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RESEARCH ARTICLE

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Lufenuron treatment temporarily represses gene expression and affects the SUMO pathway in liver of Atlantic salmon

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Abstract

Lufenuron is a benzoylurea insecticide currently in use to combat sea lice infestation in salmon aquaculture in Chile. With pending approval in Norway, the aim of this work was to study the uptake and toxicity of lufenuron in liver tissue of Atlantic salmon. Juvenile salmon weighing 40 g were given a standard 7-day oral dose, and bioaccumulation and transcriptional responses in the liver were examined 1 day after the end-of-treatment (day 8) and after 1 week of elimination (day 14). Bioaccumulation levels of lufenuron were 29 ± 3 mg/kg at day 8 and 14 ± 1 mg/kg at day 14, indicating relatively rapid clearance. However, residues of lufenuron were still present in the liver after 513 days of depuration. The exposure gave a transient inhibition of transcription in the liver at day 8 (2437 significant DEGs, p-adj < .05), followed by a weaker compensatory response at day 14 (169 significant DEGs). Pathways associated with RNA metabolism such as the sumoylation pathway were most strongly affected at day 8, while the apelin pathway was most profoundly affected at day 14. In conclusion, this study shows that lufenuron easily bioaccumulates and that a standard 7-day oral dose induces a transient inhibition of transcription in liver of salmon.

KEYWORDS
Atlantic salmon liver tissue, bioaccumulation, lufenuron, pathway analysis, RNA-seq, salmon lice drugs

1 | INTRODUCTION

There are several environmental challenges associated with marine aquaculture in Northern Europe and North America. Infestation with salmon lice represents one of the most serious threats to the salmon farming industry. The salmon louse *Lepeophtheirus salmonis* and other sea lice genera in the family Caligidae are the economically most important pathogens in salmon aquaculture (Abolofia et al., 2017; Costello, 2006; Torrissen et al., 2013). Salmon lice are ectoparasites that attach to and damage the skin of the fish. Aside from representing a threat to fish health and leading to reduced growth and increased mortality, the sea lice also pose a threat to wild populations of Atlantic salmon (*Salmo salar*) and sea trout (*Salmo trutta*). Economically, loss of income due to the downgrade of fish quality and costs of treatment amounts to several billion NOK each year for Norway's aquaculture industry (Bjørndal & Tusvik, 2020). To combat the sea lice challenge, the industry employs both medical and non-medical methods. Anti-salmon lice therapeutics in routine use in Norway today include pyrethroids such as deltamethrin, organophosphates such as azamethiphos, avermectins such as emamectin benzoate, neonicotinoids such as imidacloprid, and benzoylureas such as diflubenzuron and diflubenzuron. Additionally,

large quantities of hydrogen peroxide are used to rid the farmed fish of the ectoparasite.

Environmentally, the benzoylurea compounds are among the most controversial drugs used in aquaculture. Benzoylurea drugs are administered orally, and spill over from treatments could pose a potential threat to marine species (Langford et al., 2014; Macken et al., 2015). Non-target species are exposed to these drugs either by feeding on un-eaten medicated pellets or by particulate waste spread to the surrounding water and sediments (Samuelsen et al., 2015). Benzoylurea compounds were developed to target chitin synthesis and affect exoskeleton development in arthropods. Crustacean species are therefore especially vulnerable to exposure to these compounds (Olsvik et al., 2015, 2017, 2019). Because of the potential negative impact of benzoylureas on non-target species such as crabs, lobster and shrimps near fish farms, the industry has aimed to limit their use. However, due to a limited number of available anti sea lice agents, and problems with resistance to several of the drugs (Aaen et al., 2015), these chemicals are still being applied. The industry is searching for alternative drugs, and two new chitin synthesis inhibitors, lufenuron and hexaflumuron, have been approved by the EU. In Atlantic salmon farming, lufenuron is orally administered prior to sea transfer to provide long-term protection against sea lice infestation (Hobbs, 2014; Poley et al., 2018). Lufenuron has been evaluated by The Committee for Veterinary Medicinal Products (CVMP) in the European Medicines Agency's (EMA, 2015), and a maximum residue limit has been established for fish (EU, 2010/37). Still, the product has so far not been given market authorization in Norway. Even so, recent surveillance has documented residues of lufenuron in the filet of Norwegian-farmed Atlantic salmon (Bernhard et al., 2022). Lufenuron has been used against *Argulus* ectoparasites in Eurasian carp (*Cyprinus carpio*) (Mayer et al., 2013), and is currently in use in salmon aquaculture in Chile for the treatment of the sea lice *Caligus rogercresseyi* (Bravo & Treasurer, 2023).

EFSA (2008) consider lufenuron to be highly toxic to aquatic organisms, and that it may pose a risk to fish at environmental levels in water. In birds and mammals, acute toxicity is low, but lufenuron is very lipophilic and has shown a high potential for bioaccumulation in fat. Metabolism is minimal, and the only significant residue that will be present after exposure is lufenuron (EFSA, 2008). Lufenuron is a benzoylurea drug that acts by interfering with sea lice larval development by blocking the synthesis of chitin (Poley et al., 2018). In fish, lufenuron has been reported to cause tissue damage, induce oxidative stress, and impact immunological function (Mirghaed et al., 2020). In liver of Eurasian carp, hepatocyte necrosis was observed after waterborne exposure to lufenuron (0.1 mg/L) (Ghelichpour et al., 2019). Lufenuron exposure (0.1 mg/L) induced morphological changes in the gills and behavioural stress in tambaqui (*Colossoma macropomum*) (Soares et al., 2016). In liver of rats (*Rattus norvegicus*), lufenuron induces oxidative stress and acts genotoxic (Basal et al., 2020). Insect studies suggest that the pesticide can be metabolized and detoxified by the cytochrome P450 system (do Nascimento et al., 2015; Hafeez et al., 2019). Recent studies suggest that CYP9A enzymes are involved in the detoxification of lufenuron in insects (Zhang et al., 2023). CYP9A enzymes belong to the CYP3 clan in mammalian species (Shi et al., 2021). Although this chemical clearly can be toxic to fish after water exposure, to our knowledge no studies have evaluated the potential genome-wide toxic mode of action of lufenuron in fish after oral administration.

With authorization of lufenuron sought in Norway, this work aimed to study the toxic mode of action of lufenuron in Atlantic salmon after a standard 1-week treatment. We hypothesize that lufenuron is relatively non-toxic to salmon and that it may induce a short-lived stress response in liver tissue. Transcriptomics was selected as an endpoint to search for genome-wide effects immediately after treatment and after 1 week of depuration. Juvenile salmon were orally administered with a standard nominal dose of 5 mg/kg salmon for 7 days. Liver tissue was sampled for uptake kinetics and RNA-seq analysis. Lufenuron concentrations and transcriptional responses in liver tissue were determined on days 8 and 14 after the start of exposure. Functional pathway analysis of differentially expressed genes (DEGs) was conducted to gain mechanistic insight into molecular responses in the liver at the end-of-exposure (day 8) and after 7 days of depuration (day 14).

2 | MATERIALS AND METHODS

2.1 | Feeding trial

The study was approved by the Norwegian Food Safety Authority (FOTS ID 20391). The trial was conducted between October 2019 and March 2021 at the Institute of Marine Research station at Matre (Matredal, Norway; 60°520 N, 05°350 E). Just before the salmons were ready to smolt, 400 individuals were randomly, but evenly distributed into four 1000 L (1.5 m in diameter) flow-through indoor fibreglass tanks supplied with fresh water at a flow rate of approximately 20–40 L/min. All fish share the same genetic and environmental background. The fish were acclimated in the tanks on regular non-medicated feed for 14 days before exposure started. Two tanks were allocated for control fish and two tanks were allocated for lufenuron-medicated fish. The light regime was natural, and water temperature followed natural variation in river intake water. The mean temperature during the week of exposure was 7°C and the average water temperature throughout the experimental period was 9°C. At the beginning of the exposure experiment, the mean weight of the fish was 40 ± 7 g ($n = 30$).

The feeding trial with lufenuron lasted for 1 week (16–22 October 2019). The experimental groups were treated with lufenuron according to the recommendation from the producer; 5 g feed/kg fish per day for seven executive days (McHenery, 2016). The control groups were given the same pellets, without lufenuron. Both groups were fed twice a day with automatic feeders. Seven days after the end of exposure, the fish were given seawater in their tanks, to initiate smoltification.

2.2 | Experimental diets

The exposed group was administered commercially available feed top coated with lufenuron. The medicated feed was made by preparing a premix of 4.5 g maizena (Unilever Norge AS) and 0.5 g lufenuron (Sigma Aldrich). The premix was surface coated on a batch of 500 g of 3 mm pellets (Skretting AS) using a few drops of cod liver oil (Møllers; Orkla Health). The feed for the control group was produced correspondingly using a premix without lufenuron. The feed was then filled on automatic feeders, each programmed to administer 132.9 g over 7 days with a feed rate of 0.5%. The daily feeding periods were from 8:30 AM to 11:30 AM and from 12:15 PM to 3:15 PM.

2.3 | Sampling

Sampling was performed 1 day after the terminated medication, day 8, and 7 days after the terminated medication, day 14. The fish were sedated with Finquel, weighed, and thereafter killed with a stroke to the head. After killing, the fork length was measured, and liver samples were rapidly excised with a scalpel and stored at −20°C for chemical analysis or snap frozen in liquid nitrogen and stored at −80°C for RNA extraction. Liver tissue from 10 medicated and 10 control fish were sampled at each time point (five fish from each of the duplicate tanks). The remaining fish were transferred to seawater on day 15 and followed for a prolonged time after treatment for toxicokinetic assessment. At days 385 and 513, liver from 10 medicated fish were collected for analyses of lufenuron.

2.4 | Analysis of lufenuron in liver samples

Liver from medicated fish sampled on days 8, 14, 385 and 513 were homogenized and analysed for lufenuron concentration individually ($n = 10$). Liver from control fish, sampled on days 8 and 14, were analysed as pooled samples ($n = 2$). Each pooled sample consisted of liver tissue from five fish obtained from the same tank. Lufenuron concentration in liver samples was quantified using LC–MS/MS. The method is based on a previously described method for flubenzurons (Olsvik et al., 2015). Between 0.1 and 0.5 g of homogenized liver were weighed into plastic tubes. In addition to the samples, a matrix-matched calibration curve, quality control sample (QC), procedural blank, and matrix blank were included in each series. The calibration curve and QC were spiked with a known amount of lufenuron (Sigma Aldrich), and stable isotope-labelled internal standard, lufenuron-d3 (Toronto Research Chemicals), was added to all samples. Acetone was added for extraction, followed by shaking for 10 and 15 min in an ultrasound bath. Thereafter, the samples were centrifuged, and the extract was transferred to a new tube and concentrated at 40°C under nitrogen flow. Concentrates were dissolved in acetonitrile: water (75:25) and filtered through a 0.45 μm nylon filter prior to analysis using an Agilent 1200 LC-system (Agilent Technologies) coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies). The instrument was equipped with an ESI source operated in a negative mode. A ZORBAX SB-C18 analytical column, 2.1 mm × 50 mm, 1.8 μm (Agilent Technologies) was used, and the injection volume was 2 μL. The mobile phase was a mixture of acetonitrile and water applied at a flow rate of 0.4 mL/min. Chromatography was performed according to a stepwise gradient: 0–0.2 min, 20% acetonitrile; 3.0–5.0 min, 98% acetonitrile; 5.1–7.0 min, 80% acetonitrile. Masshunter software (Agilent Technologies) was used for instrument control and data analysis. All gradient steps were linear. The retention time was 5.2 min for lufenuron and lufenuron-d3. The following parameters were used for the ion source: drying gas temperature, 300°C; drying gas flow, 11 L/min; nebuliser pressure, 35 psi; and capillary voltage, 4000 V. Lufenuron was monitored using the following transitions: 509.0 $m/z \rightarrow$ 488.9 m/z with a collision energy of 4 V (quantifier), and 509.0 $m/z \rightarrow$ 325.9 m/z with a collision energy of 12 V (qualifier); fragmentation energy was 94 V and cell accelerator was 4 V. For the internal standard, lufenuron-d3, the following parameters were applied: 512.0 $m/z \rightarrow$ 325.9 m/z, collision energy, 18 V; fragmentation energy, 110 V; and cell accelerator, 4 V. Procedural blank, matrix blank, matrix-matched calibration curve and controls were prepared for each series. The LOQ was determined as 3.0 ng/g, and the method was linear over the range studied (3.0–50,000 ng/g). Recovery ranged from 80% to 120%, and inter-run precision was <20%.

2.5 | RNA-seq analysis

Homogenization of liver tissue was conducted with a Precellys 24 homogenizer (Bertin Technologies). Total RNA was extracted and treated with Dnase using the BioRobot EZ1 and RNA Tissue Mini Kit (Qiagen). Characterization of total RNA was done with the Agilent 2100 Bioanalyzer using the 6000 Nano LabChip kit (Agilent Technologies). The RNA integrity number (RIN), which is a measure of RNA degradation, was 9.5 ± 0.2 ($n = 40$) in the liver (mean ± SD).

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext UltraTM RNA (NEB). The Library Prep Kit for Illumina® (NEB) was used according to the manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (Rnase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and Rnase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments of preferentially

150–200 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter). Thereafter 3 μL USER Enzyme (NEB) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina NovaSeq 6000 platform and paired-end reads were generated.

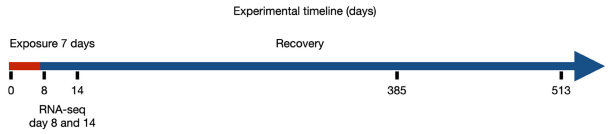
2.6 | Data analysis

Clean reads were obtained by removing adapter and poly-N sequences and read with low quality from the raw data. Paired-end clean reads were mapped to the Atlantic salmon reference genome (https://salmobase.org) using HISAT2 software. The Cufflinks Reference Annotation Based Transcript (RABT) assembly method was used to assemble the set of transcript isoforms of each bam file obtained in the mapping step. HTSeq was used to calculate the number of mapped reads to each gene. RPKM (reads per kilobase of transcript per million reads mapped) was calculated based on gene length and reads mapped to each gene. Differential expression between controls and exposed fish on days 8 and 14 was performed using the DESeq2 R package. Adjusted with Benjamini and Hochberg's FDR method, genes with an adjusted p value < .05 were assigned as differentially expressed. Gene ontology (GO) enrichment analysis was conducted with the Goseq R package, using gene length bias correction. KEGG pathway enrichment analysis was conducted with the KOBAS software.

2.7 | Statistical analyses

One-way ANOVA with Tukey's post hoc test was used to compare fish weight and chemical concentrations. In case the standard deviation differed significantly (Brown–Forsythe test), or the data did not have a normal distribution (Shapiro–Wilk test), the data were log-transformed before ANOVA. Pathway enrichment analysis was performed with Enrichr (Kuleshov et al., 2016) and Ingenuity Pathway Analysis (IPA) (Qiagen). Causal network and upstream regulator analyses were performed in IPA (Kramer et al., 2014). Upstream regulator analysis determines possible molecules or chemicals that are connected to dataset genes through a set of direct or indirect relationships. Predicted activation or inhibition was calculated by using the direction of gene regulation (upregulation or downregulation). Causal network analysis exposes causal relationships by expanding upstream analysis to include regulators not directly connected to targets in the dataset.

TABLE 1 Fish size at sampling (mean ± SEM).



	Day 8		Day 14		Day 385	Day 513
	Control	Exposed	Control	Exposed	Exposed	Exposed
Weight (g)	37.6±2.5	36.7±3.0	39.1±16.5	45.1±10.8	1118±215	2040±643
Length (cm)	15.2±0.5	15.1±0.6	16.0±1.7	16.1±1.3	43.9±2.9	54.5±4.3

Note: $n = 10$.

3 | RESULTS

3.1 | Fish growth

There were no significant differences in length and weight between the controls and the salmon exposed to lufenuron at days 8 and 14 (Table 1) (t-test, weight day 8, $p = .47$; day 14, $p = .35$). Medicated fish were followed for a prolonged time after treatment for toxicokinetic assessment. At day 385, the mean weight of the fish was 1.1 kg, while the fish weighed 2.0 kg at day 513.

3.2 | Lufenuron accumulation in liver

After a standard 7-day medication, the liver concentration of lufenuron was 29 ± 3 mg/kg 1 day after the end of medication (day 8) (mean ± SEM, $n = 10$) (Figure 1). After 7 days of depuration (day 14), the concentration of lufenuron in the liver had dropped to 14 ± 1 mg/kg ($n = 10$). At day 385, the concentration was 64 ± 7 μg/kg ($n = 10$), while at day 513, the concentration was further reduced to 22 ± 2 μg/kg ($n = 10$). Lufenuron was not detected in liver samples from the control fish at days 8 and 14 (pooled samples, $n = 2$), control fish were not examined at days 385 and 513.

3.3 | Differentially expressed genes

On days 8 and 14, there were 2437 and 169 significant DEGs in the liver, respectively (p-adj < .05, no fold change cut-off was used). Figure 2a shows the number of significant DEGs at days 8 and 14 (only DEGs with positive annotation used in downstream functional analyses are shown), while Figure 2b shows a circus plot of the same genes. At day 8, most of the significant genes were downregulated (Figure 2c), while the opposite was observed at day 14 (Figure 2d). At day 8, 95.8% of the DEGs were downregulated, while 97.0% of the DEGs were upregulated at day 14. Thus, a seven-day treatment with lufenuron reduced the transcriptional levels of many genes in the liver. This effect of the treatment was transitory, with far fewer transcripts being significantly affected 7 days after terminated treatment (day 14, 93% fewer DEGs). With almost all DEGs being upregulated, the data suggest a compensatory response at day 14.

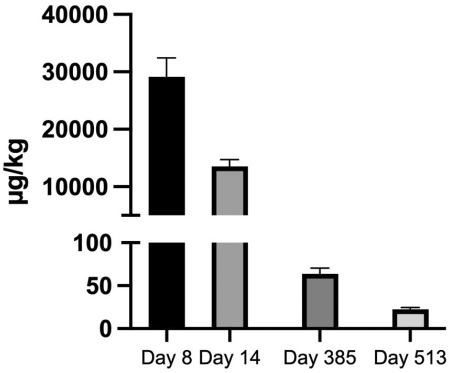


FIGURE 1 Accumulation of lufenuron in liver of Atlantic salmon on days 8, 14, 385 and 513 after dietary exposure for 7 days. Lufenuron was not detected in the controls on days 8 and 14 (lufenuron was not measured in the controls on days 385 and 513). The data are mean ± SEM ($n = 10$ for exposed animals, and $n = 2$ [pools of 5 fish] for the controls on days 8 and 14).

All DEGs from days 8 and 14, sorted by $\log_2$ fold change, are shown in File S1. Only DEGs with positive annotation and human identifiers used in the functional analyses are shown (File S1). There were 1789 significant DEGs with human identifiers at days 8 and 151 DEGs with human identifiers at day 14 (p-adj < .05). At day 8, the five DEGs with largest fold changes were phosphatase and actin regulator 3 (*phactr3*, −81.1-fold), neuronal pentraxin 2 (*nptx2*, 28.8-fold), keratin 1 (*krt1*, −19.3-fold), mucin 5B, oligomeric mucus/gel-forming (*muc5b*, 14.0-fold) and ankyrin repeat domain 52 (*ankrd52*, 12.2-fold). At day 14, the five DEGs with the largest fold changes were (*phactr3*, 92.8-fold), insulin (*ins*, 14.8-fold), cathelicidin antimicrobial peptide (*camp*, 12.8-fold), macrophage stimulating 1 receptor (*mst1r*, 10.9-fold) and cystatin SN (*cst1*, 10.0-fold).

Surprisingly, few DEGs were shared by both time-points. Of the 19 common genes, most were downregulated at day 8 and upregulated at day 14. Most profoundly, *phactr3* was 81.1-fold downregulated at day 8 and 92.8-fold upregulated at day 14, while *krt1* was 19.3-fold downregulated at day 8 and 1.4-fold upregulated at day 14. Three DEGs were upregulated at both time points. These were *mst1r*, *sox4* and *slc47a1*. The other DEGs showed opposite responses at the two sampling points. A heatmap of common DEGs is shown in Figure 3.

The GO and KEGG analyses suggested that lufenuron exposure had an inhibitory effect on transcription at day 8 (Figure 4). Many ontologies and pathways related to RNA metabolism were significantly impacted immediately after the end of the medication period. For example, the Notch signalling pathway was predicted reduced in the liver of medicated salmon. The finding further suggests that transcription and protein synthesis were impaired at day 8. In terms of potential effects on mechanisms often associated with toxicity, KEGG pathway analysis suggested that the treatment had an impact on cell differentiation and survival. Among the significant pathways at day 8 were FoxO signalling and apoptosis pathways. At day 14, there were no significant GOs or KEGG pathways (p-adj < .05).

DEGs with human identifiers were used to identify canonical pathways, diseases and functions and gene networks using IPA. At day 8, the sumoylation (small ubiquitin-like modifier, SUMO) pathway was the most significant pathway (p-value: 2.72E08) with a predicted activation. 26 DEGs in the SUMO pathway were differentially expressed at day 8 (Figure 5). There was a bias in the expected expression that genes were predicted to have if the pathway was activated, and the activation z-score for the SUMO pathway was <2. Other enriched pathways at day 8 included RAN signalling, and cell cycle and cell cycle regulation. The apelin liver signalling pathway was most significantly affected at day 14 (p-value: 5.91E04), followed by the extrinsic prothrombin activation pathway and triacylglycerol degradation. Table 2 shows the four most enriched pathways at the two sampling points with enrichment scores and the percentage of overlapping molecules. Organismal injury and abnormalities and endocrine system disorders were predicted by IPA among the top diseases at both sampling points. In terms of molecular and cellular functions, IPA predicted impacts on RNA post-transcriptional modification and gene expression at day 8, and on cellular movement, cell death and survival and gene expression at day 14. At day 8, the major outcome of lufenuron exposure was decreased transcription due to reduced processing and modification of RNA, and increased cell death and survival due to apoptosis and necrosis, while increased cell movement was the major cellular outcome at day 14.

Upstream regulator and causal network analyses were conducted to predict which molecules or chemicals could explain the direction of gene regulation. The top five upstream regulators and causal networks are shown in Table 3. File S2 shows all significant upstream regulators at days 8 and 14 with predicted activation or inhibition state. According to the top regulator networks with the highest consistency scores, lufenuron treatment-induced DNA damage, apoptosis and repressed transcription at day 8 (Figure S1A,B), followed by inhibited organismal death at day 14 (Figure S1C). Based on consistency (activation z-score) and p-value of overlap, CST5 and PHF12 (predicted activated), and MYC, MYCL and MMP3 (predicted inhibited) were among the regulators that best could explain the pattern of gene expression at day 8. Additionally, there were numerous regulators predicted activated or inhibited at day 8. IGF1, which encodes an insulin-like growth factor that promotes growth, was predicted inhibited on day 8 and activated on day 14 (Figure S2A). At day 8, several transcription factors encoding proteins that regulate endoplasmic stress and the protein unfolding response, such as XBP1, EIF2AK3, ERN1, ATF4 and AFT6, were predicted inhibited. This finding indicates the lowered degree of misfolded proteins due to reduced metabolic activity, as also suggested by the inhibitory effect on the hypoxia-inducible factor signalling pathway at day 8 (overlap 16/70 DEGs, p-value 4.63E-05). Furthermore, autophagy and FOXO1 were predicted inhibited at day 8 and activated at day 14 (Figure S2B). At day 14, a total of 10 predicted upstream regulators were significantly activated and four were predicted inhibited (with z-scores >2). Activated upstream regulators included

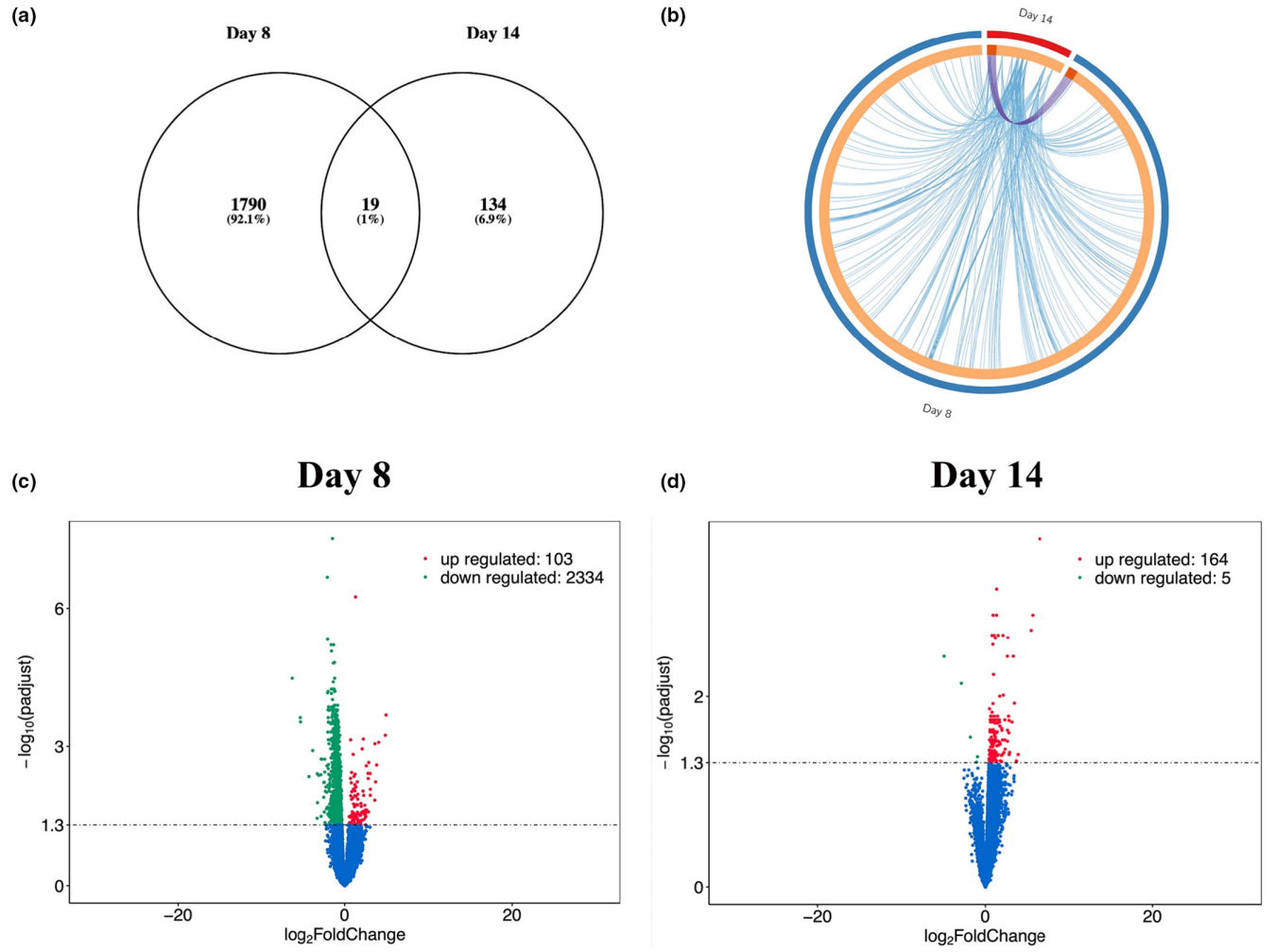


FIGURE 2 (a) Venn diagram showing the number of significant differentially expressed genes (DEGs). Only DEG with positive annotations are shown. (b) Circos plot of significant DEGs. (c) Volcano plot of all genes at day 8. (d) Volcano plot of all genes at day 14. Red (upregulated) or green (downregulated) color dots indicate significant DEGs (p-adj < .5)

CEBPA, VEGFA, RELA and IGF1. Thus, the upstream regulator analyses pointed to inhibited transcription and metabolism immediately after terminated medication followed by a compensation reaction 7 days later.

4 | DISCUSSION

This study shows that lufenuron easily accumulates in liver tissue of Atlantic salmon after standard treatment and that the compound remains in the fish for a prolonged period. Residues of the compound could impact the fish as a toxicant for a long time after exposure. Juvenile Atlantic salmon orally administered with lufenuron for 7 days experienced a repression of transcription in the liver after the end-of-treatment. This inhibitory effect on transcription was transitory. One week into the depuration period, most of the significant DEGs were upregulated, and far fewer genes were differentially regulated (more than 90% of the gene response observed on day 8 was gone by day 14). A standard nominal treatment with lufenuron for 1 week thus had a profound but short-lived impact on transcription in the liver of Atlantic salmon. Pathways analysis showed that mechanisms associated with cell cycle regulation and post translational modification of proteins were most strongly impacted immediately after treatment. One day after end-of-treatment, the SUMO pathway was most significantly affected, while the apelin signalling pathway was most significantly affected after 7 days of depuration. Induction of the SUMO pathway by lufenuron might result in adverse outcomes in fish that could be applied in the development of adverse outcome pathways (AOPs) to implement risk. Follow-up studies should aim to link the molecular initiating event with key events at a higher biological level in an AOP framework (Ankley et al., 2010).

Reversible post-translational modification by the SUMO pathway regulates many cellular functions such as protein stability, protein–protein interactions, transcription, DNA replication, cell cycle regulation and DNA repairs essential for maintaining cell homeostasis (Geiss-Friedlander & Melchior, 2007). Sumoylation consists of a series of biochemical steps catalysed by a hierarchical set of E1,

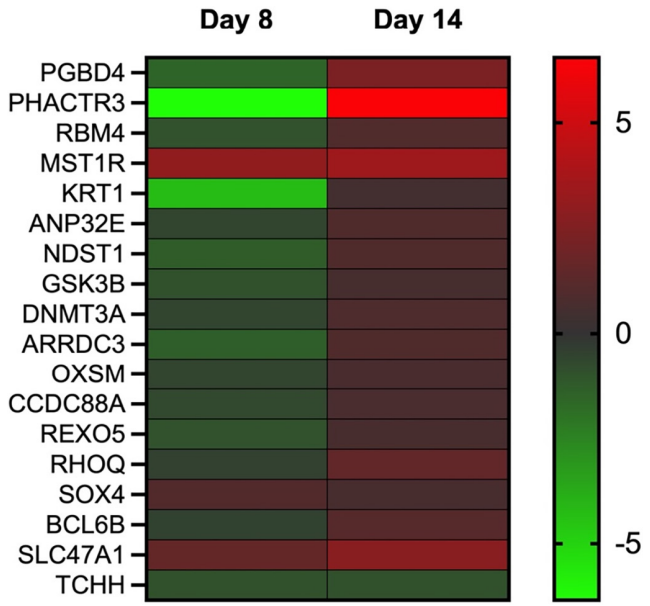


FIGURE 3 Heatmap of common genes affected by lufenuron exposure in liver of Atlantic salmon 1 day after the end of treatment (day 8) and after 7 days of recovery (day 14).

E2 and E3 enzymes, while desumoylation involves SUMO-specific proteases (Enserink, 2015). A key protein in the SUMO pathway is UBC9, encoded by the ubiquitin-conjugating enzyme E2 I (*ube2i*) gene. In this study, *ube2i* was downregulated at day 8 and was one of a total of 26 DEGs in the SUMO pathway that were differentially regulated immediately following the lufenuron exposure. Other key protein genes in the SUMO pathway that were differentially regulated at day 8 were *sumo2*, *sumo3*, *senp1*, *senp3*, *senp5*, *senp7*, *cbx4* and *pias4*. Most of these SUMO pathway genes were downregulated at day 8. Two genes associated with the SUMO pathway, *dnmt3a* and *rohq*, were differentially regulated both at days 8 and 14. Protein synthesis was predicted to decrease due to differential regulation of 235 DEGs, supporting a slowdown of metabolism. However, as one of the outcomes of the SUMO pathway itself is repressed transcription (Gill, 2005), impairment of this pathway does not necessarily reflect reduced overall metabolism. The observed response could be due to a direct impact on mechanisms linked to sumoylation and thereby on post-translational modification of proteins, or it could be a consequence of lowered overall transcription and metabolism in the liver cells. Pathway analysis predicted reduced growth of organism at day 8, and increased growth at day 14. However, no significant effect of lufenuron exposure on fish weight was observed in this study. Nutrient deprivation was therefore probably not responsible for the lufenuron-induced effect on sumoylation.

The SUMO pathway is an essential regulator of cell homeostasis following environmental stresses such as osmotic shock, hypoxia, heat, oxidative stress, nutrient deprivation, genotoxic stresses and in response to stress protein sumoylation levels usually increase sharply (Ryu et al., 2020). Chemicals known to affect the SUMO pathway in fish include dioxins, nonylphenol, PFOSA, crude oil and depleted uranium (Alexeyenko et al., 2010; Dasgupta et al., 2020; Shelley et al., 2012; Sherwood et al., 2021; Song et al., 2018). In this study, possible modes of action could be oxidative stress, DNA damage, or reduced food intake during the medicated period. It has previously been shown that sumoylation provides protection for cells against oxidative stress by repressing intracellular ROS generation (Yang et al., 2014). Several studies have shown that lufenuron can induce oxidative stress and affect the levels of antioxidants in fish after water exposure (Ghelichpour et al., 2019; Mirghaed et al., 2020). Oxidative stress can lead to the inhibition of global sumoylation due to the formation of disulfide bonds between the catalytic cysteines of SUMO E1 and E2 enzymes (Ryu et al., 2020). However, the list of significant DEGs at day 8 did not suggest a strong lufenuron-induced oxidative stress response in liver of Atlantic salmon in this study.

Genotoxicity was suggested as a possible outcome of lufenuron exposure due to the significant effects seen on day 8 on the cyclins and cell cycle regulation and the cell cycle G1/S checkpoint regulation pathways. At the G1 checkpoint, the cell must decide whether to divide or not. In case of DNA damage or replication errors, the cell cycle will stop, and the cellular outcome might be apoptosis (Pietenpol & Stewart, 2002). Activation of NF-kB is an important protective part of the DNA damage response in cells (Janssens & Tschopp, 2006). Sumoylation mechanisms can inhibit protective NF-kB activation induced by DNA damage (Lee et al., 2011). Since SENP1 and SENP2 are NF-kB-inducible proteins, it cannot be ruled out that lufenuron disturbed mechanisms linked to DNA damage protection in this study. Furthermore, MDM2 mediated p53 regulation of G1 cell cycle arrest was predicted activated at day 8. The p53 tumour suppressor is a key transcription factor that protects cells by inducing apoptosis, DNA repair and cell cycle arrest in response to a variety of stresses (Nag et al., 2013). In this work, TP53 was predicted activated both on days 8 and 14. By interfering with p53 and the protective cellular mechanism, lufenuron exposure might have made the cells more vulnerable to DNA damage.

In skin of Atlantic salmon infected with sea lice, Robledo et al. (2018) observed a repression of the SUMO pathway. Repression of the sumoylation machinery constitutes a functional antiviral response in many organisms (Yu et al., 2017). Sumoylation has been shown to coordinate the repression of inflammatory and anti-viral gene-expression programs and impact the host innate immune response to pathogens (Decque et al., 2016). The intriguing link between treatment with a chitin synthesis inhibitor and repression of sumoylation in fish liver needs further study.

An impact on RAN signalling, the second most significant pathway at day 8, further supports a lufenuron-induced effect on sumoylation. RAN is a small GTP-binding protein belonging to the RAS superfamily that is essential for the translocation of RNA and proteins through the nuclear pore complex (Celen & Sahin, 2020). RAN controls a variety of cellular functions by interacting with other proteins and is also involved in the control of DNA synthesis and cell cycle progression. Proteins in the RAN signalling pathway interact with SUMO1 and other proteins in the SUMO pathway (Celen & Sahin, 2020). On day 8, there were two overlapping genes in the SUMO and RAN signalling pathways (RAN binding protein 2 [*ranbp2*]

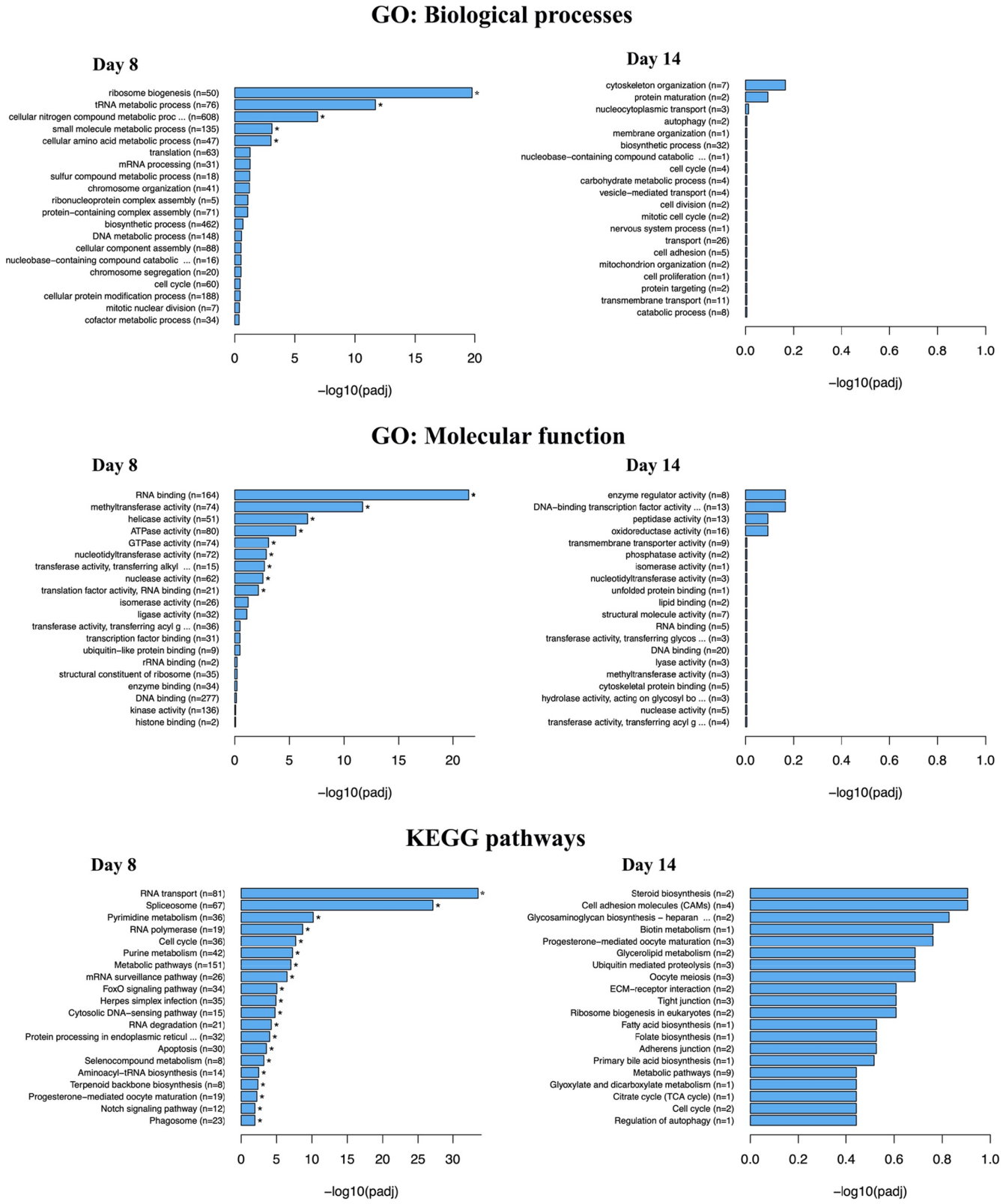


FIGURE 4 Top gene ontologies (GOs) and KEGG pathways at day 8 and day 14.

and regulator of chromosome condensation 1 [*rcc1*]). Other key RAN signalling genes regulated at day 8 included chromosome segregation 1 like (*cse1l*), RAN binding protein 1 (*ranbp1*) and exportin 1 (*xpo1*). Altogether, pathway analysis clearly suggests that lufenuron exposure influenced mechanisms associated with sumoylation in liver of Atlantic salmon.

The apelin liver signalling pathway was the most significantly affected pathway at day 14. Apelin inhibits liver regeneration while

promoting hepatic fibrosis upon injury (Lv et al., 2017). Three DEGs in the apelin pathway were significantly affected. These were the apelin receptor (*aplnr*), collagen type XVIII alpha 1 chain (*col18a1*) and glycogen synthase kinase 3 beta (*gsk3b*). Various chemicals such as benzo(a)pyrene, bisphenol A and atrazine have been shown to affect *plant* transcription in fish (Wirbisky et al., 2015; Yadetie et al., 2021; Zhang et al., 2016). An impact on the apelin liver signalling pathway suggests that lufenuron exposure affected Fas-induced signalling and mechanisms linked to liver injury (Lv et al., 2017). Apelin has an anti-apoptotic effect and attenuates oxidative stress (Mlyczynska et al., 2021). This response is mediated by the apelin receptor. An impact on the apelin liver signalling pathway could therefore reflect a protective response to prevent inappropriate programmed cell death after lufenuron exposure at day 14.

There were only 18 common DEGs for the two sampling points. Interestingly, 14 of these DEGs were downregulated at day 8 and upregulated at day 14, suggesting a compensatory response 1 week after the end of exposure. Lufenuron exposure had the most profound effect on the transcription of *phactr3*. This gene was almost 100-fold downregulated at day 8 and 100-fold upregulated at day 14. *Phactr3* encodes phosphatase and actin regulator 3 (PHACTR3), a membrane-associated enzyme of the phosphatase and actin regulator protein family. The protein might be involved in cell migration and morphogenesis by modulating the actin cytoskeleton (Itoh et al., 2014). PHACTR3 is often hypermethylated in human cancer (Bosch et al., 2012). In fish, both benzo(a)pyrene and ethynyl estradiol upregulated *phactr3* in polar cod (*Boreogadus saida*) (Yadetie et al., 2021). Interestingly, a gene encoding DNMT3A, a de novo methyltransferase which can repress transcription, was also downregulated at day 8 and upregulated at day 14. Dysregulation of these genes could imply an impact on mechanisms associated with DNA methylation. A possible epigenetic effect of lufenuron, as indicated by differential expression of *dnmt3a* and *phactr3*, should be further studied.

Only two DEGs were highly regulated in the same direction on days 8 and 14. These genes were *mst1r*, which was 8.1- and 10.9-fold upregulated at days 8 and 14, respectively, and *slc47a1*, which was 2.9- and 6.7-fold upregulated at these time-points. *Mst1r* encodes the macrophage stimulating 1 receptor (MST1R, also called RON), a cell surface receptor for macrophage-stimulating protein (MSP) with tyrosine kinase activity thought to be involved in host defence. MST1R

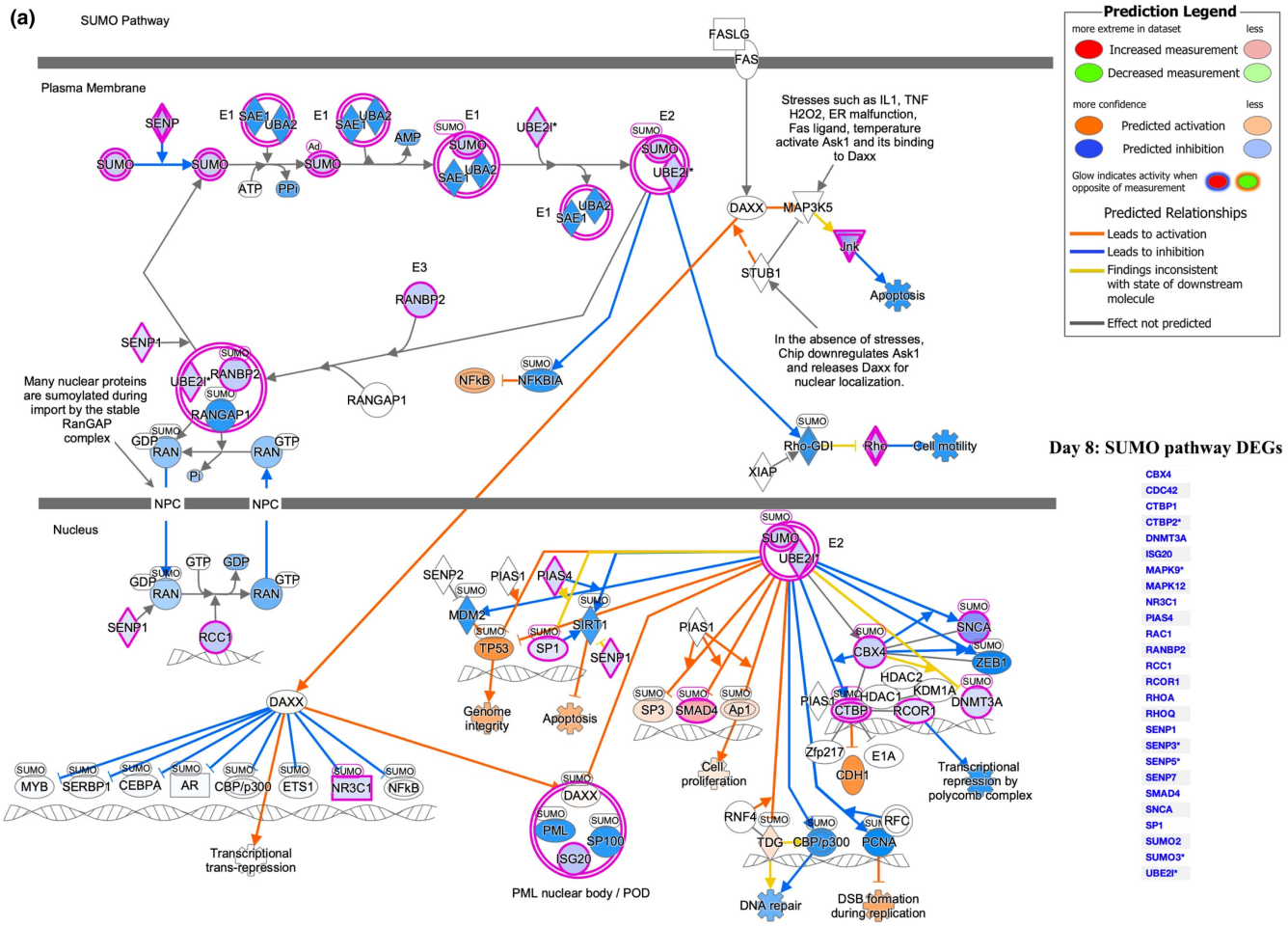


FIGURE 5 Top canonical pathways in liver of Atlantic salmon exposed to lufenuron for 7 days. (a) The SUMO pathway which was most significantly affected at day 8 and (b) the apelin liver signalling pathway that was most significantly affected at day 14.

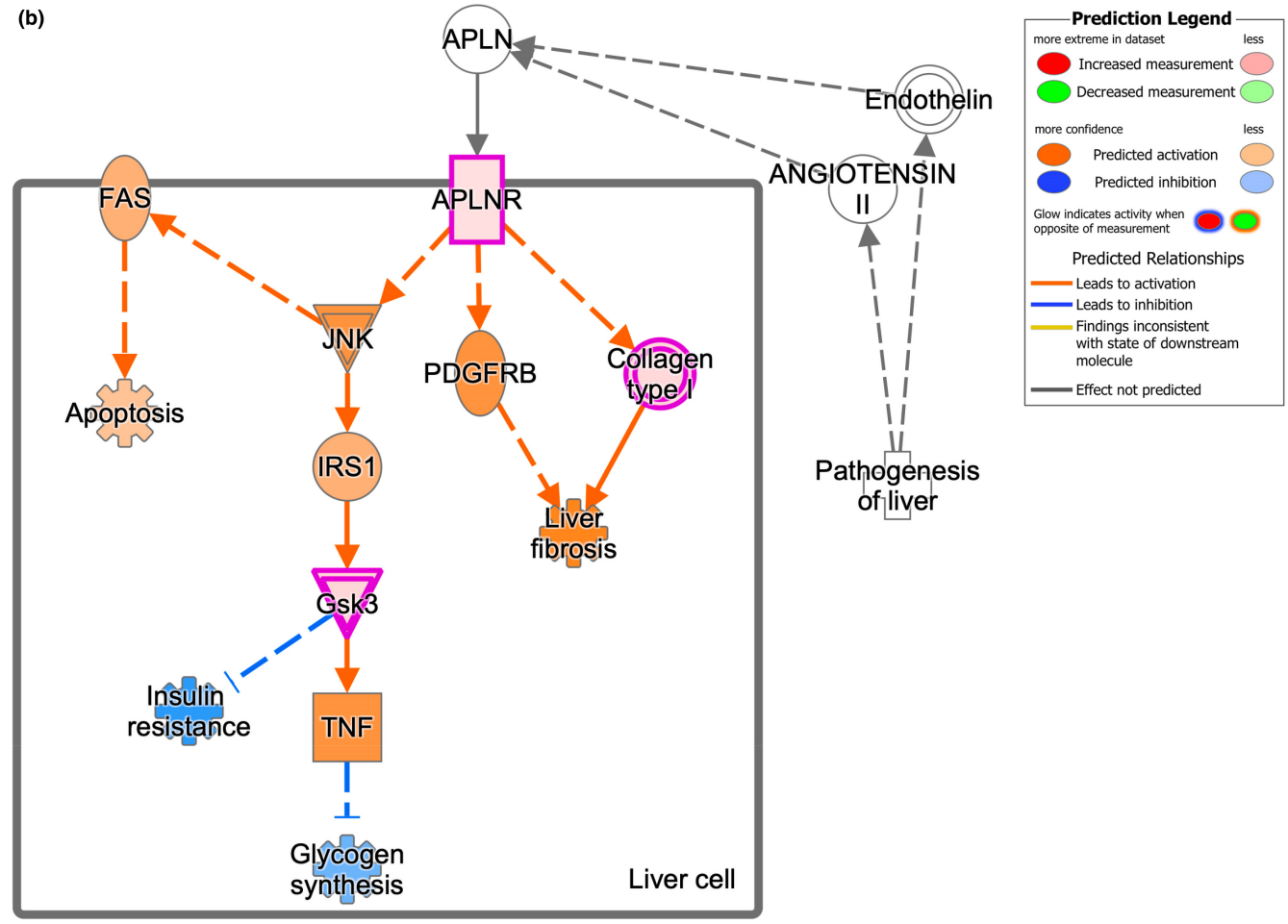


FIGURE 5 (Continued)

TABLE 2 Top canonical pathways affected in liver of Atlantic salmon exposed to 5 mg/kg lufenuron for 7 days.

Name	*p*-Value	Overlap
Day 8[a]		
Sumoylation pathway	2.63E-08	25.2% 26/103
RAN signalling	1.01E-06	52.9% 9/17
Cyclins and cell cycle regulation	2.79E-06	23.8% 20/84
Cell cycle: G1/S checkpoint regulation	7.60E-06	25.0% 17/68
Day 14		
Apelin liver signalling pathway	5.85E-04	11.5% 3/26
Extrinsic prothrombin activation pathway	4.53E-03	12.5% 2/16
Triacylglycerol degradation	5.71E-03	5.3% 3/57
Nicotine degradation III	5.99E-03	5.2% 3/58

Note: Liver tissue was collected at days 8 and 14, i.e., 1 and 7 days after the end of treatment.
[a]One pathway related to human cancer is removed.

helps regulate many physiological processes such as cell survival, migration and differentiation, and plays a role in the innate immune response (Wagh et al., 2008). MST1R has been linked to chemical and drug-induced liver injury after exposure to chemicals such as acetaminophen, bisphenol A and benzo(a)pyrene (Ali et al., 2014; Rawls et al., 2021; Yadetie et al., 2012). *Slc47a1* encodes the multidrug and toxin extrusion protein 1 (MATE1), a multidrug solute transporter of numerous cationic toxicants (Yonezawa & Inui, 2011). MATE transporters are important components of phase III cellular detoxification in vertebrates (Loncar et al., 2016). Chemicals associated with MATE1 includes bisphenol A, cadmium, and benzo(a)pyrene (Ali et al., 2014; Kreuzer et al., 2020; Yang et al., 2017). In essence, *mst1r*

TABLE 3 Top upstream regulators and causal networks at day 8 and day 14.

Regulator – day 8	*p*-Value	Predicted activation	Regulator – day 14	*p*-Value	Predicted activation
Upstream regulator					
HNF4A	4.77E-27		CEBPA	5.18E-05	Activated
CST5	4.45E-26	Activated	beta-estradiol	7.50E-05	
PHF12	1.52E-18	Activated	INHA	7.74E-05	
MYC	1.52E-18	Inhibited	AGT	9.77E-05	
MMP3	8.63E-14	Inhibited	GALNT2	2.61E-04	
Causal network					
CST5	3.4E-26	Activated	PPARA	7.42E-09	
PHF12	1.52E-18	Activated	RAP2A	9.90E-09	Activated
L-asparagine	4,11E-17	Inhibited	MAPK9	1.16E-07	Activated
CD276	1.11E-15	Inhibited	2-methyl-2-pentenoic acid	1.35E-07	Activated
RND3	3.27E-15	Inhibited	4-pentenoic acid	1.35E-07	Activated

Note: Predicted activation or inhibition is indicated when the activation *z*-score was >2/−2.

and *slc47a1* could be potential biomarkers of lufenuron exposure in fish.

Evidence suggests that benzoylurea insecticides are detoxified by the cytochrome P450 system in fish and other organisms. Exposure to diflubenzuron resulted in upregulation of *cyp3a* in liver tissue of Atlantic cod (Olsvik et al., 2013), whereas teflubenzuron exposure upregulated a gene annotated to *cyp3a* in muscle tissue of European lobster (*Homarus gammarus*) (Olsvik et al., 2015). Mammalian studies have shown similar results with increased expression of CYP3A protein after diflubenzuron exposure (Dubois et al., 1996; Sapone et al., 2005). In insects, biotransformation of lufenuron depends on CYP9 and CYP12 proteins (Bogwitz et al., 2005; Zhang et al., 2023), CYP families that belong to the mammalian CYP3 and CYP5 families. Although CYP3A appears to be involved in the detoxification of benzoylurea insecticides in vertebrates, this gene was not differentially regulated in this study (*cyp3a27* was 1.4-fold upregulated at day 14 (*p*-value = .03, *p*-adj = .22). Instead, a gene annotated to the cytochrome P450 family 2 subfamily R member 1 (*cyp2r1*) was downregulated at day 8, while several phases I and II enzyme genes were upregulated at day 14. These included *cyp1b1*, *cyp27b1*, *cyp46a1*, the UDP glucuronosyltransferase family 2 member A1 complex locus (*ugt2a1*), and four solute carriers that may be involved in the transport of organic compounds (*slc4a3*, *slc13a2*, *slc41a3*, *slc47a1*). Active biotransformation of lufenuron was therefore probably occurring in liver of salmon at day 14. However, the exact detoxification mechanism of lufenuron in fish remains unclear.

Numerous transcriptional regulators that can explain the observed gene changes were predicted as either activated or inhibited at day 8. CST5 (cystatin D, activation *z*-score 6.33) and PHF12 (PHD finger protein 12, activation *z*-score 4.80) were predicted activated on day 8. CST5 is a cysteine protease inhibitor predicted activated by differential expression of 81 DEGs, including the glucocorticoid receptor (*nr3c1*). PHF12 is a transcription repressor that interacts with chromatin and is associated with chemical and drug-induced liver injury. MYC was predicted inhibited at day 8, with an inhibition *z*-score of −8.75. A MYC-induced response on transcription suggests an effect on mechanisms associated with cell cycle progression, apoptosis and cellular transformation (Hoffman & Liebermann, 2008). According to pathway analysis, apoptosis and necrosis were predicted to be considerably increased in liver of Atlantic salmon immediately after lufenuron exposure in this study (day 8) and moderately inhibited at day 14. FOXO1 was predicted inhibited on day 8 and activated on day 14. FoxO signalling regulates processes such as apoptosis, cell cycle control, glucose metabolism, oxidative stress response, and longevity (Gross et al., 2008). As FOXO1 plays a significant role in regulating whole-body energy homeostasis (Gross et al., 2008), the result suggests an inhibitory effect on energy metabolism immediately after exposure and a compensation reaction 1 week later. A similar response was predicted for IGF1, with an inhibitory effect at day 8 and activation at day 14. The vascular endothelial growth factor A (VEGFA) was also predicted activated at day 14. Together, these findings suggest that lufenuron repressed energy metabolism and growth at day 8, followed by a compensatory response 6 days later. Taken together, the upstream regulator analyses point to hepatotoxicity and reduced metabolism immediately after exposure followed by a compensation reaction 7 days later.

In conclusion, this study shows that lufenuron easily bioaccumulates in liver of Atlantic salmon. Residues of the drug were present in liver tissue even after almost 1.5 years of depuration. Lufenuron exposure had a strong but transitory effect on hepatic transcription. Compared to the control, more than 2000 genes were downregulated at the end-of-treatment. One week into the elimination period, the transcriptional response was much weaker, and almost all genes were upregulated compared to the control. Pathway analysis predicted effects on the SUMO and apelin pathways at days 8 and 14, respectively. With a much weaker response on transcription and a compensatory response after 1 week of recovery, the study

suggests only a modest long-term lufenuron-induced effect on liver transcription.

AUTHOR CONTRIBUTIONS

Pål A. Olsvik: Writing – original draft; methodology; visualization; formal analysis; conceptualization; investigation. **Kristine E. Brokke:** Conceptualization; writing – review and editing; methodology; investigation. **Ole B. Samuelsen:** Conceptualization; writing – review and editing; investigation. **Rita Hannisdal:** Conceptualization; funding acquisition; project administration; writing – review and editing; methodology; investigation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

The RNA-seq dataset has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE199382 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199382).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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