating potential genotoxic damage and raising concerns about long-term genomic stability under continued NPs exposure (Brandts et al., 2020).

Subchronic exposure to (PS-NPs) further demonstrates that, even in the absence of overt histopathological lesions or significant changes in condition indices, substantial transcriptional reprogramming can occur (Okon et al., 2025). Alterations have been documented in genes associated with lipid metabolism, somatic growth regulation, xenobiotic detoxification, and oxidative stress responses. These findings suggest that NPs can disrupt lipid homeostasis and antioxidant balance at the molecular level before clear morphological damage becomes evident. Such early molecular biomarkers may therefore serve as sensitive indicators of sublethal stress in cultured fish (Okon et al.,2025).

Under long-term exposure scenarios, including environmentally realistic and elevated (PS-NPs) concentrations, *S. aurata* exhibits dose-dependent modifications in plasma metabolomic profiles (Blonç et al., 2025). Changes in amino acid metabolism, energy-related metabolites, and intermediary metabolic pathways indicate disturbances in energy allocation and metabolic efficiency. Moreover, significant reductions in gut microbiome diversity

have been observed, with shifts in microbial community composition that may compromise nutrient assimilation, immune competence, and overall physiological performance. These combined metabolomic and microbiome alterations suggest that chronic NPs exposure may negatively affect growth efficiency and resilience under aquaculture conditions (Blonç et al., 2025).

Importantly, the absence of pronounced external pathology in some exposure trials does not equate to biological safety. Instead, integrative approaches combining transcriptomics, metabolomics, haematological biomarkers, and microbiome analyses reveal subtle but functionally meaningful disruptions across multiple biological levels (Brandts et al., 2020; Blonç et al., 2025; Okon et al., 2025). Such multi-level responses highlight the importance of evaluating both mechanistic biomarkers and whole-organism performance endpoints when assessing NPs toxicity.

Collectively, the available evidence supports the use of *Sparus aurata* as a robust and ecologically relevant marine model for investigating the cellular, molecular, physiological, and systemic consequences of NPs contamination (Brandts et al., 2020; Blonç et al., 2025; Okon et al., 2025). By bridging mechanistic toxicology to applied aquaculture science, this species provides critical insight into how emerging pollutants may influence fish health, aquaculture sustainability, seafood safety, and the resilience of wild marine populations in NPs contaminated environments.

Oliveira et al. (2023) showed that marine nanoparticles (NPs) can harm fish reproduction and reduce reproductive success in wild populations of gilthead seabream. The study compared the effects of silver and titanium dioxide NPs at realistic and very high concentrations. High titanium dioxide levels reduced total motility without affecting velocity, while silver NPs reduced total and progressive motility at all levels and decreased velocity only at the highest concentration. NPs exposure reduced fast-moving sperm and increased slow-moving sperm. Overall, both NPs showed reproductive toxicity only at very high concentrations. These and other studies indicate that the reproductive toxicity of these marine contaminants is still poorly understood in fish sperm, and their effects on sperm subpopulations have not been previously examined

However, despite growing interest in NPs research, several gaps remain. Currently, there is a limited understanding of sperm-specific toxicity, a lack of metabolomic studies in fish sperm, insufficient data on dose-response relationships, and a scarcity of long-term exposure studies. These gaps

highlight the need for detailed mechanistic studies. Specifically in species that, like S. *aurata*, exhibit external fertilisation, where sperm activation occurs upon contact with seawater. This activation triggers rapid ATP consumption and initiates flagellar movement, allowing sperm to reach and fertilise the egg (Dzyuba and Cosson, 2014). Because of this rapid activation and limited energy reserves, sperm cells are particularly sensitive to environmental disturbances.

References

Alavi, S. M. H., Barzegar-Fallah, S., Rahdar, P., Ahmadi, M. M., Yavari, M., Hatef, A., Golshan, M., & Linhart, O. (2021). A review on environmental contaminants-related fertility threat in male fishes: Effects and possible mechanisms of action learned from wildlife and laboratory studies. *Animals, 11*(10), 2817. https://doi.org/10.3390/ani11102817

Alimi, O. S., Budarz, J. F., Hernandez, L. M., & Tufenkji, N. (2018). Microplastics and nanoplastics in aquatic environments: Aggregation, deposition, and enhanced contaminant transport. *Environmental Science & Technology, 52*(15), 8751–8760. https://doi.org/10.1021/acs.est.7b05559

Amereh, F., Babaei, M., Eslami, A., Fazelipour, S., & Rafiee, M. (2020). The emerging risk of exposure to nano(micro)plastics on endocrine disturbance and reproductive toxicity: From a hypothetical scenario to a global public health challenge. *Environmental Pollution, 261*, 114158. https://doi.org/10.1016/j.envpol.2020.114158

Bambino, K., & Chu, J. (2017). Zebrafish in toxicology and environmental health. *Current Topics in Developmental Biology, 124*, 331–367. https://doi.org/10.1016/bs.ctdb.2016.10.007

Bhagat, J., Nishimura, N., & Shimada, Y. (2021). Toxicological interactions of microplastics/nanoplastics and environmental contaminants: Current knowledge and future perspectives. *Journal of Hazardous Materials, 405*, 123913. https://doi.org/10.1016/j.jhazmat.2020.123913

Bhagat, J., Zang, L., Nishimura, N., & Shimada, Y. (2020). Zebrafish: An emerging model to study microplastic and nanoplastic toxicity. *Science of the Total Environment, 728*, 138707.https://doi.org/10.1016/j.scitotenv.2020.138707

Blonç, M., Ruiz, N., Fernando, F., Llorca, M., Farré, M., Nolis, P., Tort, L., & Teles, M. (2025). Impact of a chronic waterborne exposure to polystyrene nanoplastics on the gilthead seabream (*Sparus aurata*): Combining traditional and multi-omics approaches. *Journal of Hazardous Materials*, 139465.

Bouwmeester, H., Hollman, P. C. H., & Peters, R. J. B. (2015). Potential health impact of environmentally released micro- and nanoplastics in the human food production chain: Experiences from nanotoxicology. *Environmental Science & Technology, 49*(15), 8932–8947. https://doi.org/10.1021/acs.est.5b01090

Brandts, I., Barría, C., Martins, M.A., Franco-Martínez, L., Barreto, A., Tvarijonaviciute, A., Tort, L., Oliveira, M., & Teles, M. (2021). Waterborne exposure of gilthead seabream (Sparus aurata) to polymethylmethacrylate nanoplastics causes effects at cellular and molecular levels. *Journal of Hazardous Materials*, *403*, 123590.

Brooks, W. B. (2019). Greening chemistry and ecotoxicology towards sustainable environmental quality. *Green Chemistry, 21*, 2575–2582.

Carrete Vega, G., & Wiens, J. J. (2012). Why are there so few fish in the sea? *Proceedings of the Royal Society B: Biological Sciences, 279*(1735), 2323–2329. https://doi.org/10.1098/rspb.2012.0075

Chae, Y., Kim, D., Kim, S. W., & An, Y.-J. (2018). Trophic transfer and individual impact of nano-sized polystyrene in a four-species freshwater food chain. *Scientific Reports, 8*, 284. https://doi.org/10.1038/s41598-017-18849-y

Chatterjee, A., Maity, S., Banerjee, S., Dutta, S., Adhikari, M., Guchhait, R., Biswas, C., De, S., & Pramanick, K. (2022). *Toxicological impacts of nanopolystyrene on zebrafish oocyte with insight into the mechanism of action: An expression-based analysis*. Science of the Total Environment, 830, 154796. https://doi.org/10.1016/j.scitotenv.2022.154796

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., & Hollert, H. (2017). Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. *Science of the total environment*, *584*, 1022-1031.https://doi.org/10.1016/j.scitotenv.2017.01.156

De Sa, L. C., Oliveira, M., Ribeiro, F., Rocha, T. L., & Futter, M. L. (2018). Studies of the effects of microplastics on aquatic organisms: What do

we know and where should we focus our efforts in the future? *Science of the Total Environment, 645*, 1029–1039. https://doi.org/10.1016/j.scitotenv.2018.07.207

Dzyuba, V., & Cosson, J. (2014). Motility of fish spermatozoa: From external signaling to flagella response. *Reproduction, 147*(4), R11–R24. https://doi.org/10.1530/REP-13-0446

Feng, M., Luo, J., Wan, Y., Zhang, J., Lu, C., Wang, M., ... & Wang, Y. (2022). Polystyrene nanoplastic exposure induces developmental toxicity by activating the oxidative stress response and base excision repair pathway in zebrafish (Danio rerio). *ACS omega*, *7*(36), 32153-32163. https://doi.org/10.1021/acsomega.2c03378

Gallego, V., & Asturiano, J. F. (2018). Sperm motility in fish: Technical applications and perspectives through CASA-Mot systems. *Reproduction, Fertility and Development, 30*(6), 820–832. https://doi.org/10.1071/RD17460

Hartmann, N.B., Rist, S., Bodin, J., Jensen, L.H., Schmidt, S.N., Mayer, P., Meibom, A., & Baun, A. (2017). Microplastics as vectors for environmental contaminants: Exploring sorption, desorption, and transfer to biota. *Integrated environmental assessment and management*, *13*(3), 488-493. https://doi.org/10.1002/ieam.1904

Horvatits, T., Tamminga, M., Liu, B., Sebode, M., Carambia, A., Fischer, L., Püschel, K., Huber, S., & Fischer, E.K. (2022). Microplastics detected in cirrhotic liver tissue. *EBioMedicine*, *82*. https://doi.org/10.1016/j.ebiom.2022.104147

Koelmans, A.A.; Besseling, E.; Shim, W.J.: Nanoplastics in the aquatic environment: critical review. In: Bergmann, M., Gutow,L., Klages, M. (eds) Marine Anthropogenic Litter, pp. 325–340(2015).

Lee, W.S., Cho, H.J., Kim, E., Huh, Y.H., Kim, H.J., Kim, B., Kang, T., Lee, J.S., & Jeong, J. (2019). Bioaccumulation of polystyrene nanoplastics and their effect on the toxicity of Au ions in zebrafish embryos. *Nanoscale*, *11*(7), 3173-3185.https://doi.org/10.1039/C8NR09280A

Lehner, R., Weder, C., Petri-Fink, A., & Rothen-Rutishauser, B. (2019). Emergence of nanoplastics in the environment and possible impact on human health. *Environmental Science & Technology, 53*(4), 1748–1765. https://doi.org/10.1021/acs.est.8b05512

Liang, B., Huang, Y., Zhong, Y., Li, Z., Ye, R., Wang, B., Zhang, B., Meng, H., Lin, X., Du, J., & Hu, M., 2022. Brain single-nucleus transcriptomics highlights that polystyrene nanoplastics potentially induce Parkinson's disease-like neurodegeneration by causing energy metabolism disorders in mice. *Journal of hazardous materials*, *430*, 128459. https://doi.org/10.1016/j.jhazmat.2022.128459

MacRae, C. A., & Peterson, R. T. (2015). Zebrafish as tools for drug discovery. *Nature Reviews Drug Discovery, 14*(10), 721–731. https://doi.org/10.1038/nrd4627.

Manabe, M., Tatarazako, N., & Kinoshita, M. (2011). Uptake, excretion and toxicity of nanosized latex particles on medaka (*Oryzias latipes*) embryos and larvae. *Aquatic Toxicology, 105*(3–4), 576–581. https://doi.org/10.1016/j.aquatox.2011.08.011

May, R. M. (1994). Biological diversity: Differences between land and sea. *Philosophical Transactions of the Royal Society B: Biological Sciences, 343*(1303), 105–111. https://doi.org/10.1098/rstb.1994.0017

Okon, E., Brandts, I., Djafar, H., Tvarijonaviciute, A., Balasch, J. C., & Teles, M. (2025). Effects of sub-chronic exposure to polystyrene nanoplastics on lipid and antioxidant metabolism in Sparus aurata. *Animals*, *15*(4), 562.

Oliveira, C. C. V., Ferrão, L., Gallego, V., Mieiro, C., Oliveira, I. B., Carvalhais, A., Pacheco, M., & Cabrita, E. (2024). Exposure to silver and titanium dioxide nanoparticles at supra-environmental concentrations decreased sperm motility and affected spermatozoa subpopulations in gilthead seabream, *Sparus aurata*. *Fish Physiology and Biochemistry, 50*, 1959–1970. https://doi.org/10.1007/s10695-023-01218-0

Oliveira, M., Almeida, M., & Miguel, I. (2019). A micro(nano)plastic boomerang tale: A never ending story? *TrAC Trends in Analytical Chemistry, 112*, 196–200. https://doi.org/10.1016/j.trac.2019.01.005

Panizzolo, M., Martins, V. H., Ghelli, F., Squillacioti, G., Bellisario, V., Garzaro, G., Bosio, D., Colombi, N., Bono, R., & Bergamaschi, E. (2023). Biomarkers of oxidative stress, inflammation, and genotoxicity to assess exposure to micro- and nanoplastics: A literature review. *Ecotoxicology and Environmental Safety, 267*, 115645. https://doi.org/10.1016/j.ecoenv.2023.115645

Qiang L, Cheng J. Exposure to polystyrene microplastics impairs gonads of zebrafish (Danio rerio). Chemosphere.2021;263:128161. https:// doi. org/ 10. 1016/j. chemo sphere. 2020.128161.

Reis, L. P. G. et al. Evaluation of the toxicity of bisphenol a in reproduction and its effect on fertility and embryonic development in the zebrafish (Danio rerio). Int J Environ Res Public Health 19 (2022). https:// doi. org/ 10. 3390/ ijerp h1902 0962

Ribeiro RX, da Silva Brito R, Pereira AC, Monteiro KBS, Gonçalves BB, Rocha TL. Ecotoxicological assessment of effluents from Brazilian wastewater treatment plants using zebrafish embryotoxicity test: A multi-biomarker approach. Sci. Total Environ. 2020; 735:139036.

Teng, M., Zhao, X., Wang, C., Chen, W., White, J. C., Zhao, W., Zhou, L., Duan, M., & Wu, F. (2022). *Polystyrene nanoplastics toxicity to zebrafish: Dysregulation of the brain–intestine–microbiota axis*. ACS Nano, 16(5), 8190–8204. https://doi.org/10.1021/acsnano.2c01872

Toussaint, B., Raffael, B., Angers-Loustau, A., Gilliland, D., Kestens, V., Petrillo, M., Rio-Echevarria, I.M., & Van den Eede, G. (2019). Review of micro-and nanoplastic contamination in the food chain. *Food Additives & Contaminants: Part A*, *36*(5), 639-673.

Trevisan, R., Voy, C., Chen, S., & Di Giulio, R. T. (2019). Nanoplastics decrease the toxicity of a complex PAH mixture but impair mitochondrial energy production in developing zebrafish. *Environmental Science & Technology, 53*(14), 8405–8415. https://doi.org/10.1021/acs.est.9b02003

Trindade-Santos, I., & Freire, K. D. M. F. (2015). Analysis of reproductive patterns of fishes from three large marine ecosystems. *Frontiers in Marine Science, 2*, Article 38. https://doi.org/10.3389/fmars.2015.00038

Wang, C., Ji, X., Wang, X., Song, Y., Pan, C., Qian, M., & Jin, Y. (2025). The endoplasmic reticulum-mitochondrial crosstalk involved in nanoplastics and di (2-ethylhexyl) phthalate co-exposure induced the damage to mouse mammary epithelial cells. *Environmental Pollution*, *372*, 126014.

Wu, D., Carter, L., Kay, P., Holden, J., Yin, Y., & Guo, H. (2025). Female zebrafish are more affected than males under polystyrene microplas-

tics exposure. *Journal of Hazardous Materials*, *482*, 136616. https://doi.org/10.1016/j.jhazmat.2024.136616

Zhang, C., Li, L., Alava, J.J., Yan, Z., Chen, P., Gul, Y., Wang, L., & Xiong, D. (2024). Female zebrafish (Danio rerio) exposure to polystyrene nanoplastics induces reproductive toxicity in mother and their offspring. *Aquatic Toxicology*, *273*, 107023.https://doi.org/10.1016/j.aquatox.2024.107023

Zhao, J., Lan, R., Wang, Z., Su, W., Song, D., Xue, R., Liu, Z., Liu, X., Dai, Y., Yue, T., & Xing, B. (2024). Microplastic fragmentation by rotifers in aquatic ecosystems contributes to global nanoplastic pollution. *Nature nanotechnology*, 19(3), pp.406-414. https://doi.org/10.1038/s41565-023-01558-8

Objectives

1. **Characterize the proteomic profile of *S. aurata* seminal plasma,** to establish a baseline reference for future assessment of normal vs. pathologic seminal plasma protein composition.

2. Analyze the effects of **incremental concentrations** of **polystyrene nanoplastics** (PS-NPs) on *S. aurata* **sperm motility parameters** and **viability.**

3. Evaluate the **metabolite profiling** and potential **biochemical biomarkers** of *S. aurata* seminal plasma exposed to incremental concentrations of PS-NPs.

2

Proteomic profile of gilthead seabream (*Sparus aurata*) seminal plasma

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Abstract

Seminal plasma enables sperm storage, metabolic management, maturation, and motility. However, the molecular mechanisms that determine sperm quality and function, and in particular the proteomic composition of seminal plasma, as well as its functional implications in sperm maturation and performance, are still poorly understood. The current manuscript aimed to describe the proteome of seminal plasma in gilthead seabream (*Sparus aurata*), a species of high commercial value in the Mediterranean region, through LC-MS/MS analysis. For that purpose, the ejaculate from 4 adult gilthead seabream was obtained and analyzed through liquid chromatography-mass spectrometry techniques. One of the samples was discarded due to an immediate reaction to the protein quantification reagent. A total of 426 common proteins were present in the three remaining fish, 12 of them being uncharacterized proteins. After applying the gene ontology analysis, we observed that functions associated with protein metabolism were significantly enriched. These functions included endopeptidase and peptidase complexes, proteolysis-related hydrolases, and catalytic activities. The predominant functional categories identified were associated with immune- and stress-related defense mechanisms, coagulation cascades, and proteolytic processes. The current study shows that the main components of the seminal plasma follow the canonical protective and functional pathways described for other fish species.

1. Introduction

Seminal plasma serves as a transport medium for spermatozoa [1,2], protects the germinal epithelia from oxidative damage [3,4], and provides proper conditions for motility and further fertilization of the oocyte [5]. These proper environmental conditions are even more relevant for external fertilization species such as fish, whose sperm cells, once released, face sudden changes in osmotic pressure, pH, and ion composition and concentrations. Fish seminal plasma is involved in spermatozoa motility, capacitation, survival, longevity, and functional integrity [6,7]. The composition and functions of the seminal plasma in mammals have been extensively studied [8]. These functions include the regulation of spermatozoa capacitation, the modulation of the immune response in the uterus, and sperm transport through the female genital tract, among others [9]. However, research on the composition and detailed functions of fish seminal plasma components remains scarce. Fish seminal plasma is mainly composed of minerals (Na^+, K^+, Ca^{2+} and Mg^{2+}), hormones, pheromones, cholesterol, glycerol, vitamins, free amino acids, sugars, citric acid, lipids, and proteins [10-12]. Major proteins characterised from fish seminal plasma have been associated with viability, protection against proteolytic enzymes, oxidative stress responses and pathogen protection, among others [13]. In addition to these major proteins, seminal plasma contains numerous minor proteins involved in several biological/molecular processes, whose characterisation has been possible through proteomics.

In recent years, proteomic analysis has focused on providing a comprehensive picture of seminal plasma proteins and their potential role [14]. To date, the proteomic identification of the principal constituents of seminal plasma has been partially or completely characterised only in a few commercial and edible fish species 15-19], including rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio* L.), siberian sturgeon (*Acipenser baerii*), turbot (*Scophthalmus maximus*), pikeperch (*Sander lucioperca*), and european eel (*Anguilla anguilla*). gilthead seabream (*Sparus aurata*) is one of the primary species in Mediterranean aquaculture. Despite its high economic relevance, knowledge of spermatozoal metabolism and the effects of seminal plasma components on sperm functionality is clearly insufficient. Closing this gap may lead to relevant improvements in biotechnological techniques, such as chilling storage and cryopreservation, for this species. The composition of gilthead seabream seminal plasma has been previously studied in terms of free amino acid content [20] and metabolism of carbohydrates and lipids [21]. However, the protein composition of seminal

plasma has been poorly assessed. Against this background, the present study aimed to evaluate the proteome of seminal plasma from *S. aurata* using liquid chromatography-mass spectrometry techniquess.

2. Materials and methods

2.1. Animals and samples

Seminal plasma from 4 gilthead seabream (*Sparus aurata*) was initially included in the present study. Fish milts were obtained from the breeding stock of the "Servicios Centrales de Investigación en Cultivos Marinos" (SCI-CM, CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Facilities for Breeding, Supplying and Users of Experimental Animals; Spanish Operational Code REGA ES11028000312). Samples were collected from adult male fish weighing 1.5 ± 0.56 kg and 37.87 ± 0.87 cm in length. Males were anaesthetized with 2-phenoxyethanol at a concentration of 0.3 mL/L of water. Then, the anal pore area was cleaned to remove saltwater residues, and ejaculates were obtained by applying an abdominal massage. After semen collection and complete recovery from anesthesia, all fish were returned to their holding tanks and maintained as part of the breeding stock. No fish were euthanized for the purposes of this study. Samples were collected in Petri dishes, transferred to Eppendorf tubes, and maintained at 4 ºC until their transfer to the facilities at the Universitat Autònoma de Barcelona (UAB) under refrigerated conditions.

Upon arrival at the UAB, samples were centrifuged twice at 4 ºC to obtain the seminal plasma. The first centrifugation was performed at 3000 xg for 20 minutes. The supernatant was recovered and centrifuged a second time at 10,000 × g for 10 minutes (Nynca et al., 2017). Then, the supernatants were immediately frozen and maintained at -80 ºC until analyses were performed. Fish were kept and handled following the guidelines for experimental procedures in animal research established by the Ethics and Animal Welfare Committee of the University of Cadiz, by Spanish laws (RD 53/2013 and RD 118/2021) and European Union legislation (2010/63/UE). The Ethical Committee of the Autonomous Andalusian Government approved the experiments (Junta de Andalucía reference number 20/09/2021/134).

2.2. Proteomic analysis

Lysis

Samples were thawed at 4 ºC and centrifuged at 15,000 x g for 20 minutes. Two 100-µL aliquots from each sample were then obtained. One aliquot

was used for lysis, and the other one was kept frozen at -80ºC. For lysis, 100 µL of lysis buffer (1.1% SDS, 0.1 M Tris/HCl, pH 7.59) were added to each sample. Once diluted, samples were sonicated on ice by applying 10 cycles for 1 second and 30% amplitude. Then, the samples were incubated at 95ºC for 5 minutes in a thermomixer at 300 rpm. Afterwards, they were allowed to cool at room temperature for at least 5 minutes. A second sonication was performed, applying 5 cycles for 1 second each, also on ice. Lastly, samples were centrifuged at 13°C at 16,000 × *g* for 20 minutes. The resulting supernatants were transferred to LoBind tubes (Sigma-Aldrich-Merck, Spain) and stored at -80ºC.

Quantification

Samples were initially diluted at 1:50 and 1:100 in bidistilled water using the microBCA assay. However, the elevated concentration of the samples required bigger dilutions. In a second round of quantification, the samples were diluted to 1:200 and 1:400. Quantification was performed following the internal protocol at LP-CSIS/UAB. FASP digestion was performed following the internal procedure of the LP-CSIC/UAB.

LC-MS/MS analysis

The LC-MS/MS analysis was performed with an Orbitrap Exploris 480 (Thermofisher, Spain) equipped with a nanoESI ion source. From each digested sample, 300 nanograms were loaded into the chromatographic system. The separation of the different proteins was performed at 0.5 µL/min in a 60-minute acetonitrile gradient from 2 to 30%. The HPLC system was an ACQUITY UPLC M-Class (Waters, USA). The Orbitrap Exploris 480 was operated in the positive ion mode with a spray voltage of 1.8 kV. For the spectrometric analysis, data-dependent acquisition (DDA) was employed, followed by 15 MS/MS scans of the 15 most intense signals detected in the global list. The full MS/MS (range 400-1600) was acquired in the Orbitrap with a resolution of 120000. The MS/MS spectra were also performed with the Orbitrap at a resolution of 15000.

Database search

For DDA spectra, peptide identification was performed using the Proteome Discover v3.0 software (Thermo Scientific, Spain). The characterized proteins were queried using a combined database for *S. aurata* reference proteome (UP000472265_2025_04_09 Uniprot) and common protein contaminants (MaxQuant). Enrichment analysis was performed using the g-Profiler web server (https://biit.cs.ut.ee/gprofiler/gost) using *S. aurata* orthologs.

3. Results

3.1. Protein profile of seminal plasma

One of the samples was discarded after the first round of quantification because it immediately reacted with the microBCA reagent. After performing the DDA mode analysis, a total of 7,592 peptides belonging to 1,347 proteins were identified in the seminal plasma samples from Fish 1. In Fish 2, a total of 6,664 peptides from 1,061 proteins were identified. Finally, 7,161 peptides from 1,260 proteins were identified in the seminal plasma of Fish 3. The chromatographs obtained with this analysis are depicted in **Figure 1**. Subsequently, those proteins identified by one or two peptides were removed, yielding a total of 776, 610, and 729 proteins for fish 1, 2, and 3, respectively. The number of peptides identified from fish 1, 2, and 3 was 6,822, 6,067, and 6,447, respectively. A total of 426 common proteins were present in the three fish, 12 of them being uncharacterised proteins. Due to the extensive number of proteins identified, they are not included in the body of the manuscript.

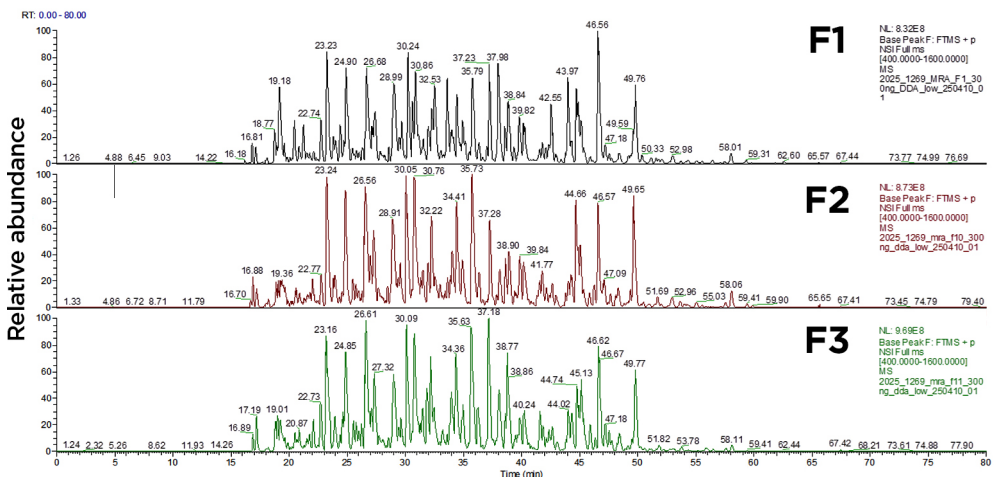


Figure 1. Chromatographs obtained by LC-MS/MS in DDA mode.
F1: Fish 1; F2: Fish 2; F3: Fish 3.

3.2. Gene Ontology (GO) analysis

The most relevant results of the g: profiler analysis are shown in **Figure 2**.

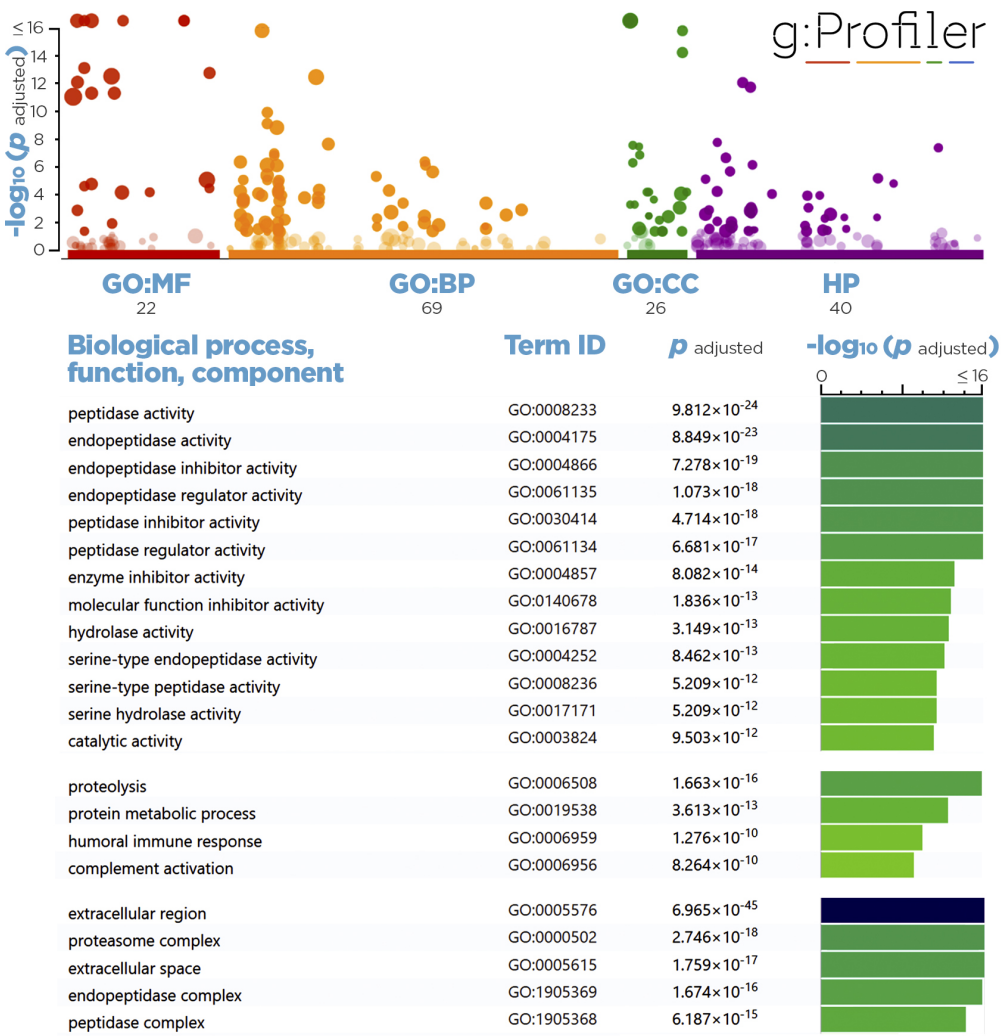


Figure 2. Manhattan plots obtained after performing the g: Profiler enrichment analysis using *Sparus aurata* orthologs.
GO terms for metabolic functions (MF) are depicted in red, for biological processes (BP) in orange and for cellular components (CC) in green.

Regarding **molecular function**, the g: profiler analysis of orthologs revealed an enrichment in GO terms related to peptidase activity (GO:0008233, n = 47 proteins identified), endopeptidase activity (GO:0004175, (n = 42 proteins), endopeptidase inhibitor activity (GO:0004866, n = 17 proteins), endopeptidase regulator activity (GO:0061135, n =17 proteins), peptidase inhibitor activity (GO:0030414, n= 17 proteins), peptidase regulator activity (GO:0061134, n = 17 proteins), enzyme inhibitor activity (GO:0004857, n = 18 proteins), molecular function inhibitor activity (GO:0140678, n = 17 proteins), hydrolase activity (GO:0016787, n = 81 proteins)), serine-type endopeptidase activity (GO:0004252, n = 22 proteins), serine-type peptide activity (GO:0008236, n = 22 proteins), serine hydrolase activity (GO:0017171, n = 21 proteins), and catalytic activity (GO:0003824, n = 134 proteins) **(Table 1)**.

For **biological processes**, an enrichment in GO terms related to proteolysis (GO:0006508, n = 42 proteins), protein metabolic process (GO:0019538, n = 62 proteins), humoral immune response (GO:0006959, n = 11 proteins), and complement activation (GO:0006956, n = 11 proteins) was observed **(Table 2)**.

Finally, **cellular components** showed enrichment in GO terms related to extracellular region (GO:0005576, n = 81 proteins), extracellular space (GO:0005615, n = 37 proteins), proteasome complex (GO:0000502, n = 14 proteins), endopeptidase complex (GO:1905369, n = 14 proteins) and peptidase complex (GO:1905368, n = 14 proteins) **(Table 3)**.

Table 1. Enrichment of GO terms related to molecular function. To facilitate visualization, only the categories with the highest number of proteins (***peptidase activity***, GO:0008233, and ***catalytic activity***, GO:0003824) are shown, together with the GO terms they share with other related categories.

Related categories: endopeptidase activity (GO:0004175), endopeptidase inhibitor activity (GO:0004866), endopeptidase regulator activity (GO:0061135), peptidase inhibitor activity (GO:0030414), peptidase regulator activity (GO:0061134), enzyme inhibitor activity (GO:0004857), molecular function inhibitor activity (GO:0140678), hydrolase activity (GO:0016787), serine-type endopeptidase activity (GO:0004252), serine-type peptide activity (GO:0008236), and serine hydrolase activity (GO:0017171).

GO:0008233. Peptidase activity

- 72 kDa type IV collagenase (**GO:0004175, GO:0016787, GO:0003824**)
- Adam metallopeptidase with thrombospondin type 1 motif 13 (**GO:0004175, GO:0016787, GO:0003824**)
- Aminopeptidase (**GO:0140678, GO:0016787, GO:0003824**)
- Angiotensinogen (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Aspartyl aminopeptidase (**GO:0016787, GO:0003824**)
- Bleomycin hydrolase (**GO:0004175, GO:0016787, GO:0003824. GO:0016787**)
- c3/c5 convertase (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Cathepsin b (**GO:0004175, GO:0016787, GO:0003824**)
- Cathepsin x (**GO:0004175, GO:0016787, GO:0003824**)
- Coagulation factor IX (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Coagulation factor VIII (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Coagulation factor Xa (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Complement c3-like (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0003824**)
- Complement c4-like (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0003824**)
- Complement component 1-s subcomponent (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Complement component c3a-duplicate 5 (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0003824**)
- Complement component C3b (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0003824**)
- Complement factor d-like (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Complement subcomponent c1r (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Cystatin fetuin-b-type domain-containing protein (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, G GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0003824**)
- Cytosolic non-specific dipeptidase (**GO:0016787, GO:0003824**)
- Dipeptidyl peptidase 1 (**GO:0004175, GO:0016787, GO:0003824**)
- Fetuin b (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0004857, GO:0140678, GO:0016787, GO:0003824**)
- Folate gamma-glutamyl hydrolase (**GO:0016787, GO:0016787, GO:0003824**)
- HtrA serine peptidase 1 (**GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Inter-α-trypsin inhibitor heavy chain h3 (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Kininogen 1 (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0003824**)
- Legumain (**GO:0004175, GO:0016787, GO:0003824**)
- Macrophage stimulating 1 (**GO:0004175, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)

Table 1. ***Cont.***

Related categories: endopeptidase activity (GO:0004175), endopeptidase inhibitor activity (GO:0004866), endopeptidase regulator activity (GO:0061135), peptidase inhibitor activity (GO:0030414), peptidase regulator activity (GO:0061134), enzyme inhibitor activity (GO:0004857), molecular function inhibitor activity (GO:0140678), hydrolase activity (GO:0016787), serine-type endopeptidase activity (GO:0004252), serine-type peptide activity (GO:0008236), and serine hydrolase activity (GO:0017171).

GO:0008233. Peptidase activity

- Matrix metallopeptidase 30 (**GO:0004175, GO:0003824**)
- Metalloproteinase inhibitor 2 (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0003824**)
- Ntr domain-containing protein (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0003824**)
- Peptidase metallopeptidase domain-containing protein (**GO:0004175, GO:0016787, GO:0003824**)
- Peptidase s1 domain-containing protein (**GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Plasminogen (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Prolyl endopeptidase (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824, GO:0016787**)
- Protein ddi1 homolog 2-like (**GO:0004175, GO:0016787, GO:0003824**)
- Prothrombin (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Serpin family g member 1 (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Serpin peptidase inhibitor, Clade a (alpha-1 antiproteinase, antitrypsin), member 10a (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Thyroxine-binding globulin (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Trypsin (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- Ubiquitin carboxyl-terminal hydrolase (**GO:0016787, GO:0003824**)
- Ubiquitin thioesterase (**GO:0016787, GO:0003824**)
- Vitamin K-dependent protein C (**GO:0004175, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- α-2-hs-glycoprotein 2 (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0003824**)
- α-2-macroglobulin bait region domain-containing protein (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)
- α-2-macroglobulin-like (**GO:0004175, GO:0004866, GO:0061135, GO:0030414, GO:0061134, GO:0004857, GO:0140678, GO:0016787, GO:0004252, GO:0008236, GO:0017171, GO:0003824**)

Table 1. ***Cont.***

Related categories: hydrolase activity (GO:0016787).

GO:0003824. Catalytic activity

- 6s proteasome non-ATPase regulatory subunit 1
- 26s proteasome non-ATPase regulatory subunit 2
- 6-pyruvoyl tetrahydrobiopterin synthase
- Adenylate kinase isoenzyme 1
- Alcohol dehydrogenase (nadp(+))
- Aldehyde dehydrogenase (nad(+))
- Aldehyde dehydrogenase 9 family-member a1a-tandem duplicate 1
- AMP deaminase (**GO:0016787**)
- Apolipoprotein a-i-like
- Aspartate aminotransferase
- ATP synthase subunit α
- ATP synthase subunit βeta (**GO:0016787**)
- ATP-citrate synthase
- Autophagy-related 7
- Biotinidase
- Carbonic anhydrase
- Chaperonin containing tcp1 subunit 6a (**GO:0016787**)
- Chitinase (**GO:0016787**)
- Coagulation factor XIII, α chain-like
- Cyclin-dependent kinase-like 1
- Cystathionine γ-lyase
- Cytosolic non-specific dipeptidase
- d-3-phosphoglycerate dehydrogenase
- Dynein axonemal heavy chain 8 (**GO:0016787**)
- EF-hand calcium-binding domain 7
- EF-hand domain-containing protein
- Fatty acid synthase (**GO:0016787**)
- Ferroxidase
- Glutathione peroxidase
- Glutathione S-transferase α-like (**GO:0016787**)
- Glutathione transferase
- Glyceraldehyde-3-phosphate dehydrogenase
- GTP-binding nuclear protein, Ran (**GO:0016787**)
- h(+)-transporting two-sector ATPase (**GO:0016787**)
- Haemoglobin subunit β-like
- Heat shock protein 90 α family class b member 1 (**GO:0016787**)
- Hypoxanthine phosphoribosyltransferase
- Interleukin-1 receptor type 1-like (**GO:0016787**)
- Isocitrate dehydrogenase [nadp]
- Lactoylglutathione lyase
- Lecithin-cholesterol acyltransferase
- Lipoprotein lipase
- Lysosomal α-glucosidase-like
- m7gpppx diphosphatase (**GO:0016787**)
- Malate dehydrogenase
- Multifunctional protein ade2-like
- Multiple inositol polyphosphate phosphatase 1-like (**GO:0016787**)
- Non-specific serine/threonine protein kinase
- ntr domain-containing protein
- Nucleoside diphosphate kinase
- Nucleoside diphosphate kinase homolog 7 (**GO:0016787**)
- Peptidase metallopeptidase domain-containing protein
- Peptidase S1 domain-containing protein
- Peroxiredoxin-1
- peroxiredoxin-5
- Phospholipase a2 (**GO:0016787**)
- Phosphopyruvate hydratase
- Phosphoserine aminotransferase
- Prostaglandin reductase 1
- Proteasome 26s subunit-ATPase 1 (**GO:0016787**)
- Proteasome 26s subunit-ATPase 5 (**GO:0016787**)
- Proteasome 26s subunit-non-ATPase 3
- Protein disulfide-isomerase
- Pyruvate kinase
- Rab guanine nucleotide exchange factor (gef) 1-like (**GO:0016787**)
- Rab10-member Ras oncogene family (**GO:0016787**)
- rab1a-member ras oncogene family b (**GO:0016787**)
- rab2a-member ras oncogene family (**GO:0016787**)
- Receptor protein-tyrosine kinase
- Receptor protein-tyrosine kinase
- s-(hydroxymethyl)glutathione dehydrogenase
- Serine hydroxymethyltransferase
- Sodium/potassium-transporting ATPase subunit α (**GO:0016787**)
- Sulfhydryl oxidase
- Superoxide dismutase [Cu-Zn]
- t-complex protein 1 subunit δ (**GO:0016787**)
- t-complex protein 1 subunit θ (**GO:0016787**)
- Thiopurine s-methyltransferase
- Transforming protein rhoa (**GO:0016787**)
- Transitional endoplasmic reticulum ATPase (**GO:0016787**)
- Transketolase
- Triosephosphate isomerase
- Tubulin α chain (**GO:0016787**)
- Tubulin β chain (**GO:0016787**)
- Ubiquitin-conjugating enzyme E2 N-like
- Ubiquitin-like modifier-activating enzyme
- α-n-acetylgalactosaminide α-2,6-sialyltransferase
- β-glucuronidase (**GO:0016787**)
- β-hexosaminidase (**GO:0016787**)

Table 2. Enrichment of GO terms related to biological processes. To facilitate visualization, the categories ***protein metabolic process*** (GO:0019538) and ***humoral immune response*** (GO:0006959) are shown, together with the GO terms they share with other related categories.

Related categories: proteolysis (GO:0006508), protein metabolic process (GO:0019538), and complement activation (GO:0006956).

GO:0019538. Protein metabolic process

- 26s Proteasome Non-Atpase Reg. Sub. 1
- 26s Proteasome Non-Atpase Reg. Sub. 11b (**GO:000658**)
- 26s Proteasome Non-Atpase Reg. Sub. 2
- 26s Proteasome Non-Atpase Reg. Sub. 8 (**GO:000658**)
- 6-Sialyltransferase
- 72 Kda Type Iv Collagenase (**GO:000658**)
- 78 KDa Glucose-Regulated Protein
- A0a671u983
- Adam Metallopeptidase with Thrombospondin Type 1 Motif 13 (**GO:000658**)
- Aminopeptidase (**GO:000658**)
- Apolipoprotein A-Ib
- Apolipoprotein A-I-Like
- Apolipoprotein C-I-Like
- Apolipoprotein Ea
- Apolipoprotein Eb
- Apple Domain-Containing Protein (**GO:000658**)
- Aspartyl Aminopeptidase (**GO:000658**)
- Bleomycin Hydrolase (**GO:000658**)
- Cathepsin B (**GO:000658**)
- Cathepsin X (**GO:000658**)
- Chaperonin Containing Tcp1 Subunit 6a
- Coagulation Factor IX (**GO:000658**)
- Coagulation Factor VIII (**GO:000658**)
- Coagulation Factor Xa (**GO:000658**)
- Complement Factor D-Like (**GO:000658**)
- Cytosolic Non-Specific Dipeptidase (**GO:000658**)
- Dipeptidyl Peptidase 1 (**GO:000658**)
- Fibrinogen α Chain
- Fibrinogen β Chain
- Fibrinogen γ Chain
- Heat Shock Cognate 71 Kda Protein-Like
- Heat Shock Protein 90 Alpha Family Class B Member 1
- Htra Serine Peptidase 1 (**GO:000658**)
- Legumain (**GO:000658**)
- Macrophage Stimulating 1 (**GO:000658**)
- Matrix Metallopeptidase 30 (**GO:000658**)
- Peptidase Metallopeptidase Domain-Containing Protein (**GO:000658**)
- Peptidase S1 Domain-Containing Protein (**GO:000658**)
- Plasminogen (**GO:000658**)
- Prolyl Endopeptidase (**GO:000658**)
- Proteasome 20s Sub. α 2 (**GO:000658**)
- Proteasome 20s Sub. α 8 (**GO:000658**)
- Proteasome 26s Sub.-Atpase 1 (**GO:000658**)
- Proteasome 26s Sub.-Atpase 5 (**GO:000658**)
- Proteasome 26s Sub.-Non-Atpase 3
- Proteasome Sub. α Type (**GO:000658**)
- Proteasome Sub. β (**GO:000658**)
- Protein Ddi1 Homolog 2-Like (**GO:000658**)
- Prothrombin (**GO:000658**)
- T-Complex Protein 1 Subunit δ
- T-Complex Protein 1 Subunit θ
- Trypsin (**GO:000658**)
- Ubiquitin Carboxyl-Terminal Hydrolase (**GO:000658**)
- Ubiquitin-Like Modifier Activating Enzyme 1 (**GO:000658**)
- Ubiquitin Thioesterase (**GO:000658**)
- Ubiquitin-Ribosomal Protein Es31 Fusion Protein (**GO:000658**)
- Vitamin K-Dependent Protein C (**GO:000658**)
- α-N-Acetylgalactosaminide α-2

GO:0006959. Humoral immune response

- C3/C5 Convertase (**GO:0019538, GO:000658, GO:0006956**)
- Complement C4-Like (**GO:0006956**)
- Complement C6 (**GO:0006956**)
- Complement Component 1-S Subcomponent (**GO:0019538, GO:000658, GO:0006956**)
- Complement Component 7a (**GO:0006956**)
- Complement Component 8-γ Polypeptide (**GO:0006956**)
- Complement Component C8 β Chain (**GO:0006956**)
- Complement Subcomponent C1r (**GO:0019538, GO:0006956**)
- Fucolectin Tachylectin-4 Pentraxin-1 Domain-Containing Protein (**GO:0006956**)
- Fucolectin-1-Like (**GO:0006956**)
- Ig-Like Domain-Containing Protein (**GO:0006956**)
- Serotransferrin (**GO:0019538**)

Table 3. Enrichment of GO terms related to cellular components. To facilitate visualization, the categories ***extracellular region*** (GO:0005576) and ***proteasome complex*** (GO:0000502) are shown, together with the GO terms they share with other related categories.

Related categories: extracellular space (GO:0005615).

GO:0005576. Extracellular region

- 72 kDa type iv collagenase
- Adam metallopeptidase with thrombospondin type 1 motif 13
- Angiotensinogen (**GO:0005615**)
- Anti-mullerian hormone
- Apolipoprotein a-ib
- Apolipoprotein a-i-like (**GO:0005615**)
- Apolipoprotein b-100-like (**GO:0005615**)
- Apolipoprotein c-i-like
- Apolipoprotein ea
- Apolipoprotein m
- Apple domain-containing protein
- C3/c5 convertase (**GO:0005615**)
- Chitinase
- Cholesteryl ester transfer protein (**GO:0005615**)
- Coagulation factor IX
- Coagulation factor VIII
- Coagulation factor Xa
- Collagen type I α1b (**GO:0005615**)
- Collagen, type I, α2 (**GO:0005615**)
- Complement c3-like (**GO:0005615**)
- Complement c6
- Complement component 1 s subcomponent
- Complement component 7a
- Complement component 8 γ polypeptide (**GO:0005615**)
- Complement component c3a duplicate 5 (**GO:0005615**)
- Complement component c3b-tandem duplicate 2 (**GO:0005615**)
- Complement component C8 β chain
- Complement subcomponent c1r
- Dipeptidyl peptidase 1 (**GO:0005615**)
- Ectonucleotide pyrophosphatase/phosphodiesterase 2
- Fam3 metabolism regulating signaling molecule c (**GO:0005615**)
- Fetuin b
- Fibrinogen α chain (**GO:0005615**)
- Fibronectin
- Folate γ-glutamyl hydrolase
- Granulin a
- Hemoglobin subunit β-like (**GO:0005615**)
- Hemopexin (**GO:0005615**)
- High mobility group protein B2
- Htra serine peptidase 1
- Ig-like domain-containing protein (**GO:0005615**)
- Immunoglobulin v-set domain-containing protein (**GO:0005615**)
- Insulin-like growth factor binding protein acid labile subunit (**GO:0005615**)
- Insulin-like growth factor-binding protein
- Inter-alpha-trypsin inhibitor heavy chain h3
- Keratin type II cytoskeletal cochleal-like (**GO:0005615**)
- Lecithin-cholesterol acyltransferase
- Lipoprotein lipase (**GO:0005615**)
- Lrrct domain-containing protein (**GO:0005615**)
- Lumican, sushi domain-containing protein
- Ntr domain-containing protein (**GO:0005615**)
- Phospholipase a2
- Phospholipid transfer protein (**GO:0005615**)
- Plasminogen (**GO:0005615**)
- Platelet glycoprotein Ib α chain-like (**GO:0005615**)
- Progranulin-like
- Prostaglandin D2 synthase a
- Proteoglycan 4b
- Retinoic acid receptor responder protein 2
- Retinol binding protein 4 (**GO:0005615**)
- Saxitoxin and tetrodotoxin-binding protein 1-like
- Selenoprotein p n-terminal domain-containing protein
- Serotransferrin (**GO:0005615**)
- Serpin family g member 1 (**GO:0005615**)
- Serpin peptidase inhibitor clade a (α-1 antiproteinase, antitrypsin) member 10a (**GO:0005615**)
- Sparc (**GO:0005615**)
- Thyroxine-binding globulin (**GO:0005615**)
- Transcobalamin-2-like
- Trypsin
- Uncharacterized loc115580777
- Uncharacterized loc115581582
- Vitamin K-dependent protein C
- Vitellogenin domain-containing protein (**GO:0005615**)
- Vitronectin-like
- Wxxw domain-containing protein
- Zgc:112492
- α-2-hs-glycoprotein 2 (**GO:0005615**)
- α-2-macroglobulin bait region domain-containing protein (**GO:0005615**)
- α-2-macroglobulin-like (**GO:0005615**)

Table 3. ***Cont.***

Related categories: endopeptidase complex (GO:1905369), and peptidase complex (GO:1905368).

GO:0000502. Proteasome complex

- 26s proteasome non-ATPase regulatory subunit 1 (**GO:1905368, GO:1905369**)
- 26s proteasome non-ATPase regulatory subunit 11b (**GO:1905368, GO:1905369**)
- 26s proteasome non-ATPase regulatory subunit 13 (**GO:1905368, GO:1905369**)
- 26s proteasome non-ATPase regulatory subunit 2 (**GO:1905368, GO:1905369**)
- 26s proteasome non-ATPase regulatory subunit 8 (**GO:1905368, GO:1905369**)
- Proteasome 20s subunit α 2 (**GO:1905368, GO:1905369**)
- Proteasome 20s subunit α 8 (**GO:1905368, GO:1905369**)
- Proteasome 26s subunit ATPase 1 (**GO:1905368, GO:1905369**)
- Proteasome 26s subunit ATPase 5 (**GO:1905368, GO:1905369**)
- Proteasome 26s subunit non-ATPase 12 (**GO:1905368, GO:1905369**)
- Proteasome 26s subunit non-ATPase 3 (**GO:1905368, GO:1905369**)
- Proteasome subunit α type (**GO:1905368, GO:1905369**)
- Proteasome subunit β (**GO:1905368, GO:1905369**)
- Ubiquitin carboxyl-terminal hydrolase (**GO:1905368, GO:1905369**)

4. Discussion

The present research work describes the first in-depth proteomic characterization of *S. aurata* seminal plasma, a major aquaculture species intensively reared in the Mediterranean aquaculture [22]. Seminal plasma is a microenvironment dedicated to maintaining the viability and motility of spermatozoa. Despite its varying composition in fish in a species-specific manner, our analysis indicates that the main components of the seminal plasma follow the canonical protective and functional pathways described for other fish species. Functions associated with protein metabolism were significantly enriched, including endopeptidase and peptidase complexes, proteolysis-related hydrolases, and catalytic activities. The predominant functional categories identified were associated with immune- and stress-related defense mechanisms, coagulation cascades, and proteolytic pathway processes.

Several acute-phase proteins (APP) were identified in the seminal plasma of *S. aurata*, notably components of the complement system and related proteins, namely fucolectins and α-2-macroglobulins. Fucolectin-1 is an F-type lectin, a phylogenetically widespread family of carbohydrate-binding proteins that has been described in several fish species [23]. Although its precise biological role in fish is still debated, the available fragmentary evidence suggests that its primary functions include antigen recognition, opsonisation, and activation of the complement system and phagocytes

[24,25]. In vertebrates, α-2-macroglobulins are multifunctional broad-spectrum proteases that participate in antigen processing, facilitate the proliferation and trafficking of cytokines and immune cells, assist in the clearance and folding of damaged or misfolded proteins during inflammation, and, due to their protease activity, modulate the activation of the complement system [25]. The complement system in fish comprises about 35 plasma- and membrane-bound proteins that interact within a proteolytic cascade of serial protein activation to recognize pathogen- or damage-associated molecular patterns (PAMPs and DAMPs, respectively), immunoglobulins or lectins through classical, alternative, or lectin-related activation pathways [27]. The activation of the complement system induces inflammatory responses, activate leukocytes, and opsonize bacteria, virus, fungi, parasites, and stressed cells, ultimately progressing to assemble the membrane attack complex (MAC) or C5b-C9 cytolytic terminal complex, by joining together the C5b, C6, C7, C8, and C9 components to form pores in the membrane of foreign cells or infected host cells [28]. The seminal plasma samples of *S. aurata* analyzed in our experiment include the pivotal C3 component, C3/C5 convertases, which cleave the C3 and C5 components to release the pro-inflammatory anaphylatoxins (C3a, C5a) and the C3b component, an opsonin that coats the foreign particles and infected cells, targeting them for phagocytosis. We also found downstream (C7a and C) and upstream components (C1 and C4-like), and SERPING1 (Serpin family g member 1), a member of the serine protease inhibitor family that inhibits C1 activation and helps reduce the uncontrolled activation of the complement system. In fish, as in mammals, most of these APPs originate mainly in the liver and, to a lesser extent, in other tissues, including the gonads [29]. Following immunisation with adjuvanted hen egg-white lysozyme, a proteomic analysis uncovered similar APPs in plasma samples from *O. mykiss* [30], which indicates that both physiological compartments, blood and seminal plasma, share a significant portion of the proteome that extends beyond the immune repertoire. In this sense, Nynca et al. [18] analyzed the transfer of proteins from the blood to the seminal fluid in *O. mykiss*, concluding that the absence of mammalian epididymis conducts and accessory glands leads to the abundance of blood proteins in fish seminal plasma.

As suggested by the presence of apolipoprotein A1 (APOA1) in our samples, the immune protection to infection in seminal plasma extends to the proteins involved in lipid metabolism. APOA1, the main component of high-density lipoproteins, regulates not only the lipid metabolism that, in turn, helps to complete the steroidogenesis during spermatogenesis [31], but also the unfolding of immune responses to pathogens in fish [32]. In this

regard, the combined antibacterial activities and lipid regulation of apolipoproteins account for the immune response to infection observed in the seminal plasma proteome of common carp (*C. carpio*) exposed to the bacterial pathogen *Aeromonas salmonicida* [33]. Similarly, we found cathepsins (CatB and CatC/dipeptidyl peptidase 1) in the seminal plasma of *S. aurata*. Both CatB and CatC are ubiquitously expressed endopeptidases that participate in lysosome-dependent cellular autophagy. In mammals, CatB not only induces the mitochondrial production of reactive oxygen species (ROS). It also activates the NFκB pathway, leading to the production of the pro-inflammatory cytokine IL-1β during programmed immune cell death processes, such as pyroptosis and pyronecrosis [34]. In fish, IL-1β also orchestrates inflammatory responses via evolutionarily conserved NFκB pathways [35]. Cathepsins are involved in the control of pathogen infection, as demonstrated by the enhanced expression of CatC transcripts in Epinephelus coioides stimulated with iridovirus (SGIV) and *Trachinotus ovatus* infected with *Vibrio harveyi* [36,37]. Our results are consistent with these and other studies. We can hypothesise that the presence of complement system components and proteins related to innate immunity in the seminal plasma of *S. aurata* may prevent inflammatory processes during the maturation of spermatozoa and guarantee a safe microenvironment during external fecundation.

However, the relative abundance and composition of immune-related proteins in fish seminal plasma are strongly dependent on breeding seasonality and reproductive peculiarities of the specific species, and this should be considered during cryopreservation procedures, either for the management of wild populations or for aquaculture practices. The artificial hormonal stimulation of *C. carpio* to control sperm production, for example, enhances the expression of immune- and stress-related proteins in the seminal fluid [38] and the balance between proteins of the innate immunity, such as complement components and APOE, and antioxidant proteins or regulators of lipid metabolism, such as APOA1 or fetuin, may affect the freezability and post-thawing efficiency of sperm in terms of mobility and performance [39]. As suggested by Vílchez et al. [19], the abundance of proteins in the seminal plasma in fish may vary during the different stages of sperm maturation, depending on the requirements of fatty acids to finalize the spermatogenesis and enhance sperm motility. In this sense, an in-depth description of dominant proteins in the seminal plasma of fish, as described here for *S. aurata*, may help to assess the viability of effective cryopreservation procedures and assess mobility performance in mature spermatozoa.

In addition, oxidative stress, induced by the production of reactive oxygen species (ROS) during cellular immunity or by enhanced spermatozoa metabolism, may impair sperm maturation, viability and cryopreservation, even during short-term storage 40]. Oxidative stress may alter the redox processes which take place in the mitochondria, leading to increased ROS production and oxidative damage, reducing the ATP available to fuel sperm motility [41]. We detected glutathione S-transferase (GST) and glutathione peroxidase (GPX) in the seminal plasma of S. aurata, two key enzymes of the antioxidant responses in fish that have also been found in the proteomic analysis of seminal fluids of other fish species [13,16]. These enzymes also protect spermatozoa from DNA damage, plasma membrane lipids and apoptosis, which may impair fertilisation success [42].

In fish, the coagulation cascade mimics the mammalian model, and both extrinsic and intrinsic triggers of hemostasis have been described [43]. We identified several proteins involved in the coagulation cascade in the seminal plasma of *S. aurata*, namely factors VIII, IX, Xa, and XIII, prothrombin, fibrinogen, kininogen, and regulators of the coagulation processes included in the fibrinolytic system, such as plasminogen, and Vitamin K-dependent protein C, that impede the colloidization of fluids [44,45]. Another member of the serpin family overrepresented in this study is Serpin peptidase inhibitor, Clade α (α-1 antiproteinase, antitrypsin), member 10a (SERPINA10), which is an inhibitor of coagulation factors Xa and Xia [46,47]. Changes in the expression of coagulation factors have also been found in carp seminal plasma exposed to *A. salmonicida* [33] and in stressed pikeperch seminal plasma [15], which suggests that coagulation components, combined with APPs, may protect the forming spermatozoa during infections. In addition, plasmin, the cleavage product of plasminogen, activates matrix metalloproteinases (MMPs) that, in turn, remodel the extracellular matrix and collagen fibers, stabilizing the germinal epithelium, thus ensuring the normal development of spermatozoa [48].

In fish, the absence of an acrosome and the protease-producing accessory glands typically found in mammals does not preclude a consistent proteolytic activity in the seminal plasma, mainly directed at remodeling the extracellular matrix and regulating spermatogenesis. In the seminal plasma of *S. aurata*, we identified several proteins related to the proteolytic machinery, which will be discussed below, including metalloproteinases, MMPs, protease inhibitors of the serpin family, and the aforementioned cathepsins. Serine proteinase inhibitors (serpins) regulate proteolytic activity in numerous processes, including inflammation, blood coagulation, fibri-

nolysis, cell migration, complement activation and embryo implantation, among others [49-52]. Proteolytic activity has been associated with semen coagulation and liquefaction, acrosome reaction and gamete fusion in the mammalian ejaculate [53-55]. Proteolytic activity has also been reported in fish seminal plasma [56-59]. Its role remains unclear to date, although some studies suggest a potential involvement in sperm motility [59].

Angiotensinogen (AGT) is a glycoprotein belonging to the non-inhibitory serpin superfamily. It is the only precursor of the angiotensin peptides composing the renin-angiotensin system (RAS) and is therefore indirectly involved in regulating blood pressure and sodium and water homeostasis [60]. The role of angiotensinogen in the ejaculate has been poorly studied and remains unclear to date. Some researchers have observed a negative correlation between angiotensinogen content and sperm motility and morphology in men [61]. In contrast, some others have stated that this glycoprotein is essential for sperm motility in mice [62]. Conversely, the renin-angiotensin system (RAS) has been reported to play a relevant role in the reproductive function in both males and females. RAS has been considered a circulating hormonal system. Nevertheless, more recent studies suggest the existence of an intracellular RAS [63] characterized by the intracellular presence of different RAS components. In this context, researchers have described the presence of several RAS components in the epididymis, vas deferens, prostate gland, testes, spermatozoa and seminal plasma. Likewise, RAS has been suggested to be involved in motility acquisition, acrosomal reaction and oocyte fertilization [64]. According to the literature, neither RAS nor angiotensinogen have been previously described in fish seminal plasma. Therefore, we can only hypothesize that angiotensinogen and RAS may also be involved in the above-mentioned reproductive functions.

Inter-α-trypsin inhibitor heavy chain h3 (ITIH3) belongs to the Inter-α-trypsin inhibitor family. This family includes plasma serine protease inhibitors that are involved in the stabilization of the extracellular matrix [65,66]. This protein is up-regulated in the follicular fluid of women with endometriosis-associated infertility [67]. However, no reference to male reproduction is present in the literature. Thus, this is the first time this molecule has been described to be present in the seminal plasma. As mentioned above, ITIH3 may be involved in the stabilization of the extracellular matrix (ECM). The presence of proteins related to ECM regulation has been previously described in fish seminal plasma, specifically the common carp [13] and the pikeperch [15]. Information about ECM in male reproduction is scarce. It is known that the basement membrane of

the seminiferous epithelium is a modified form of ECM [68], therefore suggesting a potential role in spermatogenesis. Likewise, fish eggs are surrounded by an extracellular matrix, which is involved in oogenesis, egg deposition, fertilization, and embryogenesis [69]. Fetuin B belongs to the superfamily of cysteine proteinase inhibitors that counteract the activity of serine proteinases, ensuring the protection of germinal tissues [70], and, as previously discussed, it is one of the multifunctional blood components also present in the seminal plasma of fish, performing similar functions in different tissues [1]. Fetuin seems to facilitate appropriate freezability of semen in *C. carpio* [71], but the precise role of fetuins in fish reproductive tissues is still understudied. As suggested by the downregulation of fetuin b transcripts in zebrafish infected with *Streptococcus suis* [72], immune challenges may alter the balance between proteolytic and anti-proteolytic activities in the seminal plasma. Although this study provides the first comprehensive characterization of the seminal plasma proteome of *S. aurata*, the relatively small sample size and the descriptive nature of the proteomic analysis should be considered when interpreting the findings. It is noteworthy that the proposed biological roles of the identified proteins are based on functional annotation and published literature and therefore require further validation through functional studies and larger sample sizes.

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References

1. Sharkey, D. J., Macpherson, A. M., Tremellen, K. P., & Robertson, S.A. Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. *Mol Hum Reprod* 2007, 13, 491–501.

2. Bianchi, L., Carnemolla, C., Viviani, V., Landi, C., Pavone, V., Luddi, A., Piomboni, P., Bini, L. Soluble protein fraction of human seminal plasma. *J Proteomics* 2018, 174, 85–100.

3. Crean, A. J., Adler, M. I., & Bonduriansky, R. Seminal fluid and mate choice: New predictions. *Trends Ecol Evol* 2016, 31,253–255.

4. Hopkins, B. R., Sepil, I., & Wigby, S. Seminal fluid. *Curr Biol* 2017,27, R404–R405

5. Ciereszko A., Glogowski J., Dabrowski K. Biochemical characteristics of seminal plasma and spermatozoa of freshwater fishes. In: Tiersch TR, Mazik PM, editors. *Cryopreservation in aquatic species*. Baton Rouge, Louisiana: World Aquaculture Society 2000; p. 20–48

6. Mansour, N., Richardson, G.F., McNiven, M.A. Effect of seminal plasma protein on post-thaw viability and fertility of Arctic char spermatozoa. *N Am J Aquac* 2008, 7, 92–97

7. Dietrich, M.A., Arnold, G.J., Nynca, J., Fröhlich, T., Otte, K., Ciereszko, A. Characterization of carp seminal plasma proteome in relation to blood plasma. *J Proteomics* 2012, 98, 218.232.

8. Rodríguez-Martínez, H., Kvist, U., Ernerudh, J., Sanz, L., Calvete, J.J. Seminal plasma proteins: What role do they play?. *Am J Reprod Immunol* 2011, 66 (suppl. 1), 11-22.

9. Töpfer-Petersen, E., Ekhlasi-Hundrieser, M., Kirchhoff, C., Leeb, T., Sieme, H. The role of stallion seminal plasma proteins infertilisation. *Anim Reprod Sci* 2005, 89, 159–170.

10. Linhart, O., Schleta, V., Slavik, T. Fish sperm composition and biochemistry. *Bull Inst Zool Acad Sin* 1991, 16, 285–311.

11. Ciereszko, A., Ottobre, J.S., Glogowski, J. Effects of season and breed on sperm acrosin activity and semen quality of boars. *Anim Reprod Sci* 2000, 64(1-2), 89–96.

12. Cosson, J. The ionic and osmotic factors controlling motility of fish spermatozoa. *Aquac Int* 2004, 12(1) ,69–85

13. Majewska, A.M., Kodzik, N., Dietrich, M.A., Ciereszko, A. Comprehensive proteomic characterization and functional annotation of common carp seminal plasma. *Reprod Feril Dev* 2025, 37, RD25034.

14. Ciereszko, A.; Dietrich, M.A.; Nynca, J. Fish Semen Proteomics — New Opportunities in Fish Reproductive Research. *Aquaculture* 2017, 472, 81–92.

15. Dietrich, M.A.; Judycka, S.; Żarski, D.; Malinowska, A.; Świderska, B.; Palińska-Żarska, K.; Błażejewski, M.; Ciereszko, A. Proteomic Analysis of Pikeperch Seminal Plasma Provides Novel Insight into the Testicular Development of Domesticated Fish Stocks. *Animal* 2021, 15, 100279.

16. Han, M.M.; Ding, F.H.; Li, J. Characterization of the Immunological Factors in Turbot (*Scophthalmus Maximus*) Seminal Plasma and Their Correlation with Sperm Quality. *Aquacult Int* 2019, 27, 1859–1868.

17. Kodzik, N.; Ciereszko, A.; Szczepkowski, M.; Karol, H.; Judycka, S.; Malinowska, A.; Świderska, B.; Dietrich, M.A. Comprehensive Proteomic Characterization and Functional Annotation of Siberian Sturgeon Seminal Plasma Proteins. *Aquaculture* 2023, 568, 739326.

18. Nynca, J.; Arnold, G.; Fröhlich, T.; Ciereszko, A. Proteomic Identification of Rainbow Trout Blood Plasma Proteins and Their Relationship to Seminal Plasma Proteins. *Proteomics* 2017, 17, 1600460.

19. Vílchez, M.C.; Pla, D.; Gallego, V.; Sanz, L.; Pérez, L.; Asturiano, J.F.; Calvete, J.J.; Peñaranda, D.S. Identification of the Major Proteins Present in the Seminal Plasma of European Eel, and How Hormonal Treatment Affects Their Evolution. Correlation with Sperm Quality. *Comp Biochem A Mol Integr Physiol* 2016, 201, 37–45.

20. Lahnsteiner, F. A comparative study on the composition and importance of free amino acids in semen gilthead sea bream, S*parus aurata*, and perch, *Perca fluviatilis*. *Fish Physiol Biochem* 2010, 36(4), 1297-1305.

21. Lahnsteiner, F., Mansour, N., Caberlotto, S. Composition and metabolism of carbohydrates and lipids in *Sparus aurata* semen and its relation to viability expressed as sperm motility when activated. *Comp Biochem Physiol* 2010, 157(1), 39-45.

22. Mhalhel, K.; Levanti, M.; Abbate, F.; Laurà, R.; Guerrera, M.C.; Aragona, M.; Porcino, C.; Briglia, M.; Germanà, A.; Montalbano, G. Review on Gilthead Seabream *(Sparus Aurata*) Aquaculture: Life

Cycle, Growth, Aquaculture Practices and Challenges. *J Mar Sci Eng* 2023, 11, 2008.

23. Cammarata, M.; Parisi, M.G.; Vasta, G.R. Evolution and Immune Function of Fish Lectins. In *Lessons in Immunity*; Academic Press, 2016; pp. 239–256.

24. Calduch-Giner, J.A.; Sitjà-Bobadilla, A.; Davey, G.C.; Cairns, M.T.; Kaushik, S.; Pérez-Sánchez, J. Dietary Vegetable Oils Do Not Alter the Intestine Transcriptome of Gilthead Sea Bream (*Sparus Aurata*), but Modulate the Transcriptomic Response to Infection with Enteromyxum Leei. *BMC Genomics* 2012, 13, 470.

25. Dara, M.; Giulianini, P.G.; Manfrin, C.; Parisi, M.G.; Parrinello, D.; La Corte, C.; Vasta, G.R.; Cammarata, M. F-Type Lectin from Serum of the Antarctic Teleost Fish *Trematomus Bernacchii* (Boulenger, 1902): Purification, Structural Characterization, and Bacterial Agglutinating Activity. *Comp Biochem Physiol B Biochem Mol Biol* 2021, 256, 110633.

26. Xie, W.; Gao, L.; Deng, H.; Liu, D.; Pang, Q. The Evolution, Oligomerization, Function, and Action Mechanism of A2-Macroglobulin. *Cells* 2026, 15, 353.

27. Kania, P.W.; Buchmann, K. Complement Activation in Fish with Emphasis on MBL/MASP. In *Principles of Fish Immunology : From Cells and Molecules to Host Protection*; Buchmann, K., Secombes, C.J., Eds.; Springer International Publishing: Cham, 2022; pp. 279–300 ISBN 978-3-030-85420-1.

28. Ricklin, D.; Hajishengallis, G.; Yang, K.; Lambris, J.D. Complement: A Key System for Immune Surveillance and Homeostasis. *Nat Immunol* 2010, 11, 785–797.

29. Zhang, S.; Cui, P. Complement System in Zebrafish. *Dev Comp Immunol* 2014, 46, 3–10.

30. Bakke, F.K.; Monte, M.M.; Stead, D.A.; Causey, D.R.; Douglas, A.; Macqueen, D.J.; Dooley, H. Plasma Proteome Responses in Salmonid Fish Following Immunization. *Front Immunol* 2020, 8, 11.

31. Bogevik, A.S.; Hayman, E.S.; Bjerke, M.T.; Dessen, J.-E.; Rørvik, K.-A.; Luckenbach, J.A. Phospholipid and LC-PUFA Metabolism

in Atlantic Salmon (*Salmo Salar*) Testes during Sexual Maturation. *PLOS ONE* 2020, 15, e0233322.

32. Tian, Y.; Wen, H.; Qi, X.; Mao, X.; Shi, Z.; Li, J.; He, F.; Yang, W.; Zhang, X.; Li, Y. Analysis of Apolipoprotein Multigene Family in Spotted Sea Bass (*Lateolabrax Maculatus*) and Their Expression Profiles in Response to Vibrio Harveyi Infection. *Fish Shellfish Immunol* 2019, 92, 111–118.

33. Dietrich, M.A.; Adamek, M.; Teitge, F.; Teich, L.; Jung-Schroers, V.; Malinowska, A.; Świderska, B.; Rakus, K.; Kodzik, N.; Chadzińska, M.; Karol, H.; Liszewska, E.; Ciereszko, A. Proteomic Analysis of Carp Seminal Plasma Provides Insights into the Immune Response to Bacterial Infection of the Male Reproductive System. *Fish Shellfish Immunol* 2022, 127, 822–835.

34. Xie, Z.; Zhao, M.; Yan, C.; Kong, W.; Lan, F.; Narengaowa; Zhao, S.; Yang, Q.; Bai, Z.; Qing, H.; et al. Cathepsin B in Programmed Cell Death Machinery: Mechanisms of Execution and Regulatory Pathways. *Cell Death Dis* 2023, 14, 255.

35. Zou, J.; Secombes, C.J. The Function of Fish Cytokines. *Biology* 2016, 5, 23.

36. Wei, S.; Wang, S.; Yang, M.; Huang, Y.; Wei, J.; Huang, X.; Qin, Q. Characterization of Cathepsin C from Orange-Spotted Grouper, *Epinephelus Coioides* Involved in SGIV Infection. *Fish Shellfish Immunol* 2019, 84, 423–433.

37. Diao, Q.; Du, H.; Zhao, N.; Wu, Y.; Du, X.; Sun, Y.; Zhou, Y.; Cao, Z. Cathepsin C (CTSC) Contributes to the Antibacterial Immunity in Golden Pompano (*Trachinotus Ovatus*). *Fish & Shellfish Immunology* 2022, 128, 316–326, doi:10.1016/j.fsi.2022.07.078.

38. Dietrich, M.A; Irnazarow, I.; Inglot, M.; Adamek, M.; Jurecka, P.; Steinhagen, D.; Ciereszko, A. Hormonal Stimulation of Carp Is Accompanied by Changes in Seminal Plasma Proteins Associated with the Immune and Stress Responses. *J Proteomics* 2019, 30, 202:103369.

39. Chelewani, A.P.; Shimabukuro, T.; Takahashi, E.; Nishimura, T.; Fujimoto, T. Changes in Sperm Quality and Seminal Plasma Parameters of Masu Salmon *Oncorhynchus Masou* during the

Breeding Season and Implications for Cryopreservation Success. *Fish Sci* 2025, 91, 799–809.

40. Judycka, S.; Nynca, J.; Hliwa, P.; Ciereszko, A. Characteristics and Cryopreservation of Semen of Sex-Reversed Females of Salmonid *Fish. Int J Molt Sci* 2021, 22(2), 964.

41. Contreras, P.; Dumorné, K.; Ulloa-Rodríguez, P.; Merino, O.; Figueroa, E.; Farías, J.G.; Valdebenito, I.; Risopatrón, J. Effects of Short-Term Storage on Sperm Function in Fish Semen: A Review. *Rev Aquac* 2020, 12, 1373–1389.

42. Cabrita, E.; Martínez-Páramo, S.; Gavaia, P.J.; Riesco, M.F.; Valcarce, D.G.; Sarasquete, C.; Herráez, M.P.; Robles, V. Factors Enhancing Fish Sperm Quality and Emerging Tools for Sperm Analysis. *Aquaculture* 2014, 432, 389–401.

43. Jagadeeswaran, P.; Sheehan, J.P. Analysis of Blood Coagulation in the Zebrafish. *Blood Cells Mol Dis* 1999, 25, 239–249.

44. Griffin, J.H.; Zlokovic, B.V.; Mosnier, L.O. Protein C Anticoagulant and Cytoprotective Pathways. *Int J Hematol* 2012, 95, 333–345.

45. Schaller, J.; Gerber, S.S. The Plasmin–Antiplasmin System: Structural and Functional Aspects. *Cell Mol Life Sci* 2011, 68, 785–801.

46. Van de Water, N.; Tan, T.; Ashton, F.; O'Grady, A.; Day, T.; Browett, P.; Ockelford, P.; Harper, P. Mutations within the Protein Z-Dependent Protease Inhibitor Gene Are Associated with Venous Thromboembolic Disease: a new form of thrombophilia, *Br J Haematol* 2004, 127, 190–194.

47. Wei, Z.; Yan, Y.; Carrell, R.W.; Zhou, A. Crystal Structure of Protein Z-Dependent Inhibitor Complex Shows How Protein Z Functions as a Cofactor in the Membrane Inhibition of Factor X. *Blood* 2009, 114, 3662–3667.

48. Santana, J.C. de O.; Quagio-Grassiotto, I. Extracellular Matrix Remodeling of the Testes through the Male Reproductive Cycle in Teleostei Fish. *Fish Physiol Biochem* 2014, 40, 1863–1875.

49. Potempa, J.; Korzus, E.; Travis, J. The serpin superfamily of proteinase inhibitors: structure, function and regulation. *J Biol Chem* 1994,

269:15957-15960.

50. Janciauskiene, S. Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles. *Biochem Biophys Acta* 2001, 153, 221-235.

51. Silverman, G.; Bird, P.; Carrell, R.; Church, F.C.; Coughlin, P.B.; Gettins, G.W.; Irving, J.A.; Lomas, D.A.; Luke, C.J.; Moyer, R.W.; Pemberton, P.A.; Remold-O'Donnell, E.; Salvesen, G.; Travis, J.; Whisstock, JC. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. *J biol Chem* 2001, 276, 33293-33296.

52. Van Gent, D.; Sharp, P.; Morgan, K.; Kalsheker, N. Serpins: structure, function and molecular evolution. *Int J Biochem Cell Biol* 2003, 35:1536-1547.

53. Lilja, H.; Oldbring, J.; Ramnevik, G.; Laurell, C.B. Seminal vesicle secreted proteins and their reactions during gelation and liquefaction of human semen. *J Clin Invest* 1987, 80, 281-285.

54. Wilson, M.J., Garcia, B., Woodson, M., Sinha, A.A. Gelanolytic and caseinolytic proteinase activities in the secretions of the ventral, lateral, and dorsal lobes of the rat prostate. *Biol Reprod* 1993, 48, 1174–1184.

55. Evans, J.P., 2001. Fertilin beta and other ADAMs as integrin ligands: insights into cell adhesion and fertilization. *Bioessays* 23, 628–639.

56. Breton, B., Menezo, Y., Billard, R., 1974. Mise en évidence de quelques enzymes dans le sperme de la carpe Cyprinus carpio L. et de la truite Salmo gairdneri Richardson et dans le liquide coelomatique de la truite. Serie D 278, *Comptes Rendus de L'academie des Sciences, Paris*, pp. 1285–1288.

57. Lahnsteiner, F., Patzner, R.A., Weismann, T. The spermatic duct of salmonid fishes (Salmonidae, Teleostei). Morphology, histochemistry and composition of the secretion. *J Fish Biol* 1993, 42, 79–93.

58. Lahnsteiner, F., Berger, B., Patzner, R., Weismann, T., 1995. Fine structure and motility of spermatozoa and composition of the seminal plasma in the perch. *J Fish Biol* 47, 492–508.

59. Kowalski, R., Glogowski, J., Kucharczyk, D., Goryczko, K., Dobosz,

S., Ciereszko, A. Proteolytic activity and electrophoretic profiles of proteases from seminal plasma of teleosts. *J Fish Biol* 2003, 63, 1008–1019.

60. Lu, H.; Cassis, L.A.; Kooi, C.W.V.; Daugherty, A. Structure and Functions of Angiotensinogen. *Hypertens Res* 2016, 39, 492–500.

61. Zhu, X.; Xie, T.; Zhan, X.; Liu, L.; Yang, X.; Xu, X.; Ye, W.; Song, Y. Association of seminal angiotensinogen with sperm motility and morphology in male infertility. *Andrologia* 2019, 51(11), e13421.

62. Gao, S.; Chen, Z.; Shi, J.; Chen, Z.; Yun, D.; Li, S.; Wu, X.; Fei, S. Sperm motility is associated with epididymis metabolism disorder in mice under obstructive azoospermia. *FASEB J* 2023, 37(8) e23081.

63. Re, R.N. Intracellular renin and the nature of intracrine enzymes. *Hypertension* 2003, 42, 117-122.

64. Gianzo, M.; Subirán, N. Regulation of male fertility by the renin-angiotensin system. *Int J Mol Sci* 2020, 21(21), 7943.

65. Salier, J.P.; Diarra-Mehrpour, M.; Sesboue, R.; Bourguignon, J.; Benarous, R.; Ohkubo, I.; Kurachi, S.; Kurachi, K.; Martin, J.P. Isolation and characterization of cDNAs encoding the heavy chain of human inter-alpha-trypsin inhibitor (I-alpha-TI): unambiguous evidence for multiploypeptide chain structure of I-alpha-TI. *Proc. Nat Acad Sci* 1987, 84, 8272-8276.

66. Himmelfarb, M.; Klopochi, E.; Grube, S.; Staub, E.; Klaman, I.; Hinzman, B.; Kristianse, G.; Rosenthal, A.; Durst, M.; Dahl, E. ITIH5, a novel member of the inter-alpha-trypsin inhibitor heavy chain family, is downregulated in breast cancer. *Cancer Lett* 2004, 204, 69-77.

67. Cao, X.L.; Song, J.Y.; Sun, Z.G. Quantitative label-free proteomic analysis of human follicle fluid to identify novel candidate protein biomarker for endometriosis-associated infertility. *J Proteomics* 2022, 30, 266:104680.

68. Dym, M. Basement membrane regulation of Sertoli cells. *Endocr Rev* 1994, 15, 102-115.

69. Litscher, E.S.; Wassarman, P.M. Evolution, structure, and synthesis of vertebrate egg-coat proteins. *Trends Devel Biol* 2014, 8, 65-76.

70. Nynca, J.; Słowińska, M.; Dietrich, M.A.; Bilińska, B.; Kotula-Balak, M.; Ciereszko, A. Isolation and Identification of Fetuin-B-like Protein from Rainbow Trout Seminal Plasma and Its Localization in the Reproductive System. *Comp Biochem Physiol B Biochem Mol Biol* 2011, 158, 106–116.

71. Dietrich, M.A.; Ciereszko, A. Proteomic Characterization of Fresh Spermatozoa and Supernatant after Cryopreservation in Relation to Freezability of Carp (*Cyprinus Carpio* L) Semen. *PLOS One* 2018, 13(3), e0192972.

72. Wu, Z.; Zhang, W.; Lu, Y.; Lu, C. Transcriptome Profiling of Zebrafish Infected with *Streptococcus Suis*. *Microb Pathog* 2010, 48, 178–187.

3

Short-term exposure to polystyrene nanoplastics alters sperm viability and motility, and seminal plasma composition in the gilthead seabream (*Sparus aurata*)

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Abstract

Nanoplastics (NPs) contamination poses a growing threat to aquatic environments due to their persistence, bioaccumulation, and potential to disrupt reproductive performance. In the present study, the effects of varying concentrations of polystyrene nanoplastics (PS-NPs) on sperm viability and motility, as well as biochemical indicators and metabolic profiles in seminal plasma from gilthead seabream (*Sparus aurata*) were analysed. Fish semen samples were exposed to low (1 μg/mL), medium (10 μg/mL) and high (50 μg/mL) concentrations of PS-NPs and incubated for 1 hour at 4 ºC. Sperm viability was analysed before and after exposure by cytometry techniques. Motility was analysed with a CASA system. The biochemical profile was determined using an automated clinical chemistry analyser with the corresponding reagent kits. Finally, the metabolome was determined by Nuclear Magnetic Resonance. PS-NPs uptake showed a linear relationship with respect to the PS-NPs concentrations used, being significantly elevated at higher concentrations compared to low doses. However, sperm viability decreased in a dose-dependent manner with respect to PS-NPs concentrations tested, with a significant reduction observed at the highest concentration. Motility analysis revealed that low PS-NPs exposure significantly increased sperm velocity. Cholesterol and triglyceride concentrations were significantly increased in samples exposed to high concentrations of PS-NPs, indicating altered lipid metabolism. Forty metabolites were identified in the seminal plasma from Gilthead seabream, with betaine and glycine being the most abundant. However, no statistically significant differences in the metabolome composition were observed among the tested groups. Overall, exposure to PS-NPs induces complex, concentration-dependent alterations in sperm physiology that affect viability, normal motility, and seminal plasma composition. These findings raise concerns about the potential reproductive impact of environmental PS-NPs pollution on aquatic organisms, warranting more in-depth research.

1. Introduction

Teleost fish exhibit external fertilisation, which critically depends on precise timing, as spermatozoa remain motile for a brief timeframe until their energy reserves are depleted, to reach and penetrate the oocyte through the micropyle (Iwamatsu, 2000). Once motility begins, the sperm's fertilising capacity rapidly declines in a species-specific fashion due to energy depletion and alterations in membrane structure and function (Cosson et al., 2008; Dzyuba et al., 2017). This finely tuned process is increasingly threatened by the environmental contaminants present in aquatic ecosystems. Substances such as heavy metals, pesticides, fungicides, pharmaceuticals, and plastics can impair sperm motility and reduce fertilisation success, ultimately contributing to reproductive failure and population declines (Alavi et al., 2021). Among these pollutants, nanoplastics (NPs) have emerged as particularly concerning due to their widespread presence and potential to interfere with reproductive and cellular functions in aquatic organisms (Peng et al., 2020). Marine environments are particularly vulnerable to plastic pollution, with plastic waste making up to 85 % of total marine debris, mainly originated from surface runoffs and wastewater discharges (80 %), fishing industry activities (18 %), including the use of polymer-coated cage nets and feed pipes (Mattsson et al., 2018), and littering and coastal human activities (Peng et al., 2020). Plastic contamination is particularly intense in semi-enclosed regions such as the Mediterranean Sea (Meijer, 2021; Scovazzi, 2024), where the most reported polymers are polyethylene (PE), polypropylene (PP), and polystyrene (PS) (Wang et al., 2021).

Although data from model organisms provide insight into the plethora of toxic effects ascribed to NPs, significant knowledge gaps persist (Gupta et al., 2022). There is growing experimental evidence in mammals and model fish species indicating that the reproductive system is a likely target of NPs toxicity (reviewed by Liu et al., 2024). In this sense, Qiang et al. (2020) observed that adult zebrafish (*Danio rerio*) exposed to 100 µg/L of polystyrene nanoplastics (PS-NPs) (1 µm) displayed altered steroidogenic gene expression in the gonads. However, no effects were observed in the early development of their offspring. Similarly, Yang et al. (2023) demonstrated that 100 nm NPs inhibited both spermatogenesis and oogenesis and delayed gonadal maturation in medaka (*Oryzias latipes*). PS-NPs can affect zebrafish reproductive parameters in both sexes, inducing abnormal clustering of germ cells and spermatozoa within the testis, decreased sperm motility and altered oocyte stage distribution in females, suggesting disruption of

oocyte development (Pujol et al., 2025). These findings support the idea that acute exposure to PS-NPs can negatively affect gametogenesis and gamete quality, potentially reducing reproductive performance.

Despite this accumulating evidence in model species, detailed studies assessing the effects of PS-NPs on reproduction in economically important cultured fish are still lacking. This is a critical knowledge gap, especially for species such as the gilthead seabream (*Sparus aurata*), which is widely farmed across the Mediterranean and plays a major role in regional aquaculture (Gasca-Leyva et al., 2002). Given the high burden of PS-NPs in Mediterranean waters and the reproductive importance of sperm motility in fish, it is essential to investigate whether PS-NPs pose a reproductive risk to this species.

The present study aimed to evaluate the short-term effects of PS-NPs at different concentrations on gilthead seabream milt. For that purpose, sperm viability and motility were assessed through cytometry techniques and computer-assisted semen analysis (CASA), respectively. Likewise, biochemical and metabolomic profiles of the seminal plasma were also assessed. This approach provides insight into how PS-NPs may directly interact with fish gametes and helps identify sensitive physiological pathways, allowing the development of strategies to avoid the negative effects of PS-NPs on sperm physiology and warranting further *in vivo* and long-term investigation.

2. Materials and methods

2.1. Sperm collection

The present study included 13 adult gilthead seabreams from the breeding stock of the "Servicios Centrales de Investigación en Cultivos Marinos" (SCI-CM, CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Facilities for Breeding, Supplying and Users of Experimental Animals; Spanish Operational Code REGA ES11028000312) obtained during the natural reproductive season (October-December '24) of this species. Fish were reared using underground water in an open-flow water system and were fed twice daily (9:00 and 14:00 h) with frozen mussels and squid to ensure sperm quality at a rate of 1 % of their estimated total biomass. Male fish of average 1.55 ± 0.56 kg in weight and 37.87 ± 0.87 cm in length were lightly anaesthetised with 2-phenoxyethanol (Sigma-Aldrich, Spain) at a dose of 0.3 mL/L of seawater. Subsequently, the anal pore area was cleaned to

remove saltwater residues, and a gentle abdominal massage was performed to stimulate sperm release, which was collected in Petri dishes and then transferred to Eppendorf tubes. The samples were maintained at 4 °C until their subsequent transfer to the facilities at the Autonomous University of Barcelona (UAB) under refrigerated conditions. A maximum of 24 hours elapsed from the sperm collection till its use for the different analyses performed.

Upon arrival at the UAB, sperm viability was preliminarily assessed by cytometry (CytoFlex LX Beckman Coulter, Spain). Only samples showing viability above 90 % were included in the study. After assessing viability, semen samples were diluted to a 1:100 dilution with 0.9 % NaCl and sperm concentration was determined by placing 3 µL in a Leja chamber (Imv Technologies, France), followed by its assessment with a CASA system (Motility Module; Sperm Class Analyzer®, Microptic S.L.; València, Spain). This pre-dilution was performed due to the excessively high concentration of milt samples, which would impair the CASA system's accuracy to assess sperm concentration. Once the milts' concentration was determined, samples were diluted with 0.9 % NaCl to a final concentration of 10^9 sperm cells/mL.

2.2. Sperm incubation with PS-NPs

After dilution to a final concentration of 10^9 sperm cells/mL, 1 mL of each semen sample was distributed into 12-well plates. Samples were exposed to four experimental conditions: a control group without PS-NPs, and three treatment groups with increasing concentrations of PS-NPs: i) 1 µg/mL (Low PS-NPs), ii) 10 µg/mL (Medium NP-NPs), and iii) 50 µg/mL (High NP-NPs). The content of each well was gently mixed in a crosswise motion to ensure even distribution. The plates were incubated at 4 °C for 1 hour, with gentle manual agitation every 15 minutes to maintain particle suspension and ensure uniform exposure of sperm cells to the PS-NPs. Incubation at 4 °C temperature was selected to keep sperm cells in a non-activated, quiescent state, preventing spontaneous motility and allowing controlled NP–cell interaction before activation (Yang and Tiersch, 2009; Boj et al., 2015), while 1-hour incubation was selected to allow sufficient time for nanoparticle-cell interaction and uptake. Fluorescently labelled PS-NPs (mean diameter: 50 nm – Dragon Green, FSDG001) used for exposure in all assays were purchased from Bangs Laboratories (Fisher, IN, USA).

2.3. Sperm viability and PS-NPs uptake analysis

Sperm viability was assessed by flow cytometry using a CytoFLEX cytometer (Beckman Coulter) equipped with 488 nm and 638 nm lasers. Propidium iodide (PI) was used to distinguish live and dead sperm cells, with fluorescence detected in the PerCP channel. Sperm samples were first diluted in 0.9 % NaCl at a final concentration of 1 × 10^6 sperm/mL. First, unstained samples were run to settle the negative events. Then, 1 mg/mL PI was added to the samples. A total of 20,000 events were recorded per run. Cell gating was adjusted to determine viability, expressed as the percentage of live cells in PI-stained samples.

Following the viability assay, quantified sperm cell samples were incubated under the same conditions explained earlier with green-fluorescent PS-NPs to assess nanoparticle uptake by flow cytometry. The PS-NPs were compatible with the FITC channel of the CytoFLEX cytometer (Beckman Coulter). Post-incubation samples were analysed using the same instrument settings as in the viability assay. For each condition, 20,000 single-cell events were recorded at a medium flow rate. Uptake was measured as the percentage of fluorescent (nanoplastic-positive) cells and/or mean fluorescence intensity (MFI) in the FITC channel. Gating strategies excluded debris and aggregates, with thresholds defined using negative controls. Data were processed using CytExpert software.

2.4. Sperm motility analysis

Sperm motility was assessed using the CASA system previously described. Milt samples were activated with seawater from the original tank where the male seabream specimens were housed at a 1:5 dilution (v/v). This decision was made due to the lack of activation when samples were diluted with artificial seawater. Immediately after, a 3µL aliquot of the diluted sample was placed into a Leja chamber (Imv Technologies, France) and assessed at room temperature (20–25 °C). The settings for motility assessment were fixed at 25 frames/s, 768x576 pixels, particle area 0-40 mm, connectivity 14 mm and 20x negative magnification. Progressive motility percentage (PM), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF) were assessed. Cells with VCL > 10 µm/s were considered motile.

2.5. Biochemical analysis of seminal plasma

Sperm samples were centrifuged at 3000 × g for 10 minutes at 4 °C. The obtained seminal plasma (supernatant) was then recovered and centrifuged again at 10,000 × g for 10 minutes at 4 °C. The supernatant was carefully collected and stored at −80 °C until analysis to preserve biochemical stability. Biochemical parameters were measured using an automated clinical chemistry analyser (AU 600, Olympus Diagnostica, GmbH), along with compatible commercial reagent kits supplied by Olympus Diagnostica and Beckman Coulter (Beckman Coulter S.L.U., Madrid, Spain). All assays were performed according to the manufacturer's instructions. Each reaction was carried out under controlled conditions, typically at 37 °C incubation temperature, with predefined reaction times specific to each analyte. Absorbance readings were taken at the appropriate wavelengths for each biochemical parameter, ranging from 340 nm to 600 nm, depending on the chromogenic or enzymatic reaction used in each kit. Calibration and quality control procedures were conducted regularly to ensure accuracy and consistency. The following parameters were assessed: albumin (g/L), creatine kinase (U/L), total cholesterol (mmol/L), aspartate aminotransferase (U/L), triglycerides (mmol/L), adenosine deaminase (U/L), total protein (g/L), and paraoxonase 1 (U/L). The concentrations or enzymatic activities were automatically calculated by the analyser based on standard calibration curves, ensuring high precision and reproducibility of the results.

2.6. Metabolite profiling of seminal plasma

Seminal plasma was obtained as described previously. To extract metabolites from the samples, Amicon Ultra-0.5 Centrifugal Filter Tubes (Sigma-Aldrich) were used. Since these filters contained traces of glycerol, they required a thorough washing process before filtering the plasma samples. The filters were rinsed with bi-distilled water and centrifuged for 5 minutes at 13,793 × *g*, after which the filtrate receivers were emptied. This washing step was repeated 4 times, avoiding the filters from drying during the process. Once the filters were properly rinsed, seminal plasma samples were immediately centrifuged in the filter tubes at 13,000 × *g* for 30 minutes at 4 °C. The resulting filtrate was expected to be clear and free of any colouration. Then, 500 µL of the filtrate and 50 µL of a 0.2 mM solution of Sodium trimethylsilylpropanesulfonate (DSS) were transferred into individual Nuclear Magnetic Resonance (NMR) tubes and manually mixed. 1 H NMR spectra were measured in a Bruker 600-MHz AVANCE

III NMR spectrometer equipped with a triple channel probe TXI 600 S3 H-C/N-D 05-Z-BTO (Bruker Biospin, Rheinstetten, Germany), operating at a 1H frequency of 600.13 MHz and 298 K with a 5 min temperature equilibration time for each sample. The standard 1D 1H-nuclear Overhauser effect spectroscopy (1D-NOESY) pulse sequence from the Bruker library was applied (noesygppr1d). The relevant parameters applied were: mixing time 100 ms (d8), recovery delay: 2 s (d1), 90◦ pulse calibrated for first sample at 10.5 µs (p1), spectral width: 7211.539 Hz (sw), spectral size 32 k (td), number of scans 256 (ns) and acquisition time 2.27 s (aq).

For metabolite identification and quantification, data processing and Spectra analysis were performed using the Chenomx 12.0 profiler software (Chenomx Inc., Edmonton, Canada). This software contains tools for automatic phase, baseline correction (spline), automatic shim correction, reference calibration and a library of metabolites for spectral profiling. The concentration of each metabolite in the NMR tube was determined based on the standard internal reference (concentration in the NMR tubes 0.018 mM).

2.7. Statistical analysis

For data analysis, the sperm motility, biochemical, and metabolite data were first tested for normality using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Since the data did not follow a normal distribution, a nonparametric Kruskal-Wallis test was performed to assess statistical differences among the experimental groups using GraphPad Prism. For the heatmap, metabolite values were log-transformed and autoscaled before generatin g it. Hierarchical clustering was performed with the Euclidean distance and Ward's linkage method, using MetaboAnalyst. To provide a broader overview of the sperm motility profile following NPs exposure, K-means clustering was conducted on the motility parameters using Origin 2025. Additionally, motility data were bootstrapped in Origin 2025, and Principal Component Analysis (PCA) with Spearman correlation was performed to explore relationships among metabolites, biochemical markers, and motility parameters, aiming to identify potential biomarkers of PS-NPs exposure.

3. Results

3.1. PS-NPs uptake and sperm viability analyses

PS-NPs uptake was positively correlated with exposure concentrations, being significantly higher at higher doses than at low doses (67.7 ± 30.2% vs 2.6 ± 3.0%, respectively) **(Figure 1A)**. Sperm viability decreased in a dose-dependent manner with respect to the PS-NPs concentrations tested. Only medium and high PS-NPs exposures significantly ($P < 0.05$) decreased the percentage of viability to 80.7 ± 8.9 % and 23.0 ± 24.8 %, respectively, compared with the control group (92.7 ± 6.0 %) **(Figure 1B)**.

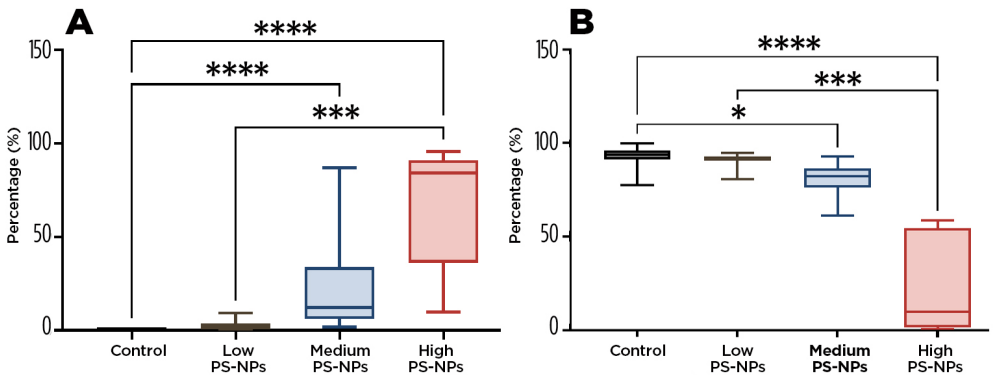


Figure 1. Effects of different concentrations of PS-NPs on sperm viability and PS-NPs uptake in sea bream.
Statistical significance is indicated as follows:
*p < 0.05, ***p < 0.001, ****p < 0.0001.

3.2. Sperm motility analysis

The effects of PS-NPs exposure on mean motility parameters are presented in **Table 1**. Briefly, exposure to low concentrations of PS-NPs (1 µg/mL) significantly enhanced velocity parameters (VCL, VAP, and VSL) and DNC. However, DNM values increased significantly in samples exposed to medium (10 µg/mL) PS-NPs concentrations but decreased with high (50 µg/mL) concentrations. K-means cluster analysis rendered 4 kinetic sperm subpopulations, or clusters.

Spermatozoa from Subpopulation 1 showed the highest values for velocity parameters, WOB index, mLAH, DNC and DNM **(Figure 2)**. Subpopulation 2 was also characterised by high values for velocity parameters and WOB index. However, mLAH, DNC and DNM values showed a 2-fold decrease compared to subpopulation 1. Subpopulation 3 showed the highest values

Table 1. Effect of polystyrene nanoplastics (PS-NPs) exposure on sperm motility parameters in gilthead seabream.

Values are presented as mean ± SEM. Different superscript letters within rows indicate significant differences (P<0.05). Abbreviations: VCL (Curvilinear Velocity); VSL (Straight-Line Velocity); VAP (Average Path Velocity); LIN (Linearity); STR (Straightness); WOB Wobble; mALH (Mean Amplitude of Lateral Head Displacement); BCF (Beat Cross Frequency); DNC (Dynamic Number of Crossings); DNM (Dynamic Number of Motions)

Parameters	Control (0 µg/mL)	Low-NPs (1 µg/mL)	Medium-NPs (10 µg/mL)	High-NPs (50 µg/mL)
VCL(µm/s)	103.38 ± 2.42^{b}	117.85 ± 2.70^{a}	105.23 ± 2.23^{b}	105.94 ± 2.62^{b}
VSL	69.09 ± 2.01^{b}	80.06 ± 2.31^{a}	69.72 ± 1.79^{b}	66.06 ± 2.22^{b}
VAP	95.02 ± 2.50^{b}	109.48 ± 2.75^{a}	95.44 ± 2.26^{b}	96.75 ± 2.68^{b}
LIN	67 ± 0.9^{ab}	68 ± 1.0^{a}	65 ± 0.8^{ab}	61 ± 1.0^{b}
STR	74 ± 0.8	75 ± 0.9	75 ± 0.7	70 ± 1.0
WOB	86 ± 0.6	89 ± 5.0^{a}	86 ± 0.5^{b}	86 ± 0.6^{b}
mALH	2.03 ± 0.05^{b}	2.04 ± 0.05^{ab}	2.16 ± 0.04^{a}	2.08 ± 5^{ab}
BCF	5.03 ± 0.16	4.75 ± 0.18	5.04 ± 0.14	5.01 ± 0.18
DNC	233.14 ± 9.51^{b}	266.37 ± 0.47^{a}	253.36 ± 8.614^{ab}	250.50 ± 11.32^{ab}
DNM	120.60 ± 2.90^{bc}	125.00 ± 3.05^{ab}	130.50 ± 2.63^{a}	113.27 ± 3.16^{c}

for the LIN, STR and WOB indexes, but velocities (VCL and VAP) slightly decreased in comparison with Subpopulations 1 and 2. Likewise, DNC and DNM showed a 2-fold decrease in comparison with Subpopulation 2. Finally, Subpopulation 4 had the lowest values for motility parameters. Additionally, the number of sperm cells composing each kinematic subpopulation was determined. The most abundant was Subpopulation 1, followed by Subpopulations 2, 3 and 4. When the percentages of each kinematic subpopulation were evaluated, a clear displacement from the fastest subpopulation (cluster 1) to the slowest subpopulation (cluster 4) was consistently observed in the exposed groups.

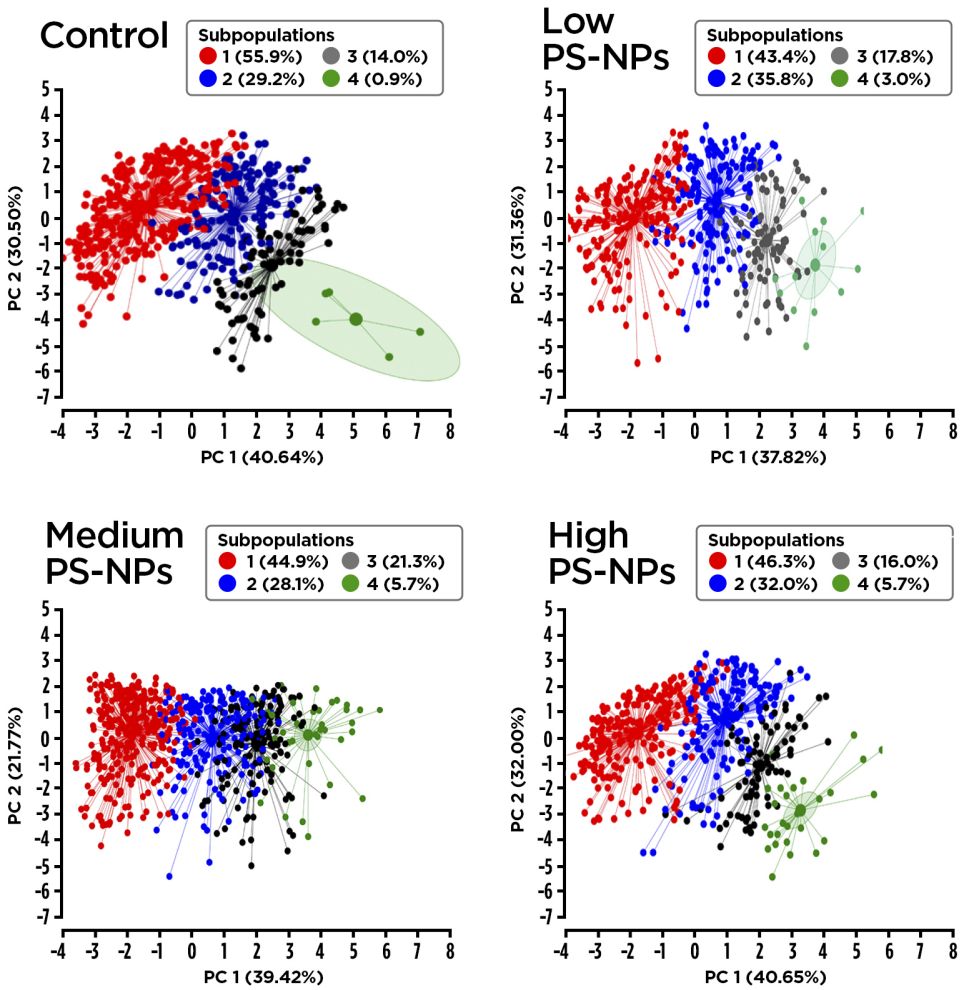


Figure 2. Principal Component Analysis (PCA) with K-means clustering of sperm motility profiles across treatment groups.
Sperm motility data were clustered into four distinct phenotypes based on velocity, trajectory, and movement dynamics using K-means clustering. Each point represents an individual sperm track, color-coded by cluster identity.

3.3. Biochemical analysis of seminal plasma

Exposure to varying concentrations of PS-NPs significantly enhanced ($P < 0.05$) seminal plasma cholesterol and triglycerides concentrations, specifically in the high PS-NPs group **(Figure 3)**. However, the remaining biochemical parameters showed no significant differences among the groups.

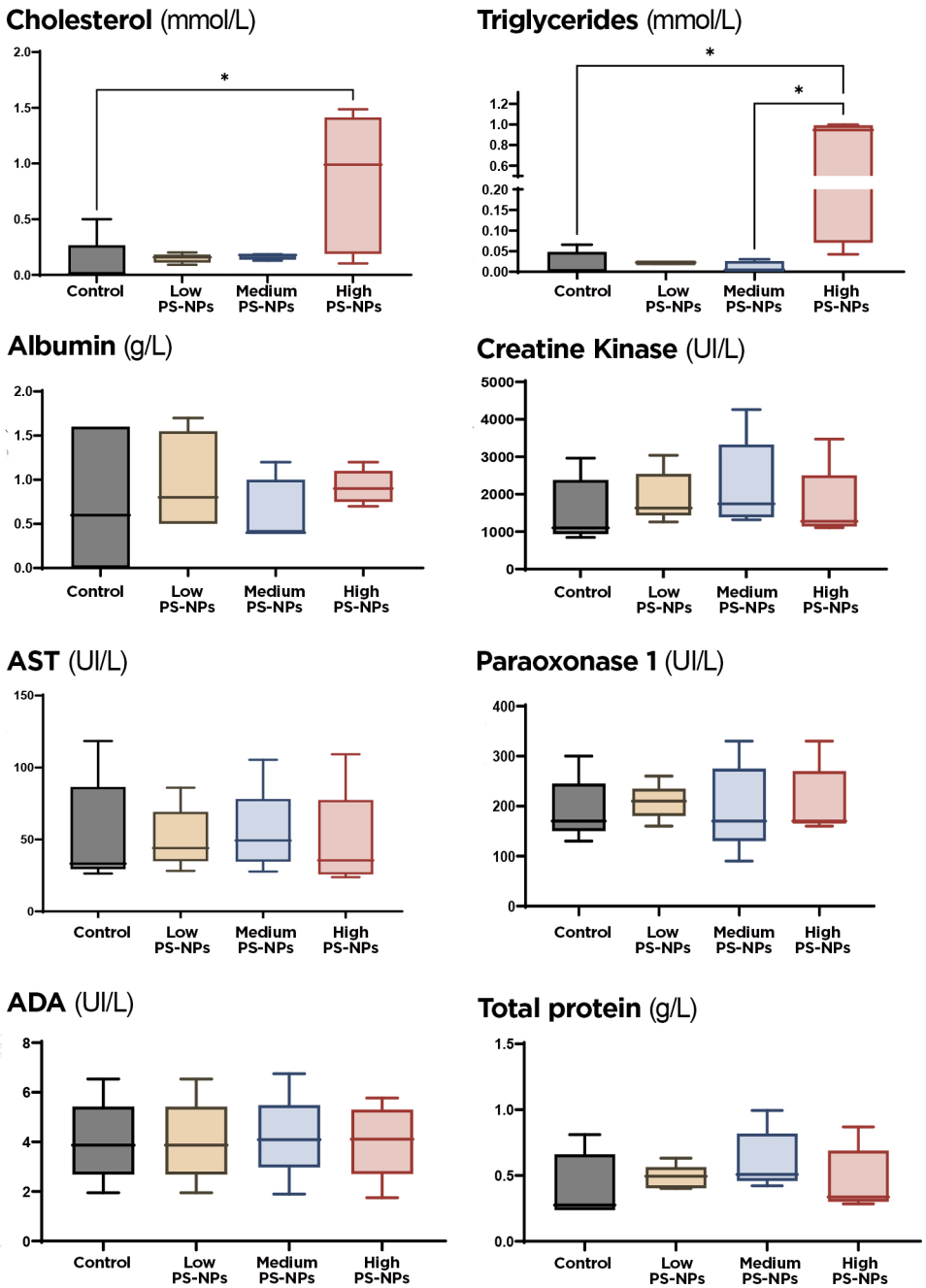


Figure 3. Biochemical parameters in sperm cells induced by PS-NPs exposure. Box plots represent the levels of Cholesterol, Triglycerides, Albumin, Creatine Kinase, Aspartate Aminotransferase, Paraoxonase 1, Adenosine Deaminase, and total Protein in sperm cells following incubation with varying concentrations of nanoparticles. Asterisks indicate statistically significant differences (*p < 0.05).

3.4. Metabolite profiling analysis of seminal plasma

A total of 40 metabolites were detected in seminal plasma samples from *S. aurata* **(Table 2)**. After Partial Least Squares Discriminant Analysis (PLS-DA) analysis, no significant difference in the overall metabolite profiles between groups was observed. Therefore, following validation, only metabolites with a VIP (Variable Importance in Projection) value greater than 1 were considered for subsequent heatmap analysis **(Figure 4)**. The most abundant metabolites were betaine and glycine, followed by aspartate, glycerol and creatine. No statistically significant differences were observed among the groups, although the concentrations of some metabolites showed a remarkable increase in the low PS-NPs group. Exclusively focusing on the most abundant metabolites, glycine, betaine, creatine, and aspartate levels were 30 % higher in the low PS-NPs group with respect to the control group. Other minor metabolites, such as glutamine, alanine, glutamate, AMP/ADP/ATP, and sarcosine, also showed increased concentrations that ranged from 25% to 65% in the low PS-NPs group compared to the control. Finally, ethanol also showed a 30-45 % increase in the medium PS-NPs and high PS-NPs groups compared to the control group **(Figure 5)**.

On the other hand, exposure to PS-NPs diminished the concentrations of glycerol, asparagine and creatine phosphate. Regarding glycerol, the decrease ranged from 25% to 50% in the exposed groups. On the other hand, the concentrations of asparagine and creatine phosphate remarkably decreased, ranging from 20% to nearly 80% in all groups compared to the control group.

Table 2. Relative concentrations (mean ± SD) of identified metabolites in sperm of gilthead seabream exposed to different concentrations of polystyrene nanoplastics (PS-NPs).

	Control (0 µg/mL)		Low PS-NPs (1 µg/mL)		Medium PS-NPs (10 µg/mL)		High PS-NPs (50 µg/mL)	
Glycine (mM)	0.706 ±	0.402	0.921 ±	0.409	0.772 ±	0.297	0.777 ±	0.297
Betaine (mM)	0.981 ±	0.618	1.335 ±	1.012	1.058 ±	0.509	0.955 ±	0.534
Creatine (mM)	0.194 ±	0.120	0.268 ±	0.181	0.215 ±	0.108	0.213 ±	0.091
Aspartate (mM)	0.143 ±	0.089	0.193 ±	0.144	0.154 ±	0.074	0.152 ±	0.065
Glycerol (mM)	0.168 ±	0.214	0.133 ±	0.073	0.111 ±	0.016	0.119 ±	0.036
Glutamine (mM)	0.032 ±	0.022	0.040 ±	0.027	0.032 ±	0.015	0.038 ±	0.023
Alanine (mM)	0.037 ±	0.031	0.057 ±	0.067	0.038 ±	0.022	0.039 ±	0.025
Taurine (mM)	0.037 ±	0.020	0.039 ±	0.024	0.036 ±	0.021	0.037 ±	0.027
Proline (mM)	0.044 ±	0.029	0.054 ±	0.054	0.046 ±	0.019	0.044 ±	0.027
Ethanol (mM)	0.096 ±	0.085	0.102 ±	0.061	0.142 ±	0.103	0.125 ±	0.062
Glutamate (mM)	0.034 ±	0.026	0.057 ±	0.061	0.041 ±	0.022	0.039 ±	0.021
DSS (chemical shape indicator) (mM)	0.017 ±	0.006	0.018 ±	0.000	0.019 ±	0.002	0.018 ±	0.000
Lactate (mM)	0.014 ±	0.015	0.016 ±	0.018	0.013 ±	0.012	0.014 ±	0.015
Asparagine (mM)	0.032 ±	0.044	0.014 ±	0.017	0.015 ±	0.012	0.016 ±	0.016
Inosinic acid (mM)	0.020 ±	0.017	0.022 ±	0.014	0.021 ±	0.018	0.023 ±	0.015
AMP/ADP/ATP (mM)	0.011 ±	0.010	0.019 ±	0.020	0.014 ±	0.011	0.012 ±	0.009
Sarcosine (mM)	0.016 ±	0.012	0.021 ±	0.019	0.017 ±	0.011	0.016 ±	0.010
β-alanine (mM)	0.018 ±	0.012	0.020 ±	0.016	0.018 ±	0.010	0.019 ±	0.011
Creatine phosphate (mM)	0.074 ±	0.173	0.022 ±	0.038	0.016 ±	0.014	0.019 ±	0.018
Acetate (mM)	0.007 ±	0.006	0.013 ±	0.016	0.010 ±	0.007	0.008 ±	0.004
Tyrosine (mM)	0.009 ±	0.009	0.014 ±	0.019	0.009 ±	0.007	0.010 ±	0.007
Formate (mM)	0.005 ±	0.003	0.005 ±	0.005	0.005 ±	0.004	0.006 ±	0.003
Leucine (mM)	0.003 ±	0.004	0.011 ±	0.030	0.002 ±	0.003	0.003 ±	0.005
Methylhistidine (mM)	0.012 ±	0.020	0.021 ±	0.041	0.013 ±	0.019	0.012 ±	0.016
Acetone (mM)	0.004 ±	0.007	0.006 ±	0.013	0.002 ±	0.004	0.003 ±	0.006
Valine (mM)	0.003 ±	0.002	0.004 ±	0.001	0.003 ±	0.002	0.004 ±	0.001
Phenylalanine (mM)	0.010 ±	0.015	0.016 ±	0.030	0.010 ±	0.012	0.010 ±	0.012
Isoleucine (mM)	0.006 ±	0.008	0.010 ±	0.018	0.006 ±	0.008	0.006 ±	0.008
Lysine (mM)	0.005 ±	0.007	0.008 ±	0.017	0.006 ±	0.011	0.008 ±	0.012
Histidine (mM)	0.013 ±	0.033	0.019 ±	0.056	0.012 ±	0.033	0.012 ±	0.035
Methionine (mM)	0.003 ±	0.007	0.003 ±	0.006	0.000 ±	0.001	0.000 ±	0.000
Isobutyrate (mM)	0.003 ±	0.006	0.005 ±	0.010	0.003 ±	0.005	0.002 ±	0.006
2′-deoxyinosine (mM)	0.003 ±	0.005	0.008 ±	0.015	0.004 ±	0.005	0.004 ±	0.005
Sucrose (mM)	0.001 ±	0.002	0.002 ±	0.002	0.001 ±	0.001	0.001 ±	0.001
Arginine (mM)	0.001 ±	0.003	0.000 ±	0.000	0.000 ±	0.000	0.000 ±	0.000
Trimethylamine (mM)	0.021 ±	0.059	0.000 ±	0.000	0.000 ±	0.000	0.000 ±	0.000
Threonine (mM)	0.000 ±	0.000	0.017 ±	0.046	0.002 ±	0.005	0.005 ±	0.007
Putrescine (mM)	0.000 ±	0.000	0.003 ±	0.009	0.000 ±	0.000	0.000 ±	0.000
Carnitine (mM)	0.000 ±	0.000	0.003 ±	0.008	0.000 ±	0.000	0.000 ±	0.000
3-hydroxyisobutyrate (mM)	0.000 ±	0.000	0.000 ±	0.000	0.002 ±	0.006	0.000 ±	0.000

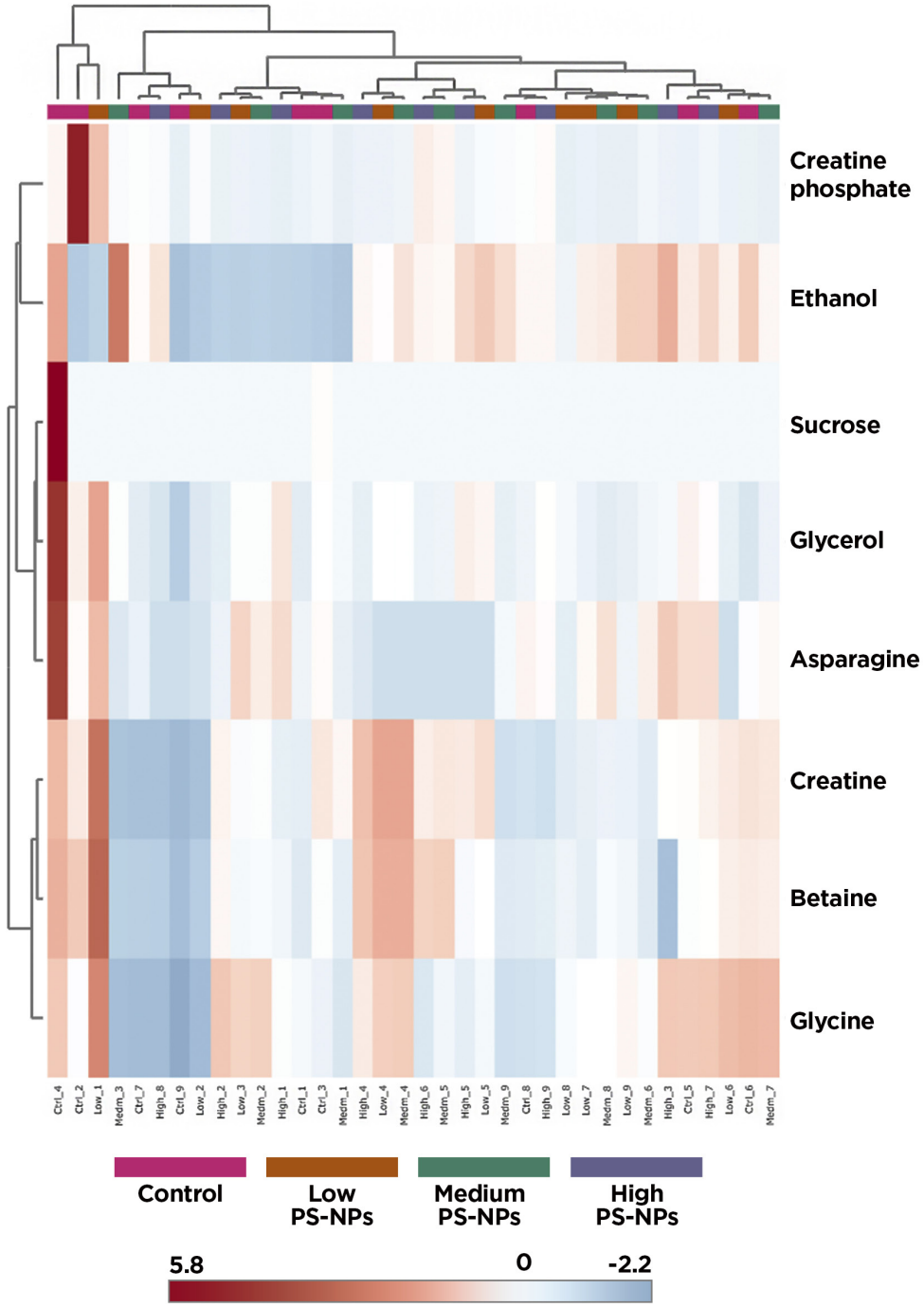


Figure 4. Metabolic changes induced by nanoplastic exposure. Heatmap shows metabolite levels across Control (0 µg/mL), Low-NPs (0.1 µg/mL); Medium-NPs (1 µg/mL); High-NPs(50 µg/mL) groups. Key changes include decreased energy metabolites (e.g., creatine derivatives) and increased stress markers (e.g., betaine, glycine) with rising NP concentration.
Colors represent relative abundance (red: high; blue: low).

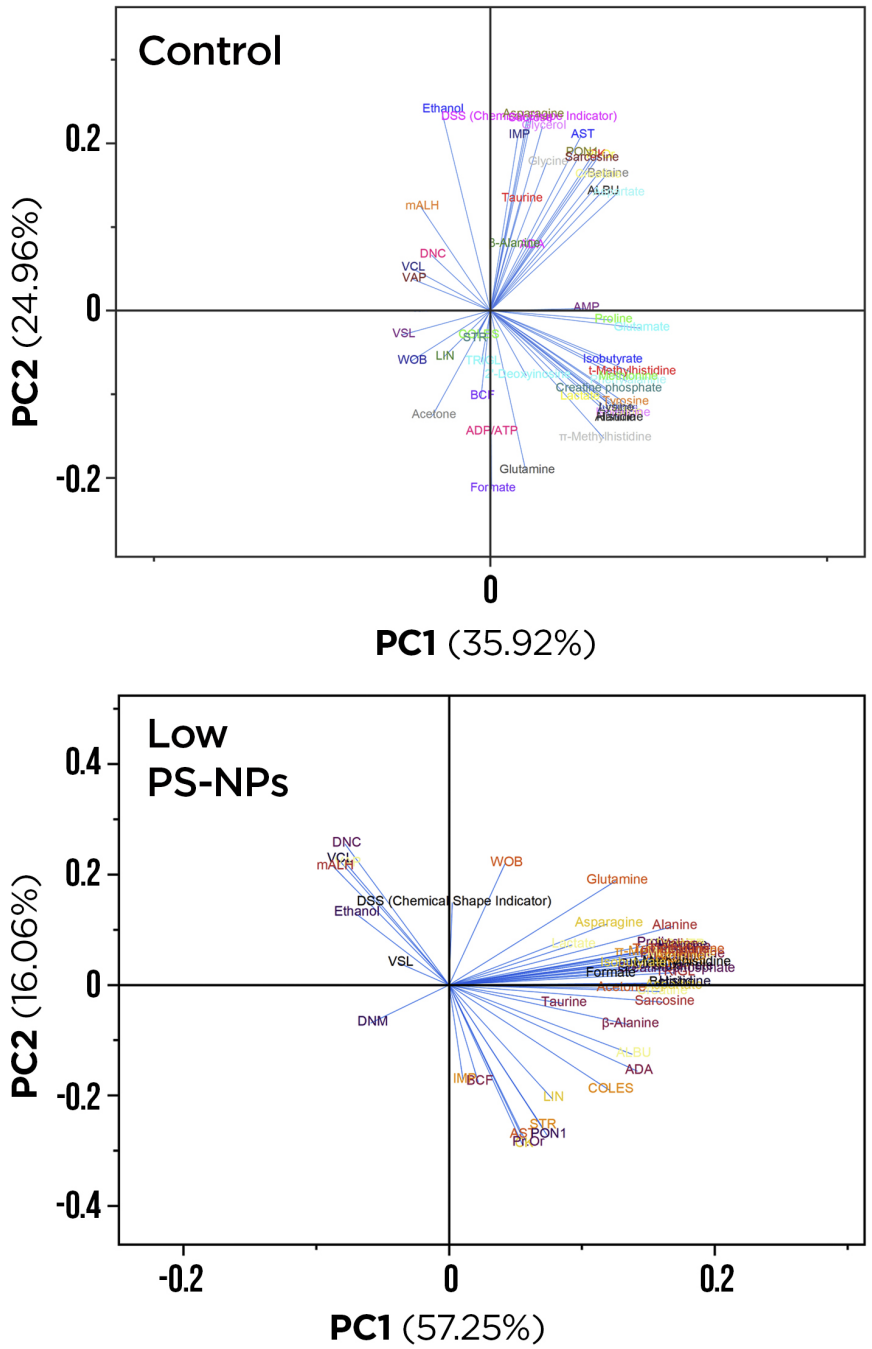


Figure 5. Principal Component Analysis (PCA) biplots illustrating biomarker patterns in sperm samples exposed to different concentrations of polystyrene nanoplastics (PS-NPs): Control (0 µg/mL), Low-NPs (0.1 µg/mL), Medium-NPs (1 µg/mL), and High-NPs(50 µg/mL)

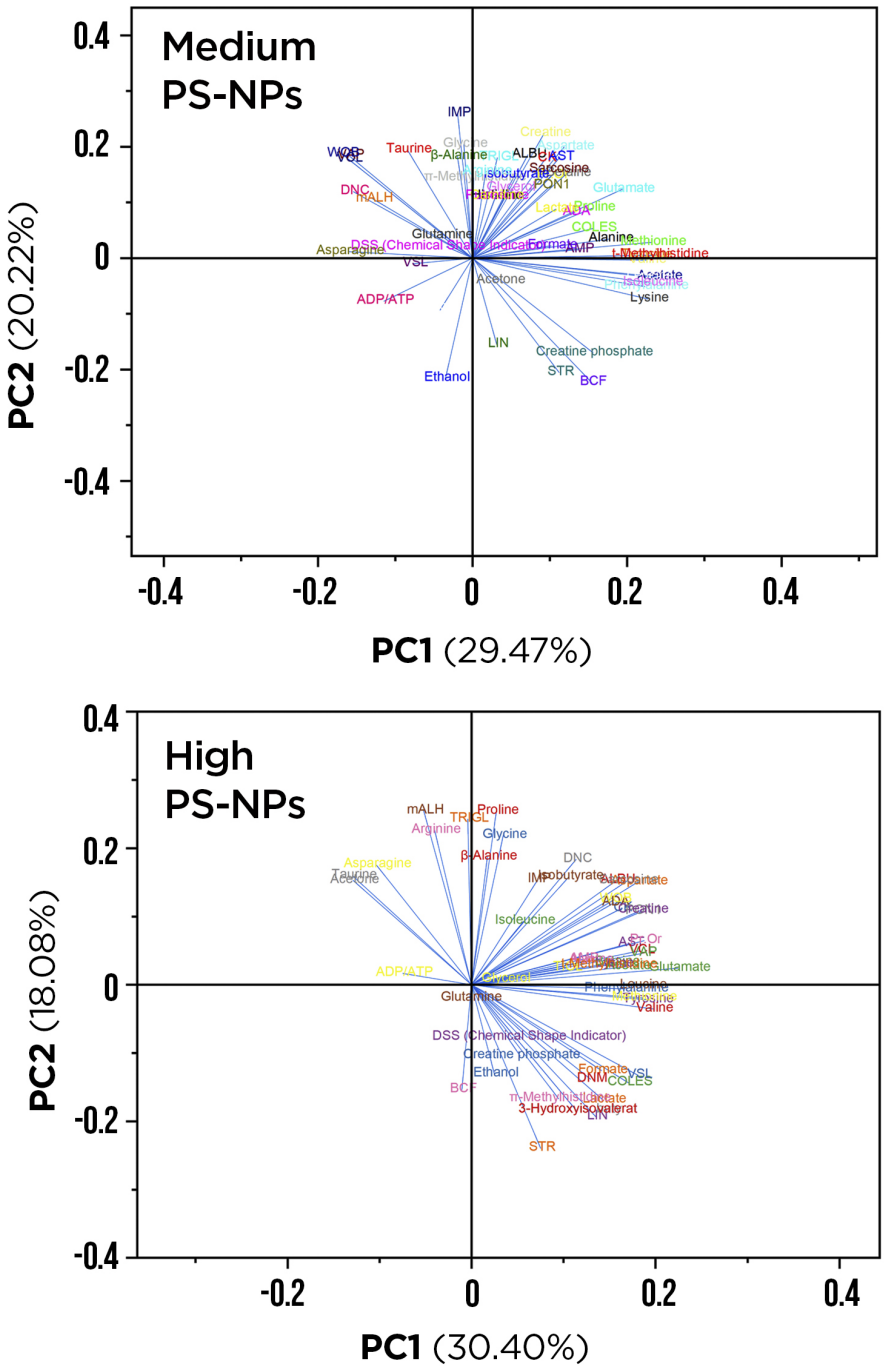


Figure 5. ***Cont.***

4. Discussion

4.1. Effect of PS-NPs exposure on sperm cells

As external fertilisers, fish sperm cells are immediately exposed to water contaminants, including NPs (reviewed by Liu et al., 2024). The effect of NPs on fish spermatozoa has been assessed in species such as *O. latipes* and zebrafish *D. rerio* (Yang et al.,2023; Pujol et al., 2025). However, no report has documented the effects of PS-NPs on *S. aurata* sperm despite the economic relevance of this species.

In the present study, we evaluated the impact of exposure to low (1 μg/mL), medium (10 μg/mL), and high (50 μg/mL) concentrations of PS-NPs on sperm viability and motility, as well as biochemical and metabolomic biomarkers in the seminal plasma from *S. aurata*. The concentration range (1–50 μg/mL) was intentionally chosen to assess dose-dependent cellular and mechanistic toxic responses under controlled *in vitro* conditions, rather than to mimic environmental exposures. Doses higher than those reported in the environment are commonly used in nanotoxicology to reveal biological sensitivity in short-term assays (Shukla et al., 2024). As expected, the uptake percentage followed a dose-dependent pattern, whereas the viability percentage followed an inverse dose-dependent pattern. Our results clearly indicate that exposing sperm cells to 50 μg/mL of PS-NPs induces sperm cell death. Thus, the high percentage of non-viable sperm cells may be related to the high uptake percentage observed in this concentration. A feasible explanation is the potential internalisation of PS-NPs into cells and the consequent membrane disruption induced by PS-NPs. This hypothesis is sustained by previous studies in hepatocytes from *D. rerio* (Brandts et al., 2020) and kidney primary macrophages and enterocytes from rainbow trout (Brandts et al., 2023). In those studies, the capacity of PS-NPs, similar to those used in our study, to internalise and alter cellular physiology was clearly demonstrated. However, more specific studies testing the effects of exposure to PS-NPs on fish sperm are needed to demonstrate this negative influence.

Fish sperm motility is a critical factor for successful fertilisation, especially in external fertiliser species such as teleost fish, including *S. aurata*. Once released into the water, sperm rapidly deplete ATP and other phosphate metabolites to enhance motility (Gallego et al., 2018) and achieve fertilisation within a highly variable, species-specific, and limited time window (Dzyuba et al., 2017).

Any reduction in sperm motility associated with environmental stressors, such as PS-NPs, can significantly affect sperm quality, impairing reproductive success and population sustainability (Lin et al., 2024).

In the present study, exposure to PS-NPs induced slight changes in motility parameters, which agrees with previous studies. In this sense, sperm quality is reduced in both mammals and aquatic species exposed to environmental contaminants, including PS-NPs. In mice, high doses of PS-NPs induced pronounced immune-inflammatory responses in testicular tissue, reduced sperm motility, and elicited metabolic disruptions, ultimately lowering fertilisation rates and impairing early embryo development (Liu et al., 2025). Likewise, PS-NPs increase oxidative stress, inflammation, DNA damage, and disruption of metabolic and hormonal pathways in rats, all of which are detrimental to sperm function and viability (Amereh et al., 2020). High concentrations of PS-NPs also decrease sperm motility and fertilisation capacity in mussels (*Mytilus galloprovincialis*), with the extent of damage depending on particle size and concentration (Contino et al., 2023). Similarly, fish respond to marine pollution by modifying sperm structure and altering the energetic resources destined to motility and viability, impairing fertilising capacity (reviewed in Alavi et al., 2021).

In our study, a clear displacement of sperm cells from the fastest (cluster 1) to the slowest (cluster 4) Subpopulation was observed after exposure to PS-NPs, demonstrating that this compound affects the distribution of the different kinematic subpopulations, despite not significantly altering mass motility. One cannot rule out the possibility that this may have a detrimental effect on spermatozoa performance in terms of fertilisation capacity, thus warranting further research in this field. The mechanism behind these changes in motility parameters remains unclear, but two hypotheses arise: i)PS-NPs may exert a direct effect on spermatozoa physiology and/or ii) PS-NPs may induce changes in the seminal plasma composition that ultimately alter sperm cell motility. However, further research is needed to elucidate these mechanisms.

4.2. Effect of PS-NPs exposure on seminal plasma composition

Seminal plasma supports the ejaculated spermatozoa, and its unique composition provides the proper conditions for sperm cell survival, motility and fertilisation capacity (Ciereszko et al., 2000). Fish seminal plasma is characterised by containing minerals such as Na^+, K^+ and Ca^{2+}, and low

concentrations of proteins, cholesterol, glucose and lipids (Rurangwa et al., 2004). In the present study, the biochemical profile of the seminal plasma from *S. aurata* was evaluated, and results similar to those previously reported in fish were observed for protein, cholesterol, and triglyceride (TAG) concentrations. TAGs are considered primary energy sources in fish spermatozoa during immotile storage, prolonging the viability of immotile sperm (Lahnsteiner et al. 2009). Likewise, cholesterol serves as an energy source for fish sperm cells (Beer-Ljubic et al. 2009). In addition, both compounds have been suggested to protect sperm cells against environmental hazards once sperm is released in the water (Bozkurt et al., 2008).

The content of TAGs and cholesterol in the seminal plasma of *S. aurata* was enhanced in samples exposed to high concentrations of PS-NPs compared to the other groups. A feasible hypothesis for this significant increase is the potential cell lysis. Cholesterol is a known component of the cell membrane of spermatozoa (Cooper, 2000). On the other hand, the presence of intracellular triglycerides has been previously described in mammal species such as humans (Rago et al., 2020; Furse et al., 2022) and rams (Evans and Setchell, 1979), and sea urchins (Mita et al., 1994; Mita et al., 1995). Thus, the cell membrane lysis would release these molecules into the seminal plasma.

Another explanation would be the disruption of sperm metabolic pathways. Chronic exposure to NPs in mice has been reported to affect pathways involved in lipid biosynthesis and metabolism, such as glycerophospholipid biosynthesis (Liu et al., 2025). However, in the current study, only the effects of a short-term exposure were assessed, making this possibility less likely. However, further research is needed to rule out these hypotheses

A total of 40 metabolites was also identified in the seminal plasma samples. Seminal plasma metabolites have been proposed as quality and/or fertility biomarkers of the ejaculate in bulls and are considered a promising alternative tool to the traditional spermiogram (Kumar et al., 2015; Velho et al., 2018; Menezes et al., 2019). Metabolomics of fish seminal plasma has been characterised in several species such as perch (*Perca fluviatilis*), burbot (*Lota lota*), rainbow trout (*Oncorhynchus mykiss*), bleak (*Alburnus Alburnus*), and chub (*Squalius cephalus*), among others (see Dietrich et al., 2019 for a review), and, nevertheless, the specific composition highly varies among species. However, to the best of our knowledge, this is the first time that the metabolomics of *S. aurata* seminal plasma has been determined.

In general terms, the metabolite composition of the seminal plasma from gilthead seabream is quite similar to that described in other fishes (Dietrich et al., 2019). It is composed of amino acids, lipids, and organic acids, among other metabolites.

In the current study, the most abundant detected metabolites were glycine and betaine. Glycine, as part of the glycine-serine-threonine metabolic pathway, plays a central role in cellular antioxidant defence and membrane regulation (Wu et al., 2021), acting as both an osmolyte and a precursor for glutathione, further enhancing the cell's antioxidant capacity in response to cellular stress and oxidative damage (Lin et al., 2022). Additionally, glycine has been demonstrated to play a role in sperm motility in *S. aurata* and *P. fluviatilis* (Lahnsteiner, 2010), clearly highlighting that seminal composition affects sperm motility in fish. Betaine is a stabiliser of proteins and cellular membranes, helping to maintain cell integrity and function under stress by regulating osmotic balance and reducing the accumulation of reactive oxygen species (ROS) (Lahnsteiner et al., 1994; Stadmiller et al., 2017). It also supports the activity of antioxidant enzymes and the synthesis of glutathione, a key cellular antioxidant (Sinesi et al., 2025).

Previous studies have reported that exposure to PS-NPs induced relevant changes in the metabolomic composition of the seminal plasma. In the present study, changes in the metabolomic composition were observed, although these changes were not statistically significant. Even though some studies have evaluated the relationship between the sperm metabolome and fertility (see Menezes et al., 2019 for a review), the role of the metabolites composing the seminal plasma is not fully understood. In animal models such as mice and *D. rerio*, exposure to PS-NPs disturbs glycolipid metabolism, induces oxidative stress, and triggers inflammation, with downstream effects on amino acid metabolism, including glycine (Pei et al., 2024; Xu et al., 2023). In the present study, samples exposed to low concentrations of PS-NPs showed increased concentrations of several metabolites, including glycine and betaine, compared to the control group. However, these variations were inconsistent within the groups exposed to medium and high concentrations of PS-NPs. By contrast, glycerol, asparagine and creatine phosphate decreased in the exposed groups compared to the control group. Regarding glycerol, a previous study showed that, in fresh semen samples, the concentration of this metabolite was lower in the seminal plasma from Holstein bulls with lower sperm motility, suggesting that high levels of this compound are necessary to support high sperm motility (Giaretta et al., 2025). Likewise, asparagine has a positive effect on perch spermatozoa

(Lahnsteiner, 2010). In the present study, decreasing concentrations of glycerol and asparagine were observed in the seminal plasma of groups exposed to PS-NPs, which could explain the decrease in sperm motility after the exposure. Considering the scarce number of studies on seminal plasma metabolomics in fish, one can only hypothesise about the significance of these observed changes. Therefore, further research is necessary to clarify the actual role of seminal plasma metabolites.

On the other hand, some metabolites showed higher concentrations only in the low-exposure group, with no changes in the medium and high-exposure groups. A feasible explanation would be that the changes observed in the seminal plasma metabolome are likely due to the interaction between sperm cells and PS-NPs. Any compound originated by this interaction is released into the seminal plasma. In the case of medium and high concentrations, the compounds released into the seminal plasma are probably products of cell degradation/death/lysis. However, the low concentration of NP-PS does not kill sperm cells, but alters their physiology, consequently releasing compounds resulting from this metabolic alteration.

5. Conclusions

It is well known that the composition of seminal plasma affects the spermatozoa in terms of viability, motility and fertility. The present results demonstrate that a short-term exposure to PS-NPs alters the viability and motility of sperm cells from *S. aurata*. Additionally, exposure to PS-NPs also modifies the biochemical composition and metabolome of the seminal plasma. Changes in viability percentage may be related to the potential crossing of the PS-NPs through the cell membrane, whereas the modifications observed in motility may be attributed to the changes occurring in the biochemical and metabolomic composition of the seminal plasma. However, further research is warranted to demonstrate these hypotheses.

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References

Alavi, S.M.H., Barzegar-Fallah, S., Rahdar, P., Ahmadi, M.M., Yavari, M., Hatef, A., Golshan, M. and Linhart, O., 2021. A review on environmental contaminants-related fertility threat in male fishes: effects and possible mechanisms of action learned from wildlife and laboratory studies. *Animals*, 11(10), p.2817.

Amereh, F., Babaei, M., Eslami, A., Fazelipour, S. and Rafiee, M., 2020. The emerging risk of exposure to nano (micro) plastics on endocrine disturbance and reproductive toxicity: From a hypothetical scenario to a global public health challenge. *Environmental Pollution,* 261, p.114158.

Aramli, M.S., Golshahi, K., Nazari, R.M., Golpour, A. and Aramli, S., 2016. Retracted: Influence of Glutamine Supplementation on Motility and Fertilization Success of Frozen–Thawed Persian Sturgeon (*Acipenser persicus)* Sperm. *Reproduction in domestic animals,* 51(4), pp.474-477.

Barman, A.S., Kumar, P., Lal, K.K. and Lal, B., 2013. Role of nitric oxide in motility and fertilizing ability of sperm of *Heteropneustes fossilis* (Bloch.). *Animal reproduction science,* 137(1-2), pp.119-127.

Barría, C., Brandts, I., Tort, L., Oliveira, M. and Teles, M., 2020. Effect of nanoplastics on fish health and performance: A review. *Marine pollution bulletin*, 151, p.110791.

Beer-Ljubic B, Aladrovic J, Marenjak TS, Laskaj R, Majic-Balic I, Milinkovic-TurS, 2009: Cholesterol concentration in seminal plasma as a predictive tool for quality semen evaluation. *Theriogenology* 72, 1132–1140.

Brandts I, Solà R, Garcia-Ordoñez M, Gella A, Quintana A, Bartin B, Esteve-Codina A, Teles M, Roher N. Polystyrene nanoplastics target lysosomes interfering with lipid metabolism through the PPAR system and affecting macrophage functionalization. *Environ Sci: Nano* 2023, 10:2245-2258.

Brandts I, Carcia-Ordoñez M, Tort L, Teles M, Roher N. Polystyrene nanoplastics accumulate in ZFL cell lysosomes and in zebrafish larvae after acute exposure, inducing a synergistic immune response

in vitro without affecting larval survival in vivo. *Enrion Sci: Nano* 2020, 7, 2410.

Bozkurt, Y., Öğretmen, F., & Erçin, U., 2008. Seminal plasma composition and its relationship with physical spermatological parameters of Grass carp (*Ctenopharyngodon idella*) semen: with emphasis on sperm motility. *Aquaculture Research*, 39(15), 1666-1672.

Chackal, R., Eng, T., Rodrigues, E.M., Matthews, S., Pagé-Lariviére, F., Avery-Gomm, S., Xu, E.G., Tufenkji, N., Hemmer, E. and Mennigen, J.A., 2022. Metabolic consequences of developmental exposure to polystyrene nanoplastics, the flame retardant BDE-47 and their combination in zebrafish. *Frontiers in Pharmacology*, 13, p.822111.

Cheng, C.H., Guo, Z.X., Luo, S.W. and Wang, A.L., 2018. Effects of high temperature on biochemical parameters, oxidative stress, DNA damage and apoptosis of pufferfish (*Takifugu obscurus*). *Ecotoxicology and environmental safety*, 150, pp.190-198.

Ciereszko A (2008) Chemical composition of seminal plasma and its physiological relationship with sperm motility, fertilizing capacity, and cryopreservation success in fish. In '*Fish spermatology*'. (Eds SMH Alavi, J Cosson, K Coward, G Rafiee) pp. 215–240. (Alpha Science International: Oxford, UK

Cnaani, A., Tinman, S., Avidar, Y., Ron, M. and Hulata, G., 2004. Comparative study of biochemical parameters in response to stress in *Oreochromis aureus, O. mossambicus* and two strains of *O. niloticus*. *Aquaculture research*, 35(15), pp.1434-1440.

Contino, M., Ferruggia, G., Indelicato, S., Pecoraro, R., Scalisi, E.M., Salvaggio, A. and Brundo, M.V., 2023. Polystyrene nanoplastics in aquatic microenvironments affect sperm metabolism and fertilization of *Mytilus galloprovincialis* (lamark, 1819). *Toxics,* 11(11), p.924.

Cooper GM. The chemistry of cells. In: *The Cell: A molecular approach*. 2nd edition. Sinauer., Sunderland, Massachusetts, p. 47..

Cosson, J., Groison, A.L., Suquet, M., Fauvel, C., Dreanno, C. and Billard, R., 2008. Studying sperm motility in marine fish: an overview on the state of the art. *Journal of Applied Ichthyology*, 24(4), pp.460-486.

Cui, L., Wang, X., Sun, B., Xia, T. and Hu, S., 2019. Predictive metabolomic signatures for safety assessment of metal oxide nanoparticles. *Acs Nano*, 13(11), pp.13065-13082.

Da Costa, B.B., Marques, L.S., Lassen, P.G., Rodrigues, R.B., Da Rosa-Silva, H.T., Moreira, J.C.F., de Oliveira, D.L. and Streit Jr, D.P., 2021. Effect of glutamine and cysteine supplementation on quality of cryopreserved sperm of South American silver catfish. *Aquaculture Research*, 52(5), pp.2173-2181.

Donnelly, E.T., McClure, N. and Lewis, S.E., 2000. Glutathione and hypotaurine in vitro: effects on human sperm motility, DNA integrity and production of reactive oxygen species. *Mutagenesis*, 15(1), pp.61-68.

Dzyuba, V., and Cosson, J., 2014. Motility of fish spermatozoa: from external signaling to flagella response. *Reproductive Biology*, 14(3), 165-175.

Dzyuba V, Cosson J, Dzyuba B, Yamaner G, Rodina M, Linhart O. The antioxidant system of seminal fluid during in vitro storage of sterlet Acipenser ruthenus sperm. *Fish Physiol Biochem* 2016;42:563e8.

Dzyuba, B., Bondarenko, O., Fedorov, P., Gazo, I., Prokopchuk, G., and Cosson, J. 2017. Energetics of fish spermatozoa: the proven and the possible. *Aquaculture*, 472, 60-72.

Evans RW, Setchell BP. Lipid changes during epididymal maturation in ram spermatozoa collected at different times of the year. *J Reprod Fert* 1979, 57:197-203.

Furse S, Kusinski LC, Ray A, Glenn-Sansum C, Williams HEL, Houlman A, Meek CL. Relative abundance of lipid metabolites in spermatozoa across three compartments. *Int J Mol Sci* 2022, 23(19):11655.

Gallego, V. and Asturiano, J.F., 2018. Sperm motility in fish: technical applications and perspectives through CASA-Mot systems. *Reproduction, Fertility and Development,* 30(6), pp.820-832.

Gao, X.Q., Fei, F., Huang, B., Meng, X.S., Zhang, T., Zhao, K.F., Chen, H.B., Xing, R. and Liu, B.L., 2021. Alterations in hematological and

biochemical parameters, oxidative stress, and immune response in *Takifugu rubripes* under acute ammonia exposure. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 243, p.108978.

Gasca-Leyva, E., León, C.J., Hernández, J.M. and Vergara, J.M., 2002. Bioeconomic analysis of production location of sea bream (*Sparus aurata*) cultivation. *Aquaculture*, 213(1-4), pp.219-232.

Giaretta E, Damato A, Zennaro L, Bonfatii V, Mislei B, Vigolo V, Falomo ME, Bertuzzo F, Gabai G, Bucci D. Metabolome and oxidative stress markers in the seminal plasma of Holstein bulls and their relationship with the characteristics of fresh and frozen/thawed sperm. *Theriognelogy* 2025, 235:262-274.

Gupta, C., Kaushik, S., Jain, S., Dhanwani, I., Garg, S., Paul, A.,, Pant, P. and Gupta, N., 2022. Bioaccumulation and toxicity of polystyrene nanoplastics on marine and terrestrial organisms with possible remediation strategies: A review. *Environmental Advance*s, 8, 100227.

He, E., Qiu, R., Cao, X., Song, L., Peijnenburg, W.J. and Qiu, H., 2020. Elucidating toxicodynamic differences at the molecular scale between ZnO nanoparticles and ZnCl2 in *Enchytraeus crypticus* via nontargeted metabolomics. *Environmental science & technology*, 54(6), pp.3487-3498.

Iwamatsu, T., 2000. Fertilization in fishes. In *Fertilization in protozoa and metazoan animals: cellular and molecular aspect*s (pp. 89-145). Berlin, Heidelberg: Springer Berlin Heidelberg.

Kamel, N.A., Bashir, D.W., EL-Leithy, E.M., Tohamy, A.F., Rashad, M.M., Ali, G.E. and El-Saba, A.A.A., 2024. Polyethylene terephthalate nanoplastics caused hepatotoxicity in mice can be prevented by betaine: molecular and immunohistochemical insights. *Journal of Biochemical and Molecular Toxicology*, 38(12), p.e70088.

Knight, L.S., Piibe, Q., Lambie, I., Perkins, C. and Yancey, P.H., 2017. Betaine in the brain: characterization of betaine uptake, its influence on other osmolytes and its potential role in neuroprotection from osmotic stress. *Neurochemical research*, 42(12), pp.3490-3503.

Kowalski, R. K., and Cejko, B. I., 2019. Sperm quality in fish: Determinants and affecting factors. *Theriogenology*, 135, 94-108.

Kowalski, R. K., Cejko, B. I., Krejszeff, S., Sarosiek, B., Judycka, S., Targońska, K., and Glogowski, J., 2014. Effect of albumin and casein supplementation on the common carp *Cyprinus carpio* L. sperm motility parameters measured by CASA. *Aquaculture international*, 22(1), 123-129.

Kumar, A.; Kroetsch, T.; Blondin, P.; Anzar, M. Fertility-Associated metabolites in bull seminal plasma and blood serum: 1H nuclear magnetic resonance analysis. *Mol. Reprod. Dev.* 2015, 82, 123–131.

Lahnsteiner F. A comparative study on the composition and importance of free amino acids in semen of gilthead sea bream, *Sparus aurata*, and perch, *Perca fluvialis. Fish Physiol Biochem* 2010, 36:1297-1305.

Lahnsteiner F. The role of free amino acids in semen of rainbow trout *Oncorhynchus mykiss* and carp *Cyprinus carpio. J Fish Biol* 2009;75:816e33.

Lahnsteiner, F., Mansour, N., McNiven, M. A., and Richardson, G. F., 2009. Fatty acids of rainbow trout (*Oncorhynchus mykiss*) semen: composition and effects on sperm functionality. *Aquaculture*, 298(1-2), 118-124.

Lahnsteiner F, Patzner RA, Weismann T. The testicular main ducts and the spermatic ducts in some cyprinid fishes-II. composition of the seminal fluid. *J Fish Biol* 1994;44:459e67.

Lee, S.E., Yi, Y., Moon, S., Yoon, H. and Park, Y.S., 2022. Impact of micro-and nanoplastics on mitochondria. *Metabolites*, 12(10), p.897.

Lever M, Slow S. The clinical significance of betaine, an osmolyte with a key role in methyl group metabolism. *Clin Biochem* 2010, 43:732-744.

Li, S., Liu, L., Luo, G., Yuan, Y., Hu, D. and Xiao, F., 2023. The crosstalk between M1 macrophage polarization and energy metabolism disorder contributes to polystyrene nanoplastics-triggered testicular inflammation. *Food and Chemical Toxicology*, 180, p.114002.

Liang, B., Huang, Y., Zhong, Y., Li, Z., Ye, R., Wang, B., Zhang, B., Meng, H., Lin, X., Du, J. and Hu, M., 2022. Brain single-nucleus transcriptomics highlights that polystyrene nanoplastics potentially induce Parkinson's disease-like neurodegeneration by causing energy metabolism disorders in mice. *J. hazardous materials*, 430, p.128459.

Lin, S., Zhang, H., Wang, C., Su, X.L., Song, Y., Wu, P., Yang, Z., Wong, M.H., Cai, Z. and Zheng, C., 2022. Metabolomics reveal nanoplastic-induced mitochondrial damage in human liver and lung cells. *Environmental science & technology,* 56(17), pp.12483-12493.

Liu, Y., Hao, F., Liang, H., Liu, W. and Guo, Y., 2025. Exposure to polystyrene nanoplastics impairs sperm metabolism and pre-implantation embryo development in mice. *Frontiers in Cell and Developmental Biology*, 13, p.1562331.

Liu, H., Li, H., Liu, Y., Zhao, H., and Peng, R., 2024. Toxic effects of microplastic and nanoplastic on the reproduction of teleost fish in aquatic environments. *Environmental Science and Pollution Research*, 31(54), 62530-62548.

Mardanyan, S., Karapetyan, L., and Antonyan, A., 2025. Adenosine and Adenosine Deaminase Contrary Manifestations in Immunity. *Scand J Immunol*. 102(1):e70038.

Martínez-Páramo, S., Diogo, P., Dinis, M.T., Soares, F., Sarasquete, C. and Cabrita, E., 2013. Effect of two sulfur-containing amino acids, taurine and hypotaurine in European sea bass (*Dicentrarchus labrax*) sperm cryopreservation. *Cryobiology*, 66(3), pp.333-338.

Mattsson, K., Jocic, S., Doverbratt, I. and Hansson, L.A., 2018. Nanoplastics in the aquatic environment. *Microplastic contamination in aquatic environments*, pp.379-399.

Meijer, P., 2021. (Paleo) oceanography of semi-enclosed seas with a focus on the Mediterranean region; Insights from basic theory. *Earth-Science Reviews*, 221, p.103810.

Menezes EB, Velho ALC, Santos F, Dinh T, Kaya A, Topper E, et al. Uncovering sperm metabolome to discover biomarkers for bull fertility. *BMC Genom* 2019;20: 1–16.

Mita M, Yasumasu I, Nakamura M. Endogenous substrate for energy metabolism and ultrastructural correlates in spermatozoa of the Sea Urchin *Diadema setosum*. *Mol Reprod Develop*, 1995, 40:103-109.

Mita M, Yasumasu I, Nakamura M. Energy metabolism of spermatozoa of the Sand Dollar *Clypeaster japoniucs*: The endogenous substrate and ultrastructural correlates. *J Biochem*, 1994, 116:108-113.

Moon, N., Morgan, C.P., Marx-Rattner, R., Jeng, A., Johnson, R.L., Chikezie, I., Mannella, C., Sammel, M.D., Epperson, C.N. and Bale, T.L., 2024. Stress increases sperm respiration and motility in mice and men. *Nature communications*, 15(1), p.7900.

Oliveira, C.C., Ferrão, L., Gallego, V., Mieiro, C., Oliveira, I.B., Carvalhais, A., Pachedo, M. and Cabrita, E., 2024. Exposure to silver and titanium dioxide nanoparticles at supra-environmental concentrations decreased sperm motility and affected spermatozoa subpopulations in gilthead seabream, *Sparus aurata*. *Fish Physiology and Biochemistry*, 50(5), pp.1959-1970.

Panizzolo, M., Martins, V.H., Ghelli, F., Squillacioti, G., Bellisario, V., Garzaro, G., Bosio, D., Colombi, N., Bono, R. and Bergamaschi, E., 2023. Biomarkers of oxidative stress, inflammation, and genotoxicity to assess exposure to micro-and nanoplastics. A literature review. *Ecotoxicology and Environmental Safety*, 267, p.115645

Peng, L., Fu, D., Qi, H., Lan, C.Q., Yu, H. and Ge, C., 2020. Micro-and nano-plastics in marine environment: Source, distribution and threats—A review. *Science of the total environment,* 698, p.134254.

Perchec, G., Jeulin, C., Cosson, J., Andre, F. and Billard, R., 1995. Relationship between sperm ATP content and motility of carp spermatozoa. *Journal of cell science*, 108(2), pp.747-753.

Płaczkowska, S., Rodak, K., Kmieciak, A., Gilowska, I., and Kratz, E. M., 2024. Exploring correlations: Human seminal plasma and blood serum biochemistry in relation to semen quality. *PLoS One*, 19(6), e0305861.

Pujol, G., Marín-Gual, L., González-Rodelas, L., Álvarez-González, L., Chauvigné, F., Cerdà, J., Teles, M., Roher, N. and Ruiz-Herrera, A.,

2025. Short-term polystyrene nanoplastic exposure alter zebrafish male and female germline and reproductive outcomes, unveiling pollutant-impacted molecular pathways. *Journal of Hazardous Materials*, 481, p.136529.

Qiang, L., Lo, L.S.H., Gao, Y. and Cheng, J., 2020. Parental exposure to polystyrene microplastics at environmentally relevant concentrations has negligible transgenerational effects on zebrafish (*Danio rerio*). *Ecotoxicology and Environmental Safety*, 206, p.111382.

Qiang, L. and Cheng, J., 2021. Exposure to polystyrene microplastics impairs gonads of zebrafish (*Danio rerio*). *Chemosphere*, 263, p.128161.

Rago V, De Rose D, Santoro M, Panza S, Malivindi R, Andò S, D'Agata R, Aquila S. Human sperm express the receptor for glucacon-like Peptide-1 (GLP-1), which affects sperm function and metabolism. *Endocrinol* 2020, 161(4):1-3.

Ribeiro, J.C., Nogueira-Ferreira, R., Amado, F., Alves, M.G., Ferreira, R. and Oliveira, P.F., 2022. Exploring the role of oxidative stress in sperm motility: A proteomic network approach. *Antioxidants & Redox Signaling*, 37(7-9), pp.501-520.

Rurangwa E, Kime DE, Olleveir F, Nash JP, 2004: The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture* 234, 1–28.

Scovazzi, T., 2024. The Regime of Enclosed or Semi-Enclosed Seas with Special Regard for the Mediterranean Sea. *Portuguese Yearbook of the Law of the Sea*, 1(1), pp.154-175.

Schreck, C. B., 2010. Stress and fish reproduction: the roles of allostasis and hormesis. *General and comparative endocrinology*, 165(3), 549-556.

Seify, M., Zarabadipour, M., Ghaleno, L.R., Alizadeh, A. and Valojerdi, M.R., 2019. The anti-oxidant roles of Taurine and Hypotaurine on acrosome integrity, HBA and HSPA2 of the human sperm during vitrification and post warming in two different temperature. *Cryobiology*, 90, pp.89-95.

Sinesi, G., Salvioni, L., Ronchi, E., Barbuzzi, E.M.G., Prosperi, D., Colombo, M. and Giustra, M.D., 2025. Exploring Bovine Serum Albumin (BSA) as a Facile Substrate for Testing of Osmolytes as Cosmetic Ingredients. *Molecules*, 30(3), p.664.

Srivastava, A. S., Oohara, I., Suzuki, T., and Singh, S. N., 1999. Activity and expression of aspartate aminotransferase during the reproductive cycle of a fresh water fish, *Clarias batrachus*. *Fish Physiology and Biochemistry*, 20(3), 243-250.

Starovlah, I.M., Radovic Pletikosic, S.M., Tomanic, T.M., Medar, M.L., Kostic, T.S. and Andric, S.A., 2022. Spermatozoa develop molecular machinery to recover from acute stress. *Frontiers in Endocrinology*, 13, p.896193.

Takei, G.L., Horibata, Y., Toyama, F., Hayashi, K., Morita, A., Ouchi, M. and Fujita, T., 2024. Hamster spermatozoa incorporate hypotaurine via TauT for self-protection. *Reproduction*, 168(2).

Tantikitti C, March BE. Dynamics of plasma free amino acids in rainbow trout (*Oncorhynchus mykiss*) under a variety of dietary conditions. *Fish Physiol Biochem* 1995;14:179e94.

Teng, M., Zhao, X., Wu, F., Wang, C., Wang, C., White, J.C., Zhao, W., Zhou, L., Yan, S. and Tian, S., 2022. Charge-specific adverse effects of polystyrene nanoplastics on zebrafish (*Danio rerio*) development and behavior. *Environment international,* 163, p.107154.

Velho, A.L.C.; Menezes, E.; Dinh, T.; Kaya, A.; Topper, E.; Moura, A.A.; Memili, E. Metabolomic markers of fertility in bull seminal plasma. *PLoS ONE* 2018, 13, e0195279.

Wallimann, T., Tokarska-Schlattner, M., and Schlattner, U. 2011. The creatine kinase system and pleiotropic effects of creatine. *Amino acids*, 40(5), 1271-1296.

Wang, H., Shi, X., Gao, Y., Zhang, X., Zhao, H., Wang, L., Zhang, X. and Chen, R., 2022. Polystyrene nanoplastics induce profound metabolic shift in human cells as revealed by integrated proteomic and metabolomic analysis. *Environment International*, 166, p.107349.

Wang, J., Zhao, X., Wu, F., Niu, L., Tang, Z., Liang, W., Zhao, T., Fang, M., Wang, H. and Wang, X., 2021. Characterization, occurrence, environmental behaviors, and risks of nanoplastics in the aquatic environment: Current status and future perspectives. *Fundamental Research*, 1(3), pp.317-328.

Wang, Y., Fu, X. and Li, H., 2025. Mechanisms of oxidative stress-induced sperm dysfunction. *Frontiers in Endocrinology*, 16, p.1520835.

Wu, X., Liu, C., Yang, S., Shen, N., Wang, Y., Zhu, Y., Guo, Z., Yang, S.Y., Xing, D., Li, H. and Guo, Z., 2021. Glycine-serine-threonine metabolic axis delays intervertebral disc degeneration through antioxidant effects: an imaging and metabonomics study. *Oxidative medicine and cellular longevity*, 2021(1), p.5579736.

Zhao, T., Tan, L., Han, X., Ma, X., Lin, K. and Wang, J., 2023. Energy metabolism response induced by microplastic for marine dinoflagellate *Karenia mikimotoi*. *Science of The Total Environmen*t, 866, p.161267.

Zhou, Y., Zhong, X., Chen, L., Gong, L., Luo, L., He, Q., Zhu, L. and Tian, K., 2023. Gut microbiota combined with metabolome dissects long-term nanoplastics exposure-induced disturbed spermatogenesis. *Ecotoxicology and Environmental Safety*, 267, p.115626.

Zhu, C., Zhou, W., Han, M., Yang, Y., Li, Y., Jiang, Q. and Lv, W., 2023. Dietary polystyrene nanoplastics exposure alter hepatic glycolipid metabolism, triggering inflammatory responses and apoptosis in *Monopterus albus—Science of The Total Environment*, 891, p.164460.

4

The impact of nanoplastics on the quality of fish sperm: a review

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Abstract

Pollution in aquatic ecosystems is intensifying under the combined pressures of climate change and anthropogenic contaminants, with nanoplastics (NPs) emerging as a critical threat to fish reproduction. Although extensive research has demonstrated the physiological impacts of NPs, their direct effects on sperm quality and functionality remain poorly characterized. This review synthesizes evidence from original research articles that specifically examined NPs' impacts on fish sperm quality and related reproductive endpoints. The findings reveal that NPs consistently impair sperm motility, viability, and fertilization capacity, while inducing oxidative stress, DNA damage, mitochondrial dysfunction, and endocrine disruption. Particle size, surface chemistry, and exposure route were identified as key determinants of toxicity, with direct sperm exposure causing immediate impairments and chronic or maternal transfer exposures leading to systemic and transgenerational effects. Notably, several studies reported reduced offspring survival, altered development, and disrupted gene expression, highlighting the intergenerational risks of NPs contamination. Despite these advances, significant knowledge gaps remain, including limited research on marine wild and cultured fish species, the effects of diverse life histories on NPs toxicity, environmentally relevant exposure levels, and the combined effects of NPs with other stressors. Overall, this review underscores that fish sperm are highly sensitive to NPs pollution, with consequences that extend across generations and threaten population stability, calling for urgent mechanistic and ecologically realistic investigations.

1. Introduction

In an ever-changing world of increased plastic contamination, pathogen load, rising temperatures, ocean acidification, and variable marine and atmospheric circulation patterns, the resilience of freshwater and marine species depends critically on the proper activation of the endocrine hypothalamic–pituitary–gonadal axis (HPG). Among the plethora of emergent contaminants described in the last decade, plastic contamination in particular is becoming increasingly severe under changing climatic conditions [1,2]. Extreme weather accelerates the degradation of plastic fragments [3] into microplastics (MPs: ranging from 1 μm to 5 mm) and, ultimately, nanoplastics (NPs: less than 1 μm). Unlike MPs, NPs due to their reduced size can cross cellular barriers and nest in gonadal tissues, interfering with the endocrine, metabolic, and stress-related hormonal axis, and, through the production of reactive oxygen species (ROS) that induces mitochondrial and DNA damage, impairing gametogenesis, fertility rates, inducing embryo mortality or malformations, and altering overall growth [4,5,6]. Environmental stressors such as elevated temperatures or high density that may increase the NPs load, may also exacerbate the activation of the endocrine stress axis and impair gonadogenesis and spermatogenesis in fish, biasing the reproductive phenotype towards masculinization [7]. It should be noted that the upper size limit used to define nanoplastics varies across studies, with some authors considering particles <100 nm and others extending the definition up to 1000 nm, which complicates direct comparisons between studies [8,9].

NPs also interact with co-contaminants, such as metals, polycyclic aromatic hydrocarbons, pharmaceuticals, pesticides, antibiotics, plastic additives, and endocrine disruptors that can further degrade the reproductive physiology, and even the behavioral courting in fish [10], inducing oxidative stress, impairing foraging activity, immunity, and lipid metabolism, and modulating steroidogenesis and thyroid function [11,12]. NPs can also be maternally transferred to embryos as described on zebrafish (*Danio rerio*) and the ovoviviparous species *Poecilia reticulata* [13], but very few studies have addressed the intergenerational effects of NPs exposure in non-model fish species. Moreover, a recent systematic review highlighted that the effects of plastic pollutants in fish have been studied mainly in adults and females, and, to a lesser degree, in males [14].

In male fish the main physiological variables analyzed in stressful, plastic-ridden environments are spermatogenesis, sperm motility, malformation,

and fertility rates, and the histopathology of the testis [15]. Spermatogenesis is a complex process dependent of the species-specific cyclic changes of the HPG axis, and the sequential activation of sex-determining genes [16], or the effect of temperature and pH in species whose sex determination depend on environmental variables acting through epigenetic modification of genes controlling gonadal differentiation and maturation [17]. However, despite increasing concern regarding NPs toxicity, a substantial gap persists in our understanding of its direct impact on fish sperm quality and functionality—a gap this review seeks to address through a critical evaluation of the existing literature.

2. Materials and methods

A comprehensive literature search was conducted to identify peer-reviewed journal articles examining the toxic effects of NPs on fish sperm. Particular emphasis was placed on outcomes related to sperm motility, viability, proteomics, and transcriptomics. Search was performed in three major electronic databases: Google Scholar, Web of Science, and PubMed, and covered studies published up to August 2025 **(Table 1)**.

Table 1. Number of research articles retrieved from PubMed, Google Scholar, and Web of Science using specific search keywords related to the effects of nanoplastics (NPs) on fish sperm and reproduction.

Keywords	PubMed	Google Scholar	Web of Science
NPs effect on fish sperm viability	3	742	4
NPs effect on fish sperm motility	3	832	9
NPs effect on fish sperm fertilization capacity	0	337	0
NPs effect on fish reproduction	12	3840	90
NPs effect on fish sperm metabolism	0	674	0

The following search terms were applied, both individually and in combination:

"NPs effect on fish sperm viability"

"NPs effect on fish sperm motility"

"NPs effect on fish sperm fertilization capacity"

"NPs effect on fish reproduction"

"NPs effect on fish sperm metabolism"

For each database, the number of results generated by these keywords is summarized in **Table 1**.

Following the initial search, a filtering process was applied to refine the literature search, such as duplicate records identified across Google Scholar, Web of Science, and PubMed, which were removed manually. We included only original research articles with experimental data on NPs and fish reproduction **(Table 2)**. Titles and abstracts were first screened to exclude non-fish species, review articles, opinion papers, and studies not addressing reproductive or sperm-related endpoints. Full-text screening was then applied to retain only original experimental studies that specifically assessed sperm quality parameters (e.g., motility, viability, fertilization capacity) or closely related reproductive outcomes directly linked to sperm function. Examples include studies focusing on gonadal development, hormone regulation, bioaccumulation, maternal transfer, immune responses, and co-exposure with other toxicants. After excluding such studies, only those articles specifically targeting sperm quality indicators, reproductive performance, or closely related endpoints were retained **(Table 2)**. No formal scoring system was applied; however, studies were evaluated for experimental clarity, relevance to NPs, and the presence of clearly defined exposure conditions and endpoints. The final dataset was narrowed to nine articles because only these met all inclusion criteria, reflecting the current scarcity of studies directly addressing NPs' effects on fish sperm. The overall literature searches and selection process followed an approach conceptually aligned with PRISMA guidelines, although implemented in a narrative rather than systematic review framework.

Studies from both marine and freshwater systems were considered. Despite the fact that these environments differ substantially in their physicochemical properties, such as salinity, ionic composition, and organic matter content, which in turn can influence NPs aggregation, stability, and bioavailability [32,33], integrating evidence from both systems allowed for a broader and more comprehensive overview of the available data. Further several studies include both microplastics and NPs but, this review focuses specifically on NPs, and results from mixed-size studies are discussed only when NPs-specific effects can be distinguished or when size-dependent differences are explicitly reported. The findings from these studies are synthesized below according to key sperm-related endpoints and biological processes, providing a structured overview of current knowledge and highlighting where gaps remain. While this review focuses on sperm quality and functionality, it is important to distinguish between studies that directly assess sperm-specific endpoints (e.g., motility, viability, fertilization capacity, mitochondrial activity, or DNA integrity) and those that evaluate broader reproductive

Table 2. Initial selection of 14 research articles retrieved from PubMed, Google Scholar, and Web of Science after the first-level filter (species restricted to fish and exclusion of review/opinion articles). Articles that met the final inclusion criteria (n = 9) are highlighted in grey.

Species	Plastic Polymer	Exposure Route	Dose	Effects (Major Findings)	Reference
Zebrafish (*Danio rerio*) (adult males and females).	Polystyrene nanoplastics (PS-NPs), ~45–50 nm diameter	Waterborne exposure (added directly into aquarium water)	An amount of 5 mg/L (5000 µg/L) PS-NPs. Exposure duration: 96 h (acute short-term exposure)	**Males**: Testicular damage, increased spermatocyte apoptosis, nanoplastic uptake by germ cells, strong gene dysregulation affecting meiosis and DNA repair, and reduced sperm motility. **Females**: Delayed oocyte development with minor, non-significant reduction in egg production. **Reproductive capacity**: Impaired fertilization success, shown by delayed hatching, reduced larval survival, and lower heart rate in offspring.	[18]
Marine medaka (*Oryzias melastigma*)	PS-NPs ~ 100 nm square fragments	Dietary (fish fed with feed mixed with SMZ, PS, or both)	A total of 3.45 mg/g dry feed	**SMZ**: ~35% GSI reduction, inhibited Aund self-renewal, and disrupted hormonal and apoptotic regulation. **PS**: No GSI or germ cell changes, but altered spermatogenesis gene expression. **SMZ and PS**: PS partially rescued GSI by promoting spermatogonia differentiation, without restoring Aund self-renewal	[19]
Zebrafish (*Danio rerio*)	PS-NPs, 50 nm	Waterborne exposure	An amount of 1 mg/L (21 days)	**Gonads**: PS-NPs impaired gametogenesis—fewer mature sperm and oocytes, delayed spermatogenesis and oocyte maturation. **Hormones:** Sex-hormone imbalance in both sexes, indicating endocrine disruption. **Genes**: Dysregulated BPG–axis and steroidogenesis genes. Outcome: Long-term PS-NP exposure caused oxidative stress and reproductive dysfunction	[20]
European whitefish (*Coregonus lavaretus*)	PS (specifically, 50 nm spherical PS-NPs coated with carboxyl groups, red fluorescent, Ex: 552 nm, Em: 580 nm)	Sperm from males was directly exposed to NPs in activation water for 10 s before being used to fertilize eggs	Low dose: 100 particles per spermatozoa High dose: 10,000 particles per spermatozoa	**Sperm**: High dose reduced motile sperm (more static cells) and altered swimming pattern; low dose had no effect. **Embryos**: No change in mortality; high dose accelerated hatching. **Offspring**: High dose reduced body mass and swimming performance, with no effect on length.	[21]
Zebrafish (*Danio rerio*)	PS (specifically, 100 nm spherical PS-NPs with green fluorescence, Ex: 488 nm, Em: 518 nm)	Chronic waterborne exposure of female adults (F0) for 42 days; offspring (F1) raised in clean water but exposed via maternal transfer (PS-NPs detected in F1 embryos and larvae)	Low dose: 200 µg/L High dose: 2000 µg/L	**Bioaccumulation**: Dose-dependent buildup with maternal transfer to embryos and larvae. **F0 reproduction**: High dose reduced egg production and ovary maturity, with E2↓, T↑, and disrupted HPG–axis genes. **F1 effects:** Faster hatching, higher larval heart rate, altered neuroendocrine and sex-differentiation gene expression, but no change in adult sex ratio or ovary structure.	[22]

Table 2. *Cont.*

Species	Plastic Polymer	Exposure Route	Dose	Effects (Major Findings)	Reference
Male zebrafish (*Danio rerio*)	PS; microplastics [PSMPs] of 5 μm diameter; NPs [PSNPs] of 80 nm diameter)	Waterborne (chronic exposure in aerated glass tanks with renewal of exposure solutions every 3 days); also in vitro exposure on mouse GC-2 spermatocytes	PSMPs/PSNPs: 1 mg/L Microcystin-LR (MCLR): 0, 5, 25 μg/L Combinations: MCLR (5 or 25 μg/L) and PSMPs (1 mg/L) or PSNPs (1 mg/L) (Exposure duration: 45 days for in vivo; various for in vitro)	**Synergy**: PS (especially PSNPs) increased MCLR bioavailability, causing stronger combined testicular toxicity than single exposures. **Mechanisms**: Co-exposure amplified oxidative stress, DNA damage, mitochondrial dysfunction, and cell-cycle arrest in spermatogenic cells. **Reproduction**: Reduced GSI, severe testicular damage, and high risk of impaired spermatogenesis and fertility.	[23]
Mediterranean mussel (*Mytilus galloprovincialis*)	PS (specifically, 50 nm amino-modified PS-NPs [nPS-NH_2]; 100 nm NPs also tested but showed no significant effects)	Waterborne exposure of sperm in controlled aquatic microenvironments (in vitro; sperm incubated with NPs for 30 min prior to fertilization)	Low: 0.1 mg/L Medium: 1 mg/L High: 10 mg/L (100 nm NPs tested at same concentrations but showed no significant effects)	**Size-dependent toxicity**: 50 nm nPS-NH_2 (not 100 nm) induced ROS, mitochondrial damage, and ATP loss in sperm. **Sperm quality:** High doses reduced motility and caused membrane and DNA damage. **Reproduction**: Fertilization success markedly decreased at ≥1 mg/L.	[24]
Zebrafish (*Danio rerio*)	PS-NPs with diameters of 80 nm, 200 nm, and 500 nm)	Waterborne exposure (chronic exposure in aquariums with water renewal every 2 days)	Single concentration: 0.5 mg/L (Exposures tested for each size: 80 nm, 200 nm, 500 nm; duration: 21 days for F0 adults, with F1 embryo assessment)	**F0 reproduction:** Delayed spermatogenesis, abnormal follicle growth, reduced GSI, strongest effects with 500 nm NPs. **F1 offspring**: Impaired development—higher mortality, delayed hatching (worst with 500 nm). **Mechanisms:** Disrupted PPAR signaling and lipid metabolism in ovaries, resembling PCOS. Size effect: 500 nm NPs caused greatest bioaccumulation and reproductive toxicity.	[25]
Pacific oyster (*Crassostrea gigas*)	Polystyrene (specifically, 50 nm polystyrene beads with amine [50-NH_2] or carboxyl [50-COOH] functional groups)	Waterborne exposure (in vitro; oyster spermatozoa incubated with NPs in filtered seawater for 1 h)	Range: 0.1 to 25 mg/mL Key tested concentrations: 0.1, 1, 10, and 25 mg/mL (for both 50-NH_2 and 50-COOH beads)	**Sperm motility**: 50-NH_2 (10 mg/mL) and 50-COOH (25 mg/mL) strongly reduced motile sperm and velocity; lower doses had no effect. **Reproduction**: 50-NH_2 decreased embryogenesis by 59%; 50-COOH had no significant effect. **Mechanism**: 50-NH_2 caused membrane-mediated spermiotoxicity; 50-COOH effects were due to aggregation/physical blockage.	[26]
Oriental river prawn (*Macrobrachium nipponense*)	Polystyrene (PS; specifically, polystyrene microplastics [PS-MPs] with a size of 5 μm)	Waterborne (acute exposure for larvae; chronic exposure for adults via immersion in contaminated water)	Acute exposure (larvae): 0, 2, 20 mg/L (24-h exposure) Chronic exposure (adults): 0, 2, 20 mg/L (4-week exposure)	**Larvae (acute):** Reduced survival and heart rate; no direct reproductive effects. **Adult males (chronic):** Impaired testicular development, oxidative stress, apoptosis, and disrupted steroid hormones. **Reproduction**: Lower hatching success, higher F1 malformations. **F1 offspring:** Reduced survival and immune function; bioaccumulation of PS-MPs observed.	[27]
Zebrafish (*Danio rerio*)	Polystyrene (PS; microplastics [MPS] of 5.8 μm diameter; NPs [NPS] of 46 nm diameter)	Waterborne exposure (chronic exposure via immersion in contaminated water)	MPS and NPS: 0, 20 μg/L Triphenyl phosphate (TPhP): 0, 200 μg/L Combinations: TPhP (200 μg/L) + MPS (20 μg/L) or NPS (20 μg/L) (Duration: 21 days)	**Acute toxicity:** MPS/NPS did not alter TPhP LC50. **Reproduction:** TPhP enlarged liver/gonads; NPS worsened effects, MPS minimal impact. **Histology:** Co-exposure impaired spermatogenesis and oogenesis, more severe with NPS. **Hormones:** NPS altered E2/T and Vtg levels in a sex-dependent manner. **Outcome:** TPhP and PS reduced egg production, fertilization, and hatchability; NPS amplified reproductive toxicity most.	[28]

Table 2. *Cont.*

Species	Plastic Polymer	Exposure Route	Dose	Effects (Major Findings)	Reference
Zebrafish (*Danio rerio*)	Polystyrene (specifically, polystyrene microplastics with a size of 5 µm)	Waterborne exposure (continuous immersion in contaminated water)	Low: 10 mg/L Medium: 100 mg/L High: 1000 mg/L (Duration: 21 days)	**Oxidative stress**: ROS increased in gonads and liver at ≥100 mg/L; no effect at 10 mg/L. **Apoptosis**: Elevated in testes at 1000 mg/L via p53 pathway; lower doses unaffected. **Histology**: Testicular basement membrane thinning at 1000 mg/L; no changes at lower doses.	[29]
Japanese medaka (*Oryzias latipes*)	Polystyrene (specifically, polystyrene NPs [NPs] with a diameter of 100 nm)	Waterborne exposure (chronic exposure via immersion in contaminated water)	Low: 10 particles/L Medium: 10^4 particles/L (10,000 particles/L) High: 10^6 particles/L (1,000,000 particles/L) (Duration: 3 months)	**Immune & oxidative stress:** Reduced LZM, SOD, CAT, GPx, and increased MDA in gonads at $\geq 10^4$ particles/L. **Gonads**: High dose (10^6 particles/L) inhibited spermatogenesis and oogenesis; lower doses had no effect. **Survival**: Three-month exposure decreased survival, worst at 10^6 particles/L.	[30]
Zebrafish (*Danio rerio*)	Polystyrene (specifically, polystyrene NPs [NPs] with a diameter of 50 nm)	Waterborne exposure (chronic exposure via immersion in contaminated water)	Polystyrene NPs (NPs): 0, 10 µg/L Triclosan (TCS): 0, 0.3 µg/L Combinations: TCS (0.3 µg/L) + NPs (10 µg/L) (Duration: 21 days, with F1 generation assessment)	**GSI**: TCS reduced GSI; NPs alone had no effect; co-exposure further decreased GSI, especially in males. **Histology**: TCS impaired spermatogenesis and oogenesis; NPs worsened testicular and ovarian damage. **Hormones**: TCS disrupted E2/T balance; NPs amplified male E2 increase and female T elevation. **Genes**: Co-exposure dysregulated sex hormone and vitellogenin genes in both sexes. **Reproduction (F1):** Lower fertilization, hatching, and larval survival, strongest from male exposure.	[31]

or gonadal-level responses, such as testicular histopathology, endocrine disruption, or whole-gonad transcriptomics. The latter provide valuable mechanistic and physiological context but represent indirect proxies of sperm function. Consequently, extrapolations from gonadal alterations to sperm quality should be interpreted with caution, particularly when direct sperm measurements are not available.

3. Effects of NPs on the quality of sperm in fish

Fish sperm, released into the surrounding water during external fertilization, are immediately exposed to contaminants, including MPs and NPs [34]. Due to small size, limited cytoplasmic content, and high membrane fluidity, sperm are particularly fragile and highly sensitive to environmental stressors [35,36]. The reviewed studies encompass both single-stressor exposures to NPs and multi-stressor scenarios in which NPs co-occur with other chemical contaminants. These two experimental approaches provide complementary insights, allowing NPs to be evaluated either as primary toxicants or as vectors and modulators of co-contaminant toxicity. Distinguishing between these roles is essential for interpreting reproductive outcomes and for understanding the ecological relevance of NPs' exposure in complex environmental settings. In single-stressor studies, NPs alone consistently impaired sperm-related and reproductive endpoints, including spermatogenesis, sperm motility, fertilization success, and offspring development, supporting their role as primary reproductive toxicants [18,20,21]. In contrast, multi-stressor studies demonstrated that NPs can exacerbate or modify the toxicity of co-occurring contaminants, such as microcystin-LR, triclosan, or sulfamethazine, often amplifying oxidative stress, endocrine disruption, and germ cell damage [23,28,31]. These findings indicate that NPs may function not only as toxicants per se, but also as vectors or stress amplifiers in chemically complex environments.

NPs, with their reactive surfaces and ability to cross biological barriers, can adhere to or penetrate sperm cells, inducing oxidative stress [37], particularly those regulated by the nuclear factor erythroid 2–related factor 2 (Nrf2), which mediates antioxidant responses to elevated reactive oxygen species (ROS). Studies show that NPs induce oxidative stress in gonadal tissues, leading to decreased antioxidant enzyme activities and impaired spermatogenesis **(Figure 1)**, as observed in medaka fish exposed to polystyrene NPs [30]. Excessive or prolonged oxidative stress can

overwhelm Nrf2 defenses, causing mitochondrial dysfunction, disrupted energy metabolism, DNA damage, and reduced sperm motility and viability [38,39]. Proteomic and transcriptomic analyses reveal that NPs exposure dysregulates pathways related to protein transport, RNA splicing, and mTOR signaling, further impairing sperm function [40]. The limited antioxidant capacity of sperm makes them particularly vulnerable to oxidative damage, which can compromise fertilization and early embryo development [39,41]. Overall, these findings highlight oxidative stress and Nrf2 pathway dysregulation as central mechanisms of NPs-induced reproductive toxicity in fish sperm **(Figure 1)**, emphasizing the need for further research on protective strategies [42].

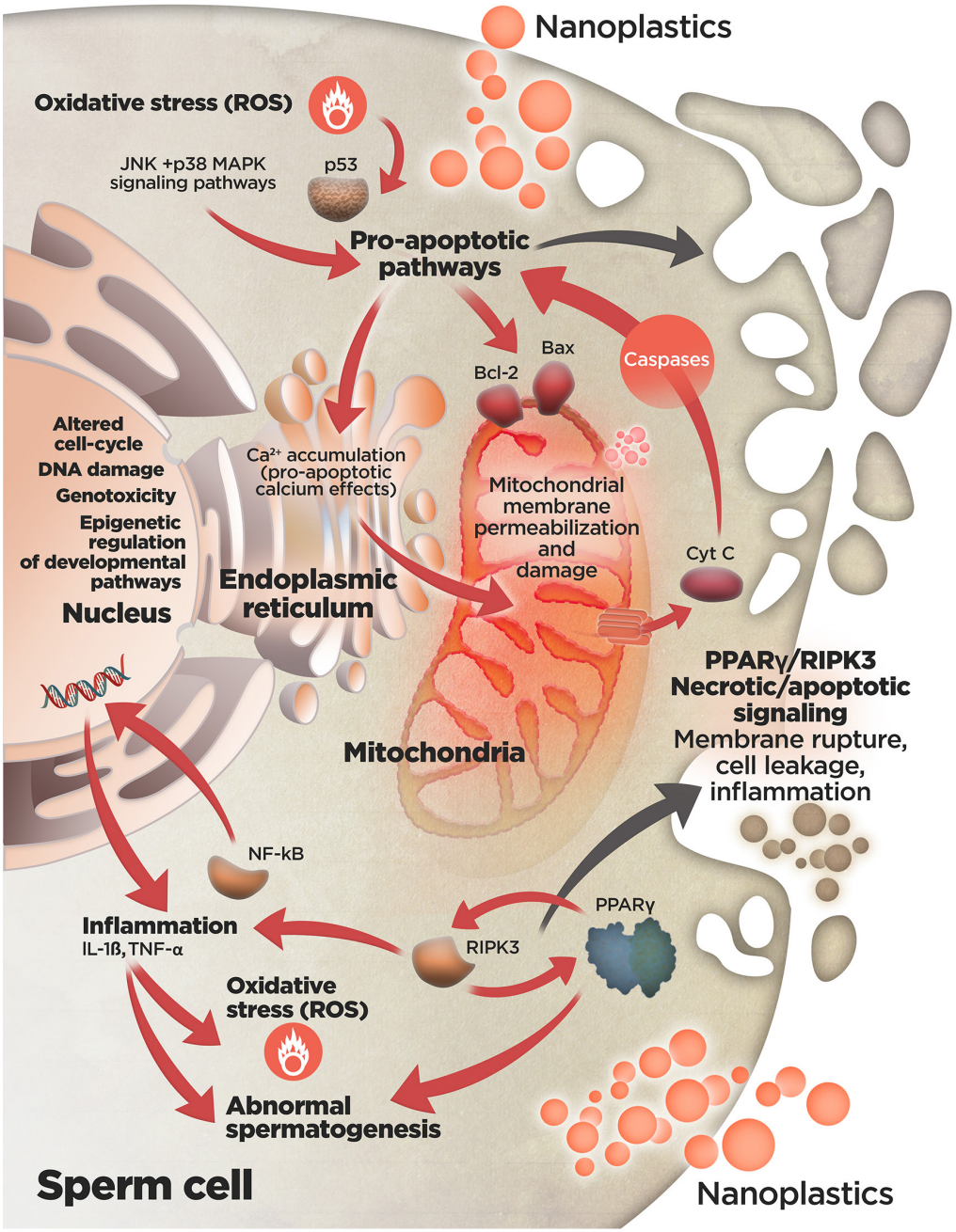
Such impairments drastically lower fertilization success, and prolonged or chronic exposure may ultimately lead to widespread reproductive failure, threatening the survival and stability of fish populations [43]. The underlying mechanisms by which NPs impair sperm function were explored in several of the included studies, revealing a combination of oxidative stress, hormonal imbalance, and transcriptional reprogramming. In zebrafish, transcriptomic analysis following NPs exposure identified the differential expression of thousands of genes involved in meiosis, DNA repair, and chromatin remodeling [18]. These large-scale molecular changes suggest that NPs interfere with the genetic and epigenetic regulation of spermatogenesis, potentially leading to defective sperm formation. Similarly, Zheng et al. [25] reported alterations in lipid metabolism pathways and abnormal folliculogenesis in zebrafish exposed to size-dependent NPs, linking metabolic disruption to impaired gamete production. Endocrine disruption was another consistent observation.

Sun et al. [20] showed that chronic exposure to NPs altered circulating levels of estradiol, testosterone, and vitellogenin in zebrafish, indicating hypothalamic–pituitary–gonadal (HPG) axis disruption. Zhang et al. [22] confirmed these findings, demonstrating that NPs transferred maternally to offspring caused hormone imbalances and disrupted sex differentiation. At the cellular level, oxidative stress and apoptosis were recurring mechanisms. For instance, Contino et al. [24] documented increased ROS production and mitochondrial dysfunction in mussel sperm, while Sun et al. [20] observed oxidative damage in zebrafish gonads. These molecular and cellular disruptions collectively explain the observed reductions in motility, viability, and reproductive success. Comparing across species highlights both shared vulnerabilities and species-specific differences in responses to NPs exposure. Freshwater fish models, particularly zebrafish, dominate

the literature and consistently show systemic reproductive impairments, including reduced sperm motility, altered gonadal histology, endocrine disruption, and multigenerational effects [18,20,22]. In contrast, European whitefish demonstrated subtler immediate effects on fertilization capacity but significant impacts on offspring development [21]. Marine invertebrates such as mussels and oysters also exhibited acute spermiotoxicity upon direct exposure to functionalized NPs, with effects evident within hours [24,26].



Figure 1. Putative mechanisms of cellular stress due to nanoplastics (NPs) exposure in fish sperm cells. NPs may cross the blood–testis barrier, triggering oxidative stress, inflammation, and the activation of intracellular necrotic and apoptotic pathways, ultimately impairing spermatogenesis. In fish, research on the cytotoxic effects of NPs in sperm cells remains limited and is generally restricted to a few laboratory model species (see **Table 1**). Furthermore, although canonical stress-related signaling pathways are broadly conserved across vertebrates, key components and their effectiveness may vary among fish species. From the studies in fish and mammalian model species, the minimum common set of molecular alterations in fish sperm cells in response to internalized NPs, as illustrated in the figure, includes: (i) the generation of reactive oxygen species (ROS) and pro-inflammatory mediators; (ii) the activation of PPARγ/RIPK3 signaling pathways involved in necrotic and apoptotic signaling; and (iii) the participation of p53, and JNK/p38 MAPK signaling in pro-apoptotic pathways. Upon NPs exposure, nuclear factor-κB (NF-κB) enhances the synthesis of pro-inflammatory cytokines (such as interleukin 1, IL-1γ; and tumor necrosis factor alpha, TNFα) which may induce oxidative stress, DNA damage (with concomitant p53 activation), and malformations in mature sperm cells. ROS production further upregulates p53 expression, which in turn activates genes encoding cell cycle arrest factors and mediates Bax/Bcl-2-dependent permeabilization of the mitochondrial outer membrane, leading to mitochondrial damage and cytochrome c (Cyt C) release to initiate caspase-dependent apoptotic signaling. Additionally, p53 activation facilitates Ca2+ accumulation in the endoplasmic reticulum, augmenting the pro-apoptotic calcium effects in the mitochondria. JNK/p38 MAPK signaling pathways, primarily activated by environmental stressors and oxidative stress, regulate cellular responses to inflammatory factors, and may also induce the apoptosis machinery by modulating the expression of other apoptotic regulators, including p53, Bcl-2, and caspases. NPs exposure usually alters the metabolism of lipids and steroidogenic hormones mediated by the activation of the peroxisome proliferator-activated receptor (PPAR) signaling pathway, potentially inducing dysgenesis during sperm-cell development. The reciprocal regulation between receptor-interacting protein kinase 3 (RIPK3) and PPARγ influences the onset of programmed necrotic or apoptotic signaling cascades, contributing to inflammatory processes. Combined with endocrine disruptors and other contaminants, the exposure to NPs may also alter the epigenome of germ cells, impairing the developmental pathways.


Nanoplastics
Oxidative stress (ROS)
JNK +p38 MAPK signaling pathways
p53
Pro-apoptotic pathways
Bax
Bcl-2
Caspases
Altered cell-cycle
DNA damage
Genotoxicity
Epigenetic regulation of developmental pathways
Nucleus
Ca2+ accumulation (pro-apoptotic calcium effects)
Endoplasmic reticulum
Mitochondrial membrane permeabilization and damage
Cyt C
PPARγ/RIPK3 Necrotic/apoptotic signaling
Membrane rupture, cell leakage, inflammation
Mitochondria
NF-kB
PPARγ
Inflammation
IL-1ß, TNF-α
RIPK3
Oxidative stress (ROS)
Abnormal spermatogenesis
Sperm cell
Nanoplastics

These results highlight the importance of the exposure route: direct sperm exposure tends to reveal immediate impairments in motility and fertilization, whereas chronic waterborne or maternal transfer exposures reveal systemic and generational consequences. The consistent finding across taxa is that NPs impair reproductive success, but the severity and manifestation of effects differ depending on species, NPs properties, and experimental context.

3.1. Sperm motility

Sperm motility was one of the most consistently assessed parameters across the included studies and appears to be a sensitive indicator of NPs toxicity. In European whitefish (*Coregonus lavaretus*), direct exposure of sperm to carboxyl-coated PS-NPs (50 nm) caused clear motility impairments in a concentration-dependent manner [21]. At higher particle concentrations, the proportion of motile sperm decreased significantly, and swimming trajectories were disrupted, suggesting that NPs' interference with sperm surface interactions or flagellar function directly compromises motility. Interestingly, although fertilization rates were not drastically reduced, the offspring derived from exposed sperm exhibited reduced body mass and impaired swimming ability, indicating that sub-lethal effects on sperm may carry consequences into early life stages.

Similar motility impairments were observed in marine invertebrate species, such as mussels and oysters, when sperm were exposed directly to NPs. In *Mytilus galloprovincialis*, amino-modified 50 nm PS particles significantly reduced sperm motility, increased ROS production, and lowered fertilization rates [24]. Likewise, in *Crassostrea gigas*, exposure to amine- and carboxyl-functionalized PS-NPs impaired sperm motility and embryogenesis, highlighting that particle surface chemistry is a critical determinant of toxicity [26]. Similarly, in zebrafish both acute [18] and chronic [20] exposures reported motility reductions associated with altered spermatogenesis and gonadal histology. Collectively, these studies provide strong evidence that motility reduction is a primary and early endpoint of NPs toxicity in aquatic organisms, although the severity of impairment varies by species, particle size, functionalization, and exposure conditions. However, the underlying mechanisms of toxicity, the effects of dose–response relationships in normal and abnormal physiology of spermatogenesis, and the potential long-term or intergenerational consequences for fish reproduction and population dynamics remain unassessed.

3.2. Effects on sperm viability and fertilization capacity

Several studies have focused on the effect of NPs on sperm viability and the ability of gametes to achieve successful fertilization in aquatic species. Yaripour et al. [21] demonstrated that while fertilization rates were not significantly impaired, the resulting offspring showed reduced body mass and lower swimming performance in European whitefish (*Coregonus lavaretus*). This indicates that even when sperm retain fertilization capacity, NPs may cause subtle cellular or molecular alterations that carry forward into offspring development. In zebrafish, both multigenerational [22] and acute exposure studies such as Pujol et al. [18] reported reduced egg production, delayed hatching, and decreased larval survival, all of which can be linked to compromised sperm or oocyte quality. These results contrast with the effect of NPs in invertebrates such as mussels and oysters, where NPs exposure led to marked decreases in fertilization rates. For example, M. galloprovincialis sperm exposed to increasing concentrations of amino-modified NPs showed not only decreased motility but also reduced viability and mitochondrial function, leading to compromised fertilization success [24]. Similarly, *C. gigas* sperm exposed to functionalized NPs exhibited dose-dependent decreases in fertilization and abnormal embryogenesis [26]. Taken together, these findings suggest that NPs affect not only the immediate functionality of sperm but also the broader reproductive outcome, including embryo quality and early survival. However, results differ by species and exposure method, indicating a need for standardized testing across taxa.

Rising water temperatures linked to global climate change can modify the toxicity of NPs in aquatic organisms [44]. Although direct experimental evidence remains limited, rising water temperatures associated with global climate change may act as an important modifying factor of NPs toxicity in fish reproduction. However, it has been documented that elevated temperatures can increase metabolic rate, membrane fluidity, and mitochondrial activity in ectothermic organisms, potentially intensifying reactive oxygen species production and reducing cellular stress tolerance [45,46,47]. In sperm cells, which rely on tightly regulated energy metabolism and possess limited antioxidant defenses, thermal stress may therefore exacerbate NPs-induced oxidative damage and functional impairment [48]. These considerations suggest that temperature could influence the magnitude of NPS effects on sperm quality, even if it has not yet been systematically evaluated.

3.3. Transgenerational and developmental effects

Evidence for transgenerational effects of NPs in fish is currently limited to a small number of studies and is primarily restricted to parental (F0) exposure with measurable effects in the first filial generation (F1). While these studies provide clear evidence of multigenerational responses, NPs-induced reproductive toxicity has been demonstrated to extend beyond the directly exposed generation, raising hypotheses regarding possible population-level impacts. Zhang et al. [22] reported maternal transfer of NPs in zebrafish, with F1 offspring displaying disrupted gene expression in sex differentiation pathways, altered hormone levels, and impaired gonadal development. This study demonstrated that NPs can accumulate in parental tissues and be transferred to gametes and embryos, resulting in persistent developmental and reproductive alterations in offspring. Similarly, Pujol et al. [18] showed that NPs-exposed zebrafish parents produced larvae with delayed hatching, reduced survival, and abnormal cardiac function. Zheng et al. [25] also observed impaired gametogenesis and disrupted lipid metabolism that persisted in the next generation, further supporting the occurrence of multigenerational effects. Collectively, these findings indicate that NPs exposure is not limited to acute, individual-level toxicity; however, extrapolation to long-term population-level consequences remains speculative and requires further multi-generational and ecologically relevant studies.

4. Knowledge gaps

Despite growing evidence, substantial gaps remain in our understanding of NPs' effects on fish-sperm quality and reproduction **(Figure 2)**. As in other studies of ecotoxicology in fish, there is a clear bias toward freshwater model species such as zebrafish, with marine fish species largely underrepresented despite their ecological and commercial importance. Few studies have specifically examined the effect of plastic pollution on spermatogenesis through the lens of proteomic and transcriptomic analysis in fish, and most investigations focus on whole gonadal tissue, which makes it difficult to isolate sperm-specific pathways of toxicity. Notably, the majority of studies did not directly expose sperm to NPs under conditions simulating the aquatic environment, even though sperm in natural waters are highly sensitive and fragile. Direct exposure of sperm to NPs may be critical because gametes in aquatic environments are often the first point of contact for pollutants and may respond differently than intact gonadal tissue or systemic exposure.

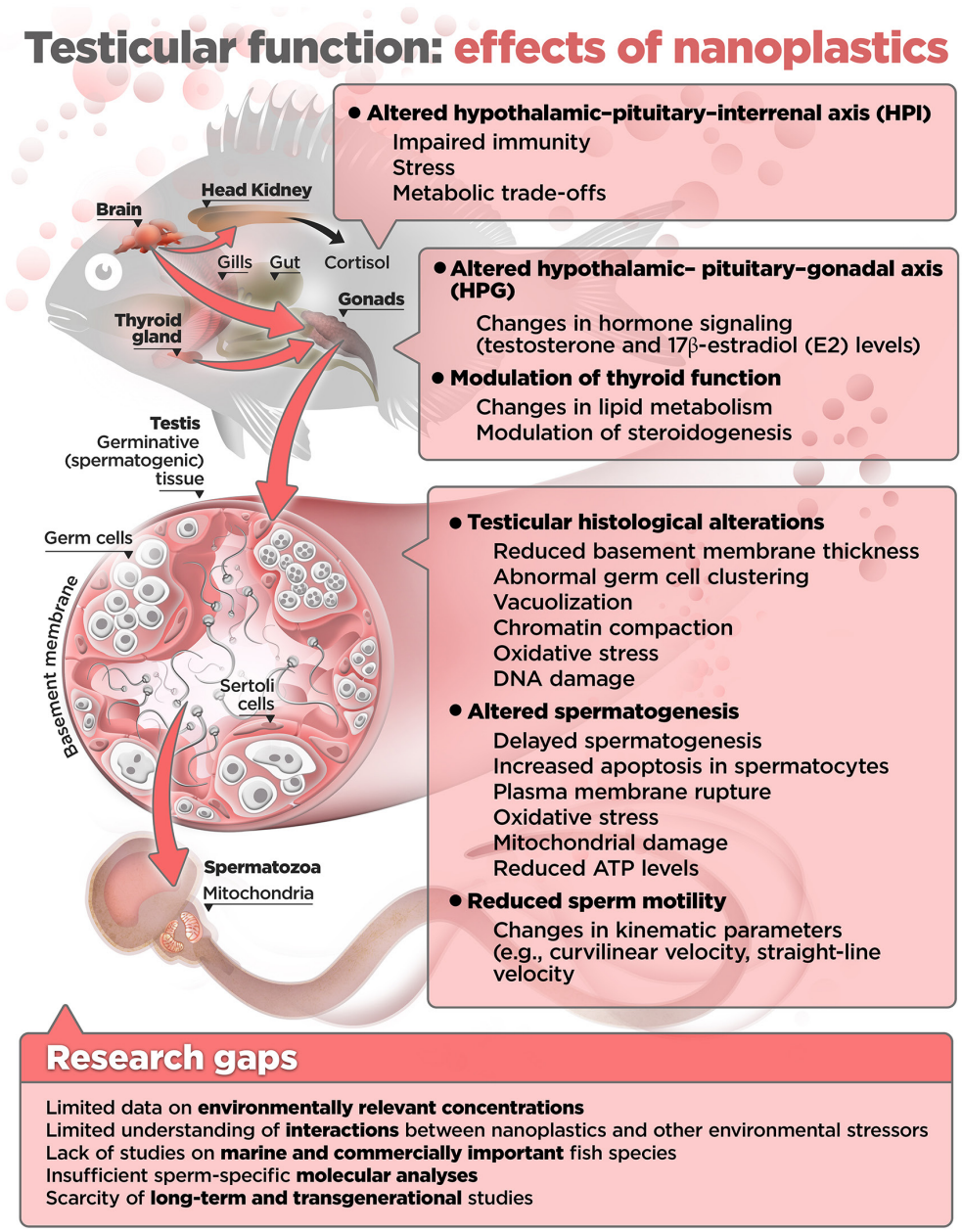


Figure 2. Summary of the effects of nanoplastics in male fish. Nanoplastics (NPs) dissolved in aquatic environments or ingested through dietary intake primarily enter fish via the gills, skin, and gastrointestinal tract, accumulating in tissues and organs. Within the testes, the small size of NPs allows them not only to be present in the seminal fluid and the testicular structures, but also to cross the cell membranes of germ cells, thereby affecting spermatogenesis. Although the effects of NPs on testicular function are species-specific, and dependent on the species analyzed, as well as on NPs size, concentration, and adherence to other compounds such as heavy metals or xenobiotics, several disruptions of reproductive homeostasis in male fish have been reported, as summarized in the figure.

A major limitation across the available literature is the frequent use of high or acute NPs exposure concentrations, often in the mg/L range, which may exceed currently reported environmental levels. Field measurements generally indicate that NPs occur at much lower concentrations, typically in the ng/L to μg/L range in surface waters [49,50], although substantial uncertainty remains due to analytical limitations and the lack of standardized detection methods [51,52]. Consequently, many experimental exposure scenarios likely represent worst-case conditions rather than average environmental exposure. Another limitation of the current literature is the reliance on gonadal, hormonal, or transcriptomic endpoints in the absence of direct sperm-specific measurements, limiting conclusions on functional sperm quality and fertilization potential. Further, relatively few studies directly compare single- and multi-stressor exposure scenarios using standardized sperm-specific endpoints, limiting the ability to disentangle whether NPs act predominantly as primary toxicants or as modulators of co-contaminant effects under environmentally realistic conditions. Further from the discussion, it can be said that temperature may modulate NPs toxicity, and empirical studies explicitly examining interactions between NPs and thermal stress on sperm quality are currently lacking. Future research should prioritize multi-stressor experiments incorporating environmentally relevant temperature scenarios to better reflect climate-driven changes in aquatic systems.

Fish reproductive sensitivity to NPs is influenced by diverse life-history traits, but this area remains underexplored. Species with external fertilization and broadcast spawning release sperm directly into water, exposing sperm immediately to NPs during activation and motility, increasing vulnerability to NPs toxicity [15,53]. In contrast, species with internal fertilization, nest-guarding, or benthic spawning may have reduced or altered exposure routes and durations, potentially lowering direct sperm–NP interactions [54]. Additionally, interspecific differences in sperm longevity from seconds to minutes or longer affect the window of susceptibility to NPs, especially during the critical fertilization period [19,53]. NPs can accumulate in gonadal tissues, disrupt the hypothalamic–pituitary–gonadal axis, induce oxidative stress, and impair spermatogenesis and oogenesis, leading to reduced fecundity and abnormal offspring across fish species [15,25,53]. These findings highlight the need for targeted research on species-specific reproductive vulnerabilities to NPs, considering reproductive strategies, sperm traits, and spawning environments to better assess ecological risks and inform aquaculture management [53]. Finally, standardized protocols

for assessing sperm endpoints, including motility, viability, fertilization success, and molecular markers are needed to enable cross-study comparisons and meta-analyses.

5. Conclusions

The evidence synthesized from the selected studies demonstrates that NPs pose a significant threat to fish reproductive health, with sperm quality emerging as a particularly sensitive target. Across multiple species, NPs exposure consistently impaired sperm motility, viability, and fertilization capacity, while also inducing molecular and cellular alterations, including oxidative stress, apoptosis, and endocrine disruption. These effects were observed not only in freshwater fish species but also in marine invertebrates such as mussels and oysters, highlighting the broad ecological relevance of NPs toxicity. Importantly, several studies revealed transgenerational consequences, showing that NPs exposure in parental generations can disrupt offspring development, hormone regulation, and gametogenesis. This underscores the long-term population-level implications of NPs pollution in aquatic environments.

The findings summarized in this review also have important implications for aquaculture and hatchery-based reproduction. In cultured species, sperm quality is a key determinant of fertilization success, larval performance, and stock sustainability. Evidence that NPs impair sperm motility, viability, and fertilization capacity suggests that male broodstock exposed to NPs either through water, feed, or contaminated rearing systems may experience reduced reproductive efficiency. Moreover, studies involving direct sperm exposure indicate that NPs present in activation water or hatchery environments could directly compromise assisted reproduction procedures, including in vitro fertilization and sperm handling protocols. These observations highlight the need for monitoring and mitigating NPs contamination in aquaculture systems, as well as for incorporating male reproductive endpoints into broodstock health assessments and risk evaluation frameworks. Despite these findings, critical knowledge gaps remain. Most notably, very few studies directly exposed sperm to NPs under conditions reflecting the aquatic environment, even though sperm are highly fragile and may respond differently than whole gonadal tissue or systemic exposures. Additionally, there is limited information on marine fish species, environmentally relevant NPs concentrations, and the combined effects of multiple stressors. Therefore, it is recommended that further

studies are conducted to investigate direct sperm exposure, mechanistic pathways, and multi-generational effects in ecologically realistic scenarios. Addressing these gaps is essential for accurately assessing the risks of NPs to fish populations and for developing strategies to mitigate their impact on aquatic ecosystems.

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References

1. Wilkinson, J.; Hooda, P.S.; Barker, J.; Barton, S.; Swinden, J. Occurrence, fate and transformation of emerging contaminants in water: An overarching review of the field. *Environ. Pollut.* 2017, 231, 954–970.

2. Zhu, X.; Yang, F.; Pang, Q.; Peng, F.; Xu, B.; Wang, L.; Xie, L.; Zhang, W.; Tian, L.; Hou, J.; et al. Fluvial dissolved organic matter quality modulates microbial nitrate transformation: Enhanced denitrification under low carbon-to-nitrate ratio. *Environ. Sci. Technol.* 2025, 59, 23456–23465.

3. Noyes, P.D.; Miranda, D.; Oliveira de Carvalho, G.; Perfetti-Bolaño, A.; Guida, Y.; Barbosa Machado Torres, F.; Barra, R.O. Climate change drives persistent organic pollutant dynamics in marine environments. *Commun. Earth Environ*. 2025, 6, 363.

4. Thushari, G.G.N.; Senevirathna, J.D.M. Plastic pollution in the marine environment. *Heliyon* 2020, 6, e04709.

5. Tekman, M.B.; Walther, B.; Peter, C.; Gutow, L.; Bergmann, M. *Impacts of Plastic Pollution in the Oceans on Marine Species, Biodiversity and Ecosystems*; WWW Germany: Berlin, Germany, 2022.

6. Gutierrez, M.F.; Andrade, V.S.; Ale, A.; Monserrat, J.M.; Roa-Fuentes, C.A.; Herrera-Martínez, Y.; Wiegand, C. Responses of freshwater organisms to multiple stressors in a climate change scenario: A review on small-scale experiments. *Environ. Sci. Pollut. Res.* 2025, 32, 4431–4444.

7. Wang, H.P.; Piferrer, F.; Chen, S.; Shen, Z.G. (Eds.) *Sex Control in Aquaculture*; John Wiley & Sons: Chichester, UK, 2018.

8. Gigault, J.; Ter Halle, A.; Baudrimont, M.; Pascal, P.Y.; Gauffre, F.; Phi, T.L.; El Hadri, H.; Grassl, B.; Reynaud, S. Current opinion: What is a nanoplastic? *Environ. Pollut.* 2018, 235, 1030–1034.

9. Jakubowicz, I.; Enebro, J.; Yarahmadi, N. Challenges in the search for nanoplastics in the environment—A critical review from the polymer science perspective. *Polym. Test.* 2021, 93, 106953.

10. Wu, B.; Yu, H.; Yi, J.; Lei, P.; He, J.; Ruan, J.; Zhang, X. Behavioral studies of zebrafish reveal a new perspective on the reproductive toxicity of micro-and nanoplastics. *Toxics* 2024, 12, 178.

11. Dasmahapatra, A.K.; Chatterjee, J.; Tchounwou, P.B. A systematic review of the effects of nanoplastics on fish. *Front. Toxicol.* 2025, 7, 1530209.

12. Zhang, J.; Zhang, Y.; Xia, X.; Ma, C.; Zhang, Q.; Li, Y.; Zhang, Q.; Wen, W.; Yang, Z. Promoting effects of lipophilic pollutants on the reproductive toxicity of proteinophilic pollutants to *Daphnia magna* under chronic exposure. *Environ. Pollut.* 2025, 372, 126013.

13. Dai, Y.; Han, R.; Yao, Z.; Yan, H.; Liu, Z.; Liu, X.; Yue, T.; Zhao, J.; Wang, Z.; Xing, B. Intergenerational transfer of micro (nano) plastics in different organisms. *J. Hazard. Mater.* 2025, 488, 137404.

14. Costa, T.C.; de Deus, B.C.T.; Altomari, L.N.; Cardoso, S.J. The effect of plastic pollution on coastal marine organisms—A systematic review. *Environ. Monit. Assess.* 2025, 197, 1014.

15. Liu, H.; Li, H.; Liu, Y.; Zhao, H.; Peng, R. Toxic effects of microplastic and nanoplastic on the reproduction of teleost fish in aquatic environments. *Environ. Sci. Pollut. Res.* 2024, 31, 62530–62548.

16. Bhat, I.A.; Dar, J.Y.; Ahmad, I.; Mir, I.N.; Bhat, H.; Bhat, R.A.H.; Ganie, P.A.; Sharma, R. Testicular development and spermatogenesis in fish: Insights into molecular aspects and regulation of gene expression by different exogenous factors. *Rev. Aquac.* 2021, 13, 2142–2168.

17. Kitano, J.; Ansai, S.; Takehana, Y.; Yamamoto, Y. Diversity and convergence of sex-determination mechanisms in teleost fish. *Annu. Rev. Anim. Biosci.* 2024, 12, 233–259.

18. Pujol, G.; Marín-Gual, L.; González-Rodelas, L.; Álvarez-González, L.; Chauvigné, F.; Cerdà, J.; Teles, M.; Roher, N.; Ruiz-Herrera, A. Short-term polystyrene nanoplastic exposure alters zebrafish male and female germline and reproductive outcomes, unveiling pollutant-impacted molecular pathways. *J. Hazard. Mater.* 2025, 481, 136529.

19. Zhang, Y.; Chen, R.; Chen, Z.; Fu, X.; Wu, Z.; Chen, J.; Xie, L.; Zong, H.; Mu, J. Dietary exposure to sulfamethazine, nanoplastics and their binary mixture disrupts the spermatogenesis of marine medaka (*Oryzias melastigma*). *Acta Oceanol. Sin.* 2024, 43, 104–110.

20. Sun, Z.; Wu, B.; Yi, J.; Yu, H.; He, J.; Teng, F.; Xi, T.; Zhao, J.; Ruan, J.; Xu, P.; et al. Impacts of environmental concentrations of nanoplastics on zebrafish neurobehavior and reproductive toxicity. *Toxics* 2024, 12, 617.

21. Yaripour, S.; Huuskonen, H.; Rahman, T.; Kekäläinen, J.; Akkanen, J.; Magris, M.; Kipriianov, P.V.; Kortet, R. Pre-fertilization exposure of sperm to nano-sized plastic particles decreases offspring size and swimming performance in the European whitefish (*Coregonus lavaretus*). *Environ. Pollut.* 2021, 291, 118196.

22. Zhang, C.; Li, L.; Alava, J.J.; Yan, Z.; Chen, P.; Gul, Y.; Wang, L.; Xiong, D. Female zebrafish (*Danio rerio*) exposure to polystyrene nanoplastics induces reproductive toxicity in mother and their offspring. *Aquat. Toxicol.* 2024, 273, 107023.

23. Duan, J.; Xiao, Y.; Wang, W.; Yuan, Y.; Ju, W.; Wu, B.; Hu, L.; Tuo, X.; Jiang, M.; Zhao, S. Synergistic reproductive toxicity of microcystin-LR and polystyrene micro/nano-plastics in male zebrafish. *Ecotoxicol. Environ. Saf.* 2025, 299, 118377.

24. Contino, M.; Ferruggia, G.; Indelicato, S.; Pecoraro, R.; Scalisi, E.M.; Salvaggio, A.; Brundo, M.V. Polystyrene nanoplastics in aquatic microenvironments affect sperm metabolism and fertilization of *Mytilus galloprovincialis* (Lamark, 1819). *Toxics* 2023, 11, 924.

25. Zheng, Y.; Gan, X.; Lin, C.; Wang, D.; Chen, R.; Dai, Y.; Jiang, L.; Huang, C.; Zhu, Y.; Song, Y.; et al. Polystyrene nanoplastics cause reproductive toxicity in zebrafish: PPAR mediated lipid metabolism disorder. *Sci. Total Environ.* 2024, 931, 172795.

26. Tallec, K.; Paul-Pont, I.; Boulais, M.; Le Goïc, N.; González-Fernández, C.; Le Grand, F.; Bideau, A.; Quéré, C.; Cassone, A.L.; Lambert, C.; et al. Nanopolystyrene beads affect motility and reproductive success of oyster spermatozoa (*Crassostrea gigas*). *Nanotoxicology* 2020, 14, 1039–1057.

27. Sun, S.; Jin, Y.; Luo, P.; Shi, X. Polystyrene microplastics induced male reproductive toxicity and transgenerational effects in freshwater prawn. *Sci. Total Environ.* 2022, 842, 156820.

28. He, J.; Yang, X.; Liu, H. Enhanced toxicity of triphenyl phosphate to zebrafish in the presence of micro-and nano-plastics. *Sci. Total Environ.* 2021, 756, 143986.

29. Qiang, L.; Cheng, J. Exposure to polystyrene microplastics impairs gonads of zebrafish (*Danio rerio*). *Chemosphere* 2021, 263, 128161.

30. Zhou, Y.; Jin, Q.; Xu, H.; Wang, Y.; Li, M. Chronic nanoplastic exposure induced oxidative and immune stress in medaka gonad. *Sci. Total Environ.* 2023, 869, 161838.

31. Li, Z.; Xian, H.; Ye, R.; Zhong, Y.; Liang, B.; Huang, Y.; Dai, M.; Guo, J.; Tang, S.; Ren, X.; et al. Gender-specific effects of polystyrene nanoplastic exposure on triclosan-induced reproductive toxicity in zebrafish (*Danio rerio*). *Sci. Total Environ.* 2024, 932, 172876.

32. Arienzo, M.; Ferrara, L. Environmental fate of metal nanoparticles in estuarine environments. *Water* 2022, 14, 1297.

33. Ding, R.; Tong, L.; Zhang, W. Microplastics in freshwater environments: Sources, fates and toxicity. *Water Air Soil Pollut.* 2021, 232, 181.

34. Chen, X.; Liu, B.; Lin, D. Sexual maturation, reproductive habits, and fecundity of fish. In *Biology of Fishery Resources*; Springer Nature: Singapore, 2022; pp. 113–142.

35. Comizzoli, P.; Amelkina, O.; Lee, P.C. Damages and stress responses in sperm cells and other germplasms during dehydration and storage

at nonfreezing temperatures for fertility preservation. *Mol. Reprod. Dev.* 2022, 89, 565–578.

36. Herrera, F.; Bondarenko, O.; Boryshpolets, S. Osmoregulation in fish sperm. F*ish Physiol. Biochem.* 2021, 47, 785–795.

37. Zhao, X.; Sun, J.; Zhou, L.; Teng, M.; Zhao, L.; Li, Y.; Wu, F. Defining the size ranges of polystyrene nanoplastics according to their ability to cross biological barriers. *Environ. Sci. Nano* 2023, 10, 2634–2645.

38. Tombul, O.K.; Akdağ, A.D.; Thomas, P.B.; Kaluç, N. Assessing the impact of sub-chronic polyethylene terephthalate nanoplastic exposure on male reproductive health in mice. *Toxicol. Appl. Pharmacol.* 2025, 495, 117235.

39. Contino, M.; Ferruggia, G.; Indelicato, S.; Pecoraro, R.; Scalisi, E.M.; Bracchitta, G.; Dragotto, J.; Salvaggio, A.; Brundo, M.V. In vitro nano-polystyrene toxicity: Metabolic dysfunctions and cytoprotective responses of human spermatozoa. *Biology* 2023, 12, 624.

40. Gao, X.; Xu, K.; Du, W.; Wang, S.; Jiang, M.; Wang, Y.; Han, Q.; Chen, M. Comparing the effects and mechanisms of exposure to polystyrene nanoplastics with different functional groups on the male reproductive system. *Sci. Total Environ.* 2024, 922, 171299.

41. Liu, Y.; Hao, F.; Liang, H.; Liu, W.; Guo, Y. Exposure to polystyrene nanoplastics impairs sperm metabolism and pre-implantation embryo development in mice. *Front. Cell Dev. Biol.* 2025, 13, 1562331.

42. Xie, X.; Deng, T.; Duan, J.; Xie, J.; Yuan, J.; Chen, M. Exposure to polystyrene microplastics causes reproductive toxicity through oxidative stress and activation of the p38 MAPK signaling pathway. *Ecotoxicol. Environ. Saf.* 2020, 190, 110133.

43. Urli, S.; Corte Pause, F.; Crociati, M.; Baufeld, A.; Monaci, M.; Stradaioli, G. Impact of microplastics and nanoplastics on livestock health: An emerging risk for reproductive efficiency. *Animals* 2023, 13, 1132.

44. Şenol, O.; Sulukan, E.; Baran, A.; Bolat, İ.; Toraman, E.; Alak, G.; Yildirim, S.; Bilgin, G.; Ceyhun, S.B. Global warming and nanoplastic toxicity; small temperature increases can make gill and

liver toxicity more dramatic, which affects fillet quality caused by polystyrene nanoplastics in the adult zebrafish model. *Sci. Total Environ*. 2023, 892, 164682.

45. Lyu, K.; Cao, C.; Li, D.; Akbar, S.; Yang, Z. The thermal regime modifies the response of aquatic keystone species *Daphnia* to microplastics: Evidence from population fitness, accumulation, histopathological analysis and candidate gene expression. *Sci. Total Environ*. 2021, 783, 147154.

46. Sanpradit, P.; Byeon, E.; Lee, J.S.; Jeong, H.; Kim, H.S.; Peerakietkhajorn, S.; Lee, J.S. Combined effects of nanoplastics and elevated temperature in the freshwater water flea *Daphnia magna*. *J. Hazard. Mater.* 2024, 465, 133325.

47. Trevisan, R.; Trimpey-Warhaftig, R.; Gaston, K.; Butron, L.; Gaballah, S.; Di Giulio, R.T. Polystyrene nanoplastics impact the bioenergetics of developing zebrafish and limit molecular and physiological adaptive responses to acute temperature stress. *Sci. Total Environ.* 2025, 958, 178026.

48. Trevisan, R.; Ranasinghe, P.; Jayasundara, N.; Di Giulio, R.T. Nanoplastics in aquatic environments: Impacts on aquatic species and interactions with environmental factors and pollutants. *Toxics* 2022, 10, 326.

49. Materić, D.; Peacock, M.; Dean, J.; Futter, M.; Maximov, T.; Moldan, F.; Röckmann, T.; Holzinger, R. Presence of nanoplastics in rural and remote surface waters. *Environ. Res. Lett.* 2022, 17, 054036.

50. Junaid, M.; Liu, S.; Liao, H.; Yue, Q.; Wang, J. Environmental nanoplastics quantification by pyrolysis-gas chromatography–mass spectrometry in the Pearl River, China: First insights into spatiotemporal distributions, compositions, sources and risks. *J. Hazard. Mater.* 2024, 476, 135055.

51. Xu, Y.; Ou, Q.; Jiao, M.; Liu, G.; Van Der Hoek, J.P. Identification and quantification of nanoplastics in surface water and groundwater by pyrolysis gas chromatography–mass spectrometry. *Environ. Sci. Technol.* 2022, 56, 4988–4997.

52. Okoffo, E.D.; Thomas, K.V. Quantitative analysis of nanoplastics in environmental and potable waters by pyrolysis-gas chromatography–mass spectrometry. *J. Hazard. Mater.* 2024, 464, 133013.

53. Yi, J.; Ma, Y.; Ruan, J.; You, S.; Ma, J.; Yu, H.; Zhao, J.; Zhang, K.; Yang, Q.; Jin, L.; et al. The invisible threat: Assessing the reproductive and transgenerational impacts of micro-and nanoplastics on fish. *Environ. Int.* 2024, 183, 108432.

54. Wong, S.; Bigman, J.S.; Dulvy, N.K. The metabolic pace of life histories across fishes. *Proc. R. Soc. B* 2021, 288, 20210910.

5

General discussion and conclusions

The reproductive versatility, diversity (in both anatomical configurations and physiological processes), and ubiquity of fish difficult the univocal and general adscription of the noxious effects of emergent contaminants to a particular organ or biological process. In the case of nanoplastics (NPs), but also for other contaminants or xenobiotics, including complex mixtures of organic and inorganic components, the main effects of NPs on the reproductive performance in male fish seem to be related to energetic imbalances mediated by oxidative stress, and changes in the metabolism of lipids, usually coupled with alterations in the immune response. However, the toxicity in fish sperm cells and/or testicular structures remains strongly species-specific and highly dependent on the classic laboratory models, with zebrafish being the gold standard against which other fish species are compared.

In recent years, commercial marine species, such as *S. aurata*, have been exposed to low, high and environmentally relevant doses of NPs to fill the information gap between laboratory models and wild species regarding the viability of male reproductive function and performance. As discussed in Chapter 4 of this thesis, the experimental bias in favour of freshwater species can be extended to the target cell and tissue of interest: sperm performance and the testicular structures that maintain spermatogenesis are the common choices for analysing the impact of NPs in fish. However, proper metabolic adjustment and the maturation processes that enable and improve sperm motility, not to mention the sperm storage, cryopreservation, and the outcomes of the artificial hormonal stimulation to enhance and control sperm production, depend strongly on the composition of the seminal plasma. Here we opted for the proteomic approach to characterise the composition of the seminal plasma in *S. aurata* (Chapter 2) due to the integrative features of "omics" technologies and their predictive value (Kajal et al. 2026). Even if the composition of seminal plasma proteins may vary during spermatogenesis, the characterisation of dominant proteins in the seminal plasma establishes a groundwork for analysing pathological or developmental deviations of the expected abundance of proteins, which, in turn, facilitates the evaluation and management of sperm performance, establishing a baseline of normal sperm biological functions. This approach also enables the direct or indirect detection of changes, the identification of proteins involved in spermatogenesis, the discovery of novel proteins, the understanding of protection against pathogens, and the clarification of how seminal plasma supports sperm survival and fertilising capacity.

1. Minimum common set of protein functionalities in the seminal plasma of fish

The proteomic profile of the seminal plasma of *S. aurata* roughly follows what has been described for other fish species, enabling the viability of developing spermatozoa, protecting against increased metabolism and related oxidative stress during spermatogenesis, and ensuring effective, mainly humoral and innate responses to inflammatory processes (Majewska et al., 2025). In this sense, our analysis indicates that the seminal plasma of *S. aurata* is particularly rich in proteins involved in acute phase response innate immunity, coagulation, and proteolytic processes, relying in the activation of several upstream, downstream, convertases, and regulators of the complement system (chapter 2) to manage and protect sperm cells and reproductive tissues from microbial threats during spawning, especially in aquatic environments, wild or farmed, prone to seasonal pathogenic outbursts (Uma et al., 2025). Our results align with earlier proteomic analyses in other teleost fish. For instance, Nynca et al. (2014) found complement C3 and several other acute-phase proteins, such as transferrin, apolipoproteins, serum albumin, hemopexin, alpha-1-antiproteinase, and precerebellin-like proteins in rainbow trout seminal plasma. Dietrich et al. (2014, 2021) also found a dominance of stress- and immune-related proteins in the seminal plasma proteome of common carp and pikeperch. Kodzik et al. (2023) showed that sturgeon seminal plasma is also rich in immune and stress-response proteins, including apolipoproteins, which were also present in our samples. This suggests that the immune capabilities of the *S. aurata* seminal plasma overlap not only with the lipid metabolic budget available for the steroidogenic activation during spermatogenesis (Bogevik et al., 2020), but also with the enhanced mitochondrial activity in resting and moving spermatozoa that may favour the production of reactive oxygen species (ROS) (Ulloa et al., 2017).

Interestingly, cathepsins were also identified in our proteomic profiling. In common carp, cathepsins, along with apolipoproteins, heat shock proteins, antioxidative enzymes, and glycolytic enzymes in the seminal plasma are associated with the regulation of sperm motility, spermatogenesis, lipid membrane stabilisation, and antioxidant defence mechanisms (Dietrich et al., 2014). In black rockfish, cathepsin paralogs (SsCTSDa, SsCTSDb) are broadly expressed and show significant upregulation in the kidney and gill following bacterial infection (Xu et al.,2025). Recombinant forms of these proteins can bind microbial ligands and cause bacterial agglutination,

supporting their immune defence function in teleost fish, which, due to the absence of an epididymal and accessory glands system, tend to share blood and seminal plasma immune proteins (Nynca et a., 2011). These and other results suggest that effective immune responsiveness is a widespread feature of fish seminal plasma that plasma functions not only as a nutrient medium, but also as a defensive system against environmental stressors and foreign entities such as nanoplastics, as discussed in chapter 3 (Nynca et al., 2017; Dietrich et al., 2022).

Similar to what has been described in carp (Majewska et al., 2025) we identified components of the ubiquitin–proteasome cellular pathway in *S. aurata* that guarantee the regulation of protein homeostasis and folding during spermatogenesis. Proteolytic enzymes likely facilitate the breakdown of damaged or foreign proteins and may modulate sperm function and antimicrobial defences, as well as regulate complement activation. Similar patterns have been noted in other fish, indicating that proteolysis is a conserved function in seminal plasma (Ciereszko et al., 2017). More intriguing is the discovery of coagulation factors, such as prothrombin and factors VIII, IX, and Xa in *S. aurata* seminal plasma. While coagulation is usually associated with haemostasis, its presence here suggests a role in stabilising the seminal plasma and forming protective barriers to restrict pathogen spread. Unlike previous studies in trout and carp, in which coagulation proteins were not addressed, these findings suggest that coagulation processes in seminal plasma may be more important than previously thought.

The co-occurrence of proteins associated with immunity, coagulation, and proteolysis points toward a highly coordinated and integrated network. Rather than operating independently, these systems likely interact to create a regulated environment that supports sperm health and shields against external threats. This integrated regulation underscores a complex biochemical landscape in fish reproductive biology that remains to be fully understood. Nevertheless, the characterization of the proteomic landscape of *S. aurata* may help to adjust and optimize cryopreservation strategies and breeding management in farmed fish. Successful short- to medium-term storage of fish sperm depends, among other factors (reviewed in Shazada et al., 2024), on the protein composition of the seminal plasma, which, in turn, is affected by the age of males, the characteristics of the spawning period, and species-specific anatomical and physiological peculiarities. While a core set of proteins is conserved, some seminal plasma components

may be unique to particular species and reflect differences in reproductive strategies. For example, Dietrich et al. (2021) identified proteins in pikeperch that appear to be species-specific, such as type-4 ice-structuring protein LS-12-like, papilin, and hyaluronan-binding protein 2. The absence of these proteins in *S. aurata* highlights the need for species-specific investigations to characterise the diversity of seminal plasma across fish. However, we can safely assume that, irrespective of the species considered, sperm motility and viability may be impaired due to the overproduction of ROS during storage, diminishing the fertilizing capabilities. In this sense, stabilising a baseline proteome of healthy seminal plasma that includes the characterization of immune and metabolic components may help to reduce or prevent short-term alterations of cryoperserved fish sperm, even if the elucidation of the precise role of the identified proteins require validation through functional annotation studies.

2. The impact of nanoplastics on sperm performance and metabolite composition in the seminal plasma

In males of *S. aurata*, the proteomic milieu described for the seminal plasma may be altered upon exposure to NPs. As described in Chapter 3, we aimed to elucidate the effects of low, medium and high concentrations (1, 10, and 50 µg/mL) of PS-NPs on sperm physiology and seminal plasma composition. These concentrations were selected to evaluate dose-dependent responses and to uncover mechanistic insights under controlled in vitro conditions. Our results indicate that PS-NPs reshaped sperm motility patterns. High concentrations of PS-NPs induced erratic movements, whereas exposure to low concentrations enhanced velocity and motility-supporting metabolites **(Figure 1)**.

As discussed in chapter 4, sperm motility is one of the most consistently measured parameters in ecotoxicological studies examining the effect of NPs on reproductive physiology in externally fertilising species such as fish. Considered a correlate of sperm viability and successful fertilization in wild environments, sperm motility in aquaculture practices is enhanced by hormonal stimulation in farmed fish to guarantee a successful fertilization, high hatching rates and normal larval development and survival (Nargesi et al. 2025). Sperm performance, including motility and viability, but also seminal plasma composition, influences the fertilization success in fish

species that endure pre-mating and post-mating evolutionary strategies of sexual selection (Morita et al., 2025). Therefore, the presence of and exposure to nanoplastics, can interfere not only with the reproductive strategies of fish in natural environments, but also with productivity in aquaculture practices. Sperm motility in fish exhibits a common trend of short-lived bursts of activity due mainly to ATP hydrolysis, creatin kinase (CK) and ROS signaling, that follows the complex and environmentally driven activation of spermatozoa (Dzyuba et al., 2017). From our data, uptake of PS-NPs by sperm cells increased with concentration, reaching the highest levels in the high-exposure group, suggesting that nanoplastics can interact directly with spermatozoa and potentially penetrate or adhere to cellular membranes. Exposure to NPs is known to cause oxidative stress, inflammation, and disrupt glycolipid and amino acid metabolism, (Panizzolo et al., 2023; Stadmiller et al., 2017; Sinesi et al., 2025; Wu et al., 2021; Knight et al., 2017; Lin et al., 2022; Pei et al., 2024; Xu et al., 2023).

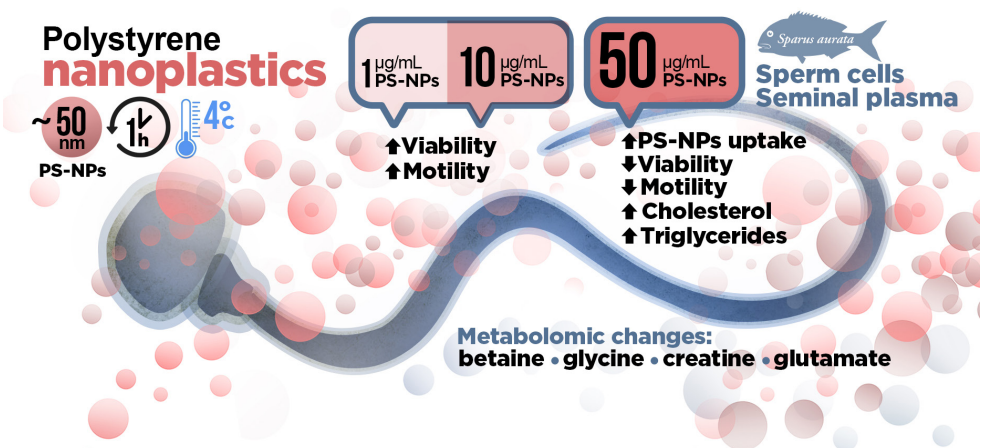


Figure 1. **Summary of the effects of incremental concentrations of PS-NPs on sperm cells and seminal plasma of gilthead seabream.**

Sperm motility analysis revealed a clear non-linear dose-response pattern, characterised by an initial enhancement of kinetic parameters followed by a pronounced decline at higher concentrations. This biphasic response suggests an adaptive stress mechanism in which cells temporarily compensate through increased metabolic turnover before reaching a threshold beyond which homeostatic regulation fails. The decline in linearity and progressive motility at elevated concentrations may disrupt flagellar coordination and ion-exchange mechanisms, particularly those governing calcium flux

and membrane polarisation. Given the narrow fertilisation window in externally fertilising marine species, such alterations are expected to have disproportionate effects on reproductive success. Importantly, the observed behavioural changes are not random but reflect structured deterioration in motility patterns, reinforcing the mechanistic basis of toxicity rather than stochastic damage (Gallego and Asturiano, 2018). Sperm viability showed a clear dose-dependent decline. While low concentrations did not significantly differ from the control group, high concentrations resulted in a marked reduction in viability. This suggests that sperm cells can tolerate low levels of nanoplastic exposure, but higher concentrations lead to membrane damage and loss of cellular integrity (Wang et al., 2022).

In our study, traditional markers of biochemical activity such as CK, AST, and ADA showed no significant changes, but we found that several metabolites involved in the regulation of energy metabolism and oxidative stress remain altered after exposure to medium and high doses of PS-NPs, which may preclude alterations in the immune function. Key metabolites that may serve as biomarkers of NPs exposure include AMP, glutamate, proline, sarcosine, and isobutyrate. Glycine and betaine concentrations increased, reflecting antioxidant and osmoregulatory responses. Both compounds help stabilise cell structure and support antioxidant defences, in part by promoting glutathione synthesis. Creatine also showed an initial increase followed by a decline, indicating increased energy demand and mitochondrial impairment. Studies across human, animal, and aquatic models show that NPs reduce energy-related metabolites, including those in the creatine phosphate system, and lower ATP production, reflecting impaired energy homeostasis (Wang et al., 2022; Lee et al., 2022). These effects are associated with inhibited mTORC1 signalling, oxidative stress, and alterations in glycolysis and oxidative phosphorylation, ultimately weakening ATP buffering capacity (Li et al., 2023; Liang et al., 2022). The extent of disruption depends on NP size, concentration, and exposure duration, with smaller particles and longer exposures causing greater effects (Zhao et al., 2023). To summarize, we can conclude that betaine and glycine emerged as key protective metabolites under PS-NP exposure. Cholesterol and triglyceride levels increased, suggesting membrane disruption or altered lipid metabolism. Wang et al. (2022) demonstrated that exposure to micro- and nanoplastics disrupts lipid metabolism in aquatic organisms. They observed changes in lipid composition crucial for preserving sperm membrane structure and fluidity, and revealed impairment of lipid pathways responsible for energy production, such as fatty acid metabolism, resulting

in decreased energy available for sperm motility. Furthermore, membrane lipid remodelling and damage were noted, often linked to oxidative stress and lipid peroxidation. This suggests that NPs disturb lipid metabolic processes vital for normal reproductive function, and may alter the immune and metabolic proteome in the seminal plasma of *Sparus aurata*, and also sperm performance.

Overall, we can conclude that the changes in the metabolomic composition of seminal plasma can influence the normal sperm development, motility, and function upon exposure to NPs. However, the precise mechanism for such alterations remains elusive in *S. aurata* and other marine fish species.

3. Conclusions

1. The proteomic characterization of gilthead seabream seminal plasma has identified key biological processes in the seminal plasma proteome, namely innate immune response, coagulation processes, and proteolytic activity.

2. The complement system and other acute-phase proteins orchestrate, judging by the abundance and composition of proteins, the immune response in *S. aurata* seminal plasma.

3. Exposure to polystyrene nanoplastics (PS-NPs) alters sperm motility and viability, and potentially fertilization success.

4. PS-NPs reshaped sperm motility patterns in a dose-dependent pattern. Exposure to high concentrations of PS-NPs (50 μg/mL) induced erratic movements, whereas low concentrations (1 μg/mL) enhanced velocity and motility-supporting metabolites.

5. Exposure to high concentrations of PS-NPs increased the levels of lipid markers (cholesterol, triglycerides) in the seminal plasma of *S. aurata*.

6. Under PS-NPs exposure, betaine and glycine emerged as key protective metabolites in the seminal plasma of *S. aurata*.

7. The characterization of the effects of nanoplastics in the reproductive system of male fish is still poorly defined, and strongly species-specific.

References

Ciereszko, A., Dietrich, M. A., & Nynca, J. (2017). Fish semen proteomics—New opportunities in fish reproductive research. *Aquaculture*, 472, 81–92.

Dietrich, M. A., Adamek, M., Teitge, F., Teich, L., Jung-Schroers, V., Malinowska, A., Świderska, B., Rakus, K., Kodzik, N., Chadzińska, M., Karol, H., Liszewska, E., & Ciereszko, A. (2022). Proteomic analysis of carp seminal plasma provides insights into the immune response to bacterial infection of the male reproductive system. *Fish and Shellfish Immunology*, 127, 822–835.

Dietrich, M. A., Judycka, S., Żarski, D., Malinowska, A., Świderska, B., Palińska-Żarska, K., Błażejewski, M., & Ciereszko, A. (2021). Proteomic analysis of pikeperch seminal plasma provides novel insight into the testicular development of domesticated fish stocks. *Animal,* 15(7), 100279.

Dietrich, M. A., Arnold, G. J., Nynca, J., Fröhlich, T., Otte, K., & Ciereszko, A. (2014). Characterization of carp seminal plasma proteome in relation to blood plasma. J*ournal of Proteomics*, 98, 218–232.

Dzyuba, B., Bondarenko, O., Fedorov, P., Gazo, I., Prokopchuk, G., & Cosson, J. (2017). Energetics of fish spermatozoa: The proven and the possible. *Aquaculture*, 472, 60–72.

Gallego, V., & Asturiano, J. F. (2018). Sperm motility in fish: Technical applications and perspectives through CASA-Mot systems. *Reproduction, Fertility and Development*, 30(6), 820–832.

Kajal, K., et al. (2026). Proteomics in aquaculture: Unravelling protein function and expression for enhanced food security. In M. Ishfaq Nazir & I. Ahmad (Eds.), *Aquatic Genomics and Food Security.* Springer.

Knight, L. S., Piibe, Q., Lambie, I., Perkins, C., & Yancey, P. H. (2017). Betaine in the brain: Characterization of betaine uptake, its influence on other osmolytes and its potential role in neuroprotection from osmotic stress. *Neurochemical Research*, 42(12), 3490–3503.

Kodzik, N., Ciereszko, A., Szczepkowska, B., Malinowska, A., Świderska, B., Dietrich, M. A., Karol, H., & Judycka, S. (2023). Comprehensive proteomic characterization and functional annotation of Siberian sturgeon seminal plasma proteins. *Aquaculture*, 568, 739326.

Lin, S., Zhang, H., Wang, C., Su, X. L., Song, Y., Wu, P., Yang, Z., Wong, M. H., Cai, Z., & Zheng, C. (2022). Metabolomics reveal nanoplastic-induced mitochondrial damage in human liver and lung cells. *Environmental Science & Technology,* 56(17), 12483–12493.

Majewska, A. M., Kodzik, N., Dietrich, M. A., & Ciereszko, A. (2025). Comprehensive proteomic characterization and functional annotation of common carp seminal plasma. *Reproduction, Fertility and Developmen*t, 37, RD25034.

Morita, M., Satoh, S., Ito, T., Kohda, M., & Awata, S. (2025). Variation in sperm motility and seminal plasma protein expression is shaped by pre- and post-mating sexual selection in the mouthbrooding cichlid (*Ophthalmotilapia ventralis*). *Evolution*, 79(12), 2807–2818.

Nargesi, E. A., Gorouhi, D., Alavi, S. M. H., & Falahatkar, B. (2025). Spermiation induction in the common bream (Abramis brama) using sGnRHa and domperidone (Ovaprim): Sperm production, seminal plasma composition, and spermatozoa motility and fertilizing ability. *Aquaculture*, 597, 741902.

Nynca, J., Słowińska, M., Dietrich, M. A., Bilińska, B., Kotula-Balak, M., & Ciereszko, A. (2011). Isolation and identification of fetuin-B-like protein from rainbow trout seminal plasma and its localization in the reproductive system. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 158, 106–116.

Nynca, J., Dietrich, G. J., Dobosz, S., Grudniewska, J., & Ciereszko, A. (2014). Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout (*Salmo trutta* m. fario) semen. *Aquaculture,* 433, 62–65.

Panizzolo, M., Martins, V. H., Ghelli, F., Squillacioti, G., Bellisario, V., Garzaro, G., Bosio, D., Colombi, N., Bono, R., & Bergamaschi, E. (2023). Biomarkers of oxidative stress, inflammation, and genotoxicity to assess exposure to micro- and nanoplastics: A literature review. *Ecotoxicology and Environmental Safety,* 267, 115645.

Shazada, N. E., Alavi, S. M. H., Siddique, M. A. M., et al. (2024). Short-term storage of sperm in common carp from laboratory research to commercial production—A review. *Reviews in Aquaculture*, 16(1), 174–189.

Sinesi, G., Salvioni, L., Ronchi, E., Barbuzzi, E. M. G., Prosperi, D., Colombo, M., & Giustra, M. D. (2025). Exploring bovine serum albumin (BSA) as a facile substrate for testing of osmolytes as cosmetic ingredients. *Molecules,* 30(3), 664.

Ulloa-Rodríguez, P., Figueroa, E., Díaz, R., Lee-Estevez, M., Short, S., & Farías, J. G. (2017). Mitochondria in teleost spermatozoa. *Mitochondrion*, 34, 49–55.

Uma, A. (2025). Pathogen and disease transmission in aquatic animals. In S. K. Mallik, N. Shahi, & P. K. Pandey (Eds.), *Management of Fish Diseases*. Springer.

Wang, H., Shi, X., Gao, Y., Zhang, X., Zhao, H., Wang, L., Zhang, X., & Chen, R. (2022). Polystyrene nanoplastics induce profound metabolic shift in human cells as revealed by integrated proteomic and metabolomic analysis. *Environment International*, 166, 107349.

Wu, X., Liu, C., Yang, S., Shen, N., Wang, Y., Zhu, Y., Guo, Z., Yang, S. Y., Xing, D., Li, H., & Guo, Z. (2021). Glycine-serine-threonine metabolic axis delays intervertebral disc degeneration through antioxidant effects: An imaging and metabonomics study. *Oxidative Medicine and Cellular Longevity*, 2021, 5579736.

Xu, X., Liu, Y., Li, X., Zhang, P., Lin, F., Chen, C., Zhang, X., Li, C., & Fu, Q. (2025). Characterization, expression profiling, and immunological role of Cathepsin D in *Sebastes schlegelii* during bacterial infection. *Developmental & Comparative Immunology*, 167, 105387.