UNIVERSITÀ DEGLI STUDI
DI NAPOLI FEDERICO II

Dottorato in Biologia

PhD Course in Biology

Dottorando / PhD Student

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Titolo / Title

Molecular biomarkers of environmental pollution linked to the reproduction of animal species

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Anno / Year

2021/2022

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Introduction

Environmental pollution: what is it?

"Introduction of contaminants into natural environments causing negative changes".

This definition traces the perimeter of a problem that is widespread globally, the common denominator of both more industrialised and developing countries. Particular attention is paid to the latter, which in most cases observe environmental regulations less strictly, in favour of an increasingly wild industrial development that is less attentive to the health of the environment and living organisms.

Pollution can take different forms: solid, liquid, gaseous, energetic (radioactivity, heat, sound, light)[1–10], and pollutants may be completely foreign to the environment or already present in it, but to a much lesser extent and with reduced diffusion.

The main forms of pollution include:

- Air pollution:
  - a major problem in large cities and conurbations, where there is a constant release of numerous pollutants into the atmosphere, such as carbon monoxide, ammonia, sulphur dioxide, methane, nitrogen compounds, particulate matter ($PM_{2.5}$, PM_{10}) that are extremely harmful to organisms[11]. Every year there are thousands of deaths due to air pollution[11];
- Light pollution:
  - due to unwanted, incorrectly implemented or excessive anthropogenic light[2];
- Abandoned waste:
  - particularly in large cities and industrialised countries, is a very pressing problem in modern societies. The accumulation and mismanagement of these causes a non-negligible release of pollutants into the environment;
- Noise pollution
  - the propagation of environmental noise, continuous and at high levels, not only causes disturbance, but is able to induce health problems for both humans and animals, leading in some cases to major health problems, such as cardiovascular problems. In animals that use sound for movement and hunting, it can lead to their death[4];
- Pollution from plastics:

        - is likely to be the environmental challenge to be faced and solved in the coming decades. The release of tons of plastic, of all kinds, into the environment causes problems at all environmental levels, from marine to terrestrial ecosystems[5];
    - » Soil contamination:
        - almost exclusively due to human activity, which causes its degradation and contamination, altering the subtle balances within it. The main cause is industrialisation and mining activities, as well as the continuous expansion of concrete and the enlargement of cities, obviously due to the increase in population[10];
    - » Radioactive contamination:
        - can be divided into two groups: natural, in that background radioactivity occurs naturally due to the presence of radioactive elements in nature; and anthropogenic, due to the use of nuclear weapons, industrial processes and the use of radioactivity in medicine[6];
    - » Thermal pollution:
        - means all those processes that lead to the degradation of water quality, resulting in an increase or decrease in environmental temperature. One example is power plants that can discharge heated water into reservoirs, thus leading to a temperature change that impacts the entire ecosystem[7];
    - » Visual pollution:
        - means all anthropogenic constructions that unnaturally alter the landscape, leading to visual degradation and deterioration of the enjoyment of that area[8];
    - » Water pollution:
        - water is a fundamental element for the life of all organisms, and the constant spillage of pollutants into it due to human activity, causes a noticeable alteration that that impacts its inhabiting organisms. In addition, pollution of groundwater and water bodies is also a serious problem for the health of humans, who very often use these same waterways as their primary source of food[9];

Therefore, pollution, in whatever form it occurs, is a serious problem for the health of ecosystems and the organisms that live and interact in them. The *Earth system* can be divided into five so-called *spheres:* the *biosphere* (living

beings), the *atmosphere* (the air), the *lithosphere* (the land), the *cryosphere* (the ice) and the *hydrosphere* (the water)[12,13]. These five compartments are in harmony with each other, a delicate balance that is increasingly being put under pressure by human activity. The constant release of pollutants into it undermines this harmony between the compartments, causing an imbalance that affects both the health of the environment and the organisms that live there, including humans.

Heavy metals

Some of the most widespread and most persistent pollutants in the environment are the so-called *heavy metals*. There is no common definition of a *heavy metal*, as this covers a wide variety of elements of the periodic table and their compounds.[14]However, an element with a density greater than 5 g/cm^3 [15] is generally considered *a heavy metal.* In recent years, more and more attempts are being made to replace this unspecific definition, *heavy metal*, with one that best renders this concept, e.g., Potencially Toxic Element(s) (PTEs). This is because so-called heavy metals do not always exhibit the typical characteristics of elements belonging to the metal group[16].

The main problem with these PTEs is their ability to accumulate in any environmental compartment and, consequently, to reach animals and humans through the trophic chain[17–19]. They are able to move with air currents, reaching distances far from the point of release, or be transported by water, as in the case of the release of these elements into the environment through agriculture.

Their bioaccumulation in organisms is capable of causing a variety of problems, ranging from cancer to immune system dysfunctions, neuronal system impairment, behavioural abnormalities, metabolic disorders, damage to specific organs, hormonal alterations, increased ROS and related disorders, and especially reproductive problems[20–22]. Examples of such elements are[12]:

- Titanium (Ti)
- Vanadium (V)
- Chromium (Cr)
- Manganese (Mn)
- Iron (Fe)
- Cobalt (Co)
- Nickel (Ni)
- Copper (Cu)
- Zinc (Zn)
- Arsenic (As)
- Molybdenum (Mo)
- Silver (Ag)
- Cadmium (Cd)
- Tin (Sn)
- Platinum (Pt)
- Gold (Au)
- Mercury (Hg)
- Lead (Pb)

Indeed, these elements are able to bind lipophilic organic molecules, or protein components, such as sulphydryl groups, and are thus able to enter cells using these molecules as transporters[12]. Toxic action, therefore, can be induced in two ways: *directly* and *indirectly*. Directly, by binding to cellular components and causing structural changes; directly affecting their function and thus altering the process in which these molecules are involved. *Indirectly*, by causing the production of radicals (ROS), which are capable of inducing oxidative damage to DNA and lipids, as well as altering the cellular homeostasis of the redox system[12]. The ability to bind specific protein groups or specific molecules, however, is also exploited in a positive manner. This characteristic is applied for detoxification of some of these elements, such as mercury, cadmium and lead, through the use of peptides containing thiol groups, the so-called TCPs (thiol-containing peptides), which can form complexes with *heavy metals* and thus act as chelators[23,24].

Model systems

Mytilus galloprovincialis (Lamarck, 1819)

The mussel, *Mytilus galloprovincialis* Lamarck, 1819, Regulation (EC) No 1638/2001 and Regulation (EC) No 216/2009), *is a* bivalve mollusc, one of the most valuable species because of its strong and intense taste, with a significant food and commercial impact. The mussel, widespread in gulfs and along coasts, lives attached to rocks or any fixed substrate in dense aggregates. It is widely found in the Mediterranean Sea, the Black Sea and the eastern Atlantic Ocean, from Morocco to the British Isles. *Mytilus galloprovincialis is* a sessile, filter-feeding organism[25]. Being a filter-feeding organism makes it an excellent bio-accumulator of xenobiotic substances, which are accumulated in tissues[26–28]. Another aspect that makes this organism an important object of study is the type of fertilization. Mussels are animals with separate sexes in which fertilization is external. The females produce a substance in their ovaries that, when dispersed in the seawater, triggers ejaculation in neighbouring males and, in turn, the sperm ejaculated in the water triggers egg-laying in the females. In this way, the release of gametes occurs directly into the seawater, resulting in their direct exposure to any contaminants that are present[26].

Mytilus galloprovincialis has a two-stage reproductive cycle, with a resting period followed by a period of gamete production and release. Specifically, sperm production begins at around the end of September - beginning of October, when the temperature of the sea water begins to drop. A first release of gametes into the water occurs between December and January, thus leading to an emptying of the gonads and the start of a new gamete production, with a subsequent release of gametes in about March. A final release of gametes occurs around the beginning of summer before of the gonad resting

phase. In this refreshment phase, the germ cells give way to adipogranular cells and connective tissue cells, which will be the majority component until the start of new gamete production[29]. The spermatozoon of *Mytilus galloprovincialis* belongs to the group of spermatozoa defined as *ect-aquasperm*[30]. These are spermatozoa that are released free in water[30] with an overall length of about 60 µm. The size of the head averages 5 µm in length and about 2 µm in width, with an acrosome that can reach 5 µm[31–33]. The spermatozoon has a single ring of five mitochondria, located below the nucleus, of spherical shape[32].

After being released into the water, the spermatozoon is able to fertilize the egg cell, which is also released into the seawater, following chemo-attractant signals, which push the spermatozoon in the right direction, until fertilization[34].

The high degree of chromatin compaction in the sperm head is achieved by the presence of highly basic proteins belonging to the *Sperm Nuclear Basic Protein* (SNBPs) family, named Protamine-Like (PL): PL-II, PL-III and PL-IV. These proteins represent approximately 76% of the nuclear basic protein component of the sperm chromatin of mussel and coexist with 20% core histones and 4% non-histone proteins[25,28,35–39]. Evolutionarily, PLs belong to the Protamine-Like type (PL-type) group, intermediate group between the Histone-type (H-type) and Protamine-type (P-type). To the latter group belong mammalian protamines [40–42], such as the human protamines (PRM 1 and PRM 2).

The main difference between the different SNBPs is the content of basic amino acids, arginines and lysines[42] are the following:

- Histone type (H-type): 2-6% arginine; 25-30% lysine
- Protamine-Like type (PL-type): 8-30% arginine; 30-40% lysine
- Protamine type (P-type): 30-70% arginine

This different amino acid composition is also reflected in the ability to structure chromatin. In fact, the presence of P-types ensures greater DNA compaction than PL-types, which in turn allow greater DNA compaction than histones (H-types)[42].

PLs are extremely important for the proper chromatin compaction of mussel spermatozoa, but are particularly susceptible to external stresses, such as presence of PTEs[26,28,35–39,43]. Alteration of these proteins, which are crucial for the proper chromatin compaction of mussel spermatozoa and, consequently, for their correct gametic function, may certainly prove to be an important factor in mussel fertility problems. In fact, in addition to being an organism that is widely used in biomonitoring programs, *Mytilus galloprovincialis* is a widespread food source in many countries bordering the Mediterranean Sea, and beyond. Thus, this is not only an environmental concern, which is certainly of weight, but also an economic issue directly related to the reproduction and then the food production of this mollusc[44].

Meiosis is an important stage in the formation of gametes; it is a process that ensures diversity in the population and, above all, allows correct separation of homologous chromosomes during the process of cell division. Correct separation of the chromosomes is a sign of good fertility and avoids future post-fertilization problems[45]. In fact, aneuploidies are a major cause of miscarriage, congenital problems, and embryonic developmental disorders[45,46].

Aneuploidies

Human aneuploidies consist of an alteration in the number of chromosomes normally present in diploid (2n) cells. There can be several underlying causes.

Problems related to embryonic development, after fertilization of the egg cell by the spermatozoon, can depend on both the egg cell and the spermatozoon. In particular, the male contribution to infertility accounts for about half of the total infertility cases in couples and about 7% of men are infertile[47]. Very often male infertility has unknown causes that cannot be directly identified, so-called *idiopathic* infertility[48]. Just as often, however, this idiopathic infertility appears to have genetic causes, particularly linked to the Y chromosome[49,50]. In fact, another of the major causes of male infertility is linked precisely to gene problems, such as translocations, deletions and incorrect chromosome number[48] which therefore lead to aberrant spermatogenesis and consequently the impossibility of fertilization.

Sertoli cells

Sertoli cells form the so-called *blood testis barrier* (BTB), isolating the inside of the seminiferous tube. Disruption of this barrier causes germ cells to shut down and the opening of this barrier is finely regulated in favour of the immune system. Sertoli cells occupy a volume of about 20% inside the seminiferous tubes and produce and release large amounts of lactate, the main form of energy they release for germ cell maintenance.

Anatomically, human spermatozoa are produced inside the testicles, which perform two main functions: synthesis of steroid hormones and sperm production[51]. The process of spermatogenesis takes place in the seminiferous tubes, starting with diploid cells. Inside the seminiferous tubes are germ cells and Sertoli cells, which help and support the development of spermatozoa[52]. In the interstitial space, on the other hand, are Leyding cells, for the production of hormones, and blood vessels for the transport of nutrients. In short, spermatogenesis begins with *spermatogonia A*, present on the inner basement membrane of the seminiferous tubes and representing the stem cells from which *spermatogonia B* constantly originate. From a genetic point of view, spermatogonia are cells with a diploid inheritance[53]. From these originate the *primary spermatocytes* that enter into meiosis giving rise to the *secondary spermatocytes* that at the end of meiosis give rise to four haploid *spermatids*. The latter undergo maturation until the complete formation of the

mature spermatozoon, which assumes its classical structure: head, neck and tail (Figure 1)[51,54,55].

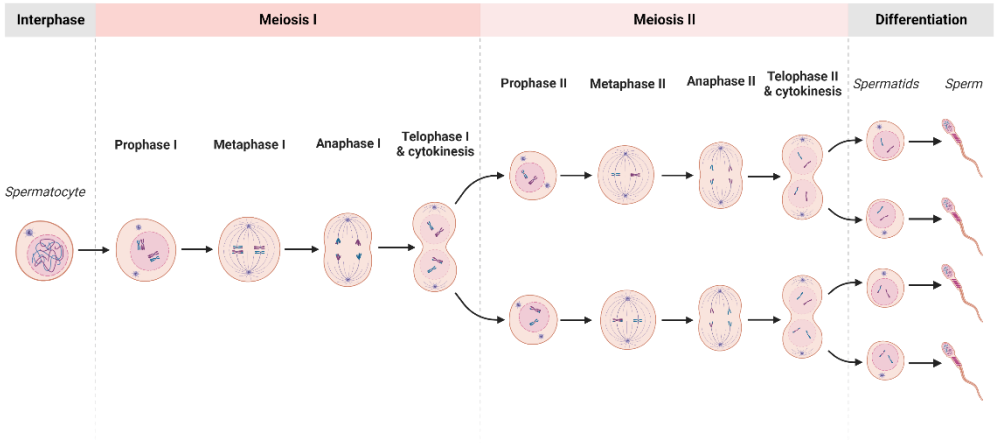

Figure 1 Meiosis process of human spermatocytes

Throughout this process, Sertoli cells play a very important role in supporting sperm development by providing nutrients and energy to the germ cells for their proper functioning. In fact, failure to supply nutrients and energy to these cells triggers programmed germ cell death through cell apoptosis[51].

At the end of the process of spermatogenesis there is a mature spermatozoon, a highly specialized and differentiated cell[56] composed of three parts:

- Head: has a size of approximately 5 µm in length and approximately 3 µm in width. Two regions can be identified: the anterior portion, consisting of the acrosome, and the posterior portion, where the nucleus is contained, which exhibits very high chromatin compaction [56] by typical spermatozoon proteins, the protamines (PRM)[57]
- Neck (intermediate region): is the part that connects the head to the tail. There are two centrioles, one proximal and one distal. They perform two important functions: first they give rise to the flagellum for the movement of the spermatozoon, and second, one of the two centrioles is transferred during fusion with the egg cell and will give rise to the centrosome and microtubules of the zygote[58]. In addition, this portion of the spermatozoon contains the mitochondria that generate the energy required for the movement of the sperm
- Tail: can be about 50 µm long and has a typical 9+2 axoneme[56] and is the structure that enables sperm movement

As in the case of spermatozoa of other species, one of the peculiarities of the human spermatozoon is the high degree of chromatin compaction within the head[59,60]. This compaction is made possible by the presence of specific highly basic proteins that represent the major protein component of sperm chromatin: protamines (PRM). The human sperm nucleus contains two types of protamines: protamine 1 (P1), encoded by a single-copy gene, and the protamine 2 (P2) family of proteins (P2, P3 and P4), also encoded by a single gene that is transcribed and translated into a precursor protein. There are two human protamines, encoded by separate single genes[61]:

- PRM 1: 51 amino acids and a mass of 6823 Da. It is synthesised directly as a functional isoform and has no other isoforms
- PRM 2: 102 amino acids and a mass of 13051 Da. It is synthesized as a precursor (pre-P2) and later matured into several isoforms (HP2, HP3, HP4). The most common isoform is HP2[62]

An important aspect to consider when dealing with protamine-induced sperm DNA compaction is the possible retention of histones and an incorrect histone-protamine transition[63]. During spermatogenesis, the core histones are replaced by the TP1 and TP2 transition proteins (TPS), then the transition proteins (TPS) are replaced by testis-specific protamines[64]. Transition proteins have the following characteristics:

- TP1: 55 amino acid residues; 6424 Da; with 18% lysine and 18% arginines [65,66]
- TP2: 138 amino acid residues; 15641 Da; with about 10% lysine, 10% arginines and 5% cysteines[65,67]

Thus, these proteins have a basicity intermediate between histones and protamines[65] and their proper functioning is associated with problems in sperm development and reduced sperm motility[63,65]. Their function, however, is still not entirely clear; there is a great deal of information on the subject in the literature and knowledge here is not yet exhaustive[68,69]. However, at the final phase of spermiogenesis in humans, spermatozoa still contain approximately 15% histones[57]. Because of histone retention in mice and humans[70] histone-bound DNA compared with protamines is less condensed and paternal genes can easily interact with transcription factors. On the other hand, sperm DNA is more vulnerable to environmental stressors in the decondensed state because the condensed sperm head protects the DNA from environmental damage. In addition, H4 hyperacetylation of core histones plays a significant role in histone-protamine exchange. Indeed, epigenetic modifications and histone variants are

involved in the histone-protamine transition[71]. What is clear, however, is the possibility of perturbation of this important process, essential for proper sperm development, by external factors, environmental pressures[72]. Indeed, some heavy metals are inhibitors of histone acetylases, key enzymes in the histone-protamine transition[73] others can bind histones or protamines, leading to alterations in their function[74–76]. The high compaction of sperm chromatin by protamines plays an important role in protecting DNA from both external and internal agents such as ROS[77]. In fact, given the important role that this cell must play, as the carrier of genetic material for zygote formation, together with the egg cell, its DNA must be intact and free of any alteration. One of the main causes of male infertility, is DNA oxidative damage[77]. ROS are important messengers in several processes affecting the spermatozoon, such as cell capacitation or apoptosis[78]. One of the main sources of ROS, however, is indeed the spermatozoon, because of the presence of mitochondria that allow its movement; the second main source of ROS, on the other hand, is the presence of active leucocytes in the semen [79,80]. These sources of ROS are capable of inducing an imbalance in the cell's redox system. Thus, oxidative stress is defined as when the production of ROS and the antioxidant capacity of a cell undergo an imbalance in favour of ROS[77]. The causes of this imbalance are many, and range from lifestyle, diet, genetic issues and pollution[77]. Protamines are also one of the most variable proteins found in nature, with data supporting positive Darwinian selection. In humans, alterations in the expression of protamines P1 and P2 have been found to be associated with infertility. Mutations in protamine genes have also been found in some infertile patients. Transgenic mice defective in protamine expression also have several structural defects in the sperm nucleus and show varying degrees of infertility. There is also evidence that altered levels of protamines can result in increased susceptibility to sperm DNA damage, causing infertility or poor outcomes in assisted reproduction.

It is the environment that very often plays a negative role on human reproductive health and semen quality , leading more frequently to reproductive and infertility-related pathologies[77,81–85]. In fact, as previously mentioned, among the pollutants capable of producing strong oxidative stress are PTEs, which are known to negatively affect the quality of semen (sperm count, motility, viability, hormonal imbalance)[86].

AIM OF THE THESIS

The aim of this thesis was to study the effects at the molecular level of certain *potentially toxic elements* (PTEs), on reproductive health in two model systems, the marine organism, *Mytilus galloprovincialis,* and human spermatozoa, with the objective of finding new molecular biomarkers of environmental pollution related to reproduction. The analyses conducted on *Mytilus galloprovincialis*, were performed by tank exposures with artificial seawater for 24 h at 18 °C, of PTE at concentrations similar to those found in the Mediterranean Sea. Mussels exposed and not exposed to PTEs were then used for subsequent analysis, both at a protein and gene level, within the reproductive sphere[26,37–39]. Specifically, the impact of some PTEs on Protamine-Like (PL), and in particular on their electrophoretic pattern on both urea acetic acid polyacrylamide gels in (AU-PAGE) and SDS-PAGE, was evaluated. In addition, their DNA-binding ability was evaluated by EMSA assays and their ability to protect DNA under pro-oxidation conditions. Fluorescence analyses with an ANS (8-anilinonaphthalene-1-sulphonic acid) probe and light scattering studies with CG-MALS (Composition Gradient- Multiple angle light scattering) were then conducted to assess how some of these PTEs are able to alter the conformation of PLs and their interaction with DNA or to form PL aggregates.

These experimental approaches, together with others, have also been used to investigate human reproductive health by assessing possible differences, particularly of PRM properties, in individuals residing in low and high environmental impact areas in various parts of Italy. Again, the status of PRMs was evaluated with particular attention to the protamine-histone ratio, their ability to bind DNA, their ability to protect DNA under pro-oxidative conditions or to be involved in oxidative DNA damage. Specifically, the areas examined were: *Terra dei Fuochi* (*Land of Fires,* Campania region) and *Valle del Sacco* (*Valley of Sacco rivers*, Lazio region), for high environmental impact; *Valle del Sele* (Campania region) for low environmental impact[81,82].

So, in detail, the precise objectives of this PhD thesis work were:

Mytilus galloprovincialis

- To define the impact of mercury on the properties of *Mytilus galloprovincialis* Protamine-Like (PL) proteins
- To define the potential effects of mercury on the reproductive system of *Mytilus galloprovincialis*
- To identify new possible reproductive-related markers of environmental pollution

Human

- » To study the impact of environment on human reproductive health using molecular biology approaches
- » To identify possible molecular alterations in individuals living in high environmental impact areas, compared to individuals living in low environmental impact areas
- » To identify a possible correlation between environment and human reproductive health

MAIN RESULTS OF THE THESIS

Mytilus galloprovincialis

In vivo studies conducted on *Mytilus galloprovincialis* have shown that despite the high importance of PLs in the proper sperm chromatin compaction, these proteins are actually very susceptible to external stressors, which can alter their characteristics and functionality[37–39]. Analyses using a fluorescent probe, ANS (8-anilinaphthalene-1-sulphonic acid), showed a clear alteration in the emission spectrum of ANS in the presence of PLs from mussels exposed to the three mercury concentrations (1, 10 and 100 pM $HgCl_2$) compared with PLs from unexposed mussels. This change in the emission spectrum of the ANS is indicative of a conformational change in the PLs of mussels exposed to mercury. Indeed, ANS is able to bind to apolar regions present on the surface of proteins. The increased fluorescence of the PLs of mussels exposed to mercury indicates an increase in apolar regions on their surface, which results in a conformational change in the PLs[37]. Given these conformational changes of the PLs, their DNA-binding capacity was evaluated by electrophoretic mobility shift assay (EMSA), which showed the inability of PLs from mercury-exposed mussels to bind DNA, compared with the non-exposed condition[38]. Further validation of the inability of PLs from mercury-exposed mussels to bind DNA was obtained through DNA absorption spectra in the presence of increasing amounts of PLs. A decrease in the DNA absorption peak was observed only in the presence of PL from mussels not exposed to mercury, implying that only these proteins were able to bind DNA[37]. The next step was to evaluate the state of sperm chromatin, by means of digestion kinetics with micrococcal nuclease, after exposure of the mussels to mercury. The results showed an increase in the sperm chromatin accessibility already after 5 min of digestion in the case of mercury-exposed mussel spermatozoa, compared with the no-exposure condition[39]. Further experiments were conducted to evaluate the DNA-protective capacity of PL from mercury-exposed mussels under pro-oxidant conditions. For all mercury exposure conditions, PLs were not able to protect DNA, compared to the non-exposure condition[38]. Next, at the gene level, three stress genes were evaluated by RT-

PCR on gonadal tissue: *hsp70*, *gst* and *mt10*. The analysis showed a general hypo-expression of these genes in the gonad, indicative of gonadal stress after exposure of mussels to mercury[38]. For this reason, a morphological analysis of the gonads of mussels exposed and unexposed to mercury was carried out, with haematoxylin-eosin staining. The results showed the appearance of vacuolization of gonadal tissue as the mercury concentration increases, particularly at the exposure dose of 100 pM $HgCl_2$[39]. Finally, immunohistochemical analysis on gonads showed a decrease in the signal of two enzymes involved in hormone synthesis, 3β-HSD (3 β-Hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase type 1) and 17β-HSD (17 β-Hydroxysteroid dehydrogenases), as the concentration of mercury exposure increased, with an almost complete disappearance of the signal on the gonads of mussels exposed to 100 pM $HgCl_2$[39].

The results summarized in this section can be found in more detail in the following publications:

- Ref. 37: Lettieri, G. et al. New Insights into Alterations in PL Proteins Affecting Their Binding to DNA after Exposure of *Mytilus galloprovincialis* to Mercury-A Possible Risk to Sperm Chromatin Structure? Int J Mol Sci 22, 5893 (2021)
- Ref. 38: Lettieri, G. et al. Spermatozoa Transcriptional Response and Alterations in PL Proteins Properties after Exposure of *Mytilus galloprovincialis* to Mercury. Int J Mol Sci 22, 1618 (2021)
- Ref. 39: Lettieri, G. et al. Morphological, Gene, and Hormonal Changes in Gonads and In-Creased Micrococcal Nuclease Accessibility of Sperm Chromatin Induced by Mercury. Biomolecules 12, 87 (2022)

Homo sapiens

The comparison between individuals residing in low environmental impact areas (*Valle del Sele*) and individuals residing in high environmental impact areas (*Land of Fires*), revealed how the environment can play an important role in human reproductive health. Specifically, the evaluation of the electrophoretic pattern of sperm nuclear basic proteins (SNBPs), protamines and histones, by urea acetic acid polyacrylamide gels (AU-PAGE), revealed that in the case of the group of individuals residing in the high environmental impact area, there was a predominant percentage of alteration in the ratio of protamines/histones, which under normal conditions is about 85% protamines and 15% histones. Furthermore, not only the electrophoretic pattern showed an alteration in the protamine/histone ratio, but in the group of individuals residing in the high environmental impact area, there was an alteration in the ratio of

the same protamines, P1 and P2, which in normal situations falls in the range between 0.8 and 1.2. Within the group of individuals residing in the high environmental impact area, the samples were classified into three distinct groups, based on the electrophoretic pattern observed in AU-PAGE, and for each of these groups, the ability of SNBP to bind DNA was assessed, using electrophoretic mobility shift assay (EMSA):

- » only Histones: samples with an electrophoretic pattern composed mainly of histones, which show low affinity for DNA binding in EMSA
- » Not canonical protamines/histones ratio: samples with an altered protamines/histones ratio, requiring more protein to achieve DNA saturation in the EMSA
- » Canonical protamines/histones ratio: samples with a correct protamine/histone ratio, but still showing a different ability to bind DNA in EMSA, compared with samples having a canonical protamine/histone ratio from the group of individuals residing in the low impact area

Furthermore, PTEs accumulation analyses conducted by ICP-MS revealed the excess presence, in the semen of individuals residing in the high environmental impact area of elements such as chromium and copper, which can generate oxidative stress and induce DNA breakage. For this specific reason, the involvement of SNBPs in oxidative DNA damage under pro-oxidative conditions was assessed for residents in the two areas under study. The results show that in the case of the group of individuals residing in the high environmental impact area, there was a marked ability to induce oxidative DNA damage[82]. In addition, our studies have shown that environmental pollutants in high-impact areas may also have transgenerational effects[87].

A further study, conducted on another area of high environmental impact, *Valle del Sacco*, focused instead, mainly on contaminants other than PTEs, specifically the so-called VOCs (volatile organic compounds, or VOCs), which are particularly widespread in this area due to the numerous chemical industries in the area. Among the main VOCs found in samples from *Valle del Sacco* are 3-methyl-1-butanol, acetone and fluoren-9-ol, 3,6-dimethoxy-9-(2-phenylethynyl). Analysis of the spermiogram of subjects resident in the *Valle del Sacco* showed a significant decrease in sperm concentration and total motility, as well as severe morphological abnormalities of the sperm head, tail and neck. This finding also agrees with a higher percentage of samples containing only histones in the spermatozoa, as shown in AU-PAGE, in subjects from the high environmental impact zone compared with the low environmental impact control area.

EMSA, to assess the ability of SNBPs to bind DNA, showed that SNBPs from *Valle del Sacco* subjects have a low DNA-binding affinity and therefore do not allow DNA saturation, regardless of their composition in protamines and histones, unlike the proteins from control subjects that are able to completely saturate DNA with a protein-to-DNA ratio of about 1.0[81].

The results obtained demonstrate that evaluation of the spermiogram alone is not always sufficient to define the fertilising capacity of a semen and that molecular analyses are therefore needed to detect more subtle alterations at the level of SNBPs and DNA related to semen quality and how the environment can play an important role on human reproductive health.

The results summarized in this section can be found in more detail in the following publications:

- Ref. 81: Perrone, P; Lettieri, G et al. Molecular Alterations and Severe Abnormalities in Spermatozoa of Young Men Living in the 'Valley of Sacco River' (Latium, Italy): A Preliminary Study. Int J Environ Res Public Health 19, 11023 (2022)
- Ref. 82: Lettieri, G. et al. Discovery of the Involvement in DNA Oxidative Damage of Human Sperm Nuclear Basic Proteins of Healthy Young Men Living in Polluted Areas. Int J Mol Sci 21, E4198 (2020)
- Ref. 87: Lettieri, G. et al. Molecular Alterations in Spermatozoa of a Family Case Living in the Land of Fires. A First Look at Possible Transgenerational Effects of Pollutants. Int J Mol Sci 21, E6710 (2020)

CONCLUSIONS

In conclusion, the main objective of the research was to study environmental pollution and how it may affect the reproductive health of organisms. To this end, the study used the semen of two model systems, *Mytilus galloprovincialis* and human, with the goal of looking for potential markers and possible correlations between the reproductive system and the environment.

As for *Mytilus galloprovincialis*, despite the crucial role played by PL proteins in sperm DNA compaction, they have been shown to be particularly sensitive to environmental pollution by mercury and other pollutants, so they could be considered promising markers of reproductive fitness in this organism and also of environmental health.

With regard to the study conducted on humans, the environment seems to play an increasingly critical role in human health in general and in the reproductive sphere in particular; in fact, the quality of human semen in recent decades has seen an increasing decline. Therefore, studying how the environment may influence the reproductive sphere may be a first step toward understanding some aspects of human infertility that are still completely unknown. The identification of possible molecular alterations, highlighted in this thesis work, not detectable by simple spermiogram, in individuals living in high-impact areas, compared to individuals living in low-impact areas, may be an important first milestone. Furthermore, in this thesis, an effective correlation between the environment and human fertility has been demonstrated, giving further confirmation to the idea that has been emerging in recent years that the spermatozoon is an early sentinel of the health status of the environment and the general and reproductive health of humans.

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International Journal of
Molecular Sciences

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Spermatozoa Transcriptional Response and Alterations in PL Proteins Properties after Exposure of *Mytilus galloprovincialis* to Mercury

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Abstract: Mercury (Hg) is an environmental pollutant that impacts human and ecosystem health. In our previous works, we reported alterations in the properties of *Mytilus galloprovincialis* protamine-like (PL) proteins after 24 h of exposure to subtoxic doses of toxic metals such as copper and cadmium. The present work aims to assess the effects of 24 h of exposure to 1, 10, and 100 pM $HgCl_2$ on spermatozoa and PL proteins of *Mytilus galloprovincialis*. Inductively coupled plasma–mass spectrometry indicated accumulation of this metal in the gonads of exposed mussels. Further, RT-qPCR analyses showed altered expression levels of spermatozoa *mt10* and *hsp70* genes. In *Mytilus galloprovincialis*, PL proteins represent the major basic component of sperm chromatin. These proteins, following exposure of mussels to $HgCl_2$, appeared, by SDS-PAGE, partly as aggregates and showed a decreased DNA-binding capacity that rendered them unable to prevent DNA damage, in the presence of $CuCl_2$ and H_2O_2. These results demonstrate that even these doses of $HgCl_2$ exposure could affect the properties of PL proteins and result in adverse effects on the reproductive system of this organism. These analyses could be useful in developing rapid and efficient chromatin-based genotoxicity assays for pollution biomonitoring programs.

Keywords: mussel; $HgCl_2$; sperm nuclear basic proteins; sperm chromatin; male gametes; reproduction

Citation: Lettieri, G.; Notariale, R.; Ambrosino, A.; Di Bonito, A.; Giarra, A.; Trifuoggi, M.; Manna, C.; Piscopo, M. Spermatozoa Transcriptional Response and Alterations in PL Proteins Properties after Exposure of *Mytilus galloprovincialis* to Mercury. *Int. J. Mol. Sci.* **2021**, *22*, 1618. https://doi.org/10.3390/ijms22041618

Academic Editor: Vladimir Isachenko
Received: 26 December 2020
Accepted: 3 February 2021
Published: 5 February 2021

1. Introduction

In recent decades, large quantities of pollutants have been released into the marine environment and estuaries [1]. Of all the pollutants, toxic metals have long been considered the main contaminants in the marine environment, representing a serious danger to marine organisms [2–4]. In marine environments, due to their benthic and sedentary way of life, bivalves are easily exposed to environmental pollution (toxic metals, persistent organic pollutants, etc.) and bioaccumulate these toxicants [5]. For this reason, these organisms are typically used as models in the field of environmental toxicology [6,7]. In particular, the mussel *Mytilus galloprovincialis* has been identified by several authors as a bioindicator that responds quickly to environmental pollution, with a wide spatial distribution and economic relevance. In the life cycle of bivalves, the early developmental stages result in being most susceptible to various pollutants, such as toxic metals [8], pesticides [9], and antifouling paints [10], and it has been demonstrated that the concentrations of toxic metals able to cause lethal toxicity in embryos and larvae are much lower than those that are lethal to adults [11,12]. Among toxic metals, mercury (Hg) is one of the most toxic nonessential metals [13]. Mercury contamination in seawater is an issue to the environment and human

health. In fact, concentrations of Hg species in seawater are very low, about 1 pM [14], but are sufficient to drive bio-accumulation and -magnification of this toxic metal to levels in marine organisms that can pose human and ecological health risks [15,16]. In addition, Hg has also resulted in being more toxic in comparison to other metals, such as lead (Pb) and cadmium (Cd), to *M. galloprovincialis* embryos [17]. The toxicity of mercury has been evaluated at high doses (micromolar), in some organisms, such as the European clam (*Ruditapes decussatus*), in which Hg significantly reduced sperm viability [18] and also in *Ciona intestinalis* on which mercury affected early developmental stages. In addition, this metal impaired the testes function of the tropical fish *Gymnotus carapo* (L.), producing a decrease in sperm count and the alteration in sperm morphology [19]. Moreover, an in vivo study performed by Lahnsteiner et al. (2004) [20] showed that mercury had significantly decreased the percentage of sperm motility and velocity of *Clarias gariepinus* and *Lota lota* [20]. Further, early transcriptional changes induced in vivo in the mussel gills by a combination of nanomolar concentration of Cd, Cu, and Hg were also reported [21]. Differential toxicity has also been observed among species. In particular, the embryos and larvae of *Crassostrea gigas* were more sensitive to copper, yet *Paracentrotus lividus* embryos and larvae were more sensitive to lead and mercury [22]. Finally, Beiras et al. [23] exposed different life stages of *M. galloprovincialis* to mercury and reported a consistent decreased sensitivity to the metal as developmental stages increased in age. To the best of our knowledge, the literature lacks information regarding the effects of mercury concentrations, similar to those present in the waters of the Mediterranean basin and the North Atlantic oceans, on *M. galloprovincialis* sperm nuclear basic proteins (SNBP) and DNA. To this aim, in the present work, we exposed *M. galloprovincialis* for 24 h to three picomolar doses (1, 10, and 100 pM) of $HgCl_2$. After exposures, we measured the accumulation of mercury in the gonads of exposed mussels and the expression of the stress genes *mt10* and *hsp70* in spermatozoa. Further, we analyzed the possible changes in the electrophoretic pattern and in the DNA binding ability of protamine-like (PL) proteins. Finally, we analyzed the ability of PL proteins to protect DNA from the action of free radicals, as sperm DNA fragmentation is one of the main causes of infertility [24].

2. Results

2.1. Evaluation of Accumulation of Mercury in Male Gonads

The possible accumulation of mercury in the gonads of male mussels exposed to $HgCl_2$ was evaluated using inductively coupled plasma–mass spectrometry (ICP-MS). These analyses showed mercury accumulation in gonads after exposure for 24 h to 1, 10 and 100 pM $HgCl_2$. In particular, the amount of mercury found in the gonads of unexposed mussels was 0.02 ± 0.01 mg/kg. As regards the exposed mussels, the values were: 0.04 ± 0.01 mg/kg for 1 pM; 0.05 ± 0.03 mg/kg for 10 pM; and 0.04 ± 0.02 mg/kg for 100 pM (Figure 1).

2.2. Spermatozoa Gene Expression

After mussel exposure, a possible stress at the *M. galloprovincialis* spermatozoa level was assessed by quantitative reverse transcription polymerase chain reaction (RT-qPCR). For this aim, we measured the level of the stress genes *hsp70* and *mt10* in spermatozoa of mussels exposed to the three different concentrations of $HgCl_2$ (1, 10, and 100 pM). It was found that *mt10* was on the order of about threefold over the control after 10 and 100 pM $HgCl_2$ exposure. *hsp70* was hyper-expressed at the same extent after 100 pM $HgCl_2$ exposure, while after 10 and 1 pM $HgCl_2$ exposure, this gene resulted in being hypo-expressed, particularly at 1 pM $HgCl_2$. In this latter condition, the hypo-expression of this gene was about 1.5 times compared with the control condition. These results showed that the exposure of mussels to these $HgCl_2$ doses produced a stress condition for *M. galloprovincialis* spermatozoa (Figure 2).

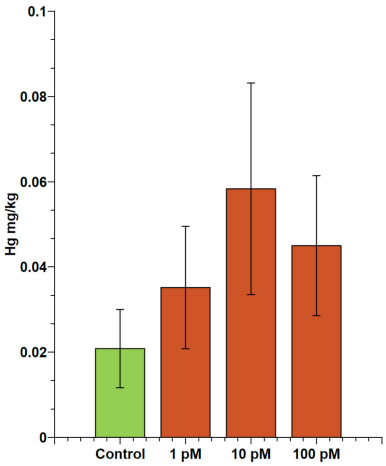

Figure 1. ICP-MS analyses of mercury bioaccumulation in gonads of *M. galloprovincialis*. Mercury concentration was evaluated after 24 h of exposure of mussels to 1, 10, and 100 pM $HgCl_2$ in laboratory tanks. The values are expressed based on the wet weight basis. All values represent the mean ± S.D. obtained from 6 gonads of mussels from the same tank.

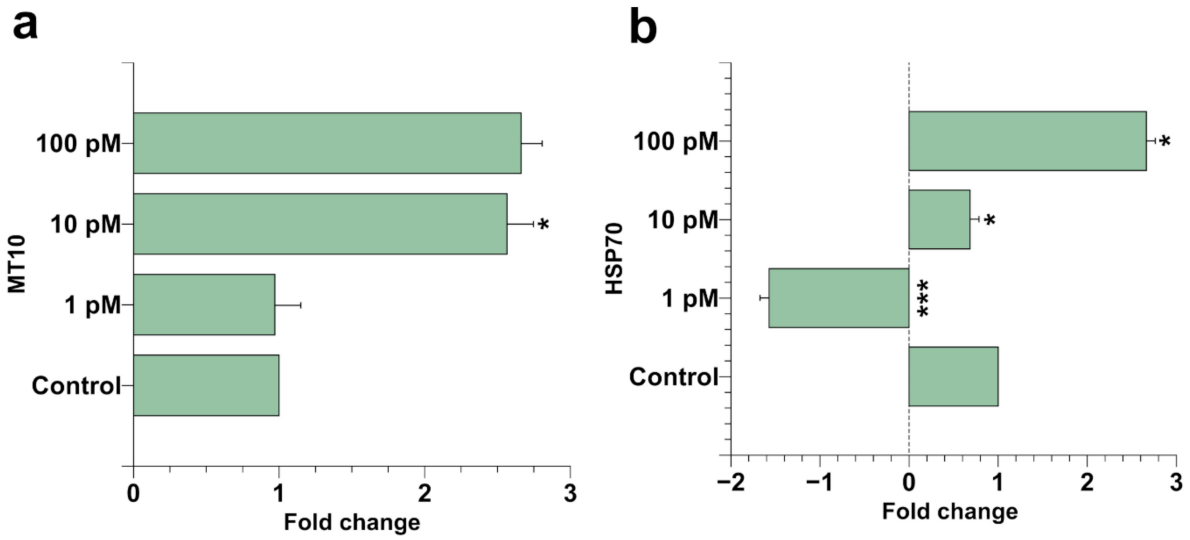

Figure 2. RT-qPCR expression analysis of *M. galloprovincialis* spermatozoa *mt10* and *hsp70*. In the figure, the fold change of the expression of the transcripts of *mt10* (**a**) and *hsp70* (**b**) is reported in the three $HgCl_2$ exposure conditions with respect to the control condition (unexposed mussels), after determining their relative expression in comparison to the reference housekeeping 18S gene. Mussels were unexposed and exposed to 1, 10, and 100 pM $HgCl_2$. Values are presented as mean ± S.D. (n = 6); asterisks indicate a statistically significant difference compared to unexposed mussels: * = $p < 0.05$; *** = $p < 0.001$.

2.3. Acid-Urea (AU)-PAGE of M. galloprovincialis PL-Proteins

The alteration of *hsp70* and *mt10* genes expression in spermatozoa was indicative of a stress condition after mussels exposure to $HgCl_2$. For this reason, the electrophoretic pattern of PL proteins extracted from mussels exposed to the three different concentrations of $HgCl_2$ was determined by acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE). No significant differences were found in the electrophoretic pattern of PL proteins extracted from mussels exposed to the three different conditions compared with unexposed mussels (Figure 3a). By contrast, differences were found by analyzing the same proteins by SDS-PAGE (Figure 3b). As shown in this figure, the samples of PL proteins extracted from exposed mussels (lanes 2–4) presented, especially at 1 and 100 pM (Figure 3b, lane 2 and 4),

additional protein bands with reduced mobility compared to control PL, likely indicative of the formation of several protein aggregates.

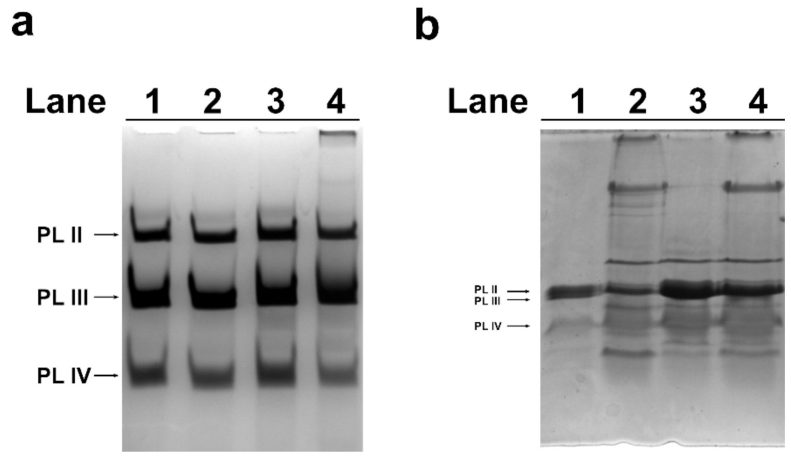

Figure 3. Electrophoretic analyses by acid-urea (AU)-PAGE (**a**) and SDS PAGE (**b**) of *M. galloprovincialis* protamine-like (PL) proteins extracted from unexposed (lane 1) and exposed mussels (lanes 2, 3, and 4): To 1, 10, and 100 pM $HgCl_2$, respectively.

2.4. Electrophoretic Mobility Shift Assay (EMSA) Assay

Because several differences were found in the SDS-PAGE analysis of PL proteins extracted from mussels exposed to the three different concentrations of $HgCl_2$, the DNA binding affinity of these PL proteins was investigated. For this aim, electrophoretic mobility shift assay (EMSA) was performed, using pGEM3 plasmid DNA as a probe, under the same conditions described in Vassalli et al. (2015) [25]. It was referred to as "DNA saturation" when all the plasmid DNA was close to the well. In the control condition (unexposed mussels), the DNA saturation was achieved at a PL proteins/DNA ratio of 1.0 (Figure 4a, lane 7), while at 1, 10, and 100 pM $HgCl_2$ conditions, DNA saturation was not observed until a PL proteins/DNA ratio of 2 (Figure 4b–d, lanes 12). These results indicated a lower DNA binding ability of the PL proteins extracted from exposed mussels with respect to those obtained from control mussels.

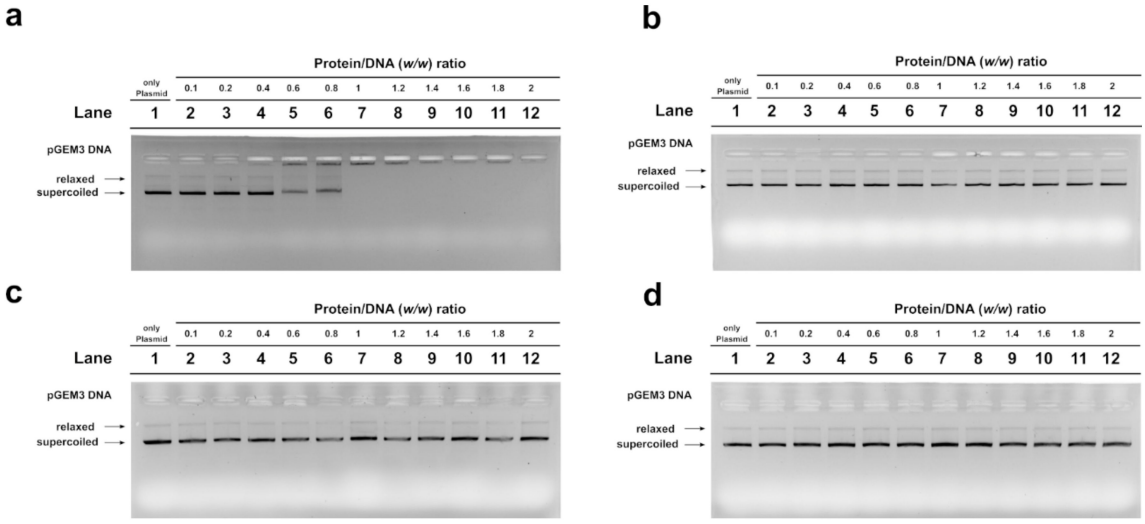

Figure 4. Electrophoretic mobility shift assay (EMSA) analyses of DNA binding ability of PL proteins from unexposed (**a**) and 1, 10, and 100 pM $HgCl_2$-exposed mussels (**b**–**d**), using plasmid DNA. Numbers on the wells indicate the PL protein/DNA (*w*/*w*) ratios used; only plasmid denotes pGEM3 plasmid DNA with no proteins added.

2.5. DNA Protection Analysis

An assay was also performed to determine the potential of *M. galloprovincialis* PL proteins to protect DNA from oxidative damage. A condition was created in which plasmid DNA damage occurred. In this condition, the plasmid DNA was placed in the presence of 10 μM H_2O_2 and 5 μM $CuCl_2$ in order to cause the Fenton reaction and produce DNA breakage. The result obtained under this condition is shown in lane 4 of all agarose gels in Figure 5, and it can be seen that more than 50% of the plasmid DNA was in the relaxed form. The addition of PL proteins from unexposed and exposed mussels to this mixture, in protein/DNA ratios of 0.4, 0.6, and 0.8, produced different effects. In particular, when PL proteins from unexposed mussels were added, already at a protein/DNA ratio of 0.4, the entity of DNA breakage was lower with respect to the damage condition (compare lanes 6 and 4 in Figure 5). At 0.6 and 0.8 protein/DNA ratios, DNA damage was not observed, suggesting that these PL proteins produced complexes capable of protecting DNA. In fact, in this latter case, at increasing PL proteins/DNA ratios, the plasmid DNA bands corresponding to supercoiled and relaxed forms became less intense as DNA saturation took place, detectable by the appearance of a high-molecular-weight DNA band close to the well. The same assays performed by using the PL proteins from exposed mussels produced different results. In fact, the PL proteins obtained from mussels exposed to all $HgCl_2$ doses were unable to produce complexes that protected DNA from oxidative damage (lanes 9–11 of panels a (1 pM), b (10 pM), and c (100 pM)).

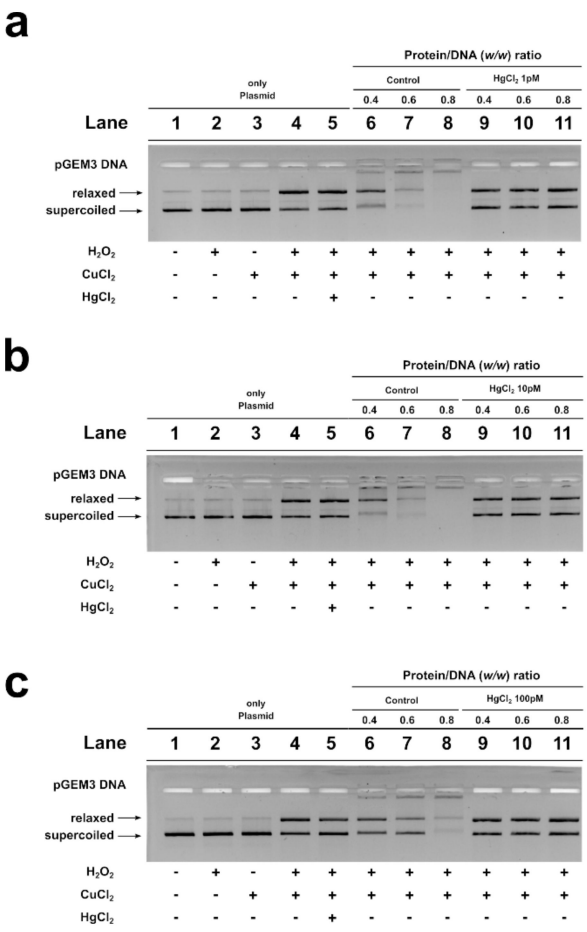

Figure 5. DNA protection analysis on 1% agarose gel of pGEM3 plasmid DNA in the presence of increasing (0.4, 0.6, and 0.8) PL/DNA ratios. Control (lanes 6, 7, and 8 of all the panels); 1 pM $HgCl_2$ (lanes 9, 10, and 11 of panel (**a**)); 10 pM $HgCl_2$ (lanes 9, 10, and 11 of panel (**b**)); 100 pM $HgCl_2$ (lanes 9, 10, and 11 of panel (**c**)).

3. Discussion

Some metals are important to maintain several biochemical and physiological functions in living organisms when they are in very low concentrations; however, they become toxic when they exceed certain threshold concentrations and represent a problem of increasing relevance for ecological, evolutionary, nutritional, and environmental reasons [26–30].

Human industrial activities have caused an increase in levels of Hg in the air, soil, and fresh and sea waters, and bioaccumulation along the food chain. Given the widespread exposure of organisms and the well-known toxicity of this metal, there is growing concern that exposure to mercury also at low levels may have many different adverse effects on different biological functions, including reproduction. In our previous works, we have reported alterations in the properties of *M. galloprovincialis* PL proteins after 24 h of exposure to subtoxic doses of toxic metals such as copper and cadmium [31,32]. Thus, in this work, we have evaluated the effects on the reproductive health of male *M. galloprovincialis* after exposure to mercury doses similar to those present in the waters of the Mediterranean basin and the North Atlantic oceans, sites where metal pollution has previously been reported [14,33,34].

For this aim, we exposed mussels for 24 h in laboratory tanks to 1, 10, and 100 pM $HgCl_2$ and measured the mercury accumulation and the *mt10* and *hsp70* expression, in the gonads and spermatozoa of exposed mussels, respectively. This is because, in our previous studies, it has been demonstrated that mussel gonad has the same accumulation capacity as gill, at least for copper. In addition, this latter metal induces alterations in the expression level of *mt10* in spermatozoa [31]. As reported by several authors [35,36], *Mytilus* species are able to synthesize the metal binding protein, metallothionein, for their detoxification.

In general, *mt10* genes appear to be highly expressed at baseline and can respond to both essential (Cu, Zn) and nonessential (Cd, Hg) toxic metals [37,38].

Indeed, our data are in accordance with the literature; the mussels showed higher expression levels of *mt10* after mercury contamination (Figure 2); in particular, the treatment of 100 pM showed an expression level increased about three times compared to the control condition (Figure 1). Expression levels of *hsp70* in mussel spermatozoa were also analyzed. HSP acts to prevent protein aggregation and to maintain functional conformations. Our RT-qPCR analysis showed significant and different responses in *hsp70* expression levels in mussel spermatozoa after the three $HgCl_2$ tested exposure doses. In particular, the extremely low expression of hsp70 was found at the lowest concentration used (Figure 2b), suggesting an impairment of the biochemical mechanism underlying the heat shock response. Other studies have suggested that the downregulation of this gene is due to some factors that influence the stability and translation of mRNA [39].

Furthermore, even at the concentration of 10 pM of $HgCl_2$, a slight downregulation of the *hsp70* gene was observed in the spermatozoa of mussels. To our knowledge, this is the first study demonstrating the downregulation of the *hsp70* gene in spermatozoa under metallic stress at low concentrations and within short acute exposure, which suggests a high sensitivity of spermatozoa in the response to mercury.

Interestingly, the increased $HgCl_2$ concentration (100 pM) upregulated the transcription of the *hsp70* gene in mussel spermatozoa, differently to low concentrations (1–10 pM). This transcriptional regulation of *hsp70* could probably be needed to improve mercury tolerance, as high levels of HSP have been shown to protect against the negative impact of metals on protein integrity [40,41].

Considering the high efficiency of spermatozoa in the response to mercury, possible alterations in the properties of PL proteins, which represent the main component of the basic nuclear proteins that organize sperm DNA, were also evaluated. In particular, the electrophoretic pattern and DNA binding affinity of these proteins were analyzed after exposure of mussels to $HgCl_2$. No significant differences in the electrophoretic pattern of PL proteins were found by AU-PAGE between unexposed and exposed mussels (Figure 3a). However, by SDS-PAGE, several protein bands with reduced mobility were found in the samples of PL proteins extracted from spermatozoa of mussels exposed to all the

$HgCl_2$ doses (Figure 3b). These protein bands could represent aggregates of PL proteins. Considering these alterations in PL proteins, their ability to bind DNA was tested. As a matter of fact, the decreased DNA binding capacity of PL proteins from exposed mussels was found for the pGEM3 DNA plasmid (Figure 4); in fact, DNA saturation was not achieved even at a proteins/DNA ratio of 2. Consistent with our previous work, it was confirmed by EMSA that the extracts of PL proteins (containing PL-II, PL-III, and PL-IV) from unexposed *M. galloprovincialis* interacted with DNA in "all or nothing" mode [4], as sperm H1 histones and *C. variopedatus* PL protein [42–46] and the DNA saturation were achieved at a proteins/DNA ratio of 1. The PL proteins obtained after all $HgCl_2$ exposures maintained the same DNA binding mode of control PL proteins, differently from those obtained after copper mussels exposure [31]. In that case, we observed an increase in DNA binding affinity and also a change in DNA binding mode from "all or nothing" to "intermediate mode," the typical DNA binding mode of somatic H1 histone [45]. These alterations could result in a change in the ability of PL proteins to bind and condense DNA and, in turn, abrogate their canonical role of DNA protection. In fact, in our previous studies, it has been reported that, in some cases, sperm nuclear basic proteins are involved in DNA oxidative damage [47].

Indeed, it was observed that all PL proteins extracted from spermatozoa of exposed mussels to mercury were unable to protect DNA from the action of copper and hydrogen peroxide (Figure 5). Taken together, our findings provide additional information that offers new insights into the mechanisms of mercury toxicity on the reproductive system of *M. galloprovincialis*. Additionally, PL protein studies could be useful for developing rapid and effective chromatin-based genotoxicity tests for biomonitoring programs for heavy metal impact assessment and species management. Therefore, further investigations are needed to understand the specific mechanisms of the action of mercury, leading to a more complete understanding of the mussel's response to stress of this toxic metal.

4. Materials and Methods

4.1. Ethics Statement

This research was performed on the marine invertebrate *M. galloprovincialis* (Lamarck, 1819), which is not protected by any environmental agency in Italy. This study was conducted in strict accordance with European (Directive 2010/63) and Italian (Legislative Decree n. 116/1992) legislation on the care and use of animals for scientific purposes.

4.2. Evaluation of Accumulation of Mercury in Male Mussel Gonads

Mercury quantification was performed as follows: Samples consisting of single gonads of male mussels were digested by using 1 mL of ultrapure nitric acid ($HNO_3 \geq 69\%$, v/v, Sigma Aldrich) in a microwave system equipped with an autosampler (CEM DISCOVER SP-D, CEM Srl, Cologno al Serio, Bergamo, Italy), according to the digestion procedure UNI EN 13805:2014 [48]. Samples were diluted to 10 mL with a solution of HNO_3, 2%, v/v. For each digestion batch, a blank digestion of ultrapure nitric acid was performed to check for metal contamination, and a quality control shellfish-based sample (QMAS, Sample 741, LGC Standard) was analyzed to control the effectiveness of digestion.

The elemental analysis was conducted by inductively coupled plasma–mass spectrometry (ICP-MS, Aurora M90 Bruker, Billerica, MA, USA). Element concentrations were determined from a calibration curve calculated on the basis of five concentrations for the analyzed elements obtained from certified standard solutions. All the materials and reagents used were checked for metal contamination through repeat analysis of method blanks. Standard samples were processed at the beginning and every 10 samples in the analytical batch to verify the instrument calibration.

The accuracy of the method was evaluated by analyzing the CRM MT-742 of the fish-based sample from interlaboratory comparison (QMAS, Round 295, LGC Standard), obtaining a recovery value of 93%.

The analyses were performed on 6 samples of each type (control, exposed to 1 pM, exposed to 10 pM, and exposed to 100 pM $HgCl_2$).

4.3. Mussels Sampling and $HgCl_2$ Exposure

To investigate the specific effects of $HgCl_2$, mixed-sex and medium-shell-size specimens of *M. galloprovincialis* were used (length 4.95 ± 0.17 cm), kindly provided by Eurofish Napoli S.R.L. Bacoli. The mussels were exposed to three concentrations of $HgCl_2$ (1, 10, and 100 pM) as previously described for other toxic metals in Piscopo et al., 2016 [5], in laboratory plastic tanks (36 × 22 × 22 cm), each containing 6 L of 33‰ artificial sea water (ASW) with the following composition for 1 L: NaCl 29.2 g; KCl 0.60 g; $MgCl_2$ 1.2 g; $NaHCO_3$ 0.20 g; and $CaCl_2$ 1.08 g. In particular, 13 mussels were placed in any tank for 24 h at 18 ± 1 °C. Water and metal salts were changed every 12 h during treatment. For each system, dissolved oxygen and temperature were recorded at predetermined time intervals. The experiments were performed in the winter period, January–February 2020. Tanks containing only ASW were used as a control for unexposed mussels. Two tanks were used for each condition for a total of eight tanks as already described in Lettieri et al., 2019 [49].

4.4. Spermatozoa Sampling and Processing

After 24 h of exposure to 1, 10, and 100 pM $HgCl_2$, collection of spermatozoa from male mussels was performed. Mussels were then opened using a knife, taking care not to cut the soft tissues. Subsequently, after stimulating male gonads with a glass Pasteur pipette and the help of seawater, gametes were obtained and subjected to microscopic examination to identify the sex of the mussels and the sexual maturity on the basis of a morphological and seminal analysis as previously described in Piscopo et al., 2018 [3]. Spermatozoa were collected as reported in Vassalli et al., 2015 [25], with a glass Pasteur pipette. In brief, the semen collected from all the male mussels contained in the tanks corresponding to a specific μM $HgCl_2$ condition were pooled and centrifuged at 1000× *g* for 2 min at 4 °C in order to remove the debris. To collect the spermatozoa, the supernatant obtained was centrifuged at 9000× *g* for 10 min at 4 °C. Pellets containing spermatozoa of about 200 mg were recovered and stored at −80 °C for the further investigations.

4.5. RNA Extraction and RT-qPCR

Total RNA was purified from spermatozoa of mussels unexposed (control) and exposed to the three different concentrations of $HgCl_2$ by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantification and quality of RNA samples were controlled with a UV-Vis spectrophotometer (NanoDropH ND-1000, Waltham, MA, USA) and by 1% agarose gel electrophoretic analysis under denaturing conditions [50]. Equal amounts of RNA obtained from the spermatozoa of mussels unexposed and exposed to $HgCl_2$ were used in qPCR analyses. We used the procedure described in Basile et al., 2017 [26] with few modifications. First, we purified the RNA samples from genomic DNA with an Ambion (Austin, TX, USA) DNA-free kit and then performed the retrotranscription with M-MLV reverse transcriptase (ImpProm II kit, Promega, Madison, WIS, USA). Then, 1 μg of RNA from each condition was used to perform cDNA syntheses using random hexamers (0.5 μg/μg RNA). For the determination of genes expression by real-time PCR, 100 ng of the cDNA was used and 10 μM of each forward and reverse primers was used in a final volume of 50 μL using SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA) with the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The conditions of qPCR were as follows: All PCR reactions were performed for 40 cycles with the following specifications: Denaturation at 95 °C for 15 min; annealing and elongation at 60 °C for 1 min. The primer set used was designed using the open-source software Primer3, starting from the sequences indicated with the accession numbers reported in the last column of the table shown in Table 1. Before real-time PCR, it was verified by PCR that each primer pair

produced a single primary amplicon. qPCR product dissociation curves for all transcripts gave single peaks.

Table 1. List of forward and reverse primers used for amplification of each gene analyzed and for the reference housekeeping 18S gene.

Gene	F-Primer	F-Primer Length	R-Primer	R-Primer Length	Accession Number
18S	GCCACACGAGATTGAGCAAT	20	CTCGCGCTTACTGGGAATTC	20	L244B9
hsp70	CGCGATGCCAAACTAGACAA	20	TCACCTGACAAAATGGCTGC	20	AY861684
mt10	GCCTGCACCTTGTAACTGTAT	21	CTGTACACCCTGCTTCACAC	20	AY566248

The results were analyzed using the ViiA™-7 Software (Foster City, CA, USA) and exported into Microsoft Excel (Redmond, WA, USA, ver. 2009—build 13231.20262). To determine relative gene expression values, the ΔΔCt method was used [51]. All samples were processed with technical triplicates. Data for each gene were normalized against 18S ribosomal RNA. The expression levels of this gene were essentially stable as also reported by other authors [38]. The change in expression of *mt10* and *hsp70* transcripts related to reference 18S rRNA in the spermatozoa samples from mussels exposed to $HgCl_2$ compared with control mussels was measured.

4.6. PL Proteins from M. galloprovincialis Spermatozoa Extraction and Analyses

Extraction of protamine-like proteins from spermatozoa was performed using 5% perchloric acid (PCA) as previously described in [52]. For this work, we used n = 10 spermatozoa pellets deriving from each tank corresponding to a specific μM $HgCl_2$ condition. Spermatozoa pellets were homogenized in a potter with 15 mL of distilled water, and then PCA was added. Acid extraction was performed as described by Vassalli and coworkers [25], and at the end of procedure, we extensively dialyzed the samples containing PCA-soluble PL-proteins against distilled water, in order to guarantee all PCA was removed. Finally, the extracted proteins were lyophilized and stored at −80 °C.

PL proteins were analyzed by AU-PAGE as previously described in Piscopo et al., 2018 [53]. Gels were then acquired using a Gel-Doc system (BioRad, Hercules, CA, USA) via Quantity One v.4.4.0 (BioRad, Hercules, CA, USA) software. The software ImageJ ver 1.50d (https://imagej.nih.gov/ij/), supported by the National Institute of Health (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA), was used for the densitometric analysis of the protein bands on gel.

SDS-PAGE of PL proteins was performed as previously described in De Guglielmo et al., 2019 [32] with a few modifications. In particular, the stacking gel was constituted by 5.0% (w/v) acrylamide (acrylamide/bis-acrylamide 30:0.15) and the separating gel was at 18.0% (w/v) acrylamide (acrylamide/bis-acrylamide 30:0.15). At the end of the run, the gels were stained with Coomassie Brilliant Blue and acquired using a GelDoc system via Quantity One v.4.4.0 software (BioRad, Hercules, CA, USA). The densitometric analysis of gel bands was carried out using the software ImageJ.

4.7. Plasmid DNA Preparation and Analysis

pGEM3 plasmid DNA was prepared according to Carbone and coworkers [54]. The quantification and quality of plasmid DNA were conducted with a UV-Vis spectrophotometer (NanoDropH ND-1000, Waltham, MA, USA) and the integrity of DNA was analyzed by gel electrophoresis on 1% agarose gels in 89 mM Tris-HCl pH 8.0, 2 mM EDTA, and 89 mM boric acid (TBE).

4.8. Analysis of the Effect of M. galloprovincialis PL Proteins on DNA Electrophoretic Mobility

The DNA binding affinity of PL proteins was evaluated by performing Electrophoretic Mobility Shift Assay (EMSA), as described in Fioretti et al., 2012 [42]. For this analysis, we used 150 ng of plasmid pGEM3 DNA in the circular form and increasing amounts of pro-

teins, expressed as protein/DNA w/w ratios in a final volume of 30 µL. The protein/DNA *w*/*w* ratios used ranged from 0.1 to 2. The results obtained were analyzed on 1% agarose gels. On the wells of the gels shown in the results, the protein/DNA *w*/*w* ratios in the different conditions tested are reported. DNA migration was visualized by staining gels with ethidium bromide (2 µg/mL) after electrophoresis.

4.9. DNA Protection Analysis

The PL ability to protect DNA from oxidative damage in the presence of 10 µM H_2O_2 and 5 µM $CuCl_2$ was performed by using 150 ng of plasmid DNA (pGEM3) and PL extracted from mussels unexposed and exposed to 1, 10, and 100 pM $HgCl_2$. The conditions of DNA oxidative damage were redefined by using a fixed amount of H_2O_2 and increasing $CuCl_2$ concentrations, in order to obtain more than 50% of the plasmid in the relaxed form, as shown in Figure S1. These conditions were obtained starting from those reported in Lettieri et al., 2020 [24]. The samples were prepared using the EMSA protocol described in paragraph 4.8. Plasmid DNA and proteins/DNA *w*/*w* ratios of 0.4, 0.6, and 0.8 were used. The interaction between DNA and PL proteins was for 5 min at room temperature, and then H_2O_2 and $CuCl_2$ were added and the samples were incubated in the dark for 30 min at 37 °C. Just before electrophoresis analysis, in order to avoid the EDTA coordination of eventual metals, a sample buffer at 1X final concentration was added to the samples. The samples obtained were then analyzed on 1% agarose gel at 100 V for 30 min in TEB 1X, staining the gels with ethidium bromide (2 µg/mL) at the end. The images of the gels were acquired at the GelDoc Biorad (Hercules, CA, USA). All experiments were performed at least three times.

4.10. Statistical Analysis

Multiple group data were analyzed using one-way ANOVA and Dunnett's test was used to compare means between the groups. Values were considered significant when $p < 0.05$. Statistically significant differences are defined at the 95% confidence interval. Data are shown as mean ± S.D. Analyses were made on the pool of spermatozoa collected from mussels of any different laboratory tank.

Supplementary Materials: Supplementary Materials can be found at https://www.mdpi.com/1422-0067/22/4/1618/s1: Figure S1. Settings of the ideal conditions for pGEM3 plasmid DNA breakage in presence of $CuCl_2$ and H_2O_2.

Author Contributions: Conceptualization, M.P.; supervision, M.P., C.M. and M.T.; investigation, G.L., R.N., A.A., A.D.B. and A.G.; formal analysis, G.L. and R.N.; visualization, M.P., G.L., R.N. and C.M.; data curation, M.P., G.L. and R.N.; writing—original draft preparation, M.P., G.L. and R.N.; project administration, M.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors express their gratitude to the EUROFISH s.r.l. fish company (Bacoli, NA) for providing the mussels used in this study.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ASW	Artificial sea water
AU-PAGE	Acetic acid-urea polyacrylamide gel electrophoresis

SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ICP-MS	Inductively coupled plasma–mass spectrometry
PCA	Perchloric acid
PL	Protamine-like
qPCR	Quantitative polymerase chain reaction
RT-qPCR	Reverse transcript quantitative polymerase chain reaction
SNBP	Sperm nuclear basic protein

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International Journal of
Molecular Sciences

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New Insights into Alterations in PL Proteins Affecting Their Binding to DNA after Exposure of *Mytilus galloprovincialis* to Mercury—A Possible Risk to Sperm Chromatin Structure?

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Abstract: Mercury (Hg) is a highly toxic and widespread pollutant. We previously reported that the exposure of *Mytilus galloprovincialis* for 24 h to doses of $HgCl_2$ similar to those found in seawater (range 1–100 pM) produced alterations in the properties of protamine-like (PL) proteins that rendered them unable to bind and protect DNA from oxidative damage. In the present work, to deepen our studies, we analyzed PL proteins by turbidimetry and fluorescence spectroscopy and performed salt-induced release analyses of these proteins from sperm nuclei after the exposure of mussels to HgCl2 at the same doses. Turbidity assays indicated that mercury, at these doses, induced PL protein aggregates, whereas fluorescence spectroscopy measurements showed mercury-induced conformational changes. Indeed, the mobility of the PLII band changed in sodium dodecyl sulphate-polyacrylamide gel electrophoresis, particularly after exposure to 10-pM $HgCl_2$, confirming the mercury-induced structural rearrangement. Finally, exposure to $HgCl_2$ at all doses produced alterations in PL-DNA binding, detectable by DNA absorption spectra after the PL protein addition and by a decreased release of PLII and PLIII from the sperm nuclei. In conclusion, in this paper, we reported Hg-induced PL protein alterations that could adversely affect mussel reproductive activity, providing an insight into the molecular mechanism of Hg-related infertility.

Keywords: mussel; $HgCl_2$; spermatozoa; PL proteins; sperm nuclei; fluorescence spectroscopy; turbidity assays; reproduction

Citation: Lettieri, G.; Notariale, R.; Carusone, N.; Giarra, A.; Trifuoggi, M.; Manna, C.; Piscopo, M. New Insights into Alterations in PL Proteins Affecting Their Binding to DNA after Exposure of *Mytilus galloprovincialis* to Mercury—A Possible Risk to Sperm Chromatin Structure? *Int. J. Mol. Sci.* **2021**, *22*, 5893. https://doi.org/10.3390/ijms22115893

Academic Editor: Gladys Mirey

Received: 15 April 2021
Accepted: 28 May 2021
Published: 31 May 2021

1. Introduction

1.1. Environmental Pollution by Mercury

Technological progress and industrialization have produced the release of several pollutants in aquatic and terrestrial environments. Among all pollutants, heavy metals are receiving a lot of attention from environmental scientists because of their toxic natures. Although heavy metals are generally present in trace amounts in natural waters, a large number of them are toxic even at very low concentrations [1], posing a serious danger to marine organisms [2–4]. Among the various heavy metals, mercury (Hg) has drawn a lot of attention, as it is the most toxic nonessential metal [5]. Mercury has captured the attention of the scientific community since the 1950s, when the first evidence of hazardous environmental impacts were reported [6], which led to policies and regulations to limit Hg emissions. The World Health Organization (WHO), in 2013, classified mercury as one of the 10 chemicals of greatest public health concern worldwide.

1.2. Seawater Mercury Pollution

Seawater mercury contamination is a problem for the environment and human health [7]. Indeed, in the aquatic cycle, this metal be transformed into MeHg by anaerobic bacteria [8]. Indeed, more importantly, the concentrations of Hg species in seawater are very low, about 1 pM [9], but these levels are enough for driving the bioaccumulation and biomagnification of this toxic metal, along the aquatic food chain up to humans [10,11]. Furthermore, mercury is well-studied in terms of its bioavailability, bioaccumulation, biomagnification and cellular toxicity, overall, in bivalves [12].

1.3. Mytilus galloprovincialis as Sentinel Organism

The value of bivalve mollusks and, particularly, mussels (*Mytilus* spp.) for use as sentinel organisms for pollution monitoring in the coastal environments has been well-established in several experimental and field studies [13]. The mussel *M. galloprovincialis*, for its wide geographic distribution, abundance, easy accessibility and other important characteristics, is commonly employed as model in the field of environmental toxicology [14,15] and considered an ideal bioindicator of pollutants on the Mediterranean coasts. This filter-feeding intertidal invertebrate, in fact, responds quickly to environmental pollution and accumulates high levels of various types of contaminants [16,17], providing a time-integrated measure of environmental contamination [18,19].

1.4. Mercury and Mytilus galloprovincialis Reproduction

In the bivalve life cycle, the early developmental stages prove to be the most responsive to several contaminants, including heavy metals [20], pesticides [21] and antifouling paints [22], and the concentrations of heavy metals capable of causing lethal toxicity in embryos and larvae have been shown to be considerably lower than the ones that are lethal to adults [23,24]. In particular, mercury was also found to be more toxic than some other metals, like lead (Pb) and cadmium (Cd), to *M. galloprovincialis* embryos [25], and Beiras et al. 1995 [26] indicated a consistent decreased sensitivity to this metal as the *M. galloprovincialis* developmental stages increased in age. We have previously shown alterations in the properties of protamine-like proteins (PL) of *M. galloprovincialis* after exposure to sublethal doses of metals such as copper and cadmium [3,4,27]. Recently, we showed that the exposure of *M. galloprovincialis* for 24 h to 1, 10 and 100 pM $HgCl_2$ caused protamine-like (PL) proteins that were partly in the form of aggregates and were unable to bind and protect DNA from oxidative damage [28,29]. These exposure doses reflected the amount of mercury present in the waters of the Mediterranean basin and the North Atlantic oceans [9,30,31]. We found these results to be extremely interesting and worthy of further investigation given the paucity of information in the literature on reproductive health effects in *M. galloprovincialis* following exposure to picomolar doses of mercury chloride. Therefore, in the present work, we expanded our investigations in order to understand the nature of mercury-induced alterations in PL proteins, affecting them binding with DNA. Specifically, we analyzed, by turbidity assays, the effect of adding increasing concentrations of $HgCl_2$, in the range of 1–100 pM, to PL proteins and evaluated, by fluorescence spectroscopy, alterations in PL proteins from mussels exposed to 1, 10 and 100-pM $HgCl_2$. Finally, we performed salt-induced release analyses of these proteins from sperm nuclei after exposure of mussels to 1, 10 and 100-pM $HgCl_2$.

2. Results

2.1. Turbidity Assays on PL Proteins

Figure 1a shows the results of the turbidity assay obtained by adding increasing amounts of $HgCl_2$, in the range of 1–100 pM, to a solution of PL proteins extracted from nonexposed mussels and measuring the changes of 420-nm absorbance. The graph describing the results obtained indicated that the absorbance at 420 nm of the PL solution slightly increased as the concentration of $HgCl_2$ incremented differently from that of Bovine Serum Albumin (BSA) (Figure 1a). This result suggests the possible formation of protein aggre-

gates, corroborating that already obtained in Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Figure 1b) [28], in which additional protein bands with reduced mobility were observed in the samples of PL proteins extracted from mussels exposed to 1, 10 and 100-pM $HgCl_2$.

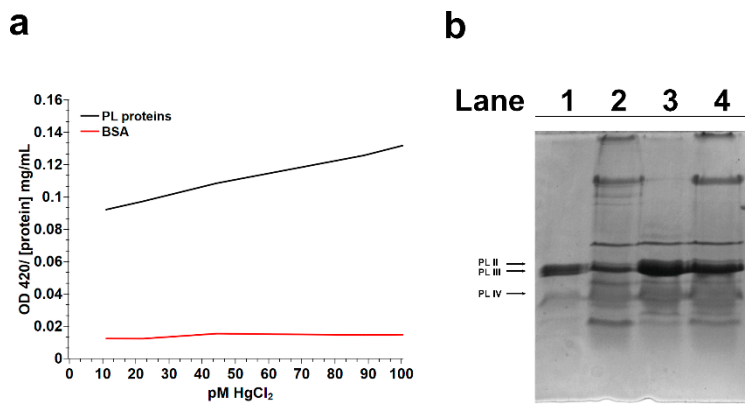

Figure 1. Turbidity assay (**a**) following 420-nm absorbance changes as a function of $HgCl_2$ adding: in black, the trend of PL proteins, and, in red, that of BSA (negative control). An electrophoretic analysis by SDS PAGE (**b**) of *M. galloprovincialis* PL proteins extracted from nonexposed (lane 1) and exposed mussels (lanes 2, 3 and 4): to 1, 10 and 100-pM $HgCl_2$, respectively. (**b**) A reused figure that corresponds to (B) of Figure 3 (Lettieri et al. 2021) obtained under the Creative Common CC BY license. Lettieri G, Notariale R, Ambrosino A, Di Bonito A, Giarra A, Trifuoggi M, Manna C, Piscopo M. Spermatozoa Transcriptional Response and Alterations in PL Proteins Properties after Exposure of *M. galloprovincialis* to Mercury. *Int J Mol Sci.* 2021 Feb 5;22(4):1618. doi: 10.3390/ijms22041618.

2.2. Fluorescence Spectroscopy

In order to investigate the mercury-induced structural changes of PL proteins, fluorescence spectra at an excitation wavelength of 350 nm were performed on these proteins from nonexposed mussels (control) and exposed to $HgCl_2$ using a hydrophobic dye such as 8-Anilinonaphthalene-1-sulfonic acid (ANS) [32]. The spectra of PL from nonexposed mussels presented a peak at 479 nm, while those of mussels exposed to all $HgCl_2$ doses showed a red shift respectively of 17.5 nm (peak at 496.5 nm, 1 pM), 23.5 nm (peak at 502.5 nm, 10 pM) and 11.0 nm (peak at 490.0 nm, 100 pM) (Figure 2). The red shift shown could be compatible with the binding of ANS to more polar regions of these proteins in accordance with those reported by Bothra et al. 1998 [33]. In addition, an increase in the ANS fluorescence intensity of about 1.3 and 1.6 times for PL from mussels exposed to 1 and 100-pM $HgCl_2$ respectively, was observed (Figure 2). These differences indicated alterations in the conformation of PL proteins from $HgCl_2$-exposed mussels.

2.3. SDS-PAGE Analysis of PL Proteins

In order to identify any changes in the PL protein electrophoretic mobility after the exposure of mussels to $HgCl_2$, we performed an electrophoretic analysis by SDS-PAGE, maintaining the same final percentage of acrylamide described by Lettieri et al. 2021 [28] but changing the Acrylamide-Bisacrylamide ratio (29:1) in order to obtain an higher resolution of the protein bands. In these conditions, the electrophoretic pattern by SDS-PAGE of the PLII and PLIII proteins (Figure 3) showed that after the exposure of mussels to 10-pM $HgCl_2$ (Figure 3b, lane 3), the band corresponding to the PLII protein did not seem to appear on the gel in the position observed in the control (Figure 3b, lane 1). This behavior was, in part, also observed in the PL samples from mussels exposed to 1 and 100-pM $HgCl_2$, in which a lower amount of PLII was revealed in comparison with nonexposed mussels (Figure 3b, lanes 2 and 4). Differently, the electrophoretic analysis carried out on the same sample by AU-PAGE showed a complete PL pattern, including PLII proteins (Figure 3a). These results suggested that mercury could influence the electrophoretic mobility of the PLII protein in

the presence of SDS. The densitometric analysis performed on the electrophoretic bands (Figure 3c) in SDS-PAGE showed that the amount of protein corresponding to the apparent PLIII band in lane 3 was higher with respect to that measured for the PLIII protein in the samples loaded in the other lanes and similar to the sum of the bands relative to the PLII and PLIII proteins. This result supports the hypothesis of a comigration of the PLII and PLIII proteins in SDS-PAGE in the sample obtained from mussels exposed to 10-pM $HgCl_2$.

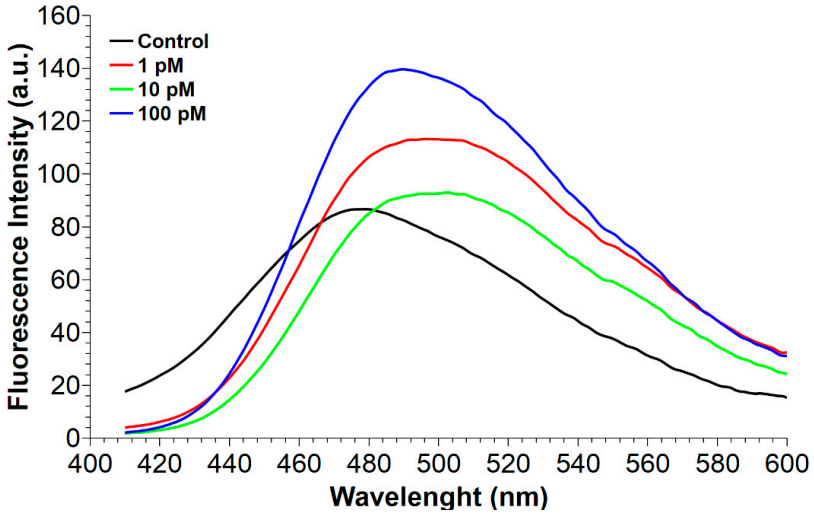

Figure 2. Fluorescence spectra of PL proteins from nonexposed (control) and $HgCl_2$-exposed mussels.

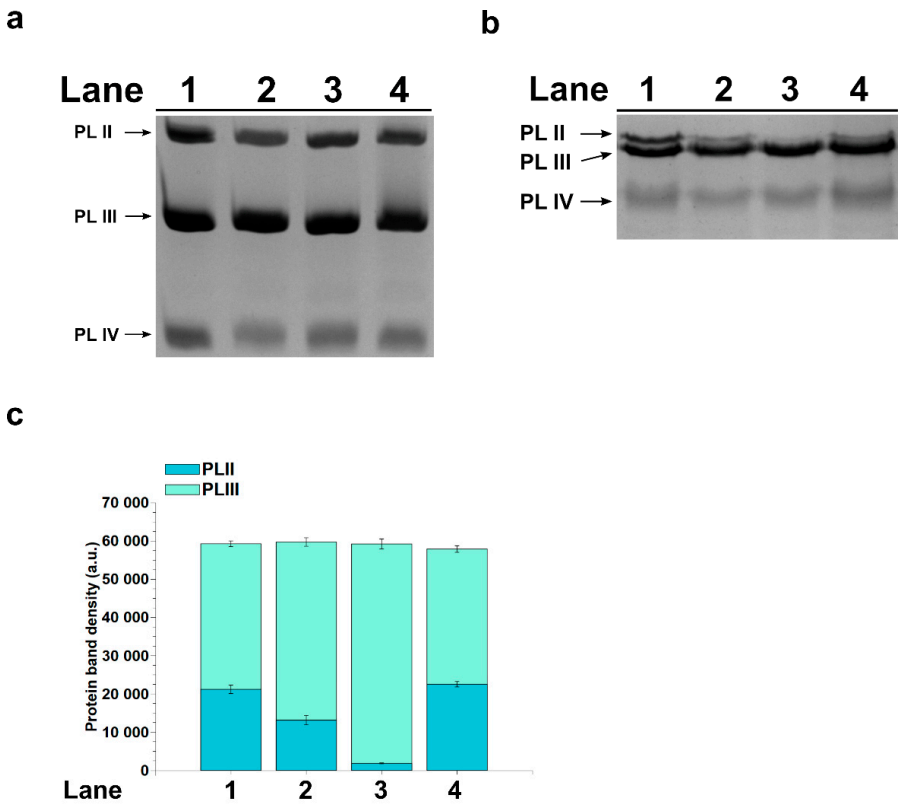

Figure 3. SDS-PAGE (**b**) and densitometric bands analyses (**c**) of the PLII and PLIII proteins, extracted from spermatozoa of nonexposed mussels (lane 1) and of mussels exposed to 1, 10 and 100-pM $HgCl_2$ (lanes 2, 3 and 4, respectively). AU-PAGE (**a**) of the PL proteins from the spermatozoa of nonexposed mussels (lane 1) and of mussels exposed to 1, 10 and 100-pM $HgCl_2$ (lanes 2, 3 and 4, respectively) (n = 6).

2.4. DNA Absorption Spectrum after PL Proteins Addition

In order to evaluate possible effects of the exposure of mussels to $HgCl_2$ on the ability of PL proteins to bind to DNA, the absorption spectrum in the 200–300-nm range of plasmid DNA alone was compared with the absorption spectra of the same following the addition of increasing amounts of PL (0.5–1–1.5–2–3 PL/DNA (*w/w*) ratios) from nonexposed and exposed $HgCl_2$ mussels. Figure 4 shows the results of these assays. The decrease of the maximum DNA absorption shown in Figure 4a is indicative that PL extracted from nonexposed mussels binds DNA. Contrarily, adding PL extracted from mussels exposed to all the $HgCl_2$ doses did not cause changes in the DNA absorption spectrum. In fact, in these cases, the absorption spectra of plasmid DNA alone and plasmid DNA in the presence of increasing amounts of PL are almost superimposable. All this could suggest any alterations in the PL–DNA binding.

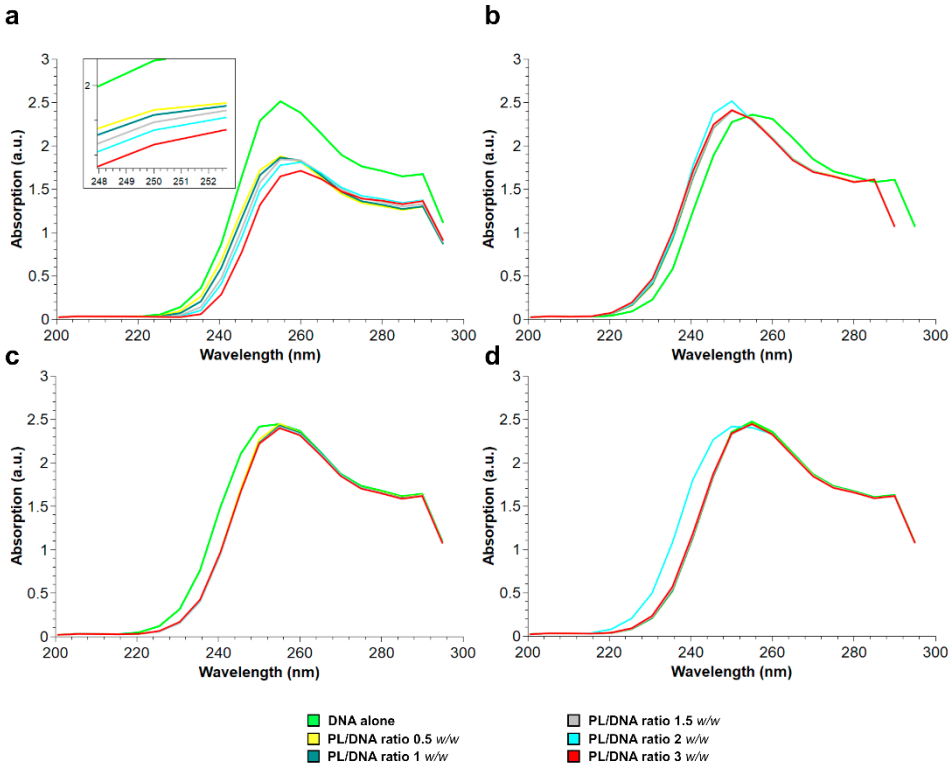

Figure 4. Absorption spectra in the range of 200–300 nm of plasmid DNA alone and PL/DNA mixtures at various PL/DNA ratios: PL extracted from nonexposed mussels (**a**) and PL extracted from mussels exposed to 1 pM (**b**), 10 pM (**c**) and 100-pM $HgCl_2$ (**d**), respectively. Green: plasmid alone, yellow: PL/DNA 0.5 ratio (*w/w*), blue: PL/DNA ratio 1 (*w/w*), grey: PL/DNA ratio 1.5 (*w/w*), blue: PL/DNA ratio 2 (*w/w*) and red: PL/DNA ratio 3 (*w/w*).

2.5. Release of PL Proteins from Sperm Nuclei

In order to confirm possible changes in the PL–DNA binding of mussels exposed to $HgCl_2$ at the three doses, an analysis of the release of these proteins from the sperm nuclei with increasing concentrations of NaCl was performed as shown in Figure 5. No significant differences in the PLIV release were observed in the control and $HgCl_2$-exposed mussels, while alterations were showed both for PLII and PLIII. In particular, PLII was released in smaller amounts after exposure of the mussels to all three doses of $HgCl_2$. About 50% of this protein was released after the exposure of mussels to 1 and 100-pM $HgCl_2$, while only about 33% after exposure to 10-pM $HgCl_2$. PLIII release from the sperm nuclei of mussel nonexposed ended at 2-M NaCl. The further addition of NaCl did not release this protein. In fact, the electrophoretic analysis of the proteins release at 3 and 4-M NaCl did not show the band corresponding to this protein. For this reason, in the graph, there are

not values of a densitometric analysis for these latter concentrations of NaCl. Regarding PLIII, after the exposure of mussels to 1 pM, a larger amount of NaCl (4 M instead of 2 M) was required for obtaining about 80% of this protein in comparison with the control condition. The exposure of mussels to 100-pM $HgCl_2$ instead caused a PLIII release of only about 55% at 2-M NaCl, and the latter additions of NaCl did not significantly increase the amount released of this protein. Finally, only 28% of PLIII was released after the exposure of mussels to 10-pM $HgCl_2$.

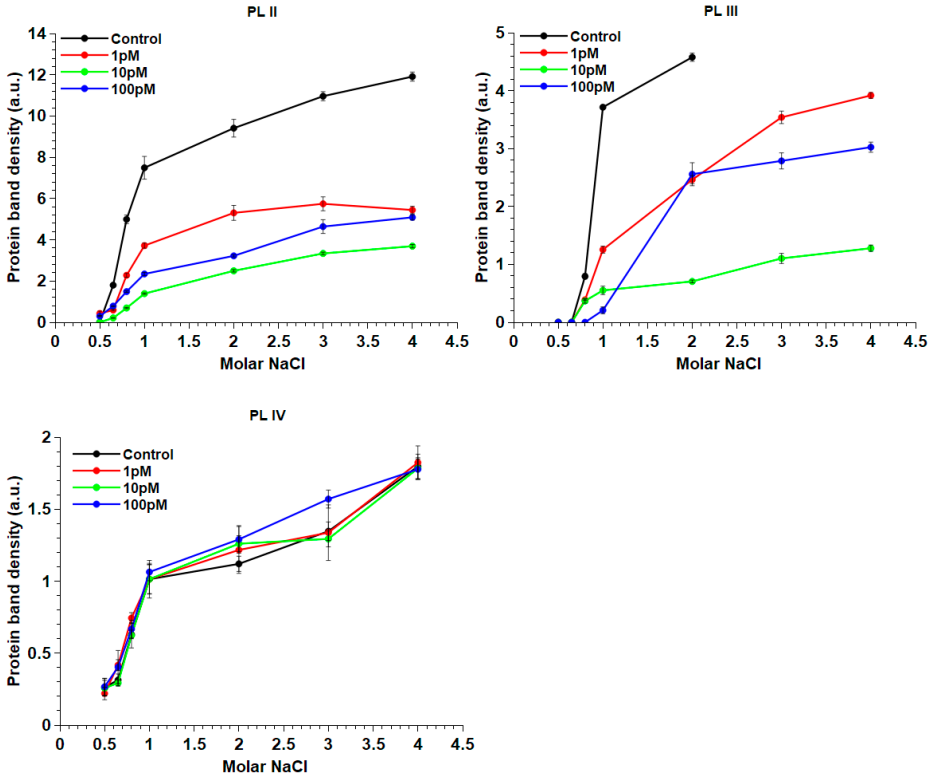

Figure 5. The release of PL from sperm nuclei at different NaCl molar concentrations in the control mussels and exposed to 1, 10 and 100-pM $HgCl_2$ (n = 6).

3. Discussion

Considering the extensive exposure of organisms and the well-recognized toxicity of mercury that causes, even with low-level exposure, several negative impacts on different biological functions, in this work, we deepened our studies on the effects of this metal on the reproductive health of *M. galloprovincialis*, having already reported not only alterations in the expression of some gonadal stress genes but, also, on the properties of the PL proteins, which represent the main nuclear basic protein component of the sperm chromatin of this organism [28]. First, we confirmed our hypothesis that mercury could induce PL protein aggregate formation. Indeed, turbidity assays showed that the addition of increasing concentrations of $HgCl_2$, in the range of 1–100 pM, to PL proteins from nonexposed mussels caused a slight increase in the absorbance at 420 nm, indicative of protein aggregate formation. That $HgCl_2$ induces protein aggregation has already been reported for other proteins. For example, Arnhold et al. 2015 [34] identified amyloid protein aggregation in the cell nucleus as a novel Hg–bio-interaction pathway [34], and we, in our previous studies, showed the formation of a mercerized tetrameric Hb as a result of treating human erythrocytes with increasing concentrations of $HgCl_2$ [5]. We also investigated the possibility of mercury-induced conformational changes in PL proteins using fluorescence spectroscopy. The fluorescence analyses indicated conformational changes in PL proteins extracted from mussel exposed to $HgCl_2$, because for these proteins, an increase in ANS fluorescence was observed. This effect was particularly relevant for PL proteins after the

exposure of mussels to 1 and 100-pM $HgCl_2$ and suggested a likely rearrangement of the proteins that produced an increase in the hydrophobic surface area available for ANS binding. It is well-known that both proteins and DNA undergo conformational changes to form functional complexes and, also, to facilitate interactions with other molecules. These modifications have a direct consequence on the stability and specificity of the complex, as well as the cooperativity of interactions between multiple entities. Generally, the proteins with the most conformational changes are those that have the most contact with DNA. In general, these proteins are those that have a lower dipole moment but a more positive net charge at the interface, just like PL proteins. Some hydrophobic residues such as Cys, Ala and Gly undergo much more conformational adaptations at the interface than others [35], and the two latter amino acids are particularly abundant in PL proteins. As is well-known, mercury is capable of binding cysteines [28] and histidines [36]. The binding of mercury ions to these amino acids may underlie the conformational changes we observed from the fluorescence analyses, but we cannot exclude that mercury could also be able to bind other amino acids in which PL proteins are richer, such as glycine and alanine [37]. Furthermore, the increase in fluorescence and, especially, the red shift of the emission maximum wavelength of ANS presented by the PL proteins obtained from mussels exposed to $HgCl_2$ confirmed the interactions between these proteins and the fluorescent probe. We can hypothesize that the exposure of mussels to $HgCl_2$ could cause a lower degree of compactness of these proteins, exposing more of their hydrophobic groups. This structural rearrangement might be possible for this type of proteins, because it has been shown that small proteins with a high number of hydrophilic residues can have a higher hydrodynamic radius than larger ones [38]. Thus, perturbations at the polar surface can result in a more compact protein, and this explains the faster migration observed in the SDS-PAGE of the PLII protein isolated from mussels exposed to $HgCl_2$. Interestingly, this behavior, under our experimental conditions, was particularly noticeable after exposure of the mussels to 10-pM $HgCl_2$ compared to when they were exposed to 1 and 100-pM $HgCl_2$. This result could suggest a possible hormesis effect. Hormesis is a dose/response characterized by a biphasic effect. Many organisms/biological systems exposed to a broad range of stressors exhibit different responses depending on the dose, and various examples of hormesis effects have been reported in the literature [39–42]. The faster migration of the PLII protein, observed in the SDS-PAGE, was already reported for this protein after the exposure of *M. galloprovincialis* to $CdCl_2$ [43]. All these effects could be the consequence of the well-known observation that chromatin-associated proteins are possible targets of mercury ion toxicity, because studies of rodent tissue culture cells exposed to $HgCl_2$ have shown that mercury ions concentrate in cell nuclei and associate with chromatin [44]. In addition, it is well-known that Hg ions can also modulate the activity of certain proteins by changing their conformation. This has been demonstrated, for example, for the bacterial homodimer metalloregulator MerR, which represses transcription in the absence of mercury and activates transcription upon binding to Hg(II) [45], and for the dual-function transcription factor MerR from *B. megaterium*, whose apo- and Hg2+-bound conformations act as repressor and activator, respectively [46]. The conformational changes in PL proteins therefore prompted us to investigate whether the exposure of mussels to $HgCl_2$ could affect their ability to bind DNA. After all, we had already shown, by EMSA, a decreased DNA-binding capacity of the PL proteins from $HgCl_2$-exposed mussels for the pGEM3 DNA plasmid [28]. In the present work, we demonstrated that the absorption spectra of plasmid DNA alone and plasmid DNA in the presence of increasing amounts of PL proteins from mussels exposed to 1, 10 and 100-pM $HgCl_2$ were almost superimposed. This behavior could indicate either that the proteins were unable to bind to DNA or that their binding to DNA was different, and to investigate this in more detail, we carried out analyses of the release of PL proteins from sperm nuclei. This analysis showed that PLIV was released in the same manner in nonexposed and $HgCl_2$-exposed mussels. Contrarily, relevant changes were observed both for PLII and PLIII. In particular, these two proteins were released in lower amounts from the sperm nuclei of mussels exposed to all $HgCl_2$

doses, a situation that we had already found after the exposure of *M. galloprovincialis* to copper chloride. [27]. Interestingly, even with this type of analysis, the 10-picomolar $HgCl_2$ exposure dose was the one that gave the greatest differences. In fact, this dose of $HgCl_2$ was the one that caused the lowest release of PLII and PLIII, which turned out to be around 33% and 28% for PLII and PLIII, suggesting a stronger DNA binding of PL proteins under this $HgCl_2$ exposure condition. Therefore, our results revealed a different DNA binding of PLII and PLIII, attributable to their mercury-induced conformational changes. This modified binding to DNA changed the canonical protective role of PL proteins for DNA, as demonstrated in our previous work [28]. In conclusion, the alterations in PL proteins induced by mercury, shown in the present work, add information about the effects of heavy metals on this type of protein. Some of these effects, we had already demonstrated with other metals, such as cadmium and copper [3,4,27], but the results of the present work show that mercury is capable of inducing these effects on PL proteins, even with much lower exposure doses (pM). In addition, the results of this work provide further insights into the mechanisms of mercury toxicity on the reproductive system of *M. galloprovincialis*, even at mercury concentrations similar to those found in the Mediterranean Sea and in the oceans. [47,48]. As reported in this work, alterations in PL proteins after exposure of *M. galloprovincialis* to these $HgCl_2$ doses alter their binding to DNA. This could adversely affect the structure of sperm chromatin, which is crucial for the swimming ability of *M. galloprovincialis* spermatozoa and for their ability to fertilize. After all, mercury is recognized as a male reproductive toxicant. In fact, in vitro studies have shown that this metal induces DNA breakage in spermatozoa and causes a decrease in sperm motility. Generally, Hg levels in semen are linked to abnormal sperm morphology, especially in head and middle defects, and low sperm viability [49]; indeed, the Hg levels in sub-fertile and infertile males are higher than in fertile males [50]. Moreover, in our previous paper, we reported the accumulation of mercury in exposed mussels after exposure to these HgCl2 doses [28]. The effects of mercury at low doses have also been found in other organisms. For example, exposure to low doses of Hg adversely affects sperm functions in rats through oxidative stress mechanisms [51], while it induces the inhibition of tyrosine phosphorylation of sperm proteins and alterations of the functional dynamics of buck spermatozoa [52]. In order to reveal possible alterations in the chromatin structure of spermatozoa from mussels exposed to these $HgCl_2$ doses and to assess their fertilizing potential, our next objective will be to perform micrococcal nuclease experiments and in vitro fertilization tests. In addition, it will be interesting to assess the possible involvement of some particular amino acid able to bind Hg and to be responsible for the formation of PL protein aggregates and/or their conformational changes. Some studies have indicated that Hg binds histidines. For example, Stratton et al. 2016 [36] found that Hg binds two histidines of chymotrypsin, inducing aggregation of this protein. Secondary Hg^{2+}-binding sites have been suggested to also contain histidines in alpha-lactalbumin [53]. Obviously, it cannot be excluded that, in interactions with mercury, there may be the involvement of other amino acids, such as arginines, in which PL proteins are particularly rich, as already reported in Tn10-en-coded metal-tetracycline/H1antiporter (TetA(B) [54], and this will be another future focus of our research. Finally, studies of PL proteins could be of interest in developing fast and reliable chromatin-based genotoxicity assays for biomonitoring programs for the assessment of heavy metal impacts and species management.

4. Materials and Methods

4.1. Ethics Statement

This research was conducted on the marine invertebrate *M. galloprovincialis* (Lamarck 1819), which is not protected by any environmental agency in Italy. This study was performed in strict accordance with European (Directive 2010/63) and Italian (Legislative Decree n. 116/1992) legislation on the care and use of animals for scientific purposes.

4.2. Mussels Sampling and Exposure to $HgCl_2$

In order to analyze the effects of $HgCl_2$, adult mussels of *M. galloprovincialis* L (mixed sex) of average size shell lengths 4.93 ± 0.17 cm were kindly provided by Eurofish Napoli S.r.l. Bacoli, Italy. Mussels were exposed to 1, 10 and 100-pM $HgCl_2$, as previously described when we exposed these organisms to other heavy metals [17], in laboratory plastic tanks (Ottavi, Cittadella, Italy) (36 × 22 × 22 cm). Each tank contained 6 L of 33‰ artificial sea water (ASW) with the following composition for 1 L: NaCl 29.2 g, KCl 0.60 g, $MgCl_2$ 1.2 g, $NaHCO_3$ 0.20 g and $CaCl_2$ 1.08 g. In any tank were placed 13 mussels that were exposed to a single dose of $HgCl_2$ for 24 h at 18 ± 1°C. Every 12 h, during exposure, the water and metal salts were changed, and dissolved oxygen and temperature were controlled at predetermined time intervals. The experiments were conducted in the winter period, January to February 2021. Tanks containing only ASW were used as a control for nonexposed mussels. For each condition, two tanks were used, for a total of eight tanks, as reported in Lettieri et al. 2019 [55].

4.3. Spermatozoa Sampling and Processing

Spermatozoa from male mussels were collected after 24-h exposure of the mussels to 1, 10 and 100-pM $HgCl_2$. To obtain spermatozoa, the mussels were opened using a knife, being careful not to cut soft tissue. Then, after stimulating male gonads using a glass Pasteur pipette and seawater, gametes were obtained and examined by microscopic analysis to check that they were spermatozoa and assess sexual maturity based on a morphological and seminal analysis, as reported in Piscopo et al. 2018 [4]. Spermatozoa were collected as reported in Vassalli et al. 2015 [47]. In short, semen pooled from all male mussels contained in tanks corresponding to a given pM $HgCl_2$ condition were centrifuged at 1000× *g* for 2 min at 4°C to discard debris. Supernatant obtained was centrifuged at 9000× *g* for 10 min at 4°C to collect spermatozoa in pellets of about 200 mg, which were recovered and stored at −80°C for further investigations.

4.4. PL Proteins from M. galloprovincialis Spermatozoa Extraction and Analyses

PL proteins were extracted from 10 sperm pellets corresponding to the exposure of mussels to a specific concentration of pM $HgCl_2$, using 5% perchloric acid (PCA) as previously described [56]. In brief, spermatozoa pellets were homogenized in a potter with 15 mL of distilled water, and then, acid extraction with PCA was performed as reported by Vassalli et al. 2015 [47]. The sample containing PCA-soluble PL proteins was then extensively dialyzed against distilled water in order to remove all PCA and then lyophilized and stored at −80°C.

Two types of electrophoretic analyses were conducted for PL proteins: AU-PAGE, as previously described by Piscopo et al. 2018 [57], and SDS-PAGE, as previously described by Piscopo et al. 2020 [58], with a few modifications. In particular, the stacking gel was constituted by 5% (*w/v*) acrylamide (acrylamide/bis-acrylamide 29:1), and the separating gel was 18% (*w/v*) acrylamide (acrylamide/bis-acrylamide 29:1). At the end of the run both by AU-PAGE and SDS-PAGE, the gels were stained with Coomassie Brilliant Blue and acquired using a GelDoc system via Quantity One v.4.4.0 software (Bio-Rad, Hercules, CA, USA). The densitometric analysis of the gel bands was carried out using the software ImageJ ver. 1.50d (https://imagej.nih.gov/ij/ accessed on 15 April 2021), supported by the National Institute of Health (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA).

4.5. Plasmid DNA Preparation and Analysis

For the analyses of the DNA–PL interaction, pGEM3 plasmid DNA was used. This plasmid was prepared following the protocol described by Carbone et al. 2012 [59]. The quantification and quality of the plasmid DNA was evaluated with a UV-Vis spectrophotometer (NanoDropH ND-1000, Waltham, MA, USA), and the integrity of the DNA was

analyzed on 1% agarose gels in 89-mM Tris-HCl, pH 8.0, 2-mM EDTA and 89-mM boric acid (TBE).

4.6. Turbidity Analysis of M. galloprovincialis PL Proteins

M. galloprovincialis PL proteins from nonexposed and $HgCl_2$-exposed and bovine serum albumin (BSA) were analyzed at 0.85 mg/mL in Tris HCl 10 mM, pH 6.8, and the optical densities were evaluated at 420 nm (OD420). To the protein solution, increasing concentrations of $HgCl_2$ (in the range from 0 to 400 µM) were added, and after each increase of $HgCl_2$ concentration, absorbance at 420 nm was measured. The increase in absorbance (at 420 nm) was indicative of protein aggregate formation. In fact, we refer to this optical density as turbidity.

4.7. Fluorescence Spectroscopy Analyses

Fluorescence measurements of PL from nonexposed (control) and exposed mussels to $HgCl_2$ were performed using a PerkinElmer (Waltham, MA, USA) luminescence spectrometer LS-55 by using 1 mL of PL solution 1 mg/mL in water in the presence of 5-µM ANS in a 1-cm optical path cuvette. After excitation at 350 nm, fluorescence spectra were obtained in the emission range of the wavelengths from 410 to 600 nm. Three measurements were performed for each sample: PL from nonexposed (control) and exposed mussels to 1 pM, 10 pM and 100-pM $HgCl_2$. Data were analyzed with the software QtiPlot 1.0.0 rc13 (ver. 5.12.8).

4.8. Effect of PL Addiction from $HgCl_2$-Exposed Mussels on the DNA Absorption Spectrum

To evaluate the DNA–PL protein interaction, absorption spectra of DNA following the adding of PL from 1 pM, 10 pM, and 100-pM $HgCl_2$-exposed mussels were conducted. For DNA spectra, 1-µg plasmid DNA in 400 µL of 1X TEB was used. Spectra were recorded in the range 200–300 nm. The absorption spectrum of plasmid DNA alone and those relative after the addition of increasing amounts of PL in the 0.5–1–1.5–2–3 protein/DNA ratios (*w/w*) were determined.

4.9. Preparation of M. galloprovincialis Sperm Nuclei and Salt-Induced Release of Nuclear Proteins

For the preparation of sperm nuclei, the procedure reported in Olivares and Ruiz 1991 [60] was followed. The release of sperm nuclear basic proteins was done following the protocol described by De Guglielmo et al. 2018 [43] using increasing NaCl concentrations: 0.2 M, 0.4 M, 0.5 M, 0.65 M, 0.8 M, 1 M, 2 M, 3 M and 4 M. For each suspension, incubation at 4°C for 30 min and then centrifugation at 13,000× *g* for 30 min was performed. Sperm nuclear basic proteins were extracted from supernatants with 0.2-N HCl (final concentration). The samples were incubated for 16 h at 4 °C and then centrifuged for 30 min at 13,000× *g*. The supernatants obtained were widely dialyzed with distilled water. Four micrograms of proteins in each sample were examined by AU-PAGE (in accordance with Fioretti et al. 2012 [61]) to assess the stepwise release of these proteins from sperm DNA as a function of the NaCl concentration. A sample containing a PL protein and core histones was obtained by extraction with 0.2-N HCl from *M. galloprovincialis* spermatozoa using the same method described for PCA extraction. This sample served as a reference in the electrophoretic analysis for protein band quantification.

4.10. Statistics Analysis

The data were analyzed using one-way ANOVA. Tukey's test and Student's *t*-test were used to compare the means between the groups. Values were considered significant when $p < 0.05$. Statistically significant differences were defined at the 95% confidence interval. Data were shown as the mean ± SD.

Author Contributions: Conceptualization, M.P.; Supervision, M.P.; C.M. and M.T.; Investigation, G.L., R.N., N.C. and A.G.; Formal analysis, G.L., R.N. and N.C.; Visualization, M.P., G.L., R.N. and C.M.; Data curation, M.P., G.L., R.N. and N.C.; writing—original draft preparation, M.P., G.L., R.N. and N.C. and Project administration, M.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ASW	Artificial sea water
AU-PAGE	Acetic acid-urea polyacrylamide gel electrophoresis
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
PCA	Perchloric acid
PL	Protamine-like
SNBP	Sperm nuclear basic protein
TBE	Tris/Borate/EDTA
OD	Optical density

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Morphological, Gene, and Hormonal Changes in Gonads and In-Creased Micrococcal Nuclease Accessibility of Sperm Chromatin Induced by Mercury

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Abstract: Mercury is one of the most dangerous environmental pollutants. In this work, we analysed the effects of exposure of *Mytilus galloprovincialis* to 1, 10 and 100 pM $HgCl_2$ for 24 h on the gonadal morphology and on the expression level of three stress genes: *mt10*, *hsp70* and *πgst*. In this tissue we also evaluated the level of steroidogenic enzymes 3β-HSD and 17β-HSD and the expression of PL protein genes. Finally, we determined difference in sperm chromatin accessibility to micrococcal nuclease. We found alterations in gonadal morphology especially after exposure to 10 and 100 pM $HgCl_2$ and hypo-expression of the three stress genes, particularly for *hsp70*. Furthermore, decreased labelling with both 3β-HSD and 17β-HSD antibodies was observed following exposure to 1 and 10 pM $HgCl_2$ and complete absence at 100 pM $HgCl_2$ exposure. Gonads of mussels exposed to all $HgCl_2$ doses showed decreased expression of PL protein genes especially for PLIII. Finally, micrococcal nuclease digestions showed that all doses of $HgCl_2$ exposure resulted in increased sperm chromatin accessibility to this enzyme, indicative of improper sperm chromatin structure. All of these changes provide preliminary data of the potential toxicity of mercury on the reproductive health of this mussel.

Keywords: mercury; *Mytilus galloprovincialis*; protamine-like proteins genes; gonad; spermatozoa; micrococcal nuclease digestion; hormones; morphology

Citation: Lettieri, G.; Carusone, N.; Notariale, R.; Prisco, M.; Ambrosino, A.; Perrella, S.; Manna, C.; Piscopo, M. Morphological, Gene, and Hormonal Changes in Gonads and In-Creased Micrococcal Nuclease Accessibility of Sperm Chromatin Induced by Mercury. *Biomolecules* **2022**, *12*, 87. https://doi.org/10.3390/biom12010087

Academic Editor: Yves Ménézo

Received: 30 November 2021
Accepted: 4 January 2022
Published: 6 January 2022

1. Introduction

The increasing worldwide levels of pollutants in the marine environment require control and monitoring strategies. Mercury is an extremely toxic heavy metal in the marine environment and its effects on marine organisms are complex and often unpredictable. The United Nations Environment Programme (UNEP)'s "Global Mercury Assessment" estimated that anthropogenic activities have increased current atmospheric mercury concentrations by approximately 450% above natural levels, resulting in a substantial increase in seawater [1]. In marine environments, mercury is subject to bioaccumulation and biomagnification, and its seawater contamination is posing a serious health problem for humans [2,3] and marine organisms [4,5].

In environmental biomonitoring programs, the use of bioindicator organisms represents one of the most suitable methods for the assessment of trace metal toxicity [6]. Bivalves are excellent organisms as bioindicators of heavy metal pollution due to their characteristics including worldwide distribution, sedentary filter-feeding, easy sampling and reasonable size [7].

Mytilus galloprovincialis (*M. galloprovincialis*) is a readily available organism and widely distributed in the Mediterranean. This bivalve mollusc accumulates contaminants in its tissues mainly in the hepatopancreas, kidney, gills and gonads [8]. This organism responds quickly and effectively to the presence of many environmental pollutants, including heavy metals [9]. Exposure of *M. galloprovincialis* to various heavy metals causes alterations in its reproductive system [9–14]. In the sperm chromatin of *M. galloprovincialis* the main basic protein component is represented by the Protamine-like (PL) proteins [15]. In previous works we have found that the exposure to subtoxic doses of copper causes an increase in the binding affinity of PL-proteins to DNA and alterations in the expression of spermatozoa *mt10* gene [12]. Finally, alterations, such as a reduced DNA binding affinity of PL-proteins [9], and an increased *mt20* gene expression [14] following exposure to subtoxic doses of cadmium, have been reported. *Mytilus* spp. have shown a greater capacity for mercury (Hg) accumulation than other bivalve species [16], and recently, we evaluated the effects of exposure to mercury doses (1, 10 and 100 pM $HgCl_2$), similar to those found in the Mediterranean basin and North Atlantic oceans, on *M. galloprovincialis* male reproductive system [17]. In particular, we showed bioaccumulation of mercury in the male gonads and alterations in the expression of spermatozoa *mt10* and *hsp70* stress genes. We also found several changes in the PL-proteins, such as a reduced ability to bind DNA and an inability to protect DNA from oxidative damage [9]. Furthermore, at these doses of $HgCl_2$ exposure, the formation of PL protein aggregates and alterations in their structural conformation was shown to result in changes in both their binding to DNA and their release from sperm nuclei [10].

In order to obtain further useful information on the effects of these $HgCl_2$ exposure doses on the male reproductive system of *M. galloprovincialis*, in the present work we evaluated possible morphological changes in the gonads by hematoxylin and eosin (H&E) staining and the levels of steroidogenic enzymes 3β-HSD and 17β-HSD by immunohistochemistry. In addition, by quantitative reverse transcription polymerase chain reaction (RT-qPCR), gene expression of *mt10*, *hsp70*, *πgst*, and genes encoding PL proteins were also measured in the gonads. Finally, the possible difference in sperm chromatin accessibility to micrococcal nuclease (MNase) in mussels unexposed and exposed to these doses of mercuric chloride was investigated.

2. Materials and Methods

2.1. Ethics Statement

This research was performed on the marine invertebrate *M. galloprovincialis* (Lamarck, 1819), which is not protected by any environmental agency in Italy. This study was conducted in strict accordance with European (Directive 2010/63) and Italian (Legislative Decree n. 116/1992) legislation on the care and use of animals for scientific purposes.

2.2. Mussels Sampling and Exposure to $HgCl_2$

Adult mussels of *M. galloprovincialis*, medium size of the shell length 4.93 ± 0.17 cm, were supplied by Eurofish Napoli S.R.L. Baia, in Naples and used in this study. Fifteen mussels of unknown sex were exposed to 1, 10 and 100 pM $HgCl_2$, as previously described for the other heavy metals [18] in laboratory plastic tanks (36 cm × 22 cm × 22 cm) containing 6 L of 33‰ artificial sea water (ASW) with the following composition for 1 L: NaCl 29.2 g, KCl 0.60 g, $MgCl_2$ 1.2 g, $NaHCO_3$ 0.20 g and $CaCl_2$ 1.08 g. In each tank, mussels were exposed to a single dose of $HgCl_2$. The exposure was performed at 18 ± 1 °C, for 24 h changing water and metal salts every 12 h during treatment and recording dissolved oxygen and temperature at predetermined time intervals. The experiments were conducted in January of the current year. Tanks containing only ASW were used as a control for nonexposed mussels.

2.3. Gonad Sampling and PL Proteins Extraction

After 24 h of exposure to the three $HgCl_2$ doses, gonads were collected from male specimens. Mussels were opened forcing the valves with a knife, being care not to cut soft tissues. Mussels' sex was initially identified observing the colour of mantle and confirmed by light microscope observation of gonads smear. Briefly, the piece of gonad was smeared on the microscope slide and as soon as it was dry, it was observed under the light microscope. Each identified gonad was stocked and stored at −80 °C.

Sperm-filled gonads were used for acid extraction of PL proteins. Briefly, one gonad from each condition was mechanically pestled in distilled water. Subsequently, centrifugation was performed at 1000× *g* for 2 min at 4 °C to remove debris, and from the obtained supernatant, PLs were extracted by 5% perchloric acid as described in Piscopo et al. 2017 [14].

2.4. RNA Extraction, cDNA Synthesis and RT-qPCR

Total RNA was purified from gonads of nonexposed mussels (control) and exposed to 1, 10, 100 pM $HgCl_2$ using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA obtained was quantified by a UV-Vis spectrophotometer (NanoDropH ND-1000, Waltham, MA, USA). Moreover, electrophoretic analysis was performed on 1% agarose gels under denaturing conditions to assess its quality. Genomic DNA was removed from the samples with an Ambion (Austin, TX, USA) DNA-free kit. cDNA was synthesized from 1 µg of RNA from each samples using M-MLV reverse transcriptase (ImpProm II kit, Promega, Madison, WI, USA). The RT-qPCR was carried out as described in Lettieri et al. [11]. In particular, to determine the gene expression was used 100 ng of the cDNA with 10 µM of each forward and reverse primers in a final volume of 50 µL using SYBRGreen PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA) with the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Each PCR reaction was conducted for 40 cycles as described in Lettieri et al., 2019 [19] maintaining the following conditions: Denaturation at 95 °C for 15 min; annealing and elongation at 60 °C for 1 min. The primer used for the reaction was engineered using the open-source software Primer3, following the sequences and the accession numbers reported in Table 1. The results were exported into Microsoft Excel (Redmond, WA, USA, ver. 2009—build 13231.20262) from the ViiA™-7 Software (Foster City, CA, USA). The relative quantification of gene expression was evaluated using the the ΔΔCt method as reported in Livak et al. [20]. The expression of *mt10*, *hsp70*, *πgst*, *PL-III* and *PL-II/IV* genes was compared to those obtained from control mussels. In Table 1 was reported the list of primers used.

Table 1. List of forward and reverse primers used for amplification of each gene analysed and for the reference housekeeping GAPDH gene.

Gene	F-Primer	F-Primer Length	R-Primer	R-Primer Length	Accession Number
Gapdh	CTGCACCACCAACTGCTT	18	TTCTGGGTGGCAGTGATG	18	SY171038758-018/019
Hsp70	CGCGATGCCAAACTAGACAA	20	TCACCTGACAAAATGGCTGC	20	AY861684
Mt10	GCCTGCACCTTGTAACTGTAT	21	CTGTACACCCTGCTTCACAC	20	AY566248
Gst	AGTTAGAGGCCGAGCTGAA	19	TGGAAACCGTCATCATCTG	19	SY140930374-050/051
Pl III	CACCCAACAAGAAGGATGCC	20	CCTTGCCCTTTTCTTTCCCC	20	SY140930274
Pl II/IV	AAGCCCAAGTAGACGTTCCA	20	TCCGAGGTGTGATGTGTTGA	20	SY140930274

2.5. Sperm Nuclei Preparation and MNase Assay

For the preparation of sperm nuclei the procedure reported in Olivares and Ruiz 1991 [21] was followed. In brief, sperm nuclei were obtained starting from spermatozoa pellet of mussels exposed to different $HgCl_2$ doses. Spermatozoa pellets were obtained centrifuging semen for 10 min at 1900× *g* at 4 °C. Each pellet was then resuspended in 1 ml of a solution (solution 1) with the following composition. -NaCl 0.15 M- EDTA pH 8 25 mM PMSF 1 mMF and the sample was centrifuged at 4 °C for 10 min at 1900× *g*.

Subsequently, the obtained pellet was resuspended in the following buffer (solution 2): 0.25 M sucrose- $MgCl_2$ 5 mM- Tris-HCl pH 8 10 mM- PMSF 1 mM- Triton X-100 0.38% and then centrifuged at 4 °C for 10 min at 1900× *g*. The pellet was recovered and washed twice with the previous buffer in the absence of Triton. The prepared sperm nuclei were resuspended in the following buffer (solution 3)-NaCl 0.15 M- Tris HCl 10 mM pH 8- $CaCl_2$ 0.5 mM and subjected to MNase assay. Digestion was carried out with 10 units of enzyme at 37 °C for 1 mg/mL DNA concentration (A260 = 20) and the reaction stopped at different times (5′, 10′, 15′, 30′ and 60′) adding 2 mM EDTA pH 8 at ice. The digestion products were centrifuged at 1900× *g* for 10 min at 4 °C. This produced a pellet (P) and a supernatant (S). P was used for DNA extraction following a common extraction phenol/chloroform/isoamyl alcohol. Briefly, 1 M NaCl and 0.5% SDS was added to P and incubated 30 min at RT. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, samples were vortexed and centrifuged at 4 °C for 5 min at 13,000× *g*. Successively, 0.3 M NaCl and 2.5 volumes of ethanol 100% was added to the aqueous phase to precipitate the DNA at −20 °C for 1 h.

2.6. Morphological Analyses of Gonad

For light microscope investigations, samples 0.5 cm × 0.5 cm were taken from the central portion of one hemi-mantle. Specimens were fixed in Bouin's solution for 24 h, dehydrated in alcohol and embedded in paraffin wax. Five µm thick sections were cut and stained with hematoxylin and eosin stain.

2.7. Immunohistochemistry Analyses

For immunohistochemistry, 5 µm thick sections of Bouin fixed specimen on poly-l-lysine slides were deparaffinized and rehydrated in xylene and a series of graded alcohols. The sections were treated with 10 mM citrate buffer pH 6.0 in the microwave for antigen retrieval and then incubated in 2.5% H_2O_2 in methanol for endogenous peroxidase blocking. Non-specific background was reduced with the incubation in 3% BSA in PBS buffer for 1 h at room temperature. Sections were then treated overnight at 4 °C with the primary rabbit antibodies diluted in 1% BSA in PBS buffer: (1) anti-human 3β-HSD (1:50), (2) anti-mouse 17β-HSD (1:50). In the first instance, antibodies specificity was tested by making an immunoblot analysis on *Mytilus galloprovincialis* gonadal proteins as previously reported by Prisco et al. (2017) [22]. The reaction was revealed with a peroxidase-conjugated goat anti-rabbit secondary antibody, using DAB (Roche) as chromogen. Negative controls were carried out by omitting primary antibodies. An immunohistochemical signal was observed using a Zeiss Axioskop microscope; images were acquired by using AxioVision 4.7 software (Zeiss).

2.8. Statistical Analysis

The ANOVA test followed Tukey's post-hoc test was performed for gene expression analysis. The statistical analyses were performed by GraphPad Prism 9 (ver. 9.1.2 (226)) (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Gonadal mt10, πgst and hsp70 Expression

The RT-qPCR was used to assess the expression of *mt10*, *πgst* and *hsp70* as a response to possible stress in male gonads after mussel exposure to 1, 10 and 100 pM $HgCl_2$. All genes were downregulated after exposure of mussels to all doses of $HgCl_2$ except for *mt10* gene after exposure to 1 pM $HgCl_2$ in which an overexpression, approximately of 2-fold, of this gene, was observed (Figure 1c).

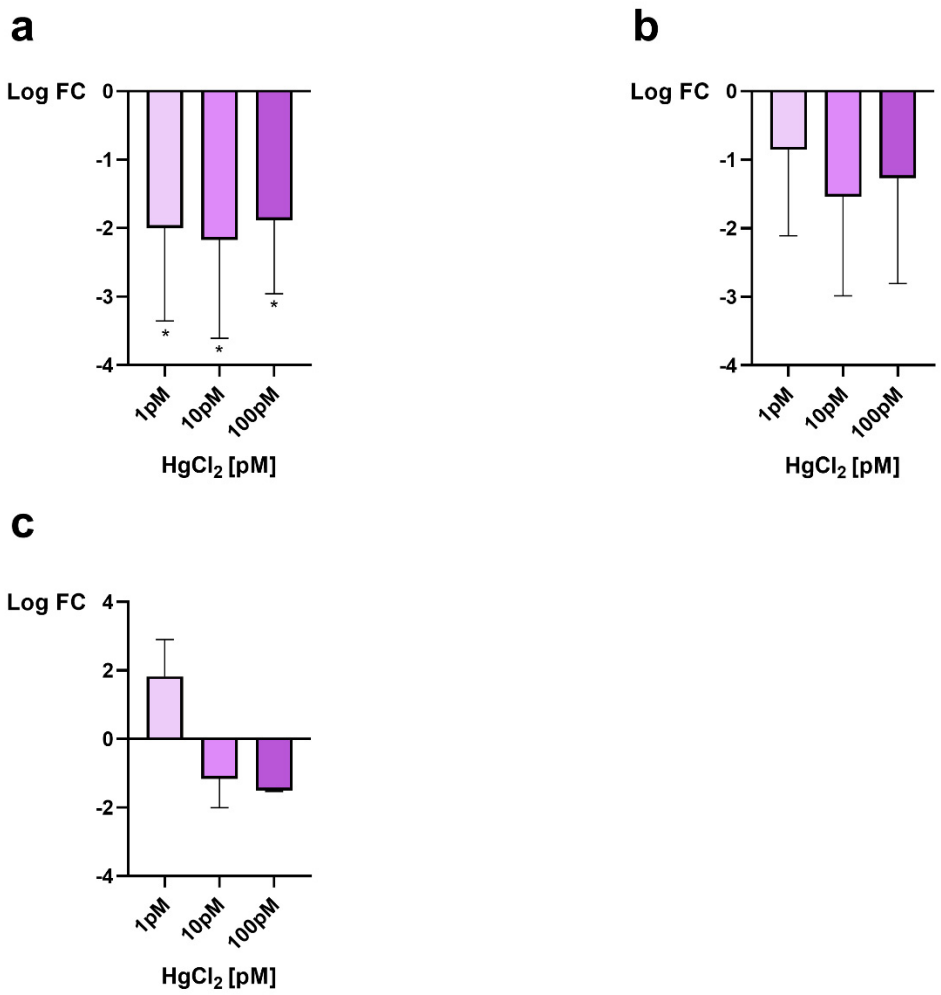

Figure 1. RT-qPCR expression analysis of *M. galloprovincialis* gonadal *hsp70*, *πgst* and *mt10*. In the figure, the change in expression, respect to the control condition (nonexposed mussels), of *hsp70* (**a**), *πgst* (**b**) and *mt10* (**c**) is reported under the three $HgCl_2$ exposure conditions. Expression was determined with respect to the GAPDH housekeeping gene. Values are presented as mean ± S.D. (n = 6); asterisks indicate a statistically significant difference from unexposed mussels: * = $p < 0.05$.

Hsp70 gene showed a similar decrease of expression for all exposure doses, a little more pronounced after exposure to 10 pM $HgCl_2$, where the reduction was of about 2.2 times compared to the control condition (Figure 1a). Downregulations were also found both for the πgst gene (Figure 1b) and for the *mt10* gene (Figure 1c) but to a lesser extent (1–1.5-fold).

3.2. Morphological Analisys of Gonad

Given the alteration of the two stress genes in the gonad after exposure of mussels to all $HgCl_2$ doses, we evaluated possible morphological alterations in this tissue. Testicular sections of control mussel showed normal spermatogenesis and cell arrangement in the spermatic follicles and the connective tissue formed by adipogranular cells (ADGs) and vesicular connective tissue cells (VTCs) resulted normally structured (Figure 2a,b). In 1 pM $HgCl_2$ treated mussels the sperm follicles resulted like the control (Figure 2c,d), whereas in 10 and 100 pM $HgCl_2$ treated mussels, degenerative vacuolized areas in spermatogenic layers were visible (stars) (Figure 2e,h). Spermatogenesis appeared to proceed normally, but connective tissue (CT) showed increasing disorganization and degeneration in relation to increasing $HgCl_2$ dose; ADGs were no longer distinguishable in samples treated with 10 and 100 pM $HgCl_2$ (Figure 2f,h). Finally, in samples treated with 100 pM $HgCl_2$ the CT appeared totally disorganized (Figure 2h).

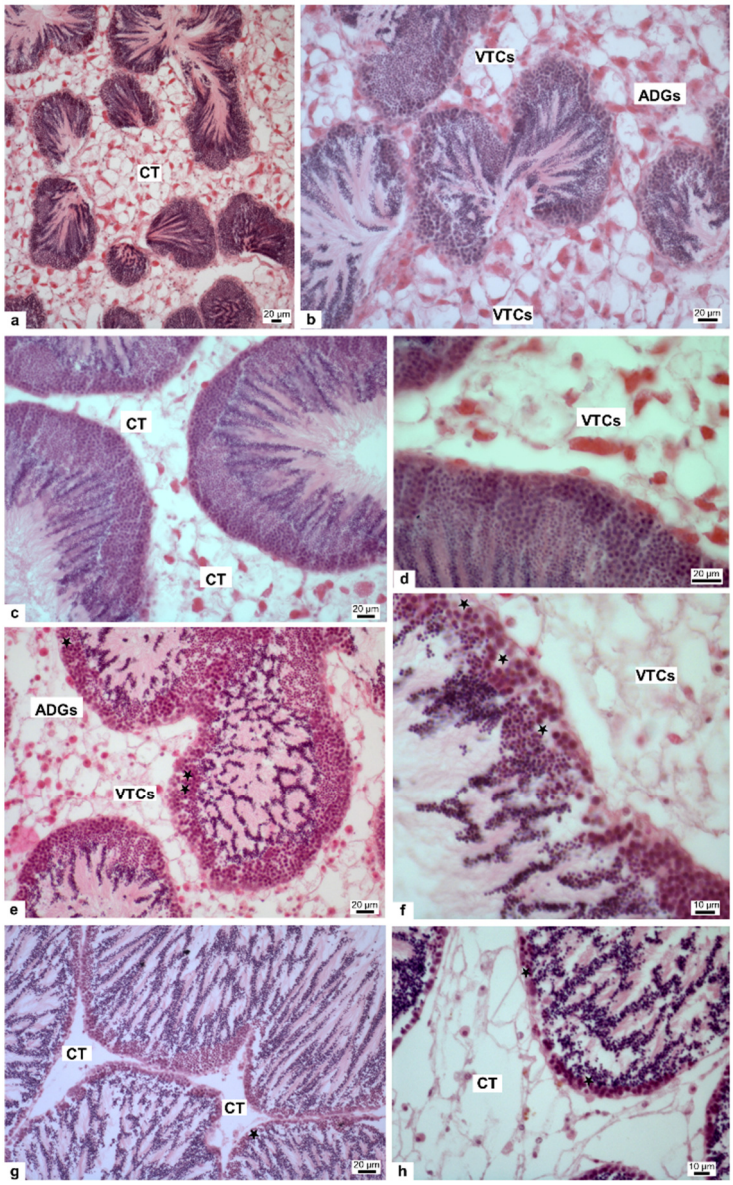

Figure 2. Staining of *M. galloprovincialis* gonad with hematoxylin and eosin. Gonad morphology of nonexposed (**a**,**b**) and exposed mussels to 1 (**c**,**d**), 10 (**e**,**f**) and 100 pM (**g**,**h**) $HgCl_2$, respectively. CT: connective tissue; VTCs: vesicular connective tissue cells; ADGs: adipogranular cells; * (star): degenerative vacuolized areas.

3.3. Immunohistochemistry

Anti-3β-HSD and anti-17β-HSD antibodies showed the presence of both enzymes in the gonad, in overlapping manner, so we present in the figure the localization of 3β-HSD only. The enzymes are present in connective tissue and in germ cells (GC) of control specimens (Figure 3a). In the specimens treated with 1 pM $HgCl_2$ (Figure 3b), the labelling on connective tissue (CT) was still present, decreasing instead in germ cells. Antibody positivity decreased even more in specimens treated with 10 pM $HgCl_2$ (Figure 3c) and completely disappeared in specimens treated with 100 pM $HgCl_2$ (Figure 3d).

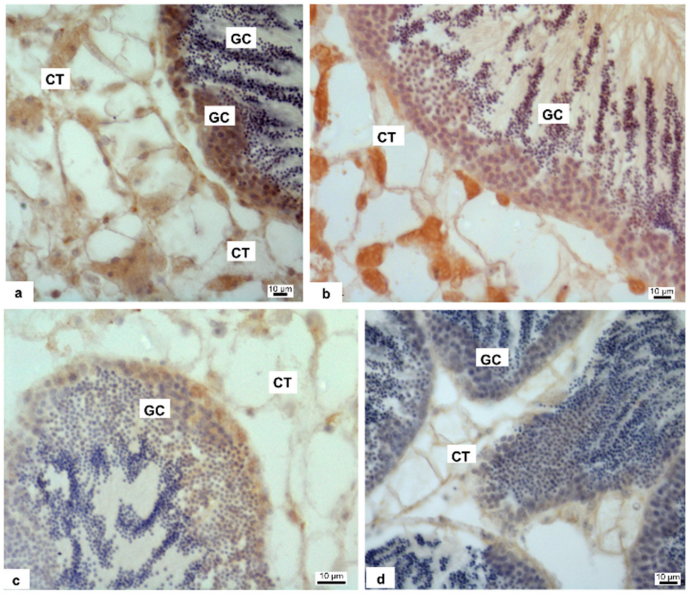

Figure 3. Immunolabeling of *M. galloprovincialis* male gonad with anti-3β-HSD antibody. Brown coloration indicates the enzyme presence. Nonexposed (**a**) and exposed mussels to 1 (**b**), 10 (**c**) and 100 pM (**d**) $HgCl_2$, respectively. Germ cells (GC) are positive to antibody in control and 1 and 10 pM treated samples (**a**–**c**); the positivity disappears almost completely in 100 pM treated samples (**d**) In the connective cells (CT, connective tissue) the 3β-HSD enzyme is recognizable in control and 1 pM treated animals (**a**,**b**); in 10 and 100 pM treated samples the antibody positivity is poor (**c**) or totally disappeared (**d**).

3.4. Gonadal PL-Proteins Genes Expression

Considering the morphological changes and enzymes changes involved in hormones synthesis found in the gonad of mussels exposed to these doses of $HgCl_2$, we also evaluated in this tissue the expression levels of genes encoding PL-proteins, as these are the major basic protein component of the sperm chromatin of this organism. In gonads, the *PLII/IV* was found to be slightly downregulated under all exposure conditions. A more marked downregulation was instead detected for the PL-III gene, under all exposure conditions but particularly at 10 pM $HgCl_2$, where an approximately 2-fold decrease was measured (Figure 4).

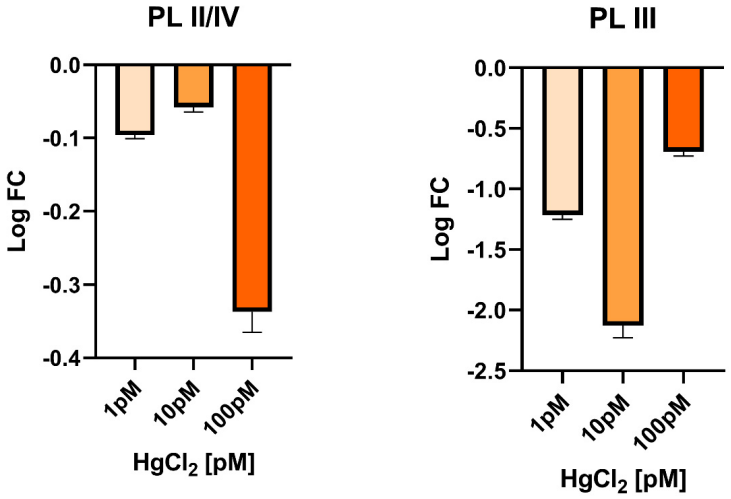

Figure 4. RT-qPCR expression analysis of *M. galloprovincialis* gonadal PLIII and PLII/IV genes. In the figure, the change in gene expression is reported under the three $HgCl_2$ exposure conditions compared to the control condition (nonexposed mussels: CTR). Expression was determined with respect to the GAPDH housekeeping gene. Values are presented as mean ± S.D. (n = 6).

3.5. MNase Digestion Pattern of M. Galloprovincialis'sperm Nuclei

Figure 5 shows the MNase digestion time course of *M. galloprovincialis'* sperm chromatin for 5, 15, 30, and 60 min. The analyses were conducted on sperm chromatin of mussels nonexposed and exposed to the three $HgCl_2$ exposure doses. In this approach, DNA contents of the fractions obtained at different times of MNase digestion were analysed. The results show that in unexposed mussels (Figure 5a) MNase digestion of sperm chromatin results in the electrophoretic pattern typical of sperm chromatin of this organism. Chromatin appears mainly as a smear, which is indicative of the absence of a typical nucleosomal organization. In contrast, following exposure to all doses of $HgCl_2$, the sperm chromatin DNA appears fully degraded upon digestion with Mnase as early as the lowest digestion time (5′), indicative of an improper sperm chromatin structure (Figure 5a,b).

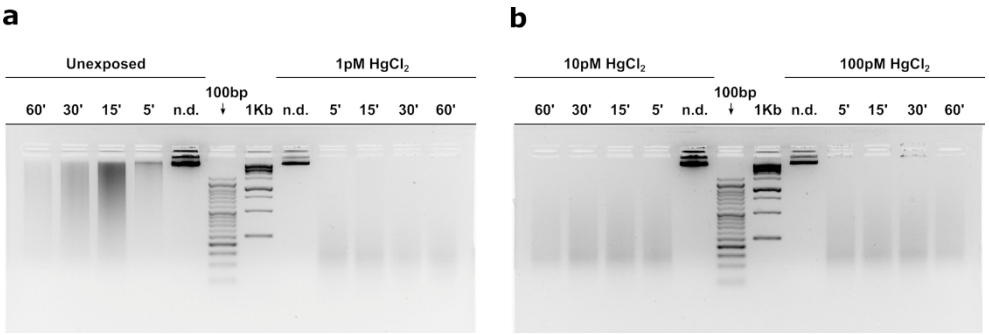

Figure 5. Micrococcal nuclease (MNase) digestion time course of *Mytilus galloprovincialis*' sperm chromatin. Analyses of DNA by electrophoresis on agarose gel: unexposed and exposed mussels to 1 pM $HgCl_2$ (Panel (**a**)), exposed mussels to 10 and 100 pM $HgCl_2$ (Panel (**b**)). n.d. = undigested sperm genomic DNA. 1Kb and 100 bp: molecular weight markers.

4. Discussion

Mercury (Hg) is a global environmental pollutant that affects ecosystem health and marine organisms [23,24], due to its significant implications on several biological processes, including growth, sexual maturation, and reproductive success [25]. Contamination of toxic substances in the environment, such as mercury, has been known to have socio-economic consequences. These effects have led to a limitation of Hg emissions from large-scale human activities. Bivalves are important species in coastal ecosystems, and play an important role particularly in food webs (as suspension feeders) and represent a significant fraction of fishery resources. In recent years, mussels have become a commercially important seafood species worldwide. Although the consumption of mussels is considered a safe food, bioaccumulation of heavy metals remains a problem for mussel consumers and this has an impact not only on human health but also on the economy [26]. Mussels, in fact, have strong interactions with the environment, water, and sediments and are considered good bioindicator species. In particular, *M. galloprovincialis* is an important food source, widespread throughout the world with high ability to bioaccumulate pollutants. So, the ingestion of drug-contaminated bivalves can pose a health risk to the human population [27], e.g., potential antibiotic resistance [28]. Thus, many pollutants can reach humans through mussels. These include: carbamazepine (CBZ), an antiepileptic drug used in the treatment of epilepsy, neuropathic pain, and psychiatric disorders [29], microplastics [30], and heavy metals, such as mercury (Hg), lead (Pb), chromium (Cr), and cadmium (Cd). Long-term exposure to these substances can produce neurological, physiological, and physical problems [31].

Mussels have also shown a greater capacity for Hg accumulation than other bivalve species [16], and the gonads have a particular affinity for mercury like other endocrine organs [32].

For these reasons and considering that the combination of morphological changes and transcriptional responses is recognized as valuable and promising tools for the study of ecotoxicological effects [33], in the present work, we first investigated changes in male

gonadal morphology and the level of steroidogenic enzymes after exposure of *M. galloprovincialis* for 24 h to 1, 10, 100 pM $HgCl_2$. In addition, we assessed both the expression levels of three stress genes and the PL protein-coding genes and determined the difference in the accessibility of sperm chromatin to micrococcal nuclease at these doses of exposure to $HgCl_2$.

First of all, we registered gonadal stress because RT-qPCR analyses showed altered expression of the stress genes, *mt10*, *π-gst* and *hsp70*. As reported by several authors [34,35] Mytilus species are able to synthesize metallothionenins, encoded by mt genes, in order to eliminate accumulated metals. Metallothionenins (MTs) are expressed in tissue, cell, and isoform specific modes depending on the type of metal they are exposed, including essential metals like copper and zinc and nonessential metals like cadmium and mercury. Banni M. et al., 2007 [36], based on laboratory exposures indicated that copper, zinc, mercury and cadmium, are able to induce the expression of this stress gene in mussels. Literature has reported work indicating alterations in *M. galloprovincialis mt10* gene expression after accumulation of various toxic metals, both essential, such as copper and zinc, and non-essential, such as cadmium and mercury but prevalently in digestive gland [37] in which, generally an increase of expression of these genes has been observed. However, there is a distinction between zinc and copper because these two metals act differently. First of all, Zn is a DNA stabilizer involved in transcription. It does not generate free radicals unlike Cu which is necessary for some redox reactions. Moreover, it can generate like Fe some free radicals chain reactions. Zn can displace Hg from interacting with proteins. In fact, it is sometimes administered in mammals as an anti Hg poisoning. Moreover, Hg uptake is decreased in the presence of Zn and recently have been reported new insight on the genetic basis of Zn and Cu accumulation in molluscs [38,39]. Zinc is an essential trace element for spermatogenesis. In mammals the Cu/Zn ratio is a marker of oxidative stress and several studies have suggested that later germ cells are less tolerant to ROS than early-type germ cells, mainly because of their limited reserve of antioxidant enzymes. This is due to the fact that zinc levels decrease during spermatogenesis. Zinc, in fact, plays an important role as a DNA stabilizer, being essential for several DNA repair enzymes that are important during early embryogenesis [40] and modulates SOD activity [41].

In the gonad of *M. galloprovincialis*, increased gene expression of *mt20* and *hsp70* was reported after exposure to cadmium in [13,14] whereas in our present study, we observed a decrease in the expression of both *mt10* and *hsp70* after mercury exposure, consistent with what was achieved after exposure of *M. galloprovincialis* to copper [12].Also π-gst genes was hypo-expressed after exposure of mussels to all doses of $HgCl_2$.

In our present study, after 1 pM $HgCl_2$ exposure, there was an approximately 2-fold increase in expression of *mt10* gene, while after 10 and 100 pM $HgCl_2$ exposure, we observed a hypo-expression of this gene which resulted of 1.2- and 1.5-fold, respectively, compared to the control condition. To our knowledge, there is no direct correlation between *mt10*, 3β-HSD and 17β-HSD expression. A possible relationship, at the transcriptional level, between these genes has never been reported in literature. However, it cannot be excluded that mercury, at this dose, may similarly affect the transcription factors regulating the expression of these genes. A hypoexpression was also observed for *mt20* gene in mussel digestive gland after exposure to copper (Cu) [36]. In spermatozoa, however, as demonstrated in Piscopo et al., 2021 [11], *mt10* gene did not show significant differences after 1 pM $HgCl_2$ exposure, while an increase in expression levels after mussels exposure to 10 and 100 pM $HgCl_2$ was found, in particular an increase of about three times in the expression of this gene.

The different response found in mussels spermatozoa and gonads exposed to $HgCl_2$ could suggest higher effectiveness of spermatozoa in the response to mercury, compared with gonads. A different response from spermatozoa and gonads was previously observed in response to subtoxic concentrations of copper [10].

Heat shock proteins (HSPs) are a group of ubiquitous proteins witch expression is activated by thermal stress [42]. Other stimuli that influence cell protein structure and

function, including oxidative stress and heavy metals, can induce heat shock genes [43]. In unstressed cells, some members of the HSP family are produced constitutively and operate as molecular chaperones, assisting protein maturation steps such as folding, unfolding, and translocation across membranes [44]. HSPs are classified based on their molecular weight. The stress-inducible *hsp70* and constitutively expressed hsc70 proteins are well-known members of the HSP70 (70 kDa) multigene family. HSP70 acts as a cell damage defence system, whereas HSC70 acts as a molecular chaperone. In our present research stress-inducible *hsp70* gene showed a hypo-expression in all exposure doses, more pronounced at 10 pM $HgCl_2$ exposure condition and in the order of approximately 2.2-fold compared to the control condition. A hypo-expression of *hsp70* gene was also find in *M. galloprovincialis* spermatozoa after 1 and 10 pM $HgCl_2$ exposure [11]. To our knowledge, this is the first time that has been demonstrated a downregulation of the *hsp70* gene in gonads under metallic stress at low concentrations and within short acute exposure, as well as in spermatozoa [11]. However, this is not the first time that downregulation of this gene has been observed in response to a xenobiotic. In fact, a decrease in the expression of *hsp70* gene has been observed in response to paracetamol in blue mussel *Mytilus edulis* [45]. *Hsp70* preserves protein integrity and suppresses apoptosis directly, whereas thermal and oxidative stress damage protein structure. *Hsp70* is implicated in inducible stress and is involved in numerous biological processes: cancer, autophagy [46], apoptosis [47] and necrosis [48]. This implies that when HSP70 is downregulated in mussels exposed to this elements, a greater risk of apoptosis and cell death is induced [45].

Hypo-expression was also found for the *π-gst* gene, the main glutathione s- transferase isoform expressed in mussel tissues [49]. Also in this case, no linearity was observed with respect to the three exposure doses. The most marked hypo-expression was obtained after 10 pM $HgCl_2$ exposure, and was of the order of 1.5-fold compared to control condition. One possible explanation for down-regulation of *π-gst* gene could reside in a potent activation of the lysosomal-vacuolar system and, subsequently, in the enhancement of autophagy [50], a condition that may mask anabolic processes such as increased gene transcription, in agreement with the hypothesis of Dondero et al., 2006 [51].

The gonadal stress responses observed by RT-qPCR prompted us to investigate on the possible damages mercury-induced on this tissue. We found that the treatment with 1 pM $HgCl_2$ did not give morphological changes in the gonad respect to the control, while the two other doses, 10 and 100 pM $HgCl_2$, caused vacuolized areas between the germ cells and the complete disorganization of the connective tissue. We used gonads at the mature stage, on mussels obtained in January and vacuolized areas were observed only in mussels exposed to $HgCl_2$ and not in control condition. The observed morphological changes of seminiferous follicles, as vacuolization and decrease of germ cell compartment thickness, were reported also in fish [52] and in mammal testis [53,54] after $HgCl_2$ treatment. A growing number of studies show that sex steroids are widespread in molluscs [55]. Initially, they were thought to be taken through the diet, since many plant species contain sex steroids similar to those in vertebrates [56]. Nevertheless, numerous studies have shown that the major classes of molluscs, i.e., cephalopods, gastropods, and bivalves, are capable of synthesizing sex steroids from precursors such as cholesterol or pregnenolone [57–59]. Actually, most of the steroidogenic pathways described for vertebrates have been shown to occur in molluscs.

In literature, it has been already demonstrated the negative impact of mercury on spermatogenesis and also steroidogenesis: in male catfish exposed to organic and inorganic mercury the activity of 3β-HSD was inhibited completely [60], as we observed in Mytilus treated with the higher $HgCl_2$ doses. 3β-HSD is implicated in the conversion of dehydroepiandrosterone (DHEA) in androstenedione. The 3-HSD family of enzymes also catalyzes the synthesis and/or degradation of 5-androstanes and 5-pregnanes. As a result, 3β -HSD regulates key steroid hormone-related responses in the adrenal cortex, gonads, placenta, liver, and other peripheral target organs. In general, all steroid hormones, including glucocorticoids, mineralocorticoids, progesterone, androgens, and oestrogens,

require 3β-HSD for production [61]. 17β-HSD, on the other hand, mediates the conversion of androstenedione (ASD) to testosterone (T). However, 17β-HSD have a wide range of functions, such as the regulation of not only steroids but also fatty acid and bile concentrations [62]. Our results showed a positive reaction after labelling with both anti-3β-HSD and anti-17β-HSD antibodies at the level of germ cells and connective tissue in the control specimen as previously reported [22], germ cells are positive to antibody in 1 pM and 10 pM treated samples also, but the positivity disappears almost completely in 100 pM treated samples. In the connective tissue the steroidogenic enzymes are recognizable in control and 1 pM treated animal, whereas in 10 and 100 pM treated samples the antibody positivity is very poor. A decrease in spermatogenesis, 17β-HSD, 3β-HSD and also testosterone level in plasma was shown in male mice after exposure to arsenic trioxide [32]. In the light of these results, we examined on gonads the expression of genes encoding PL-proteins, as these are the major component of the basic proteins that constitute the sperm chromatin of this organism [63]. A decrease of expression was found in the case of the PLIII gene in exposed mussels. The downregulation was observed at all exposure conditions but in particular after exposure to 10 pM $HgCl_2$. In addition, a slight hypo-expression of PLII-PLIV was detected. These results highlights the negative impact of mercury also on gonadal protamine-like (PL) genes, proteins essential for correct DNA compaction in *M. galloprovincialis* sperm heads. A similar result was found after mussels exposure to sub-toxic concentrations of copper [12]. These alterations in the PL gene could be associated with changes in 3β-HSD and 17β-HSD expression that we observed by immunolabelling as mercury has already been shown to disrupt hormones, including testosterone [64]. Both enzymes play an important role in hormone regulation, in particular for testosterone production and its involvement in spermatogenesis [22]. These results are in line with those observed in experiments previously conducted following exposure to sub-toxic copper concentrations [12] and those relating to the accumulation of Polycyclic Aromatic Hydrocarbons (PAH) and heavy metals in the male gonad of *M. galloprovincialis* showing that these pollutants disrupts spermatogenesis and produce alterations in somatic and germ cells [65]. The testicular toxicities of mercurials, including impaired spermatogenesis and/or steroidogenesis, have been demonstrated in a number of laboratory animal species: fish [50,66–69], fowls [67]. Furthermore, this has also been demonstrated in the mouse [66,70].

The morphological and hormonal alterations observed in the gonad and the changes found for both stress genes and those encoding PLs prompted us to investigate possible alterations in the sperm chromatin structure of mussels exposed to the three doses of $HgCl_2$. Results obtained by digestion with MNase show substantial differences between control and treated at all three doses of $HgCl_2$. In fact, the DNA of the sperm nuclei was totally hydrolyzed after digestion with MNase in mussels exposed to all exposure conditions, contrary to what was observed in unexposed mussels in which, despite there is no nucleosomal organization, and then a smear is obtained after sperm chromatin digestion of spermatozoa with MNase but not a total degradation of DNA. These results are in line with the alterations we had highlighted in our previous works showing a difference in PL conformation upon exposure of mussels to $HgCl_2$ and their different binding to DNA [10,11]. The release of PL proteins from sperm nuclei was also altered especially for PLII and PLIII at all doses of $HgCl_2$ exposure [10]. Far more significant was the evidence, we have already provided, concerning the effects of the addition of PL from mussels exposed to all three $HgCl_2$ doses to a plasmid DNA under pro-oxidant conditions. The result showed the breakage of DNA and a non-protection of the latter unlike what happened with PL from mussels not exposed [10,71,72]. Thus, the result we obtained with MNase digestion are quite predictable as it indicates that in mussels exposed to these doses of $HgCl_2$ there might be an improper structure of the sperm chromatin. This could indicate a possible risk in the fertilizing capacity of spermatozoa from mussels exposed to these doses of $HgCl_2$. After all, in literature, mercury is recognized as a male reproductive toxicant. In fact, studies performed in vitro have shown that this metal induces DNA breakage in spermatozoa and causes a decrease in sperm motility. Our previous work provides insights

into the mechanisms of mercury toxicity on the reproductive system of *M. galloprovincialis*. Specifically, the alteration of PL-proteins binding to DNA induced by exposure to these $HgCl_2$ doses [10]. This is in line with the results obtained with MNase digestion and could indicate an alteration in the structure of sperm chromatin, which is known to be critical for the swimming ability of spermatozoa and their ability to fertilize. In any case, however, our results are currently only descriptive as fertilization tests with spermatozoa of mussels exposed to these doses of $HgCl_2$ have not yet been conducted. Therefore, it cannot be predicted at this time whether this may have a negative impact from an economic point of view. Further study will allow to understand if the genotoxic effect of $HgCl_2$ in mussels could become an environmental problem since our ultimate goal is to develop genotoxicity tests based on sperm chromatin status.

In conclusion, the data obtained in this work could be an important step in understanding the mechanisms of mercury toxicity and reveal responses in a tissue such as the gonad that is generally not considered for ecotoxicological studies. In addition, our results again emphasize the importance of using *M. galloprovincialis* as a sentinel organism and suggest a possible risk on the fertilizing capacity of spermatozoa in mussels exposed to these doses of $HgCl_2$ as revealed by the results of experiments conducted with MNase. However, these results will need to be further investigated with additional studies to understand how these mechanisms impact on the reproductive health of *M. galloprovincialis*.

Moreover, heavy metal contamination can induce an ecological imbalance in the receiving environment and on the diversity of aquatic organisms. Pollutants accumulate in the food chain and are responsible for harmful effects on the occurrence of various diseases, such as Minamata disease (organic mercury poisoning). Therefore, preventive measures are needed to reduce the intensity of heavy metal pollution in the aquatic environment [73].

Author Contributions: Conceptualization, M.P. (Marina Piscopo) and G.L.; methodology, M.P. (Marina Piscopo), G.L. and R.N.; software, G.L.; validation, M.P. (Marina Piscopo) and C.M.; formal analysis, G.L.; investigation, G.L., R.N., N.C., M.P. (Marina Prisco), A.A. and S.P.; Data curation, G.L., R.N., N.C.; writing—original draft preparation, M.P., G.L., N.C., R.N., M.P. (Marina Prisco), A.A. and S.P.; writing—review and editing, M.P. (Marina Piscopo), G.L., N.C. visualization, G.L.; supervision, M.P. (Marina Piscopo); project administration, M.P. (Marina Piscopo). All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CT	Connective tissue
VTC	Vesicular connective tissue cells
ADGs	Adipogranular cells
17β-HSD	17β-Hydroxysteroid dehydrogenases
3β-HSD	β-Hydroxysteroid dehydrogenase/Δ^{5-4} isomerase
PL- proteins	Protamine-Like
H&E	Hematoxylin and Eosin
ASD	Androstenedione
T	Testosterone
DHEA	Dehydroepiandrosterone
n.d.MNase	UndigestMicrococcal nuclease

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International Journal of
Molecular Sciences

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Discovery of the Involvement in DNA Oxidative Damage of Human Sperm Nuclear Basic Proteins of Healthy Young Men Living in Polluted Areas

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Received: 11 May 2020; Accepted: 10 June 2020; Published: 12 June 2020

Abstract: DNA oxidative damage is one of the main concerns being implicated in severe cell alterations, promoting different types of human disorders and diseases. For their characteristics, male gametes are the most sensitive cells to the accumulation of damaged DNA. We have recently reported the relevance of arginine residues in the Cu(II)-induced DNA breakage of sperm H1 histones. In this work, we have extended our previous findings investigating the involvement of human sperm nuclear basic proteins on DNA oxidative damage in healthy males presenting copper and chromium excess in their semen. We found in 84% of those males an altered protamines/histones ratio and a different DNA binding mode even for those presenting a canonical protamines/histones ratio. Furthermore, all the sperm nuclear basic proteins from these samples that resulted were involved in DNA oxidative damage, supporting the idea that these proteins could promote the Fenton reaction in DNA proximity by increasing the availability of these metals near the binding surface of DNA. In conclusion, our study reveals a new and unexpected behavior of human sperm nuclear basic proteins in oxidative DNA damage, providing new insights for understanding the mechanisms related to processes in which oxidative DNA damage is implicated.

Keywords: DNA oxidative damage; protein–DNA binding; human protamines; heavy metals; EMSA; fluorescence

1. Introduction

DNA is the vital carrier of genetic information in all living cells, but its chemical stability is affected by several factors. In fact, DNA is highly susceptible to chemical modifications by exogenous agents such as ionizing radiation and ultraviolet light [1–3] and by several environmental contaminants (pesticides, hydrocarbons, and especially heavy metals), which can generate oxidative stress [4,5]. Beyond environmental agents, DNA is also subject to oxidative damage from by-products of cellular metabolism (endogenous agents). The consequential alterations of DNA structure are generally incompatible with its crucial role in the maintenance and transmission of genetic information. For this reason, cells respond to DNA oxidative damage by specified DNA repair pathways to physically remove the damages [6]. In the past 20 years, many papers regarding the involvement of DNA oxidative damage in human infertility have been published [7]. It is well known that both male and female gametes can be exposed to DNA damage, which may compromise their functionality and their capacity to produce normal embryos. In particular, DNA damage, affecting sperm quality, increases the risk of genetic and epigenetic abnormalities and can lead to some diseases. Although a small amount of Reactive Oxygen Species (ROS) is necessary for some fundamental processes for the physiological function of male gametes, high levels of ROS can cause functional failure [8]. There is a great interest in having new insights in the mechanisms of DNA oxidative damage, since it can also cause genetic alterations that may result in diseases, such as cancer and neurodegenerative syndromes [9], and contribute to some features of aging. Despite the numerous studies conducted on the matter [10], the precise molecular mechanisms that lead to DNA oxidative damage are not yet fully understood. In particular, to the best of our knowledge, no previous study has evaluated the possible involvement of Sperm Nuclear Basic Proteins (SNBP) in DNA oxidative damage. As a rule, histones and protamines, compacting DNA, protect from oxidative damage. Thus, in some stress conditions, possible functional alterations of SNBP properties should be more evident, considering the higher degree of compaction of sperm chromatin compared to that of somatic cells. As a matter of fact, in a previous work, we have demonstrated that some sperm histones, in the presence of specific heavy metals, can participate in DNA oxidative damage [4], suggesting that in particular stress conditions, their protective rule appears to be reversed. This observation prompted us to investigate through molecular-level analyses the possibility that SNBP from men living in polluted areas might have an involvement in oxidative DNA damage. In fact, we found a higher DNA fragmentation index in the spermatozoa of males recruited in the "Land of Fires", which is a high environmental impact area of Campania Region (Southern Italy) [11–14] in which resident people presented similar values for semen volume, pH, sperm cell count, and morphology, but a significant increase of immotile cells percentage [11].

To this aim, we have evaluated the protein framework, the DNA binding, and the potentiality to induce the oxidative DNA damage of SNBP from a cohort of men living in the "Land of Fires". This study was conducted as part of a biomonitoring project "EcoFoodFertility" [11] (http://www.ecofoodfertility.it/the-project.html).

2. Results

2.1. Characteristics of Impacted Areas Used for the Recruitment

The geographical areas selected for the recruitment (Figure 1) differ for the number of sites recognized by the Campania Region Environmental Protection Agency for the presence of a high concentration of toxic contaminants [15]. We considered 9 municipalities, inside the red circle in Figure 1, showing a high number of illegal disposal sites of toxic waste (Table 1). It's interesting to note that in the pattern of chemical elements of semen from males living in this area, the presence of high concentrations of Cr, Cu, and Zn was found [11]. The 7 municipalities in the area included in the green circle in Figure 1 were considered for the low number of environmental impact sites.

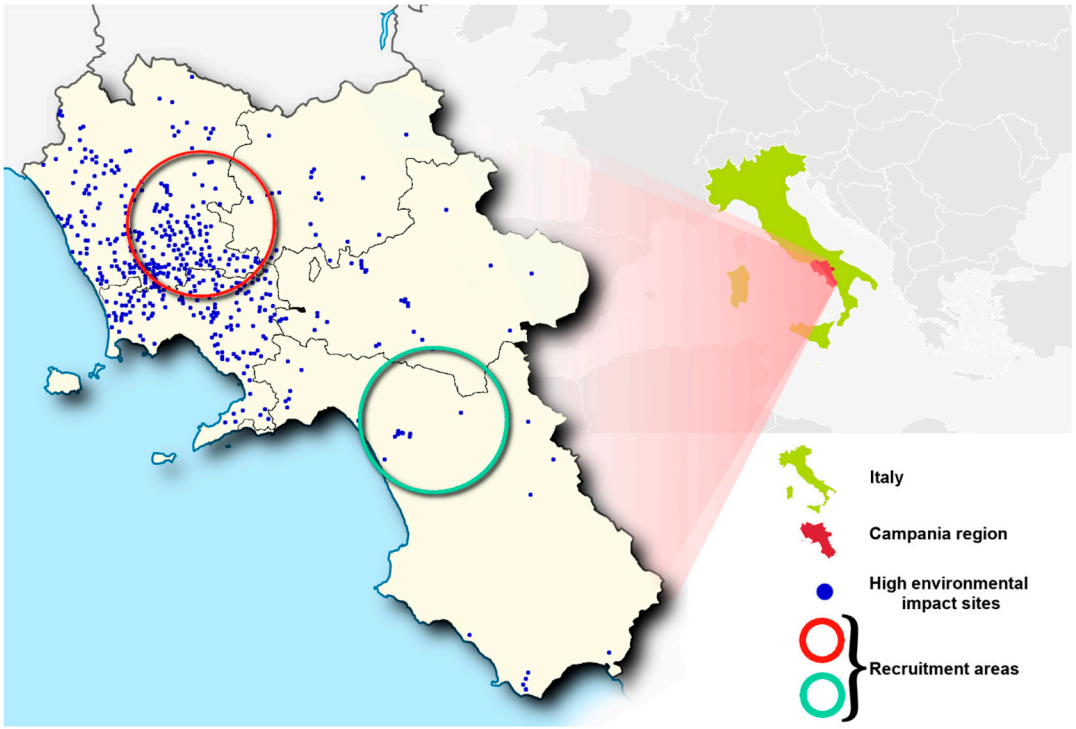

Figure 1. Map of the geographical areas selected for the recruitment. The red and green circles indicate the high and low environmental impact areas, respectively, in Campania region (Italy). Blue points indicate the sites of high environmental impact as recognized by the Campania Region Environmental Protection Agency report (2008).

Table 1. List of municipalities in low and high environmental impact areas for the recruitment.

Municipalities	
Low Impact Areas	**High Impact Areas**
Oliveto Citra–Contursi Terme–San Gregorio Magno–Buccino–Ricigliano–Valva–Colliano	Acerra–Caivano–Afragola–Casalnuovo-Pomigliano d'Arco–Brusciano–Giugliano–Cardito–Marigliano

2.2. Analysis of SNBP

We have observed different staining in the spermatozoa collected from samples of males living in the high (H-group) environmental impact area (Figure 2a). Regarding the nuclear maturity, the light, the intermediate, and the dark aniline blue-stained spermatozoa represent mature, moderately immature, and severely arrested maturity spermatozoa, as previously described [16]. We found a prevalence of samples containing dark blue-stained spermatozoa (right panel in Figure 2a), indicating the persistence of histones in the chromatin. In lower number of samples, we detect a prevalence of light and intermediate blue-stained spermatozoa (left and middle panels in Figure 2a, respectively), indicating mature and immature gametes, respectively. Instead, almost all the males living in the low (L-group) environmental impact areas had light-stained spermatozoa in their samples, showing the prevalence of mature gametes. We extracted the SNBP from samples belonging to males living in the two areas and characterized the protein content by Acid-urea Polyacrylamide Gel Electrophoresis (AU-PAGE) (Figure 2b). In lane 1 of Figure 2b, a representative electrophoretic pattern of samples belonging to the L-group is shown. In this sample, we observed the classic electrophoretic pattern of human SNBP, with the canonical protamines/histones ratio (CP/Hr), which was accordingly previously described [17]. In the samples of males belonging to the H-group, we observed several differences in the electrophoretic protein patterns. In particular, we identified samples showing only histones and other basic proteins (only-H) (lanes 2 and 3 in Figure 2b) in which protamines seem to be absent. In another group of samples, we observed the presence

of protamines and histones, but not in the canonical ratio (nCP/Hr). This latter condition was very heterogeneous, presenting a variable ratio between protamines and histones, as shown in Figure 2b (lanes 4–7). We categorized the analyzed 240 samples belonging to the two different areas based on the number and type of bands identified on the AU-PAGE, which is in accordance with the classification reported in [17]. These three types of electrophoretic profiles were differently distributed in the L- and H-groups. In particular, in males belonging to the L-group, we found only two conditions, the first represented by the 95.06% of samples shoving the CP/Hr profile, and the other represented by the 4.94% of samples showing the only-H pattern (Figure 2c). Differently, we found the CP/Hr profile in the 16.61% of sample from males belonging to the H-group, observing in the majority of this group samples (61.61%) the only-H profile. The remaining 21.78% of samples from the H-group showed a heterogeneous nCP/Hr profile (Figure 2c). The presence of bands close to the well in the samples showing altered protamine/histone ratios could be caused by the presence of substances promoting protein aggregation, such as the excess of some chemicals found in the semen of people from Land of Fire [11].

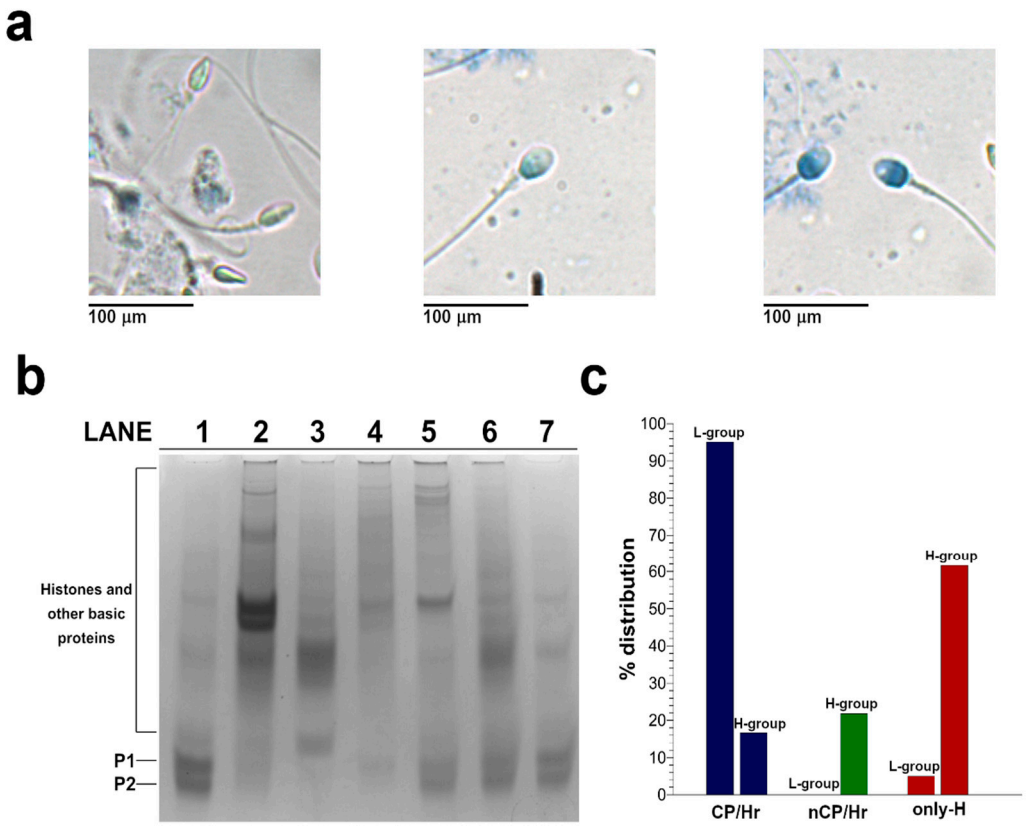

Figure 2. Characterization of human Sperm Nuclear Basic Proteins (SNBP) from samples belonging to the H- and L-groups. (**a**) Staining of spermatozoa collected from samples of H-group males. The light (left), the intermediate (middle), and dark aniline blue-stained (right) spermatozoa represent mature, moderately immature, and severely arrested maturity spermatozoa found in this group. (**b**) AU-PAGE of SNBP showing the CP/Hr (lane 1), only-H (lane 2–3), and not CP/Hr (lanes 4–7). (**c**) Percentage distribution in H-and L-groups of protamines/histones ratios found in spermatozoa. In blue, the samples containing the CP/Hr; in green, the nCP/Hr; and in red, the samples showing only-H.

2.3. DNA Binding Ability of SNBP Analyzed by EMSA

We studied, by Electrophoretic Mobility Shift Assays (EMSA), the differences in the ability to bind the DNA of the three typologies of SNBP observed in the samples of the L- and H-groups. In particular, we evaluated the ratio proteins/DNA necessary to obtain DNA saturation, which was

indicated by the formation of a high-molecular weight DNA band, close to the well, in electrophoretic pattern [18]. All the analyzed protein samples interacted with DNA in the typical "all or nothing" DNA binding mode of SNBP in agreement with data previously reported for SNBP [19,20]. Briefly, in the "all or nothing" DNA binding mode, the DNA band on the gel migrates with high mobility in the absence or in the presence of low protein (histones or protamines) concentrations. At increasing protein/DNA ratios, there are no DNA bands with intermediate electrophoretic mobility, but there begins to appear a DNA band with low mobility, close to the well, which is indicative of high molecular weight complex DNA–proteins.

We found that protein samples presenting the CP/Hr profile belonging to the H-group reached DNA saturation at a protein/DNA ratio of about 0.8 (Figure 3a), instead of the 1.2 ratio observed for the SNBP from samples of the L-group (Figure S1). As expected, samples presenting the only-H profile, regardless of whether they belonged to the L- and H-groups, showed instead low DNA binding ability, because DNA saturation did not occur even at 3.8 proteins/DNA ratio (Figure 3c,d). In accordance with the heterogeneity of the samples belonging to the third condition (nCP/Hr), we observed similar trends, but not identical DNA saturation values. However, all samples presented common features, showing a reduced DNA binding ability and a not-stable binding mode to DNA. In fact, as shown in the representative gel of Figure 3b, we observed, at the protein/DNA ratio concentrations from 0.05 to 1 (lanes 2–8), the decrease of supercoiled plasmid DNA fraction and the increase of the fraction close to the well. The next addition of proteins at protein/DNA ratio 1.2 (lane 9) results in an increase of supercoiled DNA fraction suggesting proteins detachment to DNA (compare lanes 8 and 9 in Figure 3b).

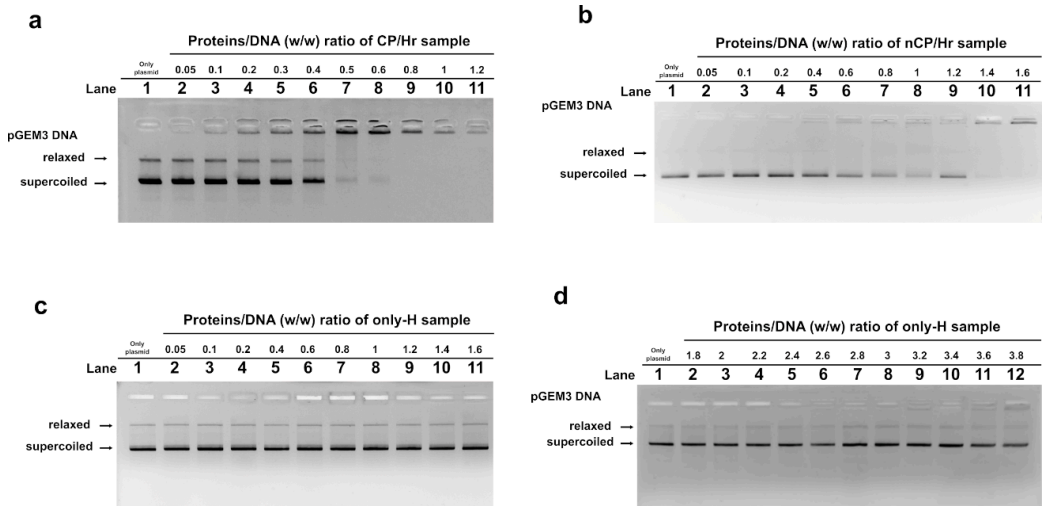

Figure 3. DNA-binding ability of SNBP obtained from H-group analyzed by Electrophoretic Mobility Shift Assays (EMSA) on 1% agarose gel. Bands on gel representing the state of pGEM3 plasmid DNA incubated in a ratio *w*/*w* with increasing amount of SNBP from samples containing CP/Hr (**a**), nCP/Hr (**b**), and only-H (**c**,**d**).

2.4. DNA Binding Ability of Sperm Proteins Determined by Fluorescence Analysis

Considering that the anomalous behaviors of the only-H and nCP/Hr conditions can easily explain the differences observed in their binding to DNA, we focused on the slight differences observed in the CP/Hr samples belonging the L- and H-groups. We analyzed the features of these latter in a more detailed manner by using extrinsic fluorescence approaches in solution. We exploited the sensitivity of fluorescent probe 8-anilinonaphthalene-1-sulfonic acid (ANS) to solvent polarity, increasing its fluorescence intensity on binding to biomolecules, in addition to a strong solvent-dependent shift in its emission spectrum [21,22]. We observed an increase in the fluorescence of ANS in the presence of sample belonging to the L-group with respect the ones from H-group; in addition, a marked blue-shift of the maximum of fluorescence was recorded for the sample collected in the low-environmental impact areas (Figure 4a). This trend was observed for all

the fluorescence-measured samples belonging the two groups, as shown by the box plot in Figure 4b. The observed difference was sufficiently significant, with a measured p-value ≤ 0.05, indicating a different accessibility of the fluorescent probe to the surface of proteins belonging to the two groups. These differences also affected the ability to bind the DNA of the proteins isolated from the two groups. In particular, measurements of ANS fluorescence in the presence of the complex protein–DNA of the proteins belonging to the L-group showed a linear decrease of the fluorescence at the increase of DNA concentration (Figure 4c). This result can be explained by a reduced protein surface available to the binding with the fluorescent probe, being the protein constantly subtracted by the binding to DNA. Although, also in the sample belonging to the H-group, we observed a similar decrease in the ANS fluorescence after the addition of increasing concentrations of DNA, the slope was not linear, but better described by a second-order function (Figure 4d). The differences in the overall fluorescence and in the interaction mode of human protamines belonging to the H-group suggested changes in the exposed surface of these proteins.

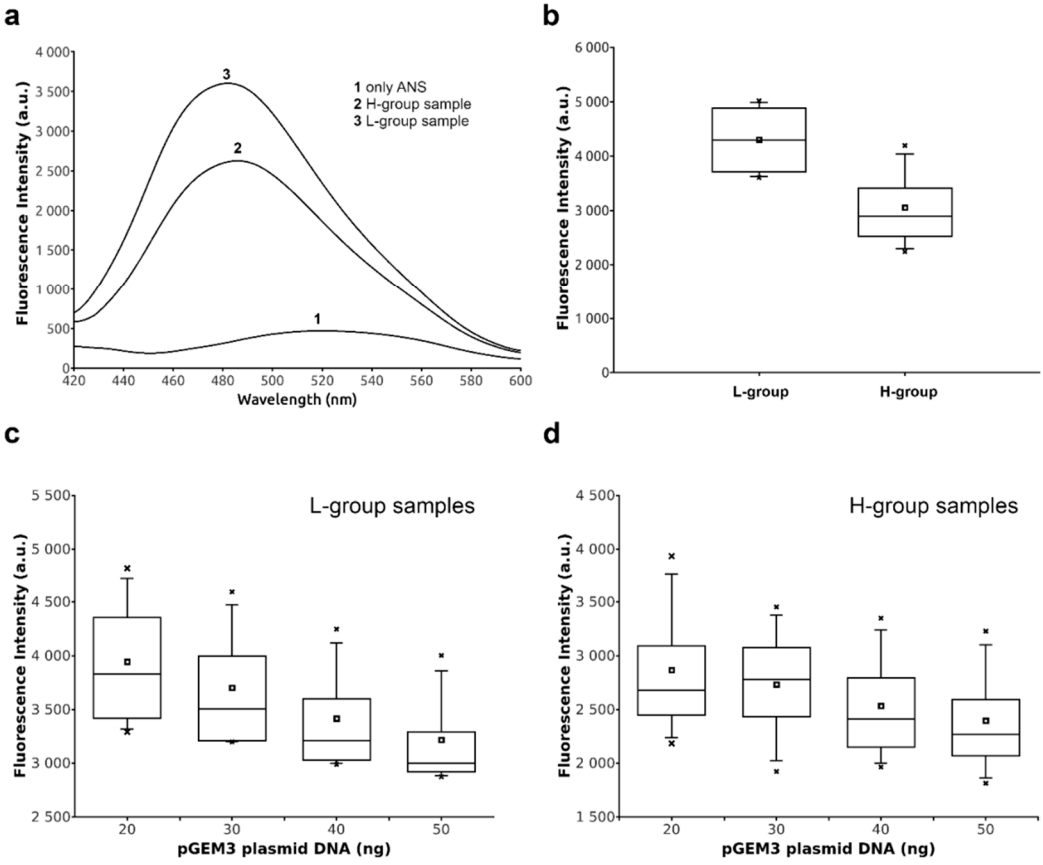

Figure 4. Fluorescence analyses of SNBP from samples containing CP/Hr belonging to the H- and L-groups. (**a**) Fluorescence spectra of ANS in the absence (curve 1) and in the presence of SNBP obtained from samples belonging to H- (curve 2) and L-groups (curve 3). (**b**) Box plot of fluorescence intensities of 8-anilinonaphthalene-1-sulfonic acid (ANS) in the presence of SNBP from samples belonging to H- and L-groups. Box plot of fluorescence intensities of the complexes ANS–SNBP belonging to the L-group (**c**) and H-group (**d**), in the presence of increasing pGEM3 plasmid DNA concentrations.

2.5. H_2O_2-Induced DNA Breakage in the Presence of Human SNBP

In Figure 5, the results of the analyses of H_2O_2-induced DNA breakage in the presence of human SNBP are shown. DNA breakage was evaluated by the conversion of a supercoiled to relaxed form of pGEM3 DNA plasmid in the presence of SNBP. In our experimental conditions, DNA breakage was not observed when plasmid was mixed with 30 μM H_2O_2, being necessary

higher H_2O_2 concentration, at least 100 μM, in order to cause DNA breakage (Figure S2). The addition of samples containing CP/Hr, at low protein/DNA ratios, to the pGEM3 DNA plasmid in the presence of H_2O_2 resulted in an increase of the relaxed plasmid DNA fraction at detriment of the supercoiled one (Figure 5a lanes 4, 6). Similar results were observed also for samples belonging to the H-group containing only-H (Figure 5d lanes 4, 6, 8). More relevant DNA damage was observed by using samples containing nCP/Hr; in fact, in this latter case, plasmid DNA appeared almost completely in the relaxed form (Figure 5b, lane 4). However, this latter condition being very heterogeneous, we have also observed differences in the protein/DNA ratio necessary to obtain DNA damage (Figure 5c, lane 6). The same analysis performed with samples of males living in low environmental areas presenting protamines and histones in canonical ratio did not show DNA breakage (Figure S3).

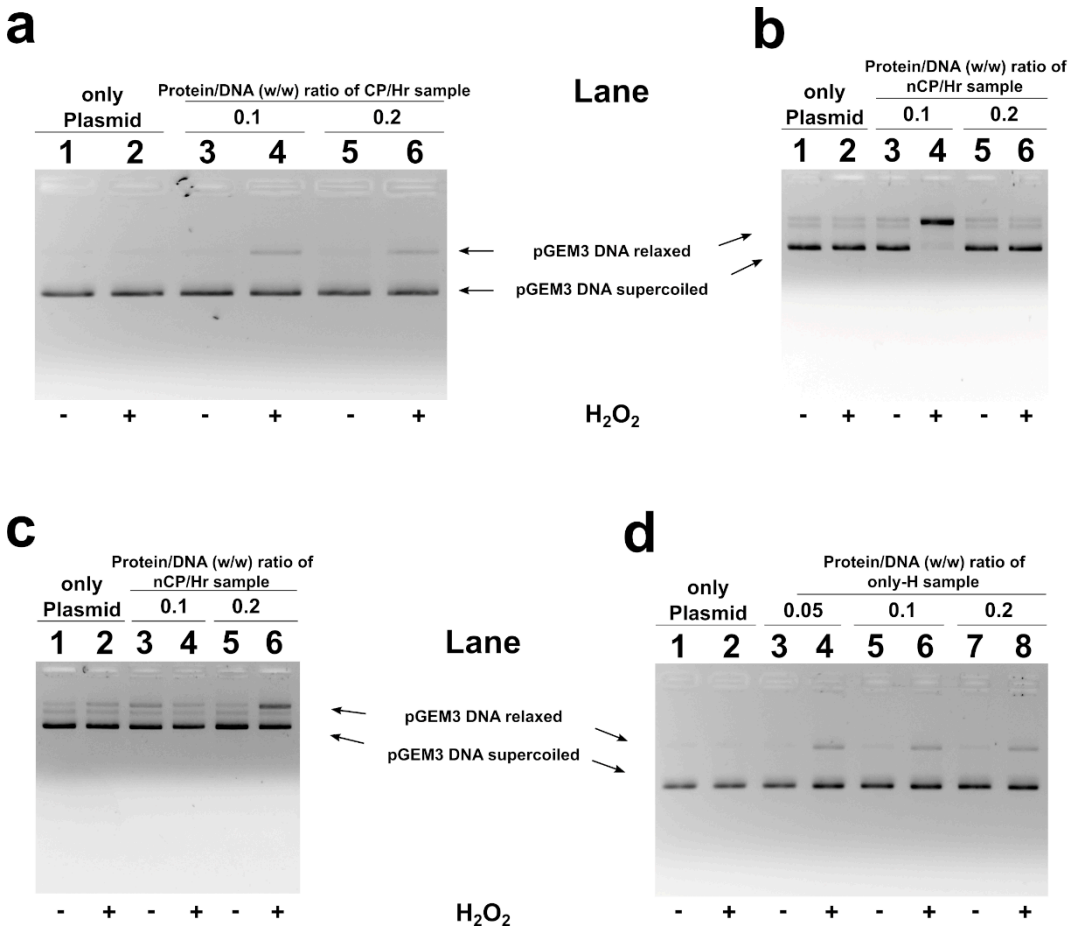

Figure 5. Analysis on 1% agarose gel of pGEM3 plamid DNA breakage induced by H_2O_2. In the presence of SNBP from H-group samples showing CP/Hr (**a**), nCP/Hr (**b**,**c**) and only-H (**d**).

3. Discussion

Living organisms are constantly exposed to numerous DNA damaging agents that can impact health and modulate disease states. DNA damage can cause genetic alterations that lead to the development of cancer, may result in cell death, as in neurodegenerative diseases, and could contribute to some features of aging [2]. However, the highest risk of DNA damage is represented by the effects at the gametes level, since this can jeopardize the possibility of fertilization and consequently the continuity of the species. Indeed, due to their characteristics, male gametes are the most sensitive cells to the accumulation of damaged DNA, considering their continuous production and exposure to environmental agents, such as oxidizing agents [23–26]. In this regard, many studies have been performed to understand the mechanisms of oxidative DNA damage, but some aspects are still unknown. We have tried to give new insights on this topic using human

spermatozoa as model cell for this study, evaluating the possible involvement of human SNBP in DNA oxidative damage. In fact, we have already reported that in the presence of heavy metals, some SNBP isolated from other organisms can participate in DNA oxidative damage, reversing their canonical protective rule [4]. However, in vitro studies are not sufficient to describe the complexity of in vivo effects on DNA oxidative damage, because DNA is not free but complexed with proteins to form chromatin in living cells [27], and it has been observed that Cu (II)/H_2O_2-induced DNA damage increases in the nucleosome compared to isolated DNA [28]. Therefore, it is of fundamental importance to conduct in vivo studies on DNA oxidative damage, since the results that can be obtained are an order of magnitude more complex than those obtained by treating only DNA in vitro. These observations are in agreement with our in vivo studies on SNBP from mussels exposed to subtoxic concentrations of heavy metals, such as copper or cadmium [5,29]. In fact, we found that the heavy metals measured in gonads accumulated mainly in the fraction of SNBP, causing their involvement in DNA oxidative damage [5,30]. Therefore, in the present study, we considered it more appropriate to use the spermatozoa of men living in high environmental impact sites, such the "Land of Fires", where some heavy metals, that participate in Fenton-like reactions, such as copper or chromium, are particularly abundant. In fact, in these subjects, a higher DNA fragmentation index [12] and alterations of specific bio-markers of DNA oxidative damages have been reported [11]. In particular, the comparison of subsets of randomly selected subjects from the L- and H-groups showed significantly lower Glutathione-S reductase and Glutathione peroxidase activities in the subset from the H-group (−32% and −25%, respectively; $p < 0.05$). Moreover, the mRNA level of γ-Glutamate cysteine ligase was also two-fold lower in the latter subset. In addition, DNA damage was measured in the same subsets, where antioxidant enzymes were assessed, reporting a DNA fragmentation index (DFI) value 2-fold higher in the H-group with respect to the L- one ($p = 0.01$) [11].

In agreement, our molecular analyses indicated an unusual distribution in the electrophoretic profiles of SNBP in men belonging to the H-group. In addition, we observed that all SNBP isolated from this group changed their protective ability, participating in DNA oxidative damage. This result was particularly marked for those samples presenting a not canonical protamine/histone ratio (Figure 6a). This strong occurrence of DNA oxidative damage in samples from men belonging to the H-group could be explained by an excess of copper and chromium found in the semen of people living in the "Land of Fires" [11]. In fact, it is well known that copper overload generally leads to oxidative stress, promoting the formation of hydroxyl radicals, which strongly reacts with practically any biological molecule, including DNA, causing severe damage to the cells [31–34]. Several studies have also demonstrated that copper can form several binary and ternary complexes with arginine residues [35–37], of which human protamines are extremely rich, promoting a site-specific damage at guanine residues of DNA by a selective binding between guanine and arginine [38]. Moreover, our recent studies have also revealed that Cu(II) interacts with arginine residues of sperm H1 histones, inducing oxidative DNA damage [4]. Moreover, Human Protamine 2 has a strong Cu(II)-binding amino acid motif at its N-terminus (Arg-Thr-His), which is able to mediate oxidative DNA double-strand scission and the generation of 8-oxo-2′-deoxyguanosine (8-oxo-dG) from free 2′-deoxyguanosine (dG) and from DNA by H_2O_2 [39,40]. Keeping in mind this evidence, it would be possible to speculate that these proteins could trap this metal, increasing the availability of Cu(II) ions near the binding surface of DNA. This condition could have as a consequence the promotion of the Fenton reaction in DNA proximity after H_2O_2 addition, determining DNA breakage and explaining the DNA oxidative damage found in CP/Hr samples of men belonging to the H-group. This finding is in accordance with the analyses carried out in the presence of Cu(II) concentrations for the in vitro determination of DNA-binding affinity of protamines and their involvement in DNA breakage. In fact, preliminary experiments indicated an increase in the DNA-binding affinity of CP/Hr proteins belonging to the L-group, in the presence of copper chloride, saturating the DNA at the protein/DNA ratio of 0.3 (lane 5, Figure S4) instead of 1.2, as observed for the CP/Hr proteins samples of the L-group in the absence of copper chloride (lane 9, Figure S1). In addition, in the presence of copper chloride, we observed an increase of relaxed DNA plasmid

(lanes 4 and 10, Figure S5), confirming the involvement of protamines in the DNA oxidative damage, as already demonstrated in our previous work on sperm H1 histones [4]. We found in the literature that also chromium, the other heavy metal found in excess in the semen of people in the H-group, could participate in Fenton-like reactions producing reactive oxygen species and could influence the structure of chromatin by binding to both DNA and histones [41,42]. The toxic effect of chromium results in radical-mediated DNA strand breakage and the formation of stable chromium–DNA complexes, including chromium–DNA adducts and protein–chromium–DNA and DNA–chromium–DNA cross-links [43,44]. In addition, histones bind chromium through lysine residues [41,45] and could determine an "indirect" DNA damage in a similar way as hypothesized for copper. These evidences could explain the effect measured in the samples from men belonging to the H-group, showing the presence of only histones. In fact, in these samples, we observed an extent of damage comparable to that found in the samples belonging to the men of the H-group, showing a canonical protamine/histone ratio (Figure 6a).

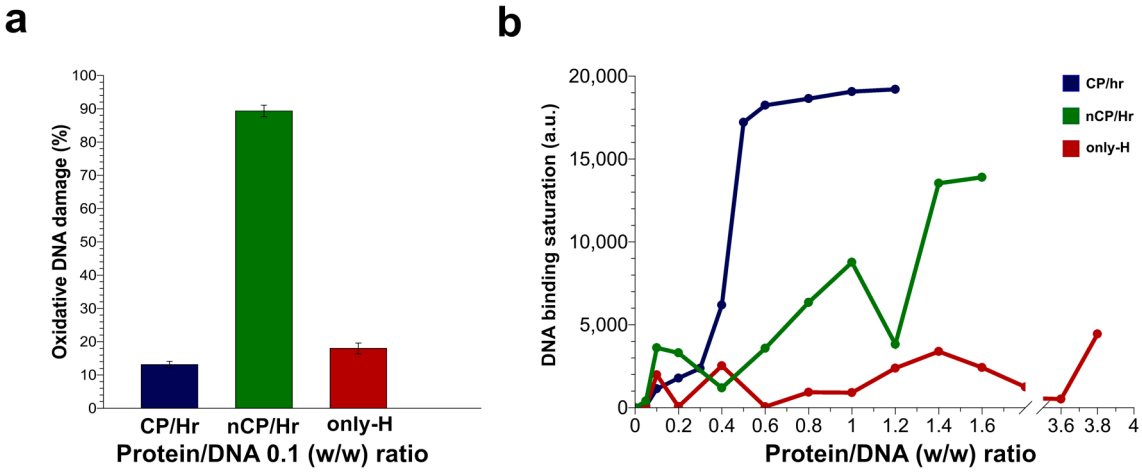

Figure 6. Graphical representation of the effects on DNA of human sperm proteins from samples belonging to the H-group showing canonical protamines/histones ratio (CP/Hr), protamines and histones, but not in the canonical ratio (nCP/Hr), and only-H. (**a**) Histograms of DNA oxidative damage, at 0.1 protein/DNA *w*/*w* ratio, quantified by densitometric analysis of bands on gels in Figure 5. (**b**) Lines chart of the DNA binding saturation, at increasing protein/DNA ratios, calculated by densitometric analysis of supercoiled bands on the gels of Figure 3.

The concomitant presence in the area under study of an excess of these heavy metals, participating in the Fenton reaction and able to bind histones and protamines respectively, can justify the more marked extend of DNA oxidative damage measured in the samples presenting histones and protamines in not canonical ratio (Figure 6a). The ability to induce DNA breakage, observed in the SNBP of men belonging to the H-group, can also be ascribed to the structural changes of these proteins due to tertiary/quaternary structure interactions. In order to study these conformational changes, we performed fluorescence measurements that are a sensitive tool to obtain information about protein–ligand interactions. However, we could not use the intrinsic fluorescence of these proteins because of their low content in aromatic amino acids. Therefore, we analyzed the features of sperm nuclear basic proteins in the samples showing the canonical ratio protamines/histones in the L and H-groups by using extrinsic fluorescence approaches with a solvatochromic dye such as ANS [21]. It is known that solvatochromic dyes are powerful tools for monitoring protein conformational changes and proteins interactions with nucleic acids, other proteins, and lipid membranes [22]. Generally, the increase of ANS fluorescence intensity and a blue shift in the emission maxima are attributed to the binding of the fluorescent probe to the hydrophobic sites on the protein and to its reduced mobility [21]. However, it has been also reported that ANS could bind arginine and lysine residues on the protein surface through ion pair formation [46], although the total fluorescence contribution of the ANS bond to these external sites is much less compared to that from the buried sites. In our experiments, we observed a reduced

fluorescence intensity of ANS in the sample from the H-group with respect to the ones from the L-group, indicating a different accessibility of the fluorescent probe to the surface of proteins belonging to the two groups. Taking into account the high extend of basic amino acid residues (arginine, lysine, and histidine residues) on the surface of these proteins, we could explain this outcome by a lesser number of arginine residues that could bind ANS in the sample from the H-group, following the binding with heavy metals. Otherwise, we can also hypothesize an indirect effect on the ANS fluorescence of the binding of protamines with the heavy metals. In fact, the increment of the total surface charge of the H-group protamines due to the addition of the positive charges of heavy metals could result in a more hydrophilic dielectric constant of the solution, quenching the fluorescence of the solvatochromic dye. Either of these hypotheses supported possible changes in the function of protamines, being the protein surface altered. In fact, these differences also affected the ability to bind the DNA of sperm protein from samples belonging to the H-group, having measured a not linear fluorescence quenching at the increased DNA concentrations with respect to the ones of the L-group. These differences in DNA binding were more evident from the plot of the band density against the protein/DNA ratio (Figure 6b). In fact, analyzing the DNA-binding ability of the SNBP from the samples of the H-group, we observed that DNA saturation was reached using a lower amount of proteins with respect to the SNBP from the samples of the L-group. This behavior supports the hypothesis of a possible alteration in the proteins' surface with an overall increase in the positive charge of the protein mediated by surface ions, determining a strong bond to DNA.

These differences in DNA binding could also explain the behavior of the samples with nCP/Hr (Figure 6b). The high content in arginine residues of protamines permits the binding both to minor and major DNA grooves, producing the adequate degree of sperm chromatin compactness, while histones interact only with the precise region of sperm DNA, producing a less compact chromatin [47]. Taking into account that 10–15% of histones are retained in human sperm chromatin [48–51], forming a heterogeneous mixture of nucleohistones and nucleoprotamines, we could hypothesize that the presence of both protamines and histones in altered ratios could determine not only an unstable binding to DNA, but also a reduced DNA protection to the external agents, such as heavy metals. In addition, in samples showing only histones, the low degree of compactness of the sperm chromatin could result in a low amount of chromium presented locally to DNA by histones, but at the same time, an increased exposure of DNA to external perturbants. Accordingly, increased concentrations of only histones samples resulted in a decreased degree of DNA breakage (Figure S6). As regards chromium, it is also important to consider that growing evidence suggests that epigenetic effects may in part be dependable for their genotoxicity and carcinogenicity [52,53]. In fact, it has been demonstrated that long-term chromium exposures may cause a significant increase in histone deacetylation. This effect may be particularly relevant in the histones–protamines transition which, as well known, requires histone acetylase activity and then could explain the high percentage of subjects (about 65%) in H areas that presented only histones in spermatozoa. In addition, the increase in histone deacetylation would lead to histone methylation in specific positions involved in gene repression and silencing, such as H3K9 [54–57].

In any case, considering that spermatozoa are produced continuously, we have no evidence that the abnormal protein patterns observed in samples isolated from men living in the "Land of Fires" cannot change over time because of the continuous changes in environmental conditions and in the quantity or types of xenobiotics accumulated in gametes.

In conclusion, in this work, we demonstrated for the first time the involvement in DNA oxidative damage of human SNBP from men exposed to pollutants, giving new insights on the toxicity mechanisms of some heavy metals. The potential implications of these findings could provide guidance in the future to better understand many mechanisms related to different diseases and processes in which oxidative DNA damage is implicated.

4. Materials and Methods

4.1. Reagents

All used reagents were of analytical grade and purchased at Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

4.2. Ethical Statements

All methods were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) guidelines and regulations. All experimental protocols were approved by the Ethical Committee of the Local Health Authority Campania Sud-Salerno (Committee code 43/2015/06). Informed consent was obtained from all recruited subjects (over 18) before sample collection.

4.3. Recruitment

The recruitment was conducted from October 2017 to November 2018, during a pilot study (EcoFoodFertility initiative, www.ecofoodfertility.it) to investigate the use of human semen as an early biomarker of pollution in healthy men [11,12,58], living in areas with low and high environmental impact in the Campania region (Southern Italy). The geographical areas selected for the recruitment are shown in Figure 1. Semen samples from the first group (n = 80 healthy males) came from San Francesco d'Assisi Hospital in Oliveto Citra-Province of Salerno, which is a municipality belonging to the low environmental impact area known as "Alto-medio Sele" (Oliveto Citra, Contursi Terme, San Gregorio Magno, Buccino, Ricigliano, Valva, and Colliano). This area has a low environmental impact (https://www.arpacampania.it/); its economy is principally based on low-to-medium scale farming and without known illegal disposal of toxic wastes (green circle in Figure 1). The semen samples of the second group (n = 160 healthy males) came from the municipalities belonging to the "Land of Fires" (Acerra, Caivano, Afragola, Casalnuovo, Pomigliano d'Arco, Brusciano, Giugliano, Cardito, and Marigliano) and the Medicina Futura center (Acerra-Province of Naples) (red circle in Figure 1). "Land of Fires" is a high environmental impact area of Campania that is officially recognized on the basis of the Campania Region Environmental Protection Agency report, as an area with the highest concentration of illegal disposal sites of toxic waste (https://www.arpacampania.it/). The participants were selected within clinically healthy male volunteers in fertile age (in the range 18–30 years old). Enrollment criteria were as follows: residence for at least 10 years in the study area, no known chronic diseases (diabetes or other systemic diseases), no varicocele, no prostatitis, and other factors that could affect semen quality (such as fever, medications, exposure to X-rays, etc.), no drinker, no smoker, no reported history of drug abuse, and no known occupational exposures to toxic chemicals. Moreover, the recruited participants follow a healthy diet and practice at least 30 min of walking at day. Data were collected by questionnaire and physical examination, including the urogenital evaluation (testis volume and transrectal prostate evaluation). Upon enrolment, a code number (HS1, HS2, HS3, …, HSn) was assigned to each volunteer by the recruiting andrologist (the recruiter) in order to preserve anonymity. Each code number was uploaded into a computer database along with personal and clinical information.

4.4. Spermatozoa Collection and SNBP Extraction

Semen samples were centrifuged at 5500 × g for 30 min at 4 °C in order to separate the spermatozoa from seminal plasma. Sperm pellets with a volume of about 50 µL were stored at −80 °C until biochemical and molecular analyses. The protocol used for SNBP extraction is based on the reports from [59], with slight changes. In brief, the sperm pellets were washed twice with 500 µL of phenylmethanesulfonyl fluoride (PMSF), resuspended with 50 µL of 1 mM PMSF and 50 µL of a solution containing 6 M guanidinium chloride and 10 mM DTT and then incubated at 20 °C for 30 min. For sperm chromatin precipitation, 5 volumes of cold absolute ethanol were added, and the

samples were incubated at least 60 min at –20 °C. After centrifugation at 13,680 g for 15 min at 4 °C, the pellet obtained was resuspended in 500 μL of 0.5 M of HCl; the sample was incubated for 5 min at 37 °C and then centrifuged a 1000× *g* for 10 min at 4 °C. The sperm nuclear basic proteins were precipitated from supernatant obtained by adding trichloro acetic acid with a final concentration of 20%; the sample was incubated 60 min at 4 °C, and then centrifuged at 14,000× *g* for 10 min at 4 °C. The pellet obtained was washed by adding 500 μL of acetone containing 1% β-mercaptoethanol. The sample was centrifuged twice at 14,000× *g* for 10 min at 4 °C and the final pellet dried in a speed-vacuum for 10–15 min or under a fume hood at room temperature. The dried proteins were resuspended in 50 μL of ultrapure water (milliQ) and used immediately or stored at −20 °C in aliquots of 50 μg.

4.5. Acid-Urea Polyacrylamide Gel Electrophoresis

Human protamines were analyzed by AU-PAGE as previously described [17]. In brief, the components of gel were 15 mL of a solution composed by 2.5 M urea, 0.9 M acetic acid, and 15% acrylamide/0.1% N,N′-Methylene-bis-acrylamide, 80 μL of TEMED and 800 μL of 10% APS. After gelification in about 1 h, at room temperature, pre-electrophoresis was performed at 150 V for 1 h 30 min, placing the negative electrode at the bottom of the gel. The buffer used was 0.9 N acetic acid and in the wells were loaded 20 μL of a solution containing 0.9 N acetic acid 2.5 molar urea. After pre-electrophoresis, the wells were washed with 0.9 N acetic acid buffer using a syringe, and the electrophoretic chamber was filled with fresh 0.9 N acetic acid buffer. Then, 2–2.5 μL per well of each sample, containing 4 μg of proteins in 0.9 N acetic acid and 2.5 molar urea, were loaded for the run, which was conducted at 150 V for about 55 min. At the end of the electrophoresis, gels were stained with Amido Black, and then with Coumassie Blue Brilliant R-250, as previously described [60]. Gels were acquired using a Gel-Doc system (BioRad, Hercules, CA, USA) through Quantity One v.4.4.0 (BioRad, Hercules, CA, USA) software. A densitometric analysis of the bands on the gel was performed using the software ImageJ ver 1.50d (Wayne Rasband, National Institute of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018).

4.6. Plasmid DNA Preparation

pGEM3 plasmid DNA (2867 bp) was purified from transformed *Escherichia coli* HB 101 cells, using the method as previously described [61]. For plasmid purification, we used the standard protocol of the QIAGEN Plasmid Midi Purification kit (QIAGEN Plasmid Midi Purification handbook, third edition © 2020) but performing all the steps at 12 °C and not at 22 °C as recommended by Qiagen handbook in order to obtain high amounts of sc pDNA. Another critical step was the pDNA pellet air drying after isopropanol precipitation and ethanol washes. During these steps, we maintain the sample on ice avoiding pipetting DNA, because this may cause shearing. Finally, the quality of plasmid DNA was evaluated by gel electrophoresis on 1% agarose gels in 89 mM Tris-HCl pH 8.0, 2 mM EDTA, and 89 mM boric acid (TBE). The obtained plasmid DNA was used, in the circular form, for Electrophoretic Mobility Shift Assays (EMSA) of DNA and DNA oxidative damage experiments.

4.7. DNA Binding Affinity of SNBP by EMSA

The effect of human SNBP, extracted from individuals living in low and high environmental impact area, on DNA was analyzed by EMSA as previously described [62], with slight modifications. In brief, mixtures DNA/proteins were prepared. Each mixture contained 150 ng of plasmid DNA (pGEM3) and an increasing amounts of proteins, which was expressed as protein/DNA wt/wt ratios (reported on the wells of the gels shown in the results section). The protein/DNA wt/wt ratios were between 0.05 and 3.8, as indicated in each experiment. At the end of the interaction between DNA and proteins (5 min at room temperature), all samples were added with TBE 10× (to obtain TBE 1× final concentration) just before running the gels and analyzed on 1%

agarose gel in TBE. DNA migration was visualized by staining agarose gels with ethidium bromide (2 μg/mL) after electrophoresis. All experiments were performed at least five times.

4.8. Fluorescence Spectroscopy

The fluorescence analyses were carried out in a 1 cm optical path length cuvette (STARNA), 0.5 mL volume, using a Jasco spectrofluorometer model FP 8200, equipped with a Julabo F25-HD temperature controller (Julabo GmbH, Seelbach, Germany). Fluorescence measurements has been carried out on human protamines at the concentrations of 0.025 mg/mL in the presence of 5 μM ANS in water. Fluorescence spectra were acquired in the emission range of wavelength from 420 to 600 nm after excitation at 350 nm. Photomultiplier absorbance did not exceed 600 V in the spectral regions measured. Each spectrum was signal averaged at least three times and smoothed with the software Spectra Manager Ver. 2.09 (Jasco Analytical Instruments, Tokyo, Japan). All measurements were performed at least three times at 25 °C.

4.9. DNA Breakage Analyses

pGEM3 plasmid DNA breakage in the presence of SNBP extracted from individuals living in low and high environmental impact area and 30 μM hydrogen peroxide (H_2O_2) was analyzed on 1% agarose gel in TBE 1× final concentration. Here, 150 ng of plasmid DNA (pGEM3) and proteins/DNA *w*/*w* ratios in a range from 0.1 to 0.4 were used. DNA and proteins were incubated at room temperature for 5 min in order to interact, after which H_2O_2 was added and the samples were incubated for 30 min at 37 °C in the dark. At the end of incubation, samples were added with TBE in order to obtain 1× final concentration just before electrophoresis analysis in order to avoid the EDTA coordination of eventual metals. Electrophoresis was carried out at 100 V for 30 min. DNA migration was visualized by staining agarose gels with ethidium bromide (2 μg/mL) after electrophoresis. All experiments were performed at least five times.

4.10. Aniline Blue Staining

The staining was performed as previously described [63], with few modifications. In brief, the fresh semen smear of each sample was air dried and then stained with 5% aqueous aniline blue stain (Histon Color Test, AB Analitica, Padua, Italy) in 4% acetic acid (pH 3.5) for 5 min. A cover slide 24 × 50mm was put on each slide. Stained and unstained spermatozoa were observed using light microscopy (Nikon Eclipse Ci) at ×1000 magnification under oil immersion (Plan 100×/1.25 oil objective).

4.11. Densitometric Gel Analysis

Gels were acquired using a Gel-Doc system (BioRad, Hercules, CA, USA) via Quantity One v.4.4.0 (BioRad, Hercules, CA, USA) software. Densitometric analysis on gel bands was carried out using the software ImageJ ver 1.50d (https://imagej.nih.gov/ij/) supported by the National Institute of Health (Wayne Rasband, National Institute of Mental Health). The quantification of DNA binding saturation by SNBP measured by EMSA was calculated by subtracting the value obtained from the densitometric analysis of each supercoiled bands to the value in the absence of proteins. The quantification of DNA oxidative damage from bands on agarose gel was determined as the percentage of the band of relaxed DNA form with respect to the total amount of DNA (supercoiled + relaxed forms) bands.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/21/12/4198/s1, Figure S1: DNA binding ability of sperm proteins obtained from L-group analyzed by EMSA; Figure S2: Evaluation of pGEM3 DNA plasmid breakage in presence of H_2O_2 concentrations; Figure S3: DNA breakage induced by H_2O_2; Figure S4: DNA binding ability of SNBP in the presence of 15 μM $CuCl_2$ analyzed by EMSA; Figure S5: DNA breakage induced by

H_2O_2 in the presence of 15 μM $CuCl_2$; Figure S6: Graphical representation of oxidative DNA damage in presence of human sperm proteins.

Author Contributions: Conceptualization, M.P., F.F. and L.M.; Supervision, M.P., F.F., S.R., M.T. and L.M.; Investigation, G.L., G.D., E.M., C.C., R.E., A.C., T.N. and A.G.; Formal analysis, M.P., F.F., L.M., S.R., T.N. and G.L.; Visualization, M.P., F.F. and G.L.; writing—original draft preparation, M.P. and F.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: We thank Liliana Parlato for her technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ROS	Reactive oxygen species
AU-PAGE	Acid-urea Polyacrylamide Gel Electrophoresis
EMSA	Electrophoretic Mobility Shift Assays
ANS	8-Anilinonaphthalene-1-sulfonic acid
SNBP	Sperm Nuclear Basic Proteins
H-group	Man living in the high environmental impact areas
L-group	Man living in the low environmental impact areas
CP/Hr	Canonical protamines/histones ratio
nCP/Hr	Not canonical protamines/histones ratio
only-H	Only histones and other basic proteins
DFI	DNA fragmentation index

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International Journal of
Molecular Sciences

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Molecular Alterations in Spermatozoa of a Family Case Living in the Land of Fires—A First Look at Possible Transgenerational Effects of Pollutants

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Received: 30 July 2020; Accepted: 11 September 2020; Published: 13 September 2020

Abstract: In our previous work, we reported alterations in protamines/histones ratio, in DNA binding of these proteins and their involvement in DNA oxidative damage in 84% of the young men living in the Land of Fires. In the present work, we extended our findings, evaluating any alterations in spermatozoa of a family case, a father and son, living in this area, to also give a first look at the possibility of transgenerational inherited effects of environmental contaminants on the molecular alterations of sperm nuclear basic proteins (SNBP), DNA and semen parameters. In the father and son, we found a diverse excess of copper and chromium in the semen, different alterations in SNBP content and low DNA binding affinity of these proteins. In addition, DNA damage, in the presence of $CuCl_2$ and H_2O_2, increased by adding both the father and son SNBP. Interestingly, son SNBP, unlike his father, showed an unstable DNA binding and were able to produce DNA damage even without external addition of $CuCl_2$, in line with a lower seminal antioxidant activity than the father. The peculiarity of some characteristics of son semen could be a basis for possible future studies on transgenerational effects of pollutants on fertility.

Keywords: human spermatozoa; human protamines; Land of Fires; DNA oxidative damage; protein-DNA binding; heavy metals; EMSA; transgenerational effects

1. Introduction

A great concern has been raised for human health and environment in the well-known "Land of Fires", in Campania (Southern Italy) [1,2]. In fact, in 2013–2014, this area, located on the northern-east side of the Campania region, was involved in illegal activities, including the uncontrolled spilling of industrial and urban waste and burning of toxic waste of various types, with release into the environmental of heavy metals and toxic chemicals that are detrimental for human health [3,4].

Over the past few decades, accumulated evidence has indicated that environmental pollution can also have a serious impact on the fitness, reproduction and survival of living organisms [5]. Pollution can increase infertility, and recent studies demonstrated that pollutants have diverse negative impacts on human spermatozoa. They, in fact, can alter not only the classic spermatozoa parameters, such as morphology, vitality, count and motility, but can also cause DNA fragmentation [6], and it would seem that reactive oxygen species (ROS) imbalance and related oxidative stress could be the principal cause through which pollutants can alter these parameters [6–8]. As a matter of fact, spermatozoa are very susceptible to environmental changes, mainly due to the pro-oxidant effects of environmental pollutants due not only to the limited volume of the cytoplasmic space, with less antioxidant [9] defense, but also because sperm membrane lipids are the target of reactive oxygen species (ROS). For these reasons, these cells are considered "ideal" bioindicators of environmental pollution and early sentinels of human health [10], and in our recent studies we provided new insights on DNA oxidative damage mechanisms in these cells. In particular, we discovered, through molecular investigations, that in young men living in the "Land of Fires", some environmental pollutants can alter the properties of the sperm nuclear basic proteins (SNBP), making these proteins able to induce DNA breakage [11]. As is well known, in humans, during spermiogenesis, the majority of the somatic histones are replaced first by testis-specific histone variants and transition proteins and subsequently by protamines, to pack the genome into the highly condensed sperm nucleus [12,13]. In human spermatozoa, there are two types of protamines, named P1 and P2 [14,15], which occur normally in a strictly regulated 1:1 ratio [16]. In particular, a lot of studies demonstrated that strict regulation of protamines P1/P2 ratio, normally falling in the range 0.8–1.2, and a canonical protamines/histones ratio are critical for the fertility status of human sperm [17], considering that 10%–15% of histones in human are retained [18]. In our previous study on spermatozoa of young men living in the "Land of Fires", we found that only 16% of the recruited subjects presented the canonical protamines/histones ratio, while the majority (about 62%) showed only histones and the remaining part (22%) had a non-canonical protamines/histones ratio [11]. It is well known that, as a result of industrialization, there has been a rapid increase in the variety of environmental pollutants which have negative repercussions on reproductive health. Moreover, several epidemiologic studies reported unexplained father–son effects from a variety of occupational or environmental exposures. Taking also into account that increasing evidence has shown that exposure to various pollutants can produce transgenerationally inherited effects and studies conducted on mice indicated that the susceptibility to certain pollutants can increase from one generation to the next [19], in the present work we deepened our studies, focusing on a family case living in the "Land of Fires", constituted by a father (50 years old) and his son (18 years old). In these subjects, we analyzed the content of SNBP, the binding of these proteins to DNA and their ability to protect DNA from the action of free radicals. In addition, we also evaluated the potentiality of SNBP to promote the breakage of DNA, the antioxidant activities in the semen and the content of copper and chromium, two heavy metals, involved in Fenton reaction. Finally, we also took a first look at the possibility of possible transgenerational hereditary effects of environmental contaminants on molecular alterations of basic sperm proteins (SNBP), DNA and seminal parameters of these two subjects, with the aim of having a starting point for future, more extensive studies, with a larger sample of fathers and sons living in the "Land of Fires".

2. Results

2.1. Characteristics of Impacted Areas Used for the Recruitment

The father and his son live in the "Land of Fires", a high environmental impact area in Southern Italy (Campania region), while the control subjects (L-groups) belong to the low environmental impact area known as "Alto-medio Sele" (https://www.arpacampania.it/). For this study, we used two control subjects: control 1 (18 years old) and control 2 (50 years old). The geographical areas of residence of the analyzed subjects have already been described in Lettieri et al., 2020 [11]. These areas differ for

the number of sites recognized by the Campania Region Environmental Protection Agency for the presence of a high concentration of toxic contaminants [20].

2.2. Aniline Blue Staining

Aniline blue stain is a specific technique able to discriminate between lysine-rich histones and arginine/cysteine-rich protamine. Aniline selectively binds the lysines on the histones, giving to the cells the typical blue stain. A sperm with a complete nuclear maturation undergoes the almost total replacement of histones with protamine and it is negative to aniline blue staining (AB−). On the contrary, a sperm that has not completed the process of replacing histones with protamine is colored pale blue (PB) or dark blue (AB+), depending on the amount of histones still present. Regarding the nuclear maturity, AB−, PB and AB+ spermatozoa represent mature, moderately immature, and severely immature spermatozoa, respectively. Aniline staining of spermatozoa showed different results in the control, son and father samples (Figure 1). In the control 1 sample, the prevalence of aniline negative stained (AB−) sperms and the presence of only very few aniline blue stain positive (AB+) sperms was observed (a). Similar results were obtained in control 2 (Figure S1). The prevalence of pale blue (PB) aniline stained sperms were observed in the son sample (b). Differently, in the father sample, aniline blue stain-positive sperms (AB+) were observed (c).

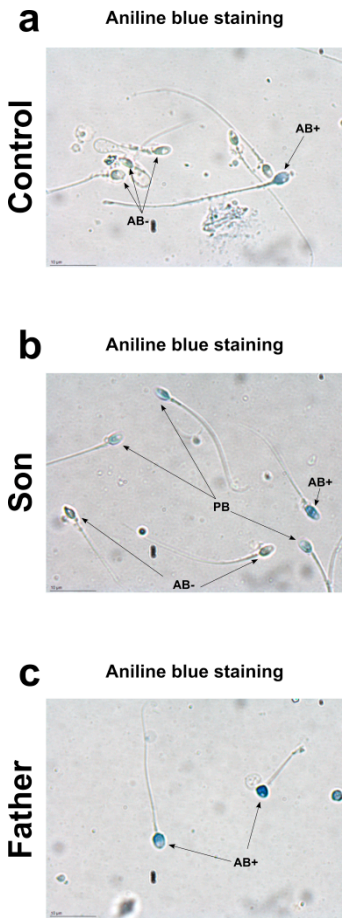

Figure 1. Aniline blue staining from control 1 sample (**a**), son (**b**) and father (**c**).

2.3. Analysis of SNBP

SNBP were extracted from spermatozoa of the father and son and from two subjects living in the low impact area (L-group) and used as controls, and the protein content was characterized by acid-urea polyacrylamide gel electrophoresis (AU-PAGE) (Figure 2). In lanes 3 and 4, the electrophoretic

pattern of control 1 and 2 samples, respectively, belonging to the L-group is shown. In these samples, we observed the classic electrophoretic pattern of human SNBP, with the canonical protamines/histones ratio (CP/Hr), which was accordingly previously described [21]. In the samples of the father and son, we observed several differences in the electrophoretic protein patterns. In fact, the SNBP of the father, shown in lane 6, consisted of only histones and other basic proteins (only-H) in which protamines seem to be absent; while the SNBP of the son showed the presence of both protamines and histones but not in the canonical ratio (nCP/Hr), (lane 5). In lanes 1, 2 and 7, instead, the electrophoretic patterns of rabbit total histones, sea urchin total histones and calf thymus H1 histone are shown, respectively.

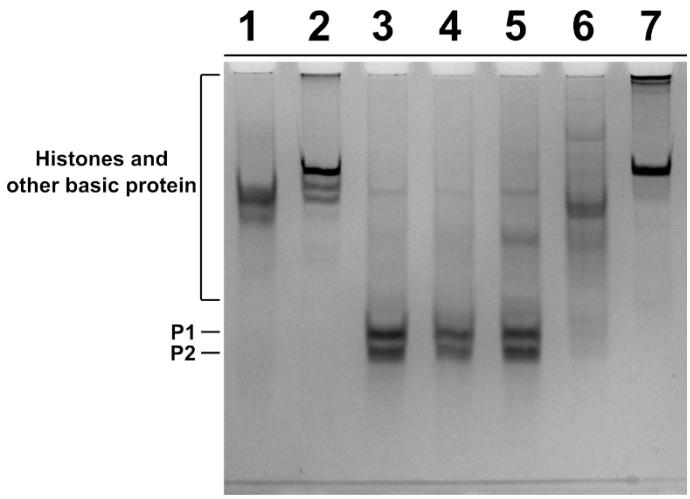

Figure 2. Characterization of human SNBP from samples belonging to two individuals of L-group (controls) and a father and his son living in the "Land of Fires". AU-PAGE of SNBP of: rabbit total histones(lane 1); sea urchin total histones (lane 2); human control 1 and 2 samples, showing the CP/Hr (lanes 3–4); son sample showing the nCP/Hr (lane 5) and father sample showing only-H (lane 6); calf thymus H1 histone (lane 7). SNBP: sperm nuclear basic proteins; L-group: low impact area group; CP/Hr: canonical protamines/histones ratio; nCP/Hr: non-canonical protamines/histones ratio; only-H: only histones and other basic proteins.

2.4. DNA Binding Ability of SNBP Analyzed by Electrophoretic Mobility Shift Assay (EMSA)

We studied, by electrophoretic mobility shift assays (EMSA), the differences in the ability to bind the DNA of the SNBP samples belonging to the control 1 subjects and of the father and son. In particular, we evaluated the proteins/DNA ratio required for obtain DNA saturation, detectable by the formation of a high-molecular weight DNA band, close to the well, in electrophoretic pattern [22]. All the analyzed SNBP samples interacted with DNA in the typical "all or nothing" DNA binding mode of SNBP in agreement with data previously reported for SNBP [23,24]. We found that SNBP samples presenting the CP/Hr profile belonging to the L-group (controls 1 and 2) reached DNA saturation at a protein/DNA ratio of about 1 (Figure 3a, lane 8 and Figure S2, lane 8, respectively).

SNBP samples from the son, instead, showed not only a reduced DNA binding ability, because DNA saturation did not occur even at the proteins/DNA ratio of about 3 (Figure 3b, lane 10), but also presented a not-stable binding mode to DNA. In fact, as shown in Figure 3b, we observed, at the protein/DNA ratio from 1.4 to 3 (lanes 2–10), a decrease in supercoiled and relaxed plasmid DNA fractions and an increase in the fraction close to the well. The next addition of proteins at protein/DNA ratio 3.2 (lane 11) resulted in a slight increase in supercoiled and relaxed DNA fractions, suggesting protein detachment to DNA (compare lanes 10 and 11 in Figure). In addition, a different binding mode to DNA appeared to be, due to the high-molecular weight DNA band, close to the well, in the electrophoretic pattern, which tended to decrease at higher protein/DNA ratios (lanes 11–12). As expected, SNBP samples from the father, presenting the only-H profile, showed low DNA binding ability; in fact, DNA saturation occurred at a 5 proteins/DNA ratio (Figure 3c, lane 11).

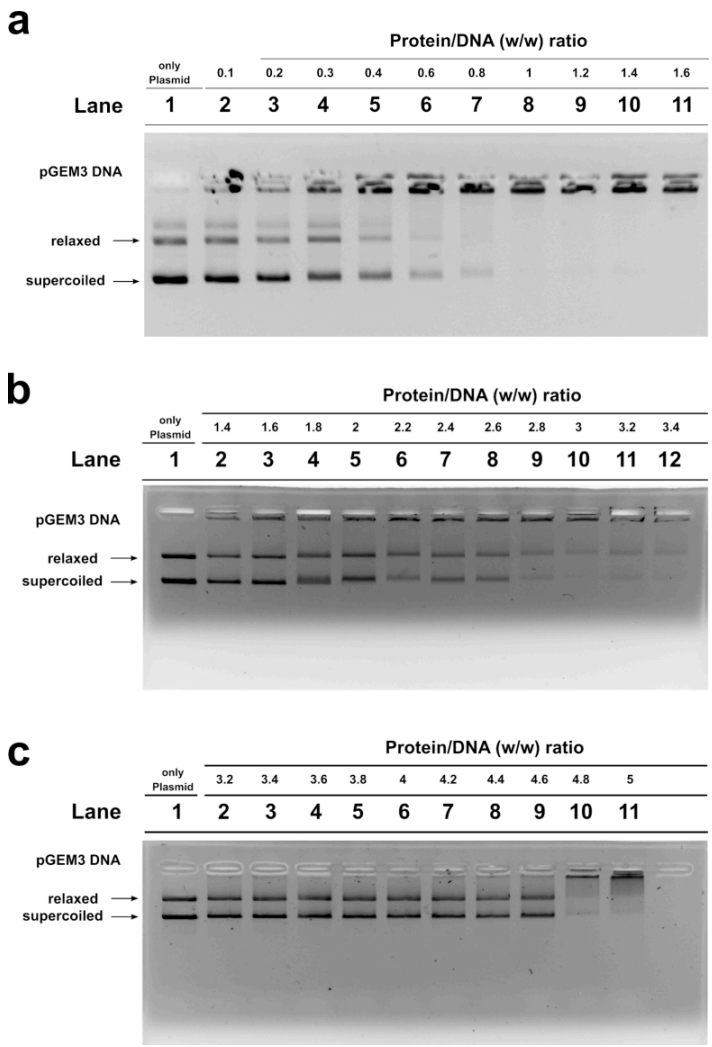

Figure 3. DNA binding ability of SNBP obtained from: control 1 (**a**); son (**b**) and father (**c**) analyzed by EMSA on 1% agarose gel. Bands on gel representing the state of pGEM3 plasmid DNA incubated in a w/w ratio with increasing amount of SNBP from samples containing: CP/Hr (**a**); nCP/Hr (**b**); only–H (**c**). Panel a from supplementary material of Lettieri et al., 2020 [11]. L-group: low impact area group; EMSA: electrophoresis mobility shift Assay; SNBP: sperm nuclear basic proteins; CP/Hr: canonical protamines/histones ratio; nCP/Hr: not canonical protamines/histones ratio; only-H: only histones and other basic proteins.

2.5. Trace Elements in Semen

We evaluated the accumulation of chromium and copper in the semen of the father, son and control 1. Control 2 presented similar values respect to control 1. We found an excess of these two metals in both the father and the son semen with respect to the controls, but the amount of the single metals was different between the father and son. In particular, as shown in Figure 4, chromium was about 17 and 10, while copper was about two and six times higher than the control in the father and son, respectively.

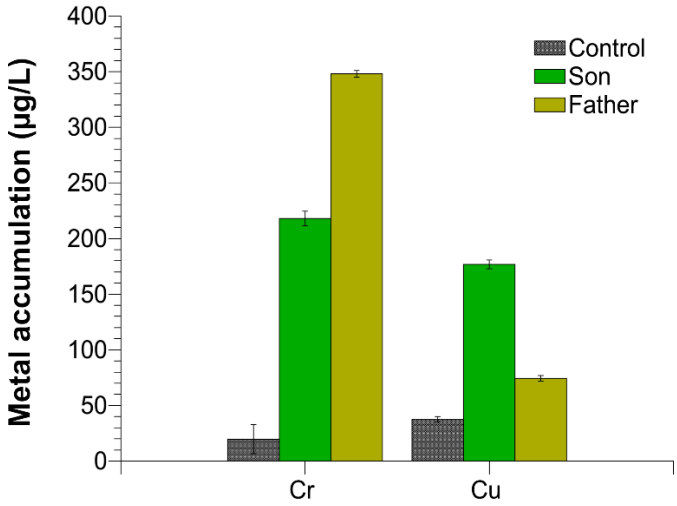

Figure 4. Chromium and copper accumulation in the semen of control 1, son and father.

2.6. H_2O_2- Induced DNA Breakage in the Presence of Human SNBP

In Figure 5, the results of the analyses of H_2O_2-induced DNA breakage in the presence of control, son and father SNBP are shown. DNA breakage was evaluated by the conversion of supercoiled to relaxed form of pGEM3 DNA plasmid in presence of SNBP. In our experimental conditions, DNA breakage was not observed when the plasmid was mixed with 30 µM H_2O_2, a higher concentration of H_2O_2 µM, of at least 100, being necessary in order to observe the breakage of DNA (Figure S3).

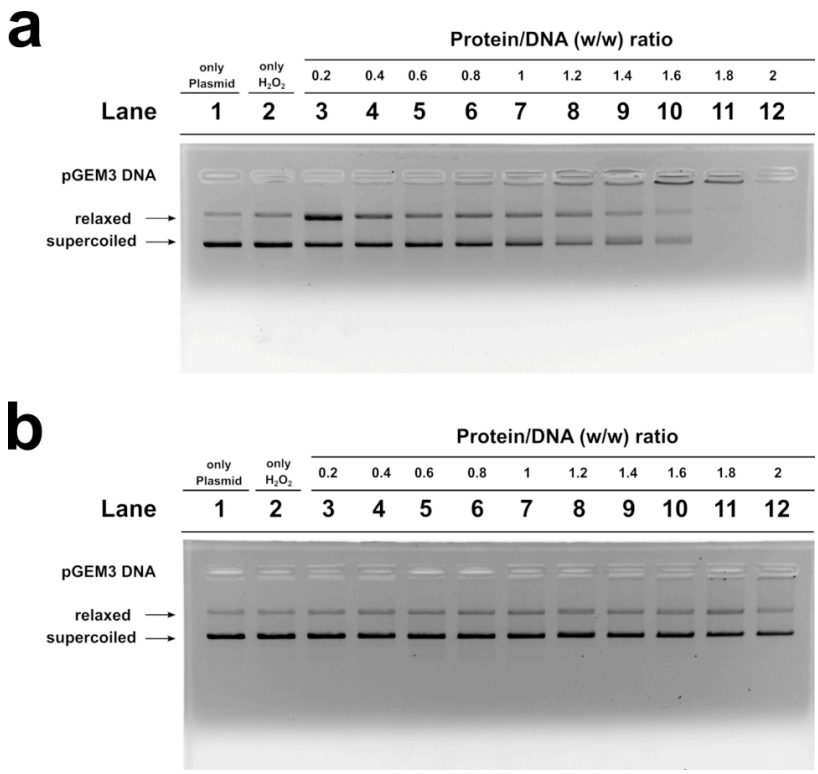

Figure 5. Analysis on 1% agarose gel of pGEM3 plamid DNA breakage induced by H_2O_2. In the presence of SNBP of the son (**a**) and father (**b**). SNBP: sperm nuclear basic proteins.

The analysis performed with the samples of males living in low environmental impact areas (controls), and presenting protamines and histones in a canonical ratio, did not produce DNA breakage (Figures S4 and S5), while the addition of the son SNBP, containing nCP/Hr, at low protein/DNA ratios (0.2), to the pGEM3 DNA plasmid in the presence of H_2O_2 resulted in an increase in the relaxed plasmid DNA fraction at the detriment of the supercoiled one (Figure 5a, lane 3). Differently, the addition of the SNBP, extracted from father spermatozoa, presenting only-H, did not cause DNA breakage, providing a very similar result to that obtained by using control SNBP (Figure 5b).

2.7. DNA Protection Analysis

We also carried out an assay to determine the potentiality of SNBP to protect DNA from oxidative damage. We created a condition in which damage to plasmid DNA occurred. In this condition, the plasmid DNA was placed in the presence of 30 µM H_2O_2 and 5 µM $CuCl_2$, so as to cause the Fenton reaction and produce DNA breakage. This condition is shown in lane 3 of the agarose gel shown in Figure 6. In this condition, more than 50% of the plasmid DNA was in the relaxed form. The addition of son or father SNBP to this mixture, in protein/DNA ratios of 0.4, 0.6 and 0.8, produced an increase in DNA breakage with respect to the damage condition shown in lane 3. In fact, in those conditions, almost all the plasmid DNA resulted in the relaxed form. When SNBP, from subjects living in the low environment impact areas and presenting a CP/H ratio, were added, completely different results were obtained. In fact, already at protein/DNA ratio of 0.4. the entity of DNA breakage was lower with respect to the damage condition (compare lanes 6 and 3 in Figure 6). At 0.6 and 0.8 protein/DNA ratios, DNA damage was not observed, suggesting that these SNBP produced complexes capable of protecting DNA. In fact, in this latter case, at increasing SNBP/DNA ratios, the plasmid DNA bands corresponding to supercoiled and relaxed became less intense as DNA saturation took place, detectable by the appearance of a high molecular weight DNA band close to the well.

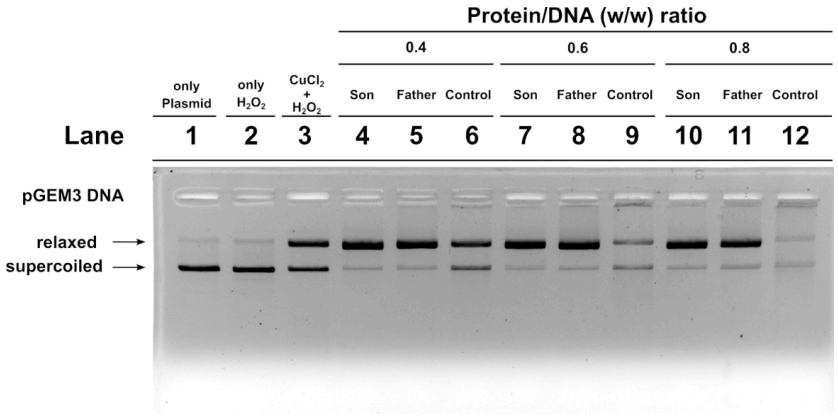

Figure 6. DNA protection analysis on 1% agarose gel of pGEM3 plasmid DNA in the presence of increasing (0.4, 0.6 and 0.8) controls (lanes 6, 9 and 12), son (lanes 4, 7 and 10) and father (5, 8 and 11) SNBP/DNA ratios. SNBP: sperm nuclear basic proteins.

3. Discussion

Living organisms are continuously exposed to numerous pollutants that can influence numerous physiological functions, including reproduction [25]. In human sperm, the highly compacted sperm chromatin structure is, in fact, achieved thanks to the association of DNA mainly with protamines, highly basic proteins which are extremely rich in arginines residues and which comprise approximately 85–90% of the human sperm DNA [18]. For a correct structure of sperm chromatin is essential not only the right content of SNBP in spermatozoa but also that these proteins maintain their properties. In the "Land of Fires", we recently found alterations in the protamines/histones ratio in the 84% of young man [11].

In the present work, we focused on a family case, constituted by a father and his son, living in the "Land of Fires", and two controls, age-matched father and son, from low environmental areas. We started from the observation of different percentages of aniline blue positive sperm stained cells in these subjects. The controls, son and father were representative of the three categories of subjects reported in Lettieri et al., 2020 [11]. Therefore, we analyzed the possible alterations of SNBP and DNA of these subjects at molecular level. As expected, by AU-PAGE analyses, the spermatozoa of controls showed a CP/H ratio—those of father contained only histones, while protamines and histones, but not in the canonical ratio, were found in the son. We also evaluated the DNA interaction of these SNBP, by EMSA, determining the proteins/DNA ratio necessary for DNA saturation, indicated by the formation of a high molecular weight DNA band, close to the well, in electrophoretic pattern. Generally, in spermatozoa of men presenting a canonical protammines/histones ratio and living in low environmental areas, we found, in our previous studies, that DNA saturation was achieved at a SNBP/DNA ratio of about 1 [11]. In EMSA experiments, we encountered a low DNA binding affinity for SNBP of both the father and son. For SNBP of the latter, an unstable bond with DNA was also observed and we noted that at the higher protein/DNA ratios tested, the high molecular weight DNA band, close to the well, was never clearly visible, suggesting the formation of different DNA–SNBP aggregates. The data obtained on the father and son indicated that the son belongs to the 22% of young men presenting a nCP/H ratio, as tested in Lettieri et al., 2020 [11]. The father, instead, presented only-H in spermatozoa, as with the majority of the samples analyzed in Lettieri et al., 2020 [11]. Father SNBP, as expected, showed a very low DNA binding affinity because of the lower basicity of histones with respect to protamines. The high level of compaction of the genome, due to protamines, confers protection from the effects of genotoxic factors, and optimizes the aerodynamic spermatozoan shape, useful for correct motility [26,27]. The electrophoretic analysis of the SNBP of the controls, father and son samples, resulted in a line with aniline blue staining.

Male gametes, for their continuous production and for exposition to environmental agents, such as oxidizing agents, are the most sensitive cells to the accumulation of damaged DNA [28–31]. Our research group has widely documented that some heavy metals are able to produce alterations in some cells [32] and to change the properties of some proteins [33], among which include human and marine organisms' SNBP [11,34,35]. In particular, in these latter proteins, some heavy metals can reverse their canonical protective rule, making them able to participate in DNA breakage [11,34,36,37]. In addition, our studies also indicated that in organisms exposed to environments polluted by heavy metals, we found accumulation of some of these metals in gonadal tissues, semen and SNBP [37–39]. As a matter of fact, in the semen of the father and son, we found different excesses of copper and chromium, two heavy metals involved in the Fenton reaction [40]. As shown in Figure 4, chromium was about 17 and 10, while copper was about two and six times higher than the control in the father and son, respectively. So, after determining the accumulation of chromium and copper in the semen of these subjects, we analyzed the potentiality of their SNBP to protect DNA or induce DNA oxidative damage.

We did not observe DNA breakage in the presence of H_2O_2 when added to plasmid DNA of both the controls and father SNBP. In contrast, son SNBP produced DNA breakage at 0.2 protein/DNA ratio. We also analyzed the ability of the SNBP of the father and son to protect DNA. We prepared a mixture containing plasmid DNA together with appropriate amount of copper chloride and hydrogen peroxide in order to induce a Fenton reaction. To this mixture we added the SNBP of controls, the father or son and obtained different results. In particular, while control SNBP were able to aggregate DNA and prevent DNA oxidative damage, the adding of son or father SNBP produced a similar increase in the fraction of the relaxed form of plasmid DNA, indicative of major DNA damage with respect to the control mixture. The ability of son SNBP to produce oxidative DNA damage without external adding of copper could be explained considering that in the semen of the son, an excess of copper and chromium was found, but especially of copper. In addition, son SNPB are constituted mainly by protamines and several studies demonstrated that copper can form several binary and ternary complexes with arginine residues [41–43], of which human protamines are extremely rich, promoting a site-specific damage

at guanine residues of DNA by a selective binding between guanine and arginine [44]. Moreover, the mediation of oxidative DNA damage by copper (II) complexes with the N-terminal sequence of human protamine P2 has been reported [45]. The involvement of SNBP of both son and father in DNA oxidative damage in the presence of H_2O_2 and copper and the more marked extent of DNA oxidative damage in the assays in which we added the SNBP of these subjects to a mixture containing plasmid DNA, copper and hydrogen peroxide could be ascribed to the concomitant presence in the semen of these subjects of an excess of chromium and copper, able to bind histones and protamines, respectively. The binding between histones and chromium happens through lysine residues [46,47] and could cause DNA damage in a similar manner as hypothesized for copper, since the toxic effect of this metal results in radical-mediated DNA strand breakage and the formation of stable chromium–DNA complexes, together with chromium–DNA adducts and protein–chromium–DNA and DNA–chromium–DNA cross-links [48,49]. The unstable DNA bond observed only for son SNBP could be due to the excess of copper, which can also determine conformational change in these proteins, as already demonstrated for protamine-like of mussels exposed to this metal [37,39]. Moreover, the presence of nCP/Hr ratio could also produce a competition between these two types of proteins for DNA binding. Taking into account that in human sperm chromatin, 10–15% of histones are retained [18,50–52], forming a heterogeneous mixture of nucleohistones and nucleoprotamines, we could hypothesize that the presence of both protamines and histones in altered ratios, in the case of the son, could determine not only an unstable binding to DNA, but also a reduced DNA protection to the external agents, such as heavy metals. There is also a need to consider that growing evidence suggests that epigenetic effects may in part be responsible for the genotoxicity and carcinogenicity of chromium [53–56], also taking into account that long-term chromium exposure may cause a significant increase in histone deacetylation. This effect certainly could affect the histones–protamines transition, which requires histone acetylase activity, and thus could explain the presence of only histones in the father spermatozoa. After all, chromium, the other heavy metal found in excess in the semen of these subjects, but in particular in the father, is also able to participate in Fenton-like reactions producing reactive oxygen species. This metal could influence the structure of chromatin by binding to both DNA and histones [46,56]. In this study, the seminal parameters and the redox status were also evaluated. Table 1 shows the data regarding the seminal parameters of the controls, father and son samples. These analyses show a poor quality of father and son semen with respect to the controls.

Table 1. Semen parameters in controls, father and son.

	Control 1		Control 2		Father		Son	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Volume (mL)	2.9	±0.2	2	±0.4	2.7	±0.6	2.3	±0.6
Sperm Concetration (million/mL)	100.7	±1.5	83.0	±10.1	13.0	±7.8	67.7	±39.1
Total sperm	288.7	±23.1	168.1	±31.7	21.7	±10.8	138.1	±52.7
Total motility %	82.7	±2.5	79.3	±4.0	66.7	±15.3	63.3	±10.4
Progressive motility %	72.7	±2.5	66.0	±1.7	40.0	±18.0	48.3	±14.4
Normal form %	11.3	±1.2	13.3	±2.1	3.7 *	±0.6	13.0	±4.6

Data are presented as mean ± SD from triplicate analyses on semen samples. Asterisk (*) indicates the significance ($p < 0.05$) between the subject (father or son) and respective age-matched control. Control 1: 18 years old; control 2: 50 years old.

Interestingly, a lower antioxidant activity in the son, even more than the father, was observed, indicative of a reduced ability to counteract oxidative stress (Table 2).

Table 2. Semen redox status.

	Control 1		Control 2		Father		Son	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TAC $^{\Delta}$	1.4	±0.1	1.3	±0.1	0.9 *	±0.1	0.7 *	±0.1
GSH	0.6	±0.1	0.5	±0.1	0.3	±0.1	0.2 *	±0.0
GSSG	0.2	±0.1	0.2	±0.0	0.1 *	±0.0	0.1	±0.0

Data are presented as mean ± SD from triplicate analyses on individual semen sample. Asterisk (*) indicates the significance ($p < 0.05$) between the subject (father or son) and respective age-matched control. Control 1: 18 years old; control 2: 50 years old; triangle ($^{\Delta}$) indicate the significance ($p < 0.05$) between father and son. TAC: total antioxidant capacity; GSH: reduced glutathione; GSSG: oxidized glutathione.

Given the dramatic increase in both the number and complexity of environmental chemical contaminants during the last several decades, our results, together with those previously published, highlight how environmental pollution can produce a deterioration in semen quality.

In conclusion, in the present work, having considered only a family case, the peculiarity of some characteristics of son semen could represent a starting point for possible future studies on transgenerational effects of pollutants on fertility, using wider sampling. After all, a study conducted on the semen quality of young adult intracytoplasmic sperm injection ICSI offspring demonstrated that the worldwide oldest ICSI-conceived adults presented significantly lower sperm concentration, lower total sperm count as well as lower total motile sperm count in comparison to a control group of spontaneously conceived peers [57]. In clinical practice when there are no conditions that indicate low sperm quality according to the classical parameters (number, motility, morphology and fragmentation state of sperm DNA), i.e., when we refer to idiopathic infertility, although there are several functional tests on the sperm, defining with certainty the fertile capacity of semen is not possible, considering further the interactions with the female part. Therefore, based on our study, the possible implications for fertility could relate to the risk of reduced reproductive capacity in the future. In fact, considering that, it is not always possible to define the state of fertility of an individual on the basis of the classic parameters of the spermiogram which could now be normal, but not subsequently; as a result, these individuals could be in a borderline condition, also taking into account that in the last 45 years, there has been a decline in semen quality. In fact, a systematic review and meta-regression analysis [58] demonstrated that sperm concentration (SC) and total sperm count (TSC) has declined significantly among men from North America, Europe and Australia between 1973 and 2011. In particular, the mean SC and TSC declined, on average, by1.4% and 1.6% per year, with an overall decline of 52.4% and 59.3%, respectively. In addition, a retrospective study reported a significant and strong decline in sperm concentration and morphology in the whole of France between 1989 and 2005 [59]. In fact, ubiquitary exposure to chemicals has been growing in the general population of France since the 1950s [59]. In particular, main endocrine disruptor chemicals (EDCs) have been found in biological matrices of French people [60], some (non-dioxin-like PCB, pesticides and triclosan) at a level higher than that in other countries. These results could therefore be consistent with the endocrine disruptor hypothesis, recently strengthened in an international report [61]. A similar situation has also been reported in the "Land of Fires", where, during the last few decades, the increasing occurrence of disorders of the male reproductive system in humans has raised attention about the possible environmental risk factors [10]. Therefore, the global decrease in sperm concentration and morphology seems most probably to be due to global changes in environmental exposure in specific areas. Therefore, it is very important to also consider the transgenerational effects that could have influenced this rapid decline in semen quality. In particular, the alterations observed in the son with our molecular approaches combined with the alterations of the redox state of his semen, as well as the excess of metals, could lead to a greater consumption of antioxidants and therefore favor a possible condition of oxidative stress, responsible for the observed alterations through these molecular approaches. Taking into account the transgenerational hypersensitivity mechanisms found in mice [19], it would also be possible to hypothesize that in the son, the antioxidant and detoxifying enzyme systems may

be less efficient due to the genetic and/or epigenetic pathways induced by the paternal gametes [62]. Therefore, as future perspectives of this study, we intend to do a more extensive one, with a larger sampling of fathers and sons resident in the "Land of Fires", to support our observations, which, however, want to represent a call to attention for a greater possible reproductive risk that requires further studies. The implications on the future reproductive capacity of young people living in this area, which for decades has shown very high pollution rates, which are reflected on the health of the resident population [63], cannot therefore be underestimated, especially if we consider that semen seems to represent an excellent marker of environmental health and general health [64–67].

4. Materials and Methods

4.1. Reagents

All used reagents were of analytical grade and purchased at Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

4.2. Ethical Statements

All methods were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) guidelines and regulations. All experimental protocols were approved by the Ethical Committee of the Local Health Authority Campania Sud-Salerno (Committee code n. 43 of 30/06/2015). Informed consent was obtained from the recruited subjects (over 18) before sample collection.

4.3. Recruitment

The semen samples of the father (50 years old) and son (18 years old) came from Casalnuovo, a municipality belonging to the "Land of Fires" and were obtained by Medicina Futura center (Acerra-Province of Naples). The "Land of Fires" is a high environmental impact area in Southern Italy (Campania region). According to the Campania Region Environmental Protection Agency report (https://www.arpacampania.it/), Campania, the "Land of Fires", is officially recognized as an area with the highest concentration of environmental disposal sites of toxic waste often associated with their combustion. Semen samples from controls came from San Francesco d'Assisi Hospital in Oliveto Citra-Province of Salerno, which is a municipality belonging to the low environmental impact area known as "Alto-medio Sele" (https://www.arpacampania.it/). The control sample used for this study belonged to the previous recruitment [11] and was representative of all semen samples presenting CP/Hr. For this study, the two control subjects considered were recruited as an age-matched father and son. In particular, control 1 was 18 years old, while control 2 was 50 years old. The economy of this area is mainly based on low-to-medium-scale farming and without known illegal disposal of toxic wastes. The samples analysis is part of a pilot study (EcoFoodFertility initiative, www.ecofoodfertility.it) which consists of the analysis of the effects produced by environmental pollution on humans, through the study of human semen that becomes an early biomarker of pollution in healthy men [6,10,68]. The father and son and control participated as volunteers at the study and they were selected according to the following criteria: residence for at least 10 years in the study area, no known chronic diseases (diabetes or other systemic diseases), no varicocele, no prostatitis, and other factors that could negatively affect semen quality (such as fever, medications, exposure to X-rays, etc.), non-drinkers, non-smokers, no reported history of drug abuse, and no known occupational exposure to toxic chemicals). In addition, the participants follow a healthy diet and practice at least 30 min of walking at day. Data were collected by questionnaire and physical examination, including the urogenital evaluation (testis volume and transrectal prostate evaluation was made). The recruiting andrologist (the recruiter) assigned a code number to each volunteer in order to preserve anonymity. Each code number was uploaded into a computer database together with personal and clinical information.

4.4. Semen Quality Evaluation

Parameters of semen quality were assessed by the examining andrologist (the evaluator), according to the World Health Organization (WHO) guidelines [69]: semen volume, pH and sperm concentration, motility and morphology were evaluated.

4.5. RedOx Status Evaluation in Seminal Plasma

TAC was measured by using the Antioxidant Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer's instructions. The determination of total and oxidized glutathione (GSH + GSSG and GSSG, respectively) was spectrophotometrically measured (at 412 nm) accordingly to the Ellman's method [70] and their concentration—upon normalization to protein content—was expressed as nmoles/mg protein.

4.6. Spermatozoa Collection and Sperm Proteins Extraction

In order to separate the spermatozoa from seminal plasma, the semen samples were centrifuged for 30 min at 5500× *g* at 4 °C and sperm pellets were stored at −80 °C in aliquots of 50 µL. Protamines extraction from spermatozoa was performed as described in Lettieri et al., 2020 [11]. In brief, the sperm pellets were washed twice with 500 µL of phenylmethanesulfonyl fluoride (PMSF), centrifuged at 10,480× *g* for 5 min at 4 °C, resuspended with 50 µL of 1 mM PMSF and 50 µL of a solution containing 6 M guanidinium chloride and 10 mM DTT and then incubated at 20 °C for 30 min. Afterwards, the samples were added with 5 volumes of cold ethanol 100% and incubated at −20 °C for 60 min to obtain the sperm chromatin precipitation. The samples were centrifuged at 13,680× *g* for 15 min at 4 °C and the pellet obtained was resuspended in 500 µL of 0.5 M HCl to solubilize SNBP. The samples were incubated for 5 min at 37 °C and then centrifuged a 1000× *g* for 10 min at 4 °C. At the end of this step, at the supernatant, trichloro acetic acid (TCA), was added for a final concentration of 20%, in order to precipitate the SNBP. The samples were incubated 60 min at 4 °C, and then centrifuged at 14,000× *g* for 10 min at 4 °C. The pellet obtained, containing SNBP was washed twice with 500 µL of acetone containing 1% β-mercaptoethanol, centrifuged twice at 14,000× *g* for 10 min at 4 °C and the final pellet was dried in a speed-vacuum for 10–15 min. The dried proteins were resuspended in 50 µL of ultrapure water (milliQ) and stored at −20 °C in aliquots of 50 µg.

4.7. Acid-Urea Polyacrylamide Gel Electrophoresis of SNBP

Acid-urea polyacrylamide gel electrophoresis (AU-PAGE) was used to analyze human SNBP as previously described in Soler-Ventura et al., 2018 [21] but with a few modifications reported in Lettieri et al., 2020 [11]. The composition of the gel for a volume of 8 mL was: 15% acrylamide/0.1% N,N'-Methylene-bis-acrylamide, (optimal acrylamide/Bis-acrylamide ratio for the separation of human SNBP), 2.5 M urea, 0.9 M acetic acid, 80 µL of TEMED and 140 µL of 10% APS.

After the electrophoresis, gels were stained with Amido Black, and then with Coumassie Blue Brilliant R-250 as previously described [71]. Gels were acquired using a Gel-Doc system (BioRad Hercules, CA, USA) by Quantity One v.4.4.0 (BioRad, Hercules, CA, USA) software. The software ImageJ ver 1.50 d (https://imagej.nih.gov/ij/) supported by the National Institute of Health (Bethesda, Maryland, USA) was used for densitometric analyses of the bands on the gel.

4.8. Plasmid DNA Preparation

pGEM3 plasmid (2867 bp) was extracted from transformed *Escherichia coli* HB 101 cells, using the standard protocol of the QIAGEN Plasmid Midi Purification kit (QIAGEN Plasmid Midi Purification handbook, third edition© 2020, Hilden, Germany), but following the precautions described in Carbone et al., 2012 [72], in order to obtain high amounts of supercoiled pDNA. In order to evaluate the quality of plasmid DNA, gel electrophoresis on 1% agarose gels in 89 mM Tris-HCl pH 8.0, 2 mM EDTA and 89 mM boric acid (TBE) was used. The circular form of the plasmid pGEM3 was used for

electrophoretic mobility shift assays (EMSA) of DNA and for the analysis of DNA breakage and DNA breakage/protection by SNBP.

4.9. DNA Binding Affinity of SNBP by EMSA

The effect of human SNBP extracted from the father, son and control samples on DNA was analyzed by EMSA as previously described [73], with slight modifications. Mixtures of DNA/proteins were prepared. In all samples, a fixed amount of plasmid DNA (pGEM3) (150 ng) and increasing amount of SNBP were used in order to obtain protein/DNA *wt/wt* ratios between 0.1 and 5, as indicated in the section results. We evaluated the protein/DNA ratio necessary to achieve DNA saturation. In the preparation of the samples, the various components were added in the following order: ultrapure water (milliQ), DNA, proteins. After that, the samples were left for 5 min at room temperature to make the proteins and DNA interact, and then all samples were added with TBE 10 X (to obtain TBE 1X final concentration) just before running the gels and analyzed on 1% agarose gel in TBE. The electrophoretic run was performed on 1% agarose gel in TBE at 100 V for about 30 min at room temperature. At the end, DNA migration was visualized by staining agarose gels with ethidium bromide (2 µg/mL) after electrophoresis. All experiments were performed at least five times. Gels were acquired using a Gel-Doc system (BioRad, Hercules, CA, USA) through Quantity One v.4.4.0 (BioRad, Hercules, CA, USA) software. A densitometric analysis of the bands on the gel was performed using the software ImageJ ver 1.50 d (Wayne Rasband, National Institute of Health, Bethesda, ML, USA, https://imagej.nih.gov/ij/, 1997–2018).

4.10. Aniline Blue Staining

The aniline staining was performed as described by Pourmsumi et al. [74], with a few modifications. In brief, the fresh semen smear of each sample was air dried and then stained with 5% aqueous aniline blue stain (Histon Color Test, AB Analitica, Padua, Italy) in 4% acetic acid (pH 3.5) for 5 min. A cover slide 24 mm × 50 mm was put on each slide. Stained and unstained spermatozoa were observed using light microscopy (Nikon Eclipse Ci) at ×1000 magnification under oil immersion (Plan 100×/1.25 oil objective). A total of 200 cells were manually evaluated on each slide for the type of staining pattern: aniline blue positive (AB+) cells, pale blue (PB) cells and aniline blue negative (AB−) unstained cells.

4.11. DNA Breakage Analyses

pGEM3 plasmid DNA breakage in the presence of SNBP extracted from the father, son and control and 30µM H_2O_2 was analyzed on 1% agarose gel in TBE 1X final concentration. The preparation of the samples was performed as described by Piscopo 2019 [39]. In brief, into any samples we put ultrapure water (milliQ), plasmid DNA pGEM3 (a fixed amount of 150 ng) and SNBP extracted from the father, son or control in increasing *w/w* protein/DNA ratios in a range from 0.2 to 2. The samples were incubated at room temperature for 5 min to make proteins and DNA interact, after which H_2O_2 was added and the samples were incubated in a Thermoblock set at 37 °C for about 30 min in the dark. At the end of incubation, sample buffer 10X (1X final concentration in the samples) was added just before electrophoresis analysis, in order to avoid the EDTA coordination of eventual metals. The electrophoretic analysis of the samples was conducted on 1% agarose gel at 100 V for 30 min in TEB 1X. After electrophoresis, agarose gels were stained with ethidium bromide (2 µg/mL) in order to visualize DNA migration and then the images of the gels were acquired at the GelDoc Biorad (Hercules, CA, USA). All experiments were performed at least five times.

4.12. DNA Protection Analysis

SNBP ability to protect DNA from oxidative damage in the presence of 30 µM H_2O_2 and 5 µM $CuCl_2$ was performed by using plasmid DNA (pGEM3) and SNBP extracted from the father, son and control samples. The samples were prepared using EMSA protocol described in the paragraph "DNA binding affinity of sperm proteins by EMSA", with slight modifications. In particular, 150 ng of plasmid

DNA (pGEM3) and proteins/DNA *w/w* ratios in a range from 0.4 to 0.8 were used. After 5 min of interaction, at room temperature, between DNA and SNBP, H_2O_2 and $CuCl_2$ were added and the samples were incubated in the dark for 30 min in a Thermoblock set at 37 °C. At the end of incubation, sample buffer 10X (1X final concentration in the samples) was added just before electrophoresis analysis, in order to avoid the EDTA coordination of eventual metals. The electrophoretic analysis of the samples was conducted on 1% agarose gel at 100 V for 30 min in TEB 1X. After electrophoresis, agarose gels were stained with ethidium bromide (2 µg/mL) in order to visualize DNA migration and then the images of the gels were acquired at the GelDoc Biorad (Hercules, CA, USA). All experiments were performed at least five times.

4.13. Trace Elements in Semen

One milliliter of nitric acid (HNO_3 ≥ 69%, *v/v* TraceSELECT®, fisher scientific, Waltham, Massachusetts, USA) was added to each tube containing aliquots of 600µL of semen and the suspension was subjected to oxidative acid digestion in a microwave system equipped with autosampler (CEM DISCOVER SP-D, CEM Srl, Cologno al Serio, Bergamo, Italy).

The elemental analysis was conducted by inductively coupled plasma mass spectrometry (ICP-MS). Element concentrations were determined from a calibration curve calculated on the basis of five concentrations for each of analyzed elements obtained from certified standard solutions and the final concentrations were expressed in µg/L. The limits of detection and quantification (LOD and LOQ) were calculated considering, respectively, 3 and 10 times the standard deviation (SD) of 10 replicates made on a negative control. ICP-MS analyses were performed for the quantification of the following elements of copper and chromium.

Supplementary Materials: Supplementary Materials can be found at http://www.mdpi.com/1422-0067/21/18/6710/s1.

Author Contributions: Conceptualization, M.P. (Marina Piscopo) and L.M.; Supervision, M.P. (Marina Piscopo), M.T., L.B.; L.M., M.P. (Marina Prisco); Investigation, F.M., C.M., G.L., T.N., A.G., M.P. (Marina Prisco); Software, G.L.; Formal analysis, M.P. (Marina Piscopo) and G.L.; Visualization, M.P. (Marina Piscopo) and G.L.; Data curation, G.L.; writing—original draft preparation, M.P. (Marina Piscopo), F.M., C.M. and G.L.; Project administration, M.P. (Marina Piscopo). All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ROS	reactive oxygen species
TCA	trichloro acetic acid
AU-PAGE	acid-urea polyacrylamide gel electrophoresis
EMSA	electrophoretic mobility shift assays
EDTA	ethylenediaminetetraacetic acid
TEB	Tris-Borate-EDTA
SNBP	sperm nuclear basic protein
pDNA	plasmid DNA
DTT	dithiothreitol
L-group	man living in the low environmental impact areas
CP/Hr	canonical protamines/histones ratio
nCP/Hr	not canonical protamines/histones ratio
only-H	only histones and other basic proteins
TAC	total antioxidant capacity
GSH	glutathione
GSSG	oxidized glutathione
SC	sperm concentration
TSC	total sperm count
ICSI	intracytoplasmic sperm injection

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Molecular Alterations and Severe Abnormalities in Spermatozoa of Young Men Living in the "Valley of Sacco River" (Latium, Italy): A Preliminary Study

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Abstract: The Valley of Sacco River (VSR) (Latium, Italy) is an area with large-scale industrial chemical production that has led over time to significant contamination of soil and groundwater with various industrial pollutants, such as organic pesticides, dioxins, organic solvents, heavy metals, and particularly, volatile organic compounds (VOCs). In the present study, we investigated the potential impact of VOCs on the spermatozoa of healthy young males living in the VSR, given the prevalent presence of several VOCs in the semen of these individuals. To accomplish this, spermiograms were conducted followed by molecular analyses to assess the content of sperm nuclear basic proteins (SNBPs) in addition to the protamine-histone ratio and DNA binding of these proteins. We found drastic alterations in the spermatozoa of these young males living in the VSR. Alterations were seen in sperm morphology, sperm motility, sperm count, and protamine/histone ratios, and included significant reductions in SNBP–DNA binding capacity. Our results provide preliminary indications of a possible correlation between the observed alterations and the presence of specific VOCs.

Keywords: sperm nuclear basic proteins; human protamines; human spermatozoa; male fertility; spermatozoa morphology; volatile organic compounds; reproduction; protein–DNA binding

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Citation: Perrone, P.; Lettieri, G.; Marinaro, C.; Longo, V.; Capone, S.; Forleo, A.; Pappalardo, S.; Montano, L.; Piscopo, M. Molecular Alterations and Severe Abnormalities in Spermatozoa of Young Men Living in the "Valley of Sacco River" (Latium, Italy): A Preliminary Study. *Int. J. Environ. Res. Public Health* **2022**, *19*, 11023. https://doi.org/10.3390/ijerph191711023

Academic Editor: Rosaria Meccariello

Received: 13 July 2022
Accepted: 31 August 2022
Published: 3 September 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

1.1. Preface

In Italy, there are some areas that have high levels of pollution. One region with substantial pollution is Italy's Land of Fires, an area between Naples and Caserta that has been overrun by illegal waste dumping and toxic fires, prompting the establishment of the EcoFoodFertility project, which is a multicenter biomonitoring study with the aim of developing a better understanding of the environmental impact of toxicants on human health (https://www.ecofoodfertility.it/, accessed on 12 July 2022).

1.2. The Issue

This study forms part of the EcoFoodFertility project and focused on another district with significant pollution named the "Valley of Sacco River" (VSR) (Latium, Italy) [1–3]. The VSR is an area located mostly in the province of Frosinone and partly in the southern

area of Rome. There is an important industrial district in the VSR. In fact, due to the intense industrial and especially chemical activity of the Colleferro plants, there has been an overload of pollutants in this area, which have contaminated the soil and groundwater over many years, creating problems throughout the food chain. The spills of illegal industrial waste have led the river to become a contaminated site; contaminated water has been used for irrigation purposes, with negative consequences on both animals and the population living in the area, and despite some reclamations, the site is under surveillance. Soil studies in the industrial area of this zone revealed the cumulative accumulation of different toxic substances and organic pesticides, especially through water and food [1], and very high levels of chromium and dioxins.

1.3. Pollution and Reproduction

In recent years, there has been a great deal of evidence that pollution has a strong effect on the reproductive health of living organisms; in fact, important effects have been demonstrated in spermatozoa [4–12], which are particularly sensitive to environmental changes [13–16]. It is well-known that various pollutants can cause an imbalance of reactive oxygen species (ROS) [5,7,17–20] and that the resulting oxidative stress events have the potential to induce alterations in sperm morphology, number, and motility [13–16]. In addition, these processes can cause oxidative damage to DNA [7]. Environmental pollution is also reported to alter disease susceptibility in different populations, and semen quality has proven to be a potential predictor of sensitivity to viral insults in highly polluted areas [21,22]. An early biomarker of environmental exposure, such as Kallikrein-related serine peptidase 3, was also recently identified in young women [23]. Moreover, volatilome fingerprinting of human biofluids by both chemical analytical techniques and gas sensor systems is attracting interest in human biomonitoring as a new approach to explore the internal chemical environment due to multiple exposomic stressors and discover potential VOC biomarkers useful in pollution health impact assessment [24]. Much research has been done in recent years because the problem of infertility due to environmental pollution is thought to be increasingly common [25]. Recently, it was also shown that reduced male fertility and gonadal development, as well as cancers of the reproductive system due to exposure to organic and inorganic pollutants, can be counteracted by flavonoids [26]. Spermatozoa are highly reactive to the pro-oxidant effects of environmental pollutants essentially for two reasons: as a result of the reduced volume of cytoplasmic space, with less antioxidant defense [27], and because sperm membrane lipids, especially rich in polyunsaturated fatty acids, are the target of reactive oxygen species (ROS) [28]. This is why spermatozoa have been recognized as an "ideal" biomarker of environmental pollution and early sentinels of human health [13]. In human spermatozoa, there are two types of SNBPs to package the genome in the highly condensed sperm nucleus: protamines (P1 and P2) and histones [29,30]. The canonical ratio between protamines and histones is about 85%:15%, and it appears essential to maintain this ratio between these two types of proteins for male fertility [31].

In a very recent work, it was demonstrated that several volatile organic compounds (VOCs) are present at high levels in the semen of young men living in the VSR [32]. VOCs are a heterogeneous category of substances that includes organic chemicals that have a high vapor pressure at room temperature. Taken together, they exhibit high volatility but show extremely different physical and chemical behaviors. VOCs are also emitted from solids or liquids, so exposure to airborne VOCs is unavoidable. Most VOCs are not acutely toxic but may have short- and long-term adverse health effects. Specifically, in the VSR area, considering the pervasive chemical exposure of the population caused by environmental pollution, exposure to VOCs via multiple exposure sources (mainly air, water, and food) has occurred with hidden dangerous cumulative effects on health.

1.4. The Aim of Work

Therefore, in the present work, we investigated whether these pollutants could produce alterations in the spermiogram parameters and SNBP properties of young men in

the VSR. To this aim, we performed molecular analyses to assess the protamine/histone ratio and the DNA binding of these proteins. Finally, we analyzed the morphology of the spermatozoa of young men in the VSR and tried to find a possible correlation between morphological, molecular, and other seminal changes and the presence of specific VOCs.

2. Materials and Methods

2.1. Reagents

All reagents used for this work were of analytical grade and were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.2. Ethical Statements

All methods were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) guidelines and regulations. All experimental protocols were approved by the Ethical Committee of the Local Health Authority Campania Sud-Salerno (Committee code n. 43 of 30 June 2015). Informed consent was obtained from recruited subjects before sample collection.

2.3. Recruitment

Subject recruitment was conducted between October 2017 and November 2018, as part of a pilot study of the EcoFoodFertility project (www.ecofoodfertility.it, accessed on 12 July 2022). The semen samples comprising the "pollution exposed" group came from individuals living in the VSR area, a region that extends principally in the province of Frosinone and to the south of Rome. The geographical areas considered in our study (Figure 1) differ considerably in the concentrations of various pollutants present. The Valley of Sele River (Control group) is a low environmental impact area (https://www.arpacampania.it/, accessed on 12 July 2022). This area has no known illegal disposal of toxic waste and has an economy based primarily on low- to medium-scale agriculture. The Valley of Sele River, based on the concentrations of pollutants detected in the area, falls into the 'maintenance' category and is included in the lower diffuse emission zones for sulfur oxide and medium diffuse emission zones for nitrogen oxide, carbon monoxide, volatile organic compounds, and suspended particles, so the risk thresholds for human health are not exceeded [33]. The VSR is an area where the concentrations of various pollutants, including heavy metals and pesticides, are much higher than the national averages [32].

Figure 1. Map of the geographical areas selected for the recruitment. The green circle indicates the Valley of Sele River in the Campania region; the red circle indicates the Valley of Sacco River (VSR) area in the Lazio region. The green and red circles indicate the geographical position of the two areas in the respective regions.

The chemical analyses carried out ascertained wide-ranging environmental pollution linked to the contamination of the Sacco River by toxic waste dumps of industrial origin to

which animals of zootechnical interest and the human population have been exposed [1]. Control semen samples used for comparison were obtained from the San Francesco d'Assisi Hospital of Oliveto Citra-Province of Salerno, a municipality belonging to a low environmental impact area called the Valley of Sele River. The selected participants were healthy males aged between 18 and 30 years and residents of at least 10 years in the areas under study with no known chronic diseases (diabetes or other systemic diseases), no varicocele, and no factors that could affect sperm quality (such as fever, medications, exposure to X-rays, etc.). Subjects were also non-drinkers and non-smokers with no reported history of drug abuse and no occupational exposure.

Data were collected by questionnaire and physical examination, including urogenital assessment (testis volume and transrectal prostate assessment). At the time of enrollment, a code number (VSR1, VSR2, VSR3, . . . , VSRn) was assigned to each volunteer by the recruiting andrologist (the recruiter) to maintain privacy. Each code number was uploaded to a computer database along with personal information and clinical information. A total of 80 samples were analyzed from the "low pollution" area and 76 samples from the "high pollution" VSR group.

2.4. Sample Collection

Human semen samples were collected upon morning masturbation after 3–4 days of sexual abstinence in sterile containers. Semen samples underwent routine tests to evaluate sperm quality (semen volume, sperm concentration, motility, and morphology) according to the World Health Organization (WHO) guidelines (World Health Organization, 2010) [34]. After incubation to 37 °C for 30–40 min and complete liquefaction, the sperm analysis was performed according to WHO [35], using both the classical microscope for optical evaluation with Makler's chamber and one automated sperm analysis using the Lenshooke Semen X1 Pro system (Bonraybio Co., Ltd., Dali Dist., Taichung City, Taiwan), an innovative automatic analytical certified CE IVD system, equipped with microscope-integrated optics and an artificial intelligence system. The following parameters were measured: sample volume, pH, sperm concentration, total and progressive motility, sperm morphology, and concentration of round cells.

2.5. SNBP Extraction from Spermatozoa

The semen was divided into 500 µL volume aliquots in 1.5 mL tubes. Semen samples were centrifuged for 30 min at 5500× *g* at 4 °C in order to purify spermatozoa. To extract protamines from spermatozoa pellets, the protocol described by Lettieri et al., 2020 [7] was used [7]. In brief, the spermatozoa pellet was resuspended in 500 µL of 1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF). Thereafter, the sample was centrifuged at 4 °C for 10 min at 10,480× *g*; the obtained pellet was washed again with 1 mM PMSF and recentrifuged as described before. A total of 50 µL of 1 mM PMSF, 50 µL of 8 M guanidine hydrochloride, and 200 mM dithiothreitol (DTT) were added to the pellet, containing DNA and protein. The sample was incubated at room temperature for 30 min, and then 5 volumes of cold ethanol were added to the sample. The sample, after being at −20 °C for 1 h, was centrifuged at 4 °C for 15 min at 13,680× *g*, and the formed pellet, containing the proteins, was resuspended with 500 µL of 0.5 M HCl and incubated in the thermoblock at 37 °C for 5 min. Thereafter, the sample was centrifuged at 1000× *g* for 10 min at 4 °C, and the obtained supernatant, containing the sperm nuclear basic proteins, was precipitated with 20% Tricloro Acetic Acid (TCA) after incubation on ice for 1 h. SNBPs were obtained in the form of a pellet after centrifugation of the sample at 4 °C for 10 min at 14,000× *g*. The pellet was washed with 500 µL of a solution containing 1% β-mercaptoheptanol and 100% acetone and then recentrifuged for 10 min at 14,000× *g* at 4 °C. The final pellet was resuspended in about 50 µL of H_2O and stored at −20 °C.

2.6. Electrophoretic Analysis of SNBPs

Acetic acid-urea gel electrophoresis (AU-PAGE) was used to analyze the SNBPs (i.e., histones and protamines) extracted from spermatozoa. AU-PAGE was performed as

previously described in Lettieri et al., 2021 [36] using 9.0% (*w/v*) acrylamide (acrylamide: bisacrylamide 25:0.67). The gel, with a final volume of 8 mL, consisted of acrylamide/*N*,*N*′-Methylene-bis-acrylamide, 8 M urea, 5% acetic acid, 100 µL TEMED, and 140 µL of 10% APS. After gel polymerization, a pre-run of approximately 1 h was performed at a constant voltage of 150 V by using 5% acetic acid as a running buffer. For the pre-run, in each well, 20 µL of a solution containing 8 M urea and 5% acetic acid was loaded. After this step, a solution containing 4 µg of protein and 20 µL of a solution containing 12.8 M β-mercaptoethanol and 8 M molar urea were loaded onto the gel, and finally, after one hour, 2 µL of 100% acetic acid and 2 µL of 0.001% pyronin were added. The electrophoresis was performed at 100 V for about 1 h. After the electrophoresis, gels were stained with Coumassie Blue Brilliant R-250 as previously described [37]. The image was acquired using GelDoc Biorad, and densitometric analysis was performed using ImageJ ver. 1.53k software (https://imagej.nih.gov/ij/, accessed on 12 July 2022) supported by the National Institute of Health (NIH) (Rockville Pike, Bethesda, MD, USA). We classified the analyzed samples in the two areas according to the type of bands identified on AU-PAGE. In particular, the band at higher mobility represented the protamines P1 and P2, while the bands at lower mobility were the histones and other basic proteins. We defined the protamines/histones ratios by the densitometric analyses of the bands obtained in the gels. When there was a prevalence of histones and other basic proteins over protamines, we classified the sample as only-H, as shown in the majority of the analyzed samples. When there was a prevalence of protamines over histones and other basic proteins, the sample was classified as nCP/Hr, as shown in the majority of the analyzed samples.

2.7. Plasmid DNA Preparation

pGEM3 plasmid (2867 bp) from *Escherichia coli* HB 101 cells was prepared by using the HiPura Endotoxin free Plasmid DNA Midiprep Kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India) following the precautions described in Carbone et al., 2012 [38], with the aim to achieve high amounts of super-coiled pDNA. After extraction of plasmid DNA, its concentration was evaluated by Spectrophotometer Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA), and the plasmid DNA topological state was verified by gel electrophoresis of 1% agarose gels in 89 mM Tris-HCl pH 8.0, 2 mM EDTA, and 89 mM Boric Acid (TBE). For the electrophoretic mobility shift assay (EMSA) and DNA breakages/protection assays, the circular form of the plasmid was used.

2.8. Analysis of SNBP/DNA Binding

The analysis of SNBP/DNA binding was performed by EMSA following the procedure described in Lettieri et al., 2021 [11]. The assay was done on 1% agarose gel, in 1× TBE buffer. The samples were prepared as previously described [39] in distilled water containing a fixed amount of plasmid DNA (150 ng) and increasing amounts of SNBPs in order to obtain samples differing in the protein/DNA *w/w* ratio. The interaction between plasmid DNA and proteins was for 5 min at room temperature, and then TBE and the sample buffer were added to a final concentration of 1×. The electrophoresis was conducted at a constant voltage of 100 V for 30 min. At the end of the electrophoresis, DNA was visualized by means of ethidium bromide. Gels were acquired using a Gel-Doc system (BioRad, Hercules, CA, USA) through Image Lab 6.0.1 software (build 34; BioRad, Hercules, CA, USA). A densitometric analysis of the bands on the gel was performed using the software ImageJ ver 1.50 d [40].

2.9. VOC Analysis

Frozen samples of semen were thawed at room temperature, and subsequently, the vials were immersed in a water bath onto a magnetic stirrer hotplate at 60 °C overnight.

After this incubation, a Carboxen®/Polydimethylsiloxane (CAR/PDMS) fiber (57318, Supelco) was exposed to headspace for 15 min, as reported in [32]. GC-MS analysis of extracted volatiles was performed using GC (6890N series Agilent Technologies) coupled

to MS (5973 series Agilent Technologies) equipped with a ZB-624 capillary column (Phenomenex); the injector temperature was set at 250 °C to allow thermal desorption of VOCs. The carrier gas was high-purity helium with a flow rate of 1 mL min^{-1}. The MS analyses were carried out in full-scan mode with a scan range of 30–500 amu at 3.2 scans/s. Chromatograms were analyzed by Enhanced Data Analysis (MSD Chemstation E.02.02, Agilent Technologies, Santa Clara, CA, USA), and the identification of the volatile compounds was achieved by comparing mass spectra with those of the data system library (NIST14, $p > 60\%$) and confirmed by the injection of external standards corresponding to most recurrent compounds. To quantify the identified VOCs, a semiquantitative method based on the internal standard (I.S.) 1,4-Dichlorobenzene-D4 (EPA-8260C) was followed. A frequency analysis was performed to identify the most frequently present VOCs.

2.10. Spermatozoa Staining

The Diff-Quik staining method was chosen because it better detects any abnormalities in sperm morphology [41]. The Diff-Quik staining test allows the assessment of human sperm chromatin status together with the assessment of sperm morphology as per WHO 2010. After evaluation of the fresh spermiogram, spermatozoa are fixed on the slide, stained by the Diff-Quik method, immersed in water for 1 min, and analyzed with NIKON Eclipse E100 optical MICROSCOPE, 10×, 20×, and 40× objectives, Plan E100X/1.25 Oil LENS, and DF-Fi3 color camera with phase contrast and BF brightfield. We counted at least 200 spermatozoa per sample in terms of normal/abnormal morphology and specified in the results the abnormalities found in terms of percentage in the VSR.

2.11. Statistical Analysis

The statistical analysis performed was the unpaired t-test for comparison of concentration and total motility between the control group and VSR group. GraphPad Prism 9.4.1 (681) was utilized for statistical analysis.

3. Results

3.1. Qualitative and Quantitative Analysis of VOCs

VOC analysis in sperm samples of young males from the VSR emphasized a very high inter-individual variability. As shown in Figure 2, the most detected VOC was 3-methylbutanal, followed by acetone and fluoren-9-ol,3,6-dimethoxy-9-2-phenylethynyl-. Several VOCs were present only in 15% of samples.

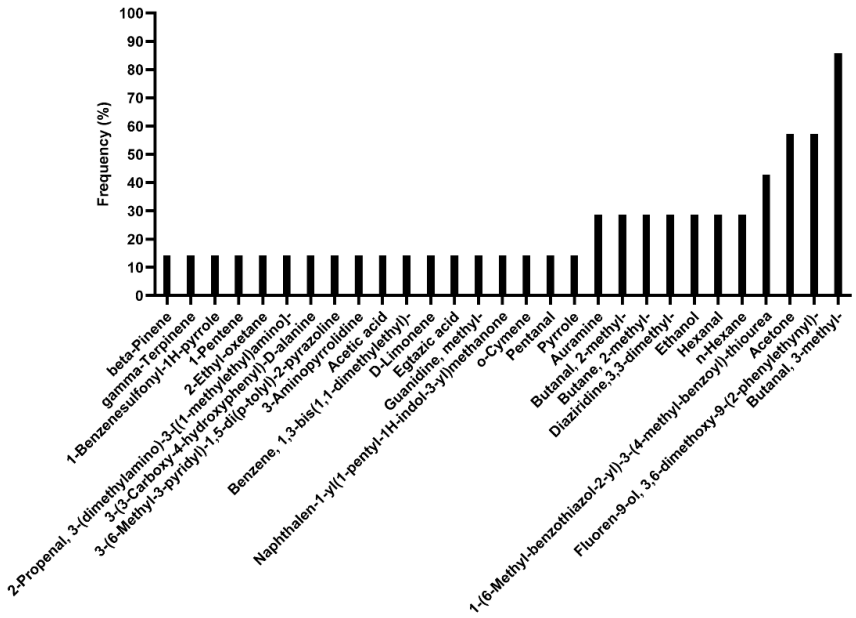

Figure 2. Frequency of VOCs which were detected in semen from the VSR group.

3.2. Anthropometric Data of Individuals and Semen Analyses

The anthropometric data of the VSR (red) displayed a very homogeneous class of subjects in terms of age (a), weight (b), height (c), and body mass index (BMI) (d) (Figure 3).

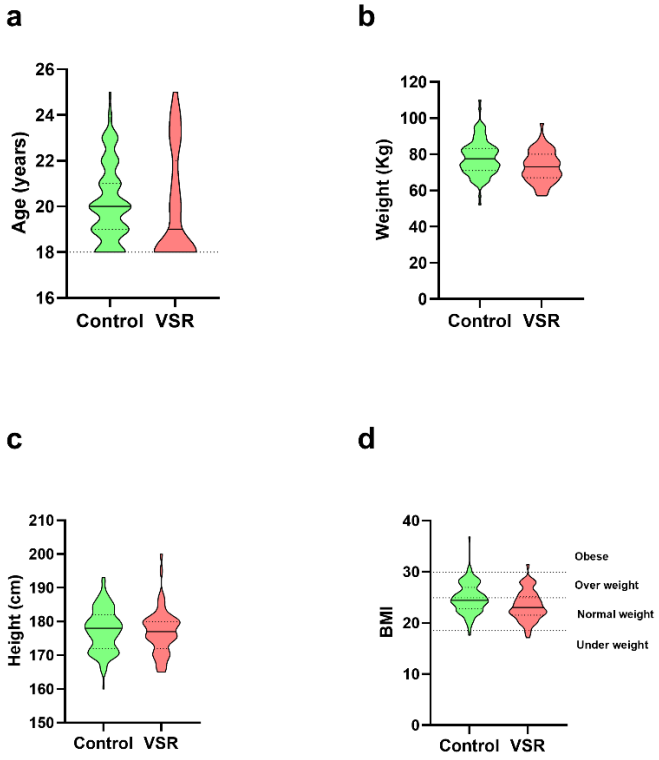

Figure 3. Anthropometric data: (**a**) age; (**b**) weight; (**c**) height and (**d**) BMI of control group (green) and VSR (red) individuals. BMI: Body Mass Index.

3.3. Semen Analysis

The results of sperm concentration and total mobility of the semen samples are shown in Figure 4. Semen analysis indicated a statistically significant reduction in sperm concentration (66.35 ± 25.10 vs. 46.03 ± 30.23) and in total sperm motility (49.79 ± 20.41 vs. 33.09 ± 22.58) in healthy young men living in the VSR. In fact, the VSR samples showed a lower total motility than the control samples. Other seminal parameters showed significant differences, in particular immotility, non-progressive motility, and round cells (Supplementary Figure S1).

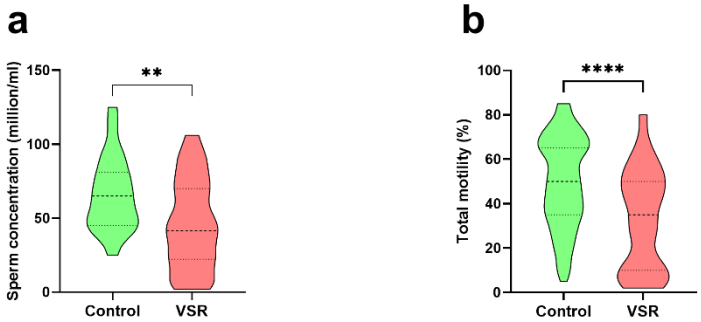

Figure 4. Statistical analysis of sperm concentration (**a**) and total motility (**b**) of the two groups. In green, control group, in red, VSR group. **: $p \leq 0.01$; ****: $p \leq 0.0001$.

3.4. Evaluation of Sperm Morphology of "Valle del Sacco" Samples

Morphological analysis of the VSR group's spermatozoa was performed by Diff-Quick staining. Figure 5 highlights the significant abnormalities seen in the VSR group's spermatozoa. In particular, while the control samples showed a clear acrosome and a dark nucleus with no morphological alterations (Figure 5a), the VSR group's spermatozoa showed a variety of different morphological alterations. They included abnormal tails and heads (Figure 5b); abnormal nuclei or absence of nuclei (Figure 5e,f,h,i); spermatozoa with a double head or double tail (Figure 5c,f) and cytoplasmatic detritus (Figure 5f); and absence of acrosomes and the presence of three tails (Figure 5d,g). In brief, about 90% of the VSR samples had various morphological changes, quantified by the sperm deformity index (SDI), such as a greater load on the head (about 40%) with altered acrosomes, pinheads, and pyriform and intermediate tract heads (about 20%) and the presence of angulations in about 10% on the tail, evaluated under the microscope by the operator and re-evaluated by photographic observation. This analysis ultimately underlined the significant alterations in spermatozoa morphology in individuals from the VSR group.

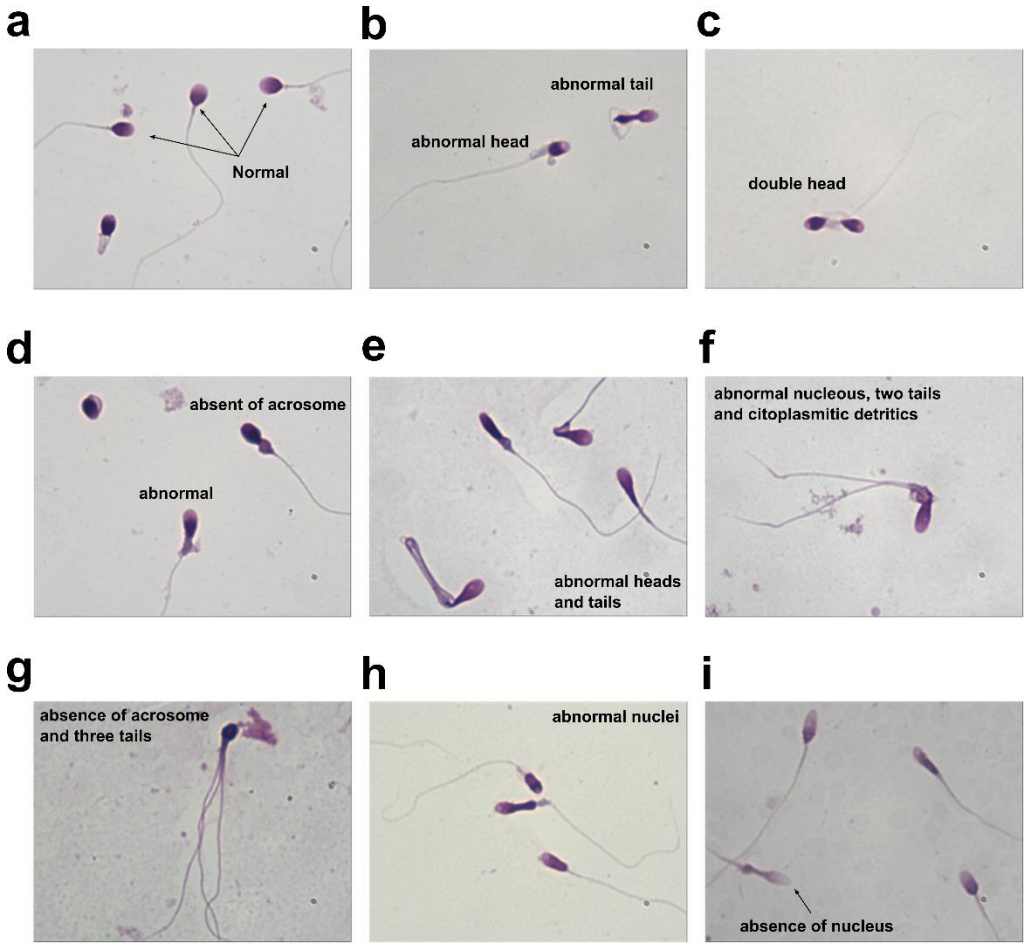

Figure 5. Diff-quick staining of VSR group's spermatozoa. (**a**): control group; (**b**–**i**): VSR group.

3.5. Analysis of SNBPs

SNBPs from samples belonging to males living in the VSR group and in the control group areas were extracted, and their protein content was characterized by acetic acid-urea gel electrophoresis (AU-PAGE) (Figure 6). The analysis by AU-PAGE showed remarkable differences between the SNBPs contained in the samples of individuals living in the two areas (Figure 6). In lane 10 of Figure 6a and in lane 1 of Figure 6b, representative electrophoretic patterns of samples belonging to the control group are shown. These samples exhibited the classic electrophoretic pattern of human SNBPs, with the canonical

protamine/histone ratio (CP/Hr), which was previously reported [42]. SNBP content in the VSR samples differed from that seen in control samples and could be grouped into two main expression patterns: the first characterized by an almost complete absence of protamines and a persistence of histones (only-H) (Figure 6a, lanes 1 to 9); the second characterized by the presence of protamines and histones but in a non-canonical ratio (nCP/Hr) (Figure 6b, lanes 2 to 10). Both conditions were extremely heterogeneous (Figure 6a,b).

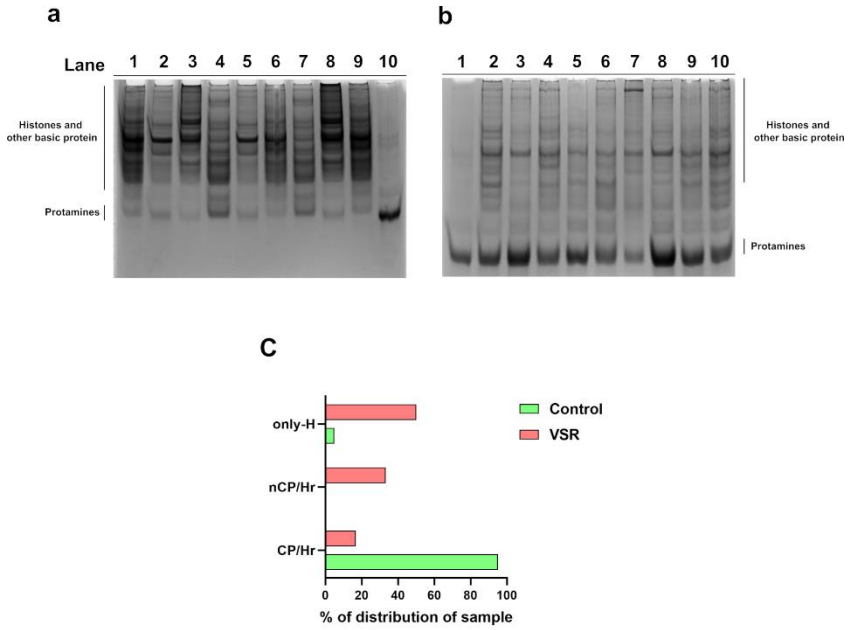

Figure 6. SNBP content as characterized by AU-PAGE displayed three different expression patterns. A normal canonical protamine/histone ratio (CP/Hr) was seen mainly in control group samples ((**a**), lane 10; (**b**), lane 1), while VSR samples more commonly displayed a histone-only ratio ((**a**), lanes 1–9) or a non-canonical protamine/histone ratio ((**b**), lanes 2–10). (**c**) Percentage distribution of protamines/histones ratios found in spermatozoa. In green, control group, in red, VSR group. CP/Hr: canonical protamine/histones ratio; nCP/Hr: non-canonical protamine/histones ratio; only-H: only histones.

We classified the samples (n = 76) analyzed in the two areas according to the type of bands identified on AU-PAGE as specified in materials and methods. The three types of electrophoretic profiles (CP/H ratio; nCP/H ratio; and only-H) were differently distributed in the VSR and in the Valley of Sele River (control group) areas. Specifically, in males belonging to the control group, we found only two conditions, the first represented by 95.06% of the samples displaying the CP/Hr profile, and the other represented by 4.94% of the samples showing the only-H pattern (Figure 6c). In contrast, in the VSR samples, we found the CP/Hr profile only in about 16% of the individuals and only-H profile in the majority of this group's samples (52%), while the remaining 32% of samples displayed a non-canonical protamine/histone ratio (nCP/Hr).

3.6. DNA Binding Ability of SNBPs Analyzed by EMSA

We analyzed, by electrophoretic mobility shift assay (EMSA), the differences in the DNA-binding ability of the types of SNBPs obtained in the control group and in the VSR samples. Specifically, we assessed the protein/DNA ratio required to achieve DNA saturation, which was indicated by the formation of a high-molecular-weight DNA band near the well in the electrophoretic gel as already reported [8]. The results indicated a very different DNA-binding capacity of SNBPs from the two sample groups. In fact, the SNBPs of control group samples, presenting the canonical protamines/histones ratio, produced DNA saturation at about a 1.2 *w/w* protein/DNA ratio as shown in Figure 7a, lane 9. In contrast, the SNBPs of the samples belonging to the VSR, regardless of their composition of

protamines and histones, showed low DNA-binding ability because DNA saturation did not occur even at a 3.8 protein/DNA ratio as shown in the representative gel indicated in Figure 7c, lane 12. In fact, panels b and c of Figure 7 show the gels of one VSR sample, but all 76 samples of the VSR group were analyzed, and this condition was found in all VSR samples, regardless of the distribution of the protamine/histone ratio.

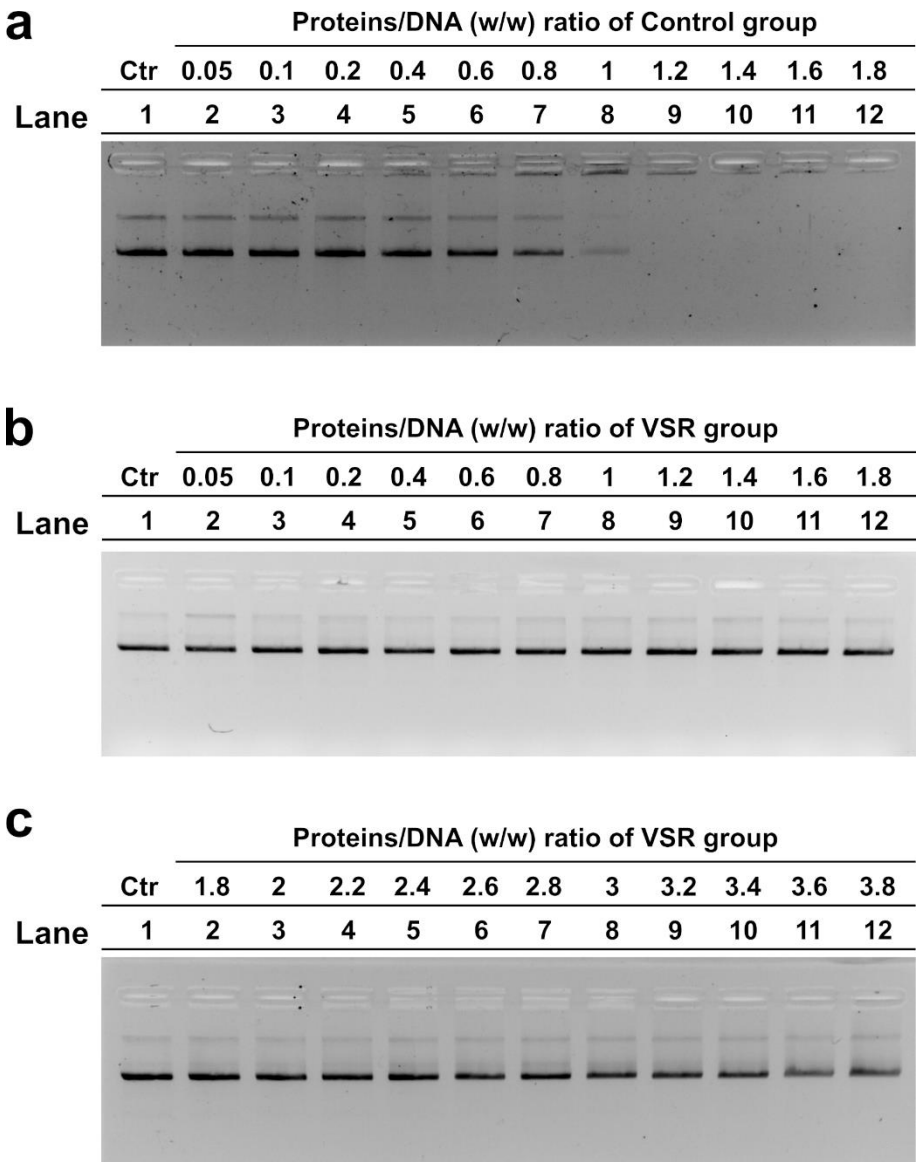

Figure 7. DNA-binding ability of SNBPs obtained from control group (**a**) and VSR group (**b**,**c**) analyzed by electrophoretic mobility shift assay (EMSA) on 1% agarose gel. Bands on gel representing the state of pGEM3 plasmid DNA incubated in a ratio *w/w* with increasing amount of SNBPs from samples. VSR: Valley of Sacco River; control: Valley of Sele River.

4. Discussion

Human sperm was proven in a recent study to be an excellent bioaccumulator of volatile organic compounds (VOCs) [43], and in a very recent paper of our research group, for the first time, the VOC composition of the semen of young men living in the VSR area was evaluated [32]. That work compared pollutants present in various matrices (semen, blood, etc.) in different areas of high environmental impact and showed that several VOCs were particularly present at high levels in the semen of young men living in the VSR area [32]. In particular, eight compounds presented a higher concentration in human semen

samples of subjects living in the VSR with respect to another polluted area of Italy (the Land of Fires in Campania) [32]. These compounds were: 1-6-Methyl-benzothiazol-2-yl3-4-methyl-benzoyl-thiourea, 2-Methylbutane, Auramine, 3,6-Dimethoxy-9-2-phenylethynyl-fluoren-9-ol, Pyrrole, Acetic acid, D-Limonene, and 3-Aminopyrrolidinein [32]. The same VSR semen samples were used in the present work to assess whether young men living in the VSR had alterations in SNBP properties. In young men living in the VSR, we found alterations in the classical seminal parameters, such as a decreased number of spermatozoa and a lower total motility, in comparison with young men living in low environmental impact areas. In addition, by molecular approaches, we highlighted changes in the state and properties of SNBPs of young men living in the VSR area. In particular, we found an altered protamine/histone ratio in the majority of the individuals. In fact, only 16% of VSR individuals showed a normal CP/Hr, while 52% showed only-H expression and 32% showed nCP/Hr.

These alterations in the protamine/histone ratio prompted us to investigate the ability of the SNBPs to bind DNA. This was because the correct protamine/histone ratio is a prerequisite for the fertilization capacity of spermatozoa [31], and also on account of our previous work on the SNBPs of young people living in the Land of Fires, which showed that an altered protamine/histone ratio produced low DNA-binding affinity [5,7]. As a matter of fact, we found, by EMSA, that the SNBP samples of all individuals living in the VSR, regardless of their SNBP composition, showed a very low DNA-binding affinity. In fact, consistent with our previous work, it was confirmed by EMSA that the extracts of sperm nuclear basic proteins from the control group presenting a canonical protamine/histone ratio interacted with DNA in an "all or nothing" mode [44], as sperm nuclear basic protein [45] and DNA saturation, i.e., the condition in which all DNA is close to the well of agarose gel, was reached at a 1.2 protein/DNA ratio. Instead, with all samples from the VSR group, DNA saturation was never reached even at a 3.8 protein/DNA ratio. The most striking finding was the severe morphological changes observed in the VSR group's spermatozoa. In more than 90% of the cases, severe morphological alterations were observed that were neither found in the control group nor in those from the Land of the Fires. These included abnormal tails and heads; abnormal nuclei and absence of nuclei; spermatozoa with a double head or double tail and cytoplasmic debris; absence of acrosomes and the presence of three tails. We tried to correlate the seminal and morphological alterations and the changes in the properties of SNBPs of the VSR group observed with the types of pollutants present in their semen, since it was recently demonstrated that human semen is an excellent bioaccumulator of VOCs [43]. It is known that many substances can cross the blood–testicular barrier and be released into the semen. This can lead to alterations in semen quality and quantity [46]. In particular, the VOCs that are most capable of crossing the blood–testicular barrier, or that result from biotransformations occurring at this anatomical site, are aldehydes, ethers, benzene derivatives, and terpenes [43]. As already mentioned, the main VOCs found in the semen of VSR individuals were: 1-(6-Methyl-benzothiazol-2-yl)-3-(4-methyl-benzoyl)-thiourea; Butane, 2-methyl-; Auramine; Fluoren-9-ol, 3,6-dimethoxy-9-2-phenylethynyl-; Pyrrole; Acetic acid, sodium salt; D-Limonene; and 3-Aminopyrrolidine [32]. The presence of these VOCs could be related to the poor semen quality found in the VSR cohort (Figure 4). The sperm concentration was higher than the reference value (>15 millions/mL) for most individuals, but these data have to be joined to the alarming occurrence of spermatozoa defects. The total motility was under the threshold value of 40% for a large part of the sample population [34].

The extremely toxic benzene derivatives that likely result from air pollution [47] are particularly present in semen as demonstrated by Longo et al., 2021 [32]. Benzene is a monocyclic aromatic hydrocarbon. It is a natural constituent of petroleum but is also synthesized from the other chemicals in the same petroleum. It is distributed in all biological fluids but seems to be particularly present in semen. Benzene and its derivatives can be highly dangerous because sperm cells, and consequently their DNA, are in close contact with these compounds, which in some cases also have mutagenic activity [48]. In

the literature, it is reported that exposure to benzene is not only associated with the onset of aneuploidy for X, Y, and 21 chromosomes in sperm [49] but also with structural aberrations of chromosome 1 in sperm cells [50]. In addition, benzene metabolite 1,2,4-benzenetriol is able to change DNA methylation and histone acetylation of specific genes [51]. It is known that these histone modifications are crucial for the transition from histones to protamines during spermatogenesis. Thus, that benzene and its derivatives can influence these histone modifications could explain the abnormal distribution in CP/H, nCP/H, and only-H that we observed in the VSR group individuals, and in particular, the finding that the majority of subjects tested in this area presented only histones. In addition, it was suggested that histones might be targets for attack by benzene or its metabolites [52]. Since histones are central to chromatin structure and function, molecular damage to histone proteins may be able to produce the cellular changes observed in benzene toxicity. Similarly, in spermatozoa, benzene may target protamines, since the cation–π interaction made by arginine residues with aromatic ligands is more robust to changes in the environment. This interaction is the most frequently found empirically and is also calculated to be stronger than for lysine in higher-polarity environments. There are relatively few cation–π interactions involving positively charged histidine residues, although the stacked $\pi + -\pi$ interaction is predicted to be of similar magnitude to that of arginine [53].

Aldehydes are organic compounds that carry in their structure the formyl functional group. In general, the levels of aldehydes can be considered as good markers to evaluate the levels of oxidative stress. Regarding the interactions between these compounds and human semen, the aldehyde of greatest concern is hexanal. Hexanal, mainly produced by the oxidation of linoleic acid, is used in the perfume industry to produce fruity fragrances. It is known that this compound, although it has no spermicidal activity, can significantly decrease sperm motility [54]. Therefore, the high percentage of immobile spermatozoa of these young men (about 70%) confirm the danger of hexanal in human semen.

Another potentially very toxic substance is auramine. This is used as a dye in the production of a large number of different items, such as paints, oils, and waxes. Several authors have indeed described the occurrence of dyes in the waters and sediments of rivers located within the area of influence of textile industry discharges [55,56], and this could explain the massive presence of this VOC in the semen of VSR individuals. Auramine is also used as an antiseptic and fungicide [41,42]. Auramine is known to affect DNA. In fact, this compound was shown to induce DNA fragmentation in the kidney, liver, and urinary bladder in rats [57]. Auramine is a diarylmethane dye, and the possibility to form complexes between p-sulfonatocalix[n]arenes and the amino acids lysine and arginine in water was demonstrated [58]. Therefore, it cannot be ruled out that these pollutants may bind to protamines via arginines and lead to the effects we demonstrated in the work.

Another compound that appears to significantly affect human semen is n-hexane. This alkane is used primarily as a fuel. It is an extremely harmful compound to the environment and very toxic to the reproductive system. In fact, even concentrations of 20 mg/mL induce irreversible immobility of the spermatozoa after 20 s. In addition, n-hexane can cause injury to the sperm plasma membrane [59] and may cause severe testicular atrophy and cessation of sperm production [60]. These effects could be responsible for the lower number of spermatozoa and the reduced mobility observed in VSR subjects.

Longo et al., 2021 [32] observed the preference of aldehyde, 3-methylbutanal, and 2-methylbutanal in the semen of these young men living in the VSR. Aldehydes may result in decreased sperm motility [61]. The decrease in motility could also be due to the epididymal dysfunction or elevated oxidative stress in the testicular environment [62].

As reported in a recent paper [63], pyrrole is another main VOC found in human semen, and in the study of Longo et al., 2021 [32], this substance was particularly present in subjects living in the VSR area [32]. Pyrrole is a flavoring agent with potential anti-inflammatory and antimicrobial properties [64]. Furthermore, it is the precursor of a wide variety of compounds that are known as pyrrole derivatives. 3-Aminopyrrolidine is another basis for a wide range of substances (3-aminopyrrolidine derivatives) of high pharmaceutical

interest. There are also many pesticides and insecticides based on pyrrole. In the literature, it is reported that pyrroles correlate directly with the extent of testicular injury. In addition, toxicity mediated by pyrrole–protein adducts was reported [65], and pyrrole–protein adducts, pyrrole–DNA adducts, and cross-linking adducts were demonstrated [65]. Pyrrole–amino acid adducts, including pyrrole–cysteine and pyrrolelysine, were synthesized by the reaction of pyrrolic metabolites with the corresponding amino acids [66]. Very recently, Fu and co-workers reported that several pyrrole–amino acid adducts, including 7-cysteine-DHP, were identified as secondary pyrrolic metabolites that could bind to DNA, leading to the formation of pyrrole–DNA adducts [67]. Among the hypothesized mechanisms for protein cytotoxicity induced by protein adducts with pyrrole is the covalent modification of proteins with consequent impairment of the normal function of target proteins [68,69].

Additionally, ethanol consumption is one of the leading causes of male infertility; however, the mechanisms that cause ethanol-induced infertility remain unclear. Studies have shown that ethanol can cause increased frequency of abnormal spermatozoa and a reduction in mean progression rate [70], complete loss of acrosomes and loss of the equatorial segment [71], and marked decreases in testosterone levels and expression of the testicular proteins AR and TyrPho [72].

Given the particularly high concentrations of VOCs found in human semen from healthy young men from the Valley of Sacco River [32], it is presumable to suppose that these contaminants could produce the morphological- and molecular-level alterations in the spermatozoa of these subjects. Indeed, both the significant reduction in motility and the high incidence of morphological abnormalities in sperm samples from the Valley of Sacco River are worrying indicators indicating a strong influence of exposure to highly contaminated living environments. Finally, our molecular biology-based approach confirmed the presence of critical alterations in SNBP properties. It is well-known that proper chromatin compaction of spermatozoa is critical for the fertilizing capacity of these cells. Of course, the alterations observed at the level of protamine/histone ratios and DNA-binding affinity of these proteins suggested that there was improper chromatin compaction in these spermatozoa, and thus, that there have been disturbances in the processes that occur at the end of spermatogenesis. The canonical protamine/histone ratio is crucial for the formation of a correct sperm chromatin structure, which is the prerequisite for the success of fertilization. Indeed, the high content of arginine residues in protamines allows them to bind to both the minor and major grooves of DNA, resulting in an appropriate compactness of the sperm chromatin, whereas histones only interact with a specific region of sperm DNA, producing less compact chromatin [45]. Considering that 10–15% of histones are maintained in the chromatin of human sperm [73–76], it may be assumed that the presence of protamines and histones in altered ratios may determine not only an unstable binding to DNA but also a decreased protection of DNA from external agents, such as various pollutants. Furthermore, in samples containing only histones, the low degree of sperm chromatin compactness could cause greater exposure of the DNA to external interferents. Indeed, it has been shown that some VOCs can cause a significant increase in histone deacetylation. This effect may be particularly significant in the histone–protamine transition, which requires histone acetylase activity, and could therefore be the reason for the high percentage of subjects (around 52%) in VSR areas who had only histones in their spermatozoa.

Given the varied negative effects of VOCs on spermatozoa, it is possible to speculate that their presence in the semen of these subjects may lead to synergistic negative effects on the functionality of these cells. Of course, this work has some limitations because we did not conduct VOC analysis on semen samples from subjects residing in the Valley of Sele River. Therefore, we can only speculate that the drastic changes observed at the morphological and molecular levels in the spermatozoa may be due to VOCs, since VOCs were found to accumulate in the sperm of these young men. As already mentioned, the Sele Valley, on the basis of the pollutant concentrations detected in the area, is included in the 'maintenance' category and is classified as a low diffuse emission area for sulfur oxide and

as a medium diffuse emission area for nitrogen oxide, carbon monoxide, volatile organic compounds, and suspended particulate matter, for which the human health risk thresholds are not exceeded [33]. Therefore, it can be supposed that since there are no excesses of volatile organic compounds in this area, high levels of VOCs should not be found in the semen of its residents. In conclusion, our findings represent preliminary data that need to be further investigated, but they nevertheless indicate great concern for the residents of that area. This is particularly noticeable taking into account that the subjects recruited in the VSR area are very young and healthy men in the prime of their fertile life. They were recruited randomly but met the selection criteria described. They are subjects who had never had a seminal examination. The context of alleged fertility problems derives from the fact that the VSR area is a site of national interest; in fact, several studies in Italy have reported that in areas of high environmental pressure, there is an increase in infertility, urogenital malformations, and chronic diseases (cancer, diabetes, etc.) [77]. Although the subject of infertility is mentioned in the text as an alarming fact in the population living in the area, we cannot absolutely state that our volunteers living in the VSR are infertile, but we simply state that their seminal quality presents alarming characteristics in terms of morphology, protamine: histone ratio, and seminal parameters. Such characteristics are crucial to understand why a couple is infertile or to suggest the couple to follow an assisted fertilization procedure [1,2,78]. For this reason, as future goals, we propose to measure the levels and types of VOCs in the young men of the Valley of Sele River of this sampling and to look for more precise correlations between particular VOCs found in the semen of the young men of the Sacco Valley and morphological and molecular alterations in the spermatozoa. An aliquot of the ejaculate was not foreseen for the VOC analysis at the time of the recruitment and collection of samples, as the authors responsible for this analysis joined the EcoFoodFertility initiative later on. However, such comparative analysis is planned to be performed in future recruitment and human biomonitoring studies in those areas. The Sele Valley is an area where VOCs are not detected in the environment at the levels found in the Sacco Valley, so presumably, they should not be present in the seminal fluid, but in any case, we will check for their presence in the next planned study. Campania, compared to Lazio, also does not have a high prevalence of VOCs, as shown by the comparison between the Land of Fires and Sacco Valley [32].

The consequences on the future reproductive potential of young people living in the VSR area therefore cannot be underestimated, especially given that semen represents an excellent marker of environmental and general health [79–82].

5. Conclusions

The limitations of this study lie in the fact that we did not carry out analyses of VOCs in the semen samples of subjects living in the Sele Valley. The fact remains, however, that despite the limitations of our study, our results showed very serious seminal alterations in these subjects, which have never been observed in other areas characterized by other types of pollutants. We therefore believe that there is a high probability that the chemical exposure of the population living in the Sacco River Valley has harmful effects on male infertility. Although our volunteer residents in the VSR cannot be defined as strictly infertile, the quality of their sperm presents alarming characteristics in terms of morphology, protamine/histone ratio, and seminal parameters. This is very relevant considering that the volunteers were very young men (18–20 years old) who should be in full reproductive capacity. These characteristics are also crucial to understand why a couple is infertile or to suggest the couple to follow an assisted fertilization procedure.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph191711023/s1, Figure S1: The figure shows the seminal parameters of two groups. Green: control group; red: VSR group. Unpaired T-test performed with GraphPad Prim ver. 9.4.1 (681). ns: $p > 0.05$; é; *: $p \leq 0.05$; **: $p \leq 0.01$; ****: $p \leq 0.0001$.

Author Contributions: Conceptualization, M.P. and L.M.; methodology, M.P. and G.L.; software, G.L.; validation, M.P., L.M. and S.P.; formal analysis, G.L., V.L., A.F. and S.C.; investigation, P.P., G.L., C.M., V.L., A.F., S.P. and S.C.; data curation, G.L., S.P., V.L., C.M., S.C. and P.P.; writing—original draft preparation, M.P., G.L. and P.P.; writing—review and editing, M.P., L.M., G.L., P.P., V.L. and S.P.; visualization, G.L. and V.L.; supervision, M.P.; project administration, M.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: All experimental protocols were approved by the Ethical Committee of the Local Health Authority Campania Sud-Salerno (Committee code n. 43 of 30 June 2015.

Informed Consent Statement: Informed consent was obtained from recruited subjects before sample collection.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

VSR	Valley Of Sacco River
EMSA	Electrophoretic Mobility Shift Assays
EDTA	Ethylenediaminetetraacetic Acid
VOCs	Volatile Organic Compounds
SNBP	Sperm Nuclear Basic Protein
CP/Hr	Canonical Protamines/Histones Ratio
nCP/Hr	Not Canonical Protamines/Histones Ratio
Only-H	Only histones and other basic proteins
BMI	Body Mass Index
AU-PAGE	Acetic Acid-Urea Gel Electrophoresis
PMSF	Phenylmethylsulfonyl Fluoride
DTT	Dithiothreitol
TCA	Trichloro Acetic Acid
TBE	Tris-Borate-EDTA

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