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The Herbicide Linuron Inhibits Cholesterol Biosynthesis and Induces Cellular Stress Responses in Brown Trout

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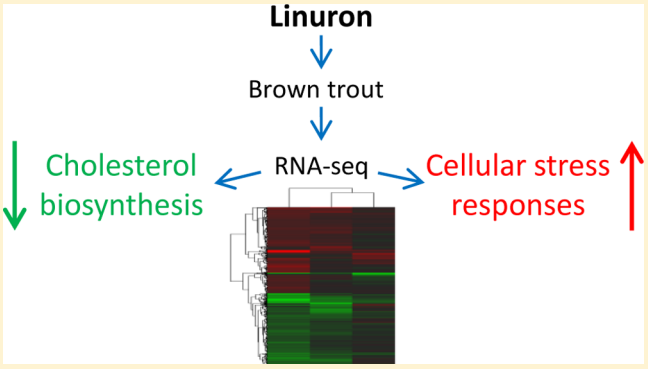
Supporting Information

ABSTRACT: The herbicide linuron is used worldwide, and has been detected in surface waters as well as in food and drinking water. Toxicological studies have reported that linuron acts as an antiandrogen in vitro and in vivo and disrupts mammalian male reproductive function. However, global mechanisms of linuron toxicity are poorly documented. We used RNA-seq to characterize the hepatic transcriptional response of mature male brown trout exposed for 4 days to 1.7, 15.3, and 225.9 μg/L linuron. We identified a striking decrease in the expression of transcripts encoding the majority of enzymes forming the cholesterol biosynthesis pathway. We also measured a very significant decrease in total hepatic cholesterol in fish exposed to 225.9 μg/L linuron and a negative correlation between total cholesterol and linuron treatment concentration. We hypothesize that inhibition of cholesterol biosynthesis may result from the disruption of androgen signaling by linuron. Additionally, there was increased expression of a number of transcripts involved in cellular stress responses, including *cyp1a* (up to 560-fold), molecular chaperones, and antioxidant enzymes. We found some evidence of similar patterns of transcriptional change in fish exposed to an environmentally relevant concentration of linuron, and further research should investigate the potential for adverse effects to occur following chronic environmental exposure.



INTRODUCTION

Linuron is a substituted phenylurea herbicide that disrupts photosynthesis by targeting protein D1, a central component of photosystem II, and inhibiting photodependent electron transport, leading to accumulation of reactive oxygen species (ROS) in plant cells.[1] This compound is used worldwide to control a number of broadleaf and grass weeds in the cultivation of a variety of crop plants, particularly vegetables and cereals. Linuron is known to enter surface waters in agricultural runoff, particularly in association with sediment, where it is moderately persistent.[2] Despite the widespread usage of this herbicide, measurements of surface water concentrations are very scarce but have been reported to occur in the nanogram to low microgram per liter range. Concentrations detected include 1.05 μg/L in a Canadian river within an agricultural catchment[3] and 4.42 μg/L in a Florida stream receiving agricultural runoff.[4] Modeling approaches have predicted peak concentrations of 31.3 μg/L in surface waters associated with application on a nearby carrot crop, highlighting the potential for short-term peaks in contamination.[2] Linuron has also been detected in drinking water and in food residues.[5,6] The potential for environmental exposure to this chemical, therefore, raises concerns about the risk linuron may pose to both human and wildlife health.

The majority of existing research investigating the toxicological effects of linuron has focused on its activity as an antiandrogenic compound. In vitro studies have shown that linuron competitively inhibits androgen binding to the androgen receptor (AR) in mammals, fish, and amphibians.[7−11]

In mammalian studies, antiandrogenic activity has been demonstrated in vivo, using the Hershberger assay, where exposure to linuron was shown to reduce the weight and development of androgen-sensitive reproductive tissues.[12−14] Linuron was also shown to have adverse impacts on male reproductive health in rats, including abnormal reproductive development following in utero exposure, Leydig cell tumorgenesis, and reduced testosterone production in vitro and in vivo.[15−17]

In stickleback, the production of spiggin, an androgen-dependent glycoprotein normally only produced by nest-building males, can be induced in females by androgen treatment, and its subsequent inhibition by antiandrogens is assumed to occur specifically through AR antagonism.[18−21]

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Linuron was reported to suppress the production of dihydrotestosterone (DHT)-induced spiggin production in cultured kidney cells, and in vivo concentrations of 100 and 250 μg/L reduced spiggin induction at both the transcript and protein level.[18−21] Recent transcriptomic and proteomic approaches in fathead minnow ovarian cells and in zebrafish embryos have demonstrated that the molecular signatures following exposure to linuron are more similar to those of the model antiandrogen, flutamide, than a model androgen (DHT) or estrogen (ethinyl-estradiol), supporting an antiandrogenic mechanism of action.[22,23]

It is hypothesized that in addition to its action as an AR antagonist linuron may also disrupt androgen synthesis and/or metabolism, but the relative contribution of each mechanism to overall antiandrogenic activity is poorly understood.[17] Linuron has also been shown to induce antiestrogenic effects, reducing plasma vitellogenin concentrations in female fathead minnows.[24,25] Linuron has therefore been shown to have multiple mechanisms of toxicity, and it is possible that others have not yet been identified. Furthermore, the majority of studies investigating the molecular mechanisms of toxicity of linuron and other antiandrogens have focused on the gonads, but androgen signaling also has an important role in regulating a range of other biological processes in different tissues, including the liver.[26] Hepatic metabolism is also the major mechanism responsible for steroid hormone degradation as well as detoxification and elimination of xenobiotics.[27] This makes the liver an important and sensitive tissue for investigating the toxic effect of chemical exposure. This study, therefore, aimed to investigate global hepatic mechanisms of toxicity of linuron in brown trout (*Salmo trutta*) using transcriptomic analysis. We hypothesized that linuron exposure would result in toxicological effects in the liver mediated through a range of molecular pathways, including antiandrogenic mechanisms of action. To address this hypothesis, we conducted an exposure of sexually mature male brown trout to three treatments of linuron (2.5, 25, and 250 μg/L), including an environmentally relevant concentration and a high concentration chosen to facilitate a mechanistic analysis of the effects seen. We then investigated the molecular mechanisms of toxicity of this herbicide in the liver of exposed fish using RNA-seq.

MATERIALS AND METHODS

Chemical Exposure. Sexually mature male brown trout were exposed to linuron via a flow-through system for a period of 4 days. The treatment groups consisted of three nominal concentrations of linuron (2.5, 25, and 250 μg/L; Pestanol Analytical Standard, Sigma) or dilution-water control alone. These included a low concentration within the range that has been measured in the most contaminated surface waters and an intermediate concentration representing concentrations that may potentially occur during peak contamination events, according to modeling scenarios. Both of these concentrations were lower than the chronic NOEC of <42 μg/L established for rainbow trout using the early life cycle test.[2] The high concentration was included in this study to facilitate a mechanistic investigation of molecular pathways disrupted by this chemical. This concentration is known to cause toxicological effects in fish, including antiandrogenic responses, but is considerably lower than the acute LC_{50} of 3 mg/L linuron established for rainbow trout.[2]

Each linuron treatment group consisted of one tank containing eight individual fish (males and sexually immature males and females, of which only sexually mature males were taken forward for analysis in order to limit variation resulting from sex and reproductive stage of maturation). Sexually mature females, which secrete high levels of natural estrogens, were excluded to limit potential interference of excreted sex steroids with the exposure (for further details see Supporting Information). Due to the size of the fish used in this experiment, it was unfortunately not feasible to employ more than one tank replicate for each linuron treatment; however, two duplicate tanks were included for the control treatment to provide us with information on intertank variation. Water samples were collected from each tank on day three of the exposure period and were analyzed using LC-MS by an accredited laboratory (South West Water, Exeter Laboratories). Fish were not fed during the exposure. Full details on fish husbandry and the exposure experiment are given in the Supporting Information.

Sampling. Fish were humanely sacrificed on day four of the exposure period by a lethal dose of benzocaine (0.5 g/L, Sigma-Aldrich) followed by destruction of the brain, in accordance with UK Home Office regulations. For each individual fish, wet weight and fork length were recorded and the condition factor (k = (weight (grams) × 100)/(fork length (centimeters)))^3 was calculated. Sex and maturity of individuals was confirmed by observation of the gonads, and mature males were selected for analysis (n = 3 − 6 per treatment group). Livers were dissected and weighed, and the hepatosomatic index (HSI, (liver weight (milligrams)/total weight (milligrams)) × 100)) and gonadsomatic index (GSI, (gonad weight (milligrams)/total weight (milligrams)) × 100)) were determined. Portions of the liver were randomly selected, snap frozen in liquid nitrogen, and stored at −80 °C prior to transcript profiling and cholesterol quantification. Statistical analysis of morphological parameters was conducted in SigmaStat (version 12.0). All morphometric data met assumptions of normality and equal variance and were analyzed using single-factor one-way analysis of variance (ANOVA). All values presented are mean ± SEM.

Illumina Sequencing and Transcriptomic Analysis. Transcript profiling was conducted on the liver of three randomly selected individual mature males per treatment group, including each duplicate control tank. RNA was extracted and spiked with External RNA Controls Consortium (ERCC) spike-in control mixes (Ambion) then prepared for sequencing using the Illumina TruSeq RNA Sample Preparation kit. The 15 individual libraries were multiplexed and sequenced on an Illumina HiSeq 2500 platform to generate 100 bp paired-end reads. Full details of the transcriptome assembly are given in the Supporting Information. Briefly, raw-sequence reads were subject to quality-related processing, filtering, and digital normalization, and then a de novo transcriptome assembly was conducted using Trinity (version r2013-02-25),[28] specifying default parameters. Transcripts were annotated using Blastx against Ensembl peptide databases using an e-value cutoff of <$1e^{-15}$, and additional annotation of previously unannotated differentially expressed transcripts was performed using Blast (e-value < $1e^{-15}$) against refseq, nr, and nt databases.

Full details of the transcriptomic analysis are described in the Supporting Information. Briefly, 83.2% of raw-sequence reads from each of the 15 individual libraries were remapped to the newly assembled brown trout liver transcriptome using Bowtie2 (version 2.1.0,[29]). Raw count data for each transcript was extracted using idxstats in samtools (version 0.1.18,[30]) and

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input into edgeR[31] for differential expression analysis. Initially, we performed expression analysis between the two independent control groups and identified only three differentially expressed transcripts. This suggests that for these groups at least there was minimal intertank variation in our experimental conditions, which only employed one replicate tank for each of the linuron treatments.

Subsequently, we conducted transcript expression analysis between the three individuals in each linuron treatment group against the combined control groups (six individuals). Transcripts with a false discovery rate (FDR) < 0.1 were considered differentially expressed (Benjamini–Hochberg correction). Heatmaps were generated via Euclidean cluster analysis, using the pheatmap package in R/Bioconductor[32] on the basis of the expression levels of all transcripts that were differentially expressed in at least one treatment group. Functional analysis was performed for differentially expressed genes from each treatment group using the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7)[33] and Ingenuity Pathways Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com). Gene ontology (GO) terms and Kegg pathways were considered significantly enriched with an adjusted P value that was <0.05 (Benjamini–Hochberg correction).

The raw sequence data and processed results from the expression analysis have been deposited in NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and are available via the GEO series accession number GSE57490.

Liver Cholesterol Measurements. Total lipids were extracted from liver samples using chloroform/methanol according to previously published protocols,[34] described in detail in the Supporting Information. Liver from all of the mature males from each treatment group were analyzed (n = 6, 4, 4, and 3, respectively, for the control, 2.5, 25, and 250 μg/L treatment groups). Briefly, liver samples (~50 mg) were ground under liquid nitrogen and extracted with a 20× (v/w) solution of chloroform/methanol (2:1). A 0.9% NaCl solution was used to isolate the lipid-containing (lower) phase, and this was washed three times with a methanol/water (1:1) solution. Lipid extracts were evaporated under a stream of nitrogen, then redissolved in 100% ethanol (0.5 mg/μL tissue) before measurement of cholesterol. Total cholesterol was measured using the enzymatic colorimetric test originally described by Allain et al.[35] on a Roche Modular P800 analyzer (Roche Diagnostics, USA) at the Blood Sciences Department, Royal Devon & Exeter Hospital, Exeter. All data met assumptions of normality and equal variance, and were analyzed using ANOVA, followed by the Tukey post hoc test. The association between the measured concentration of linuron in the water and the concentration of cholesterol in liver samples was tested using Pearson correlation. All values presented are mean ± SEM.

RESULTS

Water Chemistry and Morphological Parameters. The measured concentrations of linuron on day three of the exposure period were 1.7, 15.3, and 225.9 μg/L for the tanks exposed to nominal concentrations of 2.5, 25, and 250 μg/L, respectively. Throughout this paper we refer to these measured concentrations of linuron. Gonadal examination revealed that the number of mature males was 4, 4, and 3 in the 1.7, 15.3, and 225.9 μg/L treatment groups, respectively, and 3 in each of the duplicate control treatment groups. The mean mass and length of these mature males across treatment groups were 452.7 ± 14.3 g and 33.9 ± 0.3 cm, respectively. There were no significant differences between treatment groups in size and condition factor (mean = 1.15 ± 0.01), HSI (mean = 1.04 ± 0.04), or GSI (mean = 3.82 ± 0.27) of mature males, and we observed no alteration in the general health or behavior of the exposed fish during the experiment.

Sequencing, Transcriptome Assembly, and Quality Control. The de novo transcriptome was assembled using a total of 225.3 million 100 bp paired-reads from male brown trout liver and consisted of 172 688 transcripts (107 095 loci) with a mean length of 767.5 bp and an N_{50} of 1292 bp. A total of 62 236 transcripts were annotated using Blastx against Ensembl peptide databases, and these included a representation of 16 121 unique zebrafish transcripts. Sequencing of the liver samples of fish exposed to linuron and associated controls generated a total of 137.9 million reads, averaging 9.2 million reads per sample, and 83.3% of these remapped against the assembled transcriptome.

The ERCC spike-in controls were analyzed to provide technical validation of the quantitative expression profiling conducted in this study. Only ERCC control transcripts that were detected in a minimum of three individual libraries (at least one count) were included in the analysis. There was a strong correlation between the calculated fragments per kilobase of exon per million fragments mapped (FPKM) values and the expected concentration of control transcripts for all individual libraries (mean R^2 = 0.901 ± 0.005 SEM, Figure S3). The dynamic range was calculated individually for all samples (Table S1), and the mean dynamic range in expression levels across all libraries was 21 062 FPKM. Additionally, there was a good correlation between the calculated and expected fold-changes in expression between samples spiked with ERCC mixes 1 and 2 (R^2 = 0.58). Together, these results provide strong technical validation for the quantitative expression profiling conducted in this study.

Transcript Expression and Functional Analysis. Expression analysis using edgeR identified a total of 822 transcripts that were differentially expressed in one or more treatment group compared to the controls, 435 of which increased and 387 of which decreased in expression (FDR < 0.1). The number and the overlaps between the lists of differentially expressed transcripts for each treatment group are shown in Figure 1. A full list of differentially expressed

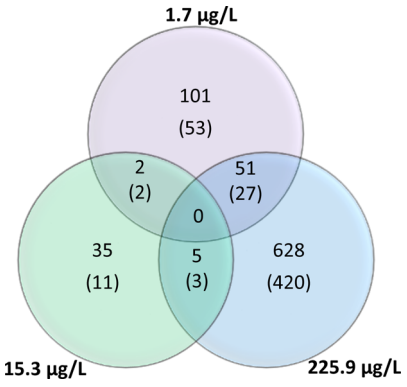

Figure 1. Venn diagram displaying the numbers of differentially expressed transcripts (false discovery rate (FDR) < 0.1 and FDR < 0.05, the latter presented in brackets) in each treatment group, obtained using edgeR.

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transcripts and associated FDR values are presented in Table S3. Clustering of all individual samples, on the basis of expression levels, is presented in Figure 2 and clustering on the basis of mean fold-change for each treatment group compared to the control is shown in Figure S1. Additionally, multidimensional scaling (MDS) plots, illustrating the similarity of individuals within each treatment group compared to the controls, are shown in Figure S2.

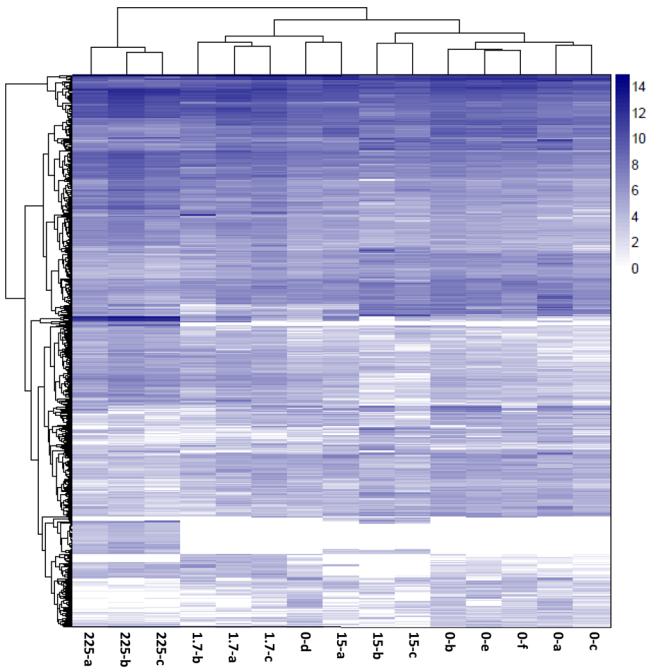

Figure 2. Heatmap illustrating the expression level of all differentially regulated transcripts in all individual samples. Individual fish are represented by the following codes: 0-a−f, control individuals; 1.7-a−c, individuals exposed to 1.7 μg/L linuron; 15-a−c, individuals exposed to 15.3 μg/L linuron; 225-a−c, individuals exposed to 225.9 μg/L linuron. Data presented are log 10 transformed read counts per transcript. The hierarchical clustering to generate gene and condition trees was conducted with an Euclidean distance metric, using the pheatmap package in R.[32]

Cluster analysis indicated that the expression profiles of all individual fish exposed to 225.9 μg/L linuron were distinct from those of the control and of the treatment groups exposed to lower concentrations of linuron, corresponding to the highest number of differentially expressed transcripts in this high treatment group (684). For the lowest concentration of linuron (1.7 μg/L), there was a similar clustering of individuals, although less distinct from the control group, reflecting the degree of transcriptional change detected following exposure to this environmentally relevant concentration (154 differentially expressed transcripts). In contrast, the individuals exposed to 15.3 μg/L linuron did not cluster together, and it was clear that there was a more variable transcript expression profile in this group (Figure S2). In particular, one individual displayed a distinct expression profile from that of the other two individuals in this group. This increased degree of biological variation between individuals reduced the statistical power for expression analysis, and subsequently resulted in the identification of fewer differentially expressed transcripts in this group (42).

The list of over-represented GO terms and Kegg pathways in fish exposed to 225.9 μg/L linuron is shown in Table S2. The most significantly enriched terms were *protein folding* and *unfolded protein binding*, reflecting an increased expression of transcripts encoding a suite of molecular chaperones involved in cellular stress response. Transcript expression of CYP1A was increased by the greatest extent (340−560-fold) in fish exposed to 225.9 μg/L linuron and was also significantly increased in fish exposed to 1.7 μg/L linuron (by 4.1−6.7-fold). In addition, there were increasing trends in the expression of *cyp1a* in fish exposed to 15.3 μg/L linuron (by 4.6−8.5-fold), albeit not significantly.

Lipid biosynthetic process and *steroid biosynthesis* were also among the most over-represented processes in fish exposed to the high treatment concentration. IPA identified a very significant over-representation of the cholesterol biosynthesis pathway in the lists of differentially expressed transcripts. This included reduced expression of transcripts encoding 14 individual enzymes involved in this pathway in fish exposed to 225.9 μg/L linuron. One of these transcripts (encoding lanosterol synthase) was also significantly suppressed in fish exposed to 1.7 μg/L linuron. A schematic illustrating the down-regulation of this pathway is shown in Figure 3a.

Liver Cholesterol Measurement. The mean concentration of total cholesterol in the liver of mature male fish included in this experiment was 2.07 ± 0.05, 1.97 ± 0.05, 1.82 ± 0.09, and 1.30 ± 0.20 mg/g of liver tissue in fish from the 0, 1.7, 15.3, and 225.9 μg/L linuron treatment groups, respectively. There was a significant decrease in the concentration of total cholesterol measured in the livers of fish exposed to 225.9 μg/L linuron compared to that of the control group and lower treatment groups (Figure 3b). Additionally, there was a strong negative correlation between the measured concentration of total cholesterol in the liver and the treatment concentration of linuron ($p = 5.88 \times 10^{-5}$).

DISCUSSION

RNA-seq analysis revealed a considerable degree of transcriptional change in fish exposed to 225.9 μg/L of linuron during this short-term (4 day) exposure. This high concentration is in a range where a number of toxicological effects have previously been reported for fish exposed to linuron, including antiandrogenic effects in stickleback following 21 day exposure to 100 and 250 μg/L linuron[18−21] and histopathological changes in the liver and kidney of rainbow trout exposed to concentrations of 30 μg/L and above for 5 weeks,[36] and is well above the established chronic LOEC for trout (<42 μg/L).[2] There were also marked transcriptional changes in fish exposed to the environmentally relevant concentration of linuron (1.7 μg/L), and we observed some broadly similar changes in the genes and pathways across treatment groups. This highlights the potential for transcriptomic analysis to identify sensitive signatures of chemical exposure when compared to higher mechanistic doses, which may be useful for assessing exposure in the environment. Previously, some evidence of antiestrogenic effects in female fathead minnows exposed to concentrations as low as 1 μg/L linuron have also been reported, although this was following a longer-term (21 day) exposure.[24] Together, these data sets suggest that environmental exposure to linuron may potentially be a cause for concern, and this should be further investigated.

The relatively low number of differentially expressed transcripts observed in the 15.3 μg/L treatment group reflects the larger degree of biological variation between individuals in this group, which reduced the statistical power for identifying

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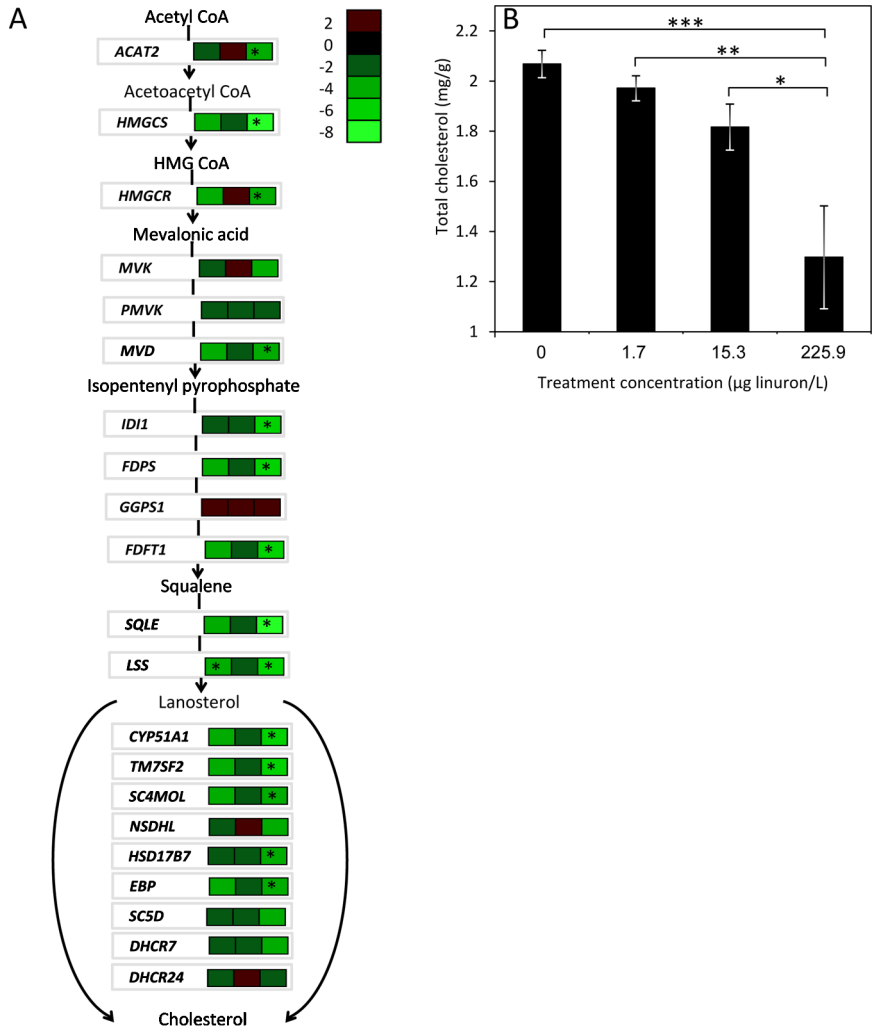

Figure 3. (A) Schematic illustration of the effects of exposure to linuron on the transcript profiles of genes encoding the cholesterol biosynthesis pathway enzymes. Each color-coded bar represents mean transcript fold change, quantified using edgeR, for each treatment compared to the control (left to right: 1.7, 15.3, and 225.9 μg/L linuron); asterisks indicate a significant decrease in expression (false discovery rate < 0.1). ACAT2 (acetyl-CoA acetyltransferase 2), HMGCS (3-hydroxy-3-methylglutaryl-coenzyme A synthase), HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase), MVK (mevalonate kinase), PMVK (phosphomevalonate kinase), MVD (mevalonate decarboxylase), IDI1 (isopentenyl-diphosphate delta isomerase 1), FDPS (farnesyl diphosphate synthase), GGPS1 (geranylgeranyl diphosphate synthase 1), FDFT1 (farnesyl-diphosphate farnesyltransferase 1), SQLE (squalene epoxidase), LSS (lanosterol synthase), CYP51A1 (cytochrome P450, family 51), TM7SF2 (transmembrane 7 superfamily member 2), SC4MOL (methylsterol monooxygenase 1), NSDHL (NAD(P) dependent steroid dehydrogenase-like), HSD17B7 (hydroxysteroid (17-beta) dehydrogenase 7), EBP (sterol isomerase), SC5D (sterol C5 desaturase), DHCR7 (7-dehydrocholesterol reductase), DHCR24 (24-dehydrocholesterol reductase). (B) Total concentration of cholesterol in the livers of fish exposed to linuron or kept under control conditions (n = 6, 4, 4, and 3 individuals for the 0, 1.7, 15.3, and 225.9 μg/L linuron treatment groups, respectively). Values presented are mean ± SEM. Asterisks indicate significant differences between treatment groups (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$) identified using an ANOVA followed by Tukey's post hoc test.

statistical differences in expression. In particular, one individual showed transcriptional changes distinguishing it from the others, perhaps reflecting different threshold concentrations of response between individuals. In this experiment, it was only feasible to perform RNA-seq analysis on three fish per treatment, restricting our ability to detect differentially expressed transcripts because of the high level of biological variation in this group. In addition, all three individuals analyzed were exposed within the same exposure tank, further limiting the power of our analysis. These results serve to highlight the importance of optimizing the number of biological replicates in RNA-seq analysis, which is rapidly becoming more practical with developments in sequencing technology. However, the robustness of our data is assured by the strong clustering of individuals in the other treatment groups and by similarities in transcriptional changes between groups exposed to the three concentrations of linuron.

Cholesterol Biosynthesis. Functional analysis revealed a striking reduction in expression of transcripts encoding for the majority of enzymes involved in the cholesterol biosynthesis pathway following exposure to the highest concentration of linuron (225.9 μg/L). Although largely not statistically significant, there were also general trends toward reduced expression of transcripts encoding the other enzymes in this pathway as well as in the lower treatment groups (Figure 3a). On the basis of these results, we hypothesized that liver cholesterol biosynthesis would be reduced following exposure to linuron, and we tested this by measuring the concentration

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of total cholesterol in the livers of exposed fish. There was a strong reduction in total cholesterol in the livers of fish exposed to 225.9 μg/L linuron, verifying this hypothesis. In addition, the hepatic cholesterol concentrations in all exposed fish were strongly negatively correlated with linuron concentrations in the exposure water. Together, these data demonstrate that the transcriptional changes observed resulted in effects at the biochemical level in liver cells of exposed fish.

Cholesterol biosynthesis is a well characterized pathway consisting of a series of complex reactions involving more than 20 enzymes. Briefly, a precursor molecule, acetyl coenzyme A (acetyl CoA), is converted through the mevalonate pathway to lanosterol. This stage includes the synthesis of mevalonic acid by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), which is generally regarded as the major irreversible, rate-limiting step in the biosynthesis of cholesterol. Lanosterol is then converted to cholesterol via a series of successive demethylation and double bond reductions, through either the Bloch or Kandutsch−Russell pathway.[37,38] Members of the sterol regulatory element binding protein (SREBP) family of transcription factors are involved in controlling a number of aspects of lipid and sterol metabolism. Specifically, the isoform SREBP-2 is a major transcriptional regulator of cholesterol biosynthesis.[37] Highly regulated feedback mechanisms are responsible for controlling the activity of SREBPs. Sterol-sensing SREBP-cleavage-activating proteins (SCAPs) bind and retain inactive SREBP precursors. When cholesterol levels are depleted, SCAP dissociates from endoplasmic reticulum (ER) membranes and transports SREBP-2 to the Golgi complex where it is cleaved and activated by site 1 and 2 proteases. Activated SREBP-2 then moves to the nucleus and induces the transcription of target genes by binding sterol response elements (SREs) in their promoter regions, together with associated cofactors. Conversely, elevated levels of cholesterol stimulate the inactivation of SREBP through its reassociation with SCAP in the ER membrane.[39] Nearly all genes encoding cholesterol biosynthesis enzymes have been shown to be regulated by SREBP-2 in mammalian studies; Sharpe and Brown[38] describe 22 enzymes involved in the cholesterol biosynthesis pathway, 21 of which are regulated by SREBP-2. These include all 14 transcripts that showed significantly reduced expression in fish exposed to 225.9 μg/L linuron. Furthermore, the expression of SREBP-2-encoding transcript (*srebf2*) was also significantly lower in fish exposed to 225.9 μg/L linuron, suggesting that linuron disrupts the regulation of cholesterol biosynthesis by reducing the transcription of *srebf2*.

We hypothesize that the antiandrogenic activity of linuron is a likely mechanism by which it down-regulates SREBP-2, and thus cholesterol biosynthesis. In prostate cancer cell lines, androgens are known to stimulate the expression of cholesterol biosynthesis enzymes by increasing the transcription of SREBP and enhancing SCAP-mediated cleavage of precursor SREBP into the mature form.[40,41] In vivo, the expression of SREBP and cholesterol biosynthesis enzymes were reduced following castration in male rats and restored following androgen treatment.[42] In fish, transcriptomic profiling of male fathead minnow exposed to pulp and paper mill effluent, which has been shown to have androgenic activity, revealed an increased expression of transcripts encoding cholesterol biosynthesis enzymes.[43] Potentially, the inhibition of cholesterol biosynthesis may be a mechanism of toxicity shared with other chemicals that antagonistically interact with the AR. Although some previous studies have reported that antiandrogen exposures modulate the expression and activity of individual enzymes involved in cholesterol metabolism and alter serum cholesterol and lipid concentrations,[23,44] the down-regulation of cholesterol biosynthesis has not been generally associated with antiandrogen toxicity in environmental studies.

Cholesterol is an essential component of cellular membranes, where it is involved in the regulation of membrane fluidity and permeability, transmembrane transport, and signaling. Cholesterol is also the precursor of a number of other essential biological molecules, including bile acids and steroid hormones. Bile synthesis in the liver is critical for the elimination of endogenous and xenobiotic metabolites.[37] We observed an increase in the expression level of *cyp7a1*, which encodes the rate-limiting enzyme responsible for biliary acid formation from cholesterol, possibly suggesting a compensatory response to the reduction in cholesterol biosynthesis. Disruption of cholesterol homeostasis has been implicated in a number of human pathologies, including prostate cancer, in which androgen signaling is a principal factor, dementia, diabetes, and Alzheimer's disease. Smith-Lemli-Opitz syndrome, which is caused by a mutation of one of the final enzymes in the cholesterol biosynthesis pathway (*DHCR7*), results in depleted cholesterol and is characterized by a wide range of clinical effects, including impaired cellular membrane function and steroid production, resulting in developmental and behavioral abnormalities.[45] The liver is the primary organ responsible for vertebrate cholesterol production; therefore, prolonged inhibition of cholesterol biosynthesis would likely result in a number of adverse health effects. To more fully assess the long-term health effects of exposure to linuron, it would be important to investigate whether the down-regulation of cholesterol biosynthesis enzymes and the depletion of hepatic cholesterol observed here is maintained following chronic exposure or is restored via compensatory cellular responses.

Although the majority of cholesterol for sex steroid production is synthesized in the gonads and adrenal gland, disruption of cholesterol biosynthesis may potentially contribute to the known antiandrogenic effects of linuron in reducing androgen synthesis, in particular if similar down-regulation of the cholesterol biosynthesis pathway is also occurring in those organs. To our knowledge, the impact of linuron on cholesterol biosynthesis in the testis has not been investigated, although Ornostay et al.[23] report reduced expression of a number of transcripts encoding cholesterol biosynthesis enzymes in fathead minnow ovarian cell cultures exposed to linuron. It is possible that an inhibition of cholesterol biosynthesis may lead to a reduction in sex steroid biosynthesis, which in turn may have also contributed to the antiestrogenic effects reported for fathead minnow by these authors in this study and in others,[23−25] potentially linking the inhibition of cholesterol biosynthesis to the reproductive toxicity of linuron.

Androgen signaling is also important in regulating other aspects of lipid metabolism, including SREBP-1 and the nuclear liver X receptor (LXR).[40,46] We found a significant increase in the expression of a transcript encoding SREBP-1 in the 225.9 μg/L linuron treatment group but a less consistent response of known SREBP-1 regulated genes. A number of GO terms relating to wider lipid and fatty acid metabolic processes were also enriched. This is consistent with the results of previous studies that have reported alteration of the metabolism and transport of lipids to be among the most common processes regulated by androgens and antiandrogens in fish.[26]

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Stress Response. Functional analysis of the lists of differentially expressed transcripts revealed that exposure to linuron caused a significant cellular stress response. Linuron induced a large and significant induction of transcripts encoding CYP1A, including at the environmentally relevant concentration. CYP1A is a primary phase 1 biotransformation enzyme involved in the detoxification or metabolic activation of a number of xenobiotics as well as many endogenous compounds.[47] It is among the most readily induced cellular proteins, and it is primarily regulated via aryl hydrocarbon receptor (AhR) signaling.[48] Planar aromatic hydrocarbons, including PCBs and PAHs, are known to be strong agonists of the AhR, and CYP1A induction (gene and protein) has been extensively used in ecotoxicology as a marker of exposure to these environmental contaminants.[49] Among commonly used pesticides, linuron was reported to be one of the most potent activators of AhR in vitro and has also been shown to strongly induce CYP1A gene expression in mouse liver, which was attributed to a structural feature (a dichlorophenyl residue).[50] In fish, CYP1A was shown to be induced by and to metabolize linuron in Japanese eel liver.[51] In mammals, linuron is metabolized to 1-(3,4-dichlorophenyl)-3-methoxyurea, 3,4-dichloroanaline, and 3,4-dichlorophenylurea, which are known to be weaker AR antagonists than the parent compound,[52,53] although little else is known about the toxicity of linuron metabolites in mammals or in fish. Some compounds are detoxified by CYP1A activity while others, including some PAHs, are bioactivated. The latter can generate highly reactive intermediates that subsequently induce cellular damage, including genotoxicity and carcinogenesis.[47] CYP1A transcription can also be regulated by hormones, directly or indirectly via upstream signaling pathways, that can affect the biological effects of xenobiotics. Androgens are known to inhibit CYP1A expression in mammals, and this is suggested to occur via AR-AhR interactions.[54] In addition to classical regulation via direct AhR activation, it is possible that the antiandrogenic effects of linuron contributed in part to the large induction of CYP1A, either through antagonism of the AR and/or the reduction in androgen production.

Protein folding was the most significantly enriched GO biological process on the list of transcripts differentially expressed following exposure to 225.9 μg/L linuron, corresponding with a consistent increase in the expression of a number of transcripts encoding molecular chaperones that can bind and stabilize damaged proteins. The chaperonin containing TCP-1 complex (CCT) is made up of eight primary subunits, six of which (*cct2*, *cct3*, *cct4*, *cct5*, *cct6a*, *cct8*), showed significantly increased expression following exposure to 225.9 μg/L linuron (by 2.3−3.6-fold). Although not statistically significant, several of these (*cct3* and *cct5*) also showed an increased expression (by more than 2-fold) in both of the lower treatment concentrations. CCT participates in normal protein metabolism by folding many proteins, particularly actin and tubulin,[55] but it has also been shown to be induced in response to stress, aiding cellular recovery. Examples include induction by chemical stress in mammalian cells[56] and by temperature stress in fish.[57] There was a strong increase in expression of transcripts encoding several heat shock proteins (HSPs) in fish exposed to 225.9 μg/L linuron (*hsp90aa1.2*, *hsp90ab1*, *hspa4a*, *hspa8*, *dnaja1l*, *dnaja4*, *dnajb1a*, *dnajb1b*). HSPs are probably the most well known stress-inducible molecular chaperones, and they have been extensively reported to respond to various environmental stressors in fish, including many pesticides. HSP90 proteins are also known to have a role in chaperoning CYP1A.[49] This strong and consistent increase in expression of transcripts encoding molecular chaperones suggests that linuron induces protein-damaging cellular stress.

There was an increase in the expression of a suite of transcripts encoding glutathione-related antioxidant enzymes (*gpx1b*, *gstal*, *gsto1*, *mgst1*, *mgst3*) and glutathione reductase (*gsr*) in fish exposed to 225.9 μg/L linuron (by 2.7−10-fold). Furthermore, there was a significant increase in expression of two members of the nuclear factor erythroid-derived 2-like family (*nfe2l1b* and *nfe2l2a*) following exposure to 225.9 μg/L linuron. These transcription factors play a key role in regulating the response of the antioxidant system.[58] This suggests that linuron generates oxidative stress, through the reactivity of the parent compound and/or metabolites. This mechanism of toxicity is common to many chemicals, particularly at high concentrations. Although evidence linking linuron with generation of oxidative stress in fish and other species is scarce, other phenylurea-based pesticides with a similar chemical structure, including diuron, have been shown to induce oxidative stress and cellular damage.[59,60] Oxidative stress has been linked to pathological changes in the liver, including necrosis, apoptosis, and carcinogenesis.[61] Linuron exposure was previously found to cause lesions and a range of adverse effects on cellular components in the liver of rainbow trout exposed to concentrations of 30 μg/L and above for 5 weeks.[36] Although we only found significant differences in the regulation of these stress-responsive processes in fish exposed to 225.9 μg/L linuron, a concentration unlikely to occur in the environment, the possibility that chronic exposure to linuron may cause oxidative stress at environmentally relevant concentrations cannot be excluded, and further research is required to investigate this.

Environmental Implications. Overall, using RNA-seq, we have demonstrated that a high concentration of linuron (225.9 μg/L) induces considerable transcriptional changes in the liver of mature male brown trout. There was evidence of a striking decrease in expression of transcripts encoding the majority of the enzymes involved in the cholesterol biosynthesis pathway, likely via SREBP-2 interaction, that resulted in a reduction in the concentration of total cholesterol in the liver following exposure to 225.9 μg/L linuron. We hypothesize that the antiandrogenic activity of linuron is likely the mechanism responsible for this effect. Additionally, we found differential expression of a number of transcripts involved in cellular stress responses. In particular, there was a considerable increase in *cyp1a* expression, including at environmentally relevant concentrations, and increased expression of molecular chaperones that bind and stabilize damaged proteins as well as a number of enzymes involved in the antioxidant system.

We note that the majority of the changes observed for these processes occurred only at a concentration far higher than those currently measured in water systems but that effects at environmentally relevant concentrations were also observed. It is possible that some of the transcriptional changes and the reduced liver cholesterol concentration observed may reflect an acute, adaptive stress response to this short-term linuron exposure and may not lead to long-term adverse effects. Toxicological responses to linuron may change over time; however, effects mediated through interaction with key transcription factors, such as AhR and SREBP-2, may be likely to continue. Future research should investigate whether similar transcriptional and biochemical changes occur after longer-term

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exposure, in particular for environmentally relevant concentrations and, importantly, if these changes result in adverse health effects.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental experimental details, heatmap of the fold changes in transcript expression between treatments (Figure S1), multidimensional scaling plots illustrating expression profiles for all treatments (Figure S2), ERCC spike-in control analysis (Figure S3 and Table S1), enriched Gene Ontology terms and Kegg pathways (Table S2), all differentially expressed transcripts (Table S3). 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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