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Proteomic Response of the Freshwater Crustacean *Hyalella azteca* to the Estrogenic Contaminant 17β-Estradiol: An Exploratory Ecotoxicological Study

Marcel L. Prokai [1], Khadiza Zaman [2,*] and Vladimir Shulaev [1,*]

[1] Department of Biological Sciences, University of North Texas, Denton, TX 76203, USA; marcelprokai@my.unt.edu
[2] Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Fort Worth, TX 76107, USA
* Correspondence: khadiza.zaman@unthsc.edu (K.Z.); vladimir.shulaev@unt.edu (V.S.)

Abstract

Estrogenic endocrine-disrupting chemicals (EDCs) are among the most pervasive contaminants in freshwater systems. Benthic amphipods live in direct contact with these agents and support the aquatic food web, yet molecular tools to detect and characterize responses to estrogenic exposure remain largely undeveloped or unvalidated, which limits their use for monitoring and risk assessment. The recent genome sequencing of *Hyalella azteca* (*H. azteca*), a key food-web species, enables proteomic approaches to help close this gap. Here we report the first exploratory, genome-enabled, whole-organism proteomics study of the response of this crustacean to the estrogenic EDC 17β-estradiol (E2) in both sexes at a nominal concentration of 100 ng/L. Using nanoflow liquid chromatography–tandem mass spectrometry, we profiled protein expressions in both sexes following acute exposure. Differentially expressed proteins were mapped to more than 100 canonical pathways by Ingenuity Pathway Analysis®. The most enriched pathways, related mainly to protein quality control, intracellular trafficking, and carbohydrate metabolism, differed in predicted direction between sexes, with directional predictions reaching the confidence threshold mainly in males. These results demonstrate the feasibility of proteomics-based molecular profiling of E2 exposure in *H. azteca*, extending beyond conventional phenotypic toxicity testing. As a hypothesis-generating dataset, our results provide a foundation for targeted validation, time-course and dose–response studies for candidate protein markers, as well as testing whether these responses extend across estrogenic EDCs.

Keywords: bioinformatics; 17β-estradiol; estrogenic endocrine disruptors; *Hyalella azteca*; IPA®; mass spectrometry; ortholog mapping; proteomics; systems biology

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1. Introduction

Concerns regarding exposure to chemicals capable of altering the normal functioning of the endocrine system in humans and wildlife were first reported over 60 years ago [1]. Since then, understanding the impacts of these agents, collectively known as endocrine-disrupting chemicals (EDCs), on human health and the environment has become an increasingly high priority [2,3]. This shift is largely driven by continued research providing a steadily growing body of scientific evidence about the health risks associated with EDCs. [4]. Exposure to these agents is ubiquitous; typical sources of exposure include contaminated food, water, or air, as well as consumer and pharmaceutical products [5,6]. The adverse impacts of these

agents may also persist across generations through epigenetic modifications [7]. While the effects of EDCs on the endocrine system are a global concern, effective national and worldwide regulation remains challenging [8,9]. This is due, in part, to the complexity of the endocrine system that EDCs can disrupt in many aspects of its normal function, such as hormone synthesis, metabolism, or binding, either individually or in concert [5]. Recently, ten characteristic mechanisms by which these agents adversely influence hormone action have been identified [10].

Among the diverse classes of EDCs, estrogenic compounds play a prominent role: they disturb reproductive and endocrine functions across species in both sexes, particularly during early developmental stages [11]. These agents mimic or block the functions of endogenous estrogens, primarily 17β-estradiol (E2), thereby interfering with the body's natural homeostasis. While E2 is known as the primary "female" hormone, playing critical roles in reproduction, sexual differentiation, and development through complex mechanisms in both sexes, it is also an environmental pollutant [12]. As an EDC, it is found primarily in water bodies near wastewater treatment plants, where it is released through effluent discharge originating from human and animal waste [13,14]. Most studies on estrogenic disruption rely on vertebrate models [15] despite invertebrates comprising over 95% of animal species on Earth [16]. While phenotypic endpoints such as growth, survival, and reproduction are well established for invertebrate sediment toxicity testing [17], no endpoint specific to estrogenic disruption exists at the molecular or mechanistic level, leaving systems-level understanding of aquatic EDC impact essentially incomplete [18]. *Hyalella azteca* (*H. azteca*), a freshwater benthic amphipod, is well suited to close this knowledge gap, as it plays an important role in nutrient cycling [19] and is already a standard model for sediment toxicity testing [20], providing a well-established foundation for assessing organism-level responses to contaminants. Tools capable of resolving proteome-level responses to estrogenic exposure specifically, however, have not yet been applied to this species. Its recently sequenced genome [21] now makes proteomic profiling possible, offering a route to molecular-level endpoints that phenotypic assays alone cannot provide. Proteomics, based primarily on mass spectrometry (MS), is an important tool for the quantitative and qualitative profiling of the proteome of an organism of interest and a fundamental contributor to the understanding of systems biology in which proteins play critical roles [22]. The objective is to interpret the roles and contributions of individual proteins within larger biological networks, rather than focusing on individual proteins isolated from biological systems. The so-called "bottom-up" approach relies on digesting proteins into peptides before analysis by tandem mass spectrometry (MS/MS). With this approach, state-of-the-art experimental methods distinguish between untargeted (global, frequently referred to as "shotgun" or discovery-driven) and targeted proteomics; both involve online separation by liquid chromatography (LC) followed by MS/MS [23]. Proteins are then identified by searching various protein databases.

Accordingly, here we report an exploratory, discovery-driven, hypothesis-generating ecotoxicological assessment of *H. azteca* proteome following exposure to E2, a representative estrogenic EDC. Given the absence of proteomics data on estrogen exposure in this species, a 48-h acute exposure aligned with previously reported toxicity testing in crustaceans [24,25] was used as a starting point for defining the acute proteome-level response to E2.

We examined protein expression changes in male and female *H. azteca*, including whether these responses differ by sex. Our overarching goal was to assess the feasibility of MS-based proteomics for identifying molecular changes beyond what phenotypic endpoints alone could capture and to generate hypotheses and candidate proteins for follow-up investigations. This work is a foundational step toward time-course, dose–response, quantitation of estrogens in the organism and targeted validation, which will be needed to evaluate

candidate protein markers of exposure across estrogenic EDCs and to establish this species as a genome-enabled molecular model for crustacean ecotoxicology.

2. Materials and Methods

2.1. Chemicals

E2, salts for the preparation of Duluth 100 hard water ($KHCO_3$, $NaHCO_3$, $MgSO_4$, NaBr, $CaCl_2 \cdot 2H_2O$, and $CaSO_4$), urea, dithiothreitol (DTT), iodoacetamide (IAA), and ammonium bicarbonate (ABC) were purchased from Millipore Sigma (St. Louis, MO, USA). Sequencing-grade trypsin was obtained from Promega (Madison, WI, USA), while Optima® LC/MS-grade water, formic acid (FA), trifluoro acid (TFA) and acetonitrile (ACN) were ordered from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. H. azteca and E2 Exposure

H. azteca culture was originally purchased from Carolina Biological Supply (Burlington, NC, USA) and has been maintained in our laboratory as a continuous culture for many years, propagated through successive generations of offspring; genetic independence between individuals therefore cannot be confirmed. Amphipods were cultured in Duluth 100 hard water under a standardized photoperiod of 16-h light:8-h dark, at a tightly regulated temperature of 23 ± 1 °C (permitted variation around the set point) [17,26], in identical incubators (Environmental Growth Chambers, Chagrin Falls, OH, USA). Aeration was provided to maintain dissolved oxygen, using a Supreme Oxy-Flo low-volume aeration system (Danner Manufacturing, Inc., Islandia, NY, USA). To ensure uniform, independent delivery across replicate chambers, the primary airflow was partitioned using an Active Aqua eight-outlet metal air manifold (Hydrofarm Holdings Group, Inc., Shoemakersville, PA, USA). During standard culture maintenance [17,26], amphipods were fed three times per week with TetraMin® (Tetra Spectrum Brands Pet, LLC, Blacksburg, VA, USA) fish food flake slurry (2 mL, 40 mg flake in 40 mL Duluth 100 hard water). Offspring were separated using a 235-µm sieve (USA Standard Test Sieve; Thermo Fisher Scientific, Waltham, MA, USA), and the collected juveniles were maintained in culture until they reached 35 days of age. At this age, amphipods were sexed under a BO61 microscope (Olympus, Hicksville, NY, USA) based on gnathopod size [27], and four amphipods (two males and two females) were placed in each of sixteen 200-mL beakers containing 100 mL of culture medium (Duluth 100 hard water), under the standardized environmental conditions specified above. Subsequently, eight beakers were spiked once with an ethanolic E2 stock solution to give a nominal exposure concentration of 100 ng/L (based on previous study using a synthetic estrogen [28]), which was not verified analytically (static exposure, no renewal). The control group (eight beakers) was kept in the same culture medium under identical conditions and received the same volume of ethanol (10 µL, solvent control). Control and E2-exposed *H. azteca* were handled identically and experiments were run concurrently, so that handling effects were shared equally by both groups. Males and females were co-exposed, reflecting their natural co-occurrence, which ensures identical exposure conditions for both sexes. Amphipods were not fed during the 48-h exposure window to prevent dietary interference with the chemical exposure or the subsequent proteomic profiles. After 48 h, sex assignments were re-confirmed under the same microscope, and one male and one female were selected at random from each beaker. This sampling strategy yielded $n = 8$ individuals per sex per group (32 in total) for proteomic analysis, one individual per beaker and sex, with no sample pooling. Each *H. azteca* was placed in a 1.5-mL microcentrifuge tube, flash frozen in liquid nitrogen, and stored at −80 °C until processing for proteomics.

2.3. Sample Preparation for MS-Based Proteomics

There was no sample pooling in the experimental setup. Each individually flash-frozen *H. azteca* was ground into fine powder using 3 repetitive cycles in a Bead Blaster™ 24 refrigerated microtube homogenizer (Millipore Sigma, Rockville, MD, USA) for 30 s, then suspended in 200 µL of 8 M aqueous urea for 60 min for protein extraction at room temperature. Quantification of the protein content from the collected supernatant was done in duplicate utilizing a BioTek Synergy H1 microplate reader outfitted with a Take3 microplate interface (Agilent, Palo Alto, CA, USA). Aliquots equivalent to exactly 100 µg of total protein/sample were brought to a uniform volume with 25 mM ABC and subjected to disulfide bond cleavage via incubation with DTT at 56 °C for 30 min, followed by covalent alkylation using 5 mM IAA at room temperature for 30 min, maintaining strictly dark conditions. To facilitate optimal enzymatic proteolysis, the reaction mixture was diluted with a 25 mM ABC buffer system until the urea concentration shifted to a threshold below 2 M. Over-night digestion was initiated at 37 °C using 2 µg of sequencing-grade trypsin per sample, and the process was terminated by adding 5 µL of FA. Desalting and peptide purification were performed by solid-phase extraction leveraging Sep-Pak™ C-18 cartridges (1 mL; Waters USA, Milford, MA, USA). Elution of captured analytes was carried out sequentially utilizing two discrete 200-µL volumes of 80% v/v aqueous acetonitrile (ACN) containing 0.1% v/v TFA, followed by a final elution with 200-µL ACN. The combined fractions were concentrated to dryness under vacuum using a CentriVap benchtop vacuum system (Labconco, Kansas City, MO, USA) and stored in clean 1.5-mL tubes at −80 °C until downstream liquid chromatography–mass spectrometry analysis [29,30].

2.4. Nanoflow Liquid Chromatography–Tandem Mass Spectrometry (nLC-MS/MS)

Individual dried samples were reconstituted in 5% ACN/0.1% FA at 1 µg/µL protein-equivalent concentration. Data-dependent acquisition (DDA) was performed on an LTQ Orbitrap Velos Pro instrument coupled to an EASY nLC-1000 nanoflow system (all from Thermo Scientific, San Jose, CA, USA). Separations were performed on a 15 cm × 75 µm i.d. column packed with 2.6-µm BioZen Peptide XB-C18 particles (Phenomenex, Inc., Torrance, CA, USA). Gradient elution was performed using water with 0.1% (v/v) FA (solvent A) and ACN containing 0.1% (v/v) FA (solvent B), as reported before [29,30]. Briefly, after the injection of 3 µL sample solution at 100% A, peptides were eluted at 300 nL/min flow rate using the following gradient program: (i) isocratic at 3% B for 2 min; (ii) linear gradient to 40% B over 60 min and then (iii) 5 min of isocratic at 40% B; (iv) scaled up to 85% B for 2 min; (v) isocratic flow at 85% B for 5 min; and (vi) returning 3% B in 8 min. Positive-ion ESI was performed using a 7-µm EasySpray emitter (ES 991, Thermo Scientific) at a source voltage of 2.2 kV and an ion-transfer tube temperature of 275 °C. Full-scan MS range was 350–1650 m/z, and recorded at nominal mass resolution of 60,000 (at m/z 400), and 20 data-dependent MS/MS spectra were acquired in the linear ion trap by collision-induced dissociation (CID) at 35% normalized collision energy, selecting only multiply charged ions ($z \geq 2$) for MS/MS acquisition, using a 2.0 m/z isolation width window, and activation time set at 10 ms. After a precursor ion was selected for fragmentation, it was dynamically excluded from MS/MS analyses for 60 s [29,30].

2.5. Data Analysis

MS/MS spectra were searched against the UniProt-TrEMBL *H. azteca* database (30,180 entries) using the SEQUEST search engine of Proteome Discoverer 2.4 (Thermo Scientific, San Jose, CA, USA) using the target decoy-based method of false-discovery rate (FDR) estimation. FDR of 1% was applied with parent ion mass tolerance of 25 ppm; fragment ion mass tolerance of 0.80 Da and one missed cleavage were allowed as search filters.

Carbamidomethylation was set as a fixed modification, while oxidation and deamidation were set as variable modifications. Raw MS files were searched using MSFragger integrated within Scaffold (version 5.3.3), and Scaffold DDA (version 6.5.0; Proteome Software, Portland, OR, USA) was used for quantitation and statistical analyses. Protein identifications were accepted at ≥99% probability by Protein Prophet, and peptide identifications at ≥95% probability by Peptide Prophet, with a minimum of two peptides required per protein. Label-free quantification (LFQ) was performed using spectral counting with the program's built-in statistical analyses, applying two-way ANOVA across all samples within groups (sex and exposure as independent variables) followed by unpaired *t*-tests for post hoc group comparisons. Individual amphipods were analyzed as units (one per beaker and sex) because beakers were set up identically and run concurrently; beaker was not modelled as a random factor in Scaffold DDA (version 6.5.0). Differentially expressed proteins (DEPs) were defined as $p < 0.05$ (Benjamini–Hochberg, BH, corrected for multiple testing) with at least a 50% change in expression between groups.

2.6. Bioinformatics

Differentially expressed proteins (DEPs) were submitted to Ingenuity Pathway Analysis® (IPA®) from QIAGEN (Redwood City, CA, USA) to seek patterns of regulation through the associated canonical pathways, diseases, and functional networks, as well as protein interaction networks. Because IPA®'s Knowledge Base is curated for vertebrates, orthologs were assigned to DEPs before pathway analysis using a reciprocal best hits (RBH) approach [31] with BLASTp (Basic Local Alignment Search Tool for proteins, version 2.17.0), part of the BLAST+ suite, against the human UniProt reference proteome, relying on 1×10^{-5} E-value cutoff. Since RBH depends on pairwise sequence similarity alone, unmatched DEPs were additionally queried against OrthoDB [32]. Proteins unmatched by either method were excluded from pathway analysis. IPA®'s Pathway Explorer module was used to build protein interaction networks, as well as disease and function categories and canonical pathways. IPA® Investigator was also utilized to search the curated database to identify conserved and divergent functions between vertebrates and crustaceans. Downstream biological consequences and network modulations were modeled using the software's Molecule Activity Predictor (MAP) function to project the activation or suppression of discrete canonical cascades, biofunctions, and disease states. These directional trends are estimated based on calculated Z-scores, which assign a predictive status to a given molecular node or network pathway. For clear visual tracking across the networks, simulated molecular activation is color-coded in orange, whereas predicted functional inhibition is depicted in blue. Pathways with a Z-score of ≥+2 are considered significantly activated, and ≤−2 significantly inhibited [33]. Overlaps of *p*-values relied on IPA®'s calculations using the right-tailed Fisher's exact test.

3. Results and Discussion

This exploratory ecotoxicological study examined responses of *H. azteca*'s proteome to a single 48-h acute E2 exposure (100 ng/L, nominal concentration [28]) under controlled laboratory conditions, establishing a baseline for molecular investigations in this species. The exposure duration is consistent with acute exposure windows used in freshwater crustacean toxicity testing [15,16,24,25] while remaining short relative to the life cycle of this species [34]. Consequently, DEPs and associated pathways characterize the acute response; determining sustained responses, concentration dependence, and causal mechanisms will require time-course and dose–response studies. High-confidence *H. azteca* protein identifications were obtained via UniProt-TrEMBL database searches using the SEQUEST algorithm and validated with MSFragger, Peptide Prophet, and Protein Prophet

procedures (Table S1). Two-way ANOVA, with sex and exposure as independent variables (Table S2a,b), followed by post hoc unpaired *t*-tests, revealed 90 DEPs in males and 55 DEPs in females in E2-exposed amphipods relative to control amphipods (Table S2c,d).

We used IPA® to derive biological annotations. As the IPA® Knowledge Base is curated primarily for vertebrates and *H. azteca* is not yet represented in it, orthologs were first assigned to DEPs using an RBH approach [31] with BLASTp against the human UniProt reference proteome; unmatched DEPs were additionally queried against OrthoDB [32], which groups proteins by evolutionary relationship, to identify further orthologs. The resulting pathway and function annotations are therefore computational predictions based on human ortholog mapping. Consistent with the exploratory aim of our study, they indicate associations with vertebrate biology, not mechanisms in *H. azteca*, and are interpreted below as candidate processes requiring experimental validation.

IPA® mapped DEPs from both sexes to more than 100 canonical pathways (Table S3); the five most significant are shown in Table 1. While these annotations originate from vertebrate-characterized pathways, the proteins contributing to their enrichment are associated with fundamental cellular processes, including protein homeostasis, carbohydrate metabolism, intracellular trafficking, and protein degradation, which are conserved across animal taxa [35–37]. Pathway enrichment does not, however, indicate that identical vertebrate signaling mechanisms operate in crustaceans.

Table 1. Top five IPA® canonical pathways, ranked by overrepresentation *p*-value, among DEPs in E2-exposed male and female *H. azteca* (Table S3a,b). Pathway names and activation Z-scores come from a predominantly vertebrate-based knowledge base after ortholog mapping and are computational predictions, not measured pathway activity. Positive Z-scores: predicted activation; negative Z-scores: predicted inhibition; $|Z| \geq 2$ is the threshold for a directional prediction; therefore, the female values are not interpreted.

Canonical Pathway	−log(*p*-Value)		Z-Score	
	Male	Female	Male	Female
Aggrephagy	11.4	11.0	−2.646	0.378
Glycolysis	10.7	10.3	−2.236	1.342
HSP90 chaperone cycle for steroid hormone receptors in the presence of ligand	10.6	10.2	−2.646	1.134
COPI-mediated anterograde transport	10.4	11.6	−2.828	1
MHC class II antigen presentation	9.68	9.2	−2.828	1.414

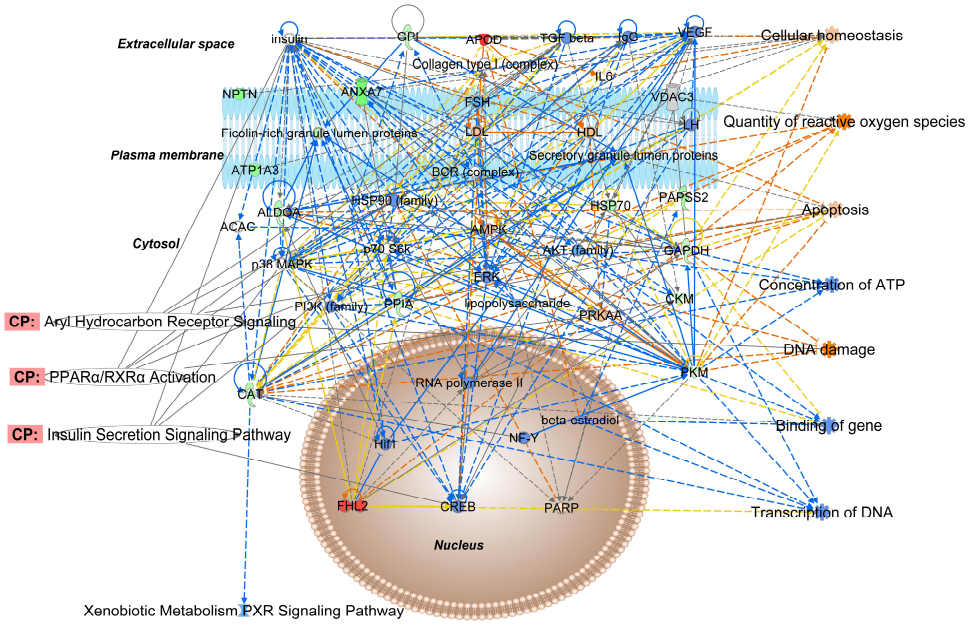
The IPA® activation Z-scores differed in sign between the sexes: negative in males and positive but of lower magnitude in females (Table 1). Across all enriched pathways, 32 of 111 (Table S3a) reached the threshold of $|Z| \geq 2$ [33] in males, compared with 15 of 121 in females (Table S3b). In males, negative Z-scores predicted decreased activity in protein quality control, intracellular trafficking, and carbohydrate metabolism, based on lower protein abundance of the annotated proteins. In females, none of the five pathways in Table 1 reached the threshold. The 15 female pathways that did reach $|Z| \geq 2$ showed opposite sign scores of similar magnitudes in males (Table S3c). However, these pathways were driven largely by a small, shared set of tubulin and actin isoforms, indicating a shift in a few core proteins rather than 15 independent processes. A predicted pathway activity must also be distinguished from measured protein abundance, since metabolic flux was not evaluated.

Glycolysis supports cellular energy requirements, and E2 is known to affect carbohydrate metabolism in vertebrates [38]. Enrichment of aggrephagy- and HSP90-associated proteins points to alterations in proteostasis and cellular stress responses. HSP90 chaperones participate in folding and quality control, but their association with the IPA® steroid hormone receptor pathway does not establish involvement of vertebrate-type estrogen receptor signaling in *H. azteca*. However, COPI-mediated transport enrichment suggested

differences in intracellular membrane trafficking. Because amphipod responses to estrogenic compounds are insufficiently characterized, these enrichments were interpreted as shifts in protein abundance rather than as evidence of conserved vertebrate endocrine pathways.

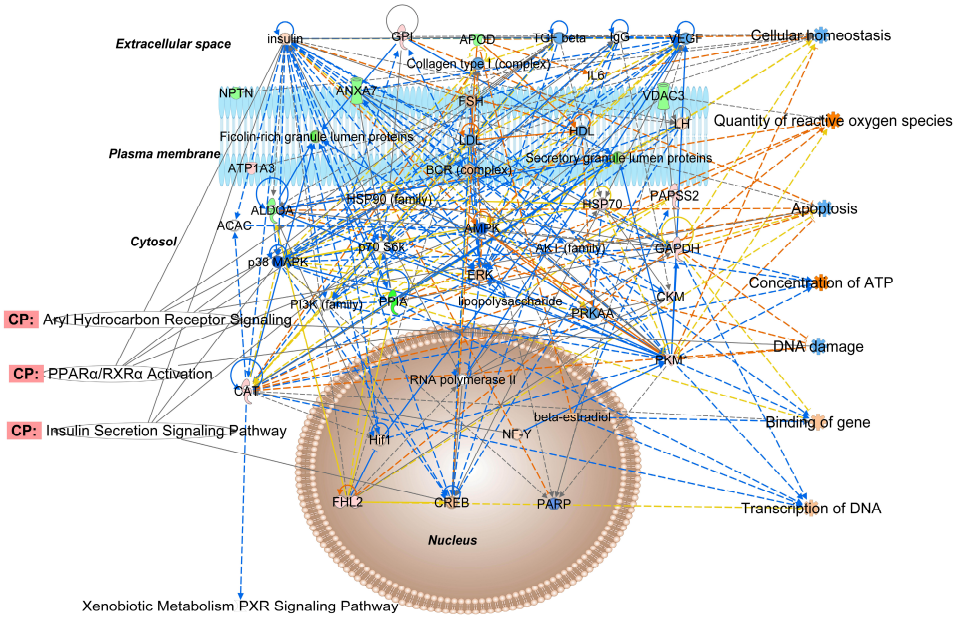
The enrichment of MHC class II antigen presentation reflects a vertebrate pathway associated with adaptive immunity [39], which crustaceans lack. This annotation was driven primarily by cathepsin A (CTSA), a protein linked to lysosomal function and protein processing [40]. Given that cathepsins play evolutionarily conserved roles in protein degradation and lysosomal homeostasis across animal taxa [41], CTSA's association is interpreted in the context of lysosomal protein processing rather than adaptive immunity.

E2-regulated DEPs associated with six IPA® protein interaction networks (Table S4). We selected the network annotated with cancer, endocrine system disorders, and organismal injury because of its relevance to exogenous estrogen exposure (Figure 1; Tables S3 and S5). These classifications reflect vertebrate functional relationships and do not imply equivalent pathological conditions in *H. azteca*. Figure 1 shows the measured DEPs (red and green nodes) together with the overlaid functions and canonical pathways. IPA®'s Investigator tool identified the functions shown on the right side of Figure 1a (male) and 1b (female), which were conserved across animal taxa. The left side shows three canonical pathways: PPARα/RXRα activation, insulin secretion signaling, and AhR signaling. Although the components of these pathways that mediate stress and xenobiotic responses are broadly conserved, their regulation in disease-specific contexts is divergent in crustaceans [42–44]. The figure also links nodes to the xenobiotic metabolism PXR signaling pathway. Because crustaceans lack a true PXR [45,46], this bioinformatic association more plausibly reflected E2-associated changes in native, functionally conserved Phase I/II xenobiotic-metabolizing enzymes.



(**a**)

Figure 1. *Cont.*



(**b**)

Figure 1. IPA®-predicted protein interaction networks for E2-exposed (**a**) male and (**b**) female *H. azteca*. Red and green nodes are DEPs measured in this study after ortholog mapping to human proteins (red, higher abundance; green, lower abundance; shade indicates magnitude). Blue and orange function nodes indicate IPA®-predicted decreases and increases, respectively, based on the abundance of the annotated DEPs. These are computational predictions and have not been demonstrated in *H. azteca*. Right side: functions identified with IPA®'s Investigator tool that are conserved across animal taxa. Left side: canonical pathways (CP) and the xenobiotic metabolism PXR signaling pathway. The components of these pathways that mediate stress and xenobiotic responses are broadly conserved, but their regulation in disease-specific contexts is divergent in crustaceans. Line style indicates relationship type (solid, direct; dashed, indirect), and line color indicates predicted direction (blue, inhibition/decrease; orange, activation/increase; yellow, undetermined). The subcellular layout (extracellular space, plasma membrane, cytosol, nucleus) reflects IPA® default annotation and does not indicate localization in *H. azteca*. Abbreviated proteins are listed in Table S6.

Based on the measured DEPs, the IPA® MAP overlay tool predicted contrasting directional responses between the sexes (Figure 1a,b, blue and orange function nodes), including ATP concentration, DNA damage, apoptosis, and transcriptional activity. ROS production was the notable exception, predicted to increase in both sexes, consistent with its known role as an early response to E2 exposure [47]. These predictions are computational hypotheses derived from vertebrate annotations and do not show that E2 caused DNA damage, apoptosis, or altered ATP levels in *H. azteca*; they require physiological and biochemical validation.

Several individual DEPs showed sex-dependent patterns. Apolipoprotein D (APOD), a protein associated with lipid transport and stress-related functions in vertebrates [48], exhibited increased abundance in males and decreased abundance in females (Figure 1). The glycolytic enzymes glucose-6-phosphate isomerase (GPI), pyruvate kinase (PKM), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed the opposite pattern, whereas aldolase A (ALDOA) was of lower abundance in both sexes. Because ALDOA is not a rate-limiting regulatory enzyme of glycolysis [49], the inverse sex-specific PKM abundance, PKM catalyzing the final, ATP-generating step, is the more plausible contributor to the divergent ATP predictions between the sexes, although ATP itself was not measured. Catalase (CAT) was of lower abundance in males and higher in females, as was ATP1A3 (a Na^+/K^+-ATPase alpha subunit), suggesting differences in antioxidant and ion-homeostasis-related proteins [50,51]. PAPSS2 followed the same pattern; while it par-

ticipates in vertebrate steroid metabolism [52,53], its physiological substrates in *H. azteca* require clarification. These sex-specific findings are consistent with prior research showing that estrogenic contaminants affect crustacean development and reproduction, such as the phenotypic effects of 17α-ethinylestradiol on *H. azteca* [28]. The present study extends these observations to molecular proteomic responses, although how protein abundance shifts translate to reproductive or developmental outcomes remains to be established.

In conclusion, this exploratory ecotoxicological study provides, to our knowledge, the first genome-enabled, MS-based proteomics profile about the response of *H. azteca* to acute E2 exposure. The protein and pathway differences are associative and cannot distinguish transient from sustained responses or establish mechanisms. Interpretation also relies on vertebrate-derived annotations that have not been confirmed in *H. azteca*. The sex-specific differences describe responses to experimental exposure and are not evidence for a physiological role of E2, which remains unresolved in crustaceans. The results therefore should be considered a hypothesis-generating dataset that identifies candidate proteins, including APOD, CAT, ATP1A3, and PAPSS2, and their associated pathways. Targeted validations, time-course and dose–response experiments, and analytical quantification of E2 in exposure media and tissues are the necessary next steps for follow-up studies.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/environments13110617/s1, Table S1: List of identified and validated proteins (SEQUEST algorithm of Proteome Discoverer 2.4 followed by Scaffold DDA 6.5.0 using MSFragger, with >95% and >99.9% probability by Peptide Prophet and Protein Prophet, respectively, with minimum of two unique peptides) from *H. azteca* obtained from all study groups; Table S2(a): List of identified and validated proteins (Scaffold DDA 6.5.0) with their orthologs. Statistical significance was determined by two-way ANOVA to observe the main effect of E2 exposure for both male and female *H. azteca*; Table S2(b): List of identified and validated proteins (Scaffold DDA 6.5.0) with their orthologs. Statistical significance was determined by two-way ANOVA to observe interaction effects: sex × exposure by comparing the E2-exposed male and female *H. azteca*; Table S2(c): DEPs between the male *H. azteca* with and without E2 exposure; proteins that met the fold change cutoffs ≥|1.5| and were statistically significant based on *t*-test considered differentially expressed; Table S2(d): DEPs between the female *H. azteca* with and without E2 exposure; proteins that met the fold change cutoffs ≥|1.5| and were statistically significant based on *t*-test considered differentially expressed; Table S3(a): IPA®-generated canonical pathways based on the submitted DEPs in the male *H. azteca*; Table S3(b): IPA®-generated canonical pathways based on the submitted DEPs in the female *H. azteca*; Table S3(c): IPA®-generated canonical pathways based on the submitted DEPs in both male and female *H. azteca* that were significant based on Z-score (|Z-score| ≥ 2) and displayed opposite regulation patterns between sexes; Table S4: List of all protein interaction network generated from DEPs from E2-exposed male and female *H. azteca*; Table S5: Disease and function associations by IPA® based on the submitted DEPs for E2-exposed male and female *H. azteca*; Table S6: List of all protein names abbreviated in Figure 1a,b with their functions.

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

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