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Long-term exposure to chemicals in sewage sludge fertilizer alters liver lipid content in females and cancer marker expression in males

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ABSTRACT

Background: The increased incidence of diseases, including metabolic syndrome and infertility, may be related to exposure to the mixture of chemicals, which are ubiquitous in the modern environment (environmental chemicals, ECs). Xeno-detoxification occurs within the liver which is also the source of many plasma proteins and growth factors and plays an important role in the regulation of homeostasis.

Objectives: The objective of this study was to investigate the effects of ECs on aspects of liver function, in a well characterized ovine model of exposure to a real-life EC mixture.

Methods: Four groups of sheep (n = 10–12/sex/treatment) were maintained long-term on control or sewage sludge-fertilized pastures: from conception to culling at 19 months of age in females and from conception to 7 months of age and thereafter in control plots until culling at 19 months of age in males. Environmental chemicals were measured in sheep livers and RNA and protein extracts were assessed for exposure markers. Liver proteins were resolved using 2D differential in-gel electrophoresis and differentially expressed protein spots were identified by liquid chromatography/tandem mass spectroscopy.

Results: Higher levels of polycyclic aromatic hydrocarbons (PAHs) and lower levels of polychlorinated biphenyls (PCBs) in the livers of control males compared to control females indicated sexually dimorphic EC body burdens. Increased levels of the PAHs Benzo[*a*]anthracene and chrysene and reduced levels of PCB 153 and PCB 180 were observed in the livers of continuously exposed females. EC exposure affected xenobiotic and detoxification responses and the liver proteome in both sexes and included major plasma-secreted and blood proteins, and metabolic enzymes whose pathway analysis predicted dysregulation of cancer-related pathways and altered lipid dynamics. The latter were confirmed by a reduction in total lipids in female livers and up-regulation of cancer-related transcript markers in male livers respectively by sewage sludge exposure.

Conclusions: Our results demonstrate that chronic exposure to ECs causes major physiological changes in the liver, likely to affect multiple systems in the body and which may predispose individuals to increased disease risks.

1. Introduction

There is increasing evidence that exposure to environmental chemicals (ECs), including Endocrine-Disrupting Compounds (EDCs), persistent organic pollutants (POPs), pharmaceuticals, their metabolites, and toxic elements such as heavy metals, contributes to the initiation and/or progression of modern human diseases including diabetes, obesity and infertility (Foulds et al., 2017; Petrakis et al., 2017; Heindel et al., 2015; Vabre et al., 2017). Human real-life EC exposure is chronic and involves exposure to a complex mixtures of chemicals, each at

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relatively low concentrations. Because of their diverse chemical structures, exposure occurs via multiple routes and, once within the body, ECs can be metabolized/stored or excreted via a variety of ways. The study of EC effects therefore presents many challenges in terms of measuring exposure or mimicking real-life exposure patterns. Human sewage sludge contains a complex mixture of chemicals which reflects human EC exposure (Venkatesan and Halden, 2014; Zhang et al., 2015; Rhind et al., 2013; Rhind et al., 2002). We have demonstrated, using sheep which are large, long-lived mammals with long gestation times as a model to human real-life exposures, that low-level exposure to such EC mixtures via grazing of pregnant ewes on pastures treated with sewage sludge as a fertilizer adversely affects thyroid, ovarian, testicular, hypothalamic and pituitary development and function and social behavior of the offspring (Lea et al., 2016; Bellingham et al., 2009; Bellingham et al., 2012; Bellingham et al., 2013; Bellingham et al., 2016; Hombach-Klonisch et al., 2013; Fowler et al., 2008; Paul et al., 2005; Lind et al., 2009; Erhard and Rhind, 2004; Evans et al., 2014). In our exposure model typical concentrations of selected polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and polycyclic aromatic hydrocarbons (PAHs) in sewage sludge are in the range of 75, 559, and 14,245 μg/kg respectively versus control inorganic fertilizer with 0.32, 9.11 and 0 μg/kg of the same pollutants (Table 2 from Lind et al., 2009).

The liver serves many roles within vertebrates, namely detoxification of substances, synthesis of proteins and clotting factors, and the production of biochemicals needed for digestion. The liver is an integral part of the endocrine system, metabolizing many circulating hormones (O'Shaughnessy et al., 2013) and secreting hormones such as insulin-like growth factors 1/2 and angiotensinogen. The liver also regulates hormonal bioavailability by secreting hormone-binding proteins including alpha fetoprotein (AFP), serum albumin (ALB), sex-hormone binding globulin (SHBG), corticosteroid binding globulin (CBG) and thyroxine binding globulin (TBG). Furthermore, the liver secretes carrier proteins, including serotransferrin (TF), ceruloplasmin (CP) and apolipoproteins to control transport of nutrients. ECs are measurable within the liver (Geens et al., 2012) where multiple classes of ECs may be detoxified. Through modification of liver functions ECs can contribute to liver disease (Yorita Christensen et al., 2013) whereas changes in liver endocrinology may contribute to other adverse EC effects, including those associated with altered metabolism and/or infertility. A recent report inventoried chemicals that are known to promote liver disease and found 123 chemicals in a range of classes, including endocrine-disrupting chemicals (EDCs), to be harmful to the liver (Al-Eryani et al., 2015).

Because sewage sludge contains many ECs and the liver has a major role in clearance of potentially harmful ECs, we have measured levels of major ECs in liver tissues and assessed liver responses to EC exposure by measurement of transcripts, proteins, and lipids. We show that proteins, transcripts, lipids and EC concentrations are altered in the livers animals chronically exposed to low level mixtures of multiple ECs.

2. Materials and methods

2.1. Ethical standards

All experiments were approved by the James Hutton Institute's Local Ethical Committee and performed according to relevant guidelines and regulations. Animal experiments were conducted under Project license (60/3356) (United Kingdom's Animals Scientific Procedures Act 1986).

2.2. Animals

This study used our well established ovine chronic EC exposure model where sheep are maintained on plots fertilized with either sewage sludge at conventional rates (exposed plots) or with inorganic fertilizer containing equivalent amounts of nitrogen (control plots) as described previously (Bellingham et al., 2016; Rhind et al., 2013; Bellingham et al., 2013; Hombach-Klonisch et al., 2013; Bellingham et al., 2012; Bellingham et al., 2009; Rhind et al., 2010; Fowler et al., 2008; Paul et al., 2005) at the Glen Saugh experimental farm near Aberdeen, Scotland. Power calculations using representative data from pilot studies (Rhind et al., 2010; Paul et al., 2005) suggested that 8 F1 animals in each of the four groups (control males, control females, exposed males, exposed females) can achieve over 80% power to detect exposure differences and that 12 F0 ewes per treatment would be required to produce at least 8 lambs of either sex.

Texel ewes (*Ovis aries*) with a regular age structure i.e. with similar numbers of ewes aged 2, 3, 4 and 5 years at the time of lambing (F0 generation) were randomly allocated in control or exposed plots (48 ewes per treatment) where they were maintained through their breeding lives. The F0 ewes were synchronized using chronogest vaginal sponges (20 mg flugestone acetate) for 14 days and mated naturally 2 days after sponge withdrawal to Texel rams. The mated ewes remained in their respective treatment plots through the pregnancy and after giving birth. Randomly selected 48 F1 lambs (12 control males, 12 control females, 12 exposed males, 12 exposed females) remained in their respective treatment plots throughout lactation, weaning and post-weaning. On their 7th month of age all the males were transferred to control plots to avoid uncontrolled mating and to meet other requirements of the experimental program whereas females remained in their allocated control or exposed plots. On the 19th month of age the F1 sheep were euthanized using indwelling jugular catheter following subcutaneous local anesthesia (lignocaine) of the site followed by barbiturate overdose as determined by Schedule 1 ASPA 1986. The 24 F1 females were synchronized using chronogest vaginal sponges (20 mg flugestone acetate) for 14 days prior to catheter insertion and euthanasia to minimize variation arising from different cycling hormonal differences. Upon euthanasia, the chronogest vaginal sponges were found absent in 3 females resulting in their subsequent exclusion from the study. Final numbers of F1 sheep analyzed (experimental units) were 12 control males, 11 control females, 12 exposed males, and 10 exposed females. Randomization of F0 ewes and F1 lambs was performed by random draws, ensuring equal number in each sex for F1 lambs. Vaginal sponge insertions, catheter insertions, euthanasia, measurements and all analyses were performed by researchers blind to exposure and during the morning hours. Body condition score (on a scale of 1–5 where 1 = emaciated and 5 = obese) was measured at euthanasia as previously described (Russel, 1991).

2.3. Liver collection and protein, DNA, and RNA extraction

Liver tissue samples were washed with phosphate buffer to remove red blood cells and then frozen at −80 °C until analysis. Qiagen AllPrep DNA/RNA/Protein mini kits (Qiagen Ltd., Crawley, UK; cat. no. 80004) were used to isolate DNA, RNA and proteins (Bellingham et al., 2013; Filis et al., 2015). DNA contamination in the RNA preparations was minimized by treating RNA with RNAse-free DNAse (Qiagen Ltd., Crawley, UK; cat. no. 79254) according to the manufacturer's guidelines. DNA and RNA concentrations were determined using Nanodrop and protein concentrations were determined by a modified Lowry assay (RC DC™ Protein Assay Kit, Bio-Rad Laboratories Ltd., Hertfordshire, UK; cat.no. 5000121).

2.4. Tissue chemical analysis

Concentrations of diethylhexyl phthalate (DEHP), selected PCB congeners (28, 52, 101,118, 138, 153, 180), PBDE congeners (28, 47, 99, 100, 153, 154, 183) and 16 PAHs were measured in male and female liver samples using gas chromatography coupled to mass spectrometry (GC/MS) following sample extraction and preparation as previously described (Lind et al., 2009; Rhind et al., 2010). Quality

control samples were included with each batch of experimental samples analyzed. Limits of detection: DEHP, 0.01 μg/g; PCBs, 0.02 μg/kg; PBDE 28, 47, 99 & 100, 0.02 μg/kg; PBDE 153, 154 & 183; 0.50 μg/kg; PAHs, 1 μg/kg, except for phenanthrene, fluoranthene, benzo[*k*]fluoranthene, indenol[1,2,3-*cd*]pyrene and dibenzo[*a*,*h*]anthracene, for which they were 5 μg/kg and pyrene, for which it was 15 μg/kg.

2.5. Superoxide Dismutase (SOD) activity and Protein Carbonyl (PC) content measurements

Approximately 100 mg of frozen liver tissue were homogenized in 1 ml cold buffer (0.05 M potassium phosphate and 0.1 mM EDTA, pH 7.8) and centrifuged at 15,000*g* for 30 min at 4 °C. Aliquots of the supernatant were diluted 1:1000 and used for determination of superoxide dismutase activity (Superoxide Dismutase Assay Kit, Cayman Chemical), and protein carbonyl content (Protein Carbonyl Colorimetric Assay Kit, Cayman Chemical) as per the manufacturer's instructions. Samples were assayed in duplicate and results were expressed relative to protein concentration, which was quantified using the Quick Start Bradford Protein microplate Assay (Bio-Rad) as per manufacturer's instructions with Bovine Serum Albumin (BSA) (range 125–2000 μg/ml) as a standard.

2.6. Real-time PCR

1 μg of the RNA from the extracted liver samples was reverse transcribed (GoScript Reverse transcription system, Promega Ltd., Southampton, UK; cat no A5001). Real-time PCRs were conducted using the SensiMix real-time PCR kit (Bioline, London, UK; cat no. QT650-05) and carried out on 384 well plates using the Roche Lightcycler 480. *B2M* and *ACTB* were identified as the most stable housekeeping genes from a group of 5 housekeeping genes tested using both GeNorm and NormFinder software (Fig. S1A). Primers for the genes of interest were designed using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exons in order to avoid amplicons from contaminating genomic DNA. Absence of amplicon generation from genomic DNA was confirmed by testing the primers on mock cDNA preparations lacking reverse transcriptase enzyme. Primer sequences, amplicon sizes and primer annealing temperatures are shown in Table S1. Each gene was quantified against a standard curve of serially diluted representative sheep liver cDNA preparations (concentration span: 5 ng/μl to 0.008 ng/μl). Relative gene amounts were normalized against the geometric mean of the relative amounts of the housekeeping genes *ACTB* and *B2M* using the formula:

$$gene\ of\ interest/\sqrt[2]{ACTB * B2M}.$$

2.7. Proteomic 2-D gel electrophoresis and DIGE analysis

Within each group, equal quantities (125 μg) of liver protein from all the F1 animals were pooled according to sex and EC exposure (control females, 11 samples; treated females, 10 samples; control males, 12 samples; treated males, 12 samples). This generated four separate protein pools which were analyzed by DIGE in quadruplicate as follows: from each pool 50 μg of protein were labelled with CY3 and another 50 μg of protein were labelled with CY5 fluorophores. CY3- and CY5-labelled proteins from each pool were combined and subjected to 2D-PAGE in sufficient permutations to ensure four individual separations for each protein pool. Fluorescent labels of the protein pools were reversed on separate gels to avoid labelling bias. For each 2D-PAGE separation, the CY3- and CY5-combined protein pools were run in the presence of a control CY2-labelled protein sample (50 μg), prepared from equal amounts of protein from each group (i.e. 12.5 μg/group) to assist spot mapping. 2D-PAGE was performed and gels were scanned using an Ettan DIGE Imager (GE Healthcare). Samespots software was used to detect spots and quantify fluorescent spot volumes. Selected spots were excised from gels and subjected to LC-MS/MS spot identification as previously described (Bellingham et al., 2013; Filis et al., 2015).

2.8. Western blot protein level quantifications

Total levels of ALB, TF, HSP90 and HSP70 proteins were quantified by Western blotting in the same 45 protein extracts used to construct the proteomics protein pools. Protein membranes were prepared, probed and quantified as previously described (Bellingham et al., 2013; Filis et al., 2015). The list of the antibodies used and their dilution is shown in Table S2. Levels of ALB and TF were normalized against ACTB signal used as loading control. ACTB levels did not vary between samples with either sex or sewage sludge exposure (Fig. S1B*vi*). 2D-western blots performed to verify the identity of additional ALB and TF spots were prepared by separating 50 μg of sheep liver protein pool prepared from equal amounts of protein from each animal.

2.9. Lipid assays

Approximately 250 mg of frozen liver tissue were homogenized in 1 ml of 0.145 M ice cold NaCl. After the addition of 10 ml of chloroform:methanol (2:1 v/v) containing 50 μg/ml butylated hydroxytoluene (BHT), the mixture was gently mixed for 10 min. Following the addition of 3 ml of distilled H_2O, the phases were separated by centrifugation for 10 min at 3000 rpm and the upper aqueous phase was removed. The organic layer was re-extracted with 3 ml of chloroform:methanol:0.145 M NaCl (3:48:47) that had 50 μg/ml BHT added. The lower lipid chloroform phase was transferred to a clean glass tube. The combined aqueous phases were washed with 3 ml of chloroform and after phase separation the chloroform was removed and combined with the rest of the chloroform fractions. The chloroform extract was evaporated under nitrogen stream and dried over desiccant. The weight of the residue represented the total lipids yield, expressed as w/w% of the wet liver weight. Liver water content was determined by drying weighed ~250 mg pieces of liver over silica in a desiccator for 2 weeks.

Phospholipids were estimated colorimetrically by forming a complex with ammonium ferrothiocyanate (Stewart, 1980). Briefly, the total lipids were resuspended in 2 ml of chloroform and duplicate 25 μl aliquots were further diluted to final volume of 2 ml with chloroform. After the addition of 1 ml of complexing reagent (0.1 M ferric chloride, 0.4 M ammonium thiocyanate) the mixture was vortexed gently. The phases were separated by centrifugation, the lower chloroform layer was removed and its absorbance was read at 488 nm. The absorbance was compared against a standard curve of phosphatidylcholine (Sigma chemical Co, Poole, Dorset UK). Phospholipid estimations were expressed as w/w% of the wet liver weight for each sample.

Triglyceride and total cholesterol levels were measured in chloroform extracts as described previously (McNeil et al., 2008). Briefly, chloroform was removed by evaporation under nitrogen and total lipids dissolved in 5 ml of ethanol. Triglycerides in 40 μl aliquots of the ethanol suspension were measured by adding 0.25 ml triglyceride reagent (ThemoElectron Triglyceride Kit cat. no. TR22421), incubating for 20 min at room temperature and measuring the absorbance at 510 nm. Triglyceride concentrations in the samples were determined from a standard curve of glycerol (0–40 nmoles) and were expressed as nmoles per mg of wet liver weight as well as w/w% of wet liver weigh by multiplying triglyceride nmoles by 0.000885. Cholesterol in 10 μl aliquots of the ethanol suspension were measured by adding 0.25 ml Cholesterol reagent (ThemoScientific cat. no. 981812), incubating for 10 min at room temperature and measuring the absorbance at 520 nm. Cholesterol concentrations in the samples were determined from a standard curve of Lipatrol (ThermoScientific cat. no. 981653, containing 4.8 mM of stock cholesterol) spanning 0–1 mM. Total

cholesterol levels were expressed as w/w% of the wet liver weight for each sample. All measurements were performed in duplicate.

2.10. Statistics and ingenuity pathway analysis

Statistical analysis of quantified ECs, transcripts, proteins and lipids was performed using 1-way ANOVA (control vs treatment for the exposure effect in females and males respectively; control females vs control males for sex differences) in R statistical software (V3.4.0). Data were log-transformed in those cases where model residuals departed from Normality (visually assessed by quantile-quantile residual plots) and/or showed heteroscedasticity (visually assessed by scatter plot of residuals vs fitted model values). Statistical significance threshold was $P < 0.05$. Spot volumes from gel-based proteomics were analyzed in R (V3.4.0) using the *limma* package following the same statistical approach as above. *P*-values were adjusted using the Benjamini-Hochberg False Discovery Rate to control for multiple testing. Statistical significance threshold was chosen to adjusted P values of < 0.05. Multidimensional scaling was produced using the *plotMDS* function in R.

Ingenuity Pathway Analysis (IPA) V9.0 (Ingenuity Systems, http://www.ingenuity.com) was used to assign affected proteins and transcripts from transcript measurements, Western blots, and proteomics to Disease or Functions Pathways (Bellingham et al., 2013; Filis et al., 2015). Statistical significance was calculated by the right-tailed Fisher Exact Test with a threshold of $P < 0.05$). Only those Disease & Functions Pathways that were predicted on the basis of at least 10 molecules or yielded activation scores above ± 1.5 were considered relevant.

3. Results

3.1. Sheep livers show sex-specific EC accumulation and xenobiotic responses

EC liver accumulation showed sex-specific patterns in control animals and male livers had higher levels of benzo[*a*]anthracene, chrysene, and total PAHs, whereas female livers had higher levels of naphthalene, PCB 101, PCB 153 and total PCBs (Table 1). Liver accumulation of benzo[*a*]anthracene and chrysene increased and of PCB 153, PCB 180, and total PCBs decreased in the sewage sludge exposed females. No differences in liver EC accumulation were observed in sewage sludge exposed males (Table 1).

Control male livers were heavier compared to control female livers and expressed higher levels of HSP90B and HSP70 proteins (Tables S3 and S4). EC exposure did not induce gross alterations in liver weight, body weight-normalized liver weight, and the average body condition score of 2.6 in sheep (Table S3). EC exposed females had higher levels of *CYP1A1* and lower levels of *CYP1B1* detoxifying enzyme transcripts and expressed higher levels of HSP90B protein (Fig. 1, Table S4). *CYP1A2* detoxifying enzyme and estrogen receptor alpha (*ESR1*) transcripts and HSP70 protein expression increased and cMOAT2 (gene: *ABCC3*) transporter transcripts decreased in EC exposed females but those differences did not reach statistical significance (Fig. 1, Table S4). Aryl hydrocarbon receptor (*AHR*), OATP8 (gene: *SLCO1B3*) importer and Phosphoenolpyruvate Carboxykinase 1 (*PCK1*) transcripts increased following EC exposure in males with the *PCK1* increase approaching statistical significance (Fig. 1, Table S4).

3.2. Large-scale molecular effects of sewage sludge exposure on the liver

445 liver protein spots were reproducible across Cy3- and Cy5-stained 2-D PAGE gel replicates. Multidimensional scaling of all protein spot volumes showed that control female livers cluster separately to control male livers and that exposed female livers cluster closer to control males in the 1st dimension (highest variation explained) but further apart from either controls females or control/exposed males in the 2nd dimension (second-highest variation explained) (Fig. 2A). Of the 445 spots analyzed, 185 spot volumes were significantly affected in females (98 up-regulated and 87 down-regulated) and 97 spot volumes were significantly affected in males (57 up-regulated and 40 down-regulated) (Fig. 2B). 145 spots exhibited sex differences in control animals, with 70 and 75 spots showing increased spot volumes in males and females respectively (Fig. 2C). 49 of the largest, most reproducible, spots were excised for LC-MS/MS identification and of these 35 spots were positively identified based on (i) high MASCOT score, (ii) agreement between estimated (i.e. from electrophoretic gel mobility) and calculated molecular weight and isoelectric point, and (iii) peptide coverage. These 35 spots gave rise to the identification of 26 proteins with some proteins appearing in more than one spot (e.g. ALDH1L1; NLTP; IDH1; ACAA2; INMT; GSTM1, Table 2). All the identified proteins and their functions are detailed in Tables 2 and S7). EC exposure altered the expression of proteins involved in detoxification; GSTT1 was upregulated by exposure by both sexes, and GSTM1 was upregulated in exposed females only (Table 2). Fatty-acid β-oxidation enzymes were also affected; ECHS1 was reduced in exposed females, ACAA2 spot volumes were predominantly increased in exposed females whereas for ACAA2 in exposed males one spot increased and one decreased (Table 2, Fig. S2).

Exposure also affected the spot volumes of the proteins secreted into the plasma; ALB was increased in both males and females, TF increased in males and APOA1 decreased in females (Table 2). 2D-Western blots probed against ALB and TF and superimposed on the DIGE scans identified additional spots that correspond to ALB and TF (Fig. 3A). Total ALB and TF levels, quantified using conventional 1D-Western blots showed similar trends to DIGE-stained summed ALB and TF spots respectively on the 2D gels (Figs. 3B, S3). All ALB and TF spots were upregulated by exposure in males (bars in Figs. 3C*i-ii*; S3). Exposure also increased the distribution of ALB spots towards more negatively-charged species in males (Fig. 3C*i*; solid lines). In females, exposure affected only a single ALB spot (Figs. 3C*i*, spot 263, S3A, Table 2) whereas female TF spots were affected in a charge-specific manner with more negatively-charged spots being downregulated and more positively-charged spots upregulated (bars in Fig. 3C*ii*; Fig. S3B). Exposure increased the distribution of TF species towards the more positively-charged species in females (Fig. 3C*ii*; dashed lines).

Transcripts for many of the identified proteins were measured to examine whether they correlate to the spot volume changes. *SCP2*, *ACAA2*, *ECHS1*, and *GSTM1* transcript changes correlated with the observed sex-differences but transcript differences did not reach statistical significance (Tables S4 and S5). *ALDH1L1*, *DES*, *ACAA2*, *TPI*, *GSTM1*, and *GSTT1*, transcripts changes correlated with spot volume exposure differences in females but none of the transcript differences reached statistical significance (Tables S4 and S5). In exposed males, *ALDH1L1*, *TF*, *GSTT*, and *FTL* transcripts changes correlated with spot volume exposure differences but only *TF* and *GSTT1* reached statistical significance (Tables S4 and S5).

3.3. EC exposure differentially affects canonical and disease pathways in both sexes and causes alterations in liver lipid content

Ingenuity Pathway Analysis of sex-different proteins and transcripts in control groups predicted that, compared to females, male livers have decreased proliferation of lymphocytes, decreased invasion of cells, increased synthesis of reactive oxygen species and increased cell viability (Table S6). Analysis of the effects of EC exposure in females predicted dysregulation of cancer-related pathways, increased production of reactive oxygen species and decreased concentration of lipids (Table S6). Pathway analysis in EC exposed males predicted reduction and dysregulation of cancer-related pathways, increased lipid synthesis and increased fatty acid metabolism (Table S6).

To examine the predicted effects of EC exposure on lipid concentrations, reactive oxygen species and cancer-related pathways, we

Table 1
Sex- and exposure-specific adult liver concentrations of environmental chemicals (ECs) following fetal and lactational exposures. EC concentrations are normalized as μg per kg of dry liver matter Data were log-transformed to adjust for those cases where model residuals departed from Normal distribution. Statistically significant differences ($P < 0.05$) are shown in bold.

Class	Chemical[a]	Sex effect[a] (♂ compared to ♀)		Exposure effect ♀		Exposure effect[b] ♂		Average value ± SEM[c]
		P value	Fold diff	*P* value	Fold diff	*P* value	Fold diff	
Phthalate	DEHP	0.95	−1.02	0.30	−1.64	0.83	1.45	0.51 ± 0.08
PAH	Naphthalene	**< 0.0001**	**−7.07**	0.18	−1.95	0.38	2.90	6.7 ± 1.14
	Acenaphthalene	*	*	*	*	0.68	1.10	1.2 ± 0.24
	Acenaphthene	0.47	2.28	*	−1.89	0.85	−1.73	2.1 ± 0.62
	Fluorene	0.44	3.32	0.58	2.00	0.79	1.64	4.2 ± 1.37
	Phenanthrene	0.29	−2.60	0.12	−4.65	0.79	1.05	14.3 ± 4.08
	Anthracene	*	−2.65	*	−4.48	*	1.03	2.0 ± 0.84
	Fluoranthene	*	*	*	*	*	*	2.7 ± 0.24
	Pyrene	*	*	*	1.79	*	*	8.2 ± 0.5
	Benzo[*a*]anthracene	**0.011**	**2.75**	**0.02**	**3.53**	0.86	2.22	9.1 ± 2.35
	Chrysene	**0.0013**	**3.60**	**0.0044**	**3.16**	0.13	2.30	71.5 ± 16.9
	Benzo[*b*]fluoranthene	*	*	*	*	*	−1.27	2.9 ± 0.29
	Benzo[*k*]fluoranthene	*	1.93	*	3.05	0.95	−1.18	1.8 ± 0.38
	Benzo[*a*]pyrene	0.087	2.37	0.25	−1.06	0.31	−2.02	38.8 ± 7.6
	Indeno[1,2,3-*cd*]pyrene	*	*	*	*	*	−3.46	5.6 ± 2.7
	Dibenzo[*a,h*]anthracene	*	*	*	*	*	*	*
	Benzo[*ghl*]perylene	*	*	*	*	*	*	0.8 ± 0.22
	Total PAHs	**0.019**	**1.98**	0.23	1.36	0.33	1.41	144 ± 20.3
PBDE	28	*	*	*	*	*	*	0.026 ± 0.01
	47	0.170	1.41	0.64	−1.10	0.54	−1.20	0.24 ± 0.02
	99	*	3.9	*	*	*	−1.55	0.03 ± 0.01
	100	*	*	*	*	*	*	*
	153	*	*	*	*	*	*	*
	154	*	*	*	*	*	−1.20	0.33 ± 0.06
	183	*	*	*	*	*	*	0.26 ± 0.014
	Total PBDE	0.28	1.15	0.49	−1.07	0.82	−1.09	1.15 ± 0.06
PCB	28	0.085	−1.68	0.09	−1.61	0.81	1.21	0.026 ± 0.002
	52	*	*	*	1.23	*	*	0.014 ± 0.001
	101	**0.008**	**−3.63**	*	−3.33	*	−1.07	0.02 ± 0.003
	118	*	−2.00	0.18	−1.86	*	1.21	0.02 ± 0.003
	138	0.53	1.38	*	−2.24	0.25	7.03	0.08 ± 0.03
	153	**0.0008**	**−3.08**	**0.022**	**−1.49**	0.52	−1.38	0.15 ± 0.014
	180	0.75	1.22	**0.026**	**−2.28**	0.80	−1.46	0.026 ± 0.005
	Total PCB	**0.0019**	**−2.39**	**0.016**	**−1.56**	0.61	−1.25	0.22 ± 0.02

[a] Only data from control males and females are compared.
[b] EC data for males have previously been reported in Bellingham et al., 2012 and are shown here to enable comparison with females.
[c] Units: μg/kg wet liver tissue except for DEHP: μg/g wet liver tissue.
* ECs with values above the limit of quantitation in < 3 animals in each group.

measured lipids, SOD activity and PC content, and the expression of transcript markers of liver cancer. The livers of EC exposed sheep had reduced total lipid levels but in males this reduction did not reach statistical significance (Fig. 4A). Liver phospholipids, cholesterol and triglyceride levels were unaltered by exposure in either sex whereas control males had higher liver cholesterol levels (Fig. 4A). Liver SOD activity and liver PC content were higher in EC exposed females but neither of those differences reached statistical significance, whereas control males had higher liver PC content (Fig. 4B, Table S3). Exposed males had increased levels of *AFP*, POU Class 5 Homeobox 1 (*POU5F1*) and Glypican 3 (*GPC3*) transcripts but only AFP transcript differences reached statistical significance (Fig. 4C, Table S4).

4. Discussion

A variety of the effects of in utero exposure to a complex mixture of ECs have been documented in the fetus using our ovine model (Lea et al., 2016; Bellingham et al., 2013; Bellingham et al., 2016; Hombach-Klonisch et al., 2013; Fowler et al., 2008; Paul et al., 2005). At least some of the effects noted in the fetus have been shown to persist into adulthood (Bellingham et al., 2012; Lind et al., 2009; Erhard and Rhind, 2004). This study demonstrates for the first time that following continuous EC exposure via sewage sludge from pre-conception into adulthood, the liver shows multiple disruptions including proteome-wide alterations and that many disruptions may be markedly persistent after exposure cessation. The liver is a metabolically and developmentally critical organ and if these results were translated to humans, who are also continuously exposed to low levels of complex mixtures of ECs, they would be expected to have serious impacts on a wide variety of aspects of adult health and wellbeing, such as liver disease and/or liver cancer.

In this study we observed sex-specific accumulation of ECs in the control animals; specifically males were seen to accumulate higher levels of PAHs and females higher levels of PCBs. Sex-specific accumulation of ECs has been observed in the organs in other animals, with a trend for higher EC accumulation in males than females (human lungs, Goldman et al., 2001; fish liver, Vives et al., 2004; human placenta, Leonetti et al., 2016; beluga blubber and liver, Becker et al., 2000). The sex-specific accumulation of ECs as well as the male-specific increase of proteins relating to stress responses (HSP70 and HSP90B) and detoxification (GSTM1 and INMT) in controls (Tables 2, S4) may reflect physiological differences between males and females in how they process xenobiotics and provides support that the liver is functionally sexually dimorphic (Roy and Chatterjee, 1983; Rando and Wahli, 2011; Filis et al., 2015; Drake et al., 2015; O'Shaughnessy et al., 2013; O'Shaughnessy et al., 2011).

Because in male sheep the chronic exposure stopped 12 months before culling, it is likely that many exposure-related differences will

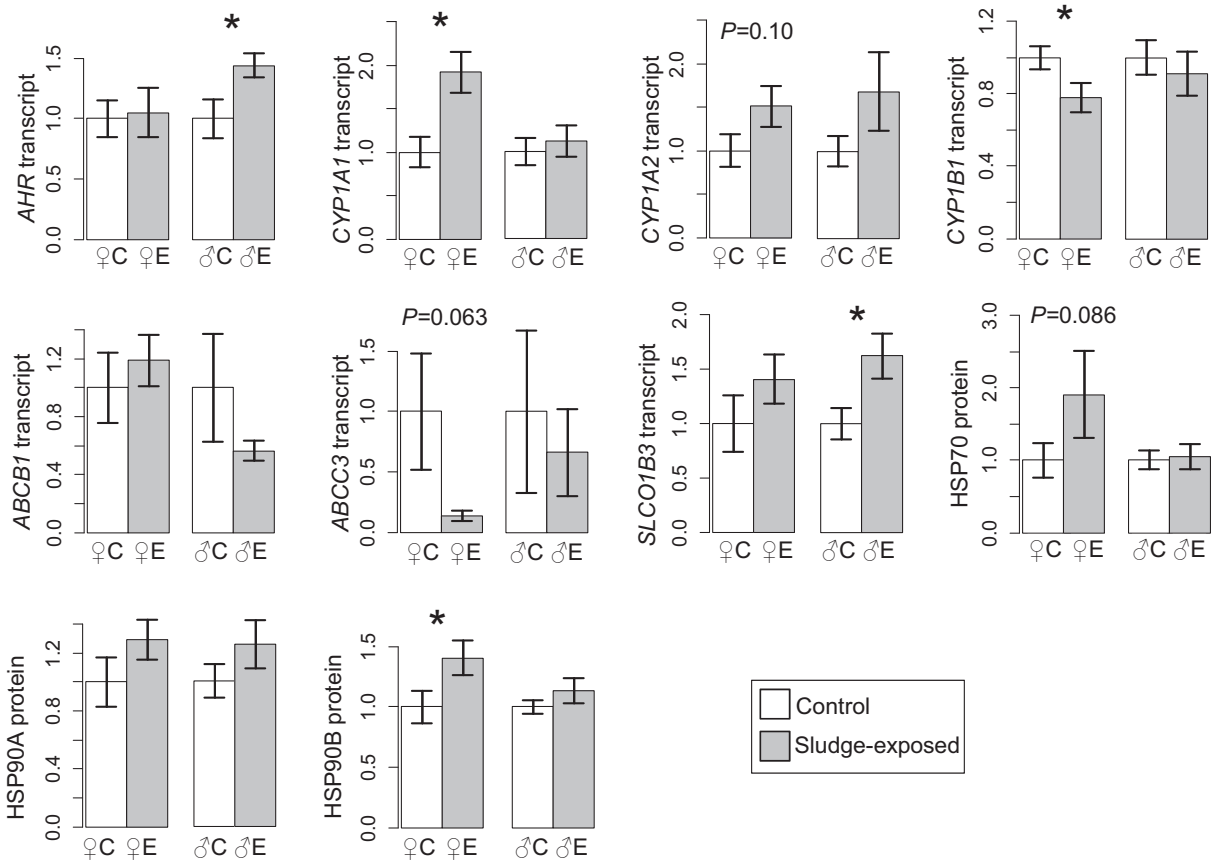

Fig. 1. Effects of sewage-sludge exposure on detoxification and xenobiotic metabolism in the sheep liver. Sewage sludge exposure was associated with increased *AHR* and *SLCO1B3* transcripts in male livers and with increased *CYP1A1* and decreased *CYP1B1* transcripts and increased HSP90B protein levels in female livers. Significant ($P < 0.05$) differences between groups are indicated by asterisks above the bars. Near-significant differences ($0.05 < P \leq 1$) are indicated as *P* values above the bars. Error bars denote ± SEM.

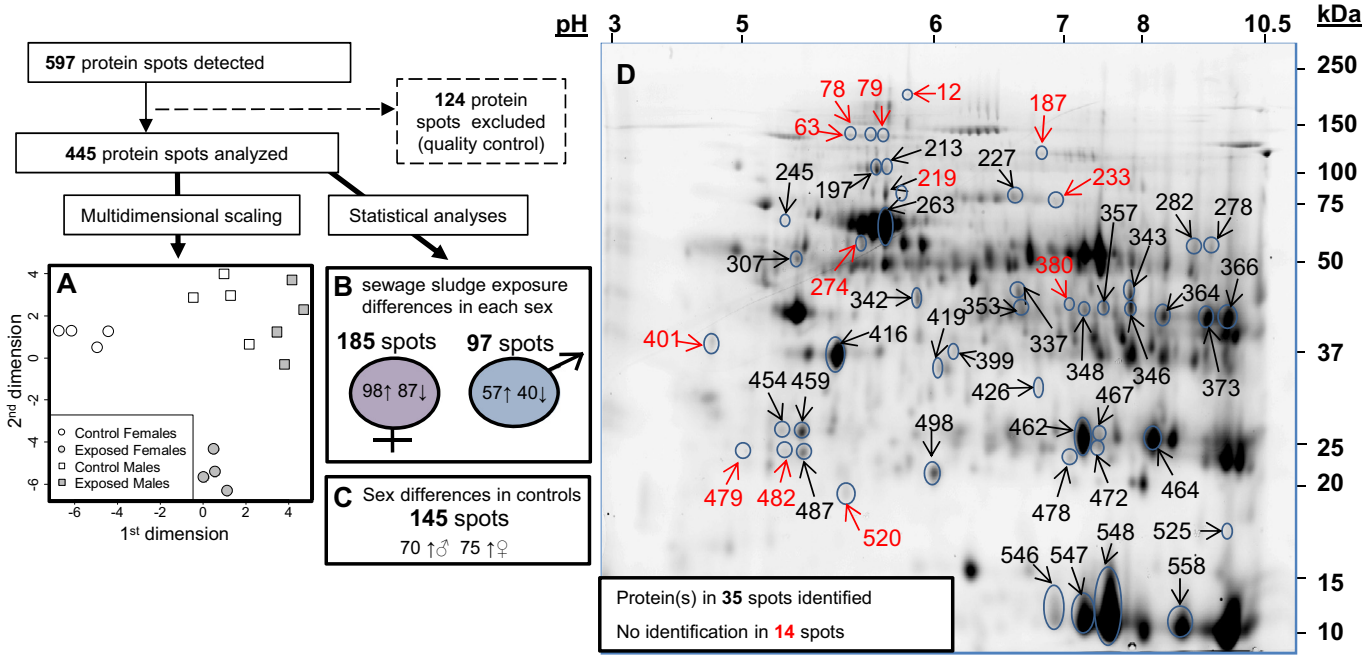

Fig. 2. Summary of proteomic analysis of sheep livers, showing an overview of spots analyzed. A. multidimensional scaling. B. numbers of differentially expressed spots in each sex. C. numbers of spots in control animals with sexually-divergent spot volumes. D. representative 2D-PAGE DIGE gel indicating the spots sent for LC-MS/MS identification.

Table 2
Protein candidates identified by LC-MS/MS analysis of protein spots. Spot numbers with an asterisk indicate detection of more than one candidate protein. For clarity, only fold-changes that achieved significance (adjusted *P* value < 0.05) are shown.

Spot #	Gene symbol	Protein name	Mascot score	Accession number	Peptide coverage (%)	Sex effect (controls only)	Exposure effect	
							♀	♂
197	*ALDH1L1*	Cytosolic 10-formyltetrahydrofolate dehydrogenase	228	XP_004018762	21		1.62	
213	*ALDH1L1*	Cytosolic 10-formyltetrahydrofolate dehydrogenase	103	XP_004018762	16		1.47	1.96
227	*TF*	Serotransferrin	77	XP_004003379	20			1.60
245	*LCP1*	Lymphocyte cytosolic protein 1 (L-plastin)	38	XP_004012112	9	−1.61		
263	*ALB*	Serum albumin	366	NP_001009376	37	2.63	1.23	1.62
278	*SCP2*	Non-specific lipid-transfer protein	78	XP_004002037	19	1.85		
282	*SCP2*	Non-specific lipid-transfer protein	89	XP_004002037	17	1.53	1.24	
307	*DES*	Desmin	262	XP_004005001	34	−1.98	−1.88	
337	*IDH1*	Isocitrate dehydrogenase 1	53	NP_001009276	19	1.48	1.30	1.38
342	*AHCY*	Adenosylhomocysteinase	92	XP_004014555	28	1.56		
343*	*ACAA2*	Acetyl-Coenzyme A acyltransferase 2	70	XP_004020712	15	1.33	1.21	1.22
	FH	Fumarate hydratase	52	NP_001155363	12			
346	*ACAA2*	Acetyl-Coenzyme A acyltransferase 2	73	XP_004020712	27	1.40	1.30	
348	*BHMT*	Betaine-homocysteine *S*-methyltransferase 1	45	XP_004010258	23	1.95		1.30
353	*IDH1*	Isocitrate dehydrogenase 1	37	NP_001009276	20	1.34	1.44	1.31
357	*ACAA2*	Acetyl-Coenzyme A acyltransferase 2	163	XP_004020712	42	1.31		−1.44
364	*ACAA2*	Acetyl-Coenzyme A acyltransferase 2	659	XP_004020712	64	1.89		
366	*ACAA2*	Acetyl-Coenzyme A acyltransferase 2	325	XP_004020712	54	2.63	1.31	
373	*ACAA2*	Acetyl-Coenzyme A acyltransferase 2	430	XP_004020712	37	3.05	1.55	
399*	*ALAD*	Delta-aminolevulinic acid dehydratase	57	XP_004004041	9	−1.52	−1.43	
	ANXA1	Annexin A1	45	XP_004004354	19			
416	*RGN*	Regucalcin	331	NP_001124407	63	1.29	1.15	1.21
419	*LDHB*	L-Lactate dehydrogenase B	55	XP_004006841	16	−1.56	1.16	1.45
426	*SULT1A1*	Sulfotransferase A1	38	XP_004020920	18	−1.48		−1.22
454	*INMT*	Indolethylamine *N*-methyltransferase	87	XP_012032256	10	1.65		
459	*INMT*	Indolethylamine *N*-methyltransferase	195	XP_012032256	16		−1.17	
462*	*GSTM1*	Glutathione S-transferase Mu 1	94	XP_004002338	30	2.42	1.35	
	TPI1	Triosephosphate isomerase	99	XP_004007628	31			
464*	*ECHS1*	Enoyl-CoA hydratase	150	XP_004020434	25	1.25	−1.35	
	IGLC2	Immunoglobulin lambda light chain constant region	117	AAU45093	32			
467*	*GSTM1*	Glutathione S-transferase Mu 1 isoform 1	105	XP_004002338	22	1.94	1.32	
	IGLC2	Immunoglobulin lambda light chain constant region	72	AAU45093	32			
472	*GSTM1*	Glutathione S-transferase Mu 1	88	XP_004002338	13	1.66	1.2	
478	*GSTT1*	Glutathione S-transferase theta 1	88	XP_004017553	40		1.62	1.55
487	*APOA1*	Apolipoprotein A1	390	XP_011973043	47	−1.65	−1.43	
498	*FTL*	Ferritin light chain	353	XP_004015414	41	−2.07		1.31
525	*HBA1*	Hemoglobin subunit alpha-1/2	50	P68240	43		−1.35	
546	*HBB*	Hemoglobin subunit beta	158	XP_004016289	75	1.62		
548	*HBB*	Hemoglobin subunit beta	300	XP_004016289	91		−1.47	
558	*HBB*	Hemoglobin subunit beta	165	XP_004016289	54		−1.79	

have been normalized following adaptive processes. Indeed, liver concentrations of selected ECs were not different to controls in males, suggesting the time spent in control pastures allowed clearance of many chemicals. The latter is not surprising as we have previously shown that there are no patterns of ECs accumulation in the liver after treatment discontinuation (Rhind et al., 2010). Despite this cessation, measurable differences remained detectable in the male livers, suggesting that the EC exposures during pre- and post-natal development imprints persistent marks in liver physiology. Consistent with the latter, we have previously reported aberrant testicular physiology in the same male sheep (Bellingham et al., 2012). In females, chrysene, which was the most abundant PAH measured, was found to be highly elevated in the exposed group and thus may serve as a likely marker of liver EC exposure. The increases in detoxification-related enzymes such as *CYP1A1* transcripts, GSTM1, GSTT1, HSP70 and HSP90 proteins in the continuously exposed females likely reflects adaptive responses to continuous EC exposure. The induction of such detoxifying enzymes which may be driven by the abundant PAHs may, at least in part, explain the drop of the less abundant PCBs in exposed females, which are metabolized by cytochrome P450 enzymes (Kaminsky et al., 1981; Duignan et al., 1987; Ariyoshi et al., 1992).

Even though our proteomic analyses were limited on the 445 most abundant proteins of the much larger total liver proteome (Chinese Human Liver Proteome Profiling Consortium, 2010), the dysregulation of 42% and 19% of the observable proteome in females and males respectively (Fig. 2B) in the exposed animals suggests that chronic EC exposure causes widespread, persistent alterations in the liver. Pathway analyses suggested that EC exposure may decrease lipid concentration in EC exposed females and this was then confirmed by total liver lipid measurements. We were however unable to determine which classes of lipids may account for the decrease in total lipids; phospholipids which account for ~50% of total lipid levels (Noble et al., 1971; Peters and Smith, 1964, Fig. 4A), cholesterol and triglycerides which account for ~2–6% of total lipids respectively (Bertolín et al., 2018; Peters and Smith, 1964; Fig. 4A) remained unchanged by exposure. It is possible that the reduction in total lipids may represent minor changes in many different classes of lipids. In male livers, the increase in *AFP* transcript levels as well as the trend for increased *POU5F1* and *GPC3* transcripts suggest that chronic exposure predisposes the liver to a pro-carcinogenic environment (Bai et al., 2017; Wu et al., 2015; El-Saadany et al., 2018). With regard to sex differences in control animals, the male livers were predicted to have reduced immunological responses, a prediction

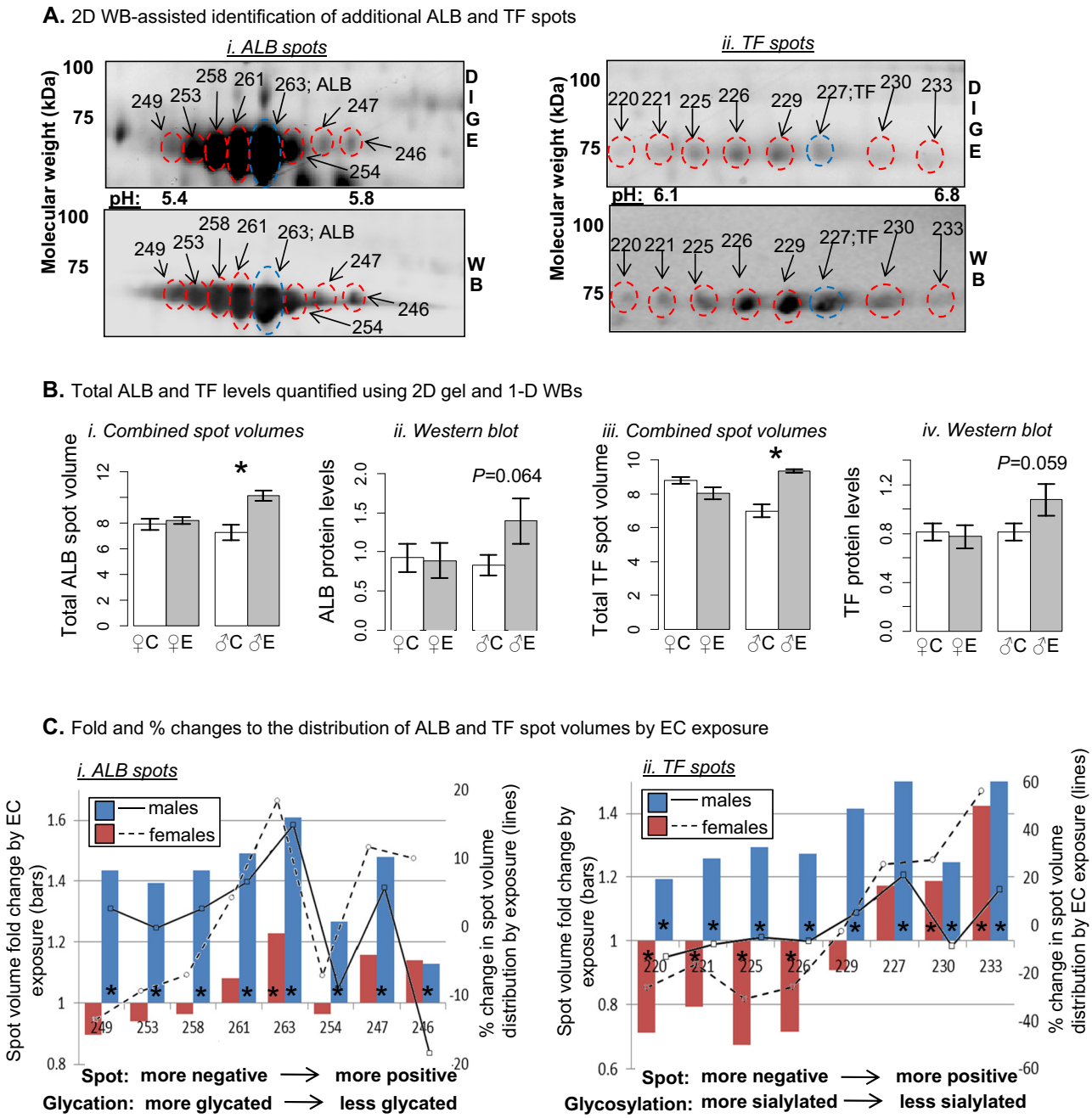

Fig. 3. In-depth examination of sheep liver ALB and TF forms. A. Identification of all ALB and TF spots by comparing DIGE-labelled 2D gels to 2D-Western blots. Blue dotted circles, spots identified by LC-MS/MS; red dotted circles, spots annotated by 2D-Western blot and DIGE comparison. B. Comparison between the sum of ALB and TF spots and total ALB and TF quantified using 1D-WBs. Significant ($P < 0.05$) differences between groups are indicated by asterisks above the bars. Near-significant differences ($0.05 < P \leq 1$) are indicated as P values above the bars. Error bars denote ± SEM. C, controls; E, exposed. C. Fold (bars) and spot distribution (lines) changes by exposure in ALB and TF spot volume levels respectively. *x*-axes are labelled according to spot number ordered by spot charge. Left *y*-axes (bars) represent change for each spot compared to control (exposed average spot volume / control average spot volume). Right *y*-axes (lines) represent % change in the distribution of each spot compared to control [(% contribution of spot average volume in total protein spot volume in exposed group / % contribution of spot volume in total protein spot volume in control group) − 1] ∗ 100. Asterisks indicate the spots that were significantly (adjusted $P < 0.05$) affected by exposure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that is has also been observed for both human and rodent livers (Klein, 2012; Scotland et al., 2011).

The results of the current study also indicated that EC exposure affected the levels and/or the post-translational modifications of a key fatty acid oxidation enzyme and three important plasma proteins, an outcome which would be expected to result in pleiotropic, organismal-wide effects. The fatty acid oxidation ACAA2 enzyme was detected in six spots spanning pI values of from 7.8 to 10, four of which were upregulated in exposed females and two of which were altered in exposed males (1 upregulated and 1 downregulated). The presence of multiple ACAA2 spots likely represent post-translational modifications that may include covalent enzyme-substrate reaction intermediates (Modis and Wierenga, 1999) which affect proteins' pI. To the best of our knowledge there is no literature on how post-translational modifications affect the pI of ACAA2 and therefore we cannot comment on the functional significance of exposure on the different ACAA2 spots. ALB is

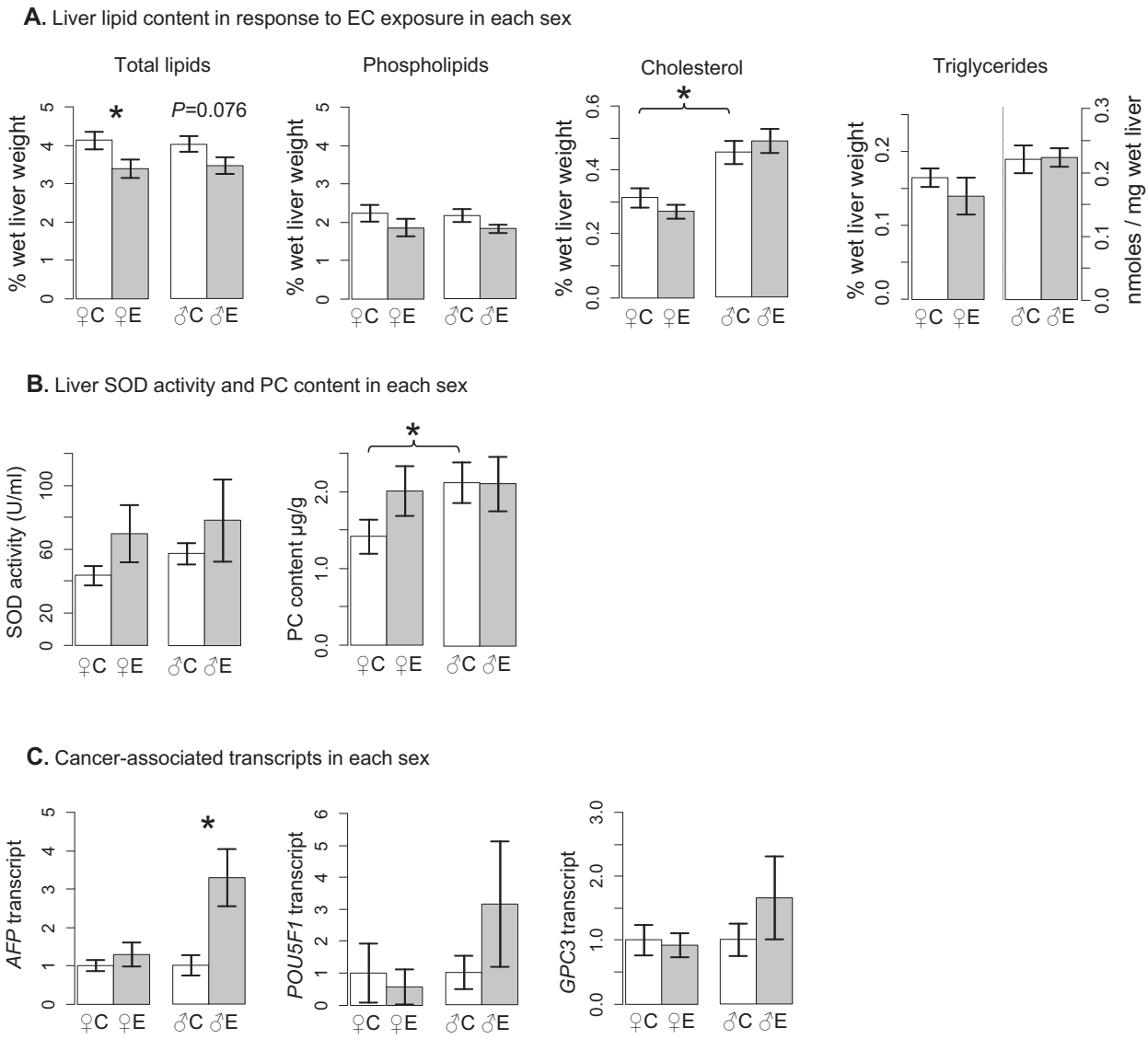

Fig. 4. Lipid measurements, oxidative stress and cancer markers in the sheep livers. A. Sewage sludge exposure decreased levels of total lipids in exposed female livers whereas phospholipids, cholesterol and triglycerides remain unaffected in both sexes. In control animals males had higher liver cholesterol levels. B. Superoxide dismutase (SOD) activity and protein carbonyl (PC) content were higher in exposed female livers but the differences do not reach statistical significance. Male control livers had higher levels of PC compared to control females. C. Exposed male livers tended to have increased transcript expression levels of the liver cancer markers *AFP*, *POU5F1* and *GPC3*, (only *AFP* reached statistical significance). Significant ($P < 0.05$) differences between groups are indicated by asterisks above the bars. Near-significant differences ($0.05 < P \leq 1$) are indicated as *P* values above the bars. Error bars denote ± SEM.

the most abundant plasma protein and is involved in the maintenance of osmotic pressure, binding of hormones and transport of fatty acids (Table S7). Even though ALB does not bind hormones with high affinity, the high levels of plasma ALB mean that ALB controls a significant fraction of total bound (i.e. inactive) and unbound (i.e. active) steroid and thyroid hormones (Baker, 2002). Alterations in ALB levels could therefore have detrimental consequences to overall health and it has been shown that low ALB levels in humans associate with liver and/or kidney disease and inflammation (Nicholson et al., 2000; Moshage et al., 1987; Spiegel and Breyer, 1994). As ALB is normally secreted by the liver without post-translational modifications it would be expected to resolve to a single spot in 2D gels, however in our 2D-WB analysis ALB resolved to at least eight spots, of similar size but of different pI (Fig. 3A). It should be noted that the samples from the current study were not perfused during collection and therefore may contain plasma and blood proteins. The presence of multiple spots likely represents alternative ALB forms, likely due to differential glycation (i.e. non-enzymatically coupled carbohydrate moieties) of circulating species (Gianazza et al., 1984; Arasteh et al., 2014). Interestingly, the distribution of these spots was affected by EC exposure in a sexually dimorphic manner; in males, ALB distribution was biased towards more positively charged spots which would suggest higher levels of glycation (Fig. 3C*i*) and would be expected to result in reduced ligand binding, increased protein stability and half-life (Rondeau and Bourdon, 2011). In addition, increased ALB glycation is thought to contribute to the development of metabolic disorders including diabetes (Cohen, 2003; Lee et al., 2014).

Transferrin (TF) is the major iron-carrying protein in the plasma; high levels of TF are associated with anemia and/or iron deficiency whereas decreased levels suggest iron overload and/or protein malnutrition (Wish, 2006). TF is variably glycosylated and isoelectric focusing normally resolves TF to at least eight glycoforms depending on the amount of terminal sialic acid sugar residues, which range from asialylated (most positively charged form) to hepta-sialylated (most negatively charged form) with the majority of TF species being tri- to penta-sialylated (Goreta et al., 2012; Sanz-Nebot et al., 2007; Fig. 3C*ii*).

EC exposure altered total TF levels in males (Fig. 3B) and also affected TF glycoform distribution in a sex-specific manner: asialylated, mono- di- and tri-sialylated species increased, and penta-, hexa- and hepta-sialylated species decreased in EC exposed females (Fig. 3C*ii*). This shift towards less sialylated TF species, mirrors the TF sialylation changes in humans with congenital disorders of glycosylation (Goreta et al., 2012), alcohol exposure (Stibler et al., 1978; Stibler, 1991; Hoefkens et al., 1997), sepsis (Piagnerelli et al., 2005) and pancreatitis (Gornik et al., 2008).

This study examined the effect of lifelong exposure to a complex chemical cocktail that reflects human EC exposures (Venkatesan and Halden, 2014; Zhang et al., 2015; Rhind et al., 2013; Rhind et al., 2002) in sheep which have long gestation and lifespan, therefore providing a good approximation to human exposures. Several study limitations however warrant discussion. Despite the similarities between sheep and humans, the EC exposure effects observed in our model may not approximate human EC exposure in every aspect. In addition, exposed males were transferred from the treatment plots and were maintained in control plots for 12 months prior to euthanasia, during which time some alterations in the liver may have reverted. However, this limitation of elapsed time between treatment and analysis is also a strength in that it enabled us to establish that persistent changes remained detectable in the livers of the animals. It is possible however that the transfer and maintenance of the exposed animals to control pastures, i.e. an environment different from which they were conceived, developed and grew, may have also caused changes in the liver that could represent physiological adaptations to the new environment.

In conclusion, this study shows that chronic EC exposure, via sewage sludge, at concentrations and complexity relevant to humans, induces persistent xenotoxicant responses in the liver, disrupts a large portion of the observable liver proteome and affects lipid levels and the expression of liver cancer markers in sheep, all of which are likely to affect many body systems. Our observations support the existing data showing that low-level EC exposure is a significant contributor to abnormal liver physiology (Foulds et al., 2017; Al-Eryani et al., 2015; Yorita Christensen et al., 2013) and expand our understanding on how EC exposure can contribute to disease. Since the sheep livers examined in this study were from young healthy animals, the presence of multiple identifiable alterations raises particular concern with regard to population health. The list of altered liver proteins, particularly plasma ALB and TF glycoforms may provide biomarkers of EC exposure while the dysregulated pathway may indicate potential routes for intervention in EC exposure-susceptible individuals.

Conflict of interest

The authors declare they have no competing interests, be it financial, personal or professional. None of the funding bodies played any role in the design, collection, analysis and interpretation of the data, in the writing of the manuscript, or in the decision to submit the manuscript for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.01.003.

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