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# High level efficacy of lufenuron against sea lice (*Lepeophtheirus salmonis*) linked to rapid impact on moulting processes

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ABSTRACT

Drug resistance in the salmon louse *Lepeophtheirus salmonis* is a global issue for Atlantic salmon aquaculture. Multiple resistance has been described across most available compound classes with the exception of the benzoylureas. To target this gap in effective management of *L. salmonis* and other species of sea lice (e.g. *Caligus* spp.), Elanco Animal Health is developing an in-feed treatment containing lufenuron (a benzoylurea) to be administered prior to seawater transfer of salmon smolts and to provide long-term protection of salmon against sea lice infestations. Benzoylureas disrupt chitin synthesis, formation, and deposition during all moulting events. However, the mechanism(s) of action are not yet fully understood and most research completed to date has focused on insects. We exposed the first parasitic stage of *L. salmonis* to 700 ppb lufenuron for three hours and observed over 90% reduction in survival to the chalimus II life stage on the host, as compared to vehicle controls. This agrees with a follow up *in vivo* administration study on the host, which showed > 95% reduction by the chalimus I stage. Transcriptomic responses of salmon lice exposed to lufenuron included genes related to moulting, epithelial differentiation, solute transport, and general developmental processes. Global metabolite profiles also suggest that membrane stability and fluidity is impacted in treated lice. These molecular signals are likely the underpinnings of an abnormal moulting process and cuticle formation observed ultrastructurally using transmission electron microscopy. Treated nauplii-staged lice exhibited multiple abnormalities in the integument, suggesting that the coordinated assembly of the epi- and procuticle is impaired. In all cases, treatment with lufenuron had rapid impacts on *L. salmonis* development. We describe multiple experiments to characterize the efficacy of lufenuron on eggs, larvae, and parasitic stages of *L. salmonis*, and provide the most comprehensive assessment of the physiological responses of a marine arthropod to a benzoylurea chemical.

## 1. Introduction

Lufenuron is a benzoylurea (or benzoylphenyl-urea; BPU) that was discovered in the 1980s by Ciba-Geigy, and subsequently marketed in animal health, bioprotection, and crop protection in products such as Sentinel™, Program™, Match™, etc. Other BPUs have been used to control ticks, mosquitos and flies of importance in companion animals, human disease, agriculture, and aquaculture (Dean et al., 1998; Merzendorfer, 2013; Ritchie et al., 2002; Sun et al., 2015).

Benzoylureas bind chitin synthase 1 in terrestrial arthropods (Douris et al., 2016) causing inhibition of chitin biosynthesis (IRAC group 15; Sparks and Nauen, 2015) in target pests. These compounds have a

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broad spectrum of activity, which can extend across generations through impacts on reproduction, egg-hatching, and moulting of larvae (Mommaerts et al., 2006) and are ideal intervention tools as they can be administered orally and have low toxicity to vertebrates (i.e. humans). The latter is exemplified by the approval and use of diflubenzuron in drinking water to control *Aedes aegypti* in Brazil (Belinato and Valle, 2015; WHO, 2008, 2006). In this and other cases, BPUs have been important alternatives for treating drug-resistant pest populations (Merzendorfer, 2013).

*Lepeophtheirus salmonis* and other ectoparasitic sea lice species (Family: Caligidae) are the most economically important pathogens of salmon farming worldwide. Infestation thresholds within the industry are set conservatively in most, but not all, farming regions with a focus on protecting wild populations of salmon from the impacts of farm-based spill over. This is accomplished by either medicinal intervention or by biological control efforts, typically in an integrated pest management strategy. A total of five pesticide or drug classes are licensed for use in Atlantic salmon culture (Igboeli et al., 2014) while the inclusion of mechanical control (Stien et al., 2016), cleaner fish (Powell et al., 2017), and other alternative measures are becoming more prominent.

Drug resistance in sea lice is a global issue for salmon aquaculture and multiple resistance has been described for most of the licenced compound classes (i.e. pyrethroids, organophosphates, avermectins and hydrogen peroxide) with the exception of BPUs (reviewed in Aaen et al., 2015). In particular, emamectin benzoate (EMB; Slice®), an in-feed avermectin treatment, was used nearly exclusively in many countries from 2000 to 2007 before resistance developed in Eastern Canada, Chile, Scotland, Norway and the Faroe Islands (Igboeli et al., 2014). No new drug therapies against sea lice have been licensed since. To target this gap in effective management tools for sea lice, Elanco Animal Health is developing an in-feed lufenuron treatment to be administered prior to seawater transfer of salmon smolts and to provide long-term protection of salmon against sea lice infestation at sea.

Lufenuron is not the first BPU to be used against sea lice, as diflubenzuron (Lepsidon™) and teflubenzuron (Calicide™) have, and are currently, being used in different salmon farming regions (Igboeli et al., 2014). However, these drugs have poor absorption across the gastrointestinal tract of salmon and represent a major ecological concern for non-target species such as lobsters (Scottish Executive, 2002; Olsvik et al., 2015). Despite prior use and research on BPUs, the mode of action for these drugs has not been characterized in crustaceans. In insects, chitin synthase, a transmembrane glycosyltransferase (family 2) responsible for the synthesis and polymerization of chitin (Merzendorfer, 2006), is a target site for BPUs (Douris et al., 2016). However, based on the multifunctional nature of this enzyme, the complexity of the moult process in general, and the large phylogenetic distance between copepods and insects, taxa-specific BPU responses are expected. Furthermore, sea lice development is poorly understood from a genomic standpoint, and may hold clues for novel drug discovery. The objectives of the current work were to (1) develop a system whereby the responses of planktonic *L. salmonis* larvae exposed to lufenuron could be studied in a physiologically meaningful way, (2) determine genes and pathways in salmon lice that are responsive to lufenuron, and (3) examine the ultrastructural impacts of lufenuron on sea lice cuticles. These lines of investigation were pursued to characterize the mode of action of lufenuron on *L. salmonis* and potentially other parasitic copepods.

## 2. Materials & methods

### 2.1. Salmon lice collection and culture

For all experiments, salmon lice (*L. salmonis*) were collected from marine aquaculture sites in Bay Management Areas 1a or 2a (BMA1a or BMA2a) of the Bay of Fundy, New Brunswick (NB) Canada between 2013 and 2015. Eggstrings were collected from gravid females for hatching at the Huntsman Marine Science Centre (HMSC) in St Andrews, NB or the Atlantic Veterinary College (AVC) in Charlottetown, Prince Edward Island, Canada. Hatching parameters are described in Poley et al. (2016) and Sutherland et al. (2015).

### 2.2. Lufenuron bioassays

Multiple bioassay experiments were carried out using larvae and adult *L. salmonis* for *in vitro* exposures to lufenuron. In all experiments, $F_0$ generation lice were collected from farms in the Bay of Fundy, NB, Canada, and eggstrings reared in the laboratory at 11 ± 1 °C, either at the AVC or at the HMSC (from where copepodids were transported to AVC for bioassay work), until desired life stages were achieved. In all cases, stock solutions of lufenuron were made by dissolving 2.5 or 5.0 mg of lufenuron in 12.5 mL of methanol (Fisher Scientific; ON, Canada) or acetone (Sigma-Aldrich; ON, Canada) before diluting 1:1 with nuclease-free water. Working solutions were made using 10 mL of stock lufenuron dissolved in 990 mL of filtered seawater from the Bay of Fundy before further dilutions to obtain desired concentrations. In the first bioassay (B1), lufenuron was dissolved in methanol before exposing copepodids to 700 ppb lufenuron or a solvent control (0.35% methanol alone) for three hours. Immediately following the bioassay, salmon lice from each condition were rinsed in SW and used to infest Atlantic salmon (*Salmo salar*) smolts (n = 4; ca. 150 g) housed in single 10 L tank systems. For infection, each salmon was removed from its tank, anesthetized using 1 ppm tricaine methanesulfonate (MS-222; Sigma-Aldrich, ON, Canada) and exposed to approximately 100 copepodids for five minutes before recovery in the original tanks. Parasite density was determined using methods described in Poley et al. (2016). Salmon were sacrificed one week later using 2.5 ppm MS-222 for lice staging and enumeration. Water temperature was maintained at 11 ± 2 °C with salinity > 32 ppt for all challenges and bioassays.

Salmon lice from the same cohort as those reported in Poley et al. (2016) were used in the second and third bioassays (B2 and B3, respectively). Similar to B1, copepodids in B2 were pre-treated with lufenuron but acetone was used instead of methanol to emulsify lufenuron for this assay. Copepodids from B2 were used to infest Atlantic salmon smolts (ca. 150 g) housed in five 30 L tanks. Three fish were housed in each tank, with three tanks used for treated copepodids and two tanks for control copepodids. Salmon were sacrificed 12 days post-infection for lice staging and enumeration as described in B1.

The B3 experiment was designed to monitor changes in gene expression related to lufenuron exposure with a commonly used *L. salmonis* microarray (described below). Triplicate pools of 500 copeopdids were used for each of seven conditions including seawater (SW) and SW + acetone controls along with five concentrations of lufenuron (30, 300, 700, 1000, and 1500 ppb) in SW + acetone. Lufenuron exposures lasted for three hours before each pool was individually rinsed and held in SW for 21 h at 10 ± 2 °C with salinity > 32 ppt. Each pool of 500 copepodids was collected at this time and stored at −80 °C for RNA extractions.

In Bioassay IV (B4), pools of 400 nauplius II staged lice were exposed to either 700 ppb lufenuron or an acetone control (n = 5 for each group) for three hours before rinsing and holding in SW. Each pool was collected separately and stored at −80 °C for RNA extractions and RT-qPCR analysis.

Two additional bioassays similar to B4 were conducted for transmission electron microscopy (TEM; B5) and metabolomics discovery (B6). Salmon lice in B5 were sampled from each group (n = 5–10 individuals per group) at 24 and 48 h post-lufenuron exposure and stored in 2% glutaraldehyde at 4 °C before processing within 24 h of collection. For B6, pools of 500 copepodid salmon lice were separated into treated and control groups (n = 6) and collected after 24 h for storage at −80 °C.

A seventh bioassay (B7) was conducted to investigate impacts of

lufenuron on eggstrings and the exposure at the first moult of the planktonic stage. Adult female salmon lice collected from farms in the Bay of Fundy, NB, were brought back to the laboratory at AVC, whereby paired eggstrings were separated between control and treatment conditions. Eggstrings (n = 25) were exposed for 24–72 h (i.e. until first emergence of nauplii I stage larvae) to either acetone controls or 500 ppb lufenuron in a 500 mL glass beaker with aeration at 12 ± 1 °C. A SW-only control was conducted using eggstrings from the same cohort (n = 25), but from different individuals than those treated with lufenuron. These exposures were replicated from three separate collections between October 1 and November 17, 2014. Following the first hatch, nauplii and remaining eggstrings were passed through a filter (100 μm) and washed with new SW prior to being placed back in a 500 mL beaker with new seawater for the remainder of the observational period (4–7 days post-treatment). Hatch rate, behaviour and developmental stages were assessed.

### 2.3. In vivo challenge with lufenuron

Atlantic salmon from a commercial hatchery in NB, Canada were transported to the AVC and acclimated at 11 ± 1 °C in a freshwater flow-through system. Smolts (n = 680; weighing 59 ± 7.3 g) were evenly distributed in two rooms with eight replicate tanks in each. Each room was on a separate biofiltration loop with four tanks per experimental condition in each system. Control fish (n = 8 tanks) were fed a base salmon diet throughout the course of the study, whereas treated fish (n = 8 tanks) were administered the same base salmon feed with the top-coated inclusion of lufenuron at a dose rate of 5 mg/kg bw/day for 7 days (total dose 35 mg/kg), before being returned to the same base salmon feed without lufenuron after 7 days treatment. After a further 7 days, all tanks underwent conversion to a recirculating SW (instant ocean) system over a 17 day period until the system could be maintained at 32 ± 3 ppt for the remainder of the study. After 5 weeks post-administration of the medicated feed, fish in all tanks were exposed to approximately 100 copepodid-staged lice per fish, cultured at the HMSC and obtained from adult female eggstrings collected from Bay of Fundy salmon farms. At 13–14 days post-infestation (dpi), 10 arbitrarily selected salmon from each tank were sampled and the sea lice enumerated and compared between groups.

### 2.4. RNA extraction

Total RNA was extracted from pooled copepodids from B3 and nauplii from B4 using TRI-Reagent as per manufacturer's instructions (Thermo Fisher Scientific, Burlington, ON, Canada) (Chomczynski, 1993; Chomczynski and Mackey, 1995). All samples were subjected to an in-solution DNase treatment (Thermo Fisher Scientific) before RNA purification using RNeasy MinElute clean-up kits (Qiagen, Toronto, ON, Canada) as per manufacturer's instructions. RNA quantity and purity was analyzed using spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific) while RNA integrity was assessed using 1% agarose gel electrophoresis. All samples were suspended in nuclease-free water and stored at −80 °C until further use.

### 2.5. Microarray

The RNA extracted from 21 pools of 500 copepodid *L. salmonis* from B3 were hybridized on a 38 K oligonucleotide microarray (eArray, Agilent; Santa Clara, CA, USA) designed with expressed sequence tags (ESTs) from Atlantic and Pacific *L. salmonis* (Sutherland et al., 2012; Yasuike et al., 2012). Sample preparation, microarray hybridization, and scanning were performed alongside samples previously reported by Poley et al. (2016). Scanning was completed using a Perkin Elmer (Waltham, MA, USA) ScanArray®, while Imagene 8.1 (Biodiscovery; Hawthorne, CA, USA) was used for image quantification. Probe filtering and statistical analyses were executed using GeneSpring GX v13.0 (Agilent). A quality control (QC) filtered probe list included probes with ≥500 Cy5 and Cy3 fluorescence intensity in at least 66% samples of at least one condition. Probes that had samples containing poor quality flags were removed from the analysis. Raw data was uploaded to Gene Expression Omnibus (GEO, NCBI) under the accession GSE99880.

A one-way ANOVA without the assumption of equal variance and post-hoc Tukey HSD ($p < 0.01$; fold change (FC) ≥ 1.5) was used to determine differentially expressed probes between groups. Probes representing the same unique contig (transcript) are displayed with a range of FC in the manuscript. Unique UniProt accessions in the differentially expressed transcript list were used for functional enrichment analyses in DAVID v6.8 (Huang et al., 2009, 2008) using a modified Fisher's exact test ($p < 0.05$; genes/enrichment category ≥4) comparing against the QC-filtered background list (Additional File 1). Transcripts with discordant differential expression between two or more concentrations of lufenuron (i.e. significant upregulation at one concentration and significant downregulation in another), or with differential expression between acetone and SW controls (see Additional File 1), were not included in this analysis. The GO Trimming program (Jantzen et al., 2011) was used to reduce redundancies in gene ontology (GO) categories using an 80% soft trim threshold. Hierarchical clustering was also used with a Euclidian distance metric and Ward's linkage rule to display similar groups of transcripts.

### 2.6. RT-qPCR

The same RNA samples used for microarray analysis, and the RNA extracted from nauplii II lice in B4, were used for RT–qPCR analysis of 15 genes. Six genes were selected for microarray validation and nine genes for further exploration (Additional File 3). Genes selected for exploration were chosen based on evidence of their role in *L. salmonis* development (Sandlund et al., 2016).

First strand synthesis of cDNA was performed as previously reported by Poley et al. (2016). Transcript-specific standard curves (5-point, 5-fold series dilution) were designed to confirm that primer efficiencies were 90%–105% with an $R^2$ of > 0.95. RT-qPCR amplification was performed in triplicate using SsoAdvanced SYBR Green Supermix (BioRad) in 11 μL reactions with 1 μL template and 0.1 μM of each primer using the following thermal regime: 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec and a combined annealing and extension step of 60 °C for 15 sec. Melt curve analysis was performed by increasing the temperature from 65 °C to 95 °C in 0.5 °C increments every 5 sec. Melt curves and gel electrophoresis confirmed single product formation for all transcripts assayed. All RT-qPCR reactions were completed using a CFX96 Thermocycler (BioRad). Each gene of interest was normalized to the geometric mean of two reference genes, *elongation factor 1-alpha* (*ef1a*) and *eukaryotic initiation factor 4* (*eif4*) (Additional File 3), in qBase-PLUS (Biogazelle; Gent, Belgium; (Hellemans et al., 2007)) with an output of $\log_2$ expression ratios. Reference gene stability was tested using geNorm (Vandesompele et al., 2002) and showed a collective M value of 0.71 and coefficient of variation (CV) of 0.24.

Microarray validation was completed using the normalized $\log_2$ expression ratios from the microarray (i.e. Cy5/Cy3) and from RT-qPCR (gene of interest/geometric mean of reference genes) for each sample using a Pearson's correlation ($p < 0.05$) (Additional File 3). Statistically significant expression differences between experimental conditions were determined using a one-way ANOVA with post-hoc Tukey's HSD ($p < 0.05$) for all comparisons. All RT-qPCR analyses were performed in R (R v.3; R Core Team, 2016).

### 2.7. Metabolomics

Twelve sea lice samples (B6: six control; six treated) were submitted (100 mg/sample) to Metabolomics Discovery (Berlin, Germany) for targeted and non-targeted metabolite screening. Non-targeted

metabolite profiling consisted of GC-MS and LC-QTOF/MS analyses. Using these methodologies, metabolites were analyzed in the range of 50–1700 Da with an accuracy up to 1–2 ppm and a resolution of mass/Δmass = 40,000. Metabolites measured in the LC were annotated according to their accurate mass and subsequent sum formula prediction. Metabolites that were not annotated in the LC-MS-analyses were listed according to their accurate mass and retention time (accurate mass@retention time, e.g. 230.9478@3.1). Target metabolites were identified by Metabolomic Discoveries proprietary databases. For analysis, the appropriate analytical platform was chosen: LC-QTOF/MS, GC-MS or both. Sample concentrations were adjusted to optimally detect necessary metabolites. Abundances of all metabolites were normalized against an internal standard, and differential abundances between conditions were calculated using Welch's *t*-test ($p < 0.05$).

### 2.8. Transmission electron microscopy

The protocol for TEM was adjusted based on the developmental stage of the lice. Artificial water or growth medium was used as a buffer. Salmon lice in nauplius stages were processed as whole organisms (see B5). Salmon lice in copepodid stages were divided into cephalothorax and abdominal sections.

Nauplii staged salmon lice were placed in 2% glutaraldehyde (SPI Supplies; London, ON, Canada) buffered in artificial SW for fixation. The organisms were left in the fixative for no more than 24 h at 4 °C. After primary fixation samples were washed in artificial SW for 10 min twice and post-fixed in buffered 1% osmium tetroxide for 1 h at room temperature. Samples were then dehydrated in ascending concentration of ethanol starting with 50% and finishing with 100% solution followed by permeation with 100% propylene oxide (PO). Each step took 10 min and was done twice. Infiltration of samples with resin comprised of three steps and took place at room temperature. Samples were infiltrated with 1:1 mixture of Spurr/PO. After 1 h the solution was changed to 2:1 mixture of Spurr/PO, which lasted for 1 h and ended with 100% Spurr overnight with infiltration under vacuum. Individual samples were embedded in flat silicone molds and left overnight in the oven at 70 °C.

For copepodid stages, processing was modified to extend dehydration in infiltration steps. Dehydration in ascending concentrations of ethanol took 12 h for each step and the solution was changed twice. Permeation in PO took 1 h with two changes of solution. Each step for infiltration with resin lasted 24 h and the last step of infiltration with 100% resin took place under vacuum.

Polymerized blocks were cut on a Reichert-Jung Ultracut E ultramicrotome. Light microscopy sections were 0.5 µm thick and were stained with 1% toluidine blue. Sections cut to 90 nm were placed on a 200 µm mesh copper super grid and stained with 5% uranyl acetate and Sato lead stain, and were viewed on a Hitachi 7500 TEM at 80 kV. Digital images were captured with an AMT XR 40 side mounted camera.

## 3. Results

### 3.1. Impacts of lufenuron on survival

Moulting was significantly disrupted in *L. salmonis* larvae exposed to lufenuron. Infections of Atlantic salmon with copepodids pre-treated with lufenuron resulted in 88 and 93% reductions of attached chalimus lice 7 and 12 dpi, respectively (Fig. 1). Additionally, only one of ca. 500 nauplii II *L. salmonis* exposed to 700 ppb lufenuron for 3 h successfully moulted to the copepodid stage. In bioassays where a moulting event did not occur during the assessment period (see methods for B3-B4 and B6), no significant differences were observed in survival (survival > 90% in both treated and control groups; data not shown). Similarly, no difference in salmon lice survival was observed when using acetone or methanol as a solvent compared to SW alone. When Atlantic salmon were fed a diet containing 5 mg/kg lufenuron for 7 days and infested with salmon lice 5 weeks later, a 96% reduction in lice was observed by 14 dpi compared with controls (Fig. 1). Lufenuron levels in the feed were confirmed through submission to the analytical laboratory at SGS Canada.

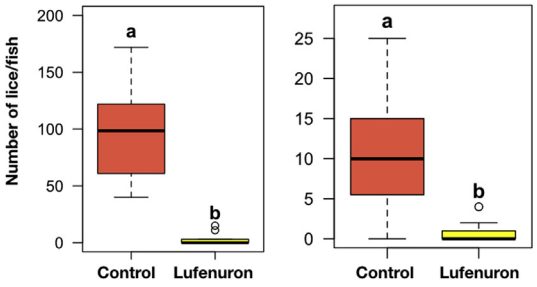


**Fig. 1.** Effects of lufenuron on *L. salmonis* infection. Lice counts were completed for two separate infection experiments between 100 and 150° days post lice infection (ddpi) of Atlantic salmon (*Salmo salar*). The first experiment (left plot; see B2), lice were pre-treated with 700 ppb lufenuron or acetone controls for 3 h before infecting salmon (n = 6) with 500 copepodids per fish. In the second experiment (right plot, see section 2.3) used salmon fed lufenuron at the recommended dose of 35 mg/kg or a control feed diet over 7 days. Five weeks post lufenuron cessation, fish (n = 640) were infected with 100 copepodids per fish. Letters denote significant differences in total live larval counts (*t*-test; $p < 0.05$).

Exposure of eggstrings for 24–72 h in 500 ppb lufenuron did not impact hatch success compared to acetone controls (i.e. all > 90% hatch rate; n = 3). Following hatch, the behaviour and development of planktonic lice differed between SW and acetone controls, such that ca. 80% of the latter were mobile within the water column while nearly 100% of the former were dispersed in the water column. Only three of ca. 1000 salmon lice from the lufenuron treated group were observed floating or swimming within the water column. The majority of salmon lice found at the bottom of the beaker in both the acetone control or lufenuron treatment were immobile. By 7 days post-hatch, the only movements of larvae in the lufenuron-treated groups were peristaltic contractions of the gut (n = 5) and some fast twitch movement of the swimming legs (n = 9). At this point, SW and acetone control lice had developed to copepodid stage larvae, successfully completing two moults (i.e. nauplii I to nauplii II; nauplii II to copepodid), whereas the lufenuron treated lice remained as nauplii I (Fig. 2). There was however significantly fewer salmon lice that developed to the infective copepodid stage in the acetone controls (mean of 11.7 with 95% CI of 10.1–13.2) compared to SW controls (mean of 7.0 with 95% CI of 5.8–8.2) in this experiment (one-way ANOVA; p = 0.009, n = 9).

### 3.2. Impacts of lufenuron on L. salmonis gene expression

Lufenuron had large impacts on the *L. salmonis* transcriptome, with 1045 differentially expressed transcripts in at least one concentration of lufenuron (30, 300, 700, 1000, and 1500 ppb) compared with acetone controls (Tukey's HSD; $p < 0.01$ and FC ≥ 1.5). The majority (61%) of these were downregulated by treatment (Table 1). Principal Component Analysis (PCA) separated acetone controls from lufenuron-treated salmon lice on the first and second principal components, which explained 31.4% and 20.4% of the variation in lice transcriptomes, respectively. Variation between biological replicates of salmon lice treated with 30–1000 ppb lufenuron was greater than differences between treatments for some samples (Fig. 3). Salmon lice in the SW control, acetone (emulsifier) control, and 1500 ppb lufenuron groups were tightly clustered within their respective conditions (Fig. 3). Salmon lice exposed to acetone alone differentially expressed 301 transcripts compared to lice maintained in SW (*t*-test without the assumption of equal variance; $p < 0.01$ and FC ≥ 1.5; Additional File 1). These transcripts were removed for gene enrichment analyses (see tags in Additional File 1). Overall, seawater and acetone controls were

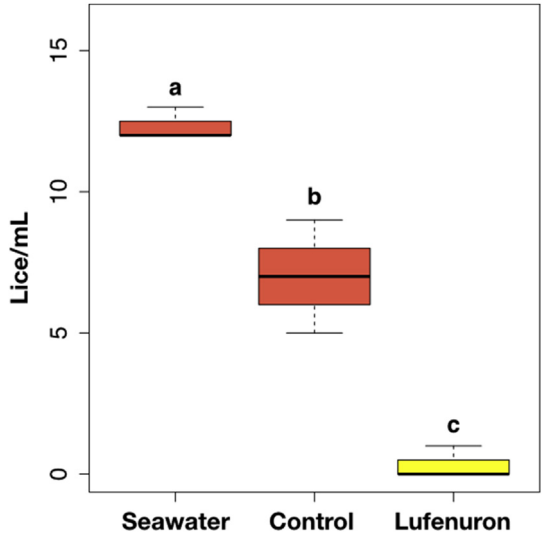


**Fig. 2.** Development of *Lepeophtheirus salmonis* following eggstring/nauplii I treatment with seawater (SW), SW + acetone (Control), or seawater + acetone + 500 ppb lufenuron (Lufenuron; B7). Lice development was assessed at 7 days post hatch as an average lice number for each stage enumerated per 5 mL count (completed in triplicate per system). Mobile copepodid lice are represented in red while immobile nauplii I-staged lice are represented in yellow. Letters denote significant differences in total live larval counts using a one-way ANOVA ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**
All differentially expressed transcripts (One-way ANOVA with $p < 0.01$; FC ≥ 1.5) in lufenuron-treated vs control lice.

| Treatment Group (ppb) | # of transcripts upregulated by lufenuron vs control | | # of transcripts downregulated by lufenuron vs control | |
|---|---|---|---|---|
| | *1.5 ≤ FC < 2* | *FC ≥ 2* | *1.5 ≤ FC < 2* | *FC ≥ 2* |
| 30 | 107 | 12 | 175 | 120 |
| 300 | 267 | 76 | 230 | 183 |
| 700 | 211 | 31 | 214 | 129 |
| 1000 | 178 | 17 | 208 | 114 |
| 1500 | 100 | 16 | 116 | 79 |

separated from all lufenuron-treated samples using 16,259 QC filtered probes (Fig. 3), indicating that lufenuron exposure causes substantial changes to the gene expression profiles of *L. salmonis* copepodid larvae.

### 3.2.1. Expression differences in early moulting and chitin synthesis

Cell signalling involved in *L. salmonis* moulting has been studied at the transcriptional level (Eichner et al., 2015a; Sandlund et al., 2016). Candidate genes described by Sandlund et al. (2016) were probed here using microarray and/or RT-qPCR (Additional Files 1 and 3) in both copepodid (B3) and nauplii II staged lice (B4) exposed to lufenuron. The *retinoic acid receptor* (*LsRXR*; Eichner et al., 2015a) transcript was only significantly induced (FC = 1.5) by 1500 ppb lufenuron compared to controls in copepodids. However, the expression of transcripts annotated to hormone receptors (e.g. *LsEcR* and *LsHR38*), transcription factors (*LsE74* and *LsE75*), and peptidases (e.g. *LsCP1*; Additional File 3) involved in *L. salmonis* development (Sandlund et al., 2016) were unchanged by lufenuron in this study (data not shown).

Based on the strong characterization of developmental genes in *Drosophila melanogaster*, sequences from the chitin synthesis pathway (KEGG: dme00520) were used as queries to identify *L. salmonis* orthologs in the Transcriptome Shotgun Assembly (TSA) database (NCBI) using a BLASTX algorithm (Fig. 4). All chitin synthesis genes were conserved between *D. melanogaster* and *L. salmonis* (Fig. 4), with between 58% and 84% positive matches in the alignments (Additional File 5). Lufenuron-treated lice differentially expressed three genes in the chitin synthesis pathway including *glucose-6-phosphate isomerase*, *glucosamine-6-phosphate-N-acetyltransferase*, and *phosphoacetylglucosamine mutase* (*PAGmut*; Fig. 5, Additional File 1). *PAGmut* showed a concentration-dependent increase in expression when exposed to lufenuron (Fig. 5), which was confirmed using RT-qPCR. However, microarray and RT-qPCR data for *G6P isomerase* were not significantly correlated ($r = 0.32$; $p = 0.2$; Additional File 3); the only instance in six genes used to validate the microarray. Other notable members of this pathway such as *chitin synthase 1* (*LsCHS1*) and the chitinases *LsChi1* and *LsChi2* (see contig ID: 5727818 and 5733254) were not differentially expressed by lufenuron. This was confirmed using specific primers obtained from Sandlund et al. (2016) for RT-qPCR.

Other transcripts with important roles in regulating moulting, but not found in the traditional chitin synthesis pathway, were differentially expressed by lufenuron. Two transcripts, *chitin deacetylase* (*CDA*) and *UDP-glucose 6-dehydrogenase*, showed similar patterns of expression to *PAGmut*, with 2.9-fold higher expression in 1500 ppb lufenuron-treated lice compared to acetone controls. The expression of nine transcripts from the solute carrier (SLC) family was also changed by lufenuron, including *SLC2A1* and *SLC35B1*, which transport glucose and sucrose, respectively. Downregulated transcripts enriched several GO categories related to transport including regulation of transport (22 transcripts; $p = 0.009$), vesicle (37 transcripts; $p < 0.05$), and transmembrane transport (16 transcripts; $p = 0.04$). Overall, only three transcripts related to chitin synthesis were induced by lufenuron treatment while more than 50 transport-related transcripts were downregulated.

### 3.2.2. Cuticle-related transcripts are downregulated by lufenuron

A group of 18 transcripts without UniProt annotations, but containing chitin-binding domains (CBD), as determined using RPS-BLAST against the Conserved Domain Database (NCBI; e < 10E-5), were downregulated between 1.5 and 3.3-fold in at least one of the treatment groups compared with controls (Fig. 6; Additional File 1). Ten of these transcripts had CBDs with conserved cysteines common to chitinases (CDD: pfam01607; cys) while eight others lacked these conserved cysteines (CDD: pfam00379; non-cys). Another seven transcripts

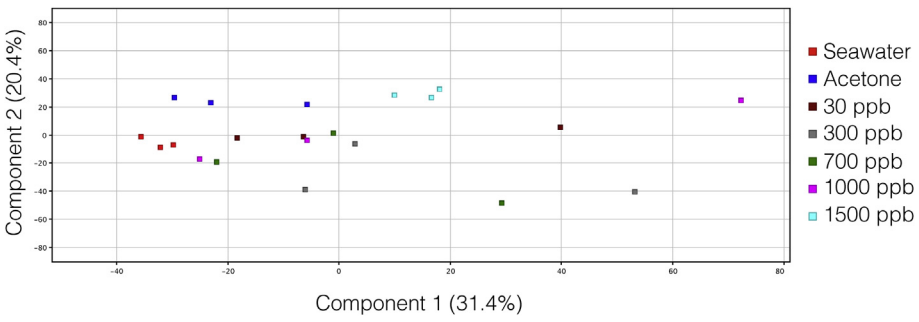


**Fig. 3.** Principal Component Analysis (PCA) of 21 samples represented by the expression of 16,259 probes passing QC filters. Each square represents a pool of approximately 500 copepodids exposed to seawater alone, acetone alone, or acetone and lufenuron (30–1500 ppb) for 3 h before a 21 h holding period in seawater, at which time lice were flash frozen and stored for RNA extractions.

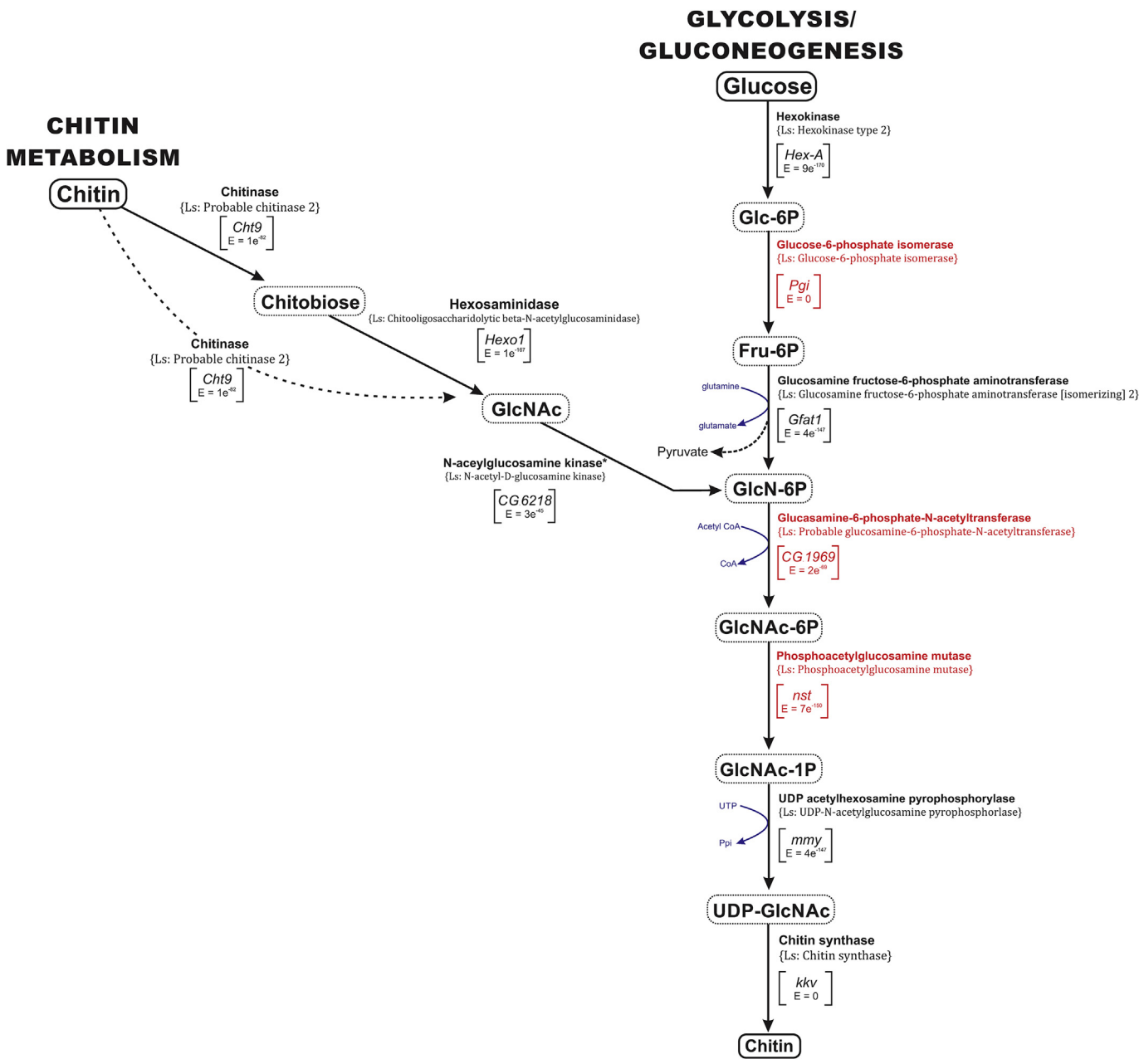


**Fig. 4.** Chitin synthesis pathway derived from *Drosophila melanogaster* (KEGG: dme00520) showing genes are conserved across insects and copepods. The gene name provided by KEGG is on top followed by the gene name for sea lice (taken from microarray annotation) and the abbreviated gene name on bottom with the corresponding e-value derived from a BLASTx search using the *D. melanogaster* sequence against the TSA database (NCBI) limited to *L. salmonis* sequences. Those sequences highlighted in red were differentially expressed in at least one condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

containing at least one C-type lectin domain (CDD: cd00037) were also downregulated between 1.5 and 4.4-fold in lufenuron-treated lice. The C-type lectin transcripts were positively correlated with transcripts containing a CBD (Fig. 7 and Additional File 4), suggesting these transcripts are co-expressed. An additional two transcripts annotated as *cuticle protein CP14.6*, a known structural component of the tobacco hornworm (*Manduca sexta*) cuticle (Rebers et al., 1997), were also downregulated by 1000 and 1500 ppb lufenuron in this study (Fig. 6). Together, 20 novel *L. salmonis* transcripts with putative roles in chitin metabolism and cuticle structure were impacted in *L. salmonis* exposed to lufenuron.

Validation of these expression patterns was completed with RT-qPCR analysis of one *CP14.6* transcript (contig 5735548), a C-type lectin transcript (contig 5732078), and one transcript with a non-cys CBD domain (contig 5729852), all of which were significantly correlated between microarray and RT-qPCR assays (Pearson's correlation, $p < 0.05$; Additional File 3). Overall, 35 transcripts with annotations (SwissProt and/or CDD) to the *L. salmonis* cuticle were differentially expressed by exposure to lufenuron (Figs. 6 and 7).

### 3.2.3. Expression of developmental genes after exposure to lufenuron

Many *L. salmonis* transcripts downregulated by lufenuron had roles in the structural integrity of epithelia and muscle based on gene annotations (Table 2). These transcripts enriched GO categories such as muscle structure development (19 transcripts; $p = 2^{-6}$) and epithelium development (23 transcripts; $p = 0.001$; Table 2). Other transcripts with roles in tissue development enriched the GO categories foregut morphogenesis (four transcripts; $p = 0.002$), neuron differentiation (21

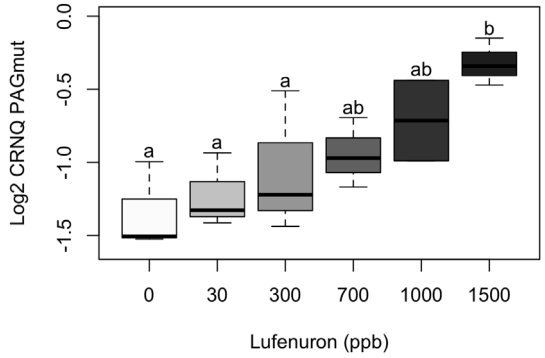


**Fig. 5.** *Lepeophtheirus salmonis* expression (calibrated normalized relative quantity – CNRQ) of *phosphoacetylglucosamine mutase* showing a concentration-dependent induction under lufenuron exposure (see B3). CNRQ calculated against *ef1α* and *eif4* reference genes. Letters denote significant differences in expression between conditions ($p < 0.05$)?

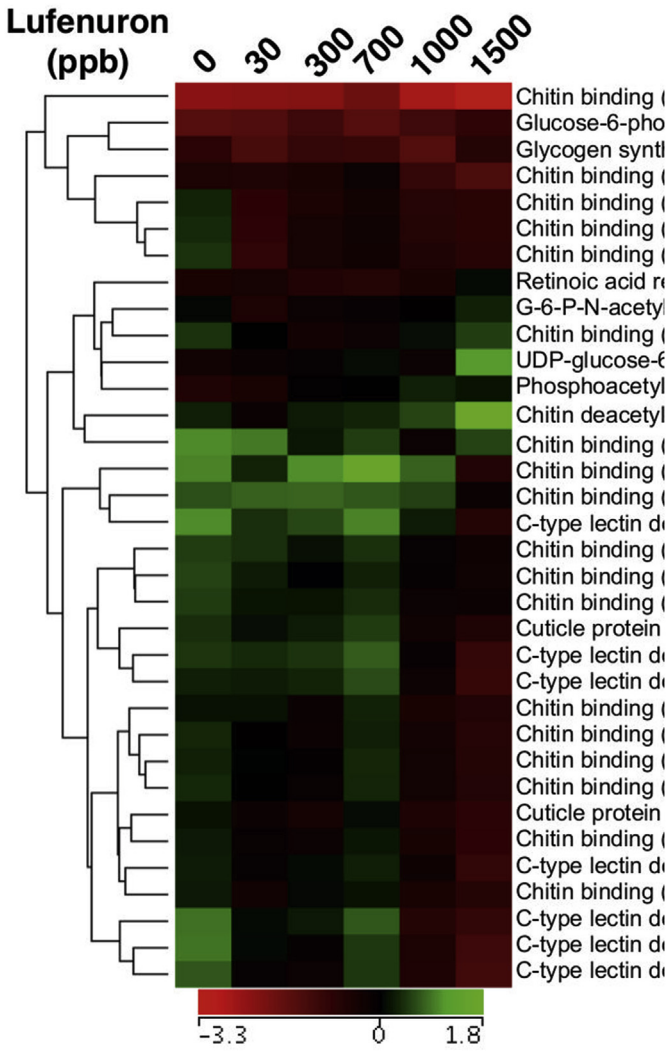


**Fig. 6.** Heatmap of chitin/cuticle-related transcripts differentially expressed by at least one concentration of lufenuron. Hierarchical clustering (Euclidian distance metric; Ward's linkage rule) clustered transcripts based on averaged expression profiles in each lufenuron treatment. Expression is represented as Log2 Cy5/Cy3 ratios.

transcripts; p = 0.01), tissue morphogenesis (17 transcripts; p = 0.0009), and growth (19 transcripts; p = 0.001; Additional File 2). Transcripts responsible for cellular development and differentiation were also downregulated by lufenuron, enriching GO categories actin cytoskeleton organization (15 transcripts; p = 0.0008) and actin filament binding (six transcripts; $p = 0.01$), among others (Additional File 2). Many of these transcripts were annotated against developmental genes of *D. melanogaster* such as *protein sarah, protein strawberry notch*, *multiple EGF-like domain*, *reticulon-4*, *ryanodine receptor*, *wing disc development protein*, and *armadillo segment polarity protein* (Additional File 1). Another eight transcripts downregulated by lufenuron enriched the GO categories chaeta development (four transcripts; p = 0.02) and epithelia-mesenchymal transition (four transcripts; p = 0.02), suggesting roles for these transcripts in *L. salmonis* moulting. Transcripts overexpressed by lufenuron largely enriched GO categories related to transcription and translation; although five transcripts did enrich the smoothened signalling pathway (p = 0.03), a pathway responsible for accurate patterning of *D. melanogaster* segments (Alcedo et al., 1996). Therefore, changes in gene expression related to lufenuron exposure in *L. salmonis* suggest a systemic impact on development, with downregulation in multiple structural constituents of the cuticle, muscle, and epithelia, as well as important effectors for cell differentiation, solute transport, and tissue morphogenesis (Table 2).

### 3.2.4. Genes related to the stress response

The identification of transcripts involved in a stress response to lufenuron exposure was difficult to quantify in this study. Exposure to 0.35% acetone (used to emulsify lufenuron) caused upregulation of known *L. salmonis* stress markers such as *heat shock beta 1* and *major vault protein* (Additional File 1; Poley et al., 2016) when compared to salmon lice maintained in SW. Of the 301 transcripts that were differentially expressed between acetone and SW controls (Additional File 1), 150 were also differentially expressed between acetone controls and at least one concentration of lufenuron. These transcripts were not included in GO analysis and are flagged in Additional File 1. Surprisingly, many transcripts related to stress that did not respond to acetone exposure were downregulated in lufenuron-treated lice, enriching GO categories such as response to stimulus (72 transcripts; p = 0.004) and response to drug (eight transcripts; p = 0.006).

### 3.3. Metabolomics discovery

In total, 997 compounds were analyzed using a metabolomics approach. Unfortunately, major compounds of interest (e.g. N-Acetyl-D-glucosamine 6-phosphate, D-Glucosamine 6-phosphate, UDP-N-acetyl-alpha-D-glucosamine) were below the limit of detection. Different normalisation methods were tested and normalisation on the total ion count (TIC) gave the best result and was used for analysis. To provide an initial overview of all analyzed samples, a PCA was calculated (Additional File 6). The majority of variation in salmon lice metabolite profiles was not explained by experimental groupings in this study. Nonetheless, 16.2% of the dataset's variance was explained by the classification into control and treated lice. A total of 36 metabolites were found to be changed significantly (*t*-test; $p < 0.05$) in the drug treated samples compared to untreated controls, most commonly phosphatidic acid and other lipids (Table 3). The profile in treated animals showed mostly a downregulation of metabolites with polyunsaturated fatty acid moieties. The metabolite with the highest increase in the drug treated salmon lice had a molecular weight of 509.98 Da corresponding to lufenuron, confirming the detection method.

### 3.4. Impacts of lufenuron on ultrastructure of the cuticle

Transmission electron microscopy was employed to look at the ultrastructural components of *L. salmonis* integument and underlying

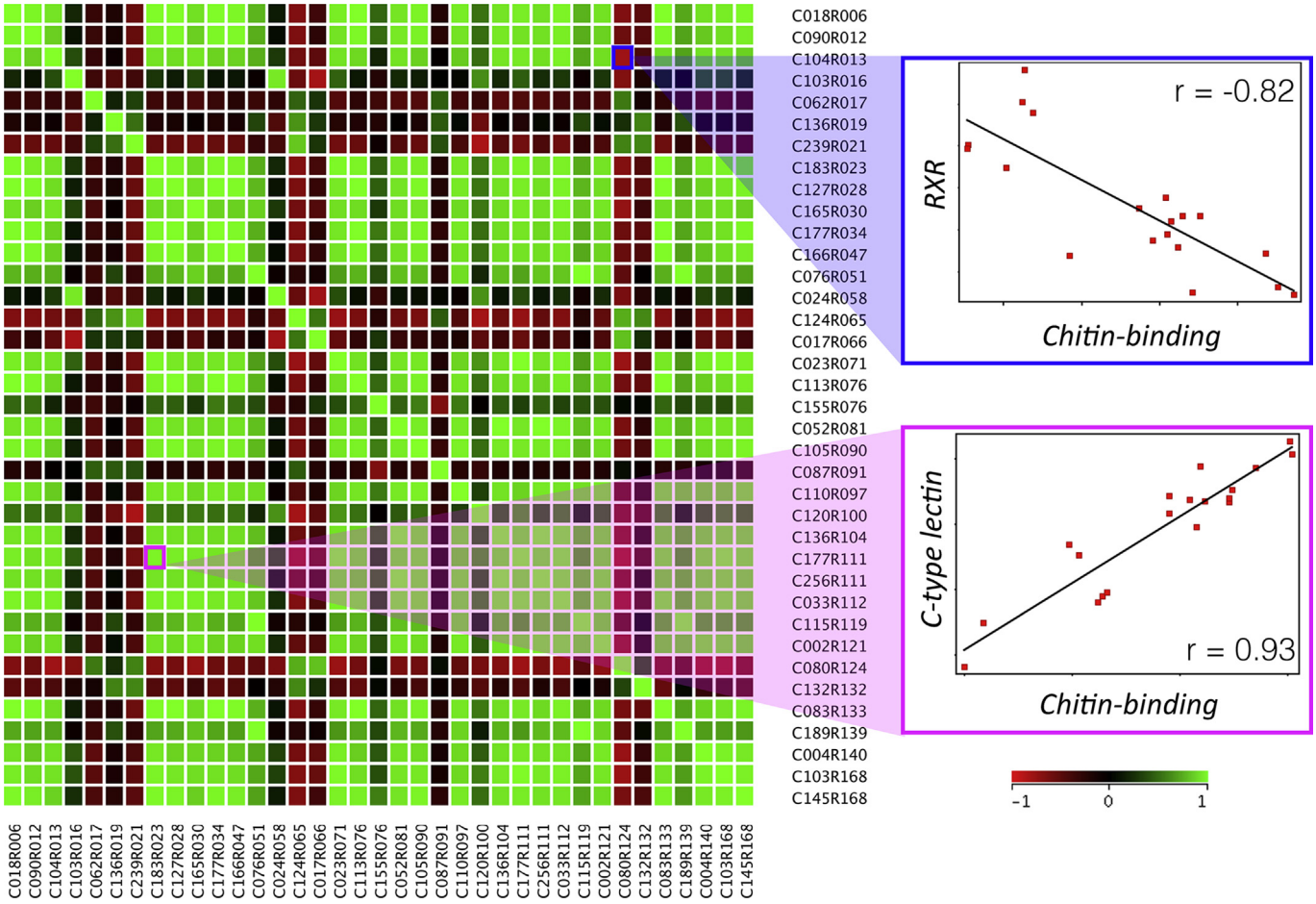


**Fig. 7.** Heatmap of correlation coefficients (Pearson's r) derived from a pairwise comparison (all against all) of cuticle/chitin-related transcripts differentially expressed by at least one concentration of lufenuron. Each transcript, represented by a probe ID (see Additional File 1), is plotted once on the x-axis and y-axis. An example of a negative (red) and positive (green) correlation is represented in the scatterplots on the right (blue and pink, respectively). All correlation coefficients from this analysis can be viewed in Additional File 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**
Transcripts downregulated by lufenuron exposure enriched numerous GO categories.

| General Functions | GO Category | GO Term | # Transcripts | p-value |
|---|---|---|---|---|
| Muscle | GO:0061061 | muscle structure development | 19 | 2E-06 |
| | GO:0055002 | striated muscle cell development | 9 | 4E-04 |
| | GO:0045214 | sarcomere organization | 6 | 6E-04 |
| | GO:0008307 | structural constituent of muscle | 4 | 0.006 |
| | GO:0030018 | Z disc | 6 | 0.01 |
| | GO:0060538 | skeletal muscle organ development | 5 | 0.02 |
| Epithelia | GO:0060429 | epithelium development | 23 | 0.001 |
| | GO:0001837 | epithelial-mesenchymal transition | 4 | 0.02 |
| | GO:0002064 | epithelial cell development | 9 | 0.02 |
| Cell Structure | GO:0030036 | actin cytoskeleton organization | 15 | 8E-04 |
| | GO:0010769 | regulation of cell morphogenesis involved in differentiation | 8 | 0.005 |
| | GO:0051015 | actin filament binding | 6 | 0.01 |
| | GO:0048468 | cell development | 41 | 8E-06 |
| Tissue Development | GO:0009887 | organ morphogenesis | 20 | 6E-04 |
| | GO:0040007 | growth | 19 | 0.001 |
| | GO:0007440 | foregut morphogenesis | 4 | 0.002 |
| | GO:0030182 | neuron differentiation | 21 | 0.01 |
| | GO:0022416 | chaeta development | 4 | 0.02 |
| Stress | GO:0042493 | response to drug | 8 | 0.006 |
| | GO:0007584 | response to nutrient | 6 | 0.004 |
| | GO:0050896 | response to stimulus | 72 | 0.004 |

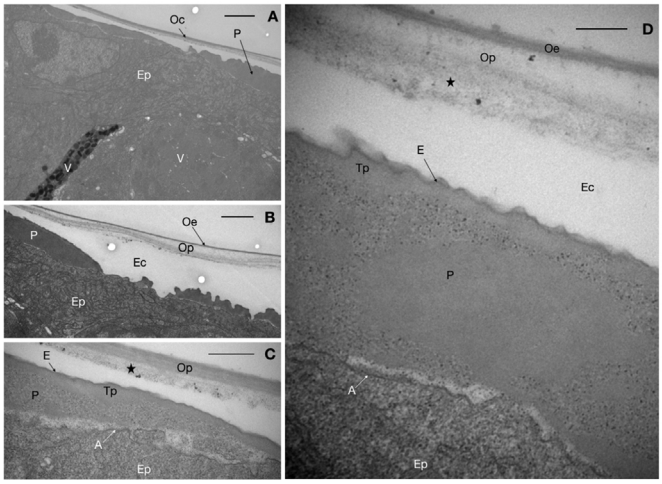


**Fig. 8.** Images of control *Lepeophtheirus salmonis* nauplii II integument and underlying tissue during apolysis using electron microscopy. A-B) 10,000× magnification with scale bar = 2 μm. C-D) 60,000× magnification with scale bar = 500 nm. Abbreviations: Old cuticle (Oc), new procuticle (P), epidermis (Ep), vesicle aggregates (V), old epicuticle (Oe), old procuticle (Op), exuvial cleft (Ec), new epicuticle (E), transitional procuticle (Tp), apical membrane (A), and diffuse substance beneath Op in the Ec (black star).

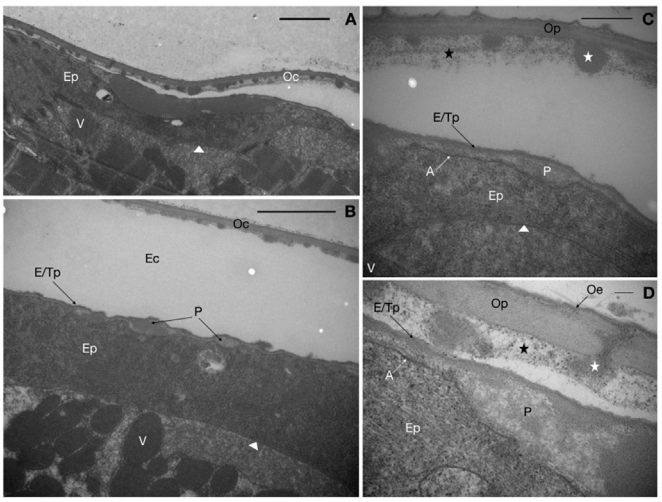


**Fig. 9.** Images of *Lepeophtheirus salmonis* nauplii II integument and underlying tissue during apolysis 24 h post lufenuron treatment using electron microscopy. A) 10,000× magnification with scale bar = 2 μm, B) 20,000× magnification with scale bar = 2 μm C) 60,000× magnification with scale bar = 500 nm, D) 120,000× magnification with scale bar = 100 nm. Abbreviations: Old cuticle (Oc), epithelium (Ep), vesicle (V), secondary membrane separating electron dense epithelium (white arrow heads), exuvial cleft (Ec), procuticle (P), poorly defined epicuticle and transitional procuticle (E/Tp), old procuticle (Op), diffuse substance beneath Op in the Ec (black star), electron-dense focii (white stars), apical membrane (A), and old epicuticle (Oe).

epithelium across stage (nauplii and copepodid) and treatment. These assessments were largely qualitative, and were aided by earlier work on the characterization of chalimus-staged *L. salmonis* cuticles during moulting (Bron et al., 2000a) and intermoult (Bron et al., 2000b). In nauplii II stage individuals, the cuticle in many regions had separated from the epithelial layer as part of apolysis, and the formation of the exuvial cleft was evident in all nauplii samples (Figs. 8–9). Formation of the new cuticle was observed between this space and the epithelial layer. The old epicuticle and procuticle remained distinct after the old cuticle was shed, with a large band of electron-lucent material beneath the procuticle in the exuvial cleft (Fig. 8C + D and 9C + D). Only

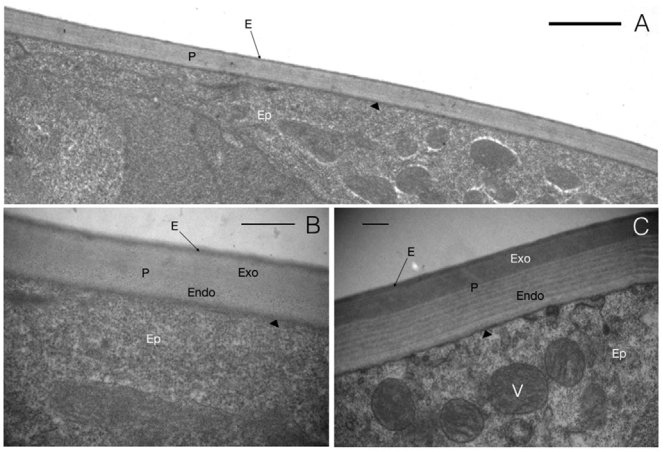


**Fig. 10.** Images of control *Lepeophtheirus salmonis* copepodid integument using electron microscopy. A) 10,000× magnification with scale bar = 2 μm B) 60,000× magnification with scale bar = 500 nm, C) 30,000× magnification with scale bar = 500 nm. Abbreviations: Epicuticle (E), procuticle (P), epithelium (Ep), electron-dense band separating cuticle and Ep (black arrow heads), exocuticle (Exo), endocuticle (Endo), and vesicles (V).

lufenuron-treated salmon lice exhibited the formation of electron-dense foci in this electron-lucent material in the exuvial cleft (Fig. 9). In some cases, the electron-dense focii were associated with fissures/pores running through the old procuticle (Fig. 9D).

In the newly synthesized cuticle of lufenuron-treated nauplii, the epicuticle and transitional procuticle were not distinct as was observed in controls (Figs. 8–9). The procuticle of all nauplii lice had not yet subdivided into the endocuticle and exocuticle, however, control lice had far more electron-dense procuticles, with some electron-lucent spaces near the apical membrane of the epithelium (Fig. 8). In lufenuron-treated nauplii, these electron-lucent spaces were interspersed throughout the procuticle and were more frequent (Fig. 9), although these observations were not quantified. In contrast, salmon lice treated with lufenuron had a more electron dense epithelium, with the appearance of a secondary membrane below which electron-dense vesicles were observed to aggregate (Fig. 9). This secondary membrane was not observed in controls, which also exhibited numerous electron-dense vesicles aggregating below the epithelium (Fig. 8). Despite the irregularities observed in both the old and new cuticles of lufenuron-treated lice, ecdysis had proceeded and parts of the new cuticle were evident in both control and treated nauplii.

At the 48 h sampling time, salmon lice in the control group had completed the moult to copepodids while only one louse in the lufenuron-treated group remained mobile, despite being deformed (data not shown). In control copepodids, an electron dense epicuticle separated the procuticle from the external environment (Fig. 10). The procuticle was further subdivided into the exocuticle (outermost) and endocuticle (innermost) layers. The endocuticle of some control animals appeared to be secreted in layers, forming overlaying laminae of electron dense chitin microfibrils (Fig. 10C). A greater electron-dense layer adjacent to the epithelium, similar in appearance to the epicuticle, was always present in control copepodids (Fig. 8C). In some sections, structure of the exocuticle and endocuticle was unclear, and appeared electron-lucid (Fig. 8B); variability of which was likely explained by differences in moult timing (age) of the copepodids and focal differences in cell populations or cellular physiology.

All treated copepodids appeared deformed macroscopically (data not shown) due to arrested moulting through the transition to copepodids (48 h). In some cases, deformed salmon lice were only early in initiating the moult as evidenced by the presence of an ecdysial membrane (Fig. 11A; see Bron et al., 2000a,b). For the louse that remained mobile 48 h post treatment (Fig. 11B + C), the epicuticle was

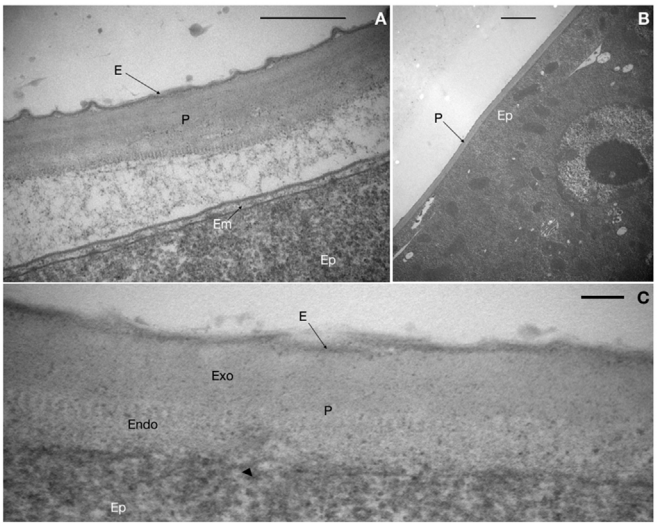


**Fig. 11.** Images of *Lepeophtheirus salmonis* copepodid integument 48 h post lufenuron treatment using electron microscopy. A) Dead, deformed copepodid at 80,000× magnification with scale bar = 500 nm. An ecdysial membrane (Em) was observed in treated lice that had arrested moulting. The epicuticle (E) often had several microridges, B) Mobile copepodid with region of completed moult at 10,000× magnification with scale bar = 2 μm. The procuticle (P) is well adhered to the epithelium (Ep), C) 120,000× magnification with scale bar = 100 nm showing mobile treated copepodid integument with an electron-lucid endocuticle (Endo), an unorganized epicuticle (E), and a poorly defined band between the cuticle and epidermis (black arrow head). The exocuticle (Exo) appeared normal.

disorganized and heterogeneous with regard to electron-density (Fig. 11C). Although the procuticle of this individual was similar to controls (Fig. 11B–C), the electron dense band between the epithelium and endocuticle was barely present (Fig. 11C). Therefore, salmon lice surviving treatment through the moult transition also exhibit physiological impacts from lufenuron exposure.

## 4. Discussion

Lufenuron is a new in-feed treatment for sea lice. It is currently marketed under the tradename Imvixa™ in Chile and is under consideration for use in North America and Europe. Lufenuron is the first new drug therapy to be licensed against sea lice in aquaculture in nearly two decades. Based on the success of lufenuron and other BPUs in veterinary medicine and agriculture, and the worsening issues with drug-resistant sea lice in salmon aquaculture, lufenuron will be an important addition to the short list of parasite management strategies currently being used in the industry. In the present study, we (1) established a system whereby the responses of the planktonic stages of the parasite to lufenuron could be studied in a physiologically meaningful way, (2) identified genes, molecular pathways, and metabolites that changed in response to lufenuron exposure, and (3) described the ultrastructural impacts of lufenuron on larval sea lice cuticles and underlying epithelia. These results are discussed in terms of drug efficacy, similarities and differences to BPU responses in insects, and the molecular mechanisms controlling *L. salmonis* development and drug resistance.

### 4.1. Efficacy of lufenuron against sea lice

A bioassay exposure model was adopted from Poley et al. (2016) to carry out short-term lufenuron immersions for different life stages of *L. salmonis*. Acetone and methanol were used as vehicles to maintain lufenuron in SW solution, however, solubility issues with methanol resulted in acetone working the best. Acetone did exhibit some toxicity to the larval sea lice, but there were no significant differences with respect to mortality in nauplii or copepodid staged lice and so this vehicle was used throughout further studies. A general stress response due to acetone exposure is measured by transcriptomics in Additional File 1.

The maximum residue limit for lufenuron in the fillets of finfish has been established at 1350 μg/kg (ppb) by several major regulatory agencies (European Food Safety Authority, 2010; European Medicines Agency, 2015; Food and Drug Administration, 2016). A concentration of 700 ppb was chosen as the dose for 3-h immersion with the initial infective copepodid stage for assessment of impact on infestation and development. Even at this concentration for a short exposure time, a major reduction in salmon lice developing through to the first moult was observed (ca. 90%). This matched well with exposure of copepodid stage lice to fish fed 5 mg lufenuron/kg bw/day for 7 days, showing 96% reductions compared to fish fed a control diet (Fig. 1). The inability of exposed eggstrings and nauplii larvae to develop to the infective copepodid, or any exposed copepodids to develop to pre-adult stages of lice suggests rapid uptake of lufenuron by both immersion and digestion. While examination of adult females on treated hosts are required to definitively determine the impact of lufenuron on egg production and progeny development, immersion of females and their eggstrings with 500 ppb lufenuron demonstrated that the drug may impact progeny of surviving adults, and extend protection into the next generation. These impacts parallel those of lufenuron in a wide range of other arthropod species. For example, *D. melanogaster* fed high doses of lufenuron were able to complete development within the instar stage, but died during ecdysis to the next instar (Wilson and Cryan, 1997). Although adult *D. melanogaster* fed the drug were not impacted, oogenesis was impaired and their eggs failed to hatch (Wilson and Cryan, 1997). Topical treatment of the potato *Solanum tuberosum* with lufenuron, resulted in 90% of the potato tuber moth, Phthorimaea operculella, unable to complete the moult through the first instar stage (Edomwande et al., 2000). Lufenuron has also reduced the size of oocytes, number of chorionated oocytes, and the incorporation of N-acetylglucosamine into chitin in the ovaries of the triatomine bug, *Rhodnius prolixus* (Mansur et al., 2010). Therefore, the general effects of lufenuron on larvae and eggs of *L. salmonis* are similar to those previously observed for insects.

### 4.2. Impacts of lufenuron on L. salmonis physiology

Nauplii II-staged lice arrested moulting between 24 and 48 h post exposure to lufenuron, and showed a variety of macroscopic deformities (data not shown). Ultrastructural differences between lufenuron-treated salmon lice and control lice were difficult to quantify, largely due to variation within and between individual cuticles. Qualitative assessments were facilitated by earlier work (Bron et al., 2000a), who also reported wide variability in cuticle morphology for chalimus-staged *L. salmonis* during moulting. Despite surviving 24 h post treatment, ultrastructural differences were observed in lufenuron-treated nauplii lice, including the formation of electron-dense focii beneath the old cuticle in the exuvial cleft, a poorly organized epicuticle, a diffuse procuticle, and lack of an electron-dense layer joining the endocuticle to the epithelium. This phenotypic aberration is likely a result of chitin synthesis being a cell autonomous process (Gangishetti et al., 2009), and developmental differences in individual moult timing (Eichner et al., 2015b) will likely contribute some variability. Ultrastructural differences in treated *L. salmonis* cuticles corresponded with the disruption of gene expression related to moulting, largely in the down-regulation of transcripts involved in the structural integrity of muscle and epithelium, as well as numerous polysaccharide-binding genes such as C-type lectins and transcripts with CBDs. Several other *L. salmonis* transcripts annotated to well-characterized *D. melanogaster* developmental genes or solute transporters were also downregulated by lufenuron. Benzoylureas generally cause a wide range of phenotypic abnormalities due to their interference with chitin formation (Post et al., 1974), abundance (Gangishetti et al., 2009; Merzendorfer et al., 2012),

and deposition (Post and Vincent, 1973), and therefore, the polygenic response observed here was expected.

The *L. salmonis* chitin synthesis pathway was described for the first time here by matching contiguous sequences from the microarray assembly (Yasuike et al., 2012) with transcripts from the well-characterized *D. melanogaster* pathway (Fig. 4 and Additional File 5). Although the mechanism of action for BPUs is not yet fully characterized, convincing evidence exists for chitin synthase 1 (*CHS*) as a binding site (Douris et al., 2016). Based on the high conservation of this gene (Merzendorfer, 2006), the *L. salmonis* chitin synthase 1 (*LsCHS-1*; GenBank: KX349436.1; Fig. 4) is a likely binding site for lufenuron in sea lice. Our analyses show that the majority of genes in the chitin synthesis pathway, including *LsCHS-1*, were not differentially expressed by lufenuron; a finding consistent with observations in BPU-treated beetles *Triboleum castaneum* (Merzendorfer et al., 2012) and flies *D. melanogaster* (Gangishetti et al., 2009). Only *PAGmut*, an isomerase residing two steps upstream of *LsCHS-1* and responsible for converting N-acetylglucosamine-6-phosphate (GlcNAc6P) to GlcNAc1P (Kato et al., 2005), was overexpressed greater than 2-fold in treated salmon lice (Fig. 5). Two additional transcripts, *CDA* and *UG6D*, showed similar responses to *PAGmut* with nearly 3-fold overexpression in the 1500 ppb lufenuron group compared to controls. To our knowledge, *CDA* is the first chitin deacetylase (contig: 5731222) to be described for this species. Studies using RNAi in *T. castaneum* revealed certain chitin deacetylases are essential for successful moulting while others are redundant (Arakane et al., 2009). Likewise, a chitin deacetylase is responsible for chitin organization of the locust *Locusta migratoria* cuticle (Yu et al., 2016). Therefore, despite few genes being induced by lufenuron exposure, *PAGmut*, *CDA*, and *UG6D* are important candidates for future studies assessing the regulation of *L. salmonis* moulting and responses to BPUs.

Chitin synthesis occurs in epithelial cells, where newly synthesized chitin is translocated across the plasma membrane for deposition and organization (reviewed in Merzendorfer, 2013, 2006). In the present study, metabolomics analysis of lufenuron-treated lice showed downregulation of several membrane constituents, namely phosphatidic acids (Table 3). Numeorus genes related to membrane structure and transport were also downregulated by lufenuron, enriching GO categories such as transmembrane transport (16 transcripts), apical plasma membrane (7 transcripts), and vesicle (37 transcripts; Table 2). These expression changes corresponded to physiological difference between lufenuron-treated lice and controls, which included vesicles aggregating below a secondary-type membrane that separated an electron-dense epithelium in treated lice only (Figs. 8–9). Another BPU, diflubenzuron, was shown to inhibit GTP-dependent $Ca^{2+}$ transport processes in intracellular vesicles of isolated cockroach *Periplaneta americana* integument cells (Nakagawa and Matsumura, 1994). As chitin is translocated across the membrane in vesicles, the molecular signals observed here might be involved in chitin transportation. Benzoylurea binding to the sulfonylurea transporter (*SUR*) was suggested as the mechanism causing vesicle inhibition (Abo-Elghar et al., 2004), although this remains ambiguous (Meyer and Moussian, 2013). Probes corresponding to the *L. salmonis SUR* (described by Carmona-Antoñanzas et al., 2015) were not differentially expressed by lufenuron despite passing QC filters for all groups (Additional File 1). Therefore, translocation of chitin from epithelia might be disrupted by lufenuron and other BPUs, however, the mechanism controlling this inhibition remains unresolved. Nonetheless, transporters described herein should be considered important candidates for studies on *L. salmonis* moulting and BPU exposure.

Understanding development of the non-model *L. salmonis* has become an important area of research in the past decade, largely due to the potential for novel drug discovery. Analyses focused on the *L. salmonis* lifecycle (Hamre et al., 2013), moulting (Bron et al., 2000a), instar growth and moult increment (Eichner et al., 2015b), intramoult transcriptome variation (Eichner et al., 2008), and characterization of

**Table 3**
Differential metabolite signatures of lufenuron-treated lice compared to controls.

| Metabolite | p-value | Log2 FC Lufenuron/ Control |
|---|---|---|
| Phosphatidic acid (33:2) | 0.03 | −1.6 |
| Phosphatidic acid (39:6) | 0.04 | −1.4 |
| Phosphatidic acid (37:6) | 0.03 | −1.4 |
| Phosphatidic acid (39:7) | 0.03 | −1.4 |
| Phosphatidylglycerol (14:0) | 0.03 | −1.3 |
| PA 1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phosphate | 0.04 | −1.3 |
| Phosphatidic acid (37:3) | 0.02 | −1.3 |
| 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-pyrophosphate | 0.03 | −1.3 |
| Phosphatidic Acid (35:3) | 0.03 | −1.3 |
| Phosphatidylglycerol (32:0) | 0.01 | −1.3 |
| Phosphatidic Acid (35:1) | 0.03 | −1.2 |
| 814.683@2 | 0.01 | −0.93 |
| Phosphatidylethanolamine (18:4/20:3) | 0.02 | −0.90 |
| 306.145@4 | 0.03 | −0.87 |
| Trp-Ser-Ser | 0.005 | −0.84 |
| Arg-Met-Phe-Asp | 0.02 | −0.78 |
| Serine | 0.004 | −0.63 |
| 389.253@0.8 | 0.04 | −0.59 |
| Asn-Ile-Ile-Val | 0.03 | −0.41 |
| 2,3-Dihydro-5,5′,7,7′-tetrahydroxy-2-(4-hydroxyphenyl)[3,8′-bi-4H-1-benzopyran]-4,4′-dione | 0.01 | −0.37 |
| Palmitoleoyl Ethanolamide | 0.02 | 0.40 |
| Citronellyl anthranilate | 0.02 | 0.44 |
| 9-Hexadecenoylcarnitine | 0.004 | 0.52 |
| 2″-O-Acetylrutin | 0.04 | 0.53 |
| Leucine | 0.02 | 0.64 |
| 435.996@1.8 | 0.03 | 0.64 |
| Ile-Arg-Lys | 0.01 | 0.69 |
| 181.988@8.6 | 0.02 | 0.69 |
| Phosphatidylglycerol (42:11) | 0.04 | 0.84 |
| Lys-Lys-Met-Phe | 0.02 | 0.86 |
| 367.214@12.3 | 0.001 | 0.87 |
| Arg-Asn-Gln-Arg | 0.05 | 1.1 |
| 894.414@0.9 | 0.01 | 1.1 |
| Ile-His-Phe | 0.04 | 1.2 |
| 678.357@0.9 | 0.04 | 1.2 |
| 509.976@0.9 (lufenuron) | 0.0 | 2.8 |
| Mannose | 0.2 | 1.1 |
| Glucose | 0.5 | −0.19 |
| Glucosamine | 0.6 | −0.052 |
| Glucose-6-phosphate | 0.7 | 0.068 |

numerous developmental genes such as *retinoic acid receptor* (*LsRXR*; (Eichner et al., 2015a), *ecdysone receptor* (*LsEcR*; (Sandlund et al., 2016), *chitinases* (*LsChi1, LsChi2,* etc.; (Eichner et al., 2015c; Sandlund et al., 2016), *yolk-associated protein* (*LsYAP*; (Dalvin et al., 2009), *heme peroxidase* (Øvergård et al., 2017), and *trypsin-like protease* (Skern-mauritzen et al., 2009) have allowed for a better understanding of development in *L. salmonis* and copepods in general. In the present study, only one gene with experimental evidence in *L. salmonis* development, *LsRXR*, was differentially expressed by lufenuron (upregulated at 1500 ppb). The 60mer probe on the microarray spans the 1409–1468 region of *LsRXR* (GenBank: KJ361516.1) common to all spliceforms of this gene (Eichner et al., 2015a), offering a generalized expression pattern for these transcripts. Interestingly, RNAi studies on *LsRXR* revealed that knockdown individuals upregulate numerous genes with chitin binding domains (Eichner et al., 2015a), similar to those described here. Chitin binding domains (CBD; pfam01607 and pfam00379) are found in numerous genes essential for the moulting process including chitinases, chitin deacetylases, peritrophic membrane proteins, cuticular proteins, and lectins and can be subdivided into two groups based on the presence or absence of a conserved cysteine motif. In this study, the expression of *LsRXR* was negatively correlated with

transcripts containing a CBD (Fig. 7), similar to observations in *LsRXR* knockdowns (Eichner et al., 2015a). The *L. salmonis* CBD transcripts did not show sequence similarity to *D. melanogaster* chitinases involved in chitin synthesis (KEGG: dme00520; see Fig. 4), suggesting these transcripts may have other functions (reviewed by Arakane and Muthukrishnan, 2010; Rebers and Willis, 2001). Although some *L. salmonis* chitinases have been characterized (Eichner et al., 2015c), a full classification of *L. salmonis* genes with CBDs like that of model insects (e.g. *T. castaneum*; Zhu et al., 2008 and *A. gambiae*; Cornman et al., 2008) are required to elucidate their exact functional relevance to BPU exposure and moulting. For example, mosquitoes (*A. gambiae*) have 156 genes with a non-cys CBD, which display a variety of functions (Cornman et al., 2008). Experimental annotation and phylogenomic studies of these genes will provide higher resolution analyses of *L. salmonis* cuticle proteins lacking sufficient annotation for future studies.

Among transcripts downregulated by lufenuron were seven transcripts containing at least one C-type lectin domain (CDD: cd00037), all of which showed patterns of co-expression with CBD genes (Fig. 4; Additional File 4). Similar to CBDs, only the conserved domain of these genes could be used for annotation as no reviewed UniProt IDs matched these *L. salmonis* sequences. The presence of C-type lectin domains in transcripts downregulated by lufenuron suggests a variety of potential interactions including those with N-acetylglucosamine (Bauters et al., 2017; Sugawara et al., 2004), calcium carbonate ($CaCO_3$) (Mann et al., 2000; Weiss et al., 2000), and mannose (Takahashi et al., 2006), among others (CDD: cd00037). In *D. melanogaster*, genes with C-type lectin domains are important for proper wing and appendage formation (Ray et al., 2015) while studies in crustaceans show that some C-type lectins are important in the moult cycle and may have roles in exoskeleton hardening and biomineralization (Inoue et al., 2001; Kuballa et al., 2011; Kuballa and Elizur, 2008). Therefore, it will be important to determine any taxa-specific effects of lufenuron as molecular responses to BPUs are not well understood in crustaceans. Transcripts with limited or no annotation are likely to perform taxa-specific functions (Khalturin et al., 2009), and will be useful for better characterizing moulting. For example, $CaCO_3$ is an important component of crustacean cuticles (Greenway, 1985), but not insect cuticles, and therefore BPUs may exert slightly different effects on *L. salmonis* physiology compared to observations in insects. Given their relevant annotation and experimental evidence to arthropod development, co-expression with *L. salmonis* CBD genes (Fig. 7), and downregulation by exposure to lufenuron (Additional File 1), transcripts with C-type lectin domains reported here should be considered important for future assessments of *L. salmonis* moulting.

### 4.3. Drug resistance in sea lice

Drug resistant strains of sea lice are a major threat to the sustainability of Atlantic salmon aquaculture (reviewed by Aaen et al., 2015). Lufenuron will be an important resource for farmers and health professionals struggling to manage multiple-resistant populations of this parasite, offering long-term protection during an important growth phase for salmon. Although the present study sheds light on the physiological responses of salmon lice to this chemical, knowledge gaps in the mechanisms controlling resistance for some of the other classes of delousing chemicals will make it difficult to assess potential issues with cross-resistance. For example, widespread resistance to EMB, the last drug to be licensed for sea lice control (Stone et al., 1999), has spread to all major salmon farming regions globally except British Columbia, Canada (Aaen et al., 2015; Saksida et al., 2013). Salmon lice used in the experiments reported here come from populations characterized as resistant to EMB (Igboeli et al., 2013; Jones et al., 2013; Sutherland et al., 2015). Benzoylureas are the only class of drugs approved for sea lice where resistance is not reported to be an issue (Aaen et al., 2015). Interestingly, when EMB-resistant strains of the armyworm *Spodoptera exiga* were re-selected in the lab over six generations with EMB, no cross-resistance with lufenuron was observed (Ishtiaq et al., 2014). However, resistance to BPUs has been observed in a few terrestrial pests and is often conferred either by a single nucleotide polymorphism (SNP) in the chitin synthase 1 gene (Douris et al., 2016), or by overexpression of metabolic enzymes such as cytochrome P450s (Bogwitz et al., 2005; Gangishetti et al., 2009), glutathione-S-transferases, and carboxylases (Nascimento et al., 2015). Selection of metabolic detoxification can have important roles in cross-resistance with other compounds as has been shown for organophosphates and pyrethroids (Rodriguez et al., 2002). Lufenuron exposure did not induce a pronounced metabolic response in *L. salmonis* in this study (Additional File 1). In contrast, proteases and other metabolic enzymes are differentially expressed under exposure to neurotoxins such as cypermethrin (Poley et al., 2016) and EMB (Carmichael et al., 2013; Sutherland et al., 2015). It is noteworthy that some enzymes putatively involved in chemical detoxification are not present on the *L. salmonis* microarray and thus remain to be analyzed regarding their response to lufenuron exposure; namely cytochrome p450s (Poley et al., 2016). Future studies assessing the efficacy and molecular responses of multiple drug treatments against sea lice are needed to better understand the interactions between treatments and selection for resistance.

### 4.4. BPUs for controlling sea lice

Diflubenzuron (Lepsidon) and teflubenzuron (Calicide) have been used for over two decades in some countries to control sea lice, yet no mechanistic data and sparse clinical efficacy data are available in the literature (Branson et al., 2000; Ritchie et al., 2002). These treatments are administered in salmon feed during the marine phase of production, and therefore uneaten feed can accumulate under cages and lead to ecotoxicological impacts on non-target organisms (Olsvik et al., 2015). However, administration of lufenuron occurs in the hatchery, thereby eliminating the most significant contribution of in-feed drug release to the environment.

This is the first report on the efficacy, molecular responses, and ultrastructural impacts of lufenuron in a crustacean, and represents the most comprehensive examination of BPU impacts on an aquatic arthropod. The first infectious stage (copepodids) was used for transcriptomics and metabolomics analyses with pools of 500 salmon lice in each sample. This stage was chosen based on it likely being the first stage to encounter the drug under field conditions. The high level of efficacy of lufenuron within a single moult in both free-living larvae and parasitic stages of salmon lice assayed here further suggests rapid removal of any stage attaching to the host salmon.

Salmon lice from the same cohort were used in both the transcriptomic analysis (B3) and the efficacy experiment (B2). We observed 93% fewer chalimus II staged lice on salmon in the lufenuron-treated group 12 dpi, a comparable reduction to salmon lice infesting lufenuron-fed Atlantic salmon (96% reduction). Responses to some BPUs are known to be dependent on temperature (Ritchie et al., 2002), concentration administered (Gangishetti et al., 2009), and timing of parasite development (Merzendorfer et al., 2012). Lufenuron will be administered in-feed to salmon at 35 mg/kg over a target 7 day period and therefore uptake of the drug by *L. salmonis* will largely occur through digestion of mucus, skin, and blood of the host, with some contact exposure through the mucus. Concentration thresholds for gene expression responses and timing of parasite collection, ideally to be completed at different time points throughout the entire moulting process, will require further attention. Moreover, as the action of BPUs is specific to those cells expressing chitin synthase 1 (i.e. largely epithelial tissue), the degree of change between certain genes described herein may be diluted if the genes are also expressed in unrelated tissues.

## 5. Conclusions

In this study, the efficacy of lufenuron treatments (~90%) was similar for *L. salmonis* pre-soaked in the drug for 3 h compared to those salmon lice infesting a treated salmon host. In all cases, lufenuron was effective against eggs, larvae, and parasitic stages of *L. salmonis*, suggesting a broad, rapid impact of this drug on sea lice.

Transcriptomics and metabolomics suggest lufenuron impacts numerous cuticle and developmental proteins as well as solute transport. However, hormonal signalling related to moulting and genes in the chitin synthesis pathway were largely unaffected by this drug. These molecular signals were linked to abnormal formation of the newly synthesized cuticle and inadequate metabolism of the old cuticle, potentially because of downregulation of novel chitin-binding and C-type lectin genes. Future studies assessing the impacts of lufenuron in salmon lice attached to the host will be important in further characterizing the mechanisms of action of this drug throughout the entire process of parasite moulting.

## Declarations

### *Ethics approval*

All experiments using Atlantic salmon followed the guidelines provided by the Canadian Council on Animal Care (http://www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf) and were approved by the UPEI Animal Care Committee (UPEI Animal Care Protocol #16–023).

### *Consent for publication*

Not applicable.

### *Availability of data and materials*

The datasets supporting the results of this article are available in the NCBI Gene Expression Omnibus (GEO) repository, GSE99880.

### *Competing interests*

Elanco Canada Ltd. is seeking to license lufenuron as an aquaculture treatment against sea lice infection, and some of the authors are scientific officers within Elanco.

### *Authors' contributions*

JDP: Wrote the manuscript with MDF, extracted RNA, analyzed microarray data, contributed to the execution of all experiments with lufenuron.

LMB: Completed RT-qPCR validation and exploration, and contributed to writing.

AMM: Completed sample labeling, hybridization, scanning, and raw data output for microarrays.

OOI: Contributed to bioassay development and execution.

SKW: Contributed to writing, study design and field applications.

AM: Contributed to study design and field applications.

JR: Contributed to study design and field applications.

MG: Contributed to study design and field applications.

LR: Contributed to study design and field applications.

JB: Contributed to study design and field applications.

DWW: Completed electron microscopy work.

BFK: Contributed to writing of the manuscript and data analysis.

BH: Contributed to writing and study design.

MDF: Contributed to writing of the manuscript, data analysis, and study design and execution.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2018.02.007.

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