



# Gonadal Histopathological Disorders in *Mytilus galloprovincialis* Male Exposed to Tars Used in Mussel Farms

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Polycyclic aromatic hydrocarbons (PAHs) and trace metals are pollutants widely distributed in the marine environment with toxic effects on live organisms, but few laboratory studies have proved their causal relationship with histopathologic alterations from invertebrates. We analyse the histopathologic effects of a tar mixture used in mussel farms on the gonad of male mussels. Mussels were exposed to water-accommodated fractions from different sub-lethal concentrations of that tar mixture during different times. The accumulation of PAHs and metals in mussels was quantified in each one tar exposure condition, and a comparative histological and morphometric analysis of the male gonad was performed, as well as of the gametogenic disorders and the cellular pathologies induced. Spermatogenesis disruption and alterations of somatic and germinal cells were observed as a direct effect of treatment. The pathologies observed followed an increasing malignity sequence depending on dose and exposure time: spawning induction; arrest of spermatogenesis and spermiogenesis; suppression of immune response; dysplasia of Sertoli and connective cells and finally, severe disorders of germinal cells. We demonstrate for the first time that tar used in waterproofing of floating rafts from the mussel farms causes severe effects on male mussel gonad, constituting a direct risk to the health of these organisms, affecting gametogenesis, quality of gametes and, ultimately, the sustainability of this resource and their quality for human consumption. Moreover, we propose the pathologies described in mussel as biomarkers of marine pollution and the mussel gonad as study model of testicular toxicity mechanisms in male reproduction.

**Keywords:** *Mytilus galloprovincialis*, histopathology, tar, mussel farms, gonad

## INTRODUCTION

Galician Rías (NW Spain) are important aquaculture areas, being mussel farming the main activity (Caballero Miguez et al., 2009). However, the pollution level in these areas rises proportionally to the industrial, tourism and port development. Moreover, the same aquaculture activities interact with the environment and can constitute a source of pollution around farms. Mussel culture is carried out in wood floating rafts, whose cleaning, and waterproofing are usually performed *in situ* with a mixture of petroleum tars and exhausted oil from the diesel engine. This mixture contains

high concentrations of polycyclic aromatic hydrocarbons (PAHs) and transition metals (Ruiz et al., 2014), and filtering organisms, such as mussels, can accumulate them to levels well-above those in the surrounding water (Farrington et al., 2016). So, the spillage of these mixtures around rafts entails a direct pollution risk on the immediate environment of farms, which can affect not only the sustainability of the mussel production but also its quality for human consumption.

Polycyclic aromatic hydrocarbons are considered among the most ubiquitous marine pollutants. Hydrocarbons and their biotransformation metabolites have harmful effects on live organisms, mainly the species with high molecular weight and benzene rings number, which have been identified as highly mutagenic, carcinogenic, and teratogenic (Arcos and Argus, 1975; Lehr and Jerina, 1977; White, 1986; IARC, 1989; Nylund et al., 1992; Boström et al., 2002; Baird et al., 2005; Haines and Hendrickson, 2009; Abbas et al., 2013; Poirier, 2016). Heavy and trace metals are also an extensively studied pollutant group in coastal environments because of their wide distribution, persistence, and toxicity. Some of them, such as Mn, Zn, and Cu, have physiological functions in living organisms but at high concentrations are also toxic (Goyer and Clarkson, 2001). Exposition of live organisms to heavy and trace metals has been related to cellular damages, carcinogenesis, and apoptosis (Gerber et al., 1980; Thompson et al., 1989; Chang et al., 1996; Wang and Shi, 2001; Beyersmann and Hartwig, 2008; Obiakor et al., 2010; Tchounwou et al., 2012; Chandel and Jain, 2014; Fatima et al., 2014). These metals are included in diverse industrial compounds, such as fuels and tars, lubricants, catalysts, paint drying agents, pigments, or pesticides (Laws, 2000; Clark, 2001), so pollution is usually occurring by combination of diverse pollutants whose toxic effects may be additive, suppressive or synergistic (Vakharia et al., 2001; Shen et al., 2006; Wang et al., 2015).

The toxic effects of PAHs and heavy metals and their action mechanisms have been widely studied in vertebrates. Among the main pathological effects usually associated with PAHs and heavy metals, are included toxicity reproductive and metabolic alterations and neoplastic diseases. These pathologies result from direct binding of these chemicals and their metabolites to macromolecules and, secondarily, from toxicity of the reactive oxygen species generated in their biotransformation (Sokol, 1987; Mortelmans and Zeiger, 2000; Adhikari et al., 2001; Ercal et al., 2001; Wang and Shi, 2001; Boström et al., 2002; Pufulete et al., 2004; Flora et al., 2008; Ono et al., 2008; Veeramachaneni, 2008; Sanders et al., 2009; Swedenborg et al., 2009; Balabanič et al., 2011; Tchounwou et al., 2012). Also in marine invertebrates, mainly molluscs, there are studies showing reproductive and neoplastic disorders related to pollution (Viarengo et al., 1990; Bolognesi et al., 1999; Matozzo et al., 2001; Creasy and Foster, 2002; Au, 2004; Pichaud et al., 2008; Jing-Jing et al., 2009; Ji et al., 2011; Ruiz et al., 2011). However, in these, much remains unknown about their biotransformation and toxic mechanisms, and few studies have proved their causal relationship under laboratory conditions (Lavado et al., 2006; Ortiz-Zarragoitia and Cajaraville, 2006; Jing-Jing et al., 2009; Schäfer and Köhler, 2009).

In previous papers, our team has documented in *Mytilus galloprovincialis* the accumulation of PAHs, trace metals and PCBs, relating it with the mutagenicity of seawater, and gonadal neoplasias development (Ruiz et al., 2011, 2013). Likewise, we have previously evaluated the mutagenic capacity of different chemicals used in mussel farms, including the used in the present work (Ruiz et al., 2014). This is a short-term toxicological study in laboratory in order to observe the histopathological effects caused by the more usually tar mixture used in mussel farms on the male gonad of mussel; prove their causal relationship in order to confirm the observed in natural environment; and propose the histological pathologies induced as possible generalist biomarkers of the aquaculture areas quality.

## MATERIALS AND METHODS

Mussels were exposed to a mixture of petroleum tars and exhausted oil the from diesel engine, generally used in cleaning and maintenance of the mussel rafts, which was kindly provided by mussel farmers from the Ría of Vigo. Our team previously analysed the trace metals and hydrocarbons content of this tar, showing a significantly high concentration of Mn, Pb, and Zn (in a proportion 1:2:7), as well as a 81.5% of PAHs species with 3 and 4 benzene rings and a 18.5% of species with 5 and 6 benzene rings (Ruiz et al., 2014).

### Mussels, Laboratory Conditions, and Acclimation

Mussels (*M. galloprovincialis*), 7–8.5 cm long, were collected from floating rafts in the Ría of Vigo in April, before starting the cleaning and waterproofing tasks of rafts in the culture area. Spring is the reproductive season of mussels in this area (Suárez et al., 2005), but also when we observed a higher PAHs accumulation and prevalence of gonadal histological disorders in these organisms in the wild (up to 739  $\mu\text{g kg}^{-1}$  dry weight and 62.5%, respectively) (Ruiz et al., 2011, 2013). The reproductive stage seems, therefore, constitute the most sensitive life-stage of mussels and the most suitable to test the toxic effects of pollutants on gametogenesis (Diepens et al., 2017). In the laboratory (Station of Marine Science of Toralla, University of Vigo), mussels were acclimated during 15 days in two polythene tanks of 150 L with 20  $\mu\text{m}$  filtered seawater, in a semi-open system of 2 mL  $\text{min}^{-1}$  flow and the same out-flow, under natural photoperiod, and constant aeration, temperature and salinity. Animals were fed every 2 days with a microalgal diet (0.0224 g per mussel) composed by *Isochrysis galbana*, *Tetraselmis suecica*, and *Chaetoceros gracilis* in a proportion of 50:30:20.

### Preparation of Water-Accommodated Fractions (WAF)

During waterproofing of rafts, a lot of tar falls to water and farmers pour the tar leftovers in the same environment, so high tar concentrations can be achieved around farms. Nevertheless, the dilutive effect of tides and currents makes their lasting become not too prolonged in time. Only the soluble fraction of these compounds, which varies depending on

their concentration and composition, is bioavailable and may exert actual toxicity on aquatic organisms (Singer et al., 2001). The WAF from different concentrations of tar were prepared in 20  $\mu\text{m}$  filtered seawater, following the recommendations by CROSERF (Chemical Response to Oil Spills: Ecological Research Forum) (Singer et al., 2000, 2001; Aurand and Coelho, 2005). Briefly: in a similar way as carried out by the mussel farmers, the tar was heated to make it more fluid. Then, each tar concentration to be tested was prepared in glass Mariotte bottles with 5 L of seawater and maintained at room temperature for 24 h under magnetic stirrer. At the beginning of the experiments, the WAFs from the different tar concentrations were similarly prepared in polythene tanks of 30 L.

### Determination of the Median Lethal Concentration (LC<sub>50</sub>)

Based on preliminary experiments, the WAF from 6 concentrations of tar between 60 and 160 mg L<sup>-1</sup> were used in order to determinate its LC<sub>50</sub> in an acute toxicity test for 96 h. For mortality test, acclimated mussels were randomly distributed in polythene tanks of 30 L (40 individuals/tank), containing the WAF from 60, 80, 100, 120, 140, and 160 mg L<sup>-1</sup> tar prepared as above described. Five litres WAF from each tar concentration was prepared daily and supplied to the corresponding tank, at a 2 mL min<sup>-1</sup> flow, replacing the seawater entry. The assay was performed in duplicate (6 × 2 tanks), and the mussels from other two tanks were maintained in seawater and taken as reference. Conditions of water flow, nutrition, temperature, aeration, and photoperiod were the same as during acclimation. Water quality in each tank was daily checked by measuring pH, dissolved oxygen (DO), salinity and ammonium concentration, using portable pH meter, oximeter and salinometer, and ammonium kit LCK 304, respectively.

Mortality data were analysed by the PROBIT regression test with IBM SPSS Statistics 23.0 package (IBM Corporation, Armonk, NY) which allowed calculating the LC<sub>50</sub> of the tar mixture for the mussels, and their associated 95% confidence limits. The adequacy of the probit model to the observed data was evaluated by Chi-square test.

### Toxicological Assay

Three sub-lethal WAF from tar concentrations were selected below the LC<sub>5</sub> value (see result section about LC<sub>50</sub> and probit test) to conduct this study: 10, 40, and 60 mg L<sup>-1</sup>. The WAF of these tar concentrations, the tanks and the mussels were prepared and disposed of as described in the above sections. The toxicological assay was also performed in duplicate. Two tanks with mussels maintained in seawater without tar were taken as reference. The conditions of the water and the mussels, as well as quality system control, were the above described. The toxic treatment lasted 17 days and samples were taken at 0, 10 and 17 days. After this time, mussels exposed to the WAF from 60 mg L<sup>-1</sup> tar were transplanted to the other two tanks under non-toxic conditions during 2 weeks before sampling (deuration time). The analysis power of the sample size (320 individuals/4 groups of study) was validated with free software G\*power ([http://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-](http://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie.html)

[arbeitspsychologie.html](http://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie.html)), giving a value of 0.96. In each sampling, 20 individuals from each treatment (10 individuals per tank) were collected and, by histological analysis, males were separated for this study.

### Biometric Analysis

In each sampling, the size, total weight (Wt), shell weight (Ws), and mantle weight (Wm) of individuals were measured. From these data, the gonadal condition index (GCI) was calculated. This index is an approximation to gametogenic development of an organism. In species of the genus *Mytilus*, the genital papilla proximity to the mantle tissue is used for the expansion of gonad, and the gametogenic development takes place in this tissue. So, in these species, the GCI is determined as the mantle condition index, which can be calculated as the ratio between the mantle weight and the weight of all soft tissues (the difference between total weight and shell weight) (Suárez et al., 2005).

### Histological and Morphometric Analysis

A cross-section (1 cm wide) of the central part of one hemimantle of each mussel was fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Sections 5  $\mu\text{m}$  thick were cut, mounted on microscope slides, and stained with haematoxylin and eosin. Additional slides were stained with modified Gomori methenamine-silver nitrate, aniline blue and periodic acid-Schiff (PAS) in order to detect basement membranes and reticulin fibres, basophilic components, and glycogen, respectively.

The slides were examined under a light microscope Nikon 90i Eclipse. Representative photomicrographs were captured by a high-resolution camera digital (Nikon DS-Fi1c) coupled to a microscope and using the image analysis system Nis-Elements BR 4.0 software (Nikon Instruments Inc., Melville, NY). Morphometric analyses were performed using the same image analysis system above: the number of gonadal follicles, vesicular cells (VC), adipogranular cells (ADG), and granulocytes, as well as the area of gonadal follicles and VCs, were quantified in 20 sections 100  $\mu\text{m}^2$  from each slide at 100 or 400x magnification (for gonadal follicles and cells, respectively). Composition of figures was performed using CorelDraw<sup>®</sup> Graphics Suite X5 (Corel Corporation, Canada).

### PAHs and Metals Extraction and Quantification

After dissection for histological analysis, soft tissues of the individuals sampled from each treatment was pooled, quickly frozen, and pulverised in liquid nitrogen using a laboratory ball mill (Fritsch GmbH, Germany). The analyse of the PAHs and trace metals levels were contracted to the Centre for Scientific and Technological Support to Research (CACTI) of the University of Vigo. PAHs were extracted with methanol in ultrasonication bath for 30 min and separated and quantified by high-performance liquid chromatography (HPLC) within tandem detectors of photodiode array and scanning fluorescence. The 16 most abundant species of PAHs in the tar composition were measured: naphthalene (Nap), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe),

anthracene (An), fluoranthene (Flt), pyrene (Py), benzo(a)anthracene (BaA), chrysene (Chry), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), dibenzo(a,h)anthracene (DBA), benzo(g,h,i)perylene (Bper), and indeno(1,2,3-c,d)pyrene (IP). The most abundant trace metals in tar (Zn, Pb, and Mn) were determined by optical emission spectroscopy with inductively coupled plasma as the excitation source (ICP-OES) after a hydrogen peroxide:nitric acid (2:5) digestion. All analyses were performed in triplicate and PAHs and metals concentrations were expressed as  $\mu\text{g kg}^{-1}$  fresh weight (fw) and  $\text{mg kg}^{-1}$  fw, respectively.

## Statistical Analysis

Statistical analyses were performed with IBM SPSS Statistics 23.0. All histological preparations were comparatively examined in relation to the reference organisms. The distribution and homoscedasticity of biometric and morphometric data were analysed by Kolmogorov-Smirnov and Levene tests, respectively. Data were assessed to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Student's *t*-test to determine the statistical significance between groups.  $p < 0.05$  was considered statistically significant.

Multivariate analysis was carried out using the software packages PRIMER6 & permanova+ (Anderson et al., 2008). The distribution of the samples based on quantitative parameters variation as the effect of tar exposure was examined by Hierarchical Cluster analysis using Bray Curtis similarity. Previously, the collinearity between variables was analysed by Pearson correlation matrix, eliminating the variables with  $r^2 > 0.95$ . The main variables explaining this distribution were identified by parsimonious models (DistLM), and represented by distance-based redundancy analysis (dbRDA) (Anderson et al., 2008).

## RESULTS

### Water Quality and LC<sub>50</sub> Determination

The water quality parameters remained within a narrow range in all tanks throughout the mortality test and the toxicological experiment:  $18 \pm 1^\circ\text{C}$ ;  $7.8 \pm 0.4$  pH;  $34.5 \pm 0.5\%$  salinity;  $87.5 \pm 5\%$  dissolved oxygen, and  $0.03 \pm 0.01$   $\text{mg L}^{-1}$  ammonium. So, the differences we described in mussels intoxicated relative to reference mussels could be considered as a consequence of tar exposition.

Survival of the reference mussels and those exposed to the WAF from  $60 \text{ mg L}^{-1}$  tar was 100%. However, mortality of the mussels exposed to WAF from tar concentrations higher than  $80 \text{ mg L}^{-1}$  increased as tar concentration increased. The log-probit regression analyse of the data allowed estimate the LC<sub>50</sub> of the tar in  $139.84 \text{ mg L}^{-1}$  for *M. galloprovincialis* (95% confidence interval:  $132.57$ – $149.08 \text{ mg L}^{-1}$ ; regression equation:  $Y = 0.028X - 3.922$ , Chi-square = 0.182,  $P = 0.67$ ). In the same analyse, the LC<sub>5</sub> (tar concentration whose WAF cause a 5% mortality of mussels) yields a value of  $81.20 \text{ mg L}^{-1}$  (95% confidence interval:  $64.02$  –  $92.23 \text{ mg L}^{-1}$ ). The toxicological assay was then performed with the WAF from tar concentrations below this value.

## PAHs and Metals Accumulation

Pollutants accumulation in the mussels exposed to tar increased significantly ( $p < 0.05$ ) in relation to reference mussels within the following ranges:  $6$ – $106 \mu\text{g kg}^{-1}$  fw total PAHs ( $\Sigma 16$  parent PAHs);  $0.08$ – $0.71 \text{ mg kg}^{-1}$  Mn;  $0$ – $1.4 \text{ mg kg}^{-1}$  Pb; and  $12$ – $75 \text{ mg kg}^{-1}$  Zn (Table 1). The total PAHs accumulation increased proportionally to tar concentration and exposure time in the mussels subjected to  $10$  and  $40 \text{ mg L}^{-1}$  of tar. However, in mussels exposed to  $60 \text{ mg L}^{-1}$  tar, the accumulation falls concerning that of the exposed to  $40 \text{ mg L}^{-1}$  tar in both two exposure times. Moreover, contrary to expected based on tar composition, the hydrocarbons with 5 and 6 benzene rings were accumulated in greater proportion (up to 30.8%) than species of 3 and 4 benzene rings. The mussels that were transplanted to non-toxic conditions for 2 weeks, after exposure to the WAF from  $60 \text{ mg L}^{-1}$  tar during 17 days, showed a small, but significant decrease ( $p < 0.05$ ) in the accumulation of PAHs of 5 and 6 benzene rings and metals, but not of hydrocarbons of 2, 3, and 4 benzene rings.

The heavy metals followed a similar pattern of accumulation, increasing proportionally with the tar concentrations supplied and exposure times ( $p < 0.05$ ). Only the mussels exposed to the highest tar concentration during longer time showed a decrease in metals accumulation. Unlike the proportion of the metals in the tar composition, the ratio Mn:Pb:Zn in the mussels exposed to tar was around  $1.2$ : $>100$ . However, the Zn and Pb proportion decreased significantly ( $p < 0.05$ ) both, in mussels exposed for 17 days to  $40$ – $60 \text{ mg L}^{-1}$  and  $60 \text{ mg L}^{-1}$  tar, respectively, as in the mussels transplanted to depuration conditions for 15 days.

## Biometric Analysis and Gonadal Condition Index

Biometric and histological analysis was performed individually in each one of 80 mussels collected in each sampling. The histological analysis allowed separating the males for this study. The ratio between sexes was almost 1.2 in all sampling, similar to that found for *Mytilus edulis* and *M. galloprovincialis* by others authors in the wild (Lubet, 1959; Villalba, 1995; Suárez et al., 2005). All male mussels showed similar size ( $7.05 \pm 0.45$  cm) and shell weight ( $8.45 \pm 0.8$  g) along the experiment. However, the total weight of soft tissues ranged between 15.98 and 5 g, and the mantle weight between 5.68 and 0.18 g, decreasing significantly as tar concentration and exposure time increased ( $p < 0.01$ ; Figures 1A,B). The mantle tissue of *Mytilus* has two interrelated physiologic functions: storage of reserve substances in specialised cells and gametogenesis at the expense of these reserves (see Supplementary Materials, "Mantle tissue of *Mytilus galloprovincialis*: histological structure"). So, *a priori*, the significant fall in mantle tissue weight could suggest an alteration of these two functions.

The GCI from the reference individuals was similar throughout the experiment (Figure 1C). The mussels exposed to  $10$  and  $40 \text{ mg L}^{-1}$  of tar mixture for 10 days showed negligible alterations ( $p > 0.05$ ) of mantle and soft tissues weights, as well as similar GCI values, compared to those of the control

**TABLE 1** | Accumulation of hydrocarbons, Mn, Pb, and Zn in *Mytilus galloprovincialis* exposed to different tar concentrations and exposure times.

| Exposure time tar concentration (mg L <sup>-1</sup> ) | 0 days | 10 days   |           |           |           | 17 days |           |           | 15 days depuration |
|---|--------|-----------|-----------|-----------|-----------|---------|-----------|-----------|--------------------|
|   | 0      | 10        | 40        | 60        | 10        | 40      | 60        |           |                    |
| <b>PAHs (μg kg<sup>-1</sup>)</b>                      |        |           |           |           |           |         |           |           |                    |
| Nap   | LD     | 1.0       | 12.0      | 2.5       | 1.6       | 13.0    | 1.3       | 1.5       |                    |
| Acy   | LD     | 5.6       | 17.2      | 8.8       | 16.5      | 11.2    | 18.4      | 16.4      |                    |
| Ace + Flu   | 1.0    | 2.5       | 3.2       | 2.2       | 4.3       | 3.3     | 1.5       | 1.6       |                    |
| Phe   | 0.1    | 2.2       | 9.6       | 2.4       | 5.0       | 4.9     | 1.8       | 2.0       |                    |
| An  | LD     | 0.4       | 1.7       | 0.5       | 0.9       | 1.0     | 0.3       | 0.9       |                    |
| Flt   | 0.7    | 4.7       | 8.9       | 7.2       | 7.5       | 12.7    | 5.0       | 6.9       |                    |
| Py  | 0.5    | 6.5       | 16.8      | 8.7       | 9.5       | 19.3    | 5.9       | 7.9       |                    |
| BaA   | 0.5    | 1.8       | 2.9       | 4.0       | 1.9       | 4.5     | 3.1       | 2.4       |                    |
| Chry  | LD     | 2.8       | 5.1       | 6.7       | 3.6       | 8.2     | 4.5       | 4.4       |                    |
| BbF   | LD     | 1.2       | 2.6       | 3.9       | 1.5       | 3.3     | 2.9       | 2.3       |                    |
| BkF   | LD     | 0.6       | 0.8       | 1.5       | 0.7       | 1.8     | 1.3       | 0.9       |                    |
| BaP   | 0.3    | 1.0       | 1.3       | 2.2       | 1.1       | 2.8     | 1.9       | 1.8       |                    |
| DBA   | 2.1    | 9.1       | 9.1       | 9.1       | 10.3      | 18.1    | 10.4      | 7.7       |                    |
| Bper + IP   | LD     | 0.4       | 0.5       | 0.9       | 0.8       | 1.5     | 0.8       | 0.6       |                    |
| ΣPAHs   | 6      | 40        | 92        | 61        | 65        | 106     | 59        | 57        |                    |
| % 2 + 3 + 4 rings PAHs                                |        | 69        | 84        | 71        | 78        | 74      | 71        | 77        |                    |
| % 5 + 6 rings PAHs                                    |        | 31        | 16        | 29        | 22        | 26      | 29        | 23        |                    |
| <b>Metales (mg kg<sup>-1</sup>)</b>                   |        |           |           |           |           |         |           |           |                    |
| Mn  | 0.08   | 0.37      | 0.42      | 0.56      | 0.40      | 0.71    | 0.41      | 0.35      |                    |
| Pb  | LD     | 0.67      | 0.75      | 0.98      | 0.76      | 1.4     | 0.7       | 0.51      |                    |
| Zn  | 12     | 46        | 63        | 69        | 61        | 75      | 48        | 35        |                    |
| Mn:Pb:Zn  |        | 1:1.8:124 | 1:1.8:150 | 1:1.8:123 | 1:1.9:152 | 1:2:106 | 1:1.7:117 | 1:1.5:100 |                    |

Each data is the average of three independent measurements. The values of all groups exposed to tar were significantly higher ( $p < 0.01$ ) than those of the reference individuals (0 mg L<sup>-1</sup> tar). The values of the mussels transplanted to non-toxic conditions (depuration sample) were significantly lower than those of the exposed to 60 mg L<sup>-1</sup> for 17 days ( $p < 0.05$ ).

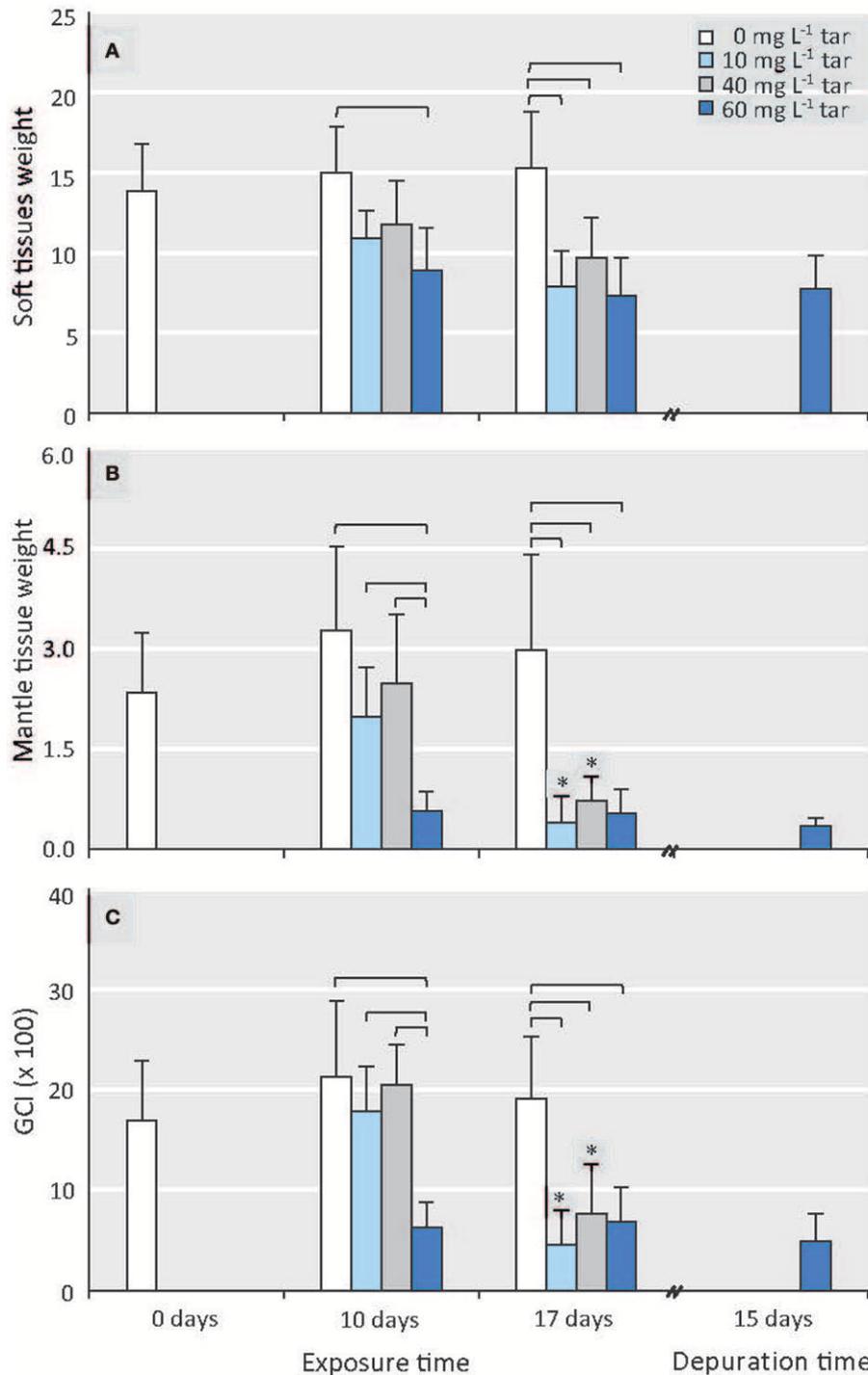
mussels. However, the mussels exposed to 60 mg L<sup>-1</sup> tar showed a significant decrease in the mantle weight and, consequently, of the GCI ( $p < 0.01$ ; **Figures 1B,C**). After 17 days' tar exposure, the mantle tissue weight and the GCI of the mussels exposed to 10 and 40 mg L<sup>-1</sup> tar fell ( $p < 0.01$ ) until similar values as those of the mussels exposed to 60 mg L<sup>-1</sup> tar during 10 days; but the mussels exposed to this last concentration showed similar values to those achieved after 17 days of exposition (**Figures 1B,C**).

### Gonadal Stage and Histological Disorders

At the onset of the toxicological assay, the acclimatized mussels were in a pre-spawn stage (see **Figure S1**). This gametogenic stage is characterised by the presence of large gonadal follicles full of ripe gametes occupying the whole mantle tissue and by a scarce storage tissue, mainly formed by vesicular cells (see **Supplementary Materials**, "Reproductive cycle of *Mytilus galloprovincialis*"). Throughout all experiment, the reference individuals showed this same stage, with a slight but significant increase of gonadal follicles number ( $p < 0.01$ ) parallel to decrease of vesicular cells (VC) area and adipogranular cells (ADG) number ( $p < 0.0001$ ; **Figures 2A1,A2, 3A–E**). In the follicular wall, auxiliary cells and some spermatogonias, spermatocytes

and spermatids can be observed (see **Figures S2A,B**). At 17 days, auxiliary cells and eosinophilic granulocytes were observed in the lumen of some follicles from the reference mussels (see **Figures S2C,D; Figure 3F**). In the seminiferous tubules, these two cellular types have a phagocytic function of cellular debris after the spawn. In bivalves, the increase in temperature is one of the main natural factors inducing spawning (Suárez et al., 2005), but during our experiment, the temperature was kept constant at 18°C. So, the lack of stimuli for spawn could have caused atresia of the over-ripened gametes, inducing the phagocytic function of auxiliary cells and the increase in the number of eosinophilic granulocytes in the follicular lumen (**Figure 3F**) as it is described in the wild under normal conditions (Suárez et al., 2005; Suárez Alonso et al., 2007).

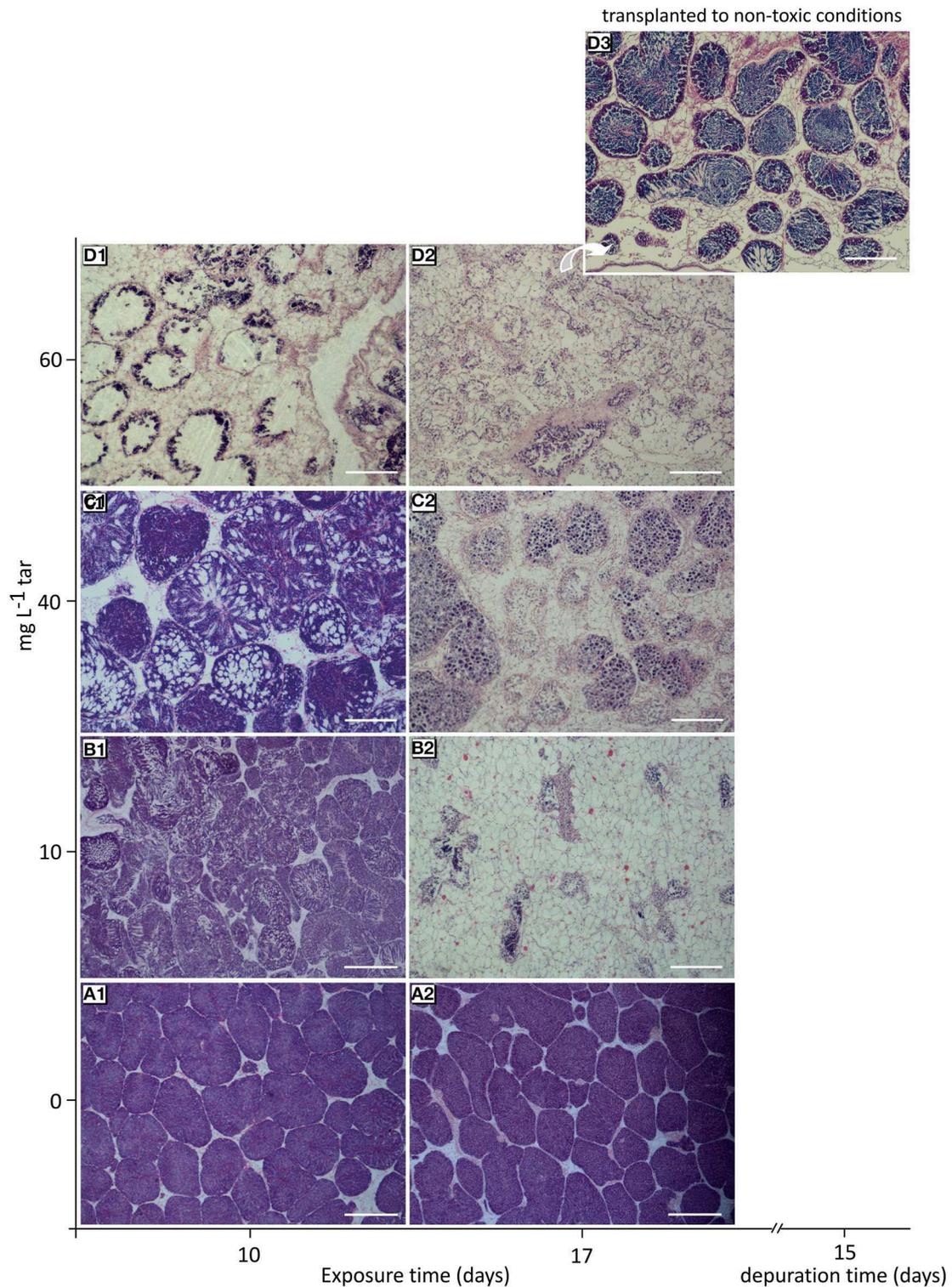
The number and area of gonadal follicles from mussels exposed to 10 and 40 mg L<sup>-1</sup> tar for 10 days were unchanged in relation to those of the reference individuals (**Figures 3A,B**). However, lumen was partially empty, and spermatozooids were present in gonoducts, evidencing clear images of spawn (**Figures 2B1,C1**). Mussels exposed to 40 mg L<sup>-1</sup> tar also showed evidence of sloughing of spermatids or emission of immature germinal cells (**Figure 2C1**). After 17 days, spawn had been massive in most individuals, and atrophy of gonadal



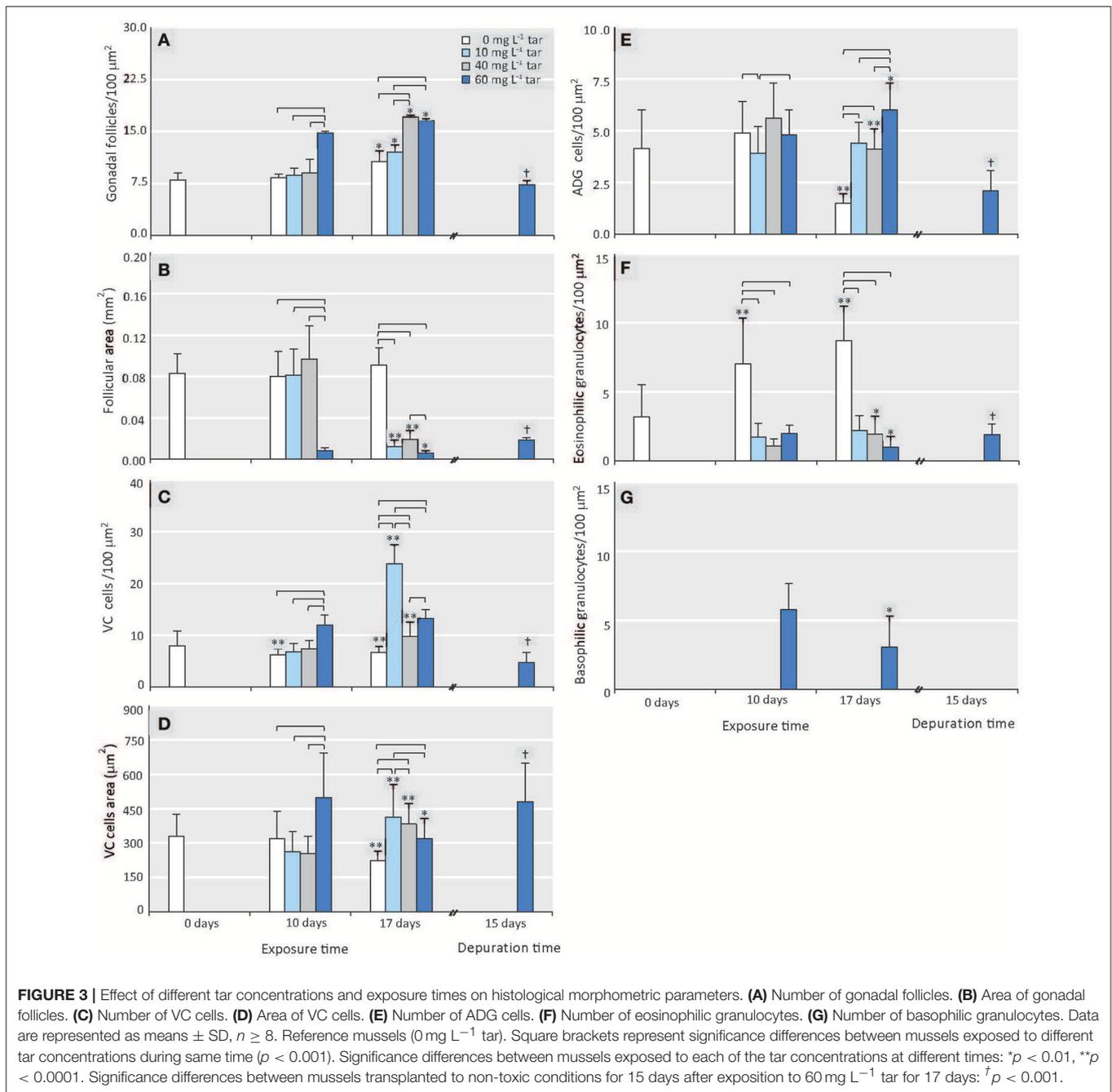
**FIGURE 1** | Effect of different tar concentrations and exposure times on biometric parameters. **(A)** Soft tissues weight. **(B)** Mantle tissue (gonad) weight. **(C)** GCI. Data are means  $\pm$  SD,  $n \geq 8$ . Reference mussels ( $0 \text{ mg L}^{-1}$  tar). Square brackets represent significance differences between mussels exposed to different tar concentrations during same time ( $p < 0.001$ ). Significance differences between mussels exposed to each of the tar concentrations at different times:  $*p < 0.01$ .

follicles was observed, decreasing their size and increasing their number per area (Figures 2B2,C2, 3A,B). The basement membrane of follicles appeared thickened acquiring a hyalinised,

appearance and the germinal epithelium lost its characteristic radial architecture (Figures 2B2,C2). Simultaneously, storage tissue was increased, mainly due to the proliferation and



**FIGURE 2** | Histological overview of the gonad from mussels exposed to different tar concentrations for 10 and 17 days. **(A1,A2)** Reference mussels ( $0 \text{ mg L}^{-1}$  tar) showing a typical pre-spawn stage. **(B1)** Spawn induction. **(B2)** Atrophy of gonadal follicles in mussels exposed to  $10 \text{ mg L}^{-1}$  tar. **(C1)** Sloughing of spermatids. **(C2)** Loss of radial architecture of germinal epithelium and hyalinisation of basement membrane of follicles in mussels exposed to  $40 \text{ mg L}^{-1}$  tar. **(D1)** Disappearance of advanced germ cells. **(D2)** Virtually empty gonadal follicles with hyperchromatic spermatocytes and proliferation of storage tissue cells in mussels exposed to 60 and  $80 \text{ mg L}^{-1}$  tar. **(D3)** Gametogenesis reactivation in mussels transplanted to non-toxic conditions for 17 days after exposition to  $60 \text{ mg L}^{-1}$  tar. Haematoxylin and eosin stain. Scale bar: 500  $\mu\text{m}$  (**A1,B1**), 200  $\mu\text{m}$  (**B2-D3**).



hypertrophy of VC cells (Figures 3C–E). The mussels exposed to 60 mg L<sup>-1</sup> tar for 10 or more days showed similar histological morphometric parameters to these described in mussels exposed to 10 and 40 mg L<sup>-1</sup> tar for 17 days, but in a more obvious way (Figures 3A–E). Besides, these individuals showed virtually empty gonadal follicles, devoid of advanced germ cells (spermatids and spermatozooids), with scarce and hyperchromatic spermatocytes, and staining of the storage tissue seemed cloudy and diffuse (Figures 2D1,D2). Concerning to immune cells, all concentrations of tar assayed

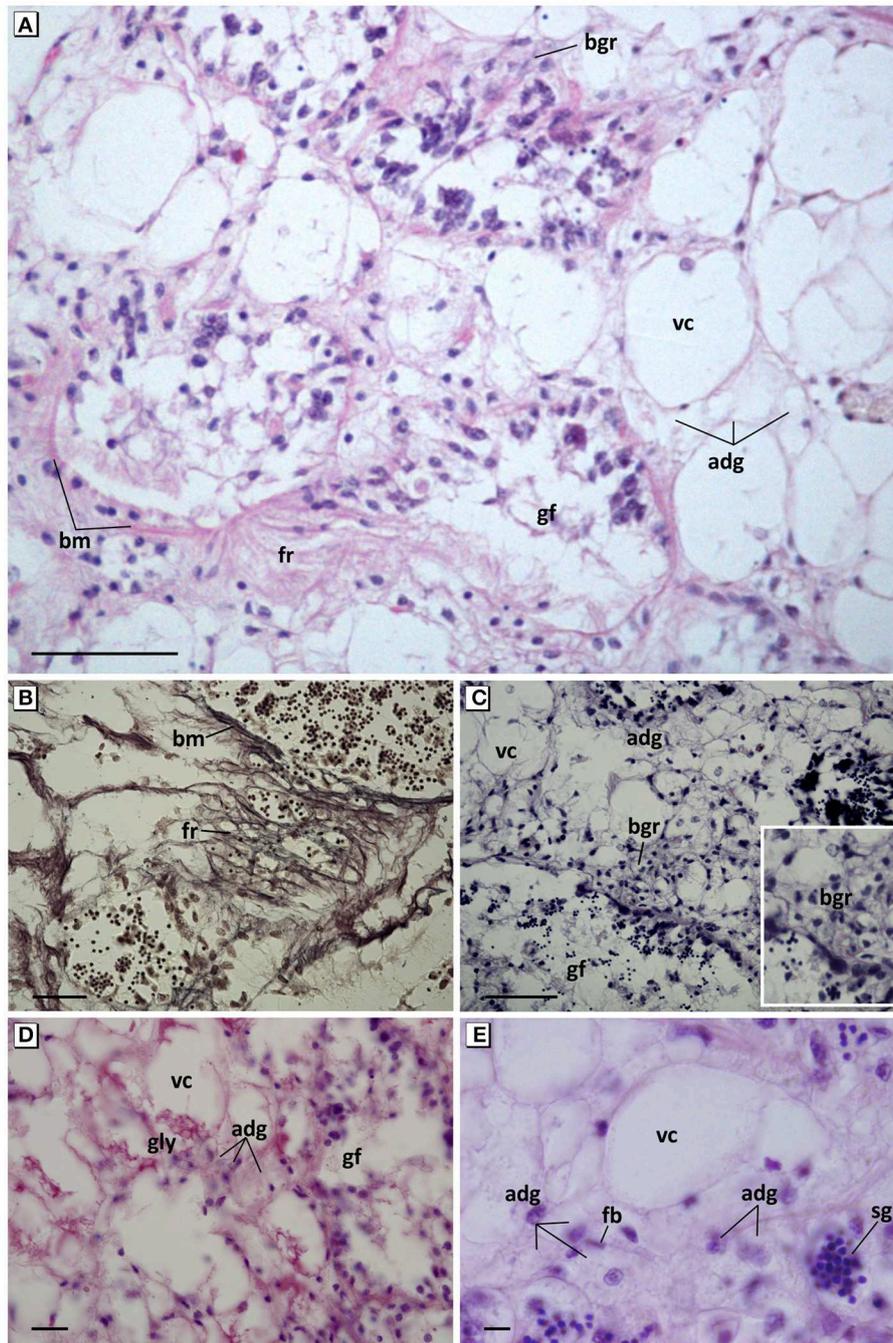
caused a significant decrease of eosinophilic granulocytes ( $p < 0.001$ ; Figure 3F), but aggregates of basophilic granulocytes were observed in the mussels exposed to 60 mg L<sup>-1</sup> tar (Figure 3G). More than 80% of the individuals exposed to each one of the tar concentrations assayed showed the histological alterations described.

### Cellular Pathological Features

The typical aspect of the different cellular types constituting the mantle tissue of *Mytilus galloprovincialis* is shown in

the **Figure S2** (see **Supplementary Materials**, “Mantle tissue of *Mytilus galloprovincialis*”), which includes representative photomicrographs of the reference individuals

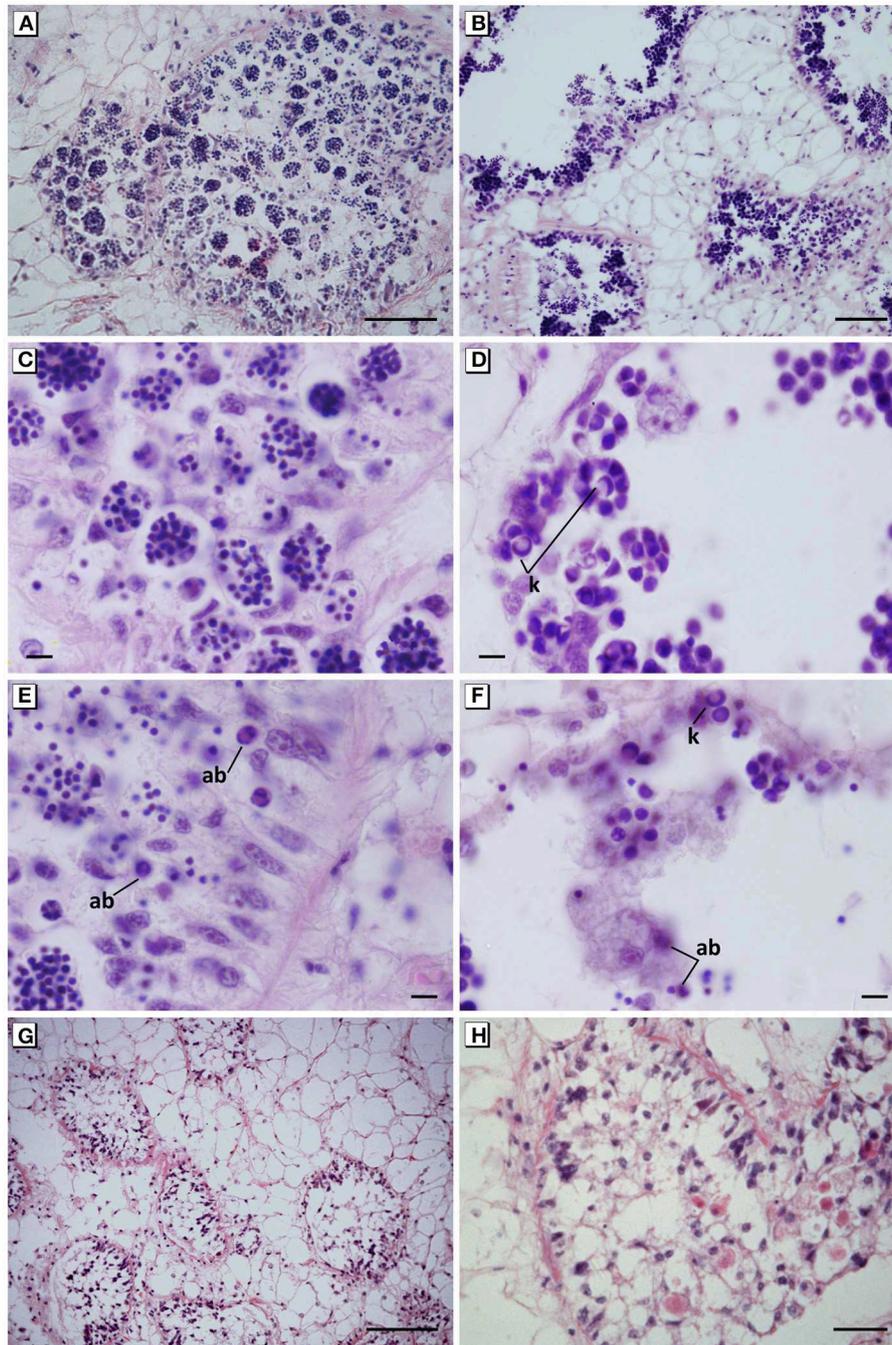
from this work. Exposure of mussels to  $10 \text{ mg L}^{-1}$  tar for 17 days and  $40 \text{ mg L}^{-1}$  tar during 10 days did not cause observable cellular alterations. However, higher



**FIGURE 4** | Pathological features of connective tissue cells. **(A)** Overview of hyperplasia, fibrotic reaction of connective tissue, and basophilic granulocytes. Haematoxylin and eosin stain. **(B)** Detail of reticulin fibers and the thickening and break of the basement membrane of the gonadal follicles. Gomori methenamine-silver nitrate stain. **(C)** Hypertrophy and hyperplasia of VC and ADG cells; appearance of cluster of basophilic granulocytes around the gonadal follicles. Aniline blue stain. **(D)** Decrease of accumulated glycogen in VC cells and increase of small granules in ADG cells. PAS stain. **(E)** Foamy aspect with refringent microvesicles and loss of eosinophilia of ADG cells. Haematoxylin and eosin stain. fr, Fibrotic reaction; fb, fibroblast; bm, basement membrane; adg, adipogranular cells; vc, vesicular cells; gly, glycogen; bgr, basophilic granulocytes; sg, spermatogenic granuloma invading connective tissue; gf, gonadal follicle. Scale bar:  $50 \mu\text{m}$  (**A,C**),  $25 \mu\text{m}$  (**B**),  $10 \mu\text{m}$  (**D,E**).

concentrations and longer exposure times induced cellular disorders of increasing intensity and malignancy in 75–82% of individuals.

After treatment with  $40 \text{ mg L}^{-1}$  of tar for 17 days and with  $60 \text{ mg L}^{-1}$  during 10 or more days, substantial changes were observed in somatic cells from the storage tissue.



**FIGURE 5 |** Maturation arrest of the germinal cells and spermatogenesis failure. **(A,C)** Disorganisation of the germinal epithelium and abnormal aggregates of hyperchromatic spermatids in mussels exposed to  $40 \text{ mg L}^{-1}$  for 17 days. **(B,D)** Disorganisation of the germinal epithelium and abnormal aggregates of apoptotic spermatocytes showing karyorrhexis in mussels exposed to  $60 \text{ mg L}^{-1}$  for 10 days. **(E)** Hypertrophy and hyperplasia of auxiliary cells (Sertoli-like cells) with an intense phagocytic activity of apoptotic spermatids in mussels exposed to  $40 \text{ mg L}^{-1}$  tar for 17 days. **(F)** Auxiliary cells in the lumen follicular engulfing apoptotic spermatocytes in mussels exposed to  $60 \text{ mg L}^{-1}$  for 10 days. In all cases, a total absence of spermatozoa in mussels was evident. Haematoxylin and eosin stain. ab, Apoptotic bodies; k, karyorrhexis. Scale bar:  $100 \mu\text{m}$  **(B,G)**,  $50 \mu\text{m}$  **(A)**,  $25 \mu\text{m}$  **(H)**,  $10 \mu\text{m}$  **(C-F)**.

The amorphous extracellular matrix increased and collagen synthesis by fibroblasts and myofibroblasts was stimulated in a possible fibrotic reaction. The fibrosis originated entailed the hyalinisation of tissue, mainly in the basement membrane of the gonadal follicles, which appeared thickened and occasionally broken (**Figures 4A,B**). The VC cells displayed hypertrophy and hyperplasia (**Figures 3C,D, 4**). Their size and number increased as the accumulated glycogen decreased, and their nucleus acquired a rounded shape and a more central position (**Figure 4**). The ADG cells changed their morphology, their size and number also increased abnormally, and their cytoplasm lost its eosinophilic character, acquiring a foamy aspect with refringent microvesicles, probably lipidic, and small PAS-positive granules (**Figure 4**). As cited above, the number of eosinophilic granulocytes decreased significantly in all mussels exposed to tar. Nevertheless, in individuals exposed to 60 mg L<sup>-1</sup>, the occurrence of another type of granulocytes with a small and slightly basophilic cytoplasm, clustered around the gonadal follicles was observed (**Figure 4C**).

In gonadal follicles, a disorganisation of the germinal epithelium was observed, with loss of germline and total absence of spermatozoa, manifesting a maturation arrest and spermatogenesis failure (**Figures 5A,B**). In the most of the follicles from mussels exposed to 40 mg L<sup>-1</sup> tar for 17 days, the only germinal cells present were abnormal aggregates of spermatids showing a large number of hyperchromatic nuclei immersed in one lightly eosinophilic cytoplasm, likely due to an incomplete cytoplasmic division (**Figures 5A,C**). In many cases, these aggregates filled the follicular lumen, and occasionally were seen invading the connective storage tissue (**Figure 4E**), like the spermatid granulomas described in vertebrates (Creasy et al., 2012; Greaves, 2012). Follicles from mussels exposed to 60 mg L<sup>-1</sup> tar for 10 days only contained spermatocytes, sometimes also forming aggregates, many of them with condensed chromatin in a peripheral position, evidencing fragmentation of the nucleus (karyorrhexis) and apoptotic processes (**Figures 5D,F**). In both cases, apoptotic bodies (fragments intensely stained by haematoxylin within a round mass of intensely eosinophilic cytoplasm) of spermatids or spermatocytes were observed into the cytoplasm of auxiliary cells. These cells (Sertoli-like cells) showed hypertrophy and hyperplasia, and their nucleus changed their original basal position elongating toward the follicular lumen perpendicularly to the follicular membrane. Their cytoplasm appeared swollen and separated from the membrane until remain free in the follicular lumen, showing an intense phagocytic activity of apoptotic germ cells (**Figures 5E,F**). By contrast, the phagocytic response of haemocytes was minimal and very few eosinophilic granulocytes were observed in the follicular lumen. Moreover, Sertoli cells with pleomorphic aspect and swollen and vacuolated cytoplasm filled a large number of gonadal follicles (**Figures 5G,H**). These morphological and positional changes of Sertoli cells hindered their quantification.

The most severe disorders caused by the tar mixture on the germinal cells were observed in mussels exposed to 60 mg L<sup>-1</sup> for 17 days. In these mussels, auxiliary (Sertoli-like) cells were not observed, or their number decreased drastically. In most individuals, the gonadal

follicles were virtually empty, showing large mononucleate and multinucleated germinal cells with prominent and irregular hyperchromatic nuclei (pyknosis), karyorrhexis, and karyolysis (**Figure 6A**). The gonadal follicles of some others individuals (30% of mussels) showed microcytic germ cells with hyperchromatic irregular nuclei, clear cytoplasm and blurred edge, forming encapsulated nodules which also invaded the connective storage tissue. These encapsulated nodules showed fibrosis and deposits PAS-positive, evidencing glycogen accumulation (**Figures 6B–D**).

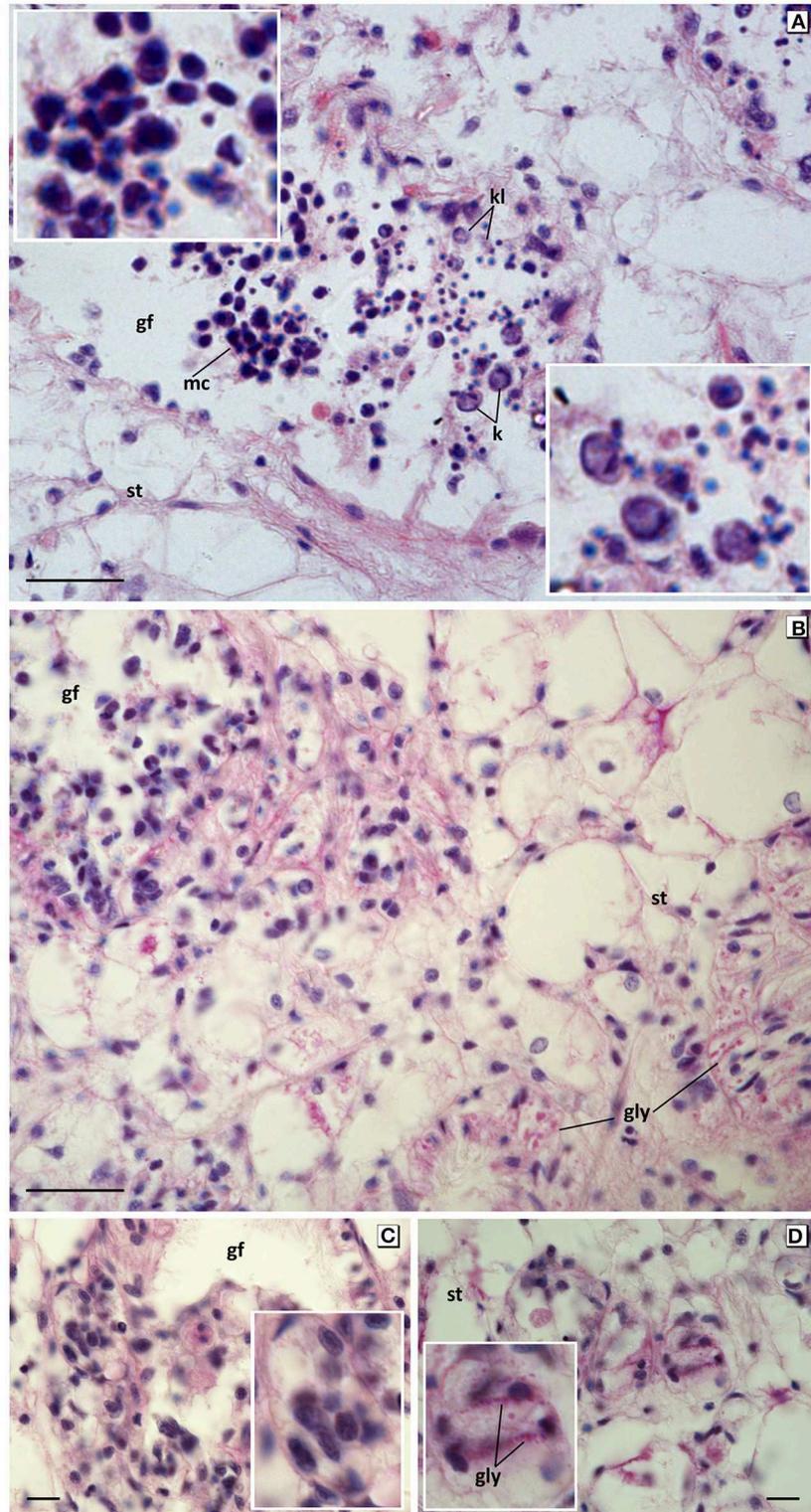
## Depuration Assay

The mussels transplanted to non-toxic conditions for 2 weeks after exposure to WAF from 60 mg L<sup>-1</sup> tar for 17 days, showed signs of an incipient reversion of the above-described disorders (**Figure 7**). The mantle weight and the GCI were maintained at similarly low values (**Figure 1**). However, a significant increase of follicular area took place, decreasing the number of the gonadal follicles per area (**Figures 2D3, 3A,B**). Spermatogenesis was reactivated, while the number of vesicular and adipogranular cells decreased significantly ( $p < 0.001$ ; **Figures 2D3, 3C–E**). Furthermore, aggregates of basophilic granulocytes disappeared at the same time as eosinophilic granulocytes increased significantly ( $p < 0.001$ ; **Figures 4E,G**). The appearance of the storage connective tissue cells was similar to those in the reference individuals, and the ADG cells started again to acquire their characteristic eosinophilia; although the VC cells area remained high and their round nucleus indicated a low glycogen accumulation. However, disorganisation and discontinuity of the germinal epithelium and some disorders of germinal cells, as aggregates of multinucleated and pyknotic spermatocytes were still evident (**Figures 2D3, 7**).

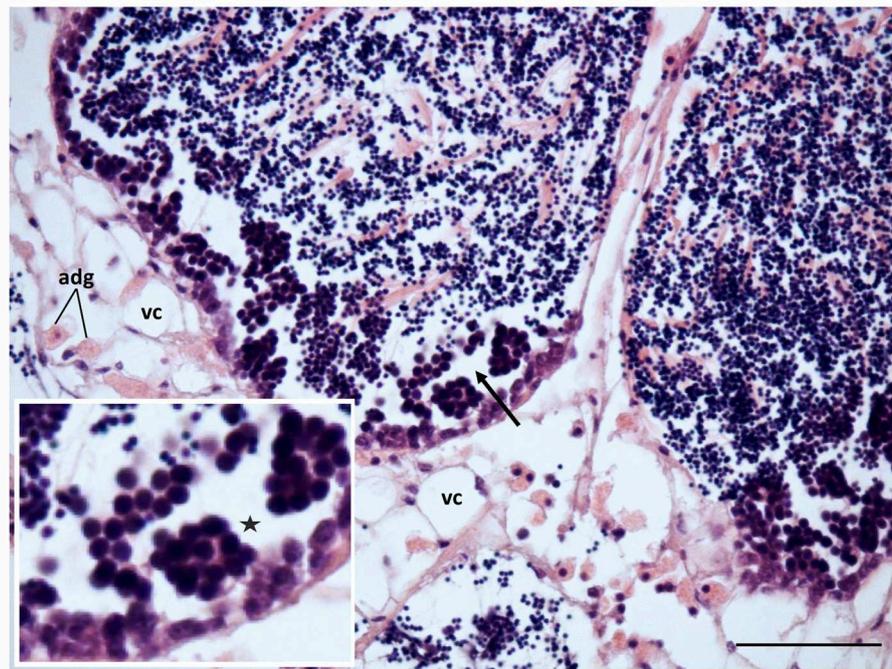
## Multivariate Analysis of the Quantitative Parameters

The Pearson correlation analysis identified a high collinearity ( $r^2 > 0.95$ ) between the PAHs and metals accumulation, and the other hand between the weight of soft tissues and mantle, GCI and follicular area. So, in the Hierarchical Cluster and DistLM analysis, the data of metals accumulation, the weight of soft tissues and mantle and follicular area were not considered.

Cluster analysis showed two significantly different groups. The first group comprised two subgroups: one included reference mussels and the other those exposed to low and medium tar concentrations for 10 days. The second group included the mussels exposed to the highest concentration for 10 and 17 days into a subgroup, and those exposed to low and medium concentrations for 17 days in other. The mussels transplanted to non-toxic conditions for 2 weeks (depuration assay) were included in the second group, although at a considerable distance of mussels exposed to medium and high doses tar. The best variables combination determined by DistLM analyses excluded the GCI, and the first two dbRDA axes explained the 87.7% of the fitted variability (**Figure 8**).



**FIGURE 6** | Apoptotic and degenerative disorders in germinal cells from mussels exposed to  $60 \text{ mg L}^{-1}$  tar for 17 days. **(A)** Large mononucleate and multinucleated germinal cells with big and irregular pyknotic nuclei (top inset), karyorrhexis and karyolysis (bottom inset). Haematoxylin and eosin stain. **(B–D)** Microcytic germ cells with hyperchromatic irregular nuclei, clear cytoplasm, and blurred edges forming encapsulated nodules, with deposits PAS positive in gonadal follicles and invading the connective tissue. PAS stain. Gf, Gonadal follicle; st, storage tissue; gly, glycogen; k, karyorrhexis; kl, karyolysis. Scale bar:  $25 \mu\text{m}$  **(A,B)**,  $10 \mu\text{m}$  **(C,D)**. Insets: 2x magnification in base image.



**FIGURE 7** | Gametogenesis reactivation and recovery of the connective tissue in mussels transplanted to non-toxic conditions after exposition to  $60 \text{ mg L}^{-1}$  tar for 17 days. adg, Adipogranular cells; vc, vesicular cells; discontinuity in the germline (\*), and aggregates of large and pyknotic spermatocytes (arrow) can still be observed. Scale bar:  $50 \mu\text{m}$ . Inset: 2x magnification in base image.

## DISCUSSION

Toxicological studies have demonstrated that hydrocarbons disrupt multiple endocrine pathways affecting hormonal regulation, metabolism and reproduction, and that several congeners have mutagenic effects contributing to carcinogenesis (Creasy and Foster, 2002; DeFur, 2004; Oetken et al., 2004; Pufulete et al., 2004; Dietrich and Krieger, 2009; Sanders et al., 2009; Balabanič et al., 2011; Creasy et al., 2012; De Falco et al., 2015).

Heavy metals have also been related to cellular injury, metabolic disorders, and DNA and proteins damage, that may lead to cell and reproductive cycle disturbance, carcinogenesis, or apoptosis (Gerber et al., 1980; Ronis et al., 1996; Wang and Shi, 2001; Tchounwou et al., 2004, 2012; Beyersmann and Hartwig, 2008; El-Sayed and El-Neweshy, 2010; Obiakor et al., 2010; Ebrahimi and Taherianfard, 2011). Although in invertebrates the number of these studies is lower, and these organisms present different susceptibility to pollutants, several authors have suggested similar effects and mechanisms than that of vertebrates and have propose them as models in endocrine disruption research (Viarengo et al., 1990; Bolognesi et al., 1999; Matozzo et al., 2001; DeFur, 2004; Porte et al., 2006; Zhou et al., 2009; Fatima et al., 2014).

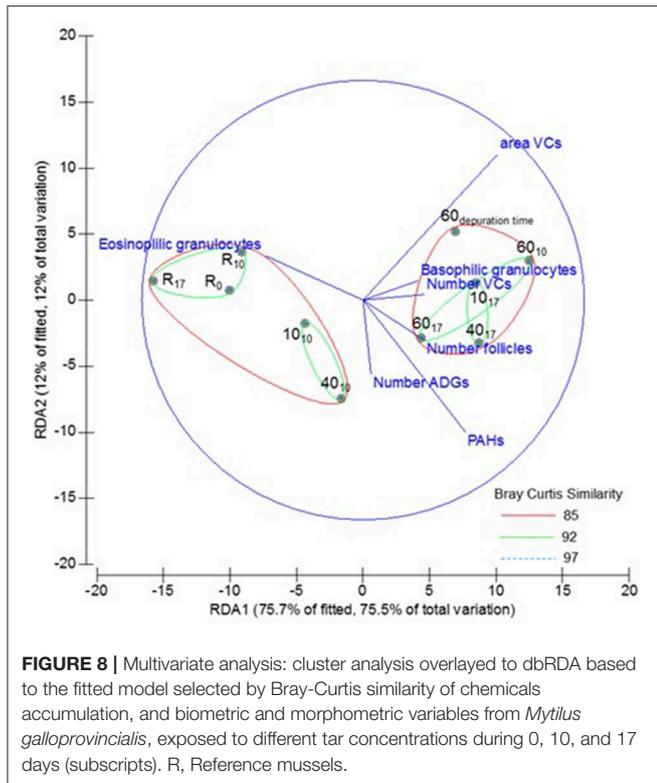
### PAHs and Metals Accumulation

Applying, where it was necessary, the conversion suggested by OSPAR (2005) to compare data given in dry or fresh weight,

the PAHs, Mn, Pb, and Zn accumulated in mussels in this study are within the range found by other authors in *Mytilus* spp, and are similar to found by our team in mussels from the Ría of Vigo (Broman et al., 1990; Baumard et al., 1998; Piccardo et al., 2001; Besada et al., 2002; O'Connor, 2002; Webster et al., 2006; Monteduro et al., 2007; Bartolomé et al., 2010; Ruiz et al., 2011). Compared to the composition of the tar used, the variation of hydrocarbons and metals in the mussels exposed to WAF of different concentrations of tar seems to indicate that PAHs with a larger number of benzene rings, Zn and Pb are more efficiently accumulated. On the other hand, the decrease of these species of PAHs and metals in the mussels after depuration for 2 weeks suggest their faster elimination, like it is also appointed by other authors (Phillips, 1976; George, 1980; Rainbow, 2002; Glad et al., 2017). These facts seem to reflect the specific metabolism of *Mytilus* and the complex relationship between bioavailability, accumulation, biotransformation, and excretion of these compounds.

### Gonadal and Histological Disorders

In the present study, cumulative gonadal disorders were observed in male mussels concerning both tar exposure and accumulation of PAHs and metals. In molluscs, similar disorders have been related to PAHs and metals pollution in wild environments (Pridmore et al., 1990; Viarengo et al., 1990; Bolognesi et al., 1999; Alonso et al., 2001; Tay et al., 2003; Aarab et al., 2004; Smolders et al., 2004; Jing-Jing et al., 2009; Yurchenko and Vaschenko, 2010; Ortiz-Zarragoitia et al., 2011; Ruiz et al.,



2011, 2013; Smolarz et al., 2017). However, this is the first study demonstrating that tar used in the waterproofing of the floating rafts from mussel farms has a direct causal relationship with the histopathological alterations observed in male mussel gonad, which affect the reproductive function, the associate storage tissue metabolism and the immune response.

The germinal epithelium of male gonad and the spermatogenesis process are, in general terms, astonishingly similar in even very different animals, and their control mechanism is also highly conserved (Roosen-Runge, 1977; Working, 1988; Bonilla and Xu, 2008; Cheng and Mruk, 2010; White-Cooper and Bausek, 2010). However, there are scarce studies about cellular disorders and pathologies of this process in invertebrates, contrarily to in vertebrates. In the latter, the testis is a known target of PAHs and metals toxicity, experiencing disruption of spermatogenesis, degeneration, and loss of germ cell, and testicular atrophy (Ronis et al., 1996; Adhikari et al., 2001; Creasy and Foster, 2002; Dietrich and Krieger, 2009; El-Sayed and El-Neweshy, 2010; Mathur et al., 2010; Ebrahimi and Taherianfard, 2011; Creasy et al., 2012; Chandel and Jain, 2014).

In this study, the first histological effect of exposure to tar in mussels was the spawning induction, like described in other invertebrates (Tay et al., 2003; Aarab et al., 2004; Jing-Jing et al., 2009; Ortiz-Zarragoitia et al., 2011). However, as tar concentration and exposure time increases, the effect observed was a gonadal atrophy, manifested as a significant decline of the GCI and a drastic reduction of gonadal follicles size.

In vertebrates, diameter reduction of seminiferous tubules is associated with loss of germ cells and constitutes a quantitative index of testicular injury (Creasy and Foster, 2002; Richburg et al., 2002; Dietrich and Krieger, 2009).

## Cell Injuries

As dose and exposure time increased, pathological disorders in different cell types were observed. The study of these cellular alterations could subsequently shed light on cellular target of tar in the gonad of molluscs, as well as on the role of these cells in the spermatogenesis regulation of these organisms.

In addition to spawning induction, exposure to 40 mg L<sup>-1</sup> tar for 10 days provoked a premature release or sloughing of immature spermatids, which resulted in cellular disorganization of germinal epithelium and loss of its centripetal radial architecture, as has also been described in others molluscs from polluted environments (Yurchenko and Vaschenko, 2010). However, higher concentrations of tar and longer exposure times originated retention of abnormal aggregates of spermatids or spermatocytes with signs of degeneration and apoptosis, which occasionally were seen invading the connective storage tissue, like some spermatid granulomas described in vertebrates (Creasy et al., 2012; Greaves, 2012). These aggregates, have been termed multinucleated giant cells in several organisms where were related with disturbances of Sertoli cells and with the arrest of spermatogenesis and spermiogenesis (Creasy and Foster, 2002; De Falco et al., 2015). Among functions of Sertoli cells are the paracrine signalling, regulation of the material passing to gametes developing, structural support of spermatogenesis and release of the mature spermatids (Creasy and Foster, 2002; França et al., 2015). These functions are accomplished through junctional complexes maintained with the developing germ cells. Several toxicants, including hydrocarbons, disturb these junctions, leading to spermatogenesis failure (Richburg et al., 2002; Salian et al., 2009; Cheng, 2014).

As described above, in mussels exposed to 40 mg L<sup>-1</sup> tar for 17 days and to 60 mg L<sup>-1</sup> tar for 10 days, auxiliary or Sertoli-like cells proliferated abnormally, their aspect changed acquiring a swelled and vacuolated cytoplasm, and basal position of their nucleus was modified. Similar injuries have been described in Sertoli cells of vertebrates caused by toxicants and related to rapid and massive degeneration of germ cells, which suggest disruption of their structural and metabolic function (Creasy and Foster, 2002; Dietrich and Krieger, 2009; Cheng, 2014). Another function of Sertoli cells is phagocytosis and removal of residual sperm and degenerating germ cells (Boekelheide et al., 2000; França et al., 2015). This function seemed highly induced in these mussels, in which an inordinate phagocytic activity of apoptotic germ cells was observed, as also has been described in fish (Dietrich and Krieger, 2009). So, the Sertoli cells hyperplasia could be both a response to counteract germinal cells degeneration through their phagocytic capacity, as also the result of a direct endocrine disruptor effect of tar. Moreover, similar as we observed in a large number of follicles from these mussels, seminiferous tubules full of pleomorphic and vacuolated Sertoli cells have been described in neoplastic and degenerative processes of these cells from vertebrates (Henley et al., 2002;

Vegter et al., 2010). According to this latter idea, the absence of Sertoli cells in mussels exposed to 60 mg L<sup>-1</sup> tar for 17 days seems to support their possible degeneration. The diminution or absence of Sertoli cells cause deregulation of germ cells and their most severe disorders, getting to form encapsulated nodules invading the connective tissue, similar to *in situ* germinal carcinomas described in vertebrates (Guminska et al., 2010).

The storage cells from interstitial connective tissue (VC and ADG cells) of the mussels exposed to 40 mg L<sup>-1</sup> of tar mixture for 17 days and to 60 mg L<sup>-1</sup> during 10 or more days showed pathological hyperplasia, hypertrophy and appearance changes compared to those of the reference mussels. These morphologic changes seem to suggest an alteration of their biochemical components and their function. In *Mytilus* spp, the VC cells function is the glycogen storage. These cells contain only a large glycogen vacuole, some mitochondria and a limited metabolic activity. However, in this work, the hyperplasia and hypertrophy of these cells as a consequence of tar exposition was parallel to depletion of glycogen store. Glycogen is the main energetic reserve in adult bivalves, and several authors have also reported its decrease as a result of oil exposure, limiting the energy available for growth and reproduction (Pridmore et al., 1990; Smolders et al., 2004). On the other hand, the ADG cells contain mainly lipid droplets and protein granules and present a high metabolic activity that varies during the gametogenic cycle under hormonal control (Pipe, 1987; Peek et al., 1989). Several authors identify the ADG cells with Leydig cells, but although there are hints on their possible role in steroidogenesis, their function (trophic and/or regulatory) has not yet been fully clarified (Croll and Wang, 2007; Lafont and Mathieu, 2007). In Leydig cells from vertebrates, similar disorders to those we described in the ADG cells from mussels in this work have been identified as pathologic: abnormal enlargement, changes in shape, and internal structure and foamy appearance of cytoplasm due to proliferation of lipid droplets (lipidosis) (Creasy and Foster, 2002; Dietrich and Krieger, 2009; Cheng, 2014). Lipidosis of Leydig cells has been related to their degeneration and with disturbance of their function (Chung et al., 2011; Oh, 2014). The Leydig cells function is the biosynthesis of testosterone, which indirectly regulates spermatogenesis in a complex cascade of chemical interactions with Sertoli and germ cells, resulting in feedback regulation of testosterone synthesis. So, disorders of Sertoli cells, such as those we described above, not only disrupt the interactions with germ cells causing the spermatogenesis failure, but also the interactions with Leydig cells, inhibiting testosterone production, which in turn induces germ cell degeneration (Creasy and Foster, 2002; Boekelheide, 2005). The sequence of disorders that we described in this work seems to point to these complex interrelations. So, the auxiliary cells of mussels, like the Sertoli cells of vertebrate, could be one of the primary targets of PAHs toxicity, whose injuries could induce sloughing of immature germ cells, as well as disturb the possible steroidogenic function of the ADG cells. Moreover, the ADG and germinal cells could also constitute secondary targets of these compounds, inducing the degeneration and the severe injuries we described in germ cells.

Fibrosis is defined as the abnormal proliferation of fibroblast resulting in an increased synthesis and deposition of collagen,

which confers a thickened appearance of the connective tissue. This phenomenon is associated to diverse pathologies as degenerative lesions and tumours coursing with inflammatory processes. In the connective tissue lining seminiferous tubules of the vertebrate testis, fibrosis has been equally described associated with injuries and degeneration of germ cells but also following exposure to endocrine disruptors (Creasy and Foster, 2002; Albrecht et al., 2006; Dietrich and Krieger, 2009). The collagen synthesis stimulation in the fibrotic reaction is mediated by the release of fibroblast growth factors from paracrine and/or tumoral cells, whose induction has been related to estrogens and oxidative stress (Walker, 2001; Albrecht et al., 2006; Dietrich and Krieger, 2009; Gómez-Sámano et al., 2017). This process is considered to be a scarring reaction aimed to maintain the structure of the tissue after degenerative injuries (Albrecht et al., 2006; Dietrich and Krieger, 2009). However, the estrogens-induced fibrosis in reproductive tract seems to suggest a different function related to the testicular development and differentiation (Watanabe and Onitake, 1995; Dietrich and Krieger, 2009; Jiang et al., 2013). Also in the gonad of bivalve molluscs, several authors have reported fibrosis associated to gonadal and haemocytic neoplasms, as well as to hydrocarbons and metals pollution and oxidative stress (Yevich and Barszcz, 1976; Peters et al., 1994; Alonso et al., 2001). In our work, the fibrotic reaction observed around gonadal follicles, related to spermatogenesis arrest and the severe disorders that we described, could be caused jointly by possible xenoestrogenic effects of tar and by the oxidative stress and cell damage generated, as described in other organisms.

The immune response is responsible for recognition, oxidation, and phagocytosis of non-self particles. The bivalve immune system is a non-specific defence mechanism known as the innate immune system, which is yet not fully understood. This is a primitive mechanism, evolutionarily conserved, triggered by intracellular molecular signalling cascades (Danilova, 2006; Song et al., 2010). The haemocytes activity from bivalves is not limited to immune response, but they are involved in different physiological processes including tissue repair, shell production, nutrition, and response to environmental and biological stresses (Song et al., 2010). The lineage of bivalve haemocytes is not clear. Several authors have suggested their origin from two different cells, precursors of granulocytes, and hyalinocytes, respectively. However, other authors indicate a single type of precursor cell of hyalinocytes, which would mature to granulocytes (Cheng, 1984; Hine, 1999). Moreover, the different types of granulocytes identified in bivalves (basophils and eosinophils) could be the result of subsequent differentiation and maturation processes (Donaghy et al., 2009).

The bivalve haemocytes seem particularly susceptible to environmental pollution by chemical or biological toxics. However, the bibliographic data are sometimes contradictory because several authors describe an increase of haemocytes number in bivalves exposed to oil spills, whereas others report their decrease (Pichaud et al., 2008; Hannam et al., 2009). Moreover, it has been described that innate immune response in bivalves is accomplished mainly by granulocytes, while hyalinocytes are the main responsible of tissues repair, requiring aggregation at a point of injury (Ruddell, 1971;

Hégaret et al., 2003). According to these assertions and with studies above cited, our results show firstly an evident decrease of the number of eosinophilic granulocytes in mussels undergoing at all tar concentrations and exposition times assayed, evidencing immunosuppression induced by treatment, although no infection was observed. Secondly, in mussels exposed to the highest tar concentration, which developed the more severe injuries, we observed an increase of basophilic granulocytes forming aggregates around the affected follicles. These data suggest that the immunotoxic effects of PAHs are dependent on concentration and exposure time, resulting in either an increase or decrease of different types of granulocytes, whose specific functions should be addressed in later works. On the other hand, the significant decrease of eosinophilic granulocytes induced by tar intoxication in mussels would explain the absence of these phagocytic granulocytes containing degradation products described by others authors as brown cells (Puy-Azurmendi et al., 2010; Bignell et al., 2011).

## Depuration Assay

Several works conclude that hormonal disturbance effects may be reverted as the xenoendocrine disrupters disappear, but it is known that processes of cell death are irreversible (Creasy and Foster, 2002; Creasy et al., 2012). In our study, the mussels transplanted to non-toxic conditions after the toxicity assay showed recovery signs of all cellular injuries.

Hydrocarbons, metals, and other chemicals exert direct effects on the function of Leydig and Sertoli cells in vertebrates, causing, among other, inhibition of testosterone synthesis, cholesterol regulation, lipid homeostasis, and insulin signalling (Jeng, 2014; De Falco et al., 2015). Testosterone inhibition, in turn, induces germ cell degeneration (Creasy and Foster, 2002; Boekelheide, 2005). In our work, the connective tissue cells (VC and ADG cells) from mussels transplanted to depuration conditions, recovered their appearance. So, the alterations described in these cells could be due to a direct xenoestrogenic effect or mediated by endocrine disruption of auxiliary cells. In these mussels, the germinal line was also partially restored, and no signs of apoptosis and degeneration were observed. In vertebrates it has been demonstrated that spermatogonial precursor cells are relatively resistant to injurious stimuli (Creasy and Foster, 2002; Creasy et al., 2012). So, in our study, some spermatogonial stem cells would have remained intact to allow resuming the gametogenesis. Nevertheless, due to the short time of transplant to non-toxic conditions, several disorders, as germinal epithelium discontinuity and aggregations of multinucleated and hyperchromatic spermatocytes were still evident.

As described in other bivalve species (Mayrand et al., 2005), we also observed granulocytes number is recovered, but we have not enough data to know if their phagocytic capacity is recovered too, neither if animals become more sensitive to stress and pathogens. Non-cellular defence responses could also be involved in this granulocytes number recovery as described in some bivalves (Charlet et al., 1996; Roch, 1999; Ortiz-Zarragoitia and Cajaraville, 2006). However, these studies are

still scarce to understand the integrated immunomodulation in these organisms.

## CONCLUSIONS

The chemicals accumulation, and biometric and morphometric analysis of the mussel male gonad presented in this study indicates quantitative specific patterns of change after exposure to tar as corroborated by multivariate analysis (concentration of PAHs accumulated; number of gonadal follicles, haemocytes, and connective interstitial cells; area of VC cells), which together with the qualitative observations of other histopathological changes (fibrosis, appearance change of the Sertoli, ADG, and germinal cells) could offer a mechanistic understanding of the joint effects of hydrocarbons and metals on reproductive function of invertebrates. This histopathological approach could support further molecular studies in order to clarify the role of the different gonadal cells in the spermatogenesis regulation of invertebrates, and be used in aquatic toxicology.

To our knowledge, this is the first study to demonstrate that tars used in the waterproofing of the floating rafts from mussel farms cause severe effects on male mussel gonad, inducing histopathological alterations, which affect the reproductive function, the associate storage tissue and the immune response. The malignancy of injuries is dependent on the concentration and exposure time and affects to different cell types in a manner comparable to that described in other organisms both vertebrates and invertebrates. So, the spillage of these mixtures around rafts entails a direct pollution risk on the immediate environment of farms, which affect the health of mussels, their reproduction and ultimately, the sustainability of this resource and its quality for human consumption.

Our findings suggest that the histopathological changes produced by the combined effects of heavy metals and PAHs tar that we described could be used as biomarkers in the monitoring of marine pollution and quality of aquaculture areas. Also, the mussel gonad could be considered as an excellent model in studies about the testicular toxicity mechanisms, disruption of spermatogenesis and metabolism in male reproduction.

## DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

## AUTHOR CONTRIBUTIONS

AA, YR, and FS designed the study. AA, PS, YR, and VD performed the data collection and wrote the draft of the manuscript. AA performed the histological and microphotographic analysis. PS performed the statistical analysis. AA, PS, YR, VD, and FS contributed to the interpretation of the results. PS and FS contributed to the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2019.00577/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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