Toxicokinetic and transcriptional effects of lufenuron on rockpool shrimp *(Palaemon elegans)*

Master thesis in bioscience
BIO5011
1st of June 2021

Anna Bentsen
Total number of pages: 74

Nord University
Faculty of bioscience and aquaculture

NORD University

INSTITUTE OF MARINE RESEARCH

Abstract

The salmon lice *Lepeophtheirus salmonis* is a global challenge for the aquaculture industry. Several pharmaceutical drugs are currently used in the treatment of lice infestations in farmed Atlantic salmon (*Salmo salar)* in Norway. Elanco Animal Health has recently developed a benzoylurea treatment with the active substance lufenuron, having the same mode of action as di- and teflubenzuron.

This study examined toxicokinetic and transcriptional effects of lufenuron in the rockpool shrimp (*Palaemon elegans*), following an extended exposure period. Adult shrimps were fed pellets coated with lufenuron (0.0001, 0.001, 0.01, 0.1, 1.0 and 10 µg lufenuron/g shrimp) twice a week for 58 days. All shrimps were measured and examined for morphological changes at the end of the experiment. In addition to the main exposure experiment, an elimination experiment was conducted to determine the half-life of lufenuron in rockpool shrimps. Sublethal effects of lufenuron were analysed by studying transcriptional responses in the hepatopancreas of shrimps that were alive when the experiment was terminated (0, 0.0001, 0.001 and 0.01 µg lufenuron/g shrimp). Markers associated with detoxification, moulting and stress were selected.

This study shows lufenuron to be lethal to rockpool shrimps when given doses of 0.01 µg lufenuron/gram shrimp and higher. Cumulative mortality reached 17.5, 15, 25% for the groups receiving the lower doses (0.0001, 0.001 and 0.01 µg lufenuron/gram shrimp, respectively) and 92.5 and 100% for the groups receiving the highest dose of lufenuron (0.1, 1.0 and 10 µg lufenuron/g shrimp, respectively). Lethal threshold concentrations (LC_x) were calculated by using a dose-response curve based on the accumulated lufenuron concentrations in the shrimps. An estimated LC_{50} were calculated to be 21.6 ng/g shrimp. An estimated half-life of 4.7 days was found using data from the elimination study. Lufenuron had no significant effect on the transcription of the selected genes in the hepatopancreas of shrimps. After 58 days of exposure, lufenuron does not seem to be potent enough to affect the transcription of detoxification, moulting and stress associated genes in rockpool shrimps receiving doses of 0.01 µg lufenuron/g shrimp or lower.

In conclusion, this study shows that lufenuron was lethal to rockpool shrimps exposed to doses equal to or higher than 0.01 µg lufenuron/g shrimp after 58 days of exposure. The 58-day LC_{50} concentration was 21.6 ng/g shrimp, and the estimated half-life was 4.7 days. Lufenuron had no effect on the transcription of the studied genes.

Abbreviations

AChE	Acetylcholinesterase
BMM	Sample with matrix
BUM	Sample without matrix
CL	Carapace length
CSI	Chitin synthesis inhibitor
dsDNA	Double stranded DNA
EMB	Emamectin benzoate
GLM	Generalized linear model
IMR	Institute of Marine Research
LOQ	Level of quantitative
m/z	Mass-to-charge Ratio
NAcGlc	N-acetylglucosamine
ssDNA	Single stranded DNA
TL	Total length
PCA	Principle component analysis
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RT	Reverse transcription

Index

1. Introduction

1.1 Salmon aquaculture in Norway

The history of Norwegian salmon farming started in the late 1960s and is regarded as one of Norway's greatest industrial success stories. In the course of 50 years, Norway has managed to become the world's top producer of farmed salmon, with an export rate of 95% (Hersoug et al., 2019; Regjeringen, 2019). Atlantic salmon accounts for close to 94% of the aquaculture industry in Norway. In 2019 approximately 1.3 million tons Norwegian salmon was produced. (Statistisk sentralbyrå, 2020). The extensive salmon production has provided an exceptional viable niche for most significant parasitic pathogen in Norwegian farmed salmon; *Lepeophtheirus salmonis,* commonly known as salmon lice.

The Norwegian Food Safety Authority promotes health and quality throughout the production chain, as well as considering an environmentally friendly production. The pharmaceutical regulations contribute to safe and rational use of drugs. Pursuant to these laws, the Norwegian Food Safety Authority have regulations of detailed provisions on registration and reporting of lice numbers, average limit lice levels, guidelines considering treatment and treatment methods, evaluation and sensitivity studies, control of residues in slaughtered fish, as well as reporting of dispensed medicines and proper drug use. The salmon farms have a responsibility when it comes to reporting to the Norwegian Food Safety Authority. From the reports they supervise that the requirements of the various regulations are met. The aquaculture industry is solely responsible for ensuring that operations take place in accordance with current regulations. The facilities must internally document assessments that have been made regarding lice control, development of resistance, fish- welfare, health and disease, etc. The decisions that are made are followed up in the industry (Nærings- og fiskeridepartementet, 2017). The economical cost of treatment against salmon lice is estimated to be ~2.45 NOK/kg. This does not include the cost of slow growth and mortality caused by the treatment. Repetitive treatments can also impact the quality of the salmon, thereby decreasing the market price (Jensen, 2013; Liu & Bjelland, 2014).

Salmon farms located at sea are often placed in wave-sheltered areas near the coast. Wild salmon that migrate to and from rivers inland, has a chance of passing the pens of farmed Atlantic salmon. In the status report for Norwegian salmon stocks from the Norwegian institute for nature science (NINA), salmon lice have the second highest degree of impact, after escaped

farmed salmon, as a threat factor for the wild Norwegian salmon stock. The density of salmon farms at sea increases the risk of infection between farmed and wild salmon populations (Mark, 2009; Thorstad & Forseth, 2019). The aquaculture industry being subjected to the animal welfare act and Aquaculture operations regulations must ensure animal welfare. This include medical treatment when needed to ensure the life quality of salmon. The industry depends on pharmaceuticals applied under veterinary prescription to manage various diseases and parasites. All pharmaceuticals used in the industry contains active ingredients with specific characteristics in terms of dilution rate and persistence in the environment. It is difficult to estimate the environmental cost aquaculture development and production cause due to the vast variation in factors affecting each aquaculture facility (Landbruks- og matdepartementet, 2009; Mark, 2009; Nærings- og fiskeridepartamentet, 2008; Urbina et al., 2019; Aaen et al., 2014).

1.2 Salmon lice (Lepeophtheirus salmonis)
Salmon lice naturally occurs in seawater and have become a global challenge for the salmon aquaculture industry. Elevated numbers of salmon lice in aquaculture facilities also cause an ecological risk of increasing numbers of parasites in wild salmonid populations. The louse has ten life stages, each separated by a moult (Fig. 1). The salmon lice nauplius is hatched from an egg and released into the water stream, being dispersed along the coast, attaching to wild salmon and sea trout (Samuelsen, 2016; Wagner et al., 2008). Depending on temperature the nauplii moult into an infective copepod after 5-15 days (Whelan, 2010). At the copepod stage it attaches to salmon, where it feeds on skin, mucous and blood of the fish. It will stay attached to the salmon until its adult stage, chalimus. Then it will breed and release several thousand offspring within a few months. During the moulting period, salmon lice produce a new, larger exoskeleton underneath the old cuticula. This process is mainly controlled by hormones, but can be affected by external factors, such as food and temperature (Eichner et al., 2014). The wounds they cause on the skin surface of salmon is the main problem with salmon lice in aquaculture. The wounds make salmon more susceptible to bacterial- and fungal infections, as well as affecting their osmoregulation ability (Igboeli et al., 2014).
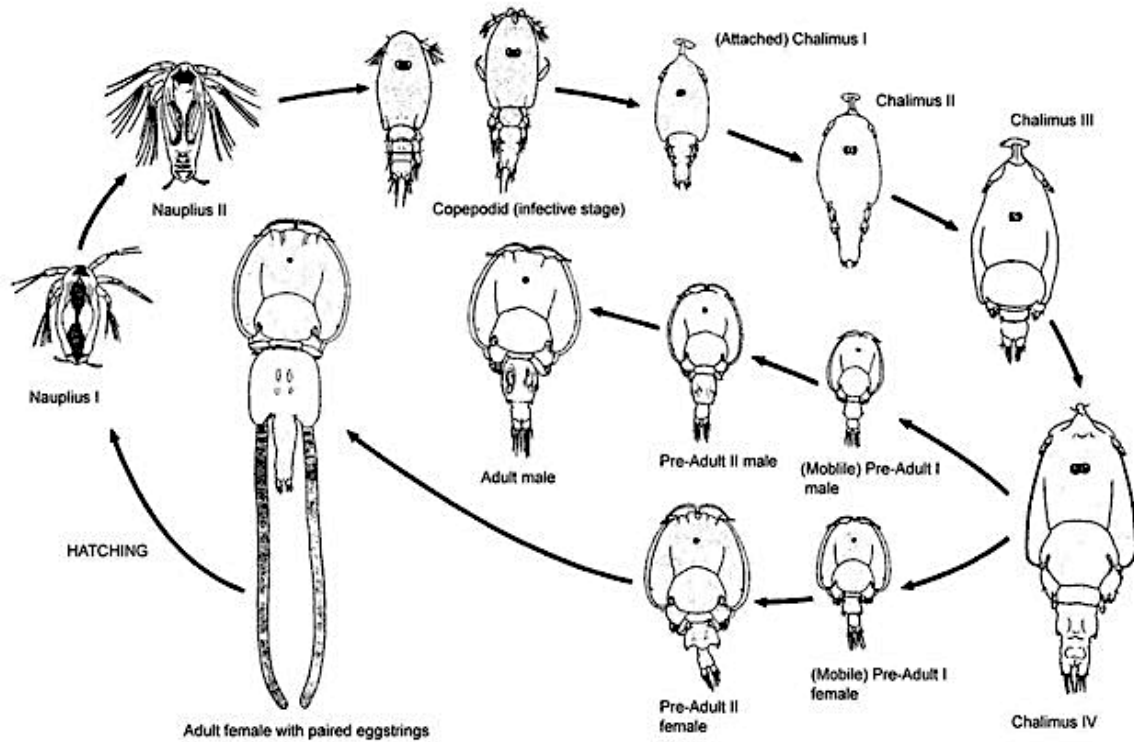
Fig. 1 Life stages of salmon lice where the naupilus and copepodid stages are planktonic. After 5-15 days, naupilus moult to copepodids. Copepodids attach to a host where it moults into the chalimus stage. In the pre-adult stage, the lice moves freely over the skin of its host to feed (Whelan, 2010).

1.3 Treatment methods against salmon lice

Several strategies have been adopted throughout the years for coping with salmon lice. Chemical treatments being the first strategy utilized in the aquaculture industry. The effectiveness of chemical treatments has shown to decrease over time, as the lice develop a resistance to them. This often leads to an increase in treatment concentrations (Cerbule & Godfroid, 2020). When chemical concentrations increased, ecological concerns arose. Which led to the introduction of biological methods as the use of cleaner fish and other non-chemical strategies, as bathing infested fish in warm- or fresh-water, and mechanically removing lice with water jets, brushes or laser technology (Cerbule & Godfroid, 2020; Overton et al., 2019). In recent years, there has been an increase in the use of non-chemical treatments, of 42% from 2016-2017, and a persistent increase from 2017-2018 of 21%. The most popular non-chemical treatment is thermal delousing. Unfortunately, studies have shown salmon present in water with a temperature above 28°C responds with signs indicating nociception or pain (Helgesen et al., 2019; Nilsson et al., 2019). Chemical bath- and in-feed treatments, as well as the use of cleaner fish preying on salmon lice, have generally been the most used strategies (Hannisdal et al., 2020; Imsland et al., 2014; Urbina et al., 2019).

3

1.4 Chemical treatments used in Norwegian aquaculture

Chemical treatments against salmon lice can be sorted into three main groups: Neurotoxins, hydrogen peroxide and chitin inhibitors (Table 1). The amount of treatment used in Norwegian industries varies yearly (Table 2). In 1999 the Norwegian government and aquaculture industry entered into an informal agreement with Kurt Oddekalv, an activist in the environmental protection association, to minimize the use of di- and teflubenzuron as treatment against salmon lice. The environmental protection association had reason to believe there was an environmental risk associated with the chemicals. In return, the association had to refrain from taking action against the industry (Blaalid, 2009). The agreement resulted in minimal usage of di- and teflubenzuron in Norwegian aquaculture in the period 1999-2008. The use of emamectin benzoate took its place, which resulted in an increase in resistance against this substance in the period 2010-2015. This led to a greater use of alternative treatment types, attempting to find one that worked. The decrease in usage after 2016 is not due to less resistance, but the use of new treatment methods such as cleaner fish, hot water, fresh water and mechanic removal (Litleskare, 2019).

1.4.1 Hydrogen peroxide

The bath treatment hydrogen peroxide (H_2O_2) was used in Norwegian aquaculture from 1993-1997. The usage of this treatment was partially terminated in 1998, when safer and more effective pharmaceuticals were introduced (Grave et al., 2004; Litleskare, 2019; Wesenberg et al., 2000). Over the years, resistance against the new pharmaceuticals grew and in 2009 hydrogen peroxide was re-introduced. The usage increased, especially in the period 2014-2016 (Table 2) (Helgesen et al., 2015; Litleskare, 2019). Inadequate delousing procedures or decreased sensitivity in salmon lice have since then led to reduced treatment efficacy of hydrogen peroxide (Denholm et al., 2002). Little is known of the function of hydrogen peroxide, but it is assumed to induce a mechanical paralysis by forming bubbles in the body (Thomassen, 1993). It is estimated to have a half-life of 8-28 days in sediment, depending on temperature, pH and density of organic matter (Lyons et al., 2014). After H_2O_2 treatment the water used is released directly into the environment from the tarpaulin or by emptying the well-boat used. Bechmann et al. (2019) found in their research evidence of tissue damage on gills and lipid peroxidation in the hepatopancreas of shrimp after exposure to hydrogen peroxide. When reaching concentrations of 15 mg/L for 1 h the tissue damage was severe, and the shrimps were not likely to recover. Regarding that many species are sensitive to the compound; the use should be limited.

1.4.2 Organophosphorus

Organophosphates are given as a bath treatment. The compound Salmosan™, with the active substance azamethiphos, was the first pharmaceuticals used to treat lice infected salmon in Norway (Urbina et al., 2019). Organophosphorus compounds were the only treatment against salmon lice in Norwegian aquaculture in the period 1989-1992. In 1999 the use stopped due to the introduction of pyrethroids, a cheaper treatment that could be used closer upon slaughter (Grave et al., 2004). Organophosphates are neurotoxins with a mode of action to inhibit acetylcholinesterase (AChE) activity in the synapse between cholinergic neurons and neuromuscular junctions, causing paralysis and eventually death to the louse (Baillie, 1985; Sparling, 2017). Organophosphates are highly water soluble and the half-life of azamethiphos in sediment, based on one report, is estimated to be 9 days (Burridge et al., 2014). Its usage has significantly decreased since 2016 (Table 2). As usage is a prerequisite of azamethiphos presence in the environment, the risk of affecting non-target species is considered to be low, even if the substance have been found to be acutely toxic to European lobster larvae (Parsons et al., 2020). There are few studies on this compound, which makes the knowledge base sparse.

1.4.3 Pyrethroids

Pyrethroids are anti-salmon lice neurotoxic compounds that have been used as a bath treatment in Norwegian aquaculture since 1994. The original compound was pyrethrum, a natural insecticide, derived from the chrysanthemum flower, that has been used as a mosquito repellent and lice remedy for thousands of years (Denholm et al., 2002). Deltamethrin and cypermethrin replaced the use of pyrethrum in Norwegian aquaculture shortly after it was introduced. In Norwegian aquaculture, the use of cypermethrin was introduced in 1996 and deltamethrin in 1998. The use of deltamethrin has declined since 2015, while the use of cypermethrin ceased in 2018 (Table 2) (Grave et al., 2004; Grefsrud et al., 2021). The chemical is absorbed over the gills of salmon. Pyrethroids` mode of action in arthropods is to block sodium channels, thereby disturbing nerve impulse transmission (Tschesche et al., 2021). Deltamethrin has been detected in the sediment near aquaculture sites, posing a risk to non-target species (Van Geest et al., 2014). Polychaetae worms are often found in the sediment in vicinity of salmon pens where they process organic matter from the facility. A study conducted by Van Geest et al. (2014) show the survival of the polychaetae worm being negatively affected in sediment with accumulated deltamethrin. Worms exposed to deltamethrin concentrations over 7.6 µg/L for 1 hour exhibited little to no movement and half of the individuals died after 48 hours, the rest were immobile or moribund. Larvae of European lobster has also found very sensitive to this

substance, while various shrimp species are somewhat sensitive. The risk of affecting non-target species is low due to reduced utilization. Should the use increase, the status will change to being a high risk on the basis that the substance is toxic to several non-target species that inhabit the Norwegian coastal area (Burridge & Van Geest, 2014). Pyrethroids are highly toxic to fish, but even more toxic to the ectoparasites, which defends the use of this compound being used as a therapeutic drug (Wesenberg et al., 2000).

1.4.4 *Avermectins*

Emamectin benzoate (EMB), an avermectin derivative given as an in-feed treatment, has been used in Norwegian aquaculture since 1999 (Grave et al., 2004). Reduced sensitivity to avermectins was suspected in salmon lice present in Norwegian salmon farms in 2008. Resistant to this treatment has since then developed throughout the industry (Espedal et al., 2013; Lam et al., 2020). Avermectins bind to the glutamate-gated chloride channels in muscle cells and synapses in the peripheral nervous system of invertebrates. Here it induces an influx of chloride ions, causing hyperpolarization of the cells. This action causes paralysis in salmon lice, and eventually death (Olsvik et al., 2008). EMB can bind to organic matter and be found in the sediment. Due to slow degradation and great usage of the substance, it is highly likely to find it in the sediment months after treatment, where it will be available for non-target species directly through consumption of organic matter and prey. There are few field studies and research regarding sensitivity towards the compound, more is needed (Scottish environment protection agency, 2005).

1.4.5 Benzoylureas

Flubenzurones are given as in-feed treatment and has been used in Norwegian aquaculture facilities since 1996 (Grave et al., 2004). Their mode of action is inhibiting the biosynthesis of chitin. It has been proven effective against salmon lice and there is no registered resistance to this type of compound. Due to its chitin synthesis inhibitory function, it can cause serious harm to crustaceans and amphipods, which has led to a lot of controversy regarding these compounds (Macken et al., 2015; Olsvik et al., 2019; Poley et al., 2018). The use of di- and teflubenzuron in Norwegian aquaculture was minimal from 1999. There was a steep increase in the use of flubenzurones when it started up again in Norwegian aquaculture facilities in 2008-2009 (Table 2) (Hannisdal et al., 2020). The compound has also been shown to build up in the sediment over time, causing a moderate threat to non-target species. The knowledge of what effect these compounds can have on the environment and species composition is moderate, and there is a need for more knowledge regarding the dissemination of flubenzurones (Samuelsen, 2016; Samuelsen et al., 2015; Scottish environment protection agency, 1999). Flubenzurones have low solubility and binds to organic matter when dissolved in water. Due to this slow degradation and the high probability of it being transferred over great distances with the current, uneaten feed and feces from treated salmon are assumed to be the main pathway into the system (Macken et al., 2015; Samuelsen et al., 2015). Traces of these chitin inhibiting pharmaceuticals have been detected in the sediments under and in vicinity of treated fish farms for several months after treatment, with an estimated half-life of 170 days in the sediment (Samuelsen et al., 2015). Lufenuron has the same mode of action as di- and teflubenzuron, which has shown to have adverse effects on crustaceans and amphipods inhabiting the benthic areas surrounding aquaculture facilities (Langford et al., 2014; Macken et al., 2015). Teflubenzuron has been shown to affect molecular mechanisms in lobsters at sub-lethal levels (Olsvik et al., 2015). Mortality in larvae of Northern shrimp has been reported to be high when exposed to diflubenzuron (Bechmann et al., 2018). As lufenuron is given in an early life stage of salmon in freshwater, and risk of affecting non-parasitic crustaceans in the sea is expected to be insignificant.

Table 1 Overview of pharmaceuticals used in Norwegian aquaculture and their active substances.

Classification	Active substance	Trade name	Treatment	Mode of action	Reference
Antiseptics	Hydrogen peroxide	Nemona™ Paramova™	Bath	Strong oxidizer that cause salmon lice to separate from host	(Thomassen, 1993)
Organophosphorus	Azamethiphos	Salmosan™ Azasure Vet™	Bath	AChE inhibitors	(Fallang et al., 2004)
Pyrethroids	Deltametrin Cypermethrin	Alpha Max™ Betamax Vet. ™	Bath	AChE inhibitors	(Fallang et al., 2004)
Avermectins	Emamectin benzoate	Slice vet. ™	Oral	Blocking nerve transmission → Paralysis/Death	(El-Saber Batiha et al., 2020)
Benzoylureas	Diflubenzuron Teflubenzuron	Lepsidon vet™ Ektobann™	Oral	Inhibit chitin synthesis	(Macken et al., 2015)

Table 2 Treatments used in Norwegian aquaculture from 2001-2019 (kg active substance) (Grave & Horsberg, 2014; Litleskare, 2019)

	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Hydrogenperoxide (100%) (tons)	-	-	-	-	308	3.071	3.144	2.538	8.262	31.577	43.246	26.597	9.277	6.735	4.523
Azamethiphos				66	1.460	3.346	2.437	4.059	3.037	4.630	3.904	1.269	204	160	154
Deltametrin	16	23	29	39	62	61	54	121	136	158	115	43	14	10	10
Cypermethrin	45	49	30	32	88	107	48	232	211	162	85	48	8	0	0
Emamectin benzoate	39	60	73	81	41	22	105	36	51	172	259	232	128	87	114
Diflubenzuron	-	-	-	-	1.413	1.839	704	1.611	3.264	5.016	5.896	4.824	1.803	622	1.296
Teflubenzuron	-	-	-	-	2.028	1.080	26	751	1.704	2.674	2.509	4.209	293	144	183

1.5 Lufenuron

Lufenuron is commonly used on dogs and cats against fleas (Fig. 2) (Legemiddelverket, 2001). It is a benzoylurea pesticide that prevent moulting in crustaceans through chitin synthesis inhibition (CSI). Moulting is a crucial step in the growing process of crustaceans and inhibition is lethal to developing individuals (Poley et al., 2018). The use of CSI pharmaceuticals in feed treatment has increased in aquaculture over the past years (Bechmann et al., 2018). When administering medicated feed there is a risk of it entering the environment as uneaten pellets or via fish excreta. Lufenuron is closely related to di- and teflubenzuron, treatments actively used in Norway since the 1990s to 2001, and then reintroduced in 2009 (Samuelsen et al., 2015). However, lufenuron has a longer duration of action, making it possible to administer the medication during early smolt phase, in freshwater. This ensures better emission control, than when medicating at the open sea (Macken et al., 2015).
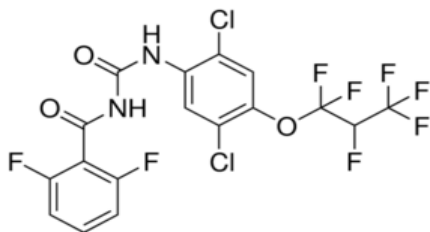

Fig. 2 Structural form of Lufenuron
($C_{17}H_8Cl_2F_8N_2O_3$).
Source: www.sigmaaldrich.com

Elanco Animal Health is the producer of the in-feed treatment containing lufenuron (Imvixa). In late 2016 the in-feed treatment got approved for prevention and control of salmon lice in aquaculture facilities in Chile (McHenery, 2016). Being the second largest salmon producing country in the world, Chile faces the same challenges with salmon lice as Norway (Regjeringen, 2019). The most common salmon lice species in Chile is *Caligus rogercresseyi*, a smaller species than *L. salmonis* (McHenery, 2016). Salmon treated with lufenuron is given a daily dose of 5 mg/kg fish once daily for 7 to maximum 14 days to ensure full therapeutic dose (35 mg/kg). After being treated, the salmon is held for approximately 7 days, to allow excretion of unabsorbed medicine, before being transferred to open sea facilities. The pre-mix of lufenuron contains 10% pure lufenuron, 88% corn starch and 2% colloidal silicon dioxide (McHenery, 2016; Rath, 2017). The treatment is expected to give long-term protection against salmon lice infestation (Poley et al., 2018). Salmon treated with lufenuron is not to be slaughtered for at least 2050 degree days after treatment is ended (McHenery, 2016). In the implementation regulation of the European commission from 2014, the maximum residue limit for lufenuron was set to 1350 μg/kg in muscle and skin of salmon (Commission implementing regulation, 2014). Salmon grow in sea pens for up to 22 months, and it is considered highly unlikely that the residue of lufenuron in salmon fillets will exceed this level (McHenery, 2016).

1.5.1 Toxicokinetics

Toxicokinetics is the study of the body's uptake, transport, storage, and excretion of a substance. Genes are up- and down regulated depending on both physical and environmental changes. Arthropods lack an adaptive immune system but have evolved an innate immunity. Their exoskeleton works as a mechanical barrier to the surroundings, while the innate immune system protects the internal environment by rapidly producing immune responses if presented with pathogens (Iwanaga & Lee, 2005). Benzoylurea pesticides present in the surrounding water, sediment and in feed items have been shown to influence the expression of genes linked to moulting, stress and detoxification (Olsvik et al., 2019; Olsvik et al., 2017).

Lufenuron is given as an in-feed treatment to salmon in the freshwater phase. Lice that attached to the salmon will take lufenuron into their body by ingesting salmon blood, then die due to the CSI function of lufenuron. The efficacy of the treatment is high (~90%) for all stages of salmon lice (Poley et al., 2018). Salmon treated with the recommended therapeutic dose of lufenuron, showed a difference between the number of lice on the untreated control group and lufenuron treated salmon after ~150 days. After 250 days there were no registered difference between the groups (Kristine Brokke, unpublished data). Elanco doesn't mention exactly how many days the treatment is estimated to last, other than it being a "long-term" (McHenery, 2016; Poley et al., 2018). If salmon that has been treated were to die after being transferred to the sea facility, would end up in a collection net at the bottom of the pen, where it is available to scavenging non-target crustaceans. This could lead to bioaccumulation of lufenuron in non-target species, depending on the degradation time in the various species.

1.6 Rockpool shrimp

The rockpool shrimp (*Palaemon elegans*), is a common littoral shrimp along the Norwegian coastal line (Fig. 3). It can be found from the south of Norway and all the way to Trondheimsfjorden in central Norway (Fig. 4). The shrimp prefer rock pools or sandy bottoms in shallow water with seaweed for hiding. It tolerates a wide variety of environmental conditions as salinity, oxygen levels and temperature (Deli et al., 2018; Samuelsen et al., 2020; Sømme, 2017). Because of its broad ecological niche, it plays an important role in the intertidal and shallow sea habitats of the European marine littoral fauna (Berglund, 1980; Berglund & Bengtsson, 1981; Reuschel et al., 2010). Shrimps are scavengers, feeding on detritus, algae and animals, and is at a potential risk of bioaccumulating chemicals from their food- and water source (Langford et al., 2011; Vogt, 2019). The exoskeleton of rockpool shrimps is hardened

by calcium carbonate and protects it from the environment, while allowing growth, mobility and respiration. It is shed several times through a lifetime (Hartnoll, 2001). The shrimp have six life stages, each separated with a moult (Fig. 5). The shrimp also moult several times after the adult stage, as it grows.
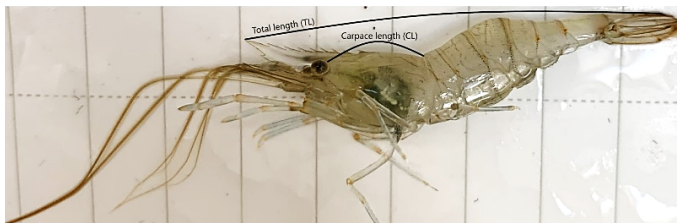
Fig. 3 Rockpool shrimp from Kumløya, Austevoll (Norway). Photo: Anna Bentsen, 2020.

Fig. 4 Distribution map of rockpool shrimp in Norway, obtained from NINAs species map service (www.https://artskart.artsdatabanken.no)

Fig. 5 Life stages of rockpool shrimp (Palaemon elegans) illustrated by Anna Bentsen

1.6.1 Moulting process

Moulting is divided into four phases; pre-moult, moult, post-moult and inter-moult (Fig. 6). The frequency of moulting is higher in larvae than adults. Rockpool shrimps usually moult each 14-20 days, varying with temperature and age. All the phases are controlled by endocrine hormones. The sinus gland, located in the eye stalk, receives secretions from the X-organs in the medulla terminalis, which starts the production of hormones that inhibit moult. In the maxillary somites, the Y-organs secrete hormones derived from cholesterol from the diet, which stimulates moulting. This action is under the control of the X-organ (Hobbs, 2001). During post-moult and inter-moult there is a neuropeptide moult-inhibiting hormone released by the X-organ/sinus gland complex. This regulates how long the inter-moult period last (Lachaise et al., 1993). When the external and internal conditions are optimal, the release of hormones in

the sinus gland is blocked. Resulting in the Y-organ no longer being suppressed, which makes it secrete moult-initiating hormones. This initiates pre-moult activity, that affects most of the body parts. The first stage of pre-moult is called proecdysis. In this stage the production and secretion of moulting hormones from the Y-organ increase into the hemolymph (Lemos & Weissman, 2021).

For the shrimp to be able to build a new, larger exoskeleton, it needs to increase glycogen reserves and reabsorb minerals like calcium from the old exoskeleton. Chitinase degrades chitin into oligosaccharides that chitobiase degrades into monomers, a key role in chitin digestion (Muzzarelli, 1977). The monomers are able to be reabsorbed into the new exoskeleton (Buchholz, 1989). An enzyme softens the cuticle at the base and the cuticle pulls away from the epidermal cells (Hobbs, 2001). This stimulates the formation of a new epicuticle unaffected by the moulting enzyme. The old exoskeleton splits so that the shrimp can emerge from it (Andrews & Dillaman, 1993). During the early post-moult period, metecdysis, the shrimp is vulnerable to its environment. The new exoskeleton gets harder and tougher as the stored minerals are deposited in the cuticle (Hobbs, 2001). The inter-moult stage, anecdysis, consist of nutrient storage and muscle build-up, as preparation for the next moult (Lemos & Weissman, 2021).
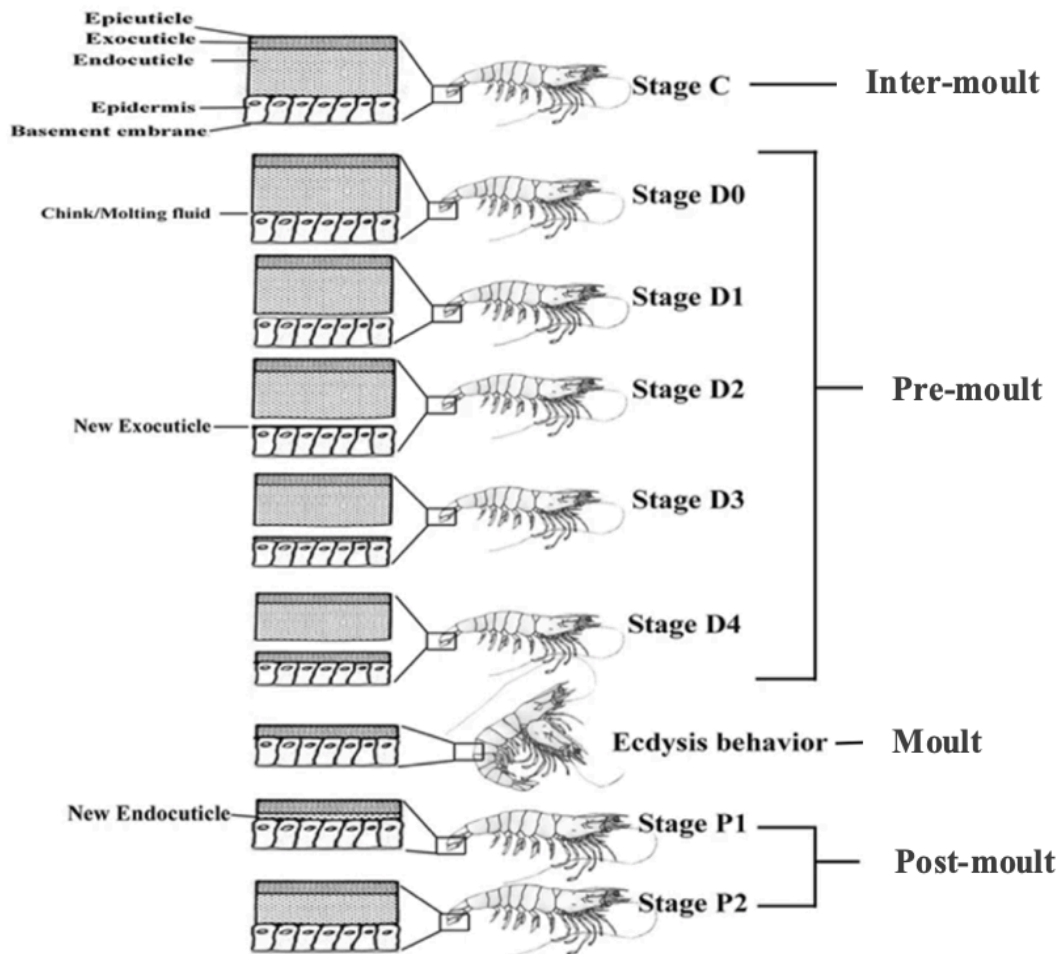
Fig. 6 Phases of the moult process with epidermal changes on the left and stages of the moulting process listed on the right (Gao et al., 2017)

1.7 Chitin

Chitin, a structural polysaccharide consisting of linear N-acetyl-D-glucosamine, is one of the fundamental components of the crustacean exoskeleton (Martin et al., 2005; Moyes & Schulte, 2014). CSIs interferes with the formation of chitin in the procuticle as well as the deposition of the epicuticle. By preventing the moulting process, they can be lethal (Harardottir et al., 2019). The environmental concern of CSIs affecting non-targeted arthropods in the marine environment is therefore a concern. CSIs such as teflubenzuron can affect molecular mechanisms in European lobsters at sub-lethal levels (Olsvik et al., 2015). Sublethal effects of CSIs can be studied by examining the transcriptional levels of selected genes (Haradottir et al., 2019).

The synthesis of chitin takes place in the epidermis and midgut. It can be divided into three steps. In the first step, enzymes form a catalytic domain that faces the cytoplasmic region, which forms the polymer. Step number two involves translocation of the initiated polymer across the membrane and into the extracellular space. The final step completes the process as the single polymers spontaneously form crystalline nanofibrils. These nanofibrils combine with other components in the extracellular matrix to form sheets of chitin. The chitin layers are cross-oriented relative to one another and forms a helicoidal bundle (Bouligand structure) that is extremely strong (Fig. 7) (João et al., 2017; Merzendorfer & Merzendorfer, 2006; Muthukrishnan et al., 2012). Alpha-chitin fibers are hydrophilic, but are impregnated by hydrophobic proteins that makes the exoskeleton hard and prevents water absorption (Andersen, 2010).
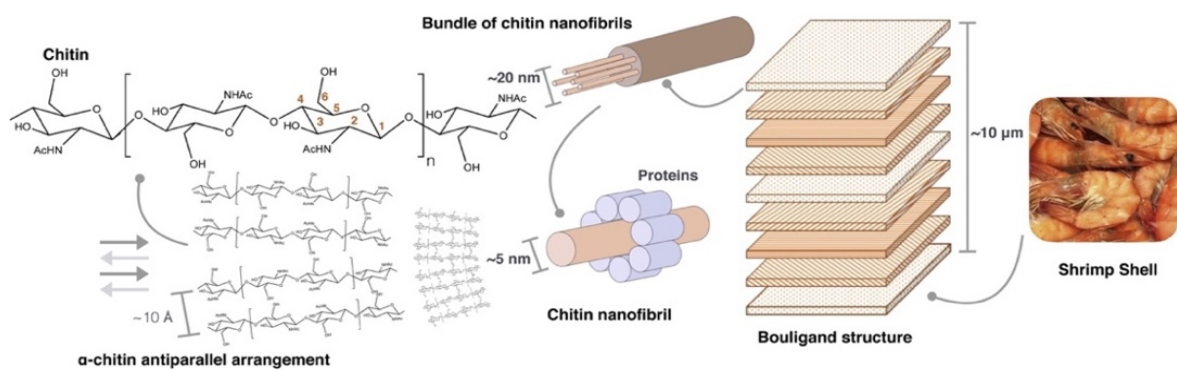
Fig. 7 Hierarchical structure of chitin in the exoskeleton of arthropods. Chitin molecules are assembled into bundles of larger nanofibrils that aligned assemble a twisted plywood structure known as the Bouligand structure (João et al., 2017)

During each moult, epidermal cells deposit new cuticle. The common biosynthetic pathway of chitin starts with glucose being converted to the polymer, glucose-6-P, with the help of glycolytic enzyme hexokinase, present in the cytosol (Fig. 8). Phosphoglucomutase catalyses the phosphate of fructose-6-P to move from C-6 to C-1, converting it to N-acetylglucosamine-1-P. This conversion leads to the formation of UDP-N acetylglucosamine, which serves as a sugar donor for the chitin synthase (CHS). Chitin synthase catalyses the polymerization of chitin. Under the pre-moult stage, endogenously secreted chitinase is involved in the degradation of chitin and reabsorption of nutrients from the old exoskeleton (Lemos & Weissman, 2021; Pedrosa-Gerasmio et al., 2019).
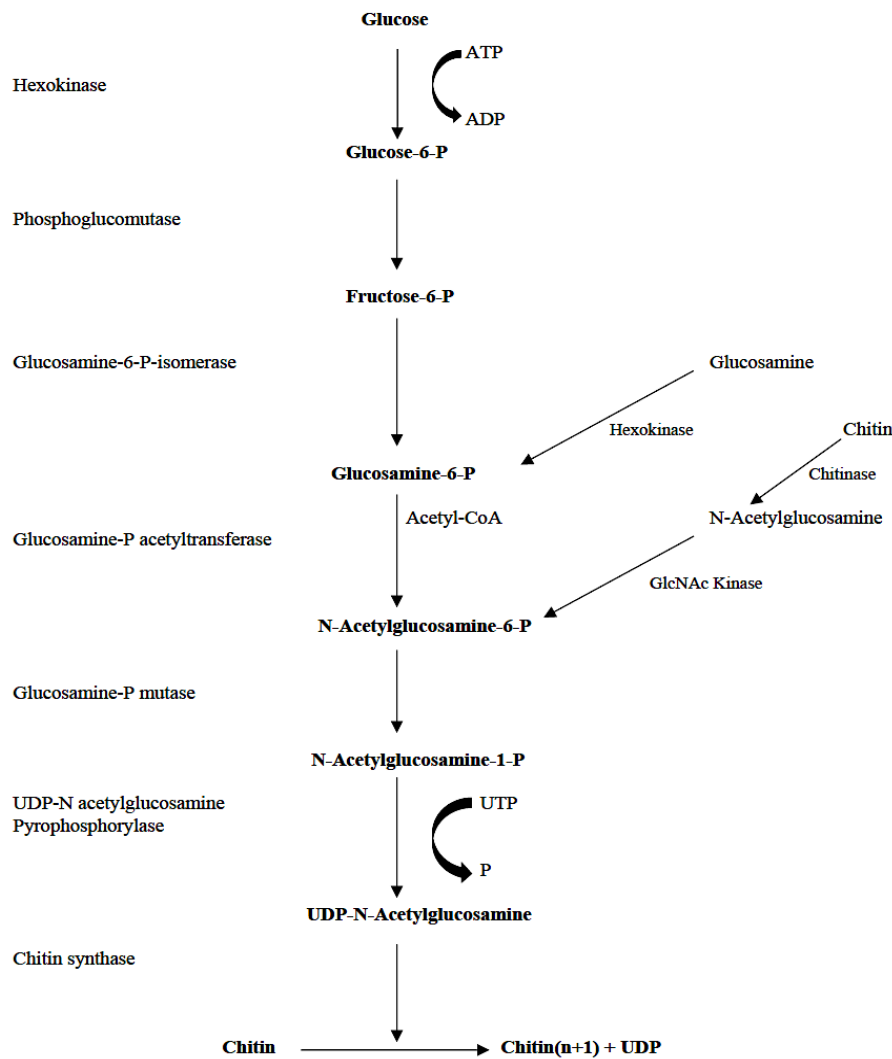
Fig. 8 The biosynthetic pathway of chitin where glycose is converted into fructose 6-P by hexokinase, phosphoglucomutase and glucosamine-6-P-isomerase. The biosynthetic pathway branches of with glutamine-fructose-6-phosphate amidotransferase converting fructose-6-P into glucosamine-6-phosphate. Then Acetyl-CoA is added by glucosamine-6-P acetyltransferase to obtain N-Acetylglucosamine-6-P. A phosphate from the N-acetylglucosamine-6-P is transferred from the C-6 to C-1 position, resulting in N-Acetylglucosamine-1-P that is uridinylated by UDP-N-acetylglucosamine pyrophosphorylase, which returns UDP-N-Acetylglucosamine that serves as a substrate for the chitin synthase.

1.8 Hepatopancreas

The hepatopancreas is the main metabolic organ of Decapoda (Fig. 9). It works as a digestive gland or midgut gland that ends in ducts that open into the stomach. It is the largest organ in the shrimp's digestive tract, located in the cephalothorax (Fig. 10). Considering it being an important detoxicating organ in shrimp, growth performance and body health of the shrimp depend on a healthy hepatopancreas. Stress, moulting and sudden changes of water quality can affect its function. Oxidative stress can occur if sufficient concentrations of toxic by-products build up. It is caused by an imbalance of harmful free radicals and detoxifying antioxidants. Complex systems as cytochrome P450 and glutathione peroxidase are detoxification systems

present in the hepatopancreas of shrimp that protect against environmental toxicants and stressors. Cytochrome P450 consist of iron-containing hemoproteins called cytochromes. The system contains thousands of enzymes able to break down contaminants and avoid oxidative stress on cells (Brignac-Huber et al., 2016; James & Boyle, 1998; Sparling, 2017; Vogt, 2019; Wang et al., 2012).

The internal digestive tract of shrimp is divided into three regions; foregut, midgut and hindgut. The cuticle of the foregut and hindgut shed during moulting. The hepatopancreas is part of the midgut, which has no cuticle. It consists of tubules with large surface epithelium that absorbs and metabolizes nutrients. The abundant R-cells are responsible for nutrient absorption and metabolization, as well as storing energy and calcium necessary for creating a new cuticle after moulting. Hormones synthesized in endocrine organs control and coordinate the activities of hepatopancreas (Štrus et al., 2019; Vogt, 2019). Recent transcriptomic studies of the hepatopancreas show that genes connected to hepatopancreatic functions are up- and downregulated under the influence of pathogens, stressors and different life stages (Chen et al., 2015; Xu et al., 2017; Zhao et al., 2018).
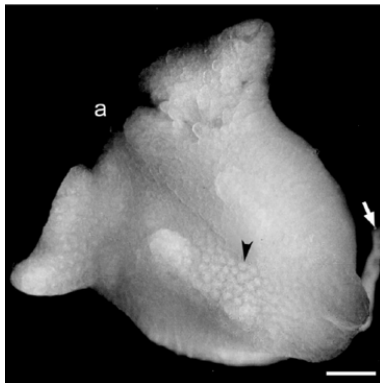
Fig. 9 Hepatopancreas of P. elegans. a: Anterior. Black arrowhead: Densely packed tubules. White arrow: Midgut. Scale: 1 mm (Vogt, 2019)

Fig. 10 Lateral view of hepatopancreas, P. elegans. Photo: Anna Bentsen

1.9 Gene expression

For a gene to be expressed, a sequence of the DNA has to be copied into mRNA by transcription. Transcription is regulated by proteins, as RNA polymerase. RNA polymerase binds to a promoter on the DNA strand. An area of the DNA strand includes an operator that regulates if the gene is expressed or not, by binding an activator or repressor. If activated, mRNA is transcribed and a mRNA strand consisting of nucleotides is made. The mRNA is transported from the nucleus to the cytoplasm where it is translated into proteins through protein synthesis (Fig. 11) (Clancy & Brown, 2008).
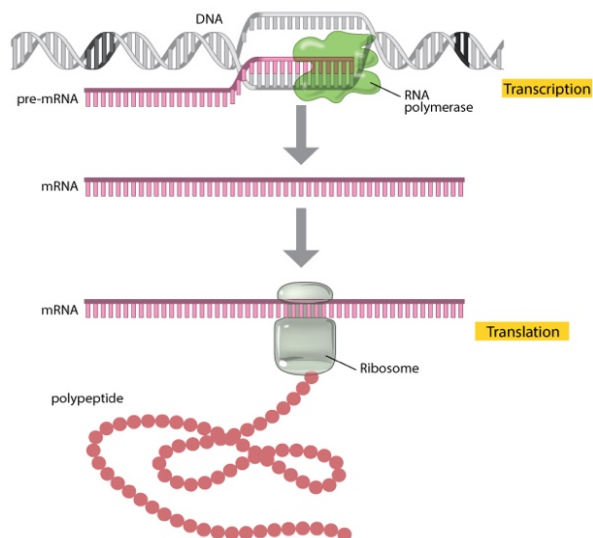
Fig. 11 RNA polymerase transcription copies the DNA sequence and creates an mRNA sequence during the process of transcription. The mRNA contains instructions for the ribosome on what amino acids to join together to make a chain of polypeptides, creating a specific protein (Clancy & Brown, 2008)

1.9.1 Genes involved in chitin synthesis

Chitin synthase (CHS) is one of the main proteins active during moulting in arthropods. It is responsible for assembling NAcGlc monomers into chitin polymers. When reabsorbing nutrients from the old skeleton, chitinase is secreted. Chitinase hydrolyses chitin and produces oligomers of NAcGlc. There are three cDNA sequences encoding for chitinase isoenzymes (*chi1, chi2* and *chi3*) in the chitin metabolism of shrimps. *Chi1* and *chi3* are present in shrimp hepatopancreas and are involved in digestion of nutrition containing chitin (Rocha et al., 2012; Watanabe & Kono, 1997; Watanabe et al., 1998). Activator molecules like trypsin and homologue transcripts are up regulated in the pre-moult phase. To prevent digestion of the new exoskeleton, digestive enzymes like chitinase are inactive when secreted and accompanied by activator molecules. Activator molecules activate digestive enzymes at the proper time and location (Seear et al., 2010).

1.9.2 *Detoxification and stress responses*

Cytochrome P450 (CYPs) are known to metabolize insecticides. Substances are converted into more water-soluble compounds by cytochrome P450 catalysing oxidation reactions, making excretion possible. The hepatopancreas is one of the major detoxification organs in shrimps with high *Cyp* expression. Research has shown that *Cyp2* and *Cyp3* genes are related to moulting and xenobiotic detoxification. The hepatopancreas of crustaceans play a central role in the biotransformation of lipophilic contaminants, making it likely that *Cyp*s expressed in hepatopancreatic tissue support this ability (Dam et al., 2008; Guenherich, 2012).

Crustaceans placed in a stressful environment or physiological conditions switch to anaerobic energy metabolism. This causes hyperglycaemia, a stress response that increases the amount of glucose in the haemolymph. In decapod crustaceans this is induced by crustacean hyperglycaemic hormone (CHH). The hormone belongs to the family of neuropeptides that include CHH and the moult-inhibiting hormone (MIH). CHH is synthesized in the eyestalks x-organ, and controls mobilization of glucose from tissue reserves to haemolymph (Chung et al., 2010; Fanjul-Moles, 2006; Mykles & Chang, 2020). The hormone is important both during development and all the life cycles of shrimp. It plays a role in metabolism of carbohydrates, inhibiting moult and reproduction, as well as affecting osmoregulatory functions (Fanjul-Moles, 2006).

Catalase (CAT) is known to protect the cell from oxidative stress by decomposing excessive hydrogen peroxide to maintain an optimum level and maintain cellular redox balance. The transcription of the gene *Cat* is upregulated if there is an excess of hydrogen peroxide in the cell. The enzyme is found in hepatopancreas and is often used to evaluate the defence ability of bacteria, plants and animals against pathogens (Zhang et al., 2008). The heath shock proteins are produced by cells exposed to environmental stress. HSP70 is involved in basic cellular processes such as correct protein folding, but it is also involved in cell- development, proliferation, apoptosis, senescent and immune responses (Baringou et al., 2016; Feder & Hofmann, 1999). HSP70 is induced in animals exposed to environmental and physiological stressors. The protein assist in repair and protection of the cell by folding proteins, assembling and disassembling multi-subunit protein complexes, moving proteins across membranes and targeting denatured- or altered proteins for degradation (Buchanan, 2000; Junprung et al., 2021) .

1.10 Research aim

The aim of this study was to examine the toxicokinetic- and transcriptional effect of lufenuron on rockpool shrimp, a common species found along the coast where salmon farms are located. The toxicokinetic aspect of the study focused on uptake and accumulation of lufenuron during the exposure experiment. Transcriptional analyses aimed to detect sublethal responses and to improve knowledge on detoxification, moulting and stress responses in rockpool shrimps exposed to lufenuron. The hypothesis was that there would be a difference in accumulated concentrations of lufenuron between treatment groups, and that the chemical induces changes in gene expression at sublethal concentrations. The result from this study will be of value when assessing the effect lufenuron has on crustaceans inhabiting areas in the proximity of aquaculture facilities with salmon treated with the salmon lice drug.

2. Materials and methods

This study was approved by the Norwegian Food Safety Authority (FOTS ID 22695). A total of 305 shrimps were used, 280 for the exposure experiment and 25 for the elimination study. For transcriptomic analyses the hepatopancreas was removed from 10 individuals each from the 0.01-0.0001 µg/g shrimp and control treatments. Toxicokinetic analyses were conducted with the remaining shrimps, as well as the remains of the shrimps with extracted hepatopancreas.

2.1 Feed experiment setup

Rockpool shrimps were collected August 10th -11th, 2020, at the west side of Kumløya, Austevoll in Vestland County (60º05′47.7″N 5º16′30.7″E) (Fig. 12). Shrimps were collected by in coastal areas with seaweed vegetation and rocks (Fig. 13). Rockpool shrimps were manually sorted out of the net and placed into buckets.

Fig. 12 Collection location of the rockpool shrimps used in this experiment (Kumløya, Austevoll, Norway) Source: Google maps

Fig. 13 Rockpool shrimps were collected inside a bay area with sandy bottom and seaweed vegetation alongside the edges of land, on Kumløya (Austevoll) Photo: Marina Mihaljevic.

At Austevoll research station (IMR), a total of 280 shrimps were randomly divided into 14 tanks, to create homogeneous treatment groups without biases. The tanks held ~50 L and had a flow rate of 20 L/min (Fig. 14). Drainpipes were covered in case shrimps escaped from their compartment, making it possible to recapture them (Fig. 15). Water temperature in the tanks, measured every day from start to the end of the experiment, was 14.7ºC ± 0.4. Each shrimp was placed in its own plastic compartment of 142 cm³ (4.5 x 4.5 x 7 cm) to control feed intake and avoid cannibalism. The bottom of each compartment was perforated to ensure water flow (<1 mm diameter) (Fig. 16). Compartments were covered with a mesh net to prevent shrimps from jumping out (Fig. 17). During the two weeks of acclimation, the shrimps were fed two 2 mm commercially produced pellets, twice a week (CLEAN Lumpfish diet, Skretting AS).
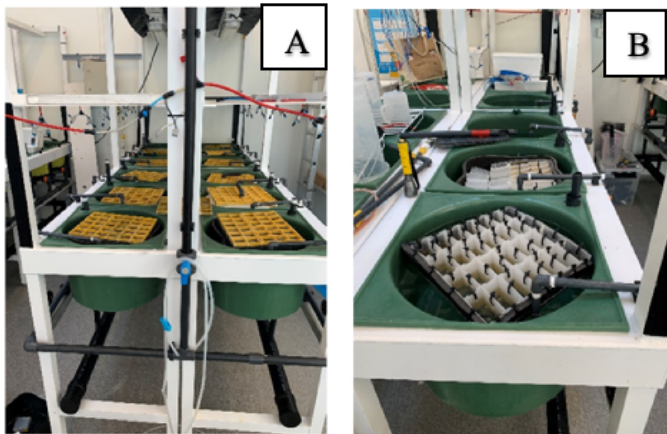
Fig. 14 Tank set-up for the feeding experiment at Austevoll research station, of the IMR, Norway.
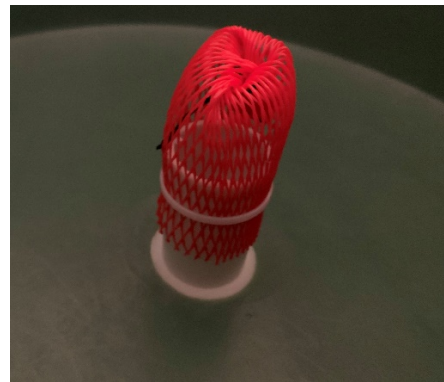
Fig. 15 The drain at the bottom of the tank was covered with a mesh netting and cable tie to prevent escapees from the containers to escape the tank.
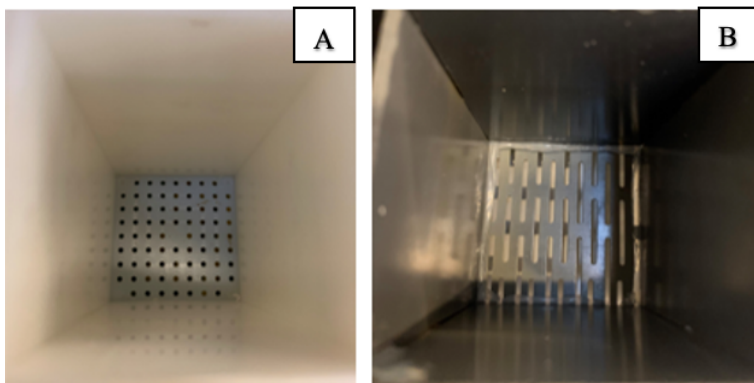
Fig. 16 Bottom of each container. The majority of the containers had the bottom showed in picture A. Two containers (B13/Dose B and B19/Dose E) had the bottom showed in picture B.
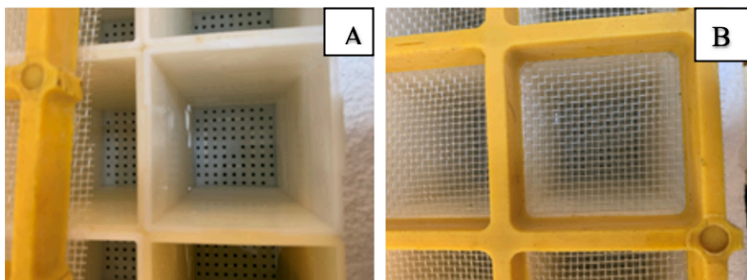
Fig. 17 Containers were covered with a mesh net to keep the shrimps from jumping out.

2.1.1 Feed exposure

The experiment consisted of seven groups with 40 shrimps each, divided into 14 tanks. All treatment groups were divided into two separate tanks to avoid external variables affecting one tank and thereby a whole treatment group. A number of shrimps were weighted, to find the mean weight of the shrimps (0.5 ± 0.4 g). The feeding of shrimps in this study, was conducted by a number of technicians at the research station. Treatment groups were fed two pellets with doses from 0-10 µg lufenuron/g shrimp (from now called µg/g shrimp) (Table 3), twice a week for 58 days (01.09.2020-28.10.2020). Pellets were given with forceps, starting with the control and ending with the highest treatment dose. The estimated number of pellets for each group was 4 pellets/week x 40 shrimps x 10 weeks, making a total of 1600 pellets. With the average weight per pellet being 6.7 mg, we prepared 25 g of lufenuron coated feed for each group.

Dose A: Shrimps were given pellets with a dose of 10 µg/g shrimp. The mean weight of the shrimps was 0.5 grams. The dose was calculated to be 5 µg lufenuron per shrimp, divided into two pellets (2.5 µg/pellets).

3731 pellets x 2.5 µg = 9.33 mg lufenuron mixed w/ 1 g maizena, coated over 25 g pellets.

Premix-1 was made of 1 mg lufenuron per gram maizena

Dose B: 933 mg premix-1 mixed with 77 mg maizena, coated over 25 g pellets.

Dose C: 93.3 mg premix-1 mixed with 906.7 mg maizena, coated over 25 g pellets.

Premix-2 was made from a 100x dilution of premix-1 (10 mg premix-1 + 990 mg maizena = 1 g premix-2)

Dose D: 933 mg premix-2 mixed with 77 mg maizena, coated over 25 g pellets.

Dose E: 93.3 mg premix-2 mixed with 906.7 mg maizena, coated over 25 g pellets.

Dose F: 9.33 mg premix-2 mixed with 990.6 mg maizena, coated over 25 g pellets.

Table 3 Concentration of lufenuron in each treatment group

Treatment group	Concentration (µg lufenuron/g shrimp)	Concentration (µg lufenuron) in each pellet	Lufenuron concentration (ng/g) in feed
Control	0	Uncoated pellet	0
Dose F	0.0001	0.000025	3.7
Dose E	0.001	0.00025	37
Dose D	0.01	0.0025	370
Dose C	0.1	0.025	3 700
Dose B	1	0.25	37 000
Dose A	10	2.5	370 000

2.1.2 Sampling for toxicokinetic and transcriptomic analyses

Dead shrimps were recorded every day and registered with picture and date, then placed in a freezer (-20ºC). Pictures obtained were used to examine morphological traits, and to compare the dead shrimps with measurements taken at start of the experiment. The study was terminated after 58 days. Shrimps alive at end of the study were euthanized with Tricaine (Finquel), weighted (Kern FWN 300 -1IP) and flash frozen in liquid nitrogen and stored at -80ºC. ImageJ was used for morphological measurements of the shrimps (Schneider et al., 2012). For transcriptomic analyses the hepatopancreas was removed after euthanizing, by using a sterile dissecting kit (forceps and scalpel). The hepatopancreas was then placed in individually labeled Eppendorf tubes, and flash frozen in liquid nitrogen and stored at -80ºC.

The elimination experiment was conducted to examine the half-life of lufenuron as a pilot study for an experiment planned to be carried out later in 2021. The experiment started November 12th, 2020, with 25 rockpool shrimps kept in separate compartments of 142cm^3 (4.5 x 4.5 x 7 cm), in a tank of ~50 L, with a flow rate of 20 L/min. They were given two pellets coated with 10 µg lufenuron/g shrimp the 12th of November. Thereafter they were fed clean pellets two times a week in the period 16th of November to 5th of December. The first sampling was conducted after 8 days. Shrimps from containers A1, A2, A4, A5 and B1 were euthanized. The second sampling was conducted after 15 days, where shrimps from containers B4, B5, C2, C4 and C5 were euthanized.

2.2 Toxicokinetics (Exposure- and elimination experiments)

The levels of lufenuron in rockpool shrimps were determined by an existing method at the IMR using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.2.1 Chemicals and work solutions

Lufenuron of analytic quality (CAS no. 103055-07-8), acetone (HPLC grade) and tetrahydrofuran (HPLC grade) were purchased from Sigma-Aldrich (Germany), acetonitrile (HPLC grade) was purchased from Honeywell (US). Water used was purified by the Milli-Q purification system (Millipore). The work solutions for standard curve and control were made with 10.00 mg ± 0.04 mg lufenuron (Sigma Aldrich) of analytic quality (CAS no. 103055-07-8) diluted with tetrahydrofuran in a 10 mL vial. For the intern standard 10.00 mg ± 0.04 mg lufenuron d-3 (CAS no. unlabelled 103055-07-8) was diluted with tetrahydrofuran in a 10 mL vial.

2.2.2 Preparation for chemical analysis

Shrimps were weighted and homogenized. Blank shrimp matter was weighted (0.25 grams ± 5%) and homogenized and used in blank, control and spiked samples. Three stock solutions were prepared, one for internal standards (IS) and two for lufenuron (control and standard curve). Work solutions were made from the stock solutions according to Table S2. 5 ml acetone was added to each sample before being vortexed for 10 min (2500 rpm). Samples were placed in an ultrasound bath for 15 min, then centrifuged (4000 rpm) for 3 min. The supernatant was transferred to 5 ml tubes and evaporated to dryness at 40°C, using nitrogen. Samples were then reconstituted with 300 µL acetonitrile:water (75:25) before being vortexed, and transferred through a 0.45 M syringe filter to a 2.0 mL HPLC vial with insert. All samples were analyzed the same day as they were prepared.

2.2.3 LC-MS/MS (QQQ) with ESI

The LC-MS/MS instrument separate compounds by liquid chromatography (LC) and detects and measures the concentration of the selected compound with triple quadrupole mass spectrometry (MS/MS). The solvent sample is forced through the column (SB C18, 2,1 x 50 mm, 1,8 µm) containing the stationary phase. The molecules are then separated based on their retention time in the column, on the basis of their affinity to the stationary phase.

Lufenuron was analyzed by using an existing method developed by the IMR (410-Flubenzuroner 190528.m), using LC-MS/MS 6410 (Agilent Technologies, Germany). Data was treated using Masshunter software (Agilent Technologies). The column temperature was set to room temperature. The injection volume was 2 µL. The mobile phases used were acetonitrile (solution A) and purified water (solution B), following the timetable: 0 min, 20% A; 0.2 min, 20% A; 3 min, 98% A; 5 min, 98% A; 5.1 min, 20% A; 7 min, 20% A. All gradient steps were linear, with a flow rate of 0.4 mL/min. The retention time was 5.2 min for both lufenuron and the IS. The analytes were ionized by Agilent Jet Stream negative electrospray (ESI) in the interface and detected using multiple reaction monitoring (MRM) mode. The mass to charge ratio (m/z) for lufenuron was 509.0/325.9 for lufenuron quantifier transition and 509.0/488.9 for lufenuron qualifier transition. For IS the m/z was 512.0/352.9. Other parameter settings were: gas temperature: 300ºC; gas flow: 11 L/min; capillary voltage: 4000 V and charging voltage: 500 V. The detection limit (LOQ) was set to 1.0 ng/g. The method used was linear up to 50 000 ng/g (R^2>0.99), with a relative standard deviation of <20%.

2.3 Transcriptomics

Real-time RT-qPCR was used to study the array of transcriptional responses of a selected set of genes to lufenuron exposure in shrimp hepatopancreas. Reverse transcriptase is responsible for copying DNA to mRNA. The gene copy is the recipe that the ribosome uses to form amino acids chains, that makes up specific proteins, that control cellular processes. By analyzing transcripts, the cellular responses to environmental changes can be studied at the molecular level. Genes often encode multiple proteins, dependent on the genomic recombination, alternative promoters for transcription initiation, alternative transcription termination and splicing of transcripts. Post-translational modifications can further modify proteins after protein biosynthesis. Responses that occur at the mRNA level will not necessarily reflect what happens at protein level. When conducting gene expression analysis, all selected transcripts are quantified, including those not translated into proteins (Campbell et al., 2011; Nørregaard Jensen, 2004). By conducting RT-qPCR analysis on a specific tissue, the function pharmaceuticals have on cellular processes in a specific tissue can be studied. In this study total RNA was isolated from hepatopancreatic samples, converted to cDNA by reverse transcriptase, and amplified with real-time RT-PCR.

2.3.1 RNA purification and quantity

To avoid RNA degradation, tissue samples were immediately flash frozen in liquid nitrogen (-80°C) and kept frozen until processing. It is crucial that the samples do not thaw, as the RNA will rapidly start degrading. Work area and equipment was cleaned using RNase Zap (Sigma). Shrimp RNA was isolated from shrimps hepatopancreatic tissue with EZ1 RNA Tissue Mini kit (Qiagen). Four ceramic beads CK28 (Bertin Technologies) were added to a Precellys tube containing 750μl QIAzol and hepatopancreas tissue. The ceramic beads were used to ensure effective disruption and homogenization of the tissue. Homogenization of the tissue was done at 6000-3x10-010 (Precellys 24 tissue homogenizer, Bertin Instruments), before being incubated for 5 min at room temperature. 150μl chloroform was added to separate RNA from protein and DNA (VWR). Samples were vortexed for 15 sec, then incubated for 3 min in room temperature, before being centrifuged at 4°C, 12000g, for 15 min (Centrifuge 5415 R, Eppendorf). Isolation of the RNA was conducted by using the BioRobot EZ1. The robot uses a magnetic-particle technology that binds RNA to magnetic beads. At the end of the program the RNA is eluted in water, releasing it from the beads (Qiagen, 2018). BioRobot EZ1 was set-up following the EZ1 RNA Handbook (Qiagen, 2012). Samples were stored at -80ºC until future analyzes.

2.3.2 Quality control of RNA

The concentration of RNA was measured by using the NanoDrop spectrophotometer (NanoDrop™ One /One^C Microvolume UV-Vis Spectrophotometer, Thermo Fisher Scientific). Samples were prepared according to the protocol of the RNA 6000 Nano LabChip Kit (Agilent Technologies), then placed into the Agilent 2100 Bioanalyzer (Agilent Technologies). The Bioanalyzer was used to evaluate the amount of degradation of RNA in the samples. Degradation is visualized in an electropherogram and reported with an RNA integrity number (RIN) and a rRNA ratio (28S/18S).

2.3.3 RT reaction

RNA was converted into cDNA with reverse transcriptase. The NanoDrop Spectrophotometer was used to measure the sample concentrations. The samples were then diluted to ensure equal concentration in all samples (50 ng/µl ± 5%). A dilution curve was made by mixing 2µl of all samples and an RNA mix of 90µl with a concentration of 100 ng/µl ± 5% into a 0.5 ml Eppendorf tube. To make a double dilution series, containing 6 tubes (A-F), 40µl of solution is transferred from tube A (100ng/µl) and added to tube B (50 ng/µl). This is done for all 6 tubes, going from tube A with 100 ng/µl to 3.125 ng/µl in tube F (Fig. 18).
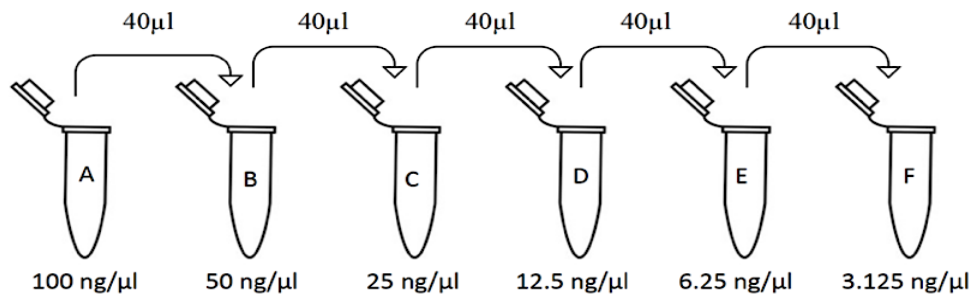
Fig. 18 Preparation of double dilution series of RNA.

RT reagents mix was made according to protocol of TaqMan reverse transcription reagents kit (Applied Biosystems). Samples were added to the 96 well reaction plate in duplicates. RNase free ddH2O (MilliQ Biocel) water was added to the no-control template (NTC) and RNA mix to the non-amplification template (NAC) (Fig. S1). The plate was centrifuged (50 x g) for 1 minute prior to PCR run. CFX384 Real-Time system was used to run the samples. Samples were incubated at 25°C for 10 min, before being transcribed at 48°C for 1 hour. After transcription, the temperature increased to 95°C for 5 min to inactivate the transcribing enzyme.

Table 4 Names, function, accession name, up- and downstream sequence, amplification size, Tm and PCR efficiency listed for all genes in the transcriptomic analyses.

Gene name	Abbrev	Marker for	Accession name	Upstream	Downstream	Ampn. size	Tm	PCR efficiency
Catalase	CAT	Oxidative/cellular stress	Unigene12260_PALA_ELEG	TCATTCGGGACCCAATTCTC	CGTGGTCTCAGGCCTTAAGG	119	78	2.114
GPX1	GPX1	Oxidative/cellular stress	Unigene38253_PALA_ELEG	GGTACGACAACCCGGGACTT	CTGATGACCAAACTGGTTGCA	102	80	2.081
Heat shock protein 70	HSP70	Oxidative/cellular stress	CL2933.Contig1_PALA_ELEG	ATTCCGCGGTACCCTTGAAC	TTTGGGATTCTGGTGGAACCT	108	78	2.087
Caspase 3	CASP3	Oxidative/cellular stress	CL2765.Contig2_PALA_ELEG	CTTTCCGAACCCCGAGCTA	CGCGAGTAACTCGGAGCAA	120	79	2.115
Cytochrome P450 1A	CYP1A	Detoxification	CL3168.Contig1_PALA_ELEG	ATTGGCACAGAACGTCATCCT	TCGAACCGTAGCGGAAGATC	91	81	2.110
Cytochrome P450 3A	CYP3A	Detoxification	Unigene44284_PALA_ELEG	AATTCGTTCAGCCGGAAATG	TGATTTTCACGCGCTTCCA	121	79	2.117
Cytochrome P450 301	CYP301	Detoxification	Unigene26826_PALA_ELEG	GCTCAGCTTCTGGCCAAGAG	AGGGTGACGACGTCCTTCCT	110	83	2.117
P53	P53	DNA damage	Unigene27470_PALA_ELEG	AGTTGATGGTGAAGGTTGTTCGT	GGCTGCTGGACAGGAACTCT	72	78	2.123
Bax	BAX	DNA damage	Unigene13881_PALA_ELEG	GCTGATGCATTTGCTGAAACA	CGGGTTATTCCCCCCATAAA	131	77	2.037
Phosphoacetylglucosamine mutase	PGM3	Immune system	CL2512.Contig2_PALA_ELEG	CAGCAGCAGGTGGAAATGAG	CAGACCAGCCTTTGGCATGT	129	78	2.069
Sulfonylurea receptor / abc transporter	SUR	Cellular functions	Unigene42051_PALA_ELEG	CTGGAAACAGGTGGCTTGGT	GCTCAGGTCCCCAAAGACAGT	107	79	2.030
Chitinase 1	CHIT1	Moulting	CL1959.Contig2_PALA_ELEG	TTGGAGGATGGAACGAAGGA	CAGACCATCGAAGCCATGTG	119	79	2.059
Trypsin	TRY	Moulting	Unigene43058_PALA_ELEG	TGACAGAGGCAGAGTGCAAGA	TCCTTCGCAGGCGTCAATAC	101	82	2.083
Cathepsin	CTSL	Moulting	Unigene33740_PALA_ELEG	GGGTCGTGTTGGGCTTTCT	ATGTTGCCGAAGTCCTTGGA	122	80	2.042

Table 5 Names, function, accession name, up- and downstream sequence, amplification size, Tm and PCR efficiency listed for all genes in the transcriptomic analyses. Ref. genes: uba52, rpl13, tuba, ldh1, actb and eef1a1.

Gene name	Abbrev.	Marker for	Accession name	Upstream	Downstream	Ampn. size	Tm	PCR efficiency
Cullin	CUL1	Moulting	Unigene4488_PALA_ELEG	GCGATCGTGCGAATAATGAA	GATGGTTGGTACCCGAGGTTT	108	77	2.023
GAP65	GAP65	Moulting	Unigene30473_PALA_ELEG	TTTGGGTCTCCGTGTTCCTAA	CAAGAAGCCCCAGTCAACCA	76	79	1.986
Chitin synthase	CHS1	Moulting	CL1391.Contig1_PALA_ELEG	CCTTTCGGCTGGATATGCAT	CCGCAGTTGCCAAAAAGAAT	113	78	2.097
ATP-binding cassette sub-family C member	ABCC8	Moulting	Unigene30559_PALA_ELEG	CGGAGAGAGCCCTTCATCAC	CAGCTTCCCATTGTCCAAGAC	125	82	2.098
Chitinobiase	CTBS	Moulting	Unigene8275_PALA_ELEG	GGGTTTTACGACGCAGACCTT	GAGCGGGATTTTGGGTTAGG	107	80	2.018
Crustacean hyperglycemic hormone	CHH	Moulting	Unigene20965_PALA_ELEG	CAGGGAGAGCTGCTACCAGAA	TTGAACAGCGTTGGCGTACT	94	81	2.115
Chitinase 1	Chitinase 1	Moulting	CL1959.Contig2_PALA_ELEG	TTGGAGGATGGAACGAAGGA	CAGACCATCGAAGCCATGTG	119	79	2.063
Ubiquitin A-52	UBA52	Reference gene	CL882.Contig1_PALA_ELEG	ATCTGGTTCTCCGCCTTCGT	GGATGAAGACGGGCATAGCA	112	80	2.128
Ribosomal protein L13	RPL13	Reference gene	Unigene41309_PALA_ELEG	CAAAAAGCGTTTGGGAAAGG	TTAGTGCGGCGATGGTCAA	108	78	2.062
Tubulin alpha	TUBA	Reference gene	Unigene12506_PALA_ELEG	TGGTGCCCTCAATGTGGATT	TGCTCGTGGTAGGCCTTCTC	120	82	2.08
Lactate dehydrogenase	LD	Reference gene	CL2402.Contig1_PALA_ELEG	GGAGGGAGAATCTCGCCTTT	AGGGTTGGAAACGACAAGCA	115	81	2.060
Beta actin	ACTB	Reference gene	CL156.Contig1_PALA_ELEG	ACCCTAAGGCCAACCGAGAA	GGCCAGAGGCGTACAGAGAA	108	80	2.096
Elongation factor 1-alpha 1	EEF1A1	Reference gene	Unigene38571_PALA_ELEG	TTCACTGCCCAGGTCATCATC	CGGTCGATCTTGCTGTGGAT	125	81	2.043

2.3.4 One step RT-PCR

One-step RT-PCR was used to test the five new PCR assays (the other PCR primes had been tested the same way earlier). A pool of RNA was created and used as input in the one-step RT-PCR reactions. After running gel electrophoresis, a clear band in the gel suggests that the PCR assay works as intended. A one-step RT-PCR kit (Qiagen) was used according to the manufacturer`s protocol. Briefly:

The mix of all RNA samples made for the dilution curve was used for the RT-PCR (Fig. 18). The concentration of the dilution curve mix was 442.5 ng/µl. To ensure 1200 ng RNA per reaction, 2.7 ng/µl of the RNA mix were added to each primer tube (in total for all six primers; 16.3 ng/µl). A master mix was made with the reagents listed in table S1. Five 0.5 ml Eppendorf tubes were added 24.2 µl of the master mix and 0.3 µl of one of the selected primers (*chit*1, *try*, *ctsl*, *pgm3* or *cul*1). Samples were placed into the CFX Maestro™ PCR machine (Bio-Rad) and run through a 7-step program. In the first step samples were held at 50°C for 30 min, making cDNA from RNA. Denaturation was initiated by increasing the temperature to 95°C for 15 min, which separated the dsDNA (Step 2). The first stage of the PCR cycle breaks the dsDNA into ssDNA at 94°C (45 seconds). The temperature was lowered to 60°C, making it possible for primers to bind to the strand, initiating the annealing stage at (45 seconds). The temperature is adjusted to 72°C for 1 minute, initiating the extending stage where bases are added to the strand one at a time (Step 3-5). The cycle is repeated for a total of 35 times, resulting in an exponential growth of new cDNA strands. The run ends by keeping the samples at 72°C for 10 min (CFX Maestro™, Bio-Rad).

A standard 2% agarose gel was made to check the quality of the selected primers. The gel was made with 2 g agarose and 100 mL TAE buffer. The solution was microwaved for 1-3 min to dissolve the agarose powder. After cooling it was poured into a gel tray with a well comb and left for approximately 30 min to solidify. A loading buffer was added to each of the primer samples. The loading buffer helps to visualize how far the amplicons have migrated in the gel, as well as increasing the density of each sample. The gel was inserted into the gel box and the well comb was removed. 1x TAE-buffer was added to the gel box until the gel was covered. GelPilot 50bp ladder (100) was pipetted into the first well, then each of the PCR products in the following wells. The gel was run on 100V for 1.5 hour, then placed into a chemiluminescence imaging system (ChemiDoc™ XRS, Bio-Rad) to visualize the amplicon fragment bands (Fig. 24).

2.3.5 Real-time PCR

Differences in gene expression in the hepatopancreatic tissue of shrimp treated with distinct concentrations of lufenuron was compared by conducting a real-time PCR. The template for this reaction is cDNA from the RT reaction (2.3.3). Samples were prepared for real-time PCR by mixing Probes master (Roche), SYBR Green Master (Roche) and primers (Table 4 and 5) separately, then transfer the samples into 8-strips tubes (110 µl/tube), following the protocol established at the IMR. Primers are used to initiate the synthesis of new cDNA copies by using DNA polymerase and a template of single-stranded cDNA. The process of copying cDNA is repeated multiple times, resulting in an exponential increase of cDNA copies. The SYBR Green Master display strong fluorescence when bound to dsDNA, and weak fluorescence in the presence of ssDNA. This helps in quantifying the amount of dsDNA that is present in each cycle.

The tubes were placed into the pipetting robot (Biomek 4000), transferring each sample into one of the wells in the 384 well plate. An optical adhesive cover was placed over the plate with applicator, before centrifuging it (1500 x g) for 2 min. Samples were incubated at 95°C for 5 min, then amplified through three stages of 95°C, 60°C and 72°C, through 45 cycles of 10 sec. The last step being one cycle of melting point analysis, with three steps of 95°C (5sek), 65°C (1 min) and 97°C. A real-time PCR curve was generated with the CFX Maestro™ (Bio-Rad) showing PCR efficiency of all the runs in the reaction. Each cycle gives a Ct value, associated with cDNA levels above the quantification threshold. An Ct value below 29 is regarded as good and reflects high amounts of target sequences. If the Ct value is higher, lower amounts of target sequences are present. Ct values below 38 indicates very low amounts of target sequences. If the reaction fail to exceed the quantification threshold, no expression is detected (Sherina et al., 2020). In this assignment these non-detects were given the value of 40. GeNorm was used to determine the most stable reference genes from a set of candidates (*uba52, rpl13, tuba, ldh1, actb* and *ef1a*) (GeNorm 3.2). The reference genes *uba52* and *rpl13,* which had a M-value of 0.3030, were the most stably expressed genes. Target genes were therefore normalized with a normalization factor based on *uba52* and *rpl13*. *Ld* and *tuba* were included as target genes in the downstream analysis, since they encode proteins that sometimes respond to chemical treatment.

2.4 Statistics

Statistical analyses of mortality between groups were conducted in Rstudio 3.5.2 (RStudio, Boston Massachusetts USA). Normality of distribution was tested by using Shapiro Wilks test of normality. The data were linearized by log transforming ($\log_{10}$) lufenuron concentrations. Lethal concentration (LC_x values) with 95% confidence interval was calculated from accumulated concentrations of lufenuron in the shrimps. With the R package "ecotox" a GLM with a binominal error structure and probit links was made (Finney & Stevens, 1948; Hlina et al., 2011). The data was plotted using the R package "ggplot2" (Wilkinson, 2011). To assess the risk of lufenuron to rockpool shrimp, the toxicological dose descriptor LC_{50} was used. The LC_{50} method is used to find the concentration of a chemical that kills 50% of the test animals during the observation time (Sparling, 2017). Welch`s t-test was conducted to compare the mortality in treatment groups fed lufenuron to the control group. For the elimination study an estimated half-life ($t_{1/2}ß$) was calculated from a linear regression analysis by logarithmically (ln) transforming the drug concentrations versus time and dividing it by the slope of the regression line ($t_{1/2}=\ln2/k$).

Statistical analyses of the transcriptional data were conducted with GraphPad Prism 9.1.0 (GraphPad software Inc., San Diego California USA). One-way analysis of variance (ANOVA) with post-hoc test was conducted on each gene to see if there was any statistical difference between the means of expression for each treatment group. Correlation analyses of genes was conducted using Spearman`s rank correlation to check for correlation between genes. Spearman`s rank correlation is a non-parametric version of Pearson`s correlation, that measures the strength and direction of monotonic association between two variables. A monotonic relationship means that as the value of one variable increase, the value of the other either increase or decrease. The method ranges the correlation with the correlation coefficient ranging from low (-1.0) to high (+1.0). R-value >0.5 is regarded as having strong positive correlation, while <-0.5 is regarded as a strong negative correlation. A principal component analysis (PCA) was conducted to analyze the relationship between the groups.

31

3. Results

3.1 Exposure experiment

3.1.1 Growth

Measurements of weight and length were recorded at the start and end of the experiment. The weights used were controlled in advance. CL and TL were not registered at end of the experiment for the highest doses (10 and 1.0 µg/g shrimp), as there were no individuals alive at that point. The pictures taken of the dead shrimps in these treatment groups did not include any length reference, making it impossible to obtain accurate measurements. A t-test was conducted for each treatment group where start weight and length were compared to the same measurements when the experiment was terminated. A significant difference was registered in mean weight, CL and TL in the shrimps from all of the treatment groups (t-test, $p<0.05$). In general, the mean weight of the shrimps was significantly higher at start of the experiment than at the end. CL and TL increased for all treatment groups, even if the weight decreased (Table 6).

The number of successful moults had a dose-dependent variation between the treatment groups, with the exception of the shrimps fed 0.0001 µg/g shrimp. The treatment group receiving 0.0001 µg/g shrimp had surprisingly few moults early in the experiment but had an increase in number of moults starting at day 20-25 (Fig. 19). For both treatment doses 10 and 1.0 µg/g shrimp, mortality was less than 1. No moults were registered 5 and 15 days after the experiment started, respectively. Shrimps fed 1.0 µg/g shrimp moulted until day 20, then the frequency gradually decreased. For shrimp fed lower doses of lufenuron, the number of moults increased exponentially until the experiment was terminated.

Only a few morphological changes were registered, mainly in the group receiving 0.01µg/g shrimp. Changes registered were bent antennae in one individual and another with damaged tail fan and deformed stiff hind legs (Fig. S3 and S4). None of the shrimps that died during the exposure experiment showed signs of morphological changes as black spots or speckled eyes. Of the shrimps that died during the exposure experiment, some looked like they were in the process of moulting, with a soft exoskeleton with flesh like colour. This applied to one shrimp exposed to 1.0 µg/g shrimp, one shrimp exposed to 0.01 µg/g shrimp, as well as 3 shrimps exposed to 0.1 µg/g shrimp (Fig. S5).

Table 6 Growth in weight, carapace length (CL) and total length (TL) in shrimps from all treatment groups. Measurements of CL and TL were not made for treatment groups receiving 1.0 and 10 µg/g shrimp as they all were dead when the experiment was terminated.

Treatment group (µg/g shrimp)	Mean weight (start) in grams	Mean weight (end) in grams	CL (start) in cm	CL (end) in cm	TL (start) in cm	TL (end) in cm
Control	1.00 ± 0.33	0.41 ± 0.15	0.69 ± 0.11	0.80 ± 0.12	3.24 ± 0.45	3.41 ± 0.41
0.0001	0.76 ± 0.50	0.45 ± 0.19	0.69 ± 0.11	0.85 ± 0.13	3.34 ± 0.33	3.74 ± 0.43
0.001	0.56 ± 0.38	0.46 ± 0.23	0.65 ± 0.14	0.85 ± 0.19	3.15 ± 0.37	3.77 ± 0.52
0.01	1.11 ± 0.48	0.44 ± 0.17	0.67 ± 0.15	0.80 ± 0.16	3.25 ± 0.57	3.73 ± 0.46
0.1	1.00 ± 0.27	0.31 ± 0.15	0.65 ± 0.11	0.73 ± 0.20	3.11 ± 0.39	3.17 ± 0.50
1.0	0.87 ± 0.60	0.32 ± 0.16	0.71 ± 0.07	NA	3.42 ± 0.36	NA
10	0.65 ± 0.51	0.35 ± 0.14	0.75 ± 0.19	NA	3.54 ± 0.85	NA
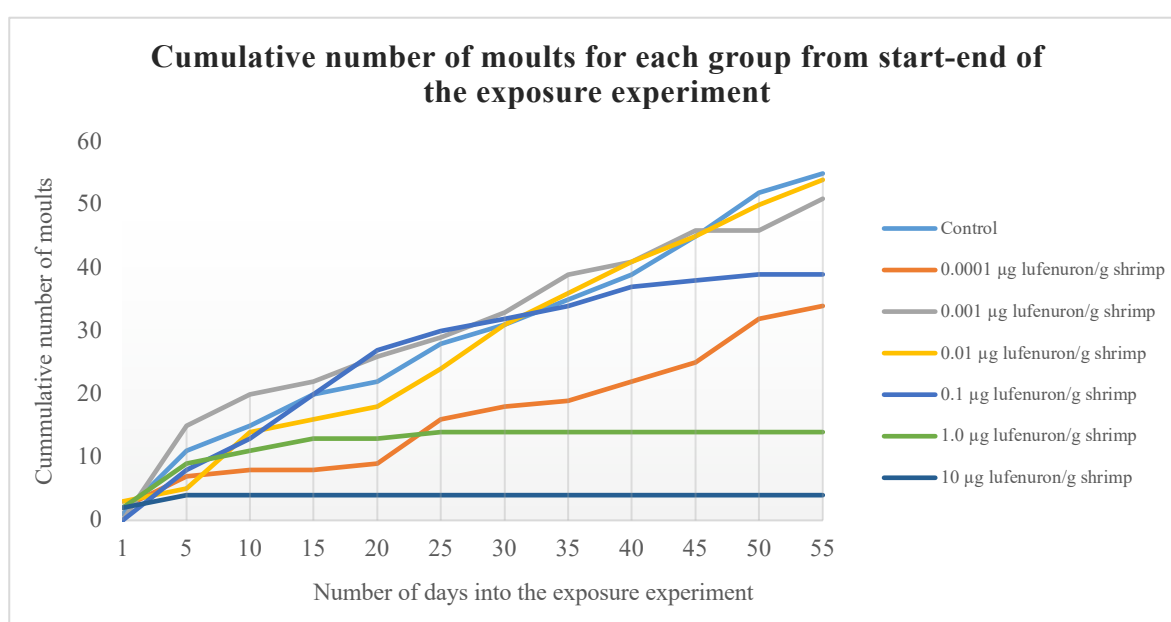
Fig. 19 The cumulative number of successful moults for shrimps in each of the treatment groups from the exposure experiment, in the period 01.09.2020-28.10.2020.

3.1.2 Mortality

A dose-dependent increase in mortality was observed (Fig. 20). At the end of the exposure experiment, shrimps exposed to less than 0.01 µg/g shrimp all had shrimps that were alive. The number of shrimps that had no registered moults between being fed pellets coated with lufenuron and until they died, was higher for shrimps exposed to 10 and 1.0 µg/g shrimp compared to shrimps exposed to lower doses (Table 7).

Total mortality in the control group was 5% (N=2) after 58 days, and mortality was observed rather late in the experiment i.e. after day 40 (Table 7, Fig. 20). Total mortality was 100% for both treatment doses 10 and 1.0 µg/g shrimp and 92.5% for shrimps exposed to 0.1 µg/g shrimp. The shrimps in these groups were all dead after 38 and 44 days, after treatment started,

respectively. For the dose of 10 µg/g shrimp the mortality started almost immediately after onset of the exposure and increased considerably until about day 30. A similar pattern in mortality was observed in shrimps exposed to 1.0 and 0.1 µg/g shrimp, but with a delay in when the mortality commenced, at day 5 and 15, respectively. Also, the mortality occurred later in time, at around day 35 in shrimps exposed to 1.0 µg/g shrimp and at day 40 in shrimps exposed to 0.1 µg/g shrimp. Total mortality in the lower doses 0.01, 0.001 and 0.0001 µg/g shrimp, was 15-25%. The mortality also started later in the experiment, after day 20 in groups receiving 0.01 µg/g shrimp and after day 30 in shrimps exposed to 0.001 and 0.0001 µg/g shrimp.

Animals in the control and groups receiving doses 0.1-0.0001 µg/g shrimp successfully went through ~2 moults, while shrimps receiving pellets with 10 and 1.0 µg/g shrimp went through <1 moult (Table 7). After day 15, moulting ceased for shrimps exposed to 1.0 and 10 µg/g shrimp and mortality increased rapidly. For shrimps receiving 0.1 µg/g shrimp mortality started at day 15, when the number of moults reached ~20. At day 20 the number of moults in this group flattened out.

Table 7 Summary with the doses of lufenuron in the pellets that was given to shrimps in each of the treatment groups compared to the measured concentration from LC-MS/MS analysis, mortality and number of moults at end of experiment.

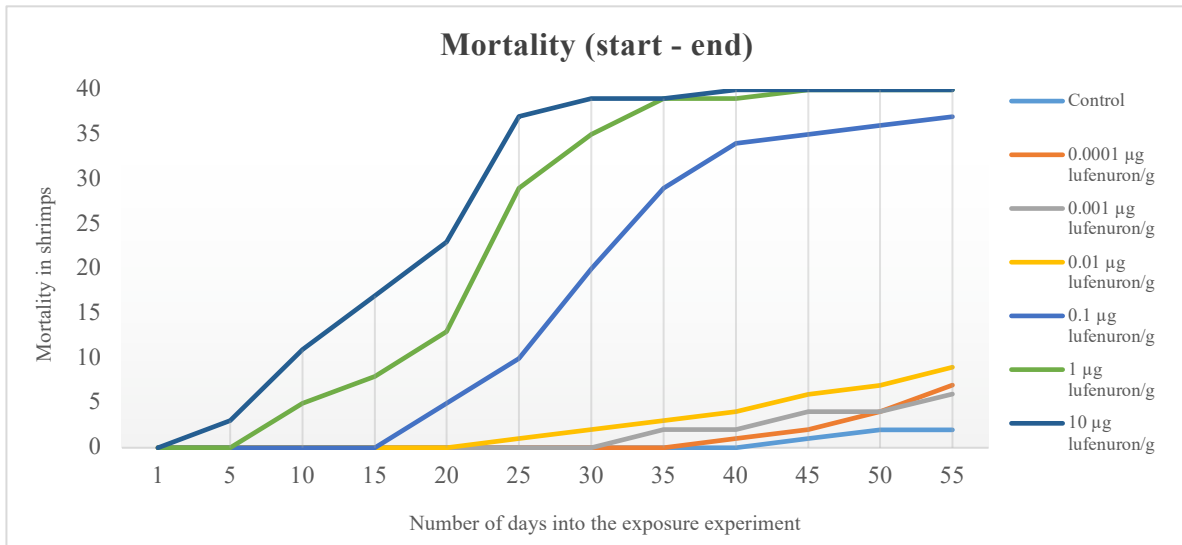| Treatment group (µg/g shrimp)	Measured concentration (ng/g)	Mortality (%)	Mean number of moults	No registered moults (%)
0	<1.0	5	1.8	0
0.0001	0.4 ± 1.6*	17.5	1.5	2.5
0.001	1.7 ± 1.3*	15	1.9	0
0.01	29 ± 17	25	2.0	5
0.1	90 ± 39	92.5	1.9	2.5
1.0	652 ± 501	100	0.8	32.5
10	14 042 ± 12 626	100	0.8	30

* Values less than LOQ was set to 0

Fig. 20 For the control, total mortality was 5% after 58 days of exposure, and mortality was observed rather late in the experiment (day 45). For the lower doses (0.01, 0.001 and 0.0001 µg/g shrimp, mortality was 15-25%, and observed at day 20-35. Mortality was 92.5% for shrimps exposed to 0.1 µg/g shrimp, with mortality commencing at day 15. For the highest doses 1.0 and 10 µg/g shrimp, mortality was 100%, and started almost immediately after onset of the exposure with an exponential increase until day 30-35. All shrimps in the two highest treatment groups were dead after 38 and 44 days, respectively.

There was a dose-dependent decrease in the total number of pellets fed to the different treatment groups (Fig. 21). Shrimps exposed to 0.001-0.0001 µg/g shrimp and the control group received an average of >30 pellets before dying. Shrimps exposed to 0.01-0.1 µg/g shrimp recieved an average of >20 pellets before dying. Shrimps in the highest doses 1.0 and 10 µg/g shrimp, received an average of 17 and 13 pellets, respectively, before dying.
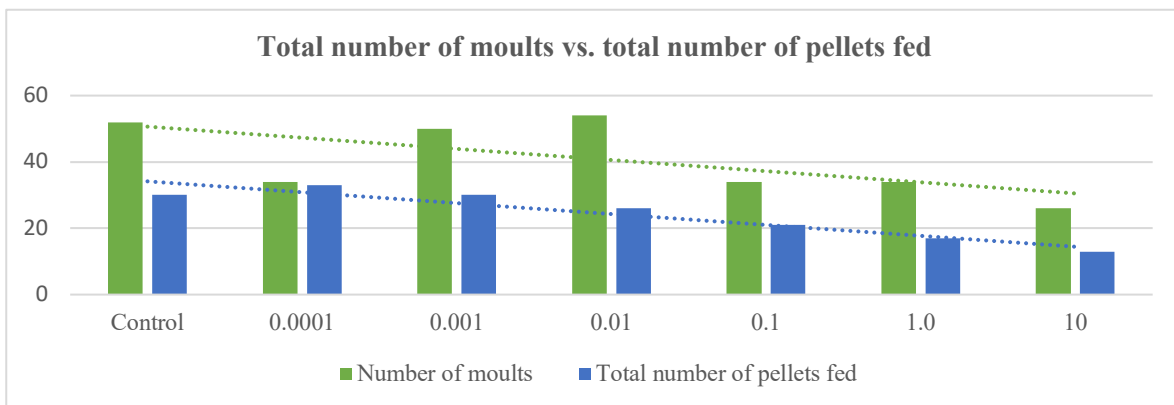

Fig. 21 Relationship between number of moults compared to the total number of pellets fed to each treatment group.

Statistical analyses conducted with Shapiro Wilks test, showed that the data from the exposure experiment was not normally distributed. A QQ-plot was created to check the distribution of the data (Fig. S6). The plot showed that the measured concentrations for shrimps exposed to 10 µg/g shrimp were considerably higher than in the other groups, causing deviation from a normal distribution. The data of accumulated lufenuron concentrations were normalized by conducting a log transformation ($\log_{10}$). The binomial general linear model with probit links confirmed an exponential increase in mortality compared to lufenuron concentrations in the treatment groups (Fig. 22) (Finney, 1971). LC_x values with a 95% confidence interval were calculated from the GLM. The estimated LC_{50} value for accumulated lufenuron concentrations in the shrimps was found to be 21.6 ng/g (Table 8). The dose-response curve of concentrations given illustrates the response shrimps in all of the treatment groups had to lufenuron exposure (Fig. 23).

Table 8 LCx values calculated for the measured concentrations found in shrimps exposed to lufenuron doses from 0-10 µg/g shrimp.

LC_x	Threshold (ng /g)
LC_5	1.3
LC_{20}	5.1
LC_{40}	14
LC_{50}	21
LC_{70}	53
LC_{80}	91
LC_{90}	193
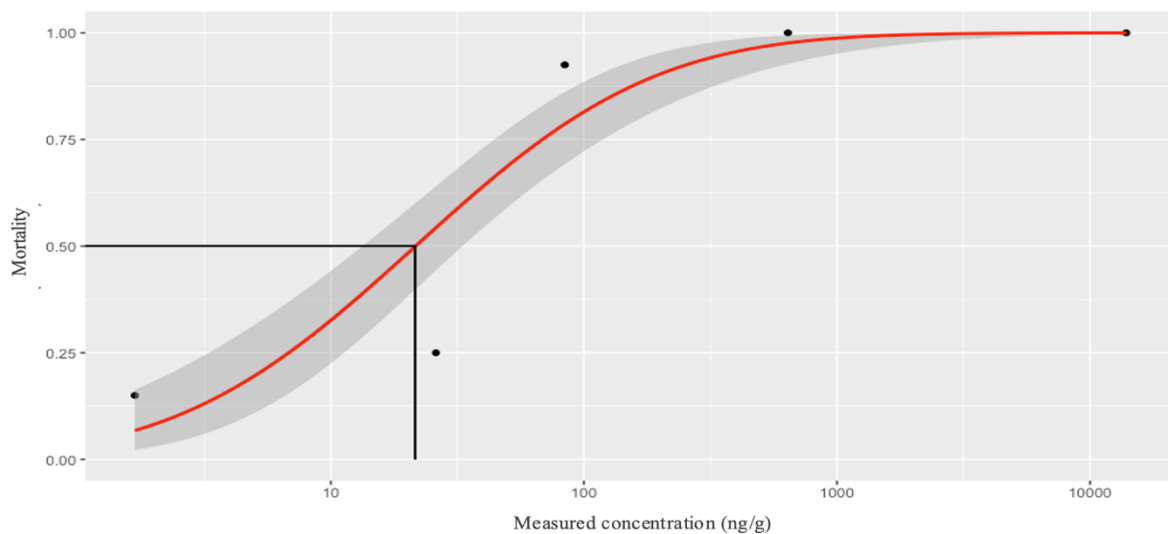
Fig. 22 Mortality in shrimps fed specific concentrations of lufenuron for 58 days, compared to the concentration measured by LC-MS/MS (ng/g). Red line was calculated with a binominal log-probit GLM, representing the best fit model for the data. Treatment groups exposed to 0.001-10 µg/g shrimp (left to right) is visualised in the plot.

Dose-response curve

Mortality

Lufenuron concentration (μg/g shrimp)
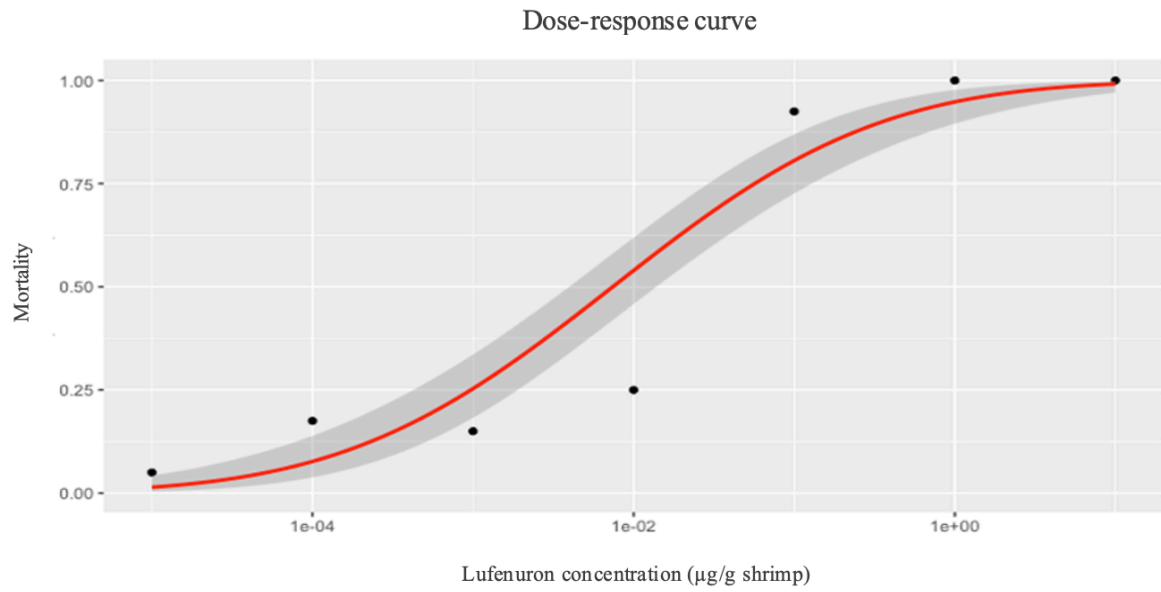
Fig. 23 The dose-response curve show the mean fraction in mortality of exposed shrimps compared to the lufenuron concentration they were exposed to (μg/g shrimp). The line was calculated with a binominal log-probit GLM and represent the best fit model for the data.

3.2 Elimination study

An elimination study was conducted as a pilot study for an upcoming experiment. Two pellets of the highest dose of lufenuron (10 µg/g shrimp) used in the main exposure experiment were fed to 25 shrimps the 12th of November. Thereafter, only pellets without lufenuron were given to monitor the clearance rate. At the first sampling, 8 days after lufenuron coated pellets had been received, the measured lufenuron concentration varied a lot between individuals (Table 9). The concentration of lufenuron measured in the shrimps gradually decreased during the period 12th November – 5th December (Fig. 24). An estimated half-life for lufenuron in shrimps was calculated to be 4.7 days based on the data collected from this pilot study (R^2=0.3414) (Fig. 25).

Table 9 Overview of sampling date, the identification mark of the shrimps sampled and their mean lufenuron concentration.

Days after treatment started	Shrimps sampled	Mean measured lufenuron conc. (ng/g)
8	A1, A2, A4, A5, B1	2651 ± 1799
15	B4, B5, C2, C4, C5	1725 ± 917
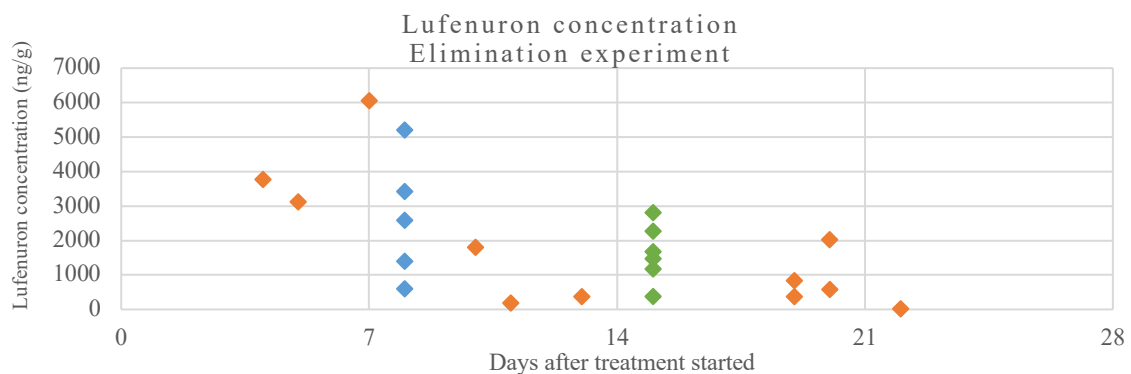
Fig. 24 Concentration of lufenuron in shrimps were analyzed by LC-MS/MS. The graph show shrimps that died (orange) and the shrimps that were sampled (19.11.2020, blue and 26.11.2020, green).
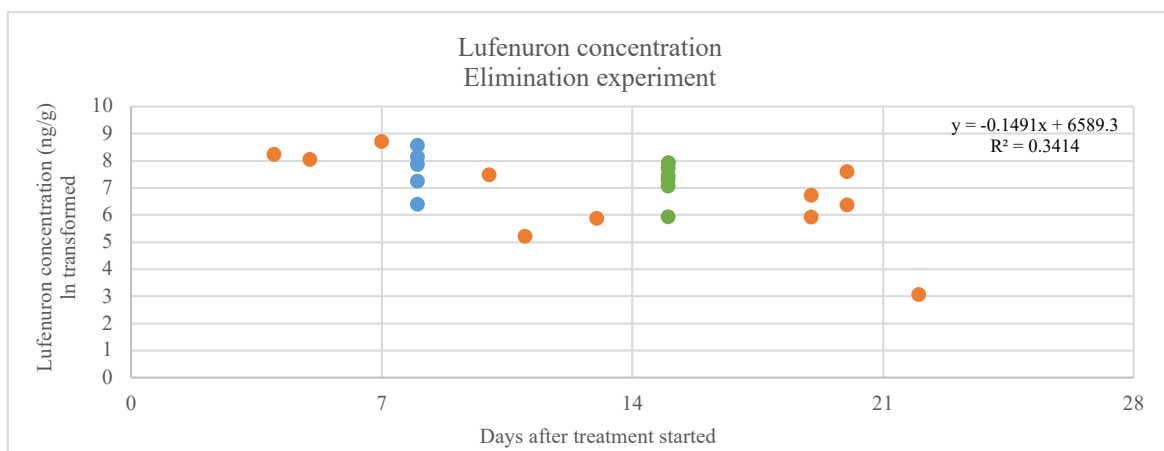
Fig. 25 Measured concentrations of lufenuron were ln transformed to get an estimated the time required to reduce the accumulated lufenuron concentrations by half ($t_{1/2}$). By using the equation $t_{1/2}$=ln2/k an estimated half-life of 4.7 days was calculated (R^2 = 0.3414).

3.3 Transcriptomics

For RNA, a 269/280 nm ratio of ~2.0 is accepted as "pure", in the sense that it´s not contaminated by reagent residues from the RNA extraction. All RNA samples used in this study had 260/280nm values were ~2.0 ± 0.02.

The RNA integrity of the samples was analyzed with an electropherogram, calculating the rRNA ratio value. The kit used in the Bioanalyzer is developed for mammalian RNA. This resulted in rRNA profiles that appeared to be degraded. Similar observations of "hidden breaks" in the 28s rRNA has been found in previous studies on crustaceans (Olsvik et al., 2015). The 28s rRNA had fragmented during heat-denaturing and migrated with the 18S rRNA, giving it a "degraded appearance". This "hidden break is common for arthropods. The integrity of the RNA (RIN) in the samples of this study was therefore not available for detection (McCarthy et al., 2015) (Fig. S2).

Most of the PCR assays used were quality-controlled in a previous study. A quality control was conducted for the remaining 5 PCR assays by running a one-step RT-PCR and agarose (2.5%) gel-electrophoresis (Fig. 26). All primers tested had a PCR product size of 100-150 bp. No double bonds were detected among the primers, suggesting that the PRC assays are working well.
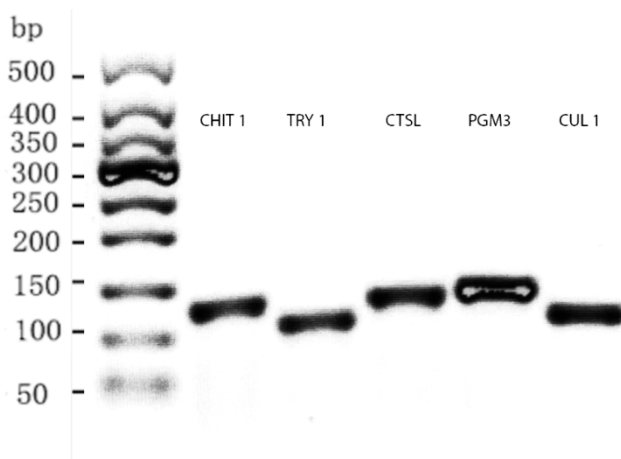

Fig. 26 Gel picture of quality check for a selected set of primers (CHIT1, TRY1, CTSL, PGM3 and CUL1).

3.3.1 One-way analysis of variance of gene expression between treatment groups

A one-way ANOVA was conducted for the transcriptional data ($\alpha = 0.05$). No significant difference was registered for any of the genes (Table 10). Dunettes post hoc test was performed for the gene *Cyp1a* which had a p-value <0.1. With a 95% confidence interval there was registered no significant differences in *Cyp1a* transcription between any of the groups compared to the control.

Table 10 Results of the one-way ANOVA conducted on the genes used for the transcriptomic analyses in this paper.

Gene	P-value	F-value
Hsp70	0.5617	0.6946
P53	0.7188	0.4502
Ctbs	0.622	0.5958
Chh	0.2100	1.589
Cyp3a	0.2160	1.564
Gpx1	0.5726	0.6763
Cat	0.4640	0.8744
Chs1	0.2246	1.530
Casp3	0.9504	0.1155
Bax	0.1058	2.202
Abcc8	0.3587	1.110
Cyp1a	0.0725	2.543
Cyp301	0.8130	0.3172
Pgm3	0.9351	0.1404
Ctsl	0.5313	0.7476
Try	0.5624	0.6336
Chit1	0.1325	0.2000
SUR	0.2507	1.431
Chitiase1	0.2216	1.541
Cul1	0.4815	0.8399
Gap65	0.2400	1.470
Tuba	0.9363	0.1384
Ld	0.7139	0.4573

3.3.2 Correlation of genes, moults and measured lufenuron concentrations

Spearman correlation matrix was derived by pairwise comparing genes to each other. None of the genes analyzed had a perfect monotonic relationship (-1.0 or +1.0). Three transcripts, *chh, cat* and *bax,* showed positive correlation to measured concentration ($p < 0.05$) (Fig. 27, Table S5). The chitin synthase encoding gene *Chs1* showed positive correlated to the number of moults ($p < 0.05$). *Sur* and *Abcc8* showed the strongest positive correlation of all tested variables ($p < 0.05$). This was expected as *Abcc8* encodes the membrane transport protein SUR, which is responsible for regulating the activity of the potassium ATP channel.
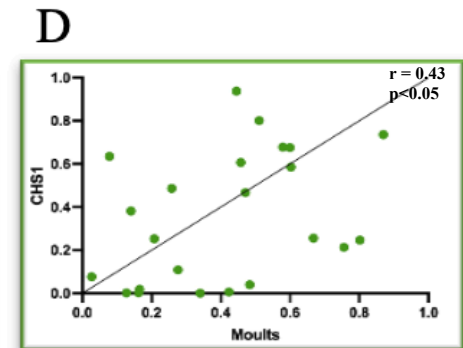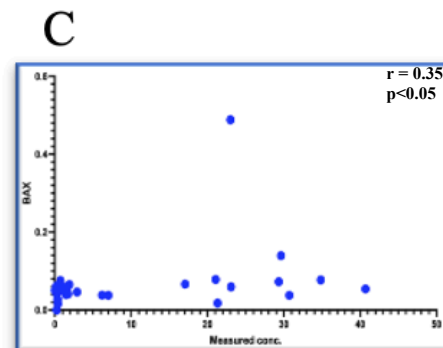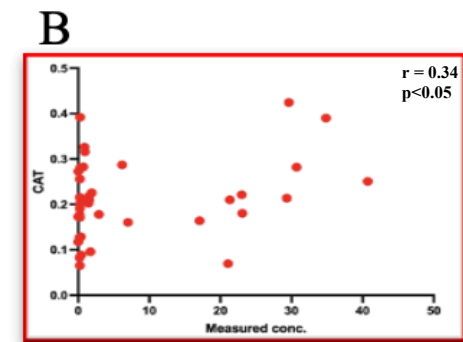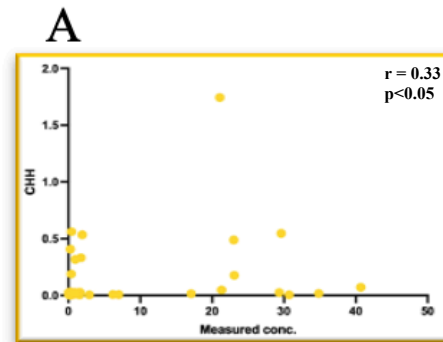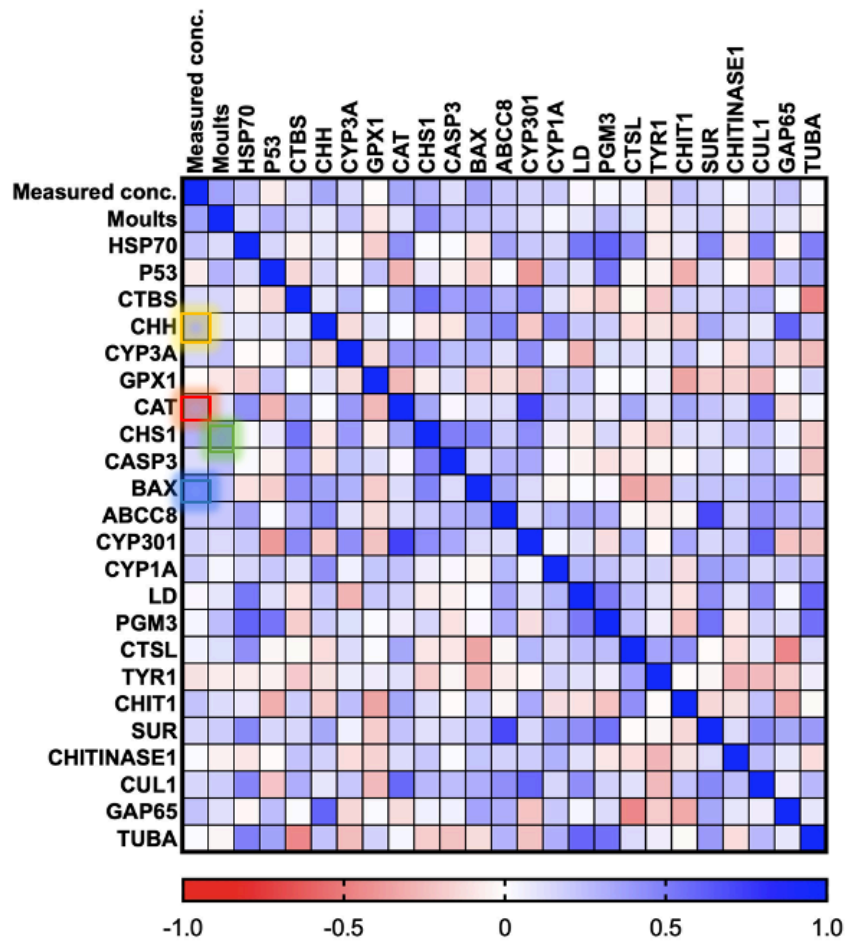
Fig. 27 Heatmap of correlation (Spearman r) comparing transcripts related to detoxification, moulting and stress against each other and the measured concentration in shrimps from treatment groups D, E and F (plus control). Scatterplots on the right show examples of transcripts with significant correlation to measured concentrations of lufenuron (chh (A), cat (B), bax (C), and chs1 (D) which had significant correlation to the number of moults.

3.3.3 Dose-response effect of lufenuron

The dose-response effect of lufenuron exposure for each treatment group is visualized in Fig. 28. As explained above, none of the treatments induced any significant effect on the studied genes.
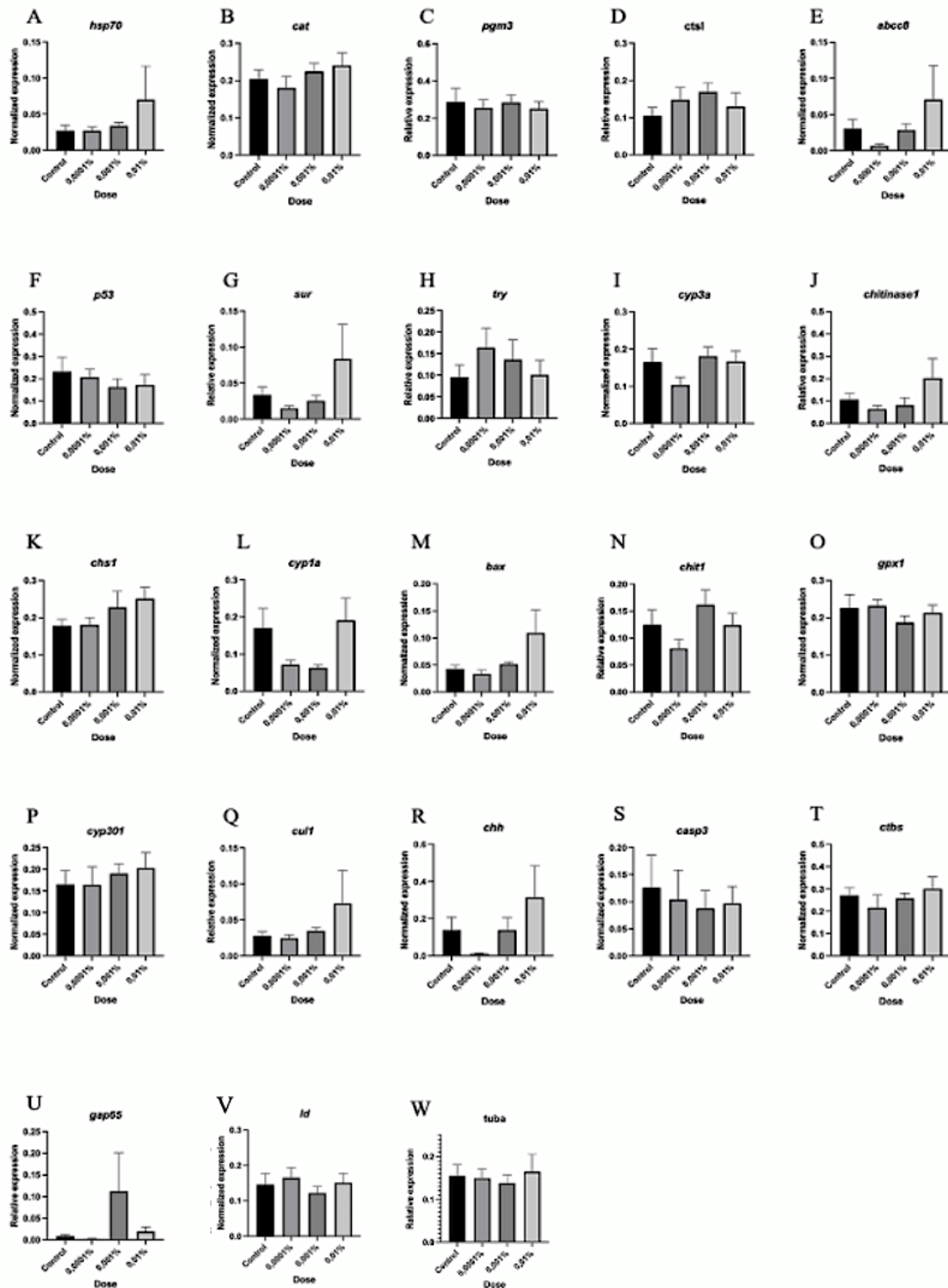
Fig. 28 Dose-response effect of exposure to lufenuron, in genes associated with moulting in shrimp hepatopancreas. Hsp70 (A), cat (B), pgm3 (C), ctsl (D), abcc8 (E), p53 (F), sur (G), try (H), cyp3a (I), chitinase1 (J), chs1 (K), cyp1a (L), bax (M), chit1 (N), gpx1 (O), cyp301 (P), cul1 (Q), chh (R), casp3 (S), ctbs (T), gap65 (U), ld (V) and tuba (W).

3.3.4 Principle component analysis of gene expression

PCA was used to look for relationships between shrimps in each treatment group (Fig. 29 and 30) (ClustVis BETA) (Metsalu & Vilo, 2015). The data were log transformed before PCA analysis (log_{10}). Shrimps given treatment doses from 0.0001-0.01 µg/g shrimp could not be differentiated based on gene expression without (Fig.29) or with accumulated lufenuron levels (Fig. 30) (Lenz et al., 2016).
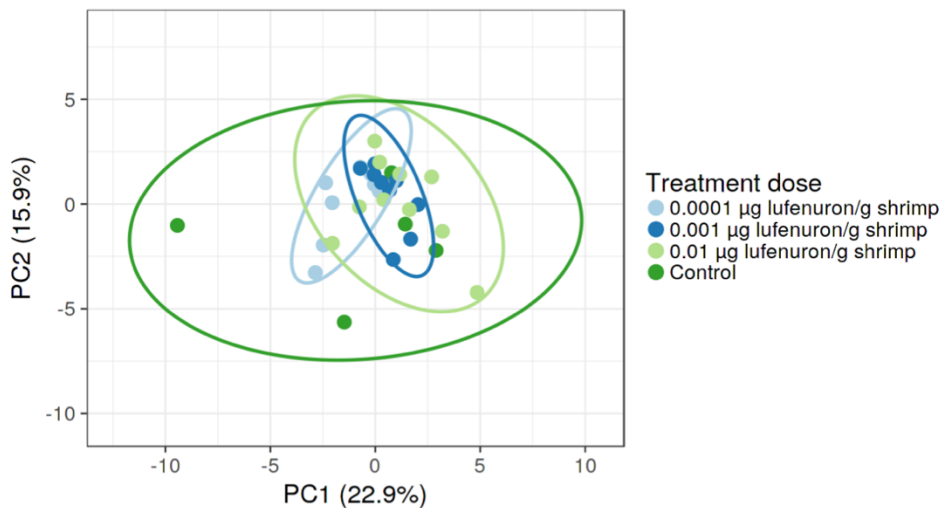
Fig. 29 X and Y axis show PC1 and PC2 that explain 15.9% and 22.9% of the total variance, respectively. With a significant level of 0.05% a new observation from the same group will fall inside the ellipse. Control (N=6), shrimps receiving 0.0001µg lufenuron/g (N=10), 0.001µg lufenuron/g (N=9) and 0.01 µg lufenuron/g (N=10).
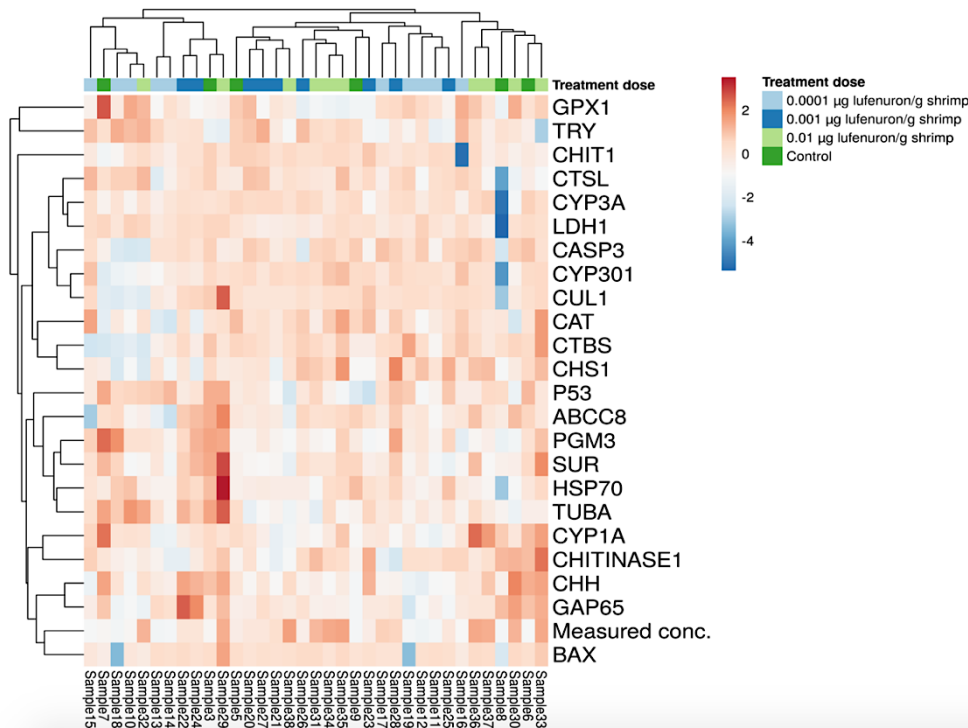
Fig. 30 Data is clustered using correlation distance and average linkage between genes tested and shrimps from control and treatment groups 0.0001, 0.001 and 0.01.

4. Discussion

The shrimps collected at Kumløya, Austevoll, were acclimated for 14 days prior to lufenuron exposure to reduce the risk of stress and mortality when adapting to the new environment. During the acclimation period water temperature and flow rate was monitored to ensure a relatively stable environment. Mortality in the control group was 5%. Similar numbers have been reported in exposure studies where wild shrimps were collected from their natural habitat and used in laboratory exposure experiments, and is thought to be a result of natural causes (Samuelsen et al., 2020). Mortality impacted by lufenuron was shown to occur in shrimps exposed to 0.01 µg/g shrimp, and higher (p<0.05). Shrimps receiving doses of 0.1 µg/g shrimp and lower, successfully went through approximately twice as many moults as the shrimps receiving higher doses. Mortality in the shrimps increased exponentially with lufenuron concentration. In the groups receiving doses higher than 0.1µg/g shrimp, mortality was high (92.5-100%). Moulting ceased at day 15 for the shrimps receiving the highest doses of lufenuron and at day 20 mortality increased exponentially (1.0 and 10 µg/g shrimp). The number of moults in shrimps fed 0.1 µg/g shrimp, went down at day 20, followed by an increase in mortality at day 25. In shrimps receiving 0.01 µg/g shrimp, the number of moults increased at the same time as mortality was induced, at day 20. These results indicate sub-lethal effects on the moulting process in the shrimps receiving lufenuron doses higher than 0.01 µg/g shrimp.

The standard deviation of accumulated concentrations was high in all the treatment groups. A reason for this could be that some of the shrimps had higher appetite or lived long enough to be fed more pellets in general, and therefore had a greater intake of lufenuron. The shrimps that died early in the experiment had less of an opportunity to eat pellets. For example, a few of the shrimps exposed to 1.0 µg/g shrimp only ate 8 pellets before dying, while the majority ate 18-20 pellets. The same was true for shrimps fed 10 µg/g shrimp, but few shrimps in this treatment group ate more than 18 pellets. An alternative reason could be individual variation in the detoxification process.

In a similar study with pink shrimp (*Pandalus montagui)* fed 0.01 and 0.1 µg teflubenzuron/g shrimp for 46 days, accumulated concentrations of 5.8 ng/g ± 2.0 and 71 ng/g ± 36 were reported, respectively (Olsvik et al., 2019). In this study the same dose of lufenuron was fed to rockpool shrimps. The accumulated concentrations of lufenuron in rockpool shrimps exposed to 0.01 µg/g shrimp was 29 ± 17 ng/g, much higher than the accumulated concentrations of

teflubenzuron in pink shrimps. When comparing the accumulated concentrations from groups receiving 0.1 μg/g shrimp, to the accumulated concentrations of teflubenzuron in pink shrimps receiving the same dose, the results were more in line with the results found in this study (90 ± 39 ng/g). Shrimps exposed to 10 μg/g shrimp quickly reached a lethal concentration, which later also was achieved in shrimps exposed to 1.0 and 0.1 μg/g shrimp. These results indicate that lufenuron accumulate relatively easily in the shrimps.

The LC_{50} value indicates the concentration of a substance that kills 50% of the test subjects after a certain exposure time. In this study it was estimated to be 21.6 ng lufenuron/g shrimp after 58 days exposure. Lethal concentrations of chemicals are normally calculated using the measured concentrations in water or air after 96 hours of exposure. Due to the design of our study, LC-values were calculated based on accumulated concentrations in the shrimp body. Many exposure studies with flubenzurones and crustaceans so far have been using waterborne exposure. For the opossum shrimp (*Mysidopsis bahia)* juveniles exposed to 1.6-2.7 μg/L diflubenzuron in seawater for 96 hours, resulted in an estimated LC_{50} value of 2.1 μg/L was obtained. In the same study a life-cycle exposure experiment was conducted, in which the opossum shrimps were exposed to 0.84-1.8 μg diflubenzuron/L seawater for 21 days, a LC_{50} of 1.24 μg/L was found (Nimmo et al., 1979). Comparison of LC_{50} values from studies with different form of exposure is complicated due to several factors coming into play. One being that flubenzurones diluted in water will be degraded by hydrolysis and photodegradation, with the dilution rate being influenced by pH and temperature (Marsella et al., 2000). In rockpool shrimp exposed to 0.0025-1.88 μg teflubenzuron/g shrimp twice a week for 66 days, an LC_{50} of 18.4 ng/g shrimp was reported. This value is close to the estimated LC_{50} calculated in this study, indicating that the lethal concentration of lufenuron and teflubenzuron are relatively similar in rockpool shrimps (Samuelsen et al., 2020).

In a study conducted by Poley et al. (2018) egg strings of salmon lice were exposed to 500 ppb of lufenuron in a 500 mL glass beaker with sea water, for 24-72 hours. Both the control- and lufenuron exposed grouped hatched successfully. Lice exposed to lufenuron remained as naupulii, while the control group completed two moults and reached copepodid stage (Poley et al., 2018). In this study, shrimps exposed to 0.01 μg/g shrimp, had no observable effect on their ability to moult. Due to differences introduced due to exposure via water and feed, comparing the studies is not easy. The observed differences in sensitivity to lufenuron in salmon lice and

rockpool shrimps could either be caused by the treatment method or variation in resilience to the drug.

The accumulated levels of lufenuron in shrimps from the elimination study showed great variation at first sampling, which could be linked to the amount of feed that was eaten by each of the shrimps. An estimated half-life of lufenuron was found to be 4.7 days. The half-life estimate was based on relatively few shrimps, and few sampling dates. A follow-up study is planned to improve the half-life calculation. The results from this elimination experiment will be helpful in designing and planning the main elimination study. Based on this study, it is recommended that the treatment dose in the follow-up study should be reduced to 0.1 ug lufenuron/g shrimp, making it possible to study the shrimps for a longer period. The shrimps from the elimination study also had an acclimation period of several months. To reduce the likelihood of shrimp dying because of age rather than lufenuron exposure, the acclimation period should be set to no longer than 2-3 weeks. In a similar study on juvenile European lobsters, the half-life of teflubenzuron, was estimated to be 3.4 days (Samuelsen et al., 2014). Another study, conducted with northern shrimp (*Pandalus borealis*) exposed to 0.1-0.3 g diflubenzuron medicated feed for 2 weeks, reported a half-life of approximately 5 days (Bechmann et al., 2017). Taken together, the half-life of flubenzurones appears to be relatively similar in shrimps.

The hepatopancreas of rockpool shrimps was used to look for transcriptional changes in rockpool shrimps exposed to different doses of lufenuron. Being the main metabolic organ of shrimps, it has a key role in detoxification, growth, and general health. When the experiment was terminated, there were no surviving individuals from treatment groups receiving 10 and 1.0 µg/g shrimp, and too few remaining individuals in the group receiving 0.1 µg/g shrimp (N=3). For this reason, hepatopancreas was only extracted from shrimps exposed to 0.01 µg/g shrimp and lower. The measured lufenuron concentration in the body of the shrimps (w/o hepatopancreas), was less than LOQ (<1.0) for control and shrimps exposed to 0.0001 µg/g shrimp. For shrimps (w/o hepatopancreas) exposed to 0.001 µg/g shrimp, the mean measured concentration was 2.1 ± 2.0 ng/g and for treatment doses of 0.001µg/g shrimp the mean concentration was 27 ± 7 ng/g. These concentrations did not vary a lot from the concentrations found when analysing the chemical content in whole-body shrimps, confirming that low amounts of lufenuron were accumulated in the hepatopancreatic tissue.

Transcriptional data from RT-qPCR was obtained from 23 target genes. The PCR efficiencies were relatively high (>2.0) for some of the assays, indicating PCR inhibitors in the RNA samples. This could be caused by excessive amounts of DNA/RNA or carry-over material in the sample, often transferred in the sample processing of the RNA isolation step (e.g. ethanol). Inhibitors can also originate from organic compounds such as bile, urea, salts, various proteins, and so on (Rådström et al., 2004; Schrader et al., 2012). Polysaccharides can also cause PCR inhibition, especially in samples containing shellfish (Atmar et al., 1995). Our samples were not checked for PCR inhibitors; however, it is unlikely that PCR inhibitors affected the transcriptional data in this study.

Previous studies where lufenuron was added to the water as a source to salmon lice, showed that it clearly was impacting their ability to moult (Poley et al., 2018). This was also indicated in this study, with the number of moults being significantly correlated with the accumulated level of lufenuron in the shrimps. A study where lobsters were fed 5% and 20% of the standard teflubenzuron concentrations used in salmon medication, showed the substance to have an impact on oxidative- and cellular stress, drug detoxification, and mechanisms associated with moulting (Olsvik et al., 2015). Very few studies have so far been conducted on the effect of lufenuron in crustaceans. Spearman`s rank correlation showed positive correlations between the measured lufenuron concentrations in the shrimps and transcriptional levels of *chh, cat* and *bax* (p<0.05). Two of these transcripts are associated with stress responses (*chh and cat)*. The crustacean hyperglycemic hormone (CHH) is responsible for elevating the glucose level of arthropods under stressful conditions (Chung et al., 2010). Catalase (CAT) catalyses the decomposition of hydrogen peroxide, thereby protecting the cell from reactive oxygen species (ROS) generated oxidative stress (Sepasi Tehrani & Moosavi-Movahedi, 2018). This result indicates that lufenuron induced a stress response in the exposed shrimps. *Bax* encodes a protein that control apoptosis, which several pharmaceuticals are known to indirectly activate. Positive correlation between *bax* and accumulated levels of lufenuron concentrations indicates a dose-dependent effect on apoptosis (Liu et al., 2016). *Chs1,* encoding the protein chitin synthase 1, showed a trend toward a significant correlation to the accumulated levels of lufenuron (p<0.08), and a significant correlation with the number of moults (p<0.007). The CHS1 protein is involved in creating the new exoskeleton through the chitin synthesis, possibly explaining the findings (Rocha et al., 2012). The expression of moult-associated genes was low in the hepatopancreas and probably dependents on what stage in the moulting process the shrimps were in when sampled. Benzoylureas generally cause morphological abnormalities, due to their

CSI function. There was a strong positive correlation between the transcriptional levels of *sur* and *abcc8* (r=0.71, p<0.05). *Abcc8* encode SUR, a membrane transport protein which is the target of the sulfonylurea class of drugs and responsible for regulating the activity of the potassium ATP channel. Benzoylureas have been shown to inhibit membrane-bound SUR receptors, thereby assisting in inhibition of the chitin synthesis in insects (Abo-Elghar et al., 2004; Gangishetti et al., 2009).

As mentioned, there was no surviving shrimps exposed to 1.0 and 10 µg/g shrimp, and to few shrimps exposed to 0.1 µg/g shrimp survived (N=3). The lack of significant gene expression responses in surviving shrimp could be because of the low doses they were given. The shrimps in this study were chronically exposed to lufenuron for almost 2 months, giving the shrimps time to adjust their metabolism to the stressors. A shorter experimental period might have resulted in more variation in gene expression. None of the selected genes showed any significant differences in gene expression. Expression of the genes *hsp70, bax, sur, cul1 and chitinase1* were all higher in shrimps from the high-dose treatment group (0.01 µg/g shrimp) compared to the lower doses (although not significantly). *Hsp70* encodes a protein linked to protein folding and oxidative stress*, bax* is associated with DNA damage and *sur* is responsible for regulating the potassium ATP channel. Two genes, linked to moulting mechanisms (*cul1, chitinase1*) had higher expression in the highest treatment dose 0.01 µg/g shrimp, although not significantly. The protein encoded by *Cul1* plays an important role in regulating the cell cycle as well as the stress response in the cell. More specifically CUL1 is engaged in degradation of proteins (Sweeney et al., 2020). Chitinase decomposes chitin to make the body able to reabsorb it and to synthesise a new cuticle. The mean number of moults in shrimps from both low dose groups and control was ~1. Shrimps exposed to 0.01 µg/g shrimp showed the highest mean number of moults, with close to 2 moults per shrimp (Table 11). This can explain the higher expression of *chitinase1* in shrimps exposed to 0.01 µg/g shrimp, considering those shrimps had to decompose chitin from almost twice as many moults as shrimps from the other treatment groups.

Table 11 Measured lufenuron concentration and mean number of moults in shrimps (without hepatopancreas) used for transcriptomic analyses.

Treatment group (µg/g shrimp)	Lufenuron concentration	Mean number of moults
Control	<1.0	0.9
0.0001	<1.0	1.1
0.001	2.1 ± 2.0	1.1
0.01	27 ± 7	1.8

For in-feed treatments administered at sea, pellets will become available to non-target crustaceans. Approximately 5-15% of orally administered flubenzurones such as teflubenzuron are left uneaten by the farmed salmon (Langford et al., 2014). Lufenuron however administered to salmon in the freshwater phase, eliminating the likelihood of uneaten pellets being accessible to non-target species. There exists no research conducted on the concentration of lufenuron in the wastewater from freshwater facilities or on salmon excretion after treatment in the marine environment. After being treated, the salmon will have elevated concentrations of lufenuron in their skin and muscle, protecting them from salmon lice infestations after being transferred to sea. Salmon is held for at least 7 days after treatment. Salmon given the recommended therapeutic dose, were found to have lufenuron concentrations of ~7000 ng/g in skin at day 8 after treatment (Kristine Brokke, unpublished data). At this stage salmon are cleared to be transferred to sea-based facilities. After being transferred, salmon that die and fall into the collection net at the bottom of the pen, are easily accessible to scavengers, as shrimps. Employees at aquaculture facilities empty the collection nets at least once daily and have strict procedures when it comes to disposal of dead salmon (Matloven, 2003; Nærings- og fiskeridepartamentet, 2008). If the collection nets only are emptied once daily short time after the treated salmon has been transferred to sea, it gives non-target species enough time to feast on treated salmon that has died and fallen into the collection net. Presenting the possibility of them accumulating concentrations that could result in either acute or chronic consequences. In our study, mortality was high (92.5%) in shrimps fed doses of half the concentration measured in salmon 8th days after treatment (Kristine Brokke, unpublished data). After ~60 days the salmon treated with lufenuron still had high concentrations of lufenuron in the skin. This concentration is similar to the lufenuron concentration in the feed of groups receiving 0.1 µg/g shrimp (3 700 ng/g), which had a mortality of 92.5%.

Concentrations measured in the sediment and water surrounding farms treated with di- and teflubenzuron have shown values higher than the measured LC_{50} of this paper (21.6 ng/g) (Langford et al., 2014). Shrimps that were analysed in treated areas did not have as high concentrations accumulated (<0.5-11 ng teflubenzuron/g w.w). In contrast to crabs, which had considerably higher concentrations accumulated (<1-537.9 ng teflubenzuron/g w.w) (Langford et al., 2014). Very different concentrations were reported in Deepwater shrimp (*Pandalus borealis)* caught less than 300m from salmon farms treated with teflubenzuron. These shrimps had median concentrations below the level of detection, the highest concentration was 200.4 ng/g (Samuelsen et al., 2015). If similar concentrations of lufenuron was to be found in the sediment, it would be fatal to rockpool shrimps. A possible solution to protect non-target species from consuming lufenuron, would be to treat the salmon in the freshwater phase, then keep the treated salmon in land-based aquaculture facilities. Then the main concerns then would be disposal of wastewater and dead treated salmon.

4.1 Methodological challenges

This study had a classic dose-response set-up with x10 increase in dose. For future studies the doses could be limited to a narrower range. The range of treatment doses resulted in a gap between mortality in shrimps fed 0.01 (25%) and 0.1 µg/g shrimp (92.5%), that had an impact on the results, making the statistics on mortality less accurate. The statistical model created in R had to use adjustments to fit the line to the data in the best possible way. If this study was to be conducted again, a treatment group with shrimps exposed to ~0.05 µg/g shrimp could have increased the accuracy of the results. The number of pellets eaten by each shrimp was not recorded daily. For future studies, uneaten pellets should be registered and removed from the tanks when adding new pellets.

At sampling it was discovered that two shrimp tanks exposed to 0.001 and 1.0 µg/g shrimp had a different bottom than the others, possibility affecting how easily pellets fall out of the containers. A Welch t-test was conducted to test whether this was the case. The mean lufenuron concentration was 1.2 ± 0.9 ng/g shrimp held in the tank with bottom B and 2.2 ± 2.3 ng/g in shrimps held in the tank with bottom A (p>0.05), in the 0.001µg/g shrimp treatment. In contrast, shrimps in the two tanks given pellets containing 1.0 µg/g shrimp had a mean lufenuron concentration of 794 ± 447 ng/g shrimp for bottom B and 510 ± 522 ng/g shrimp for bottom A (p<0.05). However, there were no statistically significant differences in accumulated levels of lufenuron in shrimps kept in tanks with different-sized holes in the treatment group with

surviving shrimps at the end of the experiment. The holes in tanks with bottom B was measured to be 1 mm wide x 9 mm long. Considering the pellets given had a diameter of 2 mm, the chances of a high amount of pellets falling through is regarded as low. Which type of bottom the tanks had thus does not appear to have affected the result.

Most of the genes selected for transcriptomic analyses were lowly expressed in the hepatopancreas. An explanation could be that the accumulated lufenuron concentrations in the shrimps were too low and the differences in expression were insufficient. Shrimps sampled for transcriptomics in this study is assumed to have accumulated too low levels of lufenuron to impact the expression of the genes analysed. In studies performed on salmon lice, genes related to the chitin synthesis pathway have been shown to be less affected by lufenuron (Poley et al., 2018). For a future study, it would be interesting to study the expression levels of these genes in surviving shrimps after shorter exposure. For this to be possible the experiment should have been terminated at an earlier stage with more surviving shrimps in the high-dose groups.

5. Conclusion

This study shows that lufenuron is lethal to rockpool shrimps exposed to doses equal to or higher than 0.01μg/g shrimp. There was a great variation in accumulated concentrations in shrimps treated with lufenuron. This could have been caused by individual differences in age, size and/or moulting stage. The 58-day LC_{50} concentration was 21.6 ng/g shrimp. From the elimination study a half-life of 4.7 days was estimated. Only non-significant differences were observed for the selected transcriptional markers between the treatment groups, which probably is due to the low concentrations of lufenuron accumulated in the shrimps after 58 days. The shrimps would also have had time to adapt to the low concentrations, reducing the differences in expression between the lower treatment groups.

6. References

Abo-Elghar, G. E., Fujiyoshi, P., & Matsumura, F. (2004). Significance of the sulfonylurea receptor (SUR) as the target of diflubenzuron in chitin synthesis inhibition in *Drosophila melanogaster* and *Blattella germanica*. *Insect Biochem Mol Biol, 34*(8), 743-752. doi:10.1016/j.ibmb.2004.03.009

Andersen, S. O. (2010). Insect cuticular sclerotization: A review. *Insect Biochemistry and insect molecular biology, 40*(3), 166-178. doi:10.1016/j.ibmb.2009.10.007

Andrews, S. C., & Dillaman, R. M. (1993). Ultrastructure of the gill epithelia in the crayfish *Procambarus clarkii* at different stages of the molt cycle. *Journal of Crustacean Biology, 13*(1), 77. doi:10.2307/1549123

Atmar, R. L., Neill, F. H., Romalde, J. L., Le Guyader, F., Woodley, C. M., Metcalf, T. G., & Estes, M. K. (1995). Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. *Applied and environmental microbiology, 61*(8), 3014-3018. doi:10.1128/AEM.61.8.3014-3018.1995

Baillie, A. C. (1985). *The Biochemical Mode of Action of Incecticides*. In Keyserling, Jäger, & Szczepanski (Eds.), *Approaches to New Leads for Insecticides*. doi:https://doi.org/10.1007/978-3-642-70821-3_2

Baringou, S., Rouault, J.-D., Koken, M., Hardivillier, Y., Hurtado, L., & Leignel, V. (2016). Diversity of cytosolic HSP70 Heat Shock Protein from decapods and their phylogenetic placement within Arthropoda. *Gene, 591*(1), 97-107. doi:10.1016/j.gene.2016.06.061

Bechmann, R. K., Lyng, E., Berry, M., Kringstad, A., & Westerlund, S. (2017). Exposing Northern shrimp (*Pandalus borealis*) to fish feed containing the antiparasitic drug diflubenzuron caused high mortality during molting. *Journal of toxicology and environmental health, Part A, 80*(16-18), 941-953. doi:10.1080/15287394.2017.1352213

Bechmann, R. K., Lyng, E., Westerlund, S., Bamber, S., Berry, M., Arnberg, M., Kringstad, A., Calosi, P., & Seear, P. J. (2018). Early life stages of Northern shrimp (*Pandalus borealis*) are sensitive to fish feed containing the anti-parasitic drug diflubenzuron. *Aquatic Toxicology, 198*, 82-91. doi:10.1016/j.aquatox.2018.02.021

Berglund, A. (1980). Niche differentiation between two littoral prawns in Gullmar Fjord, Sweden: *Palaemon adspersus* and *P. squilla*. *Ecography (Copenhagen), 3*(2), 111-115. doi:10.1111/j.1600-0587.1980.tb00716.x

Berglund, A., & Bengtsson, J. (1981). Biotic and Abiotic Factors Determining the Distribution of Two Prawn Species: *Palaemon adspersus* and *P. squilla*. *Oecologia, 49*(3), 300-304. doi:10.1007/BF00347589

Blaalid, G.-E. (2009). «Det er Oddekalv som bryter avtalen». *www.kyst.no*. Retrieved from https://www.kyst.no/article/det-er-oddekalv-som-bryter-avtalen/

Brignac-Huber, Park, J. W., Reed, J. R., & Backes, W. L. (2016). Cytochrome P450 organization and function are modulated by endoplasmic reticulum phospholipid heterogeneity. *Drug metabolism and disposition: the biological fate of chemicals, 44(12)*, 1859–1866. doi:https://doi.org/10.1124/dmd.115.068981

Buchanan, K. L. (2000). Stress and the evolution of condition-dependent signals. *Trends in ecology and evolution, 15*(4), 156-160. doi:10.1016/S0169-5347(99)01812-1

Buchholz, F. (1989). Moult cycle and seasonal activities of chitinolytic enzymes in the integument and digestive tract of the Antarctic krill, *Euphausia superba*. *Polar biology, 9*(5), 311-317. doi:10.1007/BF00287429

Burridge, L. E., Lyons, M. C., Wong, D. K. H., MacKeigan, K., & VanGeest, J. L. (2014). The acute lethality of three anti-sea lice formulations: AlphaMax®, Salmosan®, and Interox®Paramove™50 to lobster and shrimp. *Aquaculture, 420-421*, 180-186. doi:10.1016/j.aquaculture.2013.10.041

Burridge, L. E., & Van Geest, J. L. (2014). A review of potential environmental risks associated with the use of pesticides to treat Atlantic salmon against infestations of sea lice in Canada. In Oceans. (Ed.), *Canadian Science Advisory Secretariat research document 1919-50442014/002, National Capital Region*. Canada.

Campbell, N., Reece, J., Urry, L., Cain, M., Wasserman, S., Minorsky, P., & Jackson, R. (2011). Biology (9th, international ed.). In: San Francisco, CA: Benjamin Cummings/Prentice Hall.

Cerbule, K., & Godfroid, J. (2020). Salmon Louse (*Lepeophtheirus salmonis* (Krøyer)) Control Methods and Efficacy in Atlantic Salmon (*Salmo salar* (Linnaeus)) Aquaculture: A literature review. *Fishes, 5*(2), 11-10. doi:10.3390/fishes5020011

Chen, K., Li, E., Li, T., Xu, C., Wang, X., Lin, H., Qin, J. G., & Chen, L. (2015). Transcriptome and molecular pathway analysis of the hepatopancreas in the Pacific white shrimp *Litopenaeus vannamei* under chronic low-salinity stress. *PLoS ONE, 10*(7), e0131503-e0131503. doi:10.1371/journal.pone.0131503

Chung, J. S., Zmora, N., Katayama, H., & Tsutsui, N. (2010). Crustacean hyperglycemic hormone (CHH) neuropeptidesfamily: Functions, titer, and binding to target tissues. *General and comparative endocrinology, 166*(3), 447-454. doi:10.1016/j.ygcen.2009.12.011

Clancy, S., & Brown, W. (2008). *Translation: DNA to mRNA to Protein*. In Vol. 1. Moss (Ed.), *Nature Education* (pp. 101). Retrieved from https://www.nature.com/scitable/topicpage/translation-dna-to-mrna-to-protein-393/

Commission implementing regulation. (2014). *Amending Regulation (EU) No 37/2010, as regards the substance 'lufenuron'* (No 967/2014). Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32014R0967&qid=1621765589269&from=EN.

Dam, E., Rewitz, K. F., Styrishave, B., & Andersen, O. (2008). Cytochrome P450 expression is moult stage specific and regulated by ecdysteroids and xenobiotics in the crab *Carcinus maenas*. *Biochemical and biophysical research communications, 377*(4), 1135-1140. doi:10.1016/j.bbrc.2008.10.125

Deli, T., Pfaller, M., & Schubart, C. D. (2018). Phylogeography of the littoral prawn species *Palaemon elegans* (Crustacea: Caridea: *Palaemonidae*) across the Mediterranean Sea unveils disparate patterns of population genetic structure and demographic history in the two sympatric genetic types II and III. *Marine biodiversity, 48*(4), 1979-2001. doi:10.1007/s12526-017-0711-6

Denholm, I., Devine, G. J., Horsberg, T. E., Sevatdal, S., Fallang, A., Nolan, D. V., & Powell, R. (2002). Analysis and management of resistance to chemotherapeutants in salmon lice, *Lepeophtheirus salmonis* (Copepoda: Caligidae). *Pest Management Science, 58*(6), 528-536. doi:10.1002/ps.482

Eichner, C., Hamre, L. A., & Nilsen, F. (2014). Instar growth and molt increments in *Lepeophtheirus salmonis* (Copepoda: Caligidae) chalimus larvae. *Parasitology international, 64*(1), 86-96. doi:10.1016/j.parint.2014.10.006

El-Saber Batiha, G., Alqahtani, A., Ilesanmi, O. B., Saati, A. A., El-Mleeh, A., Hetta, H. F., & Magdy Beshbishy, A. (2020). Avermectin derivatives, pharmacokinetics, therapeutic and toxic dosages, mechanism of action, and their biological effects. *Pharmaceuticals (Basel, Switzerland), 13*(8), 196. doi:10.3390/ph13080196

Espedal, P. G., Glover, K. A., Horsberg, T. E., & Nilsen, F. (2013). Emamectin benzoate resistance and fitness in laboratory reared salmon lice (*Lepeophtheirus salmonis*). *Aquaculture, 416-417*, 111-118. doi:10.1016/j.aquaculture.2013.09.001

Fallang, A., Ramsay, J. M., Sevatdal, S., Burka, J. F., Jewess, P., Hammell, K. L., & Horsberg, T. E. (2004). Evidence for occurrence of an organophosphate-resistant type of acetylcholinesterase in strains of sea lice (*Lepeophtheirus salmonis* Krøyer). *Pest Management Science, 60*(12), 1163-1170. doi:10.1002/ps.932

Fanjul-Moles, M. L. (2006). Biochemical and functional aspects of crustacean hyperglycemic hormone in decapod crustaceans: Review and update. *Comparative biochemistry and physiology Part C Toxicology and pharmacology, 142*(3), 390-400. doi:10.1016/j.cbpc.2005.11.021

Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual review of physiology, 61*(1), 243-282. doi:10.1146/annurev.physiol.61.1.243

Finney, D. J. (1971). *Probit analysis* (3rd ed.). Cambridge University Press: Cambridge.

Finney, D. J., & Stevens, W. L. (1948). A table for the calculation of working probits and weights in probit analysis. *Biometrika, 35*(12), 191-201.

Gangishetti, U., Breitenbach, S., Zander, M., Saheb, S. K., Müller, U., Schwarz, H., & Moussian, B. (2009). Effects of benzoylphenylurea on chitin synthesis and orientation in the cuticle of the Drosophila larva. *European journal of cell biology, 88*(3), 167-180. doi:10.1016/j.ejcb.2008.09.002

Grave, K., & Horsberg, T. E. (2014). 2014: Forbruket av lakselusmidler er høyt og øker fortsatt. Retrieved 08.10.2020, from Folkehelseinstituttet https://www.fhi.no/hn/legemiddelbruk/fisk/forbruket-av-lakselusmidler-er-hoyt/

Grave, K., Horsberg, T. E., Lunestad, B. T., & Litleskare, I. (2004). Consumption of drugs for sea lice infestations in Norwegian fish farms: Methods for assessment of treatment patterns and treatment rate. *Diseases of aquatic organisms, 60*(2), 123-131. doi:10.3354/dao060123

Grefsrud, E. S., Karlsen, Ø., Kvamme, B. O., Glover, K., Husa, V., Hansen, P. K., Grøsvik, B. E., Samuelsen, O., Sandlund, N., Stien, L. H., & Svåsand, T. (2021). Risikorapport norsk fiskeoppdrett 2021 - kunnskapsstatus. *Rapport fra havforskningen 2021-7*.

Guenherich, P. F. (2012). *Cytochrome P450*. In Metabolism of drugs and other xenobiotics, Anzenbacher & Zanger (Eds.), (pp. 27-66). doi:https://doi.org/10.1002/9783527630905.ch2

Hannisdal, R., Nøstbakken, O. J., Hove, H., Madsen, L., Horsberg, T. E., & Lunestad, B. T. (2020). Anti-sea lice agents in Norwegian aquaculture; surveillance, treatment trends and possible implications for food safety. *Aquaculture, 521*, 735044. doi:10.1016/j.aquaculture.2020.735044

Harardottir, H. M., Male, R., Nilsen, F., & Dalvin, S. (2019). Effects of chitin synthesis inhibitor treatment on *Lepeophtheirus salmonis* (Copepoda, Caligidae) larvae. *PLoS ONE, 14*(9), e0222520. doi:10.1371/journal.pone.0222520

Hartnoll, R. G. (2001). Growth in Crustacea – twenty years on. *Hydrobiologia, 449*(1), 111-122. doi:10.1023/A:1017597104367

Helgesen, K. O., Horsberg, T. E., & Tarpai, A. (2019). The surveillance programme for resistance to chemotherapeutants in salmon lice (*Lepeophtheirus salmonis*) in Norway 2018.

Helgesen, K. O., Romstad, H., Aaen, S. M., & Horsberg, T. E. (2015). First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway. *Aquaculture reports, 1*(C), 37-42. doi:10.1016/j.aqrep.2015.01.001

Hersoug, B., Mikkelsen, E., & Karlsen, K. M. (2019). "Great expectations" – Allocating licenses with special requirements in Norwegian salmon farming. *Marine policy, 100*, 152-162. doi:10.1016/j.marpol.2018.11.019

Hlina, B. L., Birceanu, O., Robinson, C. S., Dhiyebi, H., & Wilkie, M. P. (2011). Seasonal variation in the sensitivity of invasive sea lampreys to the lampricide TFM: Importance of energy reserves and temperature. *North American Journal of Fisheries Management.*

Hobbs, H. H. (2001). *Decapoda*. In Ecology and classification of north american freshwater invertebrates, Thorp, Covich, & Thorpe (Eds.), (pp. 955-1001). doi:10.1016/B978-012690647-9/50024-7

Igboeli, O. O., Burka, J. F., & Fast, M. D. (2014). *Lepeophtheirus salmonis*: a persisting challenge for salmon aquaculture. *Animal Frontiers, 4*(1), 22-32. doi:10.2527/af.2014-0004

Imsland, A. K., Reynolds, P., Eliassen, G., Hangstad, T. A., Foss, A., Vikingstad, E., & Elvegård, T. A. (2014). The use of lumpfish (*Cyclopterus lumpus L.*) to control sea lice (*Lepeophtheirus salmonis* Krøyer) infestations in intensively farmed Atlantic salmon (*Salmo salar L.*). *Aquaculture, 424-425*, 18-23. doi:10.1016/j.aquaculture.2013.12.033

Iwanaga, S., & Lee, B.-L. (2005). Recent advances in the innate immunity of invertebrate animals. *BMB reports, 38*(2), 128-150. doi:10.5483/BMBRep.2005.38.2.128

James, M. O., & Boyle, S. M. (1998). Cytochromes P450 in crustacea. *Comparative biochemistry and physiology Part C: Pharmacology, Toxicology and Endocrinology, 121*(1-3), 157-172. doi:10.1016/S0742-8413(98)10036-1

Jensen, P. M. (2013). Lusebehandling koster flere kroner per kilo. *www.kyst.no*. Retrieved from https://www.kyst.no/article/lusebehandling-koster-flere-kroner-per-kilo/

João, C. F. C., Echeverria, C., Velhinho, A., Silva, J. C., Godinho, M. H., & Borges, J. P. (2017). Bio-inspired production of chitosan/chitin films from liquid crystalline suspensions. *Carbohydrate polymers, 155*, 372-381. doi:10.1016/j.carbpol.2016.08.039

Junprung, W., Supungul, P., & Tassanakajon, A. (2021). Structure, gene expression, and putative functions of crustacean heat shock proteins in innate immunity. *Developmental and comparative immunology, 115*, 103875-103875. doi:10.1016/j.dci.2020.103875

Lachaise, F., Le Roux, A., Hunert, M., & Lafont, R. (1993). The molting gland of crustaceans: Localization, activity, and endocrine control (A review). *Journal of Crustacean Biology, 13*(2), 198-234. doi:10.1163/193724093X00020

Lam, C. T., Rosanowski, S. M., Walker, M., & St-Hilaire, S. (2020). Sea lice exposure to non-lethal levels of emamectin benzoate after treatments: a potential risk factor for drug resistance. *Sci Rep, 10*(1), 932-932. doi:10.1038/s41598-020-57594-7

Dyrevelferdsloven, LOV-2009-06-19-97 C.F.R. (2009).

Langford, K. H., Øksnevad, S., Schøyen, M., & Thomas, K. V. (2011). *Environmental screening of veterinary medicines used in aquaculture, diflubenzuron and teflubenzuron*. (1086/2011). Norwegian Institute of Water Research: Klima- og forurensningsdirektoratet.

Langford, K. H., Øxnevad, S., Schøyen, M., & Thomas, K. V. (2014). Do antiparasitic medicines used in aquaculture pose a risk to the Norwegian aquatic environment? *Environmental science & technology*. doi:dx.doi.org/10.1021/es5005329

Legemiddelverket. (2001). Terapianbefaling: Antiparasittærbehandling av hund, katt og hest. *Fellesekspedisjonen for medisinsk informasjon.* Retrieved from http://www.legemiddelverket.no/terapi/publiserte_terapi.htm

Lemos, D., & Weissman, D. (2021). Moulting in the grow-out of farmed shrimp: a review. *Reviews in aquaculture, 13*(1), 5-17. doi:10.1111/raq.12461

Lenz, M., Müller, F.-J., Zenke, M., & Schuppert, A. (2016). Principal components analysis and the reported low intrinsic dimensionality of gene expression microarray data. *Scientific reports, 6*(1), 25696-25696. doi:10.1038/srep25696

Litleskare, I. (2019). Bruk av legemidler i fiskeoppdrett. *Legemidler i fiskeoppdrett.* Retrieved from https://www.fhi.no/hn/legemiddelbruk/fisk/2019-bruk-av-legemidler-i-fiskeoppdrett/

Liu, Y., & Bjelland, H. v. (2014). Estimating costs of sea lice control strategy in Norway. *Preventive veterinary medicine, 117*(3-4), 469-477. doi:10.1016/j.prevetmed.2014.08.018

Liu, Z., Ding, Y., Ye, N., Wild, C., Chen, H., & Zhou, J. (2016). Direct activation of BAX protein for cancer therapy. *Medicinal research reviews, 36*(2), 313-341. doi:10.1002/med.21379

Lyons, M. C., Wong, D. K. H., & Page, F. H. (2014). Degradation of hydrogen peroxide in seawater using the anti-sea louse formulation interox paramove 50. In *Canadian Technical Report of Fisheries and Aquatic Sciences 3080*. Fisheries and Oceans Canada, Maritimes Region: St. Andrews Biological Station.

Macken, A., Lillicrap, A., & Langford, K. (2015). Benzoylurea pesticides used as veterinary medicines in aquaculture: Risks and developmental effects on nontarget crustaceans. *Environmental Toxicology and Chemistry, 34*(7), 1533-1542. doi:10.1002/etc.2920

Mark, J. C. (2009). How sea lice from salmon farms may cause wild salmonid declines in Europe and North America and be a threat to fishes elsewhere. *Proceedings of the royal society B: Biological sciences, 276*(1672), 3385-3394. doi:10.1098/rspb.2009.0771

Marsella, A. M., Jaskolka, M., & Mabury, S. A. (2000). Aqueous solubilities, photolysis rates and partition coefficients of benzoylphenylurea insecticides. *Pest Management Science, 55,56*(12,9), 789-794. doi:10.1002/1526-4998(200009)56:9<789::AID-PS209>3.0.CO

2-L

Martin, G. G., Castro, C., Moy, N., & Rubin, N. (2005). N-acetyl-D-glucosamine in crustacean hemocytes; possible functions and usefulness in hemocyte classification. *Invertebrate biology, 122*(3), 265-270. doi:10.1111/j.1744-7410.2003.tb00090.x

Matloven. (2003). *Lov om matproduksjon og mattrygghet mv.* (LOV-2003-12-19-124). Retrieved from https://lovdata.no/dokument/NL/lov/2003-12-19-124/KAPITTEL_3#%C2%A719.

McCarthy, S. D., Dugon, M. M., & Power, A. M. (2015). 'Degraded' RNA profiles in Arthropoda and beyond. *PeerJ, 2015*(12), e1436-e1436. doi:10.7717/peerj.1436

McHenery, J., G. (2016). *Lufenuron for salmonids - Environmental assessment in support of an import tolerance request*. Retrieved from US Import Tolerance EA: https://www.fda.gov/media/100121/download

Merzendorfer, H., & Merzendorfer, H. (2006). Insect chitin synthases: a review. *J Comp Physiol B, 176*(1), 1-15. doi:10.1007/s00360-005-0005-3

Metsalu, T., & Vilo, J. (2015). ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Research, 43*(1), W566-W570. doi:10.1093/nar/gkv468

Moyes, C. D., & Schulte, P. M. (2014). *Principles of Animal Physiology* (Vol. 2). San Francisco, CA: Pearson/Benjamin Cummings.

Muthukrishnan, S., Merzendorfer, H., Arakane, Y., & Kramer, K. J. (2012). *Chitin metabolism in insects*. In Gilbert (Ed.), *Insect molecular biology and biochemistry* (pp. 575).

Muzzarelli, R. A. A. (1977). *Chitinases and related enzymes*. In Chitin, Vol. 1. Muzzarelli (Ed.), (pp. 155-181).

Mykles, D. L., & Chang, E. S. (2020). Hormonal control of the crustacean molting gland: Insights from transcriptomics and proteomics. *General and comperative endocrinology, 294*, 113493-113493. doi:10.1016/j.ygcen.2020.113493

Nilsson, J., Moltumyr, L., Madaro, A., Kristiansen, T. S., Gåsnes, S. K., Mejdell, C. M., Gismervik, K., & Stien, L. H. (2019). Sudden exposure to warm water causes instant behavioural responses indicative of nociception or pain in Atlantic salmon. *Veterinary and Animal Science, 8*, 100076-100076. doi:10.1016/j.vas.2019.100076

Nimmo, D. R., Hamaker, T. L., Moore, J. C., & Sommers, C. A. (1979). Effect of diflubenzuron on an estuarine crustacean. *Bulletin of Environmental Contamination and Toxicology, 22*(1), 767-770. doi:10.1007/BF02027022

Akvakulturdriftforskriften, FOR-2008-06-17-822 C.F.R. (2008).

Nærings- og fiskeridepartementet. (2017). *Handlingsplan mot resistens mot legemidler mot lakselus*. www.regjeringen.no: Nærings- og fiskeridepartementet Retrieved from https://www.regjeringen.no/contentassets/bac8a85fb17145939d38ce038130ce0e/w-0016_handlingsplan-mot-resistens-mot-legemidler-mot-lakselus.pdf.

Nørregaard Jensen, O. (2004). Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. *Current opinion in chemical biology, 8*(1), 33-41. doi:10.1016/j.cbpa.2003.12.009

Olsvik, P. A., Aulin, M., Samuelsen, O. B., Hannisdal, R., Agnalt, A. L., & Lunestad, B. T. (2019). Whole-animal accumulation, oxidative stress, transcriptomic and metabolomic responses in the pink shrimp (*Pandalus montagui*) exposed to teflubenzuron. *Journal of applied toxicology, 39*(3), 485-497. doi:10.1002/jat.3739

Olsvik, P. A., Lie, K. K., Mykkeltvedt, E., Samuelsen, O. B., Petersen, K., Stavrum, A.-K., & Lunestad, B. T. (2008). Pharmacokinetics and transcriptional effects of the anti-salmon lice drug emamectin benzoate in Atlantic salmon (*Salmo salar L.*). *BMC Pharmacology and toxicology, 8*(1), 16-16. doi:10.1186/1471-2210-8-16

Olsvik, P. A., Lunestad, B. T., Agnalt, A.-L., & Samuelsen, O. B. (2017). Impact of teflubenzuron on the rockpool shrimp (*Palaemon elegans*). *Comparative biochemistry and physiology Part C: Toxicology and pharmacology, 201*, 35-43. doi:10.1016/j.cbpc.2017.09.005

Olsvik, P. A., Samuelsen, O. B., Agnalt, A.-L., & Lunestad, B. T. (2015). Transcriptional responses to teflubenzuron exposure in European lobster (*Homarus gammarus*). *Aquatic Toxicology, 167*, 143-156. doi:10.1016/j.aquatox.2015.07.008

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K., & Stien, L. H. (2019). Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. *Reviews in aquaculture, 11*(4), 1398-1417. doi:10.1111/raq.12299

Parsons, A. E., Escobar-Lux, R. H., Sævik, P. N., Samuelsen, O. B., & Agnalt, A.-L. (2020). The impact of anti-sea lice pesticides, azamethiphos and deltamethrin, on European lobster (*Homarus gammarus*) larvae in the Norwegian marine environment. *Elsevier Ltd.* doi:http://doi.org/10.1016/j.envpol.2020.114725

Pedrosa-Gerasmio, I. R., Kondo, H., & Hirono, I. (2019). Dietary 5-aminolevulinic acid enhances adenosine triphosphate production, ecdysis and immune response in Pacific white shrimp, *Litopenaeus vannamei* (Boone). *Aquaculture research, 50*(4), 1131-1141. doi:10.1111/are.13987

Poley, J. D., Braden, L. M., Messmer, A. M., Igboeli, O. O., Whyte, S. K., Macdonald, A., Rodriguez, J., Gameiro, M., Rufener, L., Bouvier, J., Wadowska, D. W., Koop, B. F., Hosking, B. C., & Fast, M. D. (2018). High level efficacy of lufenuron against sea lice (*Lepeophtheirus salmonis*) linked to rapid impact on moulting processes. *International Journal for Parasitology: Drugs and Drug Resistance, 8*(2), 174-188. doi:10.1016/j.ijpddr.2018.02.007

Qiagen. (2012). EZ1 RNA Handbook. *Sample & Assay Technologies, Third Edition*.

Qiagen. (2018). All insights start with the sample: Your comprehensive guide for isolating top-quality RNA. doi:PROM-7288-002 1113999 09/2018

Rath, S., Erdely, H., and Reuss, R. (2017). Residue monograph prepared by the meeting of the joint FAO/WHO expert committee on food additived (JECFA), 85th Meeting 2017. Lufenuron [Press release]. Retrieved from http://www.fao.org/3/ca3708en/ca3708en.pdf

Regjeringen. (2019). *Skattlegging av havbruksvirksomhet*. (NOU 2019: 18). www.Regjeringen.no Retrieved from https://www.regjeringen.no/no/dokumenter/nou-2019-18/id2676239/?ch=5.

Reuschel, S., Cuesta, J. A., & Schubart, C. D. (2010). Marine biogeographic boundaries and human introduction along the European coast revealed by phylogeography of the prawn *Palaemon elegans*. *Molecular phylogenetics and evolution, 55*(3), 765-775. doi:10.1016/j.ympev.2010.03.021

Rocha, J., Garcia-Carreño, F. L., Muhlia-Almazán, A., Peregrino-Uriarte, A. B., Yépiz-Plascencia, G., & Córdova-Murueta, J. H. (2012). Cuticular chitin synthase and chitinase mRNA of whiteleg shrimp *Litopenaeus vannamei* during the molting cycle. *Aquaculture, 330-333*, 111-115. doi:10.1016/j.aquaculture.2011.12.024

Rådström, P., Knutsson, R., Wolffs, P., Lövenklev, M., & Löfström, C. (2004). Pre-PCR processing: Strategies to generate PCR-compatible samples. *Molecular biotechnology, 26*(2), 133-146. doi:10.1385/MB:26:2:133

Samuelsen, O. (2016). Persistence and stability of teflubenzuron and diflubenzuron when associated to organic particles in marine sediment. *Bulletin of Environmental Contamination and Toxicology, 96*(2), 224-228. doi:10.1007/s00128-015-1707-1

Samuelsen, O., Parsons, A., Agnalt, A.-L., Tjensvoll, T., Lunestad, B. T., & Hannisdal, R. (2020). Mortality in the rockpool shrimp *Palaemon elegans* following long-term exposure to low doses of the anti-parasitic drug Teflubenzuron. *Aquaculture Environment Interactions*, 23-29. doi:https://doi.org/10.3354/aei00343

Samuelsen, O. B., Lunestad, B. T., Farestveit, E., Grefsrud, E. S., Hannisdal, R., Holmelid, B., Tjensvoll, T., & Agnalt, A.-L. (2014). Mortality and deformities in European lobster (*Homarus gammarus*) juveniles exposed to the anti-parasitic drug teflubenzuron. *Aquatic Toxicology, 149*, 8-15. doi:10.1016/j.aquatox.2014.01.019

Samuelsen, O. B., Lunestad, B. T., Hannisdal, R., Bannister, R., Olsen, S., Tjensvoll, T., Farestveit, E., & Ervik, A. (2015). Distribution and persistence of the anti sea-lice drug teflubenzuron in wild fauna and sediments around a salmon farm, following a standard treatment. *Science of the Total Environment, 508*, 115-121. doi:10.1016/j.scitotenv.2014.11.082

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods, 9*, 671-675.

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors – occurrence, properties and removal. *Journal of applied microbiology, 113*(5), 1014-1026. doi:10.1111/j.1365-2672.2012.05384.x

Scottish environment protection agency. (1999). *Calicide (teflubenzuron) - Autorisation for use as an infeed sea lice treatment in marine cage salmon farms*. Retrieved from Risk

assessment, EQS and recommendations: https://www.fluoridealert.org/wp-content/pesticides/teflubenzuron.scotlandepa99.pdf

Scottish environment protection agency. (2005). *Ecological effects of sea lice medicines in Scottich sea lochs*. Retrieved from http://randd.defra.gov.uk/Document.aspx?Document=VM0293_2415_FRP.pdf

Seear, P. J., Tarling, G. A., Burns, G., Goodall-Copestake, W. P., Gaten, E., Özkaya, Ö., & Rosato, E. (2010). Differential gene expression during the moult cycle of Antarctic krill (Euphausia superba). *BMC Genomics, 11*(1), 582-582. doi:10.1186/1471-2164-11-582

Sepasi Tehrani, H., & Moosavi-Movahedi, A. A. (2018). Catalase and its mysteries. *Progress in biophysics and molecular biology, 140*, 5-12. doi:10.1016/j.pbiomolbio.2018.03.001

Sherina, V., McMurray, H. R., Powers, W., Land, H., Love, T. M. T., & McCall, M. N. (2020). Multiple imputation and direct estimation for qPCR data with non-detects. *BMC Bioinformatics, 21*(1), 545-545. doi:10.1186/s12859-020-03807-9

Sparling, D. W. (2017). *An Introduction to Ecotoxicology*. In Basics of Ecotoxicology, Sparling (Ed.), (pp. 237). doi:https://doi.org/10.1201/9781315158068

Statistisk sentralbyrå. (2020, 29.10.2020). Akvakultur (opphørt), endelige tall 2020. Retrieved from https://www.ssb.no/fiskeoppdrett

Štrus, J., Žnidaršič, N., Mrak, P., Bogataj, U., & Vogt, G. (2019). Structure, function and development of the digestive system in malacostracan crustaceans and adaptation to different lifestyles. *Cell and tissue research, 377*(3), 415-443. doi:10.1007/s00441-019-03056-0

Sweeney, M. A., Iakova, P., Maneix, L., Shih, F.-Y., Cho, H. E., Sahin, E., & Catic, A. (2020). The ubiquitin ligase Cullin-1 associates with chromatin and regulates transcription of specific c-MYC target genes. *Scientific reports, 10*(1), 13942-13942. doi:10.1038/s41598-020-70610-0

Sømme, L. S. (2017). *Strandreker*. In Store norske leksikon, Søvik (Ed.). Retrieved from https://snl.no/strandreker

Thomassen, J. M. (1993). *Hydrogen peroxide as a delousing agent for Atlantic salmon*. In Boxshall & Defaye (Eds.), *Pathogens of wild and farmed fish: Sea lice* (pp. 378). Retrieved from http://www.int-res.com/articles/dao/17/d017p197.pdf

Thorstad, E. B., & Forseth, T. (2019). *Status for norske laksebestander i 2019*. Retrieved from Norsk institutt for naturforskning: https://brage.nina.no/nina-xmlui/bitstream/handle/11250/2619889/Rapport%2012.pdf?sequence=1&isAllowed=y

Tschesche, C., Bekaert, M., Bassett, D. I., Mitchell, C., North, B., Boyd, S., Carmona-Antoñanzas, G., Bron, J. E., & Strum, A. (2021). Investigation of deltamethrin resistance in salmon lice (*Lepeophtheirus salmonis*) provides no evidence for roles of mutations in voltage-gated sodium channels. *Pest Management Science, 77*(2), 1052-1060. doi:10.1002/ps.6120

Urbina, M. A., Cumillaf, J. P., Paschke, K., & Gebauer, P. (2019). Effects of pharmaceuticals used to treat salmon lice on non-target species: Evidence from a systematic review. *Science of the Total Environment, 649*, 1124-1136. doi:10.1016/j.scitotenv.2018.08.334

Van Geest, J. L., Burridge, L. E., & Kidd, K. A. (2014). The toxicity of the anti-sea lice pesticide AlphaMax® to the polychaete worm *Nereis virens*. *Aquaculture, 430*, 98-106. doi:10.1016/j.aquaculture.2014.03.044

Vogt, G. (2019). Functional cytology of the hepatopancreas of decapod crustaceans. *J Morphol, 280*(9), 1405-1444. doi:10.1002/jmor.21040

Wagner, G. N., Fast, M. D., & Johnson, S. C. (2008). Physiology and immunology of *Lepeophtheirus salmonis* infections of salmonids. *Trends in Parasitology, 24*(4), 176-183. doi:10.1016/j.pt.2007.12.010

Wang, L., Wu, J., Wang, W.-N., Cai, D.-X., Liu, Y., & Wang, A.-L. (2012). Glutathione peroxidase from the white shrimp *Litopenaeus vannamei*: characterization and its regulation upon pH and Cd exposure. *Ecotoxicology, 21*(6), 1585-1592. doi:10.1007/s10646-012-0942-z

Watanabe, T., & Kono, M. (1997). Isolation of a cDNA encoding a chitinase family protein from cuticular tissues of the Kuruma prawn *Penaeus japonicus*. *Zoological Science, 14*(1), 65-68. doi:10.2108/zsj.14.65

Watanabe, T., Kono, M., Aida, K., & Nagasawa, H. (1998). Purification and molecular cloning of a chitinase expressed in the hepatopancreas of the penaeid prawn *Penaeus japonicus*. *Biochimica et biophysica acta, Protein structure and molecular enzymology, 1382*(2), 181-185. doi:10.1016/S0167-4838(97)00184-2

Wesenberg, G. R., Bredal, W., Vågen, I., & Reinnel, H. (2000). Terapianbefaling: Behandling mot lakselus i oppdrettsanlegg. (2000:02), 47.

Whelan, K. (2010). A review of the impacts of the salmon louse, *Lepeophtheirus salmonis* (Krøyer, 1837) on wild salmonids. *Atlantic Salmon Trust, School of Biology & Environmental Science*, 27.

Wilkinson, L. (2011). ggplot2: Elegant graphics for data analysis by Wickham, H. *Biometrics, 67*(2), 678-679. doi:10.1111/j.1541-0420.2011.01616.x

Xu, Y., Li, X., Deng, Y., Lu, Q., Yang, Y., Pan, J., Ge, J., & Xu, Z. (2017). Comparative transcriptome sequencing of the hepatopancreas reveals differentially expressed genes in the precocious juvenile Chinese mitten crab, *Eriocheir sinensis* (Crustacea: Decapoda). *Aquaculture research, 48*(7), 3645-3656. doi:10.1111/are.13189

Zhang, Q., Li, F., Zhang, X., Dong, B., Zhang, J., Xie, Y., & Xiang, J. (2008). cDNA cloning, characterization and expression analysis of the antioxidant enzyme gene, catalase, of Chinese shrimp *Fenneropenaeus chinensis*. *Fish and Shellfish Immunology, 24*(5), 584-591. doi:10.1016/j.fsi.2008.01.008

Zhao, C., Fu, H., Sun, S., Qiao, H., Zhang, W., Jin, S., Jiang, S., Xiong, Y., & Gong, Y. (2018). A transcriptome study on *Macrobrachium nipponense* hepatopancreas experimentally challenged with white spot syndrome virus (WSSV). *PLoS ONE, 13*(7), e0200222-e0200222. doi:10.1371/journal.pone.0200222

Aaen, S. M., Helgesen, K. O., Bakke, M. J., Kaur, K., & Horsberg, T. E. (2014). Drug resistance in sea lice: a threat to salmonid aquaculture. *Trends in Parasitology, 31*(2), 72-81. doi:10.1016/j.pt.2014.12.006

7. Appendix

Table S1.List of reagents used to make a master mix for RT-qPCR

Reagents	µl	Producer
Q-Solution	30	Qiagen®
One-Step RT-PCR Buffer	30	Qiagen®
dNTP Mix (10mM)	6	Qiagen®
TAQ DNA Polymerase	6	Qiagen®
RNase-free Milli-Q water	58,2	Biocel®
RNA sample	16.3	

Table S2 Solutions for the toxicokinetic analyses. For the control and standard curve work solutions, 50 µL lufenuron from each stock solution were transferred to a 10 mL vial, before being diluted with acetonitrile:water (1:1) (concentration: 5µg/mL). A standard work solution was made for control and standard curve, by diluting the work solutions further. This was done by adding 50 µL work solution and diluting it with acetonitrile:water (1:1) in a 10 mL vial (concentration 50 ng/mL). A intermediate solution was made for the standard curve by diluting 1000 µl of the standard curve work solution with acetonitrile:water (1:1) in a 5 mL vial. The same procedure was done for the control. For the internal standard, 500 µl was diluted by acetonitrile:water (1:1) in a 10 mL vial.

Work solutions	
Standard curve (1)	50 µl of 1.0 mg/ml lufenuron into a 10 ml measuring flask. Add acetonitrile:water (1:1) to the mark. Mix well.
Control (1)	50 µl of 1.0 mg/ml lufenuron into a 10 ml measuring flask. Add Acetonitrile:Water (1:1) to the mark. Mix well.
Internal standard	50 µl of 1.0 mg/ml lufenuron-d3 into a 10 ml measuring flask. Add acetonitrile:water (1:1) to the mark. Mix well.
Standard solution	
Standard curve (2)	Pipette 100 µl from the work solution to a 10 ml measuring flask. Add acetonitrile:water (1:1) to the mark. Mix well.
Control (2)	Pipette 100 µl from the work solution to a 10 ml measuring flask. Add acetonitrile:water (1:1) to the mark. Mix well
Intermediate solution	
STD(M)	Pipette 1000 µl from the standard solution to a 5 ml measuring flask. Add acetonitrile:water (1:1) to the mark. Mix well.
K(M)	Pipette 1000 µl from the standard solution to a 5 ml measuring flask. Add acetonitrile:water (1:1) to the mark. Mix well
IS(M)	Pipette 1000 µl from the standard solution to a 10 ml measuring flask. Add acetonitrile:water (1:1) to the mark. Mix well

Table S3Overview of sample standards and their added solutions and concentration (Exposure experiment). For the elimination study K (High) had a concentration equal to N14 (10 000 ng/g).

	Sample concentration (µl)
BMM	0
BUM	0
K (LOQ)	1.0
K (Medium)	500
K (High)	1500
N1	4
N2	10
N3	100
N4	300
N5	500
N6	700
N7	900
N8	1100
N9	1300
N10	1500
N11	2000
N12	4000
N13	7000
N14	10 000
N15	20 000
N16	30 000

2020-1854/   reke hepatopancreas

A	Delution curve 1000	Delution curve 1000	Delution curve 1000	Delution curve 500 ng	Delution curve 500	Delution curve 500	Delution curve 250 ng	Delution curve 250 ng	Delution curve 250 ng	Delution curve 125 ng	Delution curve 125 ng	Delution curve 125 ng
B	Delution curve 62.5 ng	Delution curve 62.5 ng	Delution curve 62.5 ng	Delution curve 31.25 ng	Delution curve 31.25 ng	Delution curve 31.25 ng	162 500 ng	162 500 ng	163 500 ng	163 500 ng	164 500 ng	164 500 ng
C	165 500 ng	165 500 ng	167 500 ng	167 500 ng	168 500 ng	168 500 ng	169 500 ng	169 500 ng	170 500 ng	170 500 ng	171 500 ng	171 500 ng
D	172 500 ng	172 500 ng	173 500 ng	173 500 ng	174 500 ng	174 500 ng	175 500 ng	175 500 ng	176 500 ng	176 500 ng	177 500 ng	177 500 ng
E	178 500 ng	178 500 ng	179 500 ng	179 500 ng	180 500 ng	180 500 ng	181 500 ng	181 500 ng	182 500 ng	182 500 ng	184 500 ng	184 500 ng
F	185 500 ng	185 500 ng	186 500 ng	186 500 ng	187 500 ng	187 500 ng	188 500 ng	188 500 ng	189 500 ng	189 500 ng	190 500 ng	190 500 ng
G	191 500 ng	191 500 ng	192 500 ng	192 500 ng	193 500 ng	193 500 ng	194 500 ng	194 500 ng	195 500 ng	195 500 ng	196 500 ng	196 500 ng
H	197 500 ng	197 500 ng	198 500 ng	198 500 ng	199 500 ng	199 500 ng	200 500 ng	200 500 ng	166 500 ng	166 500 ng	ntc	nac

Dilution curve: mix of all RNA in plate
cDNA syntese 25.11.2020

Fig. S1 Dilution curve (cDNA synthesis 25.11.2020)
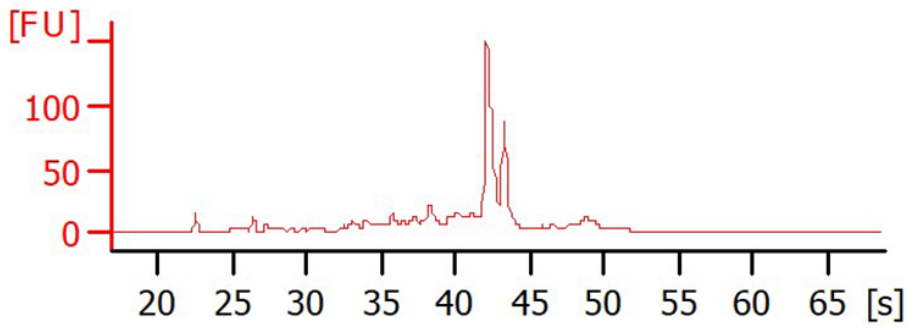
b

Fig. S2 Electropherogram example (sample 2020-1854/170)
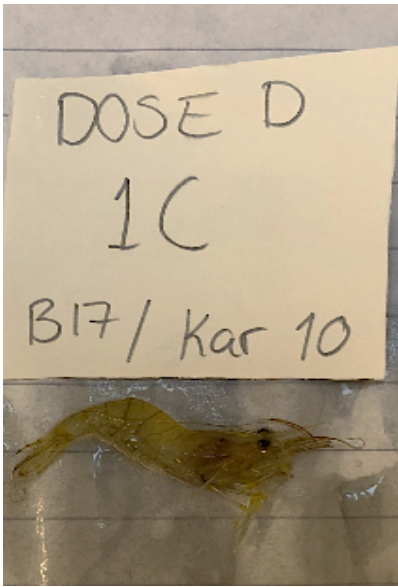
Fig. S3 Morphological changes on shrimps from exposure experiment (shrimp 1C (B17) exposed to 0.01 µg lufenuron/g), w/bent antennae.
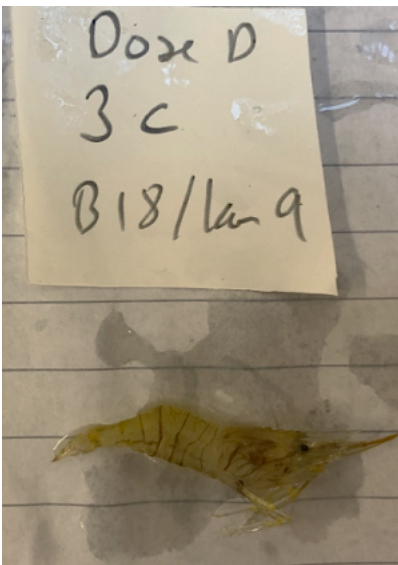
Fig. S4 Morphological changes on shrimps from exposure experiment (shrimp 3C (B18) exposed to 0.01 µg lufenuron/g, w/broken tail fan and deformed, stiff hind legs)

c

Fig. S5 Shrimps w/flesh colour, looking like they`ve moulted before dying. From top left; shrimp D3 (B13) exposed to 1.0 µg//g shrimp, shrimp D3 (B16) exposed to 0.1 µg/g shrimp, shrimp A1 (B15) exposed to 0.1 µg/g shrimp, shrimp A3 (B18) exposed to 0.01 µg/g shrimp. Bottom left; shrimp 3A (B16) exposed to 0.1 µg/g shrimp.

Table S4 Measured concentrations(ng/g) for each individual in the elimination study.

Date of death	Lufenuron concentration (ng/g)
19. nov. 2020	2590
19. nov. 2020	3437
19. nov. 2020	5217
19. nov. 2020	1403
19. nov. 2020	606
24. nov. 2020	360
16. nov. 2020	3116
26. nov. 2020	2276
26. nov. 2020	1683
22. nov. 2020	186
26. nov. 2020	378
18. nov. 2020	6044
26. nov. 2020	2813
26. nov. 2020	1474
3. des. 2020	21
30. nov. 2020	835
21. nov. 2020	1797
15. nov. 2020	3759
1. des. 2020	2021
26. nov. 2020	1175
30. nov. 2020	371
26.nov.20	583

Fig. S6 Normality of mortality data before (A) and after (B) log10 transforming.

e

Table S5 P-values for Spearman`s rank correlation of measured concentration, number of moults and transcripts analysed.

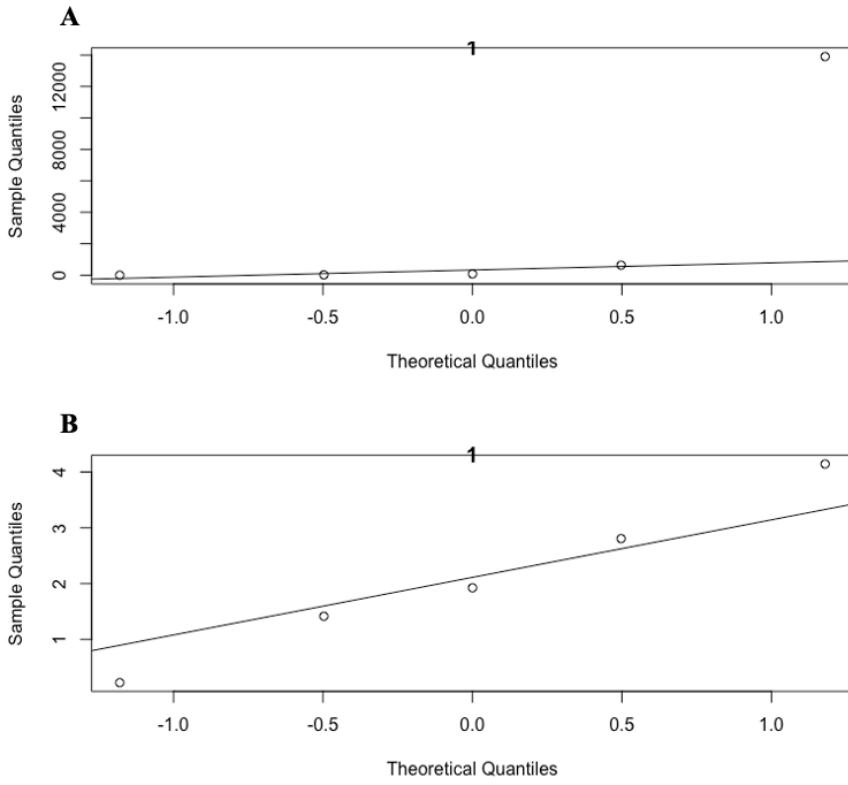|	Measured conc.	Moults	HSP70	P53	CTBS	CHH	CYP3A	GPX1	CAT	CHS1	CASP3	BAX	ABCC8	CYP301	CYP1A	LD	PGM3	CTSL	TYR1	CHIT1	SUR	CHITINASE1	CUL1	GAP65	TUBA
Measured conc.		0.0264	0.1777	0.6312	0.4020	0.0402	0.3568	0.9216	0.0394	0.0766	0.4737	0.0329	0.2171	0.3084	0.2678	0.9040	0.8772	0.7997	0.4827	0.1725	0.3424	0.9319	0.3932	0.1557	0.9112
Moults	0.0264		0.4451	0.0781	0.3405	0.6026	0.1653	0.5790	0.4837	0.0075	0.1271	0.1622	0.2076	0.4232	0.8699	0.5997	0.1401	0.4578	0.6677	0.4711	0.2579	0.7558	0.2763	0.5108	0.8021
HSP70	0.1777	0.4451		0.3404	0.7447	0.6192	0.9558	0.2885	0.0092	0.9371	0.9652	0.5127	0.0298	0.2121	0.3595	0.0008	7.1799E-05	0.0078	0.6387	0.6275	0.0036	0.6219	0.0032	0.8844	0.0019
P53	0.6312	0.0781	0.3404		0.3701	0.3794	0.9133	0.1561	0.0792	0.6350	0.7319	0.2783	0.9174	0.0160	0.2121	0.4976	0.0005	0.8659	0.7537	0.0607	0.3779	0.9164	0.1902	0.1397	0.0287
CTBS	0.4020	0.3405	0.7447	0.3701		0.6136	0.1203	0.9891	0.0393	0.0005	0.0216	0.0081	0.0752	0.0048	0.5419	0.5026	0.2609	0.8906	0.2273	0.2766	0.3772	0.1821	0.0521	0.9704	0.0039
CHH	0.0402	0.6026	0.6192	0.3794	0.6136		0.4005	0.5367	0.9444	0.5854	0.5559	0.0289	0.0040	0.2043	0.0085	0.2221	0.2690	0.4441	0.4909	0.2661	0.0363	0.3084	0.5953	6.2E-05	0.1790
CYP3A	0.3568	0.1653	0.9558	0.9133	0.1203	0.4005		0.4133	0.0172	0.0177	0.1529	0.0712	0.5463	0.0084	0.7866	0.0787	0.4552	0.4425	0.6920	0.1445	0.7836	0.4464	0.1939	0.3687	0.1246
GPX1	0.9216	0.5790	0.2885	0.1561	0.9891	0.5367	0.4133		0.0959	0.6775	0.4778	0.2497	0.4140	0.1589	0.1893	0.2151	0.9433	0.9714	0.7260	0.0338	0.2330	0.3232	0.1129	0.9413	0.3324
CAT	0.0394	0.4837	0.0092	0.0792	0.0393	0.9444	0.0172	0.0959		0.0402	0.8988	0.4794	0.3998	2.49E-07	0.1799	0.3096	0.7104	0.0398	0.5307	0.0402	0.1799	0.4672	0.0001	0.4262	0.8434
CHS1	0.0766	0.0075	0.9371	0.6350	0.0005	0.5854	0.0177	0.6775	0.0402		0.0017	0.0028	0.2531	0.0060	0.7359	0.6756	0.3815	0.6063	0.2559	0.4672	0.4860	0.2131	0.1083	0.8007	0.2464
CASP3	0.4737	0.1271	0.9652	0.7319	0.0216	0.5559	0.1529	0.4778	0.8988	0.0017		0.4208	0.0851	0.0472	0.8947	0.7806	0.5264	0.5728	0.8078	0.9579	0.3701	0.9943	0.1232	0.7997	0.1617
BAX	0.0329	0.1622	0.5127	0.2783	0.0081	0.0289	0.0712	0.2497	0.4794	0.0028	0.4208		0.0366	0.3983	0.8007	0.9081	0.8978	0.0347	0.0886	0.2736	0.1734	0.1898	0.0578	0.0340	0.4148
ABCC8	0.2171	0.2076	0.0298	0.9174	0.0752	0.0040	0.5463	0.4140	0.3998	0.2531	0.0851	0.0366		0.4496	0.0902	0.0334	0.0521	0.8690	0.6862	0.8311	6.3132E-07	0.3008	0.0066	0.0586	0.0836
CYP301	0.3084	0.4232	0.2121	0.0160	0.0048	0.2043	0.0084	0.1589	2.4895E-07	0.0060	0.0472	0.3983	0.4496		0.8710	0.5272	0.4680	0.0918	0.8741	0.0412	0.3954	0.2581	0.0001	0.1852	0.1557
CYP1A	0.2678	0.8699	0.3595	0.2121	0.5419	0.0085	0.7866	0.1893	0.1799	0.7359	0.8947	0.8007	0.0902	0.8710		0.0945	0.1613	0.3765	0.3310	0.4778	0.0197	0.0695	0.3478	0.2257	0.0626
LD	0.9040	0.5997	0.0008	0.4976	0.5026	0.2221	0.0787	0.2151	0.3096	0.6756	0.7806	0.9081	0.0334	0.5272	0.0945		0.0010	0.1456	0.5102	0.5247	0.0063	0.5093	0.0083	0.8854	0.0001
PGM3	0.8772	0.1401	0.0001	0.0005	0.2609	0.2690	0.4552	0.9433	0.7104	0.3815	0.5264	0.8978	0.0521	0.4680	0.1613	0.0010		0.1307	0.7270	0.1848	0.0005	0.5728	0.3186	0.4512	0.0004
CTSL	0.7997	0.4578	0.0078	0.8659	0.8906	0.4441	0.4425	0.9714	0.0398	0.6063	0.5728	0.0347	0.8690	0.0918	0.3765	0.1456	0.1307		0.0350	0.0087	0.9133	0.4504	0.5411	0.0039	0.3998
TYR1	0.4827	0.6677	0.6387	0.7537	0.2273	0.4909	0.6920	0.7260	0.5307	0.2559	0.8078	0.0886	0.6862	0.8741	0.3310	0.5102	0.7270	0.0350		0.9683	0.8240	0.0913	0.1142	0.2357	0.7250
CHIT1	0.1725	0.4711	0.6275	0.0607	0.2766	0.2661	0.1445	0.0338	0.0402	0.4672	0.9579	0.2736	0.8311	0.0412	0.4778	0.5247	0.1848	0.0087	0.9683		0.3801	0.4893	0.1687	0.0411	0.8885
SUR	0.3424	0.2579	0.0036	0.3779	0.3772	0.0363	0.7836	0.2330	0.1799	0.4860	0.3701	0.1734	6.3132E-07	0.3954	0.0197	0.0063	0.0005	0.9133	0.8240	0.3801		0.4005	0.0044	0.0361	0.0170
CHITINASE1	0.9319	0.7558	0.6219	0.9164	0.1821	0.3084	0.4464	0.3232	0.4672	0.2131	0.9943	0.1898	0.3008	0.2581	0.0695	0.5093	0.5728	0.4504	0.0913	0.4893	0.4005		0.1378	0.5782	0.4425
CUL1	0.3932	0.2763	0.0032	0.1902	0.0521	0.5953	0.1939	0.1129	0.0001	0.1083	0.1232	0.0578	0.0066	0.0001	0.3478	0.0083	0.3186	0.5411	0.1142	0.1687	0.0044	0.1378		0.7084	0.1104
GAP65	0.1557	0.5108	0.8844	0.1397	0.9704	0.0001	0.3687	0.9413	0.4262	0.8007	0.7997	0.0340	0.0586	0.1852	0.2257	0.8854	0.4512	0.0039	0.2357	0.0411	0.0361	0.5782	0.7084		0.5962
TUBA	0.9112	0.8021	0.0019	0.0287	0.0039	0.1790	0.1246	0.3324	0.8434	0.2464	0.1617	0.4148	0.0836	0.1557	0.0626	0.0001	0.0004	0.3998	0.7250	0.8885	0.0170	0.4425	0.1104	0.5962	

f

g