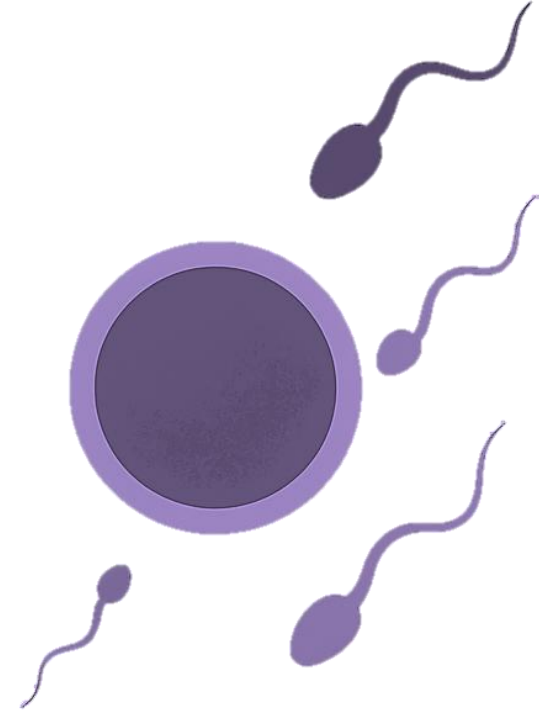


BIOLOGICAL EFFECTS OF IN VITRO NANOPLASTICS INTERNALIZATION IN MAMMALIAN GAMETES



PhD Student: Sofia Dindo 40° ciclo

Supervisor: Prof.ssa Marcella Spinaci

Co-supervisor: Prof. Salvatore Nesci



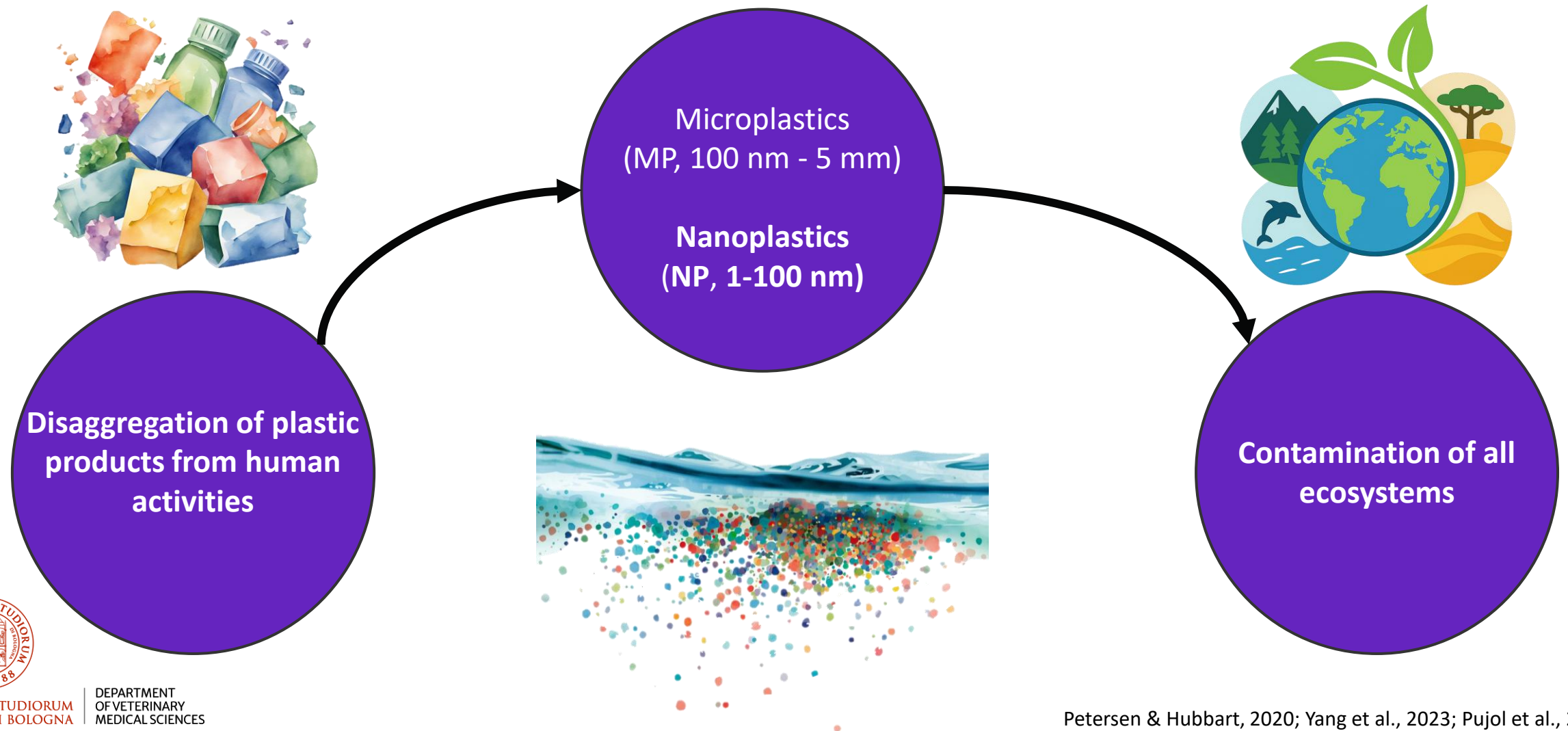


ACTIVITIES 1ST YEAR of my PhD

- ✓ Focus on mammalian semen physiology, capacitation processes, and reproductive biotechnologies.
- ✓ **In vitro gamete manipulations** and **flow cytometry** assays to investigate the biological effects of **nanoplastics (NP)** on **mammalian gametes**

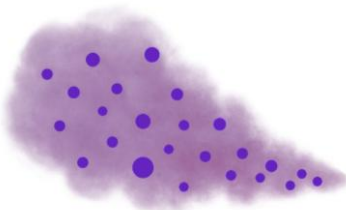


INTRODUCTION – NANOPLASTICS (NP)



INTRODUCTION – NANOPLASTICS (NP)

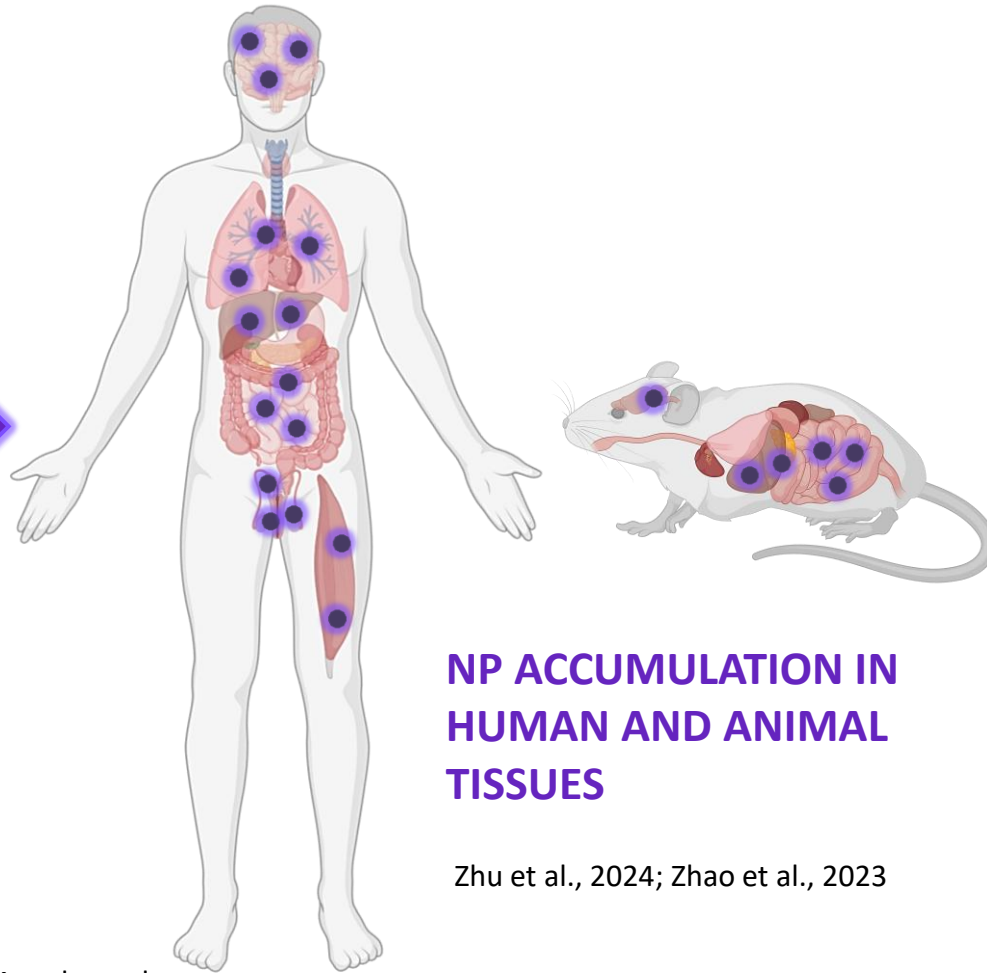
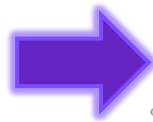
INHALATION



INGESTION



DERMAL CONTACT



NP ACCUMULATION IN HUMAN AND ANIMAL TISSUES

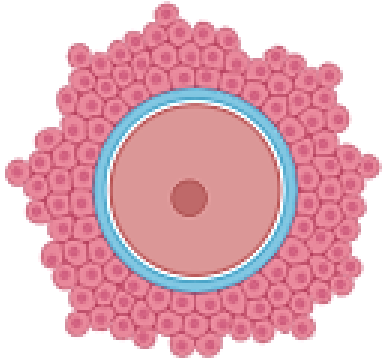
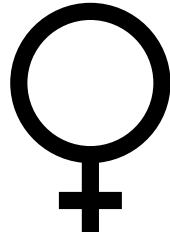
Zhu et al., 2024; Zhao et al., 2023

- ✓ oxidative stress
- ✓ inflammation
- ✓ immune dysfunction
- ✓ alterations in biochemical and energy metabolism
- ✓ impaired cell proliferation
- ✓ disruption of microbial metabolic pathways
- ✓ potential carcinogenicity

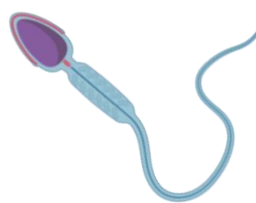
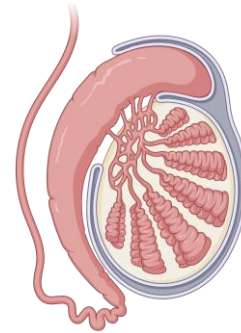
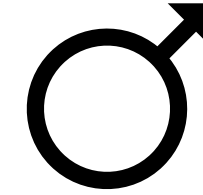
Ali et al., 2024; Zhang et al., 2022; Goodman et al., 2021; Xu et al., 2019; Dong et al., 2019; Xiao et al., 2022; Li et al., 2019; Huang et al., 2021



INTRODUCTION – NANOPLASTICS (NP)



- ✓ **Impaired meiotic progression** and chromosome organization
- ✓ **Impaired mitochondrial activity**
- ✓ Increased **oxidative stress, apoptosis** and **DNA damage**
- ✓ **Negative effect on nuclear and cytoplasmic maturation**



- ✓ Damages in testicular structure
- ✓ Disruption of blood–testis barrier (BTB)
- ✓ Testis inflammation → **spermatogenesis disorders** and **increased sperm abnormalities**
- ✓ Increased acrosomal damage
- ✓ **Oxidative stress** marked by ROS generation
- ✓ **DNA fragmentation**
- ✓ **Impaired mitochondrial activity**



AIM of the STUDY

NP are supposed to be one of the causes of the worldwide increasing human subfertility



Could nanoplastics pose a threat to mammalian reproductive performance?



Evaluate the effect of NP on mammalian gametes



PROJECT 1



CERTIFICATE

Mar
Arti
Key
Cor
Firs
Ord

This is to certify that:

Sofia Dindo

Won the ESDAR Student Competition on 26th March 2025 and is thus entitled to FREE entry to the Joint ESDAR and ECAR conference in Albena, Bulgaria on 11-13th September 2025.

Eilidh Thomson
Eilidh Thomson
ESDAR Student
Representative

Renée Båge
Renée Båge
ESDAR President

Internal activity in equine sperm

pollution, mitochondria, oxidative stress, fertility,

PhD
Veterinary Medical Sciences



OC 5.3 | Polystyrene nanoplastics impair stallion sperm function

S. Dindo¹; L. Tovar-Pascual¹; G. Mari²; M. Spinaci¹;
J. Ortiz-Rodriguez¹

Internalization of Nanoplastics in equine spermatozoa

Jose Manuel Ortiz-Rodriguez¹, Sofia Dindo¹, Vito Antonio Baldassarro¹, Laura Tovar-Pascual¹, Beatrice Misllei^{1,2}, Diego Buccì¹, Marcella Spinaci¹

¹Department of Veterinary Medical Sciences, Alma Mater Studiorum-University of Bologna, Bologna, Italy
²National Institute of Artificial Insemination (AUB-INFA), University of Bologna, Bologna, Italy

Introduction

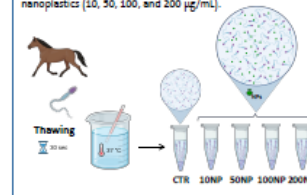
Plastic pollution is a growing global concern due to its potential effects on both human and animal health. The disaggregation of large plastic fragments into microplastics (MPs) and nanoplastics (NPs) facilitates their widespread dissemination in the environment. These particles can be internalized by organisms and subsequently accumulate in biological tissues, where they may induce toxicological effects such as inflammation and oxidative stress. While the mechanisms underlying the interaction between plastic particles and cells remain uncertain, recent human studies point to a potential association between increased subfertility and the exposure of gametes to NPs.

OBJECTIVE

The aim of this study was to evaluate the in vitro internalization of nanoplastics (NPs) in mature equine spermatozoa

Methodology

Five frozen-thawed ejaculates from different stallions were divided into five aliquots and incubated either without (CTR) or with different concentrations of 30 nm green-fluorescent nanoplastics (10, 50, 100, and 200 µg/mL).



Incubation for 1 h before analysis

Flow cytometry
• Sperm viability (DAPI/7)
• Sperm green mean fluorescence intensity (NPs)

Confocal microscopy
• NPs localization in spermatozoa stained with Zombie Violet (viability marker) and Vybrant DiI (membrane marker)

Results

A significant, dose-dependent increase in green mean fluorescence intensity (MFI) was detected in live sperm by flow cytometry (Figure 1), indicating greater NPs internalization. Sperm viability showed a decreasing trend with higher NP uptake. Confocal microscopy further revealed a preferential accumulation of NPs in the midpiece and/or post-acrosomal regions of the spermatozoa (Figure 2).

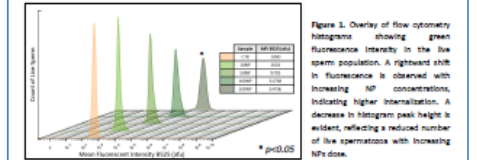


Figure 1. Overlay of flow cytometry histograms showing green fluorescence intensity in the live sperm population. A rightward shift in fluorescence is observed with increasing NP concentrations, indicating higher internalization. A decrease in histogram peak height is evident, reflecting a reduced number of live spermatozoa with increasing NP dose.

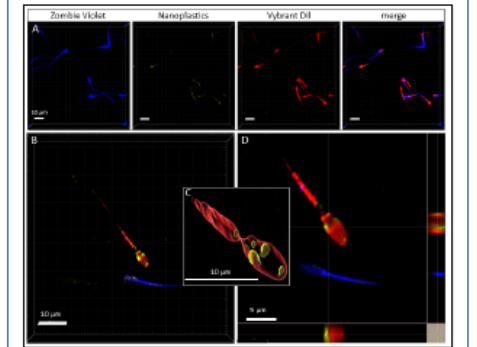


Figure 2. Representative images of internalization and accumulation of NPs in live spermatozoa. Fluorescent NPs (yellow); dead sperm (blue); sperm membrane (red). (A) 2-stack acquired by confocal microscopy and processed with IMARIS voxel-based software. (B) Higher magnification image. (C) 3D reconstruction of the head and midpiece from a live spermatozoa, with NPs localization using the fluorescence rendering. (D) Projections of the 2-stack images in which it is possible to recognize the NPs inside the live sperm.

CONCLUSION

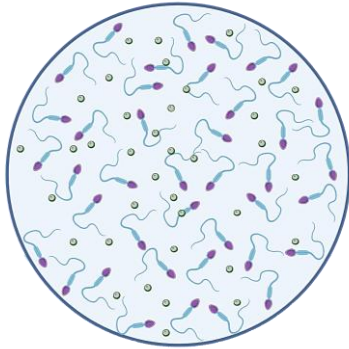
Sperm internalize NPs in a dose-dependent manner, with preferential accumulation in the post-acrosomal and midpiece regions. This distribution could suggest potential interference with mitochondrial function and fertility. Further studies, including ongoing work by our group, aim to clarify these effects.



EXPERIMENT 1



15 frozen-thawed ejaculates
from five stallions



Incubated at **38°C** with different concentrations of **30nm PS-NP**

T0, T1, T3

CTR
10, 50, 100, 200 µg/mL

Sperm kinetic parameters (CASA)
&

FLOW CYTOMETRY

Sperm marker (Hoechst 33342)
Sperm **viability** (PI)



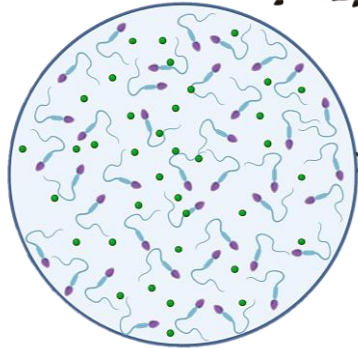
Hydrogen Peroxide production (H₂DCFDA)
Superoxide ion production (DHE)
Mitochondrial activity (JC1)



EXPERIMENT 2



5 frozen-thawed ejaculates from five stallions



Incubated at 38°C with different concentrations of 30nm Fluorescent yellow-green PS-NP

CTR
10, 50, 100, 200 µg/mL

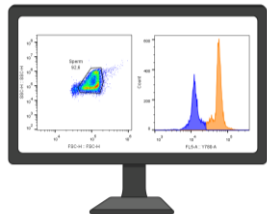
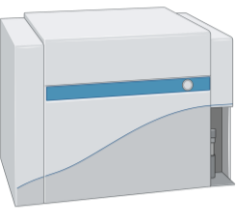


FLOW CYTOMETRY

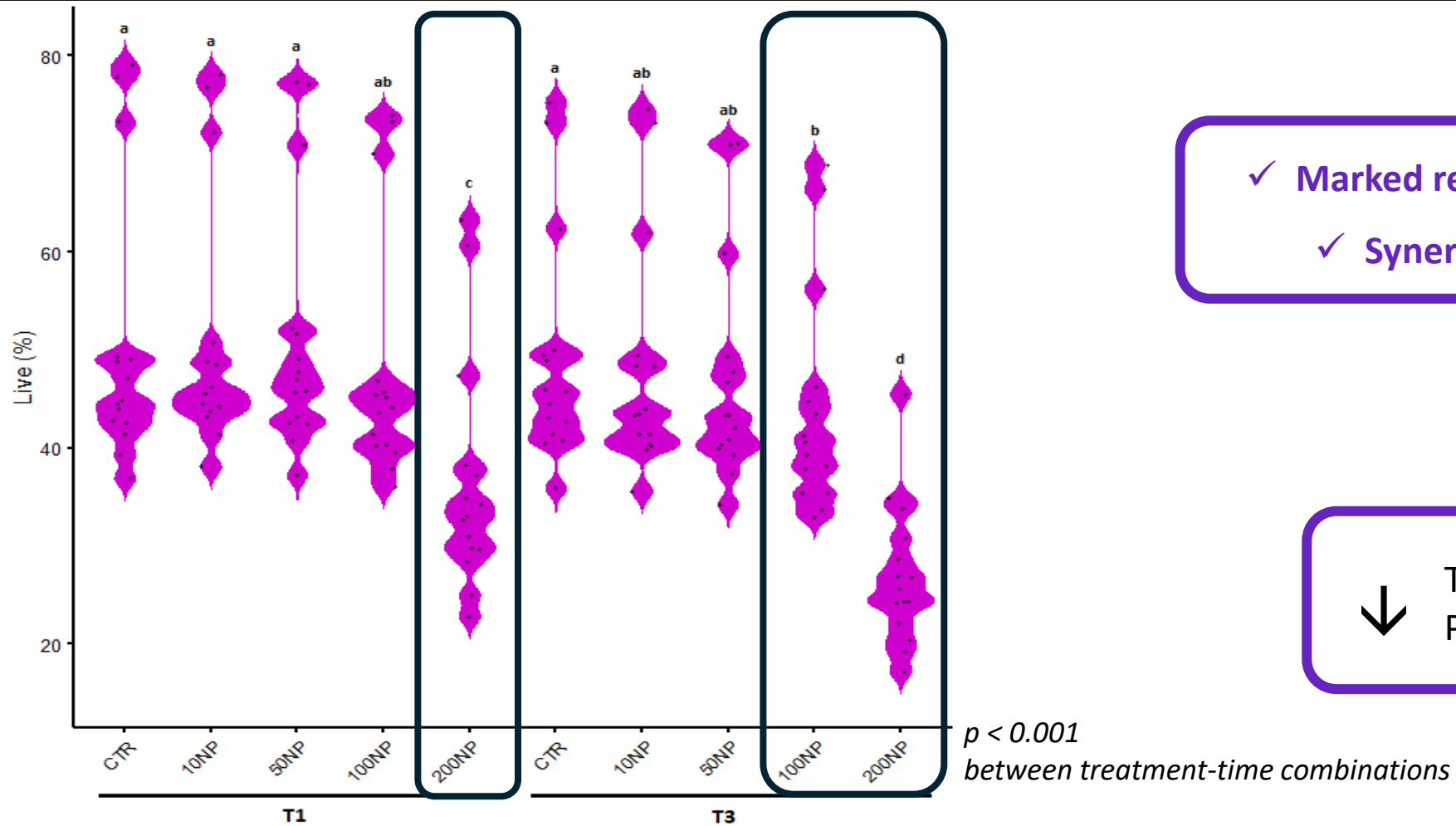
CONFOCAL MICROSCOPY

Green Mean Fluorescence Intensity (MFI) within the live sperm population to evaluate intracellular NP levels

Zombie Violet (viability)
Vybrant Dil (membrane marker)



VIABILITY & MOTILITY

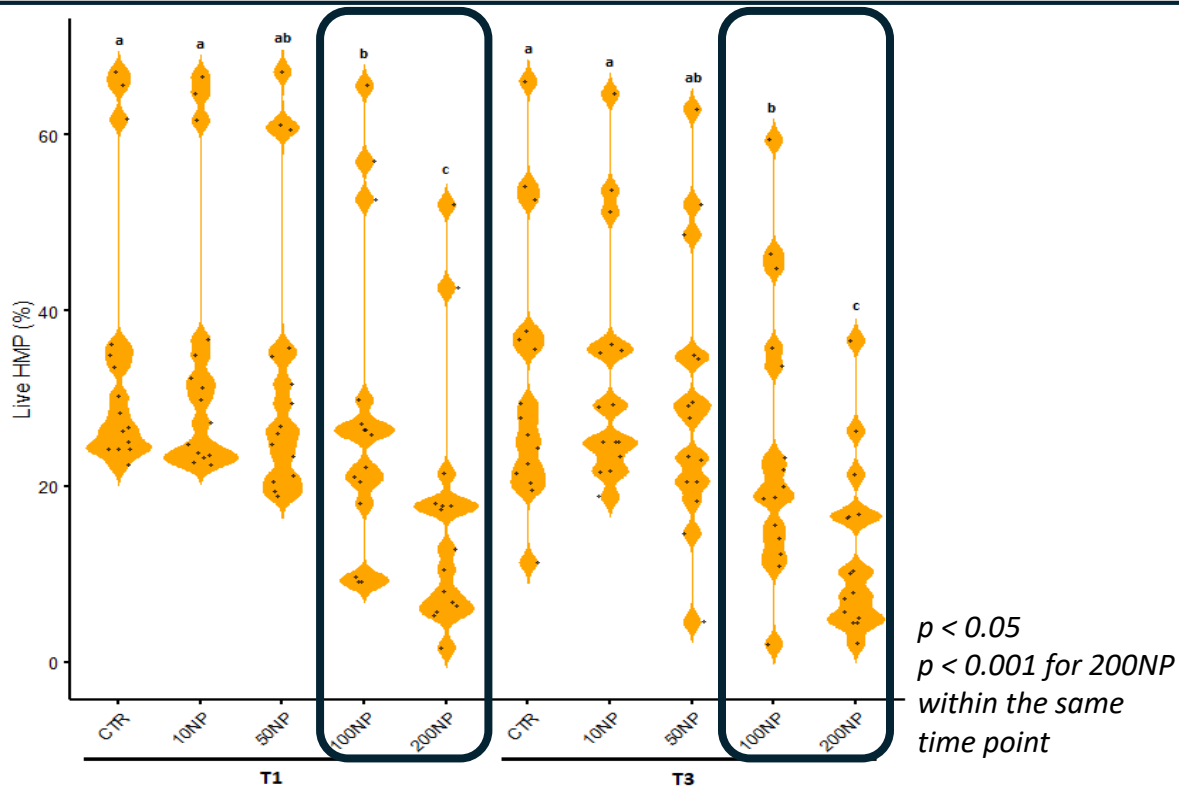


- ✓ Marked reduction in sperm survival
- ✓ Synergistic effect over time

↓ Total motility
Progressive motility

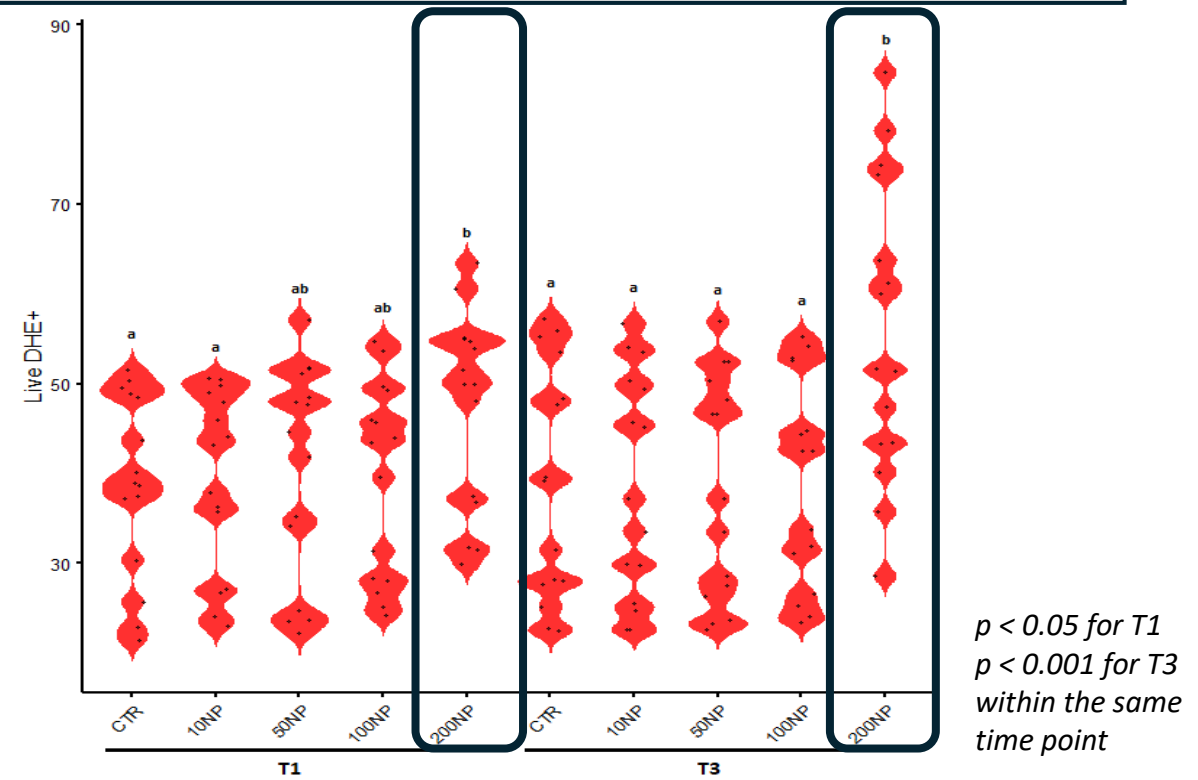


MITOCHONDRIAL ACTIVITY



Significant reduction in % of live sperm exhibiting high mitochondrial membrane potential

ROS PRODUCTION



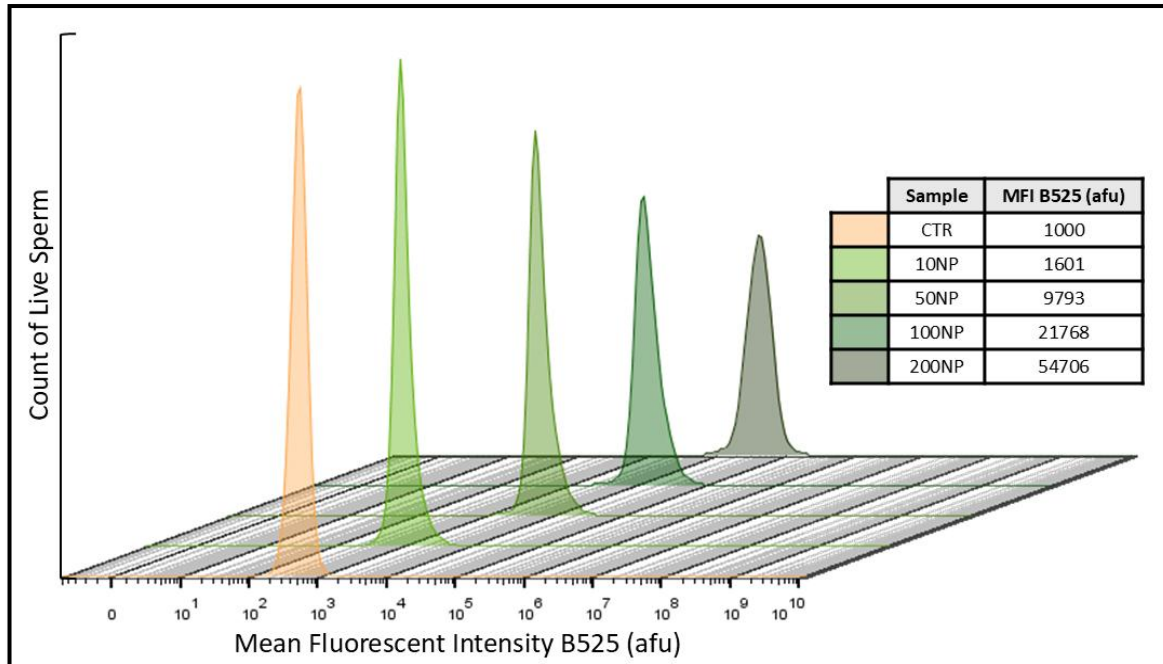
Marked increase in Superoxide ion concentration

*no significant differences in Hydrogen Peroxide concentration

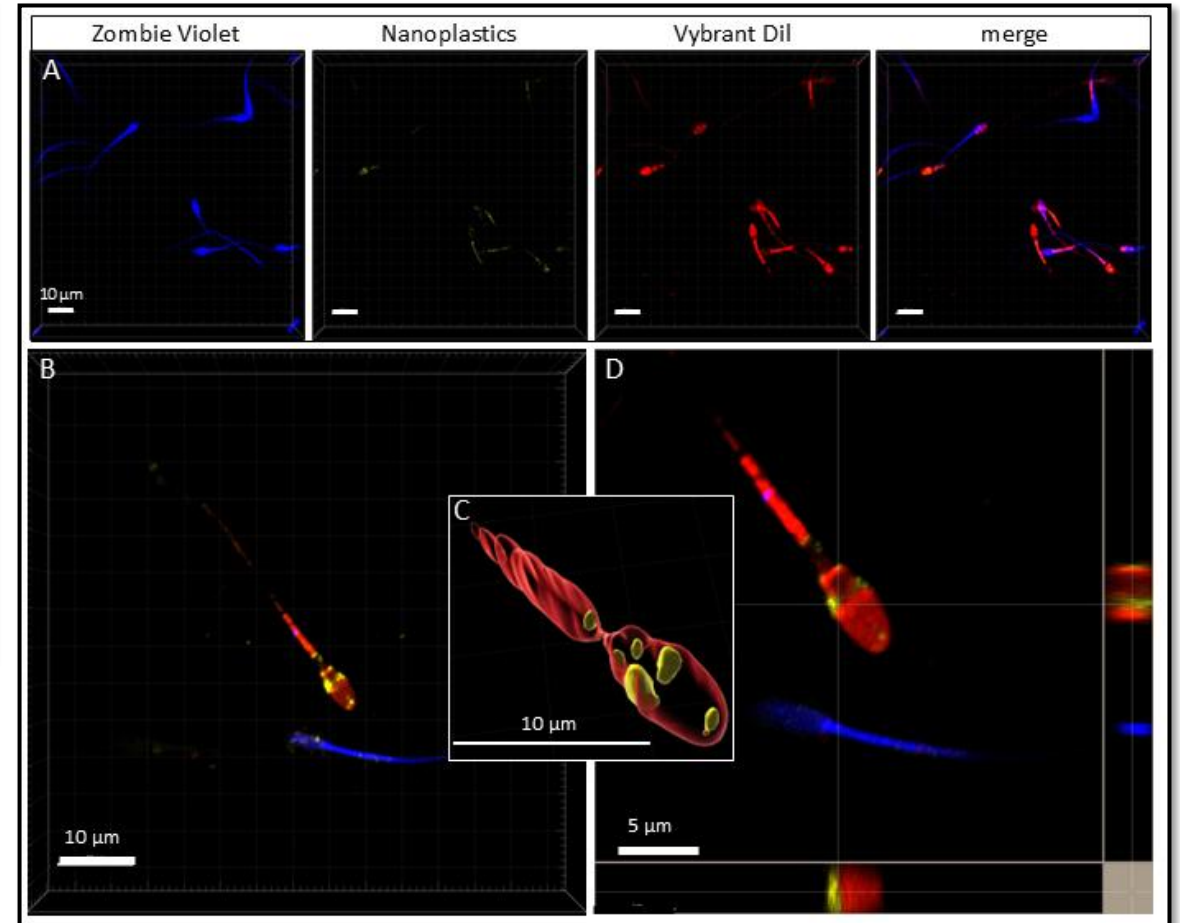
RESULTS



NP INTERNALIZATION



Sperm internalize NP in a dose-dependent manner, with a higher concentration observed in the **post-acrosomal** and **midpiece** regions



RESULTS



PROJECT 2

Research in Veterinary Science

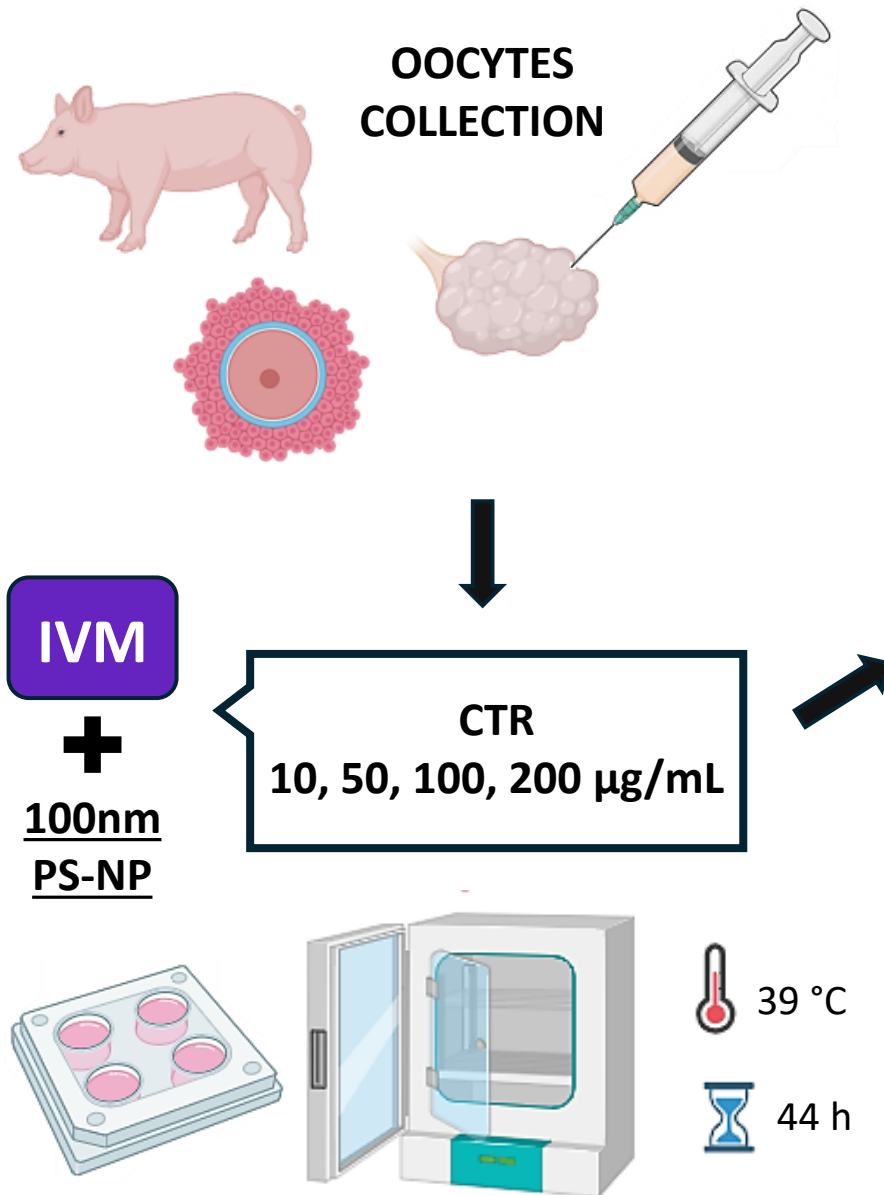
EFFECT OF POLYSTYRENE NANOPLASTICS ON IN VITRO MATURATION OF PIG CUMULUS-ENCOSED OOCYTES

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Section/Category:	Physiology
Keywords:	oocyte; nuclear maturation; cytoplasmic maturation; oocyte developmental competence; ROS; cumulus cells steroidogenesis
Corresponding Author:	Marcella Spinaci University of Bologna Department of Veterinary Medical Sciences Ozzano Emilia (Bologna), Bologna ITALY
First Author:	Marcella Spinaci
Order of Authors:	Marcella Spinaci Sofia Dindo Nadia Govoni Laura Tovar Alessandro Marino Volsa Cinzia Cappannari Diego Bucci Jose Manuel Ortiz-Rodriguez

**UNDER
REVIEW**



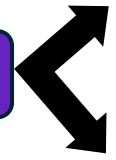


NUCLEAR MATURATION

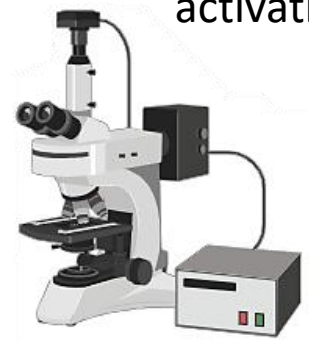


CYTOPLASMIC MATURATION

IVF
with frozen-thawed
semen



GSH and ROS levels



Parthenogenetic
activation



CellTracker Blue
H2DCFDA

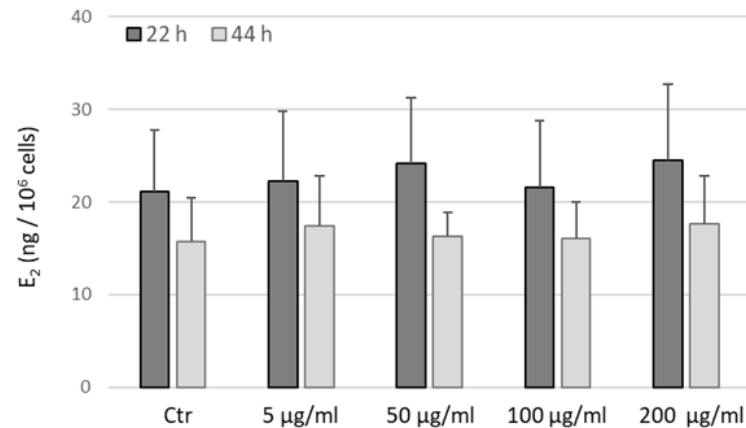
CCs STEROIDOGENESIS

Progesterone (P4)
estradiol-17 β (E2)

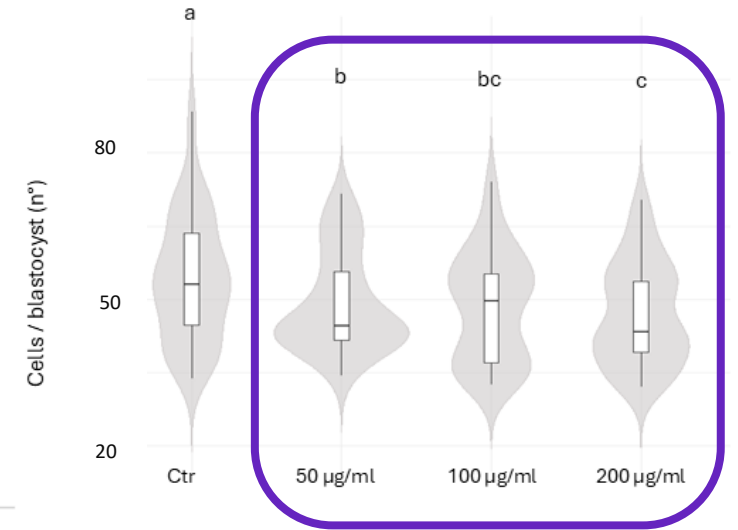
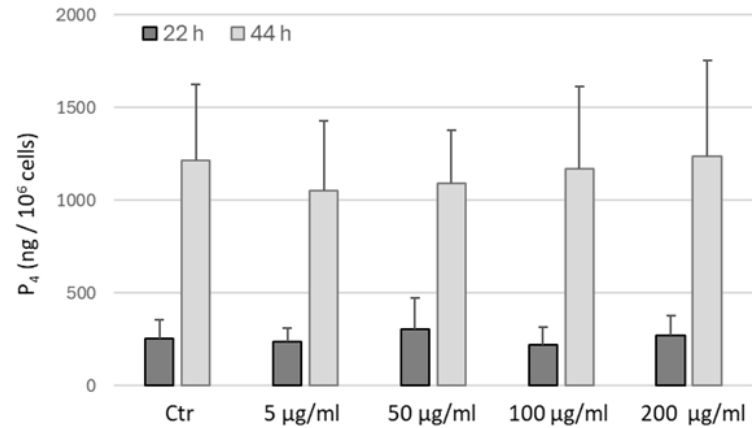
NUCLEAR and CYTOPLASMIC MATURATION, E2 & P4 levels

No influence:

- ✓ % MII
- ✓ % fertilized oocytes
- ✓ Cleavage rate, Blastocyst rate



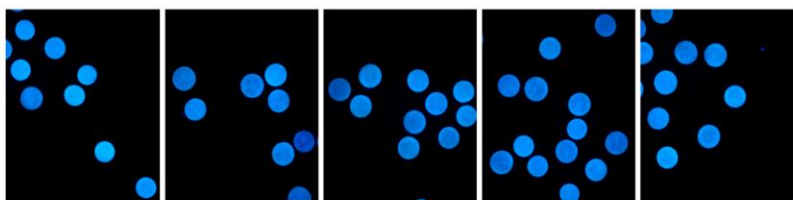
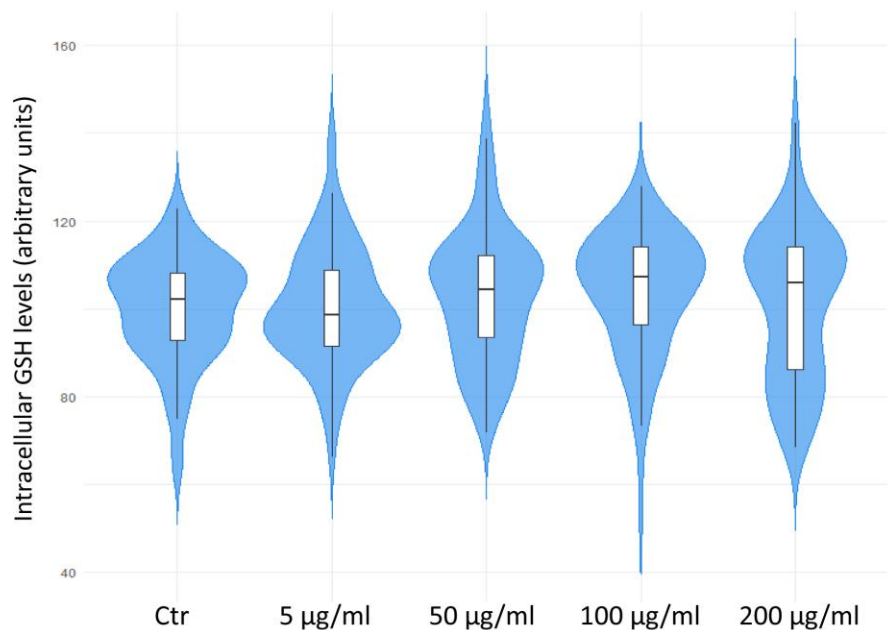
Dose dependent significant decrease in the mean number of blastomere per blastocyst



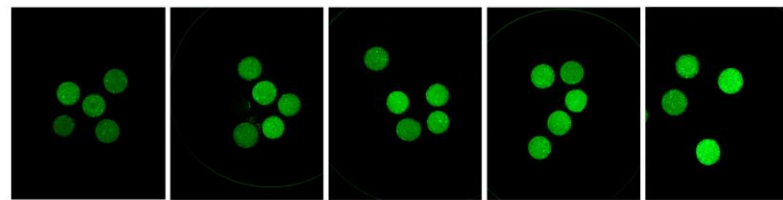
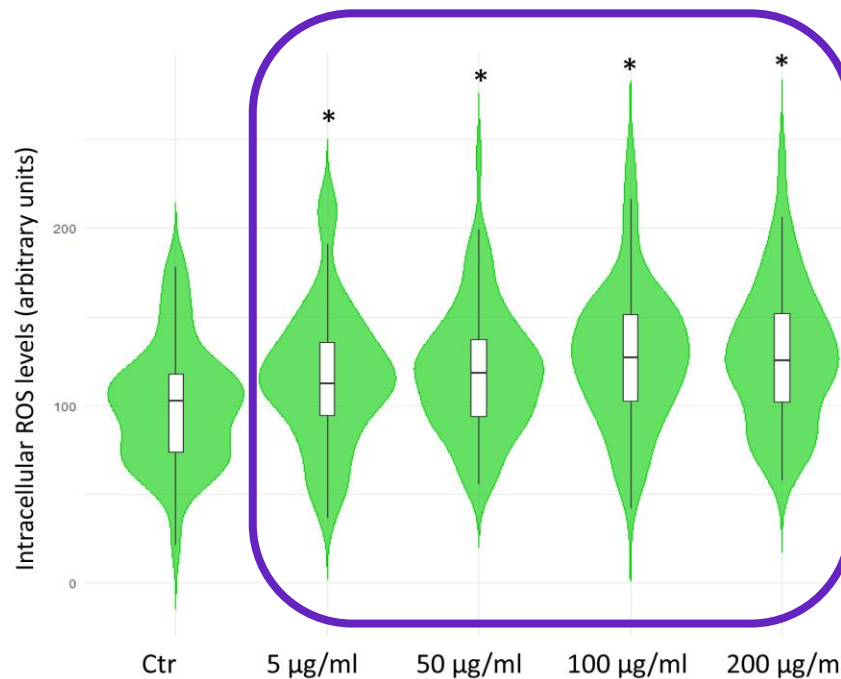
No influence on E₂ and P₄ levels



GSH and ROS levels



CellTracker Blue



H2DCFDA

Increased intracellular ROS levels in exposed oocytes ($p < 0.01$)

No influence on GSH

RESULTS



What is the role of cumulus cells in the uptake of NP?

WORK IN PROGRESS

Ctrl

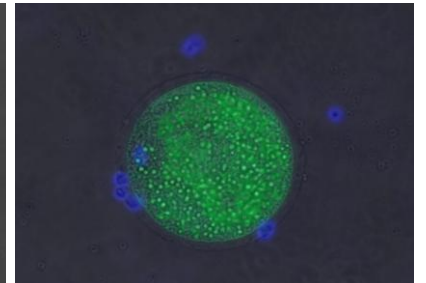
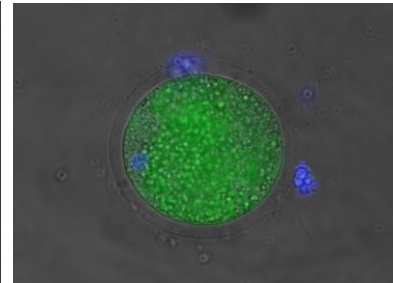
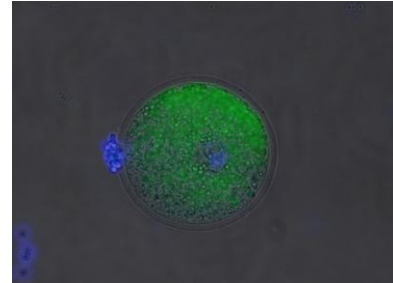
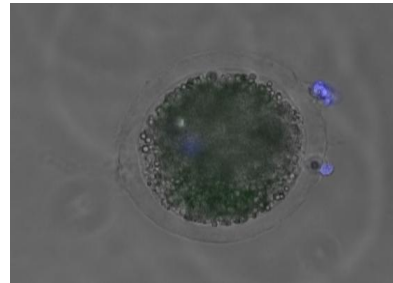
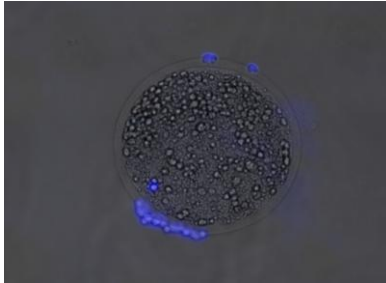
5 $\mu\text{g/ml}$

25 $\mu\text{g/ml}$

50 $\mu\text{g/ml}$

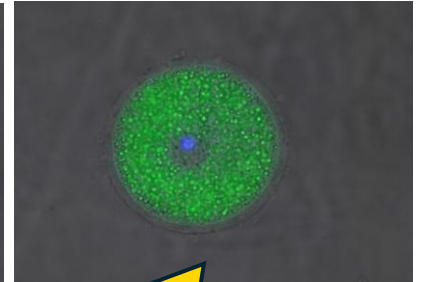
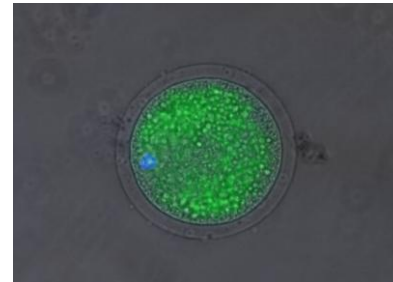
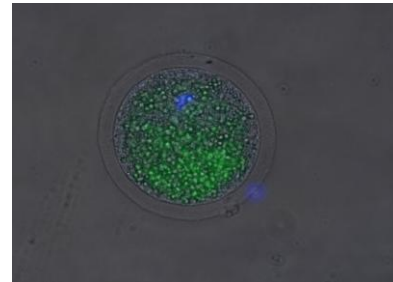
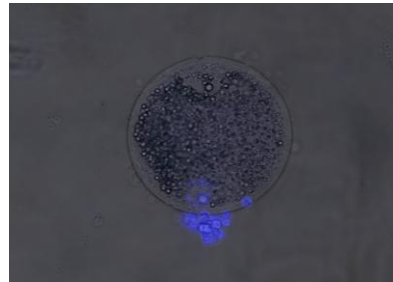
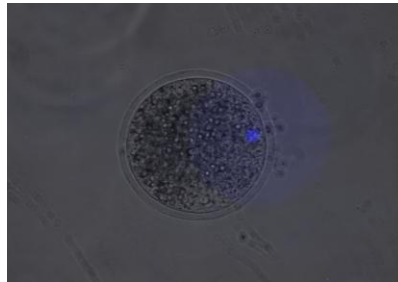
100 $\mu\text{g/ml}$

DENUDED OOCYTES



4 h
IVM

CUMULUS ENCLOSED OOCYTES



30 nm Fluorescent
yellow-green PS-NP

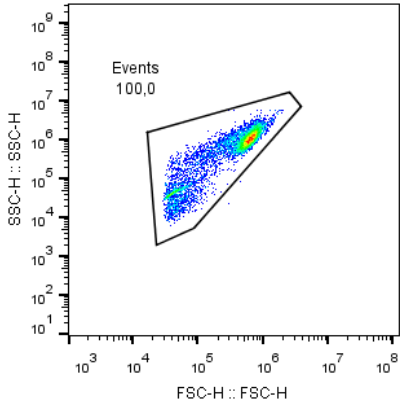
RESULTS



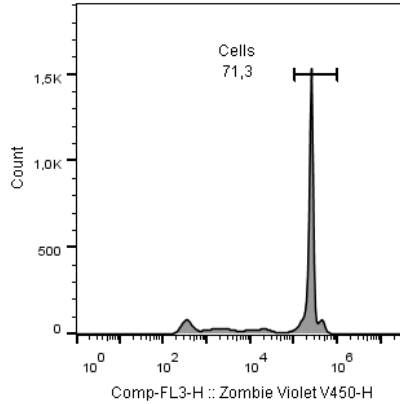
CUMULUS CELLS – IVM 44h

WORK IN PROGRESS

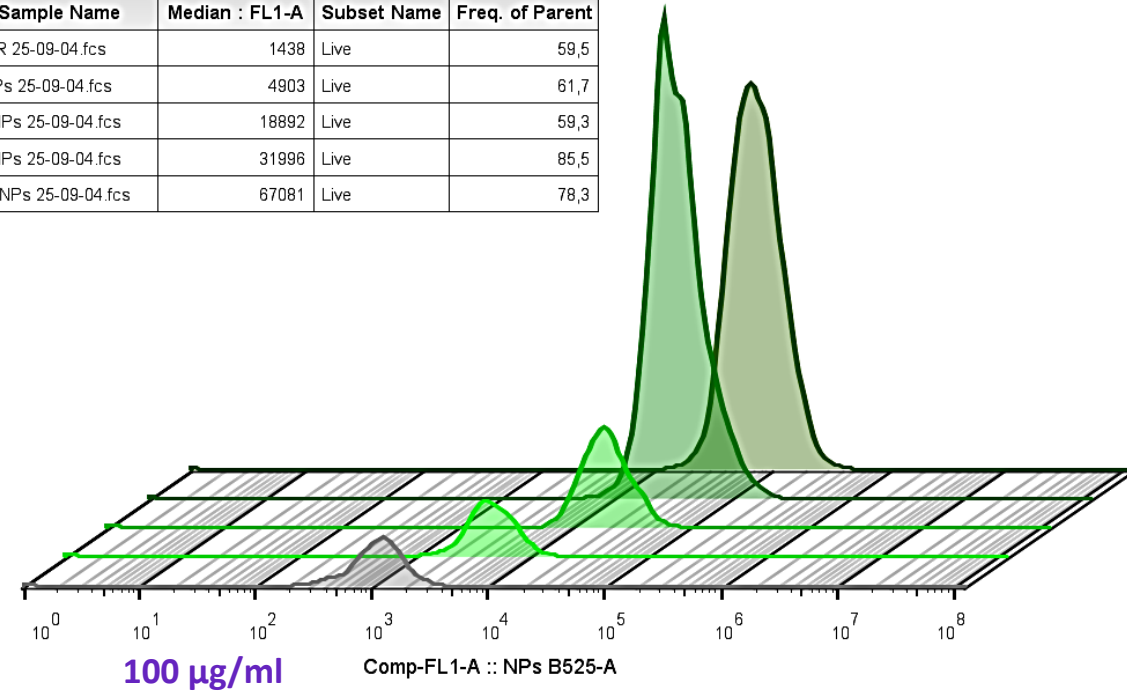
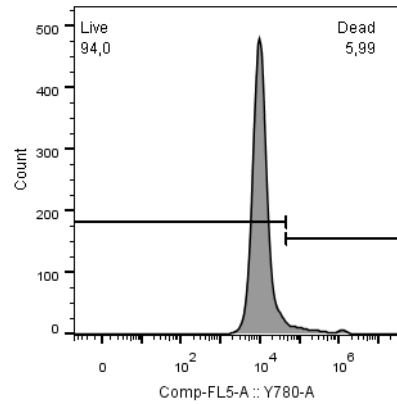
Sample Name	Median : FL1-A	Subset Name	Freq. of Parent
CTR 25-09-04.fcs	1438	Live	59,5
5NPs 25-09-04.fcs	4903	Live	61,7
25NPs 25-09-04.fcs	18892	Live	59,3
50NPs 25-09-04.fcs	31996	Live	85,5
100NPs 25-09-04.fcs	67081	Live	78,3



Cells marker (Hoechst 33342)



Cells viability (Draq7)



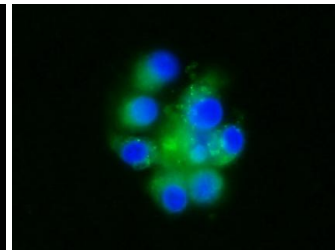
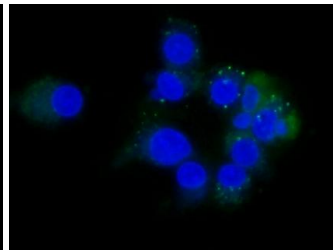
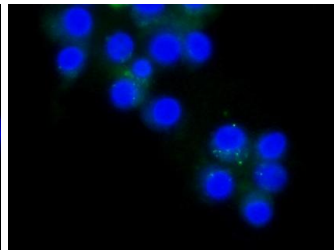
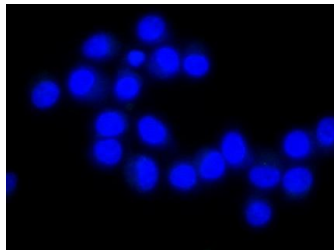
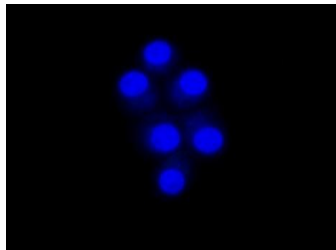
Ctr

5 µg/ml

25 µg/ml

50 µg/ml

100 µg/ml



**30 nm Fluorescent
yellow-green PS-NP**

RESULTS



Conclusions

- ✓ Both **stallion sperm** and **pig oocytes** can **internalize PS-NP**
- ✓ PS-NP **negatively** affect stallion **sperm mitochondrial activity**, potentially impairing sperm survival
- ✓ PS-NP cause **oxidative stress** in sperm and oocytes, by **increasing ROS** production and can possibly **interfere with embryonic cell proliferation**
- ✓ Further studies are necessary to explore the consequences of the global spread of NP on reproductive physiology



Project Collaborations

Assessing the impact of seasonal heat stress on sperm cryopreservation in Holstein bulls	Ghent University, Belgium
Seasonal influence on the oxidative status of seminal plasma proteins and semen parameters of Belgian Blue bulls	University of Padova, Italy
The effects of co-incubating spermatozoa with seminal extracellular vesicles (sEVs) from different ejaculate fractions on fertilization, early embryo quality, and development	University of Murcia, Spain
Use of natural extracts as a substitute for antibiotics in preserving bull semen	University of León, Spain



Thank you for your attention

sofia.dindo2@unibo.it

