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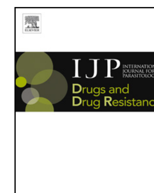

High level efficacy of lufenuron against sea lice (*Lepeophtheirus salmonis*) linked to rapid impact on moulting processes

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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

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