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Proteomic Investigation of Male *Gammarus fossarum*, a Freshwater Crustacean, in Response to Endocrine Disruptors

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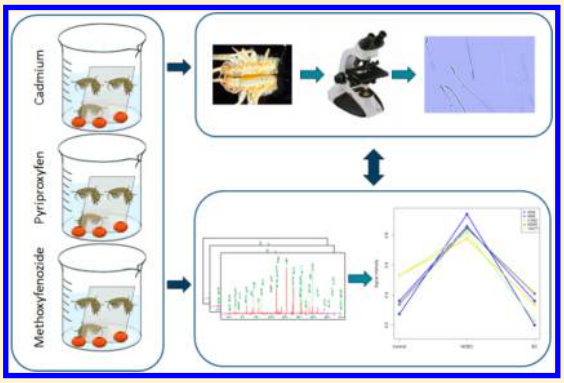
Supporting Information

ABSTRACT: While the decrease in human sperm count in response to pollutants is a worldwide concern, little attention is being devoted to its causes and occurrence in the biodiversity of the animal kingdom. Arthropoda is the most species-rich phyla, inhabiting all aquatic and terrestrial ecosystems. During evolution, key molecular players of the arthropod endocrine system have diverged from the vertebrate counterparts. Consequently, arthropods may have different sensitivities toward endocrine disrupting chemicals (EDCs). Here alteration of sperm quality in a crustacean, *Gammarus fossarum*, a popular organism in freshwater risk assessment, was investigated after laboratory exposure to various concentrations of three different xenobiotics: cadmium, methoxyfenozide, and pyriproxyfen. The integrity of the reproductive process was assessed by means of sperm-quality markers. For each substance, semiquantitative/relative proteomics based on spectral counting procedure was carried out on male gonads to observe the biological impact. The changes in a total of 871 proteins were monitored in response to toxic pressure. A drastic effect was observed on spermatozoon production, with a dose−response relationship. While exposure to EDCs leads to strong modulations of male-specific proteins in testis, no induction of female-specific proteins was noted. Also, a significant portion of orphans proved to be sensitive to toxic stress.



KEYWORDS: *ecotoxicology, crustacean, shotgun proteomics, expression profile, reproduction, endocrine disruption*

INTRODUCTION

Because spermatozoa perform the unique and critical function of propagating DNA for the development of the next generation, the global decline in human sperm count observed over the past half century is of great concern,[1] with environmental contamination being particularly incriminated. Compared with oocytes, spermatozoa are highly sensitive to xenobiotics due to their lack of defensive systems such as DNA repair or antioxidant enzymes.[2] Male reproductive disorders have been reported for several vertebrate phyla, as exemplified by testicular abnormalities and impaired fecundity in U.S. Lake Apopka alligators[3] or by feminized fish found downstream of a wastewater treatment plant in the U.K.[4] Among all of the contaminants that are able to alter sperm production, endocrine-disrupting chemicals (EDCs), especially estrogenic-like or anti-androgen-like compounds, are diverse and abundant in the environment.[5]

As highlighted by Lewis and Ford,[6] invertebrate organisms have been considerably less well studied than vertebrates despite their ecological importance. Arthropods (i.e., insects, crustaceans, and arachnids) make up >80% of all described animal species,[7] inhabiting all aquatic and terrestrial environments. There is a patent lack of knowledge regarding endocrine regulation of reproduction in small aquatic invertebrates and in ecotoxicologically relevant species, such as amphipods, mysids, and daphnids. As reported by Lye et al.,[8] some evidence suggests that crustaceans are less vulnerable to known vertebrate EDCs, such as those that mimic estrogen compounds, compared with other invertebrates, including echinoderms and mollusks. The hormonal regulation of arthropods is not based on vertebrate-like steroids but on ecdysteroids, juvenoid hormones (JH), and for males, the androgenic gland hormone.[9] This high divergence of endocrine systems suggests that possible invertebrate-targeted EDCs are different and exhibit a more diverse chemical nature than vertebrate EDCs. In arthropod pest control management, some pesticides are designed to specifically interact with arthropod hormone nuclear receptors (i.e., ecdysteroids and JH nuclear receptors) through agonist or antagonist action. Thus, these molecules act as endocrine disruptors in a variety of targeted insect species by disrupting embryogenesis and preventing metamorphosis. Different observations suggest that wild

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populations of aquatic invertebrates, especially crustaceans, can be affected as nontarget organisms.[10−12] In the same way, field evidence of alterations of sexual characteristics has been reported, especially in amphipods. For example, Jungmann et al.[13] and Ford et al.[14] collected *Gammarus fossarum* and *Echinogammarus marinus* organisms, respectively, that exhibited intersex phenotypes, that is, the co-occurrence of male and female sexual characteristics. In the same vein, Schirling et al.[15] observed abnormal structures of oocytes in *Gammarus pulex* and *G. fossarum* collected from streams impacted by sewage treatment plant effluents. Crustacean amphipods are popular organisms in ecotoxicological risk assessment and are currently used as sentinel organisms because of their ecological relevance in relation to their key role in the food chain and their contribution to the decomposition process (leaf litter breakdown).[16] In addition to its use in the laboratory, these organisms may also be easily employed in in situ bioassays for sensing bioavailable pollutants and their effects[17] through the assessment of associated toxic effects, that is, induction of detoxification mechanisms,[18] neurotoxicity,[19] or energy acquisition impairments.[20] Furthermore, physiological parameters related to the gammarid reproductive process, that is, spermatogenesis,[21] and to the female reproductive cycle[22] have been well-documented.

To date, no specific markers of exposure to endocrine disruptors were developed for aquatic invertebrates and particularly for amphipods. In recent decades, two main strategies have been employed to improve the assessment of the impact of EDCs on crustaceans. First, bioassays have been proposed for measuring the impact of pollutants on various physiological processes that are under endocrine regulation, such as growth, molting, embryonic development, vitellogenesis, and fertility. In this context, for the water flea, *Daphnia magna* (Crustacea, Cladocera), with parthenogenic reproduction, the offspring sex ratio was proposed as an end point to screen chemicals for juvenoid-related endocrine activity.[23] Geffard et al.[22] also developed a reproductive toxicity test in *G. fossarum*, for which some synchronous physiological events related to reproduction are measured, such as molting cycle, follicle growth, oocyte production, and embryonic development. Geffard et al. proposed to use the specific effect on either molting or follicle growth, and their desynchronization, as an indicator of a potential specific exposure to EDCs. Another strategy has been to rely on the development of molecular markers. However, because of the lack of genomic or proteomic data for aquatic invertebrates, specific and direct methods for the identification and quantification of protein biomarkers are scarce. Until now, biomarkers employed for invertebrate species are rather the result of a direct transposition of assays previously developed and validated in vertebrates, for which molecular biology is better understood.[24] Hence, based on developments in vertebrates, research groups in aquatic ecotoxicology have mainly focused their activities on the measurement of vitellogenin to propose this protein as a specific EDC biomarker in invertebrates. Vitellogenin and vitellogenin-like proteins (Vtg) are precursor molecules of yolk proteins that provide the energy reserves needed for embryonic development in oviparous organisms. In vertebrates, Vtg production is under the control of the estrogen pathway, and its levels are high in sexually mature females, displaying fluctuations during gametogenesis.[25] Conversely, the Vtg content is generally very low in juveniles and males, and in these organisms its induction has been employed as a robust biomarker for diagnosing the presence of estrogen mimetics and their associated effects, that is, feminization.[25] With regard to research performed in fish, Vtg has been monitored in several invertebrate species as a biomarker of EDC exposure,[26] and recently a selected reaction monitoring assay for the quantitation of *G. fossarum* Vtg was developed. Nevertheless, while the Vtg protein in *G. fossarum* females is a reliable indicator of egg quality demonstrating functionality of this protein in the reproductive process,[27] its potential use as an EDC biomarker in males is compromised. Indeed, in laboratory experiments and in a large-scale field experiment using caged organisms, a strong interindividual variability, very low or no induction under contamination exposure, and a strong impact of unidentified environmental factors, were shown.[28] Similar observations were obtained for the marine amphipod, *Echniogammarus marinus*, where expression of the orthologous gene was monitored in females, males, and intersex males.[29] Such findings and phylogenetic analysis of vitellogenin in metazoans[30] highlight the difference between invertebrate and vertebrate Vtgs, demonstrating pleiotropic functions not strictly related to reproduction. These studies underline the need for the specific identification of new key molecular players in gammarid reproductive biology for use as relevant biomarkers in ecotoxicology. We thus recently proposed a proteogenomic strategy for discovering proteins involved in *G. fossarum* reproductive function.[31] A shotgun proteomic analysis was carried out on the proteomes of three major tissues involved in the organism's reproductive function: the male and female reproductive systems and the cephalon. Proteins were identified based on a generic RNA-seq-derived protein sequence database. On the basis of the work of Chen et al.,[32] key proteins with strong sexual dimorphism were listed by performing comparative proteomics between the male and female reproductive organs. Additionally, protein expression profiles over seven spermatogenesis stages were carried out to pinpoint new proteins involved in spermatogenesis, uncovering a substantial proportion of orphan proteins.[31]

The present study aimed to discover potential new biomarkers for the monitoring of reproductive disorders and EDC exposure in male gammarids. In mammals, proteomics showed to be a valuable tool for addressing normal and pathological spermatogenesis,[33,34] and in nonmodel fish, proteomic profiling proved to be a powerful approach for studying endocrine disruption.[35] Thus, we simultaneously investigated, in response to a metal (cadmium, Cd) and two xenobiotics (methoxyfenozide, Met, and pyriproxyfen, Pyr), both the proteome modifications occurring in the male reproductive organ and the alterations in reproductive health by means of sperm-quality parameters. Here we employed a semiquantitative/relative proteomics based on spectral counting procedure as it proved to be a straightforward strategy for characterizing proteome alteration in toxicoproteomics.[36,37] Cd is a nonessential heavy metal that is highly toxic to gammarids and is associated with deleterious effects on iono-osmoregulation,[38] feeding rate, vitellogenesis, and embryonic development.[22] Met and Pyr are potent arthropod EDCs, employed as growth regulators in agriculture, the former targeting ecdysteroid signaling (molt hormone), while the latter interferes with JH signaling. Experiments were conducted using three different concentrations of each pollutant. First, sperm-quality markers in response to toxic exposure were assessed by the numeration of spermatids (i.e., immature reproductive cells) and spermatozoa. For each substance tested,

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on the basis of the measured physiological end points, proteomic investigations were then carried out at two different concentrations, a no observed effect concentration (NOEC) and an effect concentration (EC) in terms of the reproductive physiology, and compared with the corresponding control condition. Protein dynamics over the different concentrations were analyzed for the identification of proteins modulated by toxic exposure. We identified several proteins as candidate biomarkers of reproductive disorders and EDC exposure, predominantly orphans, and we discuss their perspectives in terms of ecotoxicological risk assessment.

EXPERIMENTAL SECTION

Exposure of Gammarids to Toxic Compounds

Gammarids were collected from a river in mid-Eastern France and acclimatized to laboratory conditions as previously described.[31] Males of homogeneous size (8−11 mm) paired with females at D2 molt stages (i.e., hatched juveniles in brood pouches, visible oocytes)[22] were selected and isolated in a 30 L tank. Just after copulation (separation of the amplexus), males at the beginning of spermatogenesis were collected for chemical exposure during two consecutive spermatogenesis cycles (15 days of exposure). Organisms were exposed to cadmium (Cd) (0.3, 1, and 3 $\mu g \cdot L^{-1}$), methoxyfenozide (Met) (0.001, 0.1, and 10 $\mu g \cdot L^{-1}$), and pyriproxyfen (Pyr) (0.5, 5, and 50 $\mu g \cdot L^{-1}$). The three concentration ranges were chosen based on previous work.[22,27,39] Stock solutions for organic compounds were prepared in acetone. Contaminated media were obtained by dilution of stock solutions into uncontaminated, drilled groundwater. Solvent-free (i.e., water) and solvent (i.e., 0.005% acetone) controls were also included. For each condition tested, 21 males of similar size were placed into three 500 mL glass (Cd) or polyethylene (Met and Pyr) beakers (seven animals per beaker) under constant oxygenation. A piece of net (mesh size: 200 μm; 6 × 5 cm) was added into each beaker as a resting surface. Organisms were fed ad libitum with conditioned alder leaves (*Alnus glutinosa*). Exposure media and alder leaves were renewed every 2 days, while water-quality parameters (pH, conductivity, temperature, and dissolved oxygen) and survival were recorded daily. After 5 days of exposure, sexually mature females at D2 stage were added into the beakers (an equal number of females as surviving males). The occurrence of copulation was checked daily. When at least 70% of the males had copulated (Day 7 of the experiment), single females and males still in amplexus were discarded. At the end of the exposure (Day 15 of the experiment), organisms were culled. Male gonads were sampled under stereomicroscopic magnification using fine forceps. The testis of five animals were analyzed by shotgun proteomics, while the remaining organisms (n = 7−10) were culled for the assessment of sperm-quality markers.

Sperm-Quality Indicators

Under stereomicroscopic magnification, gonads were dilacerated with a scalpel, transferred into a microtube containing 30 μL of phosphate-buffered saline (10 mM, pH 7.4 + 0.3% bovine serum albumin), kept on ice, and gently mixed 20 times with a micropipette, as described by Lacaze et al.[40] For spermatozoon numeration, 20 μL of well-mixed spermatozoon suspension was deposited on the surface of a hemocytometer and observed under optic microscopy (Leica DM 2500, magnification ×100). The total number of cells was calculated by multiplying the sperm count per microliter by the volume of sperm suspension. Statistical analyses were carried out with R software.[41] Normality and variance homogeneity were checked using the Shapiro−Wilk and Bartlett tests, respectively. Intergroup comparisons were then performed by the ANOVA test and the post hoc Tukey HSD test for parametric conditions as well as the Kruskal−Wallis test and associated post hoc test for nonparametric conditions.

Shotgun LC−MS/MS Analysis

For shotgun proteomics, each testis to be analyzed was directly dissolved in 40 μL of LDS sample buffer (Invitrogen). The sample was subjected to 1 min of sonication (transonic 780H sonicator) and boiled for 5 min at 95 °C. The testis was totally dissolved in the LDS sample buffer, and 35 μL of each sample was then subjected to SDS-PAGE on a 10-well NuPAGE 4−12% gradient (Invitrogen) for 10 min at 150 V with MES buffer. Protein samples (35 μL) were then subjected to SDS-PAGE on a 10-well NuPAGE 4−12% gradient (Invitrogen) for 10 min at 150 V with MES buffer. Gels were stained with Coomassie Blue Safe stain (Invitrogen) and destained overnight with water. Protein concentration was not assessed as the testis proteins account for only 4 μg per organ in average. The variation in protein amount per gonad tissues is relatively low, as judged from the Coomassie staining. The whole protein content from each well was extracted as a single polyacrylamide band and processed for further destaining and iodoacetamide treatment, as previously described. Proteins were proteolyzed with sequencing grade trypsin (Roche) using 0.01% ProteaseMAX surfactant (Promega). The resulting peptide mixtures were analyzed in data-dependent mode with an LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher) coupled to an UltiMate 3000 LC system (Dionex-LC Packings), operated as previously described. In brief, 2 μL from each sample was loaded and first desalted online on a reverse-phase precolumn, C18 PepMap 100 column (LC Packings). Then, the peptides were separated using a 90 min gradient on a nanoscale C18 PepMapTM 100 capillary column (LC Packings) at a flow rate of 0.3 μL/min with a gradient of CH_3CN, 0.1% formic acid prior to injection into the ion trap mass spectrometer. Full-scan mass spectra were measured from m/z 300 to 1800 using the TOP3 strategy. In brief, a scan cycle was initiated with a full scan of high mass accuracy in the Orbitrap at a resolution of 30 000 and with the lock mass option, which was followed by MS/MS scans in the linear ion trap on the three most abundant precursor ions with a signal of at least 15 000 and potential charge states of 2+ and 3+. A dynamic exclusion of previously selected ions was set for a 60 s duration.

Protein Identification and Spectral Counting Quantitation

Peak lists were generated with the Mascot Daemon software (version 2.3.2; Matrix Science) using the extract_msn.exe data import filter (Thermo). Data import filter options were set to 400 (minimum mass), 5000 (maximum mass), 0 (grouping tolerance), 0 (intermediate scans), and 1000 (threshold), as previously described.[42] MS/MS spectra were assigned to peptide sequences with the Mascot Daemon 2.3.2 search engine (Matrix Science) against the customized RNA-seq-derived database containing 1 311 444 sequences and totalling 289 084 257 amino acids, as previously described.[31] MS/MS spectra were assigned with the following parameters: full-trypsin specificity, maximum of two missed cleavages, mass tolerances of 5 ppm on the parent ion and 0.5 Da on the MS/MS, static modification of carboxyamidomethylated cysteine (+57.0215), and oxidized methionine (+15.9949) as dynamic

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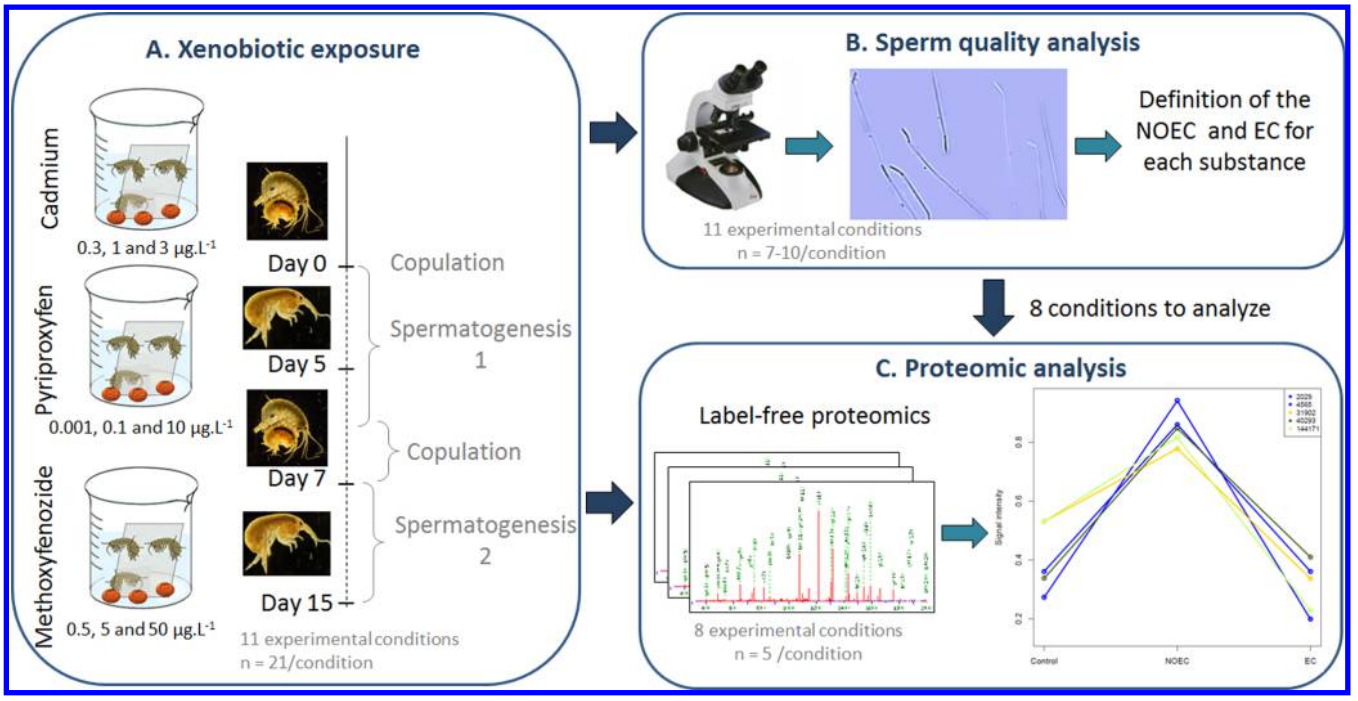

Figure 1. General strategy for male reproductive disorders assessment in *G. fossarum*. (A) Male organisms are exposed during two consecutive spermatogenesis cycles to three different xenobiotics, each at three different concentrations, including one solvent control and one solvent-free control. (B) Sperm-quality analysis by numeration of spermatids and spermatozoa. (C) Proteome analysis on the most interesting conditions for each chemical by shotgun proteomics and clustering procedures for the identification of proteome dynamics.

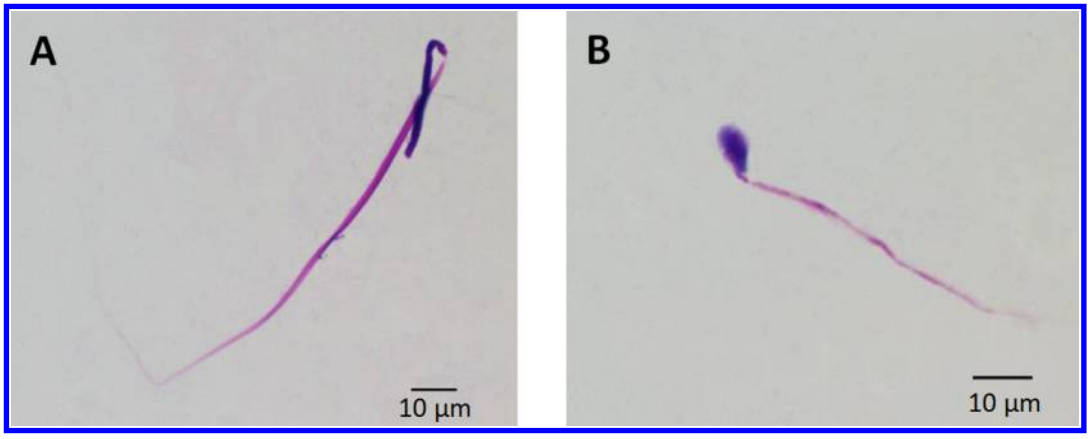

Figure 2. Microscopic observations of *G. fossarum* male reproductive cells (×100). (A) Spermatozoa and (B) spermatids after May–Grunwald–Giemsa staining.

modification. All peptide matches with a MASCOT peptide score below a p value of 0.05 were filtered and assigned to a protein according the principle of parsimony.[42] A protein was considered to be validated when at least two different peptides were detected. The false discovery rate for protein identification was estimated by employing the decoy search option of MASCOT (Matrix Science) to be <0.1%. Each xenobiotic-dependent proteome response was analyzed with the TrendQuest module of the PatternLab program.[43] For this, spectral counts were not normalized. Proteins observed in at least five of the replicate samples were considered for clustering; the minimum signal required was five, and the minimum items per cluster was four.

MS/MS Data Repository

The mass spectrometry proteomics data have been deposited with the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[44] with the data set identifier PXD001267.

RESULTS

Experimental Strategy for Comparative Proteomics of Toxicant-Exposed Animals

Figure 1 presents the global strategy employed for the assessment of male reproductive disorders in *G. fossarum* in response to toxic pressure. For each xenobiotic, male organisms were exposed to three concentrations during two consecutive spermatogenesis cycles, that is, 2 weeks, and compared with controls. During the exposure, no significant mortality was observed for the overall conditions, with survival values higher than 85%. At the end of the experiment, when the gonads were retrieved under stereomicroscope magnification, no intersex individuals were observed. We then constructed a two-step

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strategy for the analysis of the biological parameters. First, the relevance and sensitivity of sperm quality markers in response to toxicant were assessed by the numeration of spermatids and spermatozoa. Then, for each tested substance, we defined the most interesting condition to analyze the proteomes of the organisms, where sperm-quality markers revealed reproductive disorders, that is, the EC. Furthermore, because modifications at the molecular level are supposed to occur earlier than those visible at the cellular level, we also included in our study a NOEC for investigating early proteome modifications in response to toxic stress. Proteomic analyses were carried out on the testis of individual animals with a semiquantitative/relative proteomics based on spectral-counting procedure. Protein dynamics over the different concentrations were analyzed by clustering to reveal proteins modulated by toxic exposure.

Impacts of Cd, Met, and Pyr on Reproductive Physiology

Sperm-Quality Markers. Two flagellated cell types were counted under optic microscopy: the egg-shaped spermatids and the oblong-shaped spermatozoa. Their respective morphologies are presented in Figure 2A,B. No specific morphological abnormalities were observed upon toxic pressure. The numbers of spermatids in males exposed to the three xenobiotics are presented in Figure 3A. In the control condition, the mean spermatids number was $9.5 \times 10^2 \pm 2.3 \times 10^2$ and the mean spermatozoon number was $100 \times 10^2 \pm 28 \times 10^2$. Thus, spermatids represent only ~9% of the flagellated cells in the gammarid gonad. Cd and Met did not impact significantly on spermatid production (Kruskal–Wallis rank sum test, $p > 0.05$). Conversely, a significant impact on the production of spermatids was observed for the organisms exposed to Pyr (ANOVA, $p = 0.03$). Post hoc comparisons showed that only males exposed to 50 $\mu g \cdot L^{-1}$ Pyr had significantly fewer spermatids (mean ± S.E.M.: $6.7 \times 10^2 \pm 4.5 \times 10^2$) compared with the animals from the solvent control ($13 \times 10^2 \pm 4.2 \times 10^2$) (Tukey HSD, $p = 0.05$). Whereas overall spermatid production was not significantly impacted upon exposure of any of the three toxicants, spermatozoon production was shown to be more sensitive. The numbers of spermatozoa in males exposed to the three xenobiotics are presented in Figure 3B. In the control conditions, the average spermatozoon numbers (mean ± S.E.M) were $100 \times 10^2 \pm 28 \times 10^2$ and $88 \times 10^2 \pm 10 \times 10^2$ in the solvent-free control and solvent-control, respectively. For Met, sperm production was characterized by strong interindividual variability, especially for the lowest concentration of toxicant (0.001 $\mu g \cdot L^{-1}$, mean ± S.E.M.: $88 \times 10^2 \pm 53 \times 10^2$). Although a slight downward trend was observed, the spermatozoon count for organisms exposed to this substance was not significantly impacted (Kruskal–Wallis rank sum test, $p = 0.07$). A significant impact on spermatozoon production was observed in organisms exposed to Cd and Pyr (Kruskal–Wallis rank sum test, $p = 0.6 \times 10^{-3}$ and $p = 1.2 \times 10^{-5}$, respectively), both in a dose-dependent relationship. Compared with control conditions, the mean inhibition of spermatozoon production ranged from 58 (1 $\mu g \cdot L^{-1}$) to 75% (3 $\mu g \cdot L^{-1}$) for Cd, while for Pyr it ranged from 40 (5 $\mu g \cdot L^{-1}$) to 73% (50 $\mu g \cdot L^{-1}$).

Toxic Compound Concentrations for Shotgun Proteomics. For Cd, the lowest concentration of 0.3 $\mu g \cdot L^{-1}$ was chosen as the NOEC on the basis of the observation of sperm quality markers, while the highest concentration of 3 $\mu g \cdot L^{-1}$ was set as the EC. In the same way, for Pyr, the NOEC and EC were set as 0.5 and 50 $\mu g \cdot L^{-1}$, respectively. For Met, such clear-cut responses were not observed, but rather there was a slight downward trend. Thus, we chose to study the testis proteomes of organisms exposed to two concentrations of Met, that is, 0.001 and 0.1 $\mu g \cdot L^{-1}$. As a result, accounting for the solvent-free and solvent controls, proteomic analysis was carried out on a total of eight conditions. For each condition, the male gonads of five exposed animals were analyzed to obtain statistically robust results.

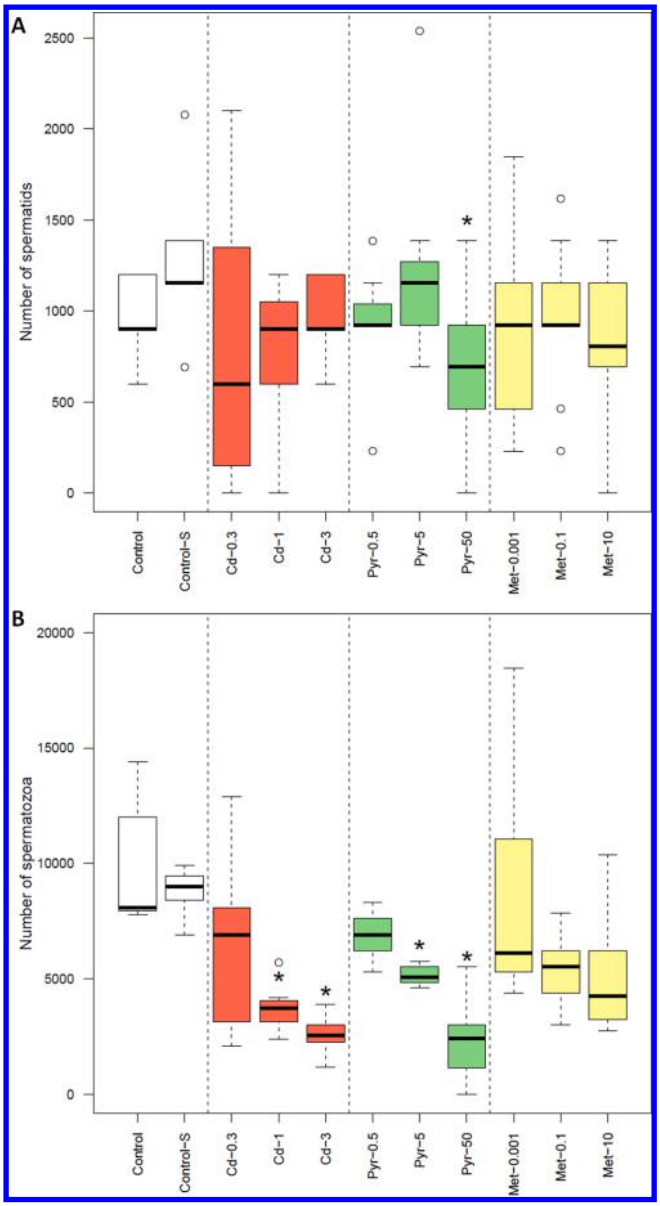

Figure 3. Number of male reproductive cells in *G. fossarum* gonads after 15 days exposure to pollutants. Number of (A) spermatid and (B) spermatozoa. Control and solvent-free control (Control-S) in white; Cd (0.3, 1, and 3 $\mu g \cdot L^{-1}$) in red; Pyr (0.5, 5, and 50 $\mu g \cdot L^{-1}$) in green; and Met (0.001, 0.1, and 10 $\mu g \cdot L^{-1}$) in yellow. Boxes extend from the first to the third quartile with a bold segment for the median value; the whiskers extend to the most extreme data points, no more than 1.5 times the interquartile range. Data points of more than 1.5 times the interquartile range are represented by empty circles. The p values of the global effect of treatment are reported in the text. * = significant difference ($p < 0.05$) from the overall control groups (solvent-free control and solvent control). n = 7–10 males per condition.

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Impact of Pollutants on the Testis Proteome

Global View of the Testis Proteome. After electrophoresis under denaturing conditions, the proteome of each of the 40 samples was retrieved in a single polyacrylamide gel band and digested in the gel. The resulting peptide mixtures were analyzed by nanoLC−MS/MS with a high-resolution Orbitrap hybrid mass spectrometer. This experiment led to a global data set of 237 950 MS/MS spectra recorded. A total of 57 558 MS/MS spectra could be assigned after querying an RNA-seq-derived protein sequence database, indicating the existence of 3020 unique peptide sequences. These allowed the identification of 871 mRNA-translated products, among which 66 (8%) orphans were listed. A complete inventory of peptides and proteins identified in this study is provided in the Supporting Information (Appendices 1 and 2) as well as their quantitation based on spectral counts and normalized spectral abundance factors (NSAFs). Remarkably, four abundant proteins made up 10% of the organ proteome: the histones H2A (ID_13250; mean NSAF percentage 4.1 ± 1.1) and H4 (ID_100349; mean NSAF percentage 2.3 ± 0.6), an arginine kinase (ID_14404; mean NSAF percentage 2.7 ± 0.5), and a protein with no predicted function (ID_128511; mean NSAF percentage 2.0 ± 0.8). The reproducibility between each replicates was evaluated from the total number of assigned spectra per sample (Appendix 2 in the Supporting Information), 1200 on average, as shown in Figure 4. On the basis of ANOVA analysis, no significant differences were observed between the different conditions ($p > 0.05$).

Identification of Temporal Variations of Testis Proteomes. For each toxicant, the proteome dynamics along the different concentrations were analyzed using the clustering method specifically developed for shotgun proteomics.[45] Across all of the experiments, a total of 70 proteins showed significant clustered temporal variations. As displayed in Figure 5, among these proteins, 44 were common to the three toxicants, 12 were shared by two conditions (three Cd-Met, three Cd-Pyr, and six Pyr-Met), and 14 were unique to a single condition. For each substance, a total of six clustered temporal patterns were recorded. The characteristics of the corresponding proteins are listed in Appendix 2 in the Supporting Information. Among the 70 proteins exhibiting temporal variations, 14 are devoid of functional annotation (20%) because of the low sequence similarity to known proteins, with three orphans. An important proportion is involved in cytoskeletal structure maintenance (22 proteins) and energy metabolism (10 proteins). The others are involved in calcium homeostasis (four proteins), heat-shock response (three proteins), or immunity (three proteins). Interestingly, one protein, functionally annotated as vitellogenin (ID_198421), is present in clusters Cd_A and Pyr_D. On the basis of the phylogenetic analysis reported by Hayward et al.,[30] this protein is included among the decapod clottable protein family, which is involved in immunity. In the Cd series, a total of 630 proteins were compared, resulting in six TrendQuest clusters comprising 55 proteins: 14 in cluster A, ten in cluster B, nine in cluster C, nine in cluster D, five in cluster E, and five proteins in cluster F. In the Pyr series, 722 proteins were compared, resulting in five clusters containing a total of 63 proteins: 19 proteins in cluster A, 16 in cluster B, 12 in cluster C, 5 proteins in cluster D, 4 in cluster E, and 4 in cluster F. Finally, in the Met series, 716 proteins were compared, resulting in six clusters containing a total of 61 proteins: 15 in cluster A, 15 in cluster B, 11 in cluster C, 8 in cluster D, 5 in cluster E, and 4 in cluster F. Figure 6 shows, for each xenobiotic, the cluster corresponding to the trend, where proteins are first induced in response to chemical stress and then decrease when physiological effects appear and thus which could be specific of an early effect of toxicants. The 18 different clusters are all available in Appendix 3 in the Supporting Information.

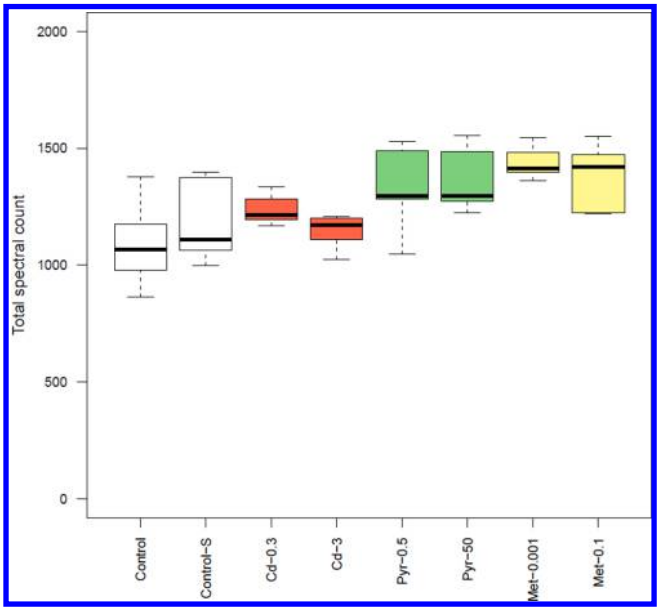

Figure 4. Analysis of reproducibility between replicates for each condition in terms of total number of assigned spectra per sample. Boxes extend from the first to the third quartile with a bold segment for the median value; the whiskers extend to the most extreme data points, no more than 1.5 times the interquartile range. Data points of more than 1.5 times the interquartile range are represented by empty circles. $n = 5$ males per condition.

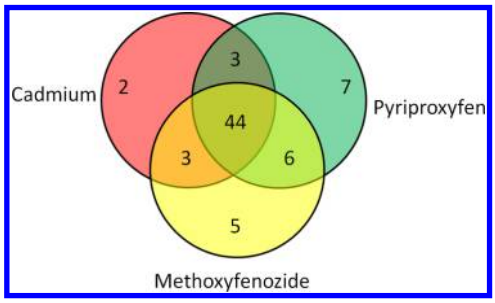

Figure 5. Differential expression of the proteins that showed clustered temporal variations in response to xenobiotic exposure..

DISCUSSION

Using Sperm Parameters to Assess Reproductive Health Status

Male reproductive disorders in invertebrates have been an underexplored issue in environmental risk assessment. Here we aimed to study male reproductive disorders in the amphipod, *G. fossarum*, a keystone organism in European freshwater ecosystems, in response to three toxicants. For this, we measured first sperm-quality markers and then global proteome alterations, in response to toxic pressure. Reproductive cell viability is commonly measured in the evaluation of sperm

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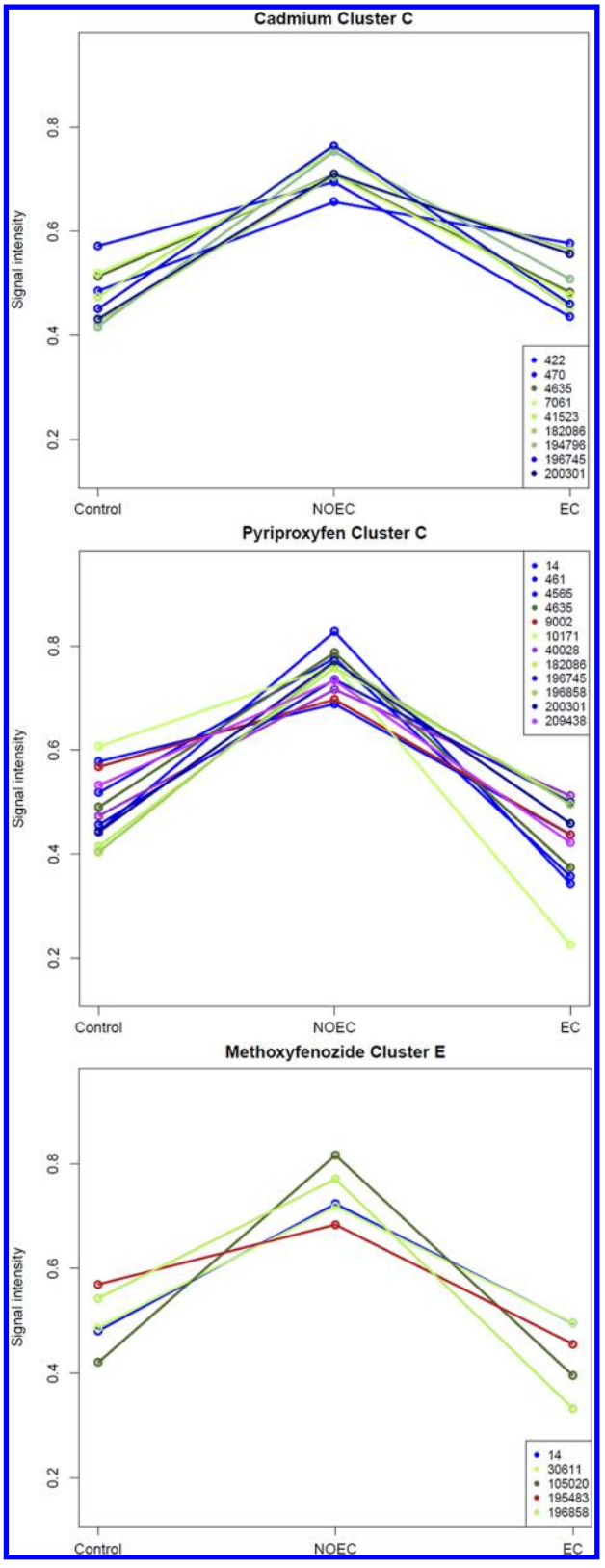

Figure 6. Examples of clustered temporal variations of proteins in response to Cd, Pyr, and Met exposure. The different trends were obtained after clustering the proteins with their spectral counts using the TrendQuest module of the PatternLab program, and minimum items per cluster was set to three. A total of 18 trends were delineated. All are displayed in Appendix 3 in the Supporting Information. For Pyr and Met, the control condition is the solvent control. The NOEC was set at 0.3, 0.5, and 0.001 $\mu g \cdot L^{-1}$ for Cd, Pyr, and Met, respectively. The EC was set at 3, 50, and 0.1 $\mu g \cdot L^{-1}$ for Cd, Pyr, and Met, respectively. Color legends are blue for proteins classified as male-specific,[31] green for proteins showing temporal variations among all xenobiotics, brown for proteins showing clustered temporal variations in Pyr and Cd, yellow for proteins showing clustered temporal variations in Cd and Met, purple for proteins showing clustered temporal variations in Met and Pyr, and red when the protein is uniquely modulated by the studied xenobiotic. n = 5 males per condition.

quality and has proved to be sensitive for fish,[46] urchins, and bivalves.[47] However, previous investigations on *G. fossarum* spermatozoa in response to genotoxic agents, or in field experiments, have shown no significant impact on cell viability.[21,48] Thus, in *G. fossarum*, the measurement of sperm viability does not appear to be a sensitive end point for the assessment of male reproductive disorders. Here we choose to monitor the production of reproductive cells and to differentiate their associated maturation states.

Spermatogenesis takes place in seminiferous tubules and is divided into three phases: (i) the proliferation phase, where intense mitotic division from spermatogonia to spermatocytes takes place, (ii) the meiotic phase producing spermatids, and (iii) the differentiation phase leading to spermatozoa.[33] Apoptosis is a key phenomenon in the control of sperm production, surplus and abnormal cells being eliminated by this mechanism. In mammals, the phase that is the most susceptible to toxic insult is believed to be the meiotic phase.[49] Apoptotic phenomena are most commonly observed in dividing spermatocytes and less frequently in the other cell types.[50] Thus, the production of both spermatids and spermatozoa usually decreases in response to toxicants. Surprisingly, the production of spermatids in *G. fossarum* was not significantly affected by exposure to any of the three toxicants, while spermatozoon production was impacted in a dose−response relationship. Thus, in crustaceans, the most susceptible phase toward toxic pressure is the differentiation phase, leading to a failure of spermatozoa maturation. Such a result has also been observed in the freshwater prawn, *Macrobrachium rosenbergii*, exposed to tributyltin.[51] At a 10 $ng \cdot L^{-1}$ treatment, a decrease only in spermatozoon production was recorded, while higher concentrations caused a decreased abundance of immature sperm cells concomitant with histological disorders in seminiferous tubules.[51] The latter observation suggests a massive toxic impact on testis tissue, preventing the production of reproductive cells, rather than a specific control mechanism within cell development.

In crustaceans, investigations on sperm quality have essentially been carried out in an agronomic perspective, while few studies have been carried out on environmentally challenged organisms. The marine amphipod, *Echinogammarus marinus*, collected at industrially impacted sites in Scotland showed a 15% decrease in sperm count compared with samples collected from reference sites.[14] In the present study, we measured, under controlled laboratory conditions, an important dose-dependent decrease in spermatozoon production in response to Cd and Pyr, up to 75%. To our knowledge, no data regarding the environmental relevance of the Met concentrations used are available for reference, but for Pyr and Cd, the chosen concentrations are environmentally realistic.[17,39] From our results, we can deduce that such a drastic impact on sperm production can lead to dramatic effects

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at the demographic level. In the amphipod, *Gammarus duebeni*, the relationship between sperm count and fertilization success was modeled by Ford et al.,[52] theorizing that a 10% reduction in the sperm count leads to population collapse in less than a decade. We recently proposed a robust population model for capturing *G. fossarum* annual population dynamics in response to modifications of juvenile survival, adult survival, and female fertility.[53] Thus, the spermatozoon count is a relevant parameter to implement for apprehending population vulnerability related to male reproductive disorders.

Impact of Pollutants on Reproductive Physiology

The arthropod endocrine system relies on the action of hormones belonging to two distinct chemical classes: ecdysteroids also called the molting hormone, and terpenoids, known as the JH. Chemicals for arthropod pest control are designed to interfere with these signaling pathways by directly interacting with the hormone nuclear receptors through either agonist or antagonist actions. Met exhibits a high affinity to the ecdysone receptor complex (EcR:USP) of lepidopteran insects (caterpillar pest), as it is a potent mimic of one of the active forms of ecdysteroids, 20-hydroxyecdysone. Induced effects include molting irregularities in caterpillars, such as abnormal cuticle development or premature apolysis. This pesticide is believed to have an excellent margin of safety to nontarget organisms,[54] although available data are limited. Remarkably, no significant impact on spermatozoon production was observed in our survey, while sperm counts from organisms exposed to Met were characterized by strong interindividual variability. Under exposure to the lowest concentration, individual organisms could exhibit a two-fold (either greater or lesser) variation in sperm count as compared with the control condition (mean = 88×10^2; min = 44×10^2; max = 190×10^2; %RSD = 60% compared with the control condition, mean = 100×10^2; %RSD = 12%). While ecdysteroids are known to control various physiological processes related to reproduction, such as molting or embryonic development, knowledge about their actions on spermatogenesis is scarce. In the shrimp, *Litopenaeus vannamei*, Parnes et al.[55] showed that males go through reproductive cycles that are strictly associated with their molt cycles, suggesting a possible hormonal regulation by ecdysteroids. In the European corn worm, *Ostrinia nubilalis*, physiological doses of ecdysteroids were administrated onto in-vitro-cultured testes to determine their effects on the final stage of spermatogenesis. A stimulatory effect was detected on testes from 4 to 5 day postecdysis larvae, while the treatment produced no stimulation on 3 day postecdysis larvae.[56] Here interindividual variability can be explained by the occurrence of different molt stages among our pool of organisms, resulting in a different sensitivity toward Met. A temporal window of effects due to specific physiological status is a characteristic of EDCs.

Pyr is a member of the insecticidal JH analogues, which inhibit the larval development and maturation of many insects (ticks, mosquitos, ants, houseflies, and fleas).[57] In our survey, exposure to this pesticide inhibited sperm production in a dose−response relationship. In decapods, JH analogues have been proven to affect metamorphosis and to induce morphological abnormalities and reduce growth rates (reviewed by ref 9). In daphnids, exposure to Pyr reduces female fecundity and stimulates male production.[12,39] Compared with ecdysteroids, JH functions have to date been elusive. Moreover, the JH receptor is still uncharacterized. In males, this hormone is believed to play a role in reproduction and behavior. In decapods, hormonal treatment resulted in the stimulation of testis growth.[58] One characteristic of hormone mimetics is their nonmonotonic dose−response curves, where exposure to low concentrations leads to physiological stimulatory effects, while higher doses may actually be inhibitory. Here organisms exposed to Pyr showed clear inhibition of spermatozoon production. Thus, exposure to lower concentrations of toxicant may give a stimulatory response.

Finally, we showed that in amphipods sperm production is highly sensitive to Cd exposure. Freshwater crustaceans are among the most Cd-sensitive macroinvertebrate species, as shown by previous studies that have documented impairments of female reproduction, embryonic development, energy acquisition,[22] and iono-osmoregulation[38] triggered by Cd exposure. Because of its electronic structure, Cd acts through a process of "ionic mimicry", interacting with transporters involved in the uptake of calcium, iron, and zinc and thus leading to a wide range of adverse effects (reviewed by ref 59). Because the integrity of calcium homeostasis is crucial for sperm maturation, capacitation, and sperm−egg interaction, sperm production is thus strongly impacted by low Cd doses.

Identification of Molecular Responses Triggered by Toxic Exposure

On the basis of the physiological end points, we analyzed the dynamics of testis proteomes for each xenobiotic in three different stages. Proteins for which modulation was only demonstrated between the NOEC and the EC were disregarded because this pattern indicates that the organism's defensive capacity was already overwhelmed in this stage. Concerning exposure to pesticides, six proteins were modulated by both the Met and Pyr compounds, including (i) an Na^+/K^+ ATPase, which suggests a disturbance of iono-osmoregulation (increase at the NOEC and decrease at the EC for Pyr, irrelevant pattern for Met), and (ii) a factor of hemocyte aggregation, a hemocytin,[60] which showed an induction followed by a decreased abundance in response to both pollutants. Five proteins were uniquely modulated upon Met exposure. Two heat shock proteins (HSPs) were induced and a glucan-pattern-recognition protein, involved in the recognition of foreign invaders, was downregulated. In this survey, seven proteins were uniquely modulated by Pyr. Among these, in addition to housekeeping proteins, an HSP 70 and a protein without functional annotation (ID 17884) were induced, while another protein with no predicted function (ID 3678) and an orphan (ID 110918) were less abundant. Surprisingly, we have previously sequenced several proteins involved in JH metabolism in *G. fossarum* testes but no variation of these occurred during spermatogenesis[31] or xenobiotic exposure, suggesting that this hormone may not directly control spermatogenesis but rather may maintain male sexual characteristics.

Only two proteins involved in cytoskeletal structure maintenance were modulated uniquely by Cd exposure. In male reproduction, the effects of Cd have been intensively studied in model mammals. These effects include decrease in testicular growth rate, failure of spermatozoon maturation, induction of oxidative stress in sperm cells, and alteration of cytoskeletal protein assembly by junctional disruption. Sperm production was observed to be significantly altered in organisms exposed to Pyr and to Cd. Three proteins were modulated by these two xenobiotics: (i) a protein with no

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Table 1. Proteins with Clustered Temporal Variations among Toxic Exposure Previously Identified as Sex Specific

	best match				proteogenomic experiment		TrendQuest cluster		
GFOSS contig ID (protein no.)	probable function	origin of the closest protein	Blast *E*-value	total spectral count	spermatogenesis	comparative	Cd	Pyr	Met
14	slow muscle myosin S1 heavy chain	*Homarus americanus*	2.64×10^{-123}	1071		male	B	C	E
422	myosin heavy chain	*Bombyx mandarina*	4.23×10^{-64}	287		male	C	A	B
461	hemocytin-like	*Apis florea*	1.73×10^{-19}	221	X	male		C	D
470	spectrin beta chain-like	*Apis florea*	0.00×10^{0}	218		male	C		
2029	projectin	*Procambarus clarkii*	0.00×10^{0}	147		male	F		
2853	sarcoplasmic calcium-binding protein	*Procambarus clarkii*	3.22×10^{-79}	318		male	B	B	C
4227	calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum	*Acyrthosiphon pisum*	0.00×10^{0}	350	X	male	A	F	B
4565	calmin-like protein	*Tigriopus californicus*	6.72×10^{-21}	333		male	F	C	C
9438	no predicted function	*Bactrocera oleae*	1.62×10^{-118}	405		male	B	F	D
22255	myosin heavy chain	*Drosophila melanogaster*	1.35×10^{-104}	498		male	B	A	B
23428	heat shock cognate 70	*Aedes aegypti*	1.30×10^{-140}	132		female			D
34845	copine-8	*Crassostrea gigas*	5.64×10^{-88}	331		male	D	B	C
69913	no predicted function	*Daphnia pulex*	6.28×10^{-84}	211		male	D	A	
196745	myosin heavy chain	*Marsupenaeus japonicus*	1.30×10^{-51}	382		male	C	C	B
200301	myosin heavy chain	*Gammarus duebeni*	1.33×10^{-102}	334		male	C	C	B

predicted function (ID 135487) that was induced upon exposure to Cd and to Pyr, (ii) another, male-specific, protein (ID 69913), which was repressed in response to Cd (irrelevant pattern for Pyr), and (iii) a protein annotated as vitellogenin, repressed in response to Pyr (irrelevant pattern for Cd). Of note, this functional prediction is misleading because it is only based on phylogenetic analysis. Because of the absence of specificity toward the female reproductive tissue, this protein is more likely to be a clottable protein, involved in innate immunity. In fact, in our survey, several proteins involved in immunity were repressed in response to pollutants. As is the case for other arthropods, the testis of gammarids can be infected by parasites that are known to actively convert males into females for enhancing the parasite transmission to the offspring, leading to a feminized phenotype.[61] Thus, intersexuality in a field population from an anthropically impacted site can also be an indirect consequence of immunosuppression caused by pollutants.

Among the proteins modulated by all three pollutants, the majority are housekeeping proteins involved in cytoskeletal structure maintenance or energy metabolism. Four proteins are calcium-dependent or involved in calcium homeostasis, including three that were previously classified as male specific. Interestingly, these three proteins, including two Ca^{2+} transporters, are all induced in response to xenobiotic exposure. Hughes et al.[62] showed that a wide range of xenobiotics inhibit Ca^{2+} ATPase in Sertoli testicular cells and that dysregulation of Ca homeostasis leads to Ca^{2+}-dependent cell death via apoptosis. Thus, overexpression of Ca^{2+} transporters may be a compensatory mechanism. The other male-specific protein induced is annotated as Copine-8. In humans, the associated gene is predominantly expressed in prostate and testis, suggesting an important role in the development and regulation of male sexual characteristics, although its function is to date elusive.[63] Finally, two orphans (ID 41523, 196558) and six proteins also without functional annotation (ID 9438, 7061, 4635, 128511, 5492, 40293) showed clustered temporal variations with all three xenobiotics. Three of them showed relevant pattern only in the Cd condition. Proteins ID 41523 and 7061 were induced when comparing the NOEC to the control, while the protein ID 128511 was repressed. The other remaining proteins, including one male specific (ID 9438), were all induced when comparing the NOEC with the control in response to all three xenobiotics.

Identification of Endocrine Disruptor Biomarkers

Traditionally, endocrine disruption in crustaceans has been studied through the prism of vertebrate endocrine disruption, that is, monitoring the induction of vitellogenin. However, this biomarker has been proven over the years to be inappropriate for this objective.[28,29] Previous comparisons of male and female reproductive proteomes allowed the identification of proteins with strong sexual dysmorphism: 129 and 75 proteins for males and females, respectively. Table 1 shows the proteins with clustered temporal variation among toxic exposure that were previously identified as sex-specific and modulated during spermatogenesis.[31] In this survey, the modulation of 14 male-specific proteins was recorded in response to EDCs exposure, while only one protein, an HSP 70, previously classified as more abundant in female tissue, was found induced. This chaperon protein has versatile roles,and its accumulation in the ovaries may be a maternal factor involved in various aspects of embryonic development, such as cell movement, proliferation, morphogenesis, and apoptosis.[64] Here this protein, along with other modulated HSPs in the testis, may have been induced for the promotion of sperm cell survival and to ensure cellular homeostasis.

Compared with vertebrates, where the proportion of steroid hormones differs between males and females, crustacean sex

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determination is under the control of a specific hormone, the androgenic gland hormone, which controls the expression of male sexual characteristics. In the absence of this hormone, organisms spontaneously develop into females. Thus, in crustaceans, the intersexual phenotype is rather the result of a demasculinization process than of a feminization one.[61] Here exposure to EDC modulated several male-specific proteins, apart from housekeeping proteins. For example, two Ca^{2+} transporters and the protein, Copine-8, were upregulated. Additionally, homologues of a protein from *Daphnia pulex* and of a protein from the olive fruit fly, *Bactrocera oleae*, both with no predicted function, were induced in response to EDCs. It is worth noting that proteins devoid of functional prediction were preponderant in this study, accounting for 20% of the proteins showing temporal variations. In our global protein data set, 8% of the identified proteins are orphans and a third were not functionally annotated. Reproductive proteins are highly diversified among the tree of life as a result of sexual selection and are consequently highly species-specific. Thus, these proteins may be involved in either sperm navigation toward the egg or sperm−egg interactions. These surface receptors play an essential role in the fertilization process, consequently any defects or abnormalities may lower the organism's reproductive potential.[65] Furthermore, compared with our previous spermatogenesis experiment, a significant portion of proteins without predicted function were newly detected, including 24 *Daphnia pulex* homologues and 18 orphans. Transcriptomic analysis on the *Daphnia pulex* genome has shown that orphan genes are among the most ecoresponsive,[66] and thus they are interesting biomarker candidates. In any case, highly divergent proteins are generally present in low abundance in the proteomes of organisms. Standard shotgun proteomic strategies allow the preferential detection of the abundant proteins, for which the functions are more commonly shared across the tree of life.[67] Additionally, in mammals spermatogenesis, "low copy number proteins" are believed to be crucial for germ cell differentiation.[33] Thus, while our strategy enabled the discovery of these proteins as potential biomarkers, measuring their sexual dysmorphism, such as monitoring their modulation toward the organism's physiological stage or xenobiotic exposure, requires more targeted proteomics approaches. Consequently, on the basis of our previous work for the quantitation of vitellogenin by LC−MS/MS,[68] a multiple-reaction-monitoring strategy should be developed for further biomarker validation. This could also be applied for routine biomonitoring using *G. fossarum* as a sentinel organism.[24]

■ ASSOCIATED CONTENT

Supporting Information

Supplementary data concerning raw peptide identification, protein identification, and quantitation can be found, respectively, in Appendices 1 and 2. All clustered temporal trends in response to xenobiotic exposure are displayed in Appendix 3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EcR:USP: ecdysone receptor complex; EDC: endocrine-disrupting chemical; HSP: heat shock protein; JH: juvenile hormone; Met: methoxyfenozide; NOEC: no observed effect concentration; LOEC: lowest observed effect concentration; Pyr: pyriproxyfen

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