Pharmacokinetics, transcriptional changes and oxidative stress in pink shrimp (*Pandalus montagui*) exposed to teflubenzuron

Master thesis in Pharmacy

By

Marte Aulin

Center for Pharmacy, University of Bergen (UiB)

National Institute of Nutrition and Seafood Research (NIFES)

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Marte Aulin

ABSTRACT

In the recent years, infestation with salmon lice has become a major problem in salmonid farms, and has resulted in great economic losses and reduced welfare for farmed salmonids. To enhance the salmon lice combat, there has been an increased use of antiparasitic drugs in salmonid farms, raising concerns about the impact of these chemicals in the environment. In this study, an experiment with the non-target species pink shrimp (*Pandalus montagui*) was carried out to evaluate the effects of the antiparasitic drug teflubenzuron to crustacean living close to the farms. The pink shrimps (14.0±1.2 mm carapax length) were fed two times weekly for 46 days, with two doses of teflubenzuron. The two doses, termed *low dose* and *high dose* contained 0.1% and 1% of a normal fish therapy dose, respectively. At the end of the feeding experiment, surviving shrimps were examined for morphological changes, like speckled eyes and deformities, before tissue from the whole shrimp were analysed by LC-MS/MS to measure accumulated teflubenzuron in the shrimps. Gene-expression analyses was performed to study the effect of teflubenzuron exposure at the transcriptional level, in addition to colorimetric analyses to monitor changes in protein carbonyl concentration and malondialdehyde levels, both indicating oxidative stress.

Overall, the studies showed a high mortality rate (20%) among the shrimps exposed to high dose of teflubenzuron, whereas the small dose yield no mortality. Deformities like stiff and crocked legs were seen in both group receiving teflubenzuron. In the pharmacokinetic analyses, the mean concentration of teflubenzuron was significantly higher (10.5-fold) in the high dose group (70.39 ng/g w.w) compared to the low dose group (6.65 ng/g w.w). However, concentrations of teflubenzuron above LOQ (<0.2 ng/g w.w) were obtained in the control groups, suggesting that there might have been a contamination during the experiment. For this reason, extra shrimps from the same catch were added as an additional control to all sample analyses in this work, and all results are presented with initial controls and additional controls. The transcriptional data indicated that exposure of teflubenzuron at the studied concentrations could have a weak effect on antioxidative defence and detoxification mechanisms in shrimps. Moreover, correlation analyses suggested that teflubenzuron might have an impact on growth in shrimps. However, due to concentrations of teflubenzuron above LOQ in the controls, the results from the molecular part of this work are non-conclusive.

ABBREVATIONS

Actb	Actin beta
Bclx	Bcl2-related protein
BMM	Tube with matrix
BUM	Tube without matrix
Cat	Catalase
Chit1a	Chitinase
CL	Carapax lenght
Ctbs	Chitobiase
Cyp3a	Cytochrome p450 -3a
Ef1A	Elongation factor 1 alpha
EMA	The European Medicines Agency
EMEA	The European Agency for the Evaluation of Medicinal products
Gclc	Glutamate-cysteine ligase catalytic subunit
Gpx	Se-glutathione peroxidase
Gss	Glutathione synthase
Gstm3	Glutathione S-transferase mu3
Hsp70	Heat shock protein – 70
IMR	Institute of Marine Research
LOD	Level of detection
LOQ	Level of quantitative
MDA	Malondialdehyde
MNE	Mean normalized expression
m/z	Mass-to-charge Ratio
NIFES	National Institute of Nutrition and Seafood Research, Norway
ROS	Reactive Oxygen Species
Rpl13	Ribosomal 1 protein L13
Sod	Superoxide dismutase
SEM	Standard error of the mean
Uba 52	Ubiquitin A-52
w.w	Weight per weight

LIST OF CONTENT

1. INTRODUCTION

1.1 THE ATLANTIC SALMON INDUSTRY

For centuries, seafood has been an important part of the Norwegian cuisine as well as an important commodity product. However, it was not until mid-19th century that fish farming was established as a commercial important sector, when the first hatcheries with eggs from salmonids were established in Norway (NLVF, 1981). In the beginning of the 20th century, farming of rainbow trout (*Oncorhynchus mykiss*) was organized, but the new commitment was less successful, and the interest for salmonid farming dropped. In the beginning of 1950, fish farming was re-established in Norway, with inspiration from the growing industry in Denmark. As there were some challenges posed by the cold climate in Norway, most of the full-year farming of fish in fresh water was limited to Hordaland and Rogaland (NLVF, 1981). The breakthrough of salmonid farming came in the beginning of 1970, as more effective methods like the use of floating pens were developed (Hovland et al., 2014; Steinset, 2017).

Today, most of the salmonid farming is located along the coast from Rogaland to Finnmark (Svåsand et al., 2016) and for the last four decades, the salmon industry has been growing to become one of the largest industrial sectors in Norway. Worldwide, Norway is the leading exporter of Atlantic salmon (*Salmo salar*) and according to Statistic Norway (SSB), the total amount of traded salmon for 2015 was 1 303 346 ton, to a first-hand value of 44 439 million NOK. In the beginning of 2016, a five-kilo salmon had the same value as a barrel of oil (SSB, 2016; Wig, 2016).

However, infestation with salmon lice is a major challenge and causes severe problems for the aquaculture in Norway. Farmed fish are particularly vulnerable due to high density in the net pens, increased stress levels and at some areas, poor water quality (Langford et al., 2014). A positive correlation between the amount of farmed fish and grade of infecting lice at the same area is documented (Svåsand et al., 2016), and the annual economic loss is estimated to be 2.45 NOK per kg salmon produced. This includes direct losses due to mortality and reduced growth, cost of treatment and extra manpower (Liu and Bjelland, 2014). The sea lice most frequently occurring in Norway is the salmon louse *Lepeophteirus salmonis*, and thus the background information given in this thesis, will concern this species. The salmon louse is a host specific ectoparasite, belonging to the order Decapoda. During its eight-stage lifecycle, the louse infects the fish and starts feeding of mucus, skin and blood. The eight stages can be divided into the platonic stages (nauplis 1&2), the infective stage (copepodit), the parasitic chalimus stage

(chalimus 1&2), the pre-adult stages (1&2) and an adult stage (Hamre et al., 2013). During the first two stages, the lice feed on their stored nutrients, before they moult to the infective stage and search for a host. At the chalimus stage the parasites are attached to the epidermis of the fish by a proteinous filament, before moulting to preadult stage (mobile stage) where they can move around and swim in the water column. The generation time for sea lice are depending on factors like salinity and temperature of the seawater. The newly hatched larvae develop poorly when salinity is below 25‰, and when salinity decreases to 15‰, the larvae do not survive (Marine-Institute, 2017). Increased water temperature results in faster production of adult lice, and greater chances of survival in the post-infection stage (Groner et al., 2016; Marine-Institute, 2017). At 6°C, sea lice develops to mature lice within 8-9 weeks while at 12°C, sea lice can develop within 4 weeks (Marine-Institute, 2017). However, if the water temperature increases too much (30-34°C), the louse dies (Steinsvik, 2017).

Salmon lice can cause wounds, reduced welfare and even death if the numbers are high enough. Mortality is often caused by secondary infections of pathogenic bacteria or virus, or by osmotic stress or anaemia (Devine et al., 2000). Smolt are more vulnerable than adult salmons when infected by sea lice, as the they are smaller in size and less tolerant to physiological changes (Liu and Bjelland, 2014).

The Norwegian government has introduced legislation to enhance the salmon lice combat. As the fish industry mainly uses open net pens (Steinset, 2017), salmon lice can easily move between farmed to wild fish, and negatively affects the wild salmonids. Infestation with sea lice in wild salmons has been related to intensive salmon production (Svåsand et al., 2016). The legislation require that farmers have to report to the Norwegian Food Safety Authority the total number of salmon lice per fish every 14th day if the temperature is below 4°C. When temperatures are at or above 4°C, the larvae develop faster and the farmers have to report every week. A mandatory treatment threshold has been set, and varies from seasoning and location of farm. Nord-Trøndelag and further south has a limit of 0.2 mature female lice per fish from Monday in week 16 to Sunday in week 23, whereas the threshold limit is 0.5 the rest of the year. In Nordland, Troms and Finnmark the limit is 0.2 mature female lice per fish from Monday in week 21 to Sunday in week 26, and 0.5 the rest of the year (NFD, 2017).

1.2 TREATMENT AGAINST SALMON LICE

There are several ways to threat salmonids infected by parasites. The most common are either by chemicals, mechanic removal or biological removal by using cleaner fish (lumpfish or wrasse) who feed on the attached lice. Cleaner fish is not considered by the Norwegian legislation as treatment, but as a method to keep the amount of lice under control (NFD, 2017). Mechanic removal involves all none-chemical methods like delousing laser, plankton shielding skirts (FHL, 2012; Aaen et al., 2015) or hot water (Steinsvik, 2017). Chemical treatment includes delousing in baths in an enclosed system and oral treatment with medicated feed. Use of chemotherapeutics has been a fast and effective treatment, however, problems with drug-resistant parasites have led to an increased use of chemicals per kg the last years, and the use of drugs in combination have become more common (Table 1.2.1) (Svåsand et al., 2016). Changes in the legislation and lowered threshold for chemical treatment can also explain the increased sales. The numbers in Table 1.2.1, obtained from the Institute of Public Health (NIPH et al., 2017), shows the sale of antiparasitic agents used in treatment of salmon louse in selected years from 2006 to 2016. As can be seen, there have been an increased sale of antiparasitic agents per kg by 2015. In 2016, the sale of chemicals were reduced (a reduction of approximately 50% compared to 2015), most likely due to extended use of other methods, like mechanic removal, or the use of more potent chemicals (NIPH et al., 2017). Note that the numbers in Table 1.2.1 are given as kg active substance, and the doses given per m^3 in bath treatment differs from 2 mg deltamethrin to approximately 2-3 g hydrogen peroxide.

In contrast to other chemotherapeutics, the sale of the chemical teflubenzuron is still increasing. Teflubenzuron and diflubenzuron belongs to the benzoyl ureas, and were frequently used in the 1990s and until 2001, before they were temporarily out of use from 2002 to 2008 due to environmental concerns and the effectiveness of other chemical treatments (Samuelsen et al., 2014). However, the extended use of chemicals like the emametin benzoate and pyrethroids in the beginning of 2000, led to concerns about resistance in sea lice. In 2008 ineffective treatments with the pyrethroids and emamectin benzoate were reported, and the year after, there were also reports of failed treatments with azamethiphos (Aaen et al., 2015). As a result of this, teflubenzuron and diflubenzuron were reintroduce in 2009 (Samuelsen et al., 2014).

Table 1.2.1: The sale of chemical against salmon lice. The numbers are given as active substance per kg of drugs (NIPH, 2017). b= bath treatment, d= in diet.

Drug	2006	2008	2010	2012	2014	2015	2016
Hydrogenperoxide[b]			3071	2538	31577	43246	26597
Organophosphates Azamethiphos[b]	-	66	3346	4059	4630	3904	1269
Pyrethroids: Cypermethrin[b]	49	33	107	232	162	85	48
Deltamethrin[b]	23	39	61	121	158	115	43
Benzoyl urea Diflubenzuron[d]	-	-	1839	1611	5016	5896	4824
Teflubenzuron[d]	-	-	1080	751	2674	2509	4209
Avermectin Emamectin benzoate[d]	60	81	22	36	172	259	232

1.3 TEFLUBENZURON

Teflubenzuron [1-(3,5-dichloro-2,4-difluorophenyl)-3-(2,6-difluorbenzyol) urea] can be distinguished from diflubenzuron by the additional halogenated functional group (Figure 1.3.1), and is considered more potent than diflubenzuron (Langford et al., 2011). Teflubenzuron is sold on the Norwegian marked as Ektoban vet® manufactured by Skretting. The drug is laced with fish oil to the surface of the feeding pellets, and given in doses of 10 mg per kilo fish for seven days (Felleskatalogen, 2016). The European Medicines Agency (EMA), formerly called The European Agency for the Evaluation of Medicinal products (EMEA) has performed a study with a single oral administrations of 10 mg teflubenzuron/kg salmon at different temperatures, indicating a low bioavailability and a temperature-dependent absorption.

In a single oral treatment of 10 mg ^{14}C-teflubenzuron/kg salmon at 10°C, the highest concentrations were found in the gall bladder, liver and kidney, and the chemical was distributed to muscle and skin with the highest concentration after 24 hours. In studies with repeated doses of 10 mg teflubenzuron/kg salmon, the highest concentration of teflubenzuron was detected in liver the first day. The major component detected for all tissue in both studies was unchanged teflubenzuron (EMEA, 1999), but five metabolites have been identified (SPC, 2016). Moreover, studies of rats exposed to teflubenzuron, showed a rapid excretion of the drug and no signs of accumulation. The maximum residue limit (MRL) for teflubenzuron in salmonidae is set by EMEA to 500 μg/kg in muscle and skin in natural proposition (EMEA, 1999).

Salmon lice are affected by teflubenzuron when feeding on drug containing skin and blood, and the substance interrupts the synthesis of chitin [β-1, 4-N acetyl glucosamine linked polymer] in the exoskeleton of the louse (Felleskatalogen, 2016). Chitin occurs as an ordered, crystalline microfibril polymer and is an important part of the exoskeleton in crustacean, as it gives protection and stability. In many ways, chitin can be compared to collagen in vertebrates (Davis, 2011). Inhibiting the synthesis of chitin before ecdysis (moulting), stops the organism from casting its exoskeleton (exuvium) and cause mortality for the louse. For this reason, teflubenzuron is only effective to lice that undergo moulting. A salmon louse moults several times during its generation time and the drug is most effective during the larvae stage where the moulting is most frequent. Adult lice do not undergo moulting and will be unaffected by the chemical (Langford et al., 2011).
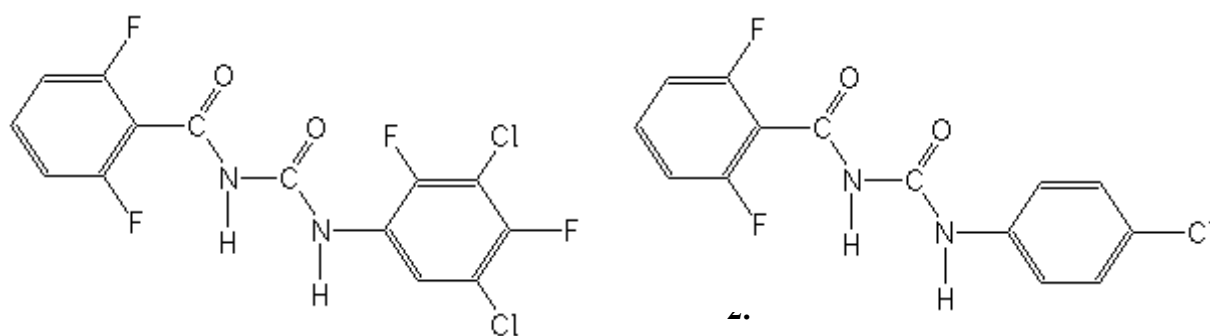
Figure 1.3.1 The chemical structure of teflubenzuron (1) and diflubenzuron (2). The structures are obtained from alanwood.net.

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1.4 Environmental concerns

While the pharmaceutical is almost non-toxic to other sea dwelling animals like fish, algae and shellfish, the benzoylurea pesticide can be highly toxic to animals depending on chitin. Given in diet, uneaten pellets (about 5-15%) represent a threat to benthic crustaceans like crabs, shrimps and lobster living close to the farming area. Furthermore, pharmacokinetic studies show a bioavailability of approximately 10% in salmon, which means that approximately 90% of the drug is excreted in its active form in faeces (Olsvik et al., 2015). Due to low water solubility (0.0094 mg/L at 20 °C) and high lipophility (log K_{ow} = 5.39) the majority of the active component is released bounded to particles in faeces and will degrade slowly in the sediment under and close to the farm (Samuelsen et al., 2015). It has been reported that teflubenzuron has an estimated half-life of 115 days in marine sediments, which indicates that the substance is present in the sediment for a long time after treatment (Langford et al., 2011). According to the summary of product characteristics (SPC), treatment with teflubenzuron should not be given at intervals less than 12 weeks, and used with caution from June to August when crustaceans go through moulting (SPC, 2016). However, there are few studies available on the ecotoxicological effects of teflubenzuron in marine crustaceans. Samuelsen et al. (2014) published a study on mortality and deformities in juvenile European lobster (*Homarus gammarus*) exposed to teflubenzuron, which showed that under controlled laboratory conditions, oral intake induced mortality and deformities in lobster during ecdysis (Samuelsen et al., 2014). There has also been a study on brown crabs (*Cancer pagurus*) and deep-water shrimps (*Pandalus borealis*) by Langford et al. (2014), were the authors caught crabs 100 m and 300 m from farming area, and deep-water shrimps 1-5 km away. The crabs contained up to 538 ng/g teflubenzuron, whereas the shrimps only contained minor concentration (Langford et al., 2014).

1.5 Detoxification kinetics

As most shrimps are omnivorous, teflubenzuron can be consumed when feeding on excess feed, faeces, plants or small animals close to the farm. There is little information available about the accumulation of teflubenzuron in shrimps. However, for chemicals in general, the hepatopancreas is the major organ for detoxification and elimination in shrimps. The hepatopancreas is surrounded by a thin membrane of connective tissue and is located in the cavity of the cephalothoracic. Five types of cell has been identified in the hepatopancreas, all

preforming individual task in the process of digestion, absorption and elimination of substances in shrimps (Sreeram and Menon, 2005). Elimination of some toxic substances have been suggested through faeces, gills or kidney in crustaceans (Ahearn et al., 2010), and there have been assumed that the enzymes of the cytochrome P450 and the glutathione-s-transferase (GST) family are important regulators of the detoxification processes for organic toxicants in shrimps (James and Boyle, 1998; Ren et al., 2014). As moulting in crustaceans has an impact on metabolism and other cell activity, stage of moulting can influence the toxicological effects of teflubenzuron (Faroongsarng et al., 2007).

Toxicology experiments show mortality at seawater concentration of 5 μg/L diflubenzuron in crabs and pharmacological studies have shown structural deformities and an impact on reproduction and swimming behaviour due to exposure. The same studies also indicate that adult crustaceans were more tolerant than larval forms (Christiansen et al., 1978; Roth et al., 1993). In studies of insects exposed to benzoylureas, the chemical have caused deformation and structural changes of the peritrophic membrane (PM) surrounding the midgut epithelium, the epidermis and the tracheal system (Merzendorfer, 2013). The PM is found in a variety of invertebrate, including shrimps, and the membrane separates the ingested materials from the midgut epithelium (Martin et al., 2006). The influence on numerous tissues and cells indicates that the antiparasitic agent is widely distributed to tissues and cells within the arthropod (Merzendorfer, 2013), suggesting that the whole body could be examined during kinetic studies of teflubenzuron in arthropods.

1.6 TRANSCRIPTIONAL CHANGES AND OXIDATIVE STRESS

When a gene is expressed in the nucleus of the cell, the base sequence (DNA) is copied by RNA polymerase to a molecule of pre messenger-RNA (mRNA), before it is spliced to the mature RNA in a process involving removal of the non-coding sequences introns. The first step in gene-expression (Figure 1.6.1) where the base sequence is copied to the RNA, is transcription of genes (NCBI, 2014). This step is essential for the translation of the encoded genes (exons) into proteins in ribosomes. Since it is relatively easy to measure the levels of mRNA in cells, transcriptional changes have often been used as biomarkers of exposure or effect in animals exposed to stress, environmental change or contaminants. Changes at the transcriptional level due to exposure to antiparasitic agents can lead to an increased expression of various genes, and provides important knowledge for the understanding of biological processes. In this thesis, 13

target genes were selected for the study of molecular responses in shrimps exposed to teflubenzuron.
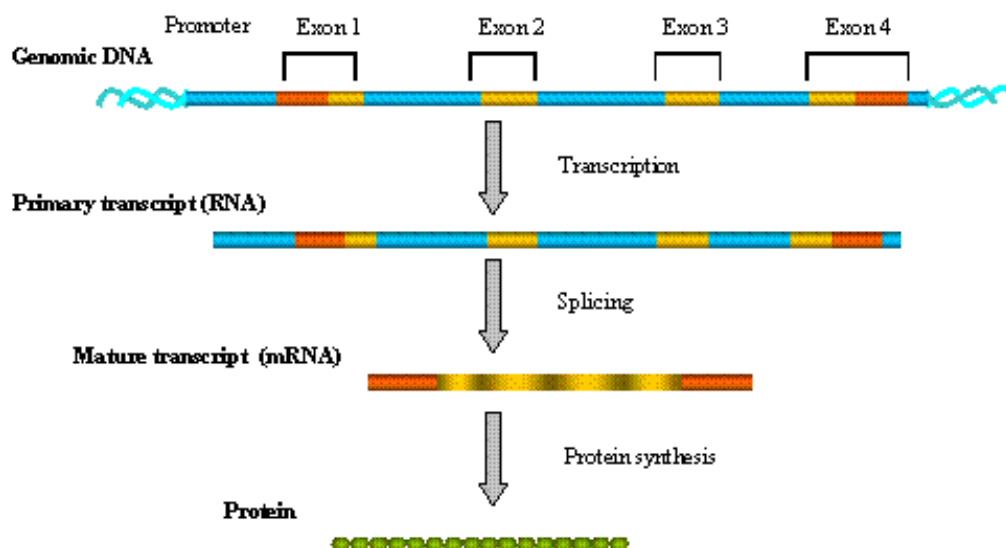
Figure 1.6.1 Gene expression: the pathway from DNA to protein. The genomic DNA is transcribed, spliced and translated for the synthesis of proteins to occur. This figure is obtained from the National Center for Biotechnology Information (NCBI).

1.6.1 DETOXIFICATION GENES

The detoxification processes in cells involves activation of several defence regulators. The information about the defence mechanism in shrimps are limited, hence the information given will concern insects and crustacean in general. However, studies on elimination of toxicants in shrimps assume that enzymes like the cytochrome 3A (CYP3A) is an important regulator of detoxification processes in cells (James and Boyle, 1998). Cytochrome P450 are a family of heme proteins catalysing the oxidative metabolism of many chemicals within the lipophilic membrane of the endoplasmic reticulum (Gates, 2017). The proteins are often used as biomarkers in environmental toxicology studies, mostly because they are sensitive to stress and are easily induced. Studies have shown that the CYP3A subfamily proteins play an important part in the metabolism of xenobiotics in fish (Ku et al., 2014). Glutathione-S-transferase Mu 3 (GSTM3) is known to be involved in detoxification processes in humans, and belongs to a group of glutathione-S-transferases (GST) responsible for phase II biotransformation in drug

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metabolism and detoxifying of electrophilic substances. Three different classes of the GST enzymes are identified in insects, crustacean and humans, based on their location in cell. The GSTM3 enzymes are located in the cytosol and belongs to the mu class found in humans (Roncalli et al., 2015). In humans, the GSTM3 enzymes play a crucial role in detoxification and conjugation of toxins. The gene-expression of GSTM3 can be affected by DNA injury, leading to methylation of GST which reduce the expression rate of GSTM3, and can therefore be an indication of DNA damage (Qi et al., 2012).

1.6.2 GENERAL STRESS

When exposed to toxic substances, unfolded proteins trigger heat shock responses, which can lead to cell damage as defects in the cytoskeleton, fragmentation and dissembling of organelles, and changes in membrane morphology (Richter et al., 2010; Toivola et al., 2010). The RNA splicing is also affected by heat shock responses, when stress granulates in cytosol, containing non-translation mRNA, interfere with the transcriptions of genes (Buchan and Parker, 2009; Richter et al., 2010). In response to these changes, the synthesis of heat shock proteins, or stress proteins is carried out (Richter et al., 2010). Among the stress proteins, heat shock protein-70 (HSP70) is often used as a biomarker, including in crustaceans, as it might be strongly induced during stress (Ahamed et al., 2010). The HSP70 belongs to a family of heat shock proteins, and are located in the cytosol and in organelles like the mitochondria and the endoplasmic reticulum (Richter et al., 2010). Under stressful condition the HSP70 proteins are activated to inhibit the process of unfolding proteins and to enfold or repair aggregated proteins (Mayer and Bukau, 2005).
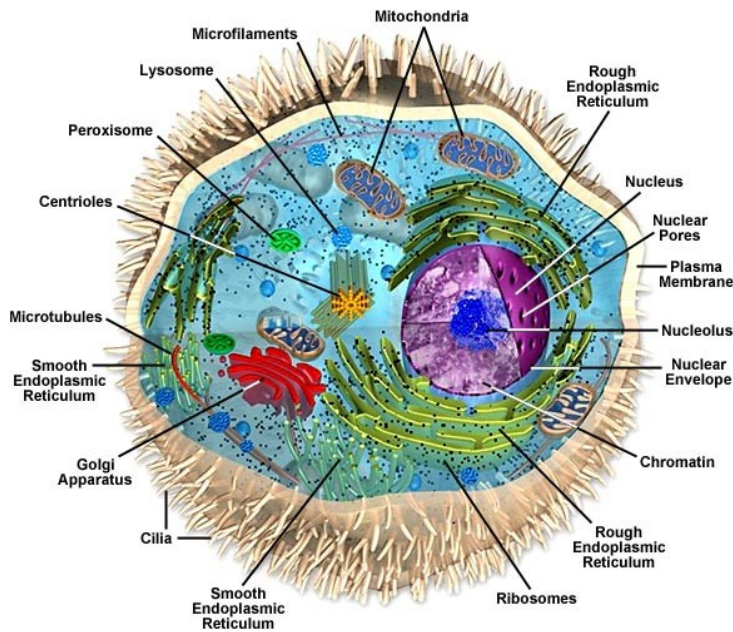
Figure 1.6.2.1: Cell structure. The endoplasmic reticulum (ER) and the mitochondria are a part of the cytosol surrounding the nucleus in the cell. Many proteins involved in detoxification and defence mechanism, like the hsp70, are located in the ER, mitochondria or other organelles in the cytosol. The Figure is obtained from: https://studyfaq.com/blog/animal-cell-anatomy-and-structure.

Accumulation of misfolded proteins, or other factors like exposure of xenobiotic and increased ROS activity, can lead to cell apoptosis (cell death) (Menze et al., 2010). Cell apoptosis is highly regulated by enzymes and the mitochondria plays an important part of signalling apoptosis when cellular stress occurs. Enzymes of the B-cell lymphoma 2 (BCL-2) protein family regulates the traffic across the outer mitochondrial membrane (OMM), and upon apoptosis, the permeability of OMM increases. The increased permeability leads to a release of pro-apoptotic proteins from the mitochondria. Seven proteins of the BCL-2 family have been identified in crustaceans, but within the crustacean clan, there seems to be a high amount of diversity in BCL-2 proteins (Menze et al., 2010). A member of the BCL-2 protein family, the BCL-2 related protein (BCLX) is located in the mitochondrial membrane and ER, and belongs to antiapoptotic proteins within the family. Overexpression of BCLX affects the transmission of Ca^{2+} between mitochondria and endoplasmic reticulum by reducing the content of Ca^{2+} in endoplasmic reticulum, which make the cell more resistant to apoptosis. An increase of calcium on the other hand, trigger apoptosis (Contreras et al., 2010).

1.6.3 CHITIN SYNTHESIS

Teflubenzuron is known to inhibit the chitin synthesis in crustaceans, but the mechanism is still unclear. Some studies have claimed that benzoylureas inhibit chitin synthase during moulting (Langford et al., 2011), whereas some studies indicate that benzoylureas acts by inhibiting the conversion of chitin synthase into its active form (Leighton et al., 1981). In either way, teflubenzuron inhibit the biosynthesis for chitin, and the pathway was first characterized in insects. In 1962, a synthesis mechanism was proposed by Candy and Kilby, starting with glucose, and ending with UDP-N-acetylglucosamine (UDP-Glnac), before the final step from UDP-Glnac to chitin was established by Jarowski et al. in 1965 (Merzendorfer and Zimoch, 2003). Today, a common biosynthesis pathway for insects, fungi and crustaceans is known, and can be seen in Figure 1.6.3.1. The first step is to convert glucose into a polymer N-acetylglucosamine-6-P (GlcNac-6-P) and changing position of phosphate from C-6, to C-1 to form N-acetylglucosamine-1-P (GlcNac-1-P). GlcNac-1-P is then urinylated by UDP-GlcNAc pyrophoshorylase yielding UDP-N-Acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc serves as a sugar donor to the growing chitin chain. The last reaction, polymerization of chitin is catalysed by chitin synthase. As can be seen from this pathway, chitin can also be reused in the new exoskeleton. When shrimps cast their exoskeleton, the old skeleton is often digested to recover minerals used in the new exoskeleton. Many enzymes are involved at different stages of this process, like chitinase (CHIT1A) which are responsible for the degradation of chitin to *N*-Acetylglucosamine (Figure 1.6.3.1) (Merzendorfer, 2011) and the chitobiase (CTBS). Both enzymes can be found in the moulting fluid, secreted by the epidermis. In addition to the epidermis, CTBS is also present in digestive juice secreted by hepatopancreas, and the activity of CTBS is higher in the pre-moulting phase (Zou and Fingerman, 1999). Several steps of degradation are required before chitin is reabsorbed into the new cuticle (Avila et al., 2011).
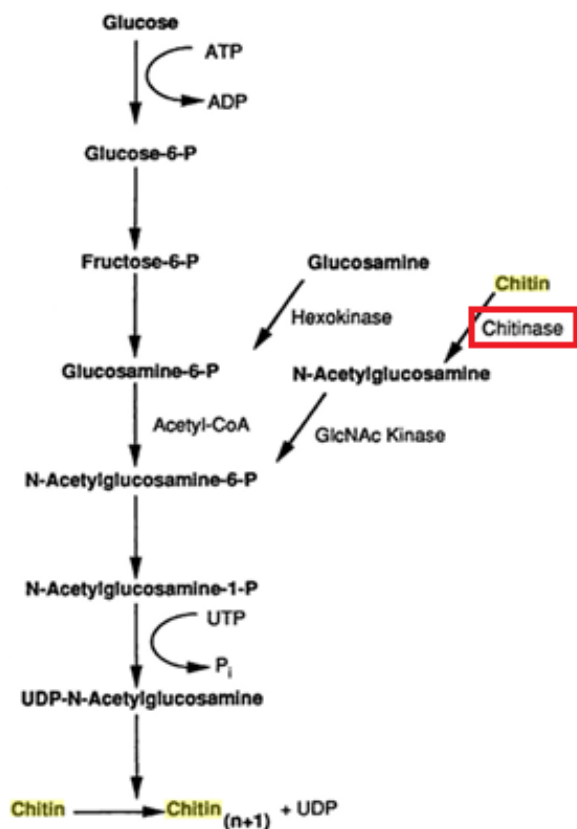
Figure 1.6.3.1: The synthesis of chitin. The enzyme studied in this thesis is marked with a red border. The figure is obtained from a report by Metabolon on global metabolic profiling data for NIFES.

1.6.4 OXIDATIVE STRESS

Reactive oxygen species (ROS) is the collective term of free radicals and reactive molecules produced during ordinary aerobic metabolism. Within the ROS term, there are several molecules having different chemical properties and biological activities. It is important to keep a low level of ROS in cells, as some of these species influence cellular signalling (Figure 1.6.4.1), and can cause oxidation of lipids, proteins and DNA. Superoxide anion ($O_2 \bullet^-$) and hydrogen peroxide (H_2O_2) are ROS affecting cellular signalling and the main source for production of these ROS are the mitochondria and the family of NADPH oxidases (NOXs). $O_2 \bullet^-$ is produced through a one-electron reduction of O_2. In order to maintain a low level of ROS, the body has established an antioxidative defence (Glasauer and Chandel, 2013). However, when the balance between pro-oxidants and antioxidants is interrupted, favouring the oxidants, a condition called oxidative stress occur. This unbalance can cause an increased ROS

production or damage to the antioxidative defence (Qiu et al., 2011; Shao et al., 2012). Prolonged oxidative stress can lead to DNA damage, pathological changes in tissue and dysfunction in the body (Li et al., 2016).
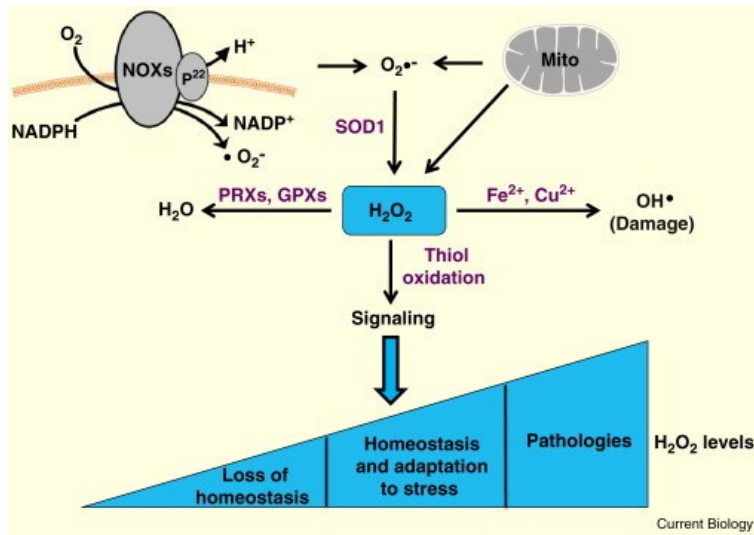
Figure 1.6.4.1: Cellular signalling can be affected by ROS released from mitochondria and enzymes of the NADPH family (NOXs). Mitochondria can either release O2*-, (being convert to H_2O_2 in cytosol by SOD1) or H_2O_2 directly in cytosol. H_2O_2 control cell signalling trough thiol oxidation within proteins, and the level of H_2O_2 determinates the input on homeostasis and adaption to stress. The figure is obtained from the journal article of Glasauer and Chandel (2013).

An increase in ROS can be induce by environmental changes, or by contaminates, like teflubenzuron. Increased ROS formation can occur both directly (contaminant stimulate ROS production) or indirectly (contaminant cause tissue injury leading to release of ROS). There are a great variety of mechanism increasing ROS formation during oxidative stress. When teflubenzuron, or any other organic lipophilic compound is accumulated, it can stimulate ROS production by disturbing electron transport systems e.g. in mitochondria and ER (Livingstone, 2001).

In crustacean, the antioxidant defence against ROS consist of a variety of enzymatic and non-enzymatic agents like the superoxide dismutase (SOD), catalase (CAT), Se-glutathione peroxidase (GPX), gluthatione synthease (GSS) and glutamate-cysteine ligase catalytic subunit (GCLC). The two latter enzymes regulate the levels of glutathion (GSH), an important cellular antioxidant protecting cells by maintaining the thiol groups in proteins, regulating the protein function after translation and detoxification of xenobiotics (Nichenametla et al., 2011). The antioxidant (GSH) is produced within the cell, and has the advantage of reacting with both oxide and hydroxyl radicals, leaving water as the waste product (Cole et al., 2011; Thorstensen, 2014). As shown in Figure 1.6.4.2, glutathione peroxidase (GPX) utilize 2 GSH to turn hydrogen

peroxide with the help of Se into oxidized glutathione (GSSG) and water. Catalase also has a role in catabolizing hydrogen peroxide, in peroxisomes. As can be seen in Figure 1.6.4.1, the SOD-enzymes are responsible for the conversion of superoxide anions ($O_2\bullet^-$) into hydrogen peroxide (H_2O_2) (Shao et al., 2012). In cytosol, the zink-SOD (Zn-SOD) is responsible for this transformation, while manganese-SOD (Mn-SOD) is responsible in the mitochondria (Lubos et al., 2011; Thorstensen, 2014). Comparison studies regarding some of these antioxidative enzymes have shown a lower GPX activity in invertebrates in contrast to vertebrates, while the CAT and SOD activities were similar or higher. This may suggest that CAT and SOD plays an important part of the ant oxidative defence in aquatic invertebrates (Livingstone, 2001).
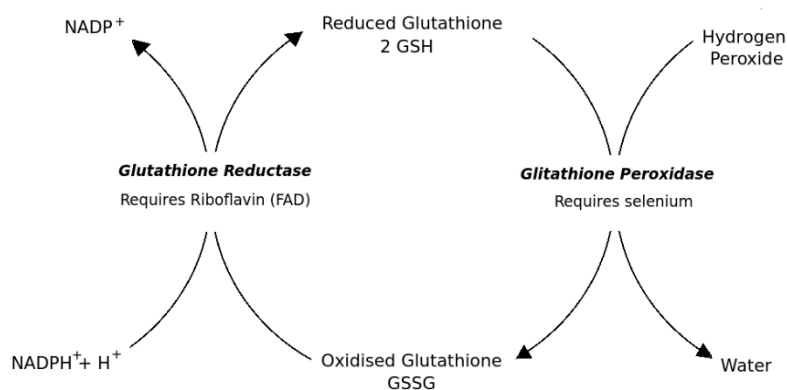
Figure 1.6.4.2: The catabolism of hydrogen peroxide by glutathione peroxidase. The figure is obtained from http://www.robertbarrington.net/wp-content/uploads/2014/01/Glutathione-Metabolism.png.

1.6.5 PROTEIN CARBONYL AND LIPID PEROXIDATION

The method of measuring content of protein carbonyl is one of the most common for determining protein oxidation. The formation of protein carbonyl occurs when ROS attacking the side-chains of proteins leading to an oxidation of amino acids residues and backbone. Derivate of protein carbonyl can also occur indirectly through oxidative cleavage of proteins, or by a secondary reaction of nucleophilic side chains of amino acid with reactive carbonyl compounds like aldehyde or ketone (Dalle-Donne et al., 2003; Kolgiri and Patil, 2017). Protein carbonyls are chemically stable and many commercial assays are available. However, the level of protein carbonyl in different tissues can vary depending on the assay used (Dalle-Donne et al., 2003). Determination of malondialdehyd (MDA) is also used to measure oxidative stress. MDA is the product of lipid peroxidation when polyunsaturated lipids are attacked by ROS, and can react with side chains of amino acid to form protein carbonyl. MDA reacts with

thiobarbituric acid at high temperature, and produce a red malondialdehyde-thiobarbuturic acid product and the absorbance can be measure by a spectrophotometer (Qiu et al., 2011).

1.7 PINK SHRIMPS

According to Langford et al. (2014), only minor concentrations of teflubenzuron were found in deep-water shrimps caught 1-5 km from a farm where treatment with teflubenzuron had occurred less than a month ago. Pink shrimp lives closer to the shore, at 4-700 m depth, and is commonly observed between 100 and 200 m depth. As the farming pens often are located close to the shore, pink shrimps are more exposed to chemical substances used in salmonid farms. Therefore, pink shrimps (*Pandalus montagui*) were studied in this thesis. The pink shrimp belong to the order Decopoda, and like other shrimps they undergo moulting several times during their lifecycle (Britannica, 2017). The pink shrimp is characterized by their semi-transparent pink/brown appearance and the shape of the rostrum witch is slightly bend upward (Ruiz, 2008). The shrimps are located in northern Atlantic, Canada, The British isles and the southern North Sea near Belgium (Holthuis, 1980).

Figure 1.7.1: Pink shrimps from Matre Research Station, photographed by Pål A. Olsvik.

1.8 THE AIM OF THIS STUDY

The main aim of this study was to examine pharmacokinetics and transcriptional changes/oxidative stress in pink shrimps exposed to teflubenzuron.

The pharmacokinetic aspect involved examination of uptake and accumulation of teflubenzuron during the feeding trial, while the oxidative stress analyses gained insight into the mechanistic effect of the antiparasitic compound. A few molecular markers for detoxification, cellular stress and exoskeleton change were also studied.

The results of the current study will be useful for the evaluation of effects of teflubenzuron on crustacean living close to Atlantic salmon farms.

2. MATERIALS AND METHODS

The methodological description in this thesis will be divided into three parts. The first part describes the pharmacokinetic studies carried out at the Contaminants laboratory at NIFES, whereas the second and third parts describe gene-expression analyses and oxidative stress assays preformed at the Molecular Biology laboratory at NIFES.

2.1 THE FEEDING EXPERIMENT

In this experiment, 52 pink shrimps (*Pandalus montagui*) from Hjeltefjorden, in Hordaland, were placed in individual cages (Figure 2.1.1) in three different tanks at the Matre Research Station, Institute of Marine Research (IMR), Norway. The acclimation period was set to 14 days. The shrimps were fed two times per week, Monday and Thursday, for forty-six days. The pellets were 3 mm and contained either no teflubenzuron, a low dose of 0.01 µg/g pellets or a high dose of 0.1 µg/g teflubenzuron per pellets. To prepare the given doses of teflubenzuron in the diet, a 1 mg/g mix (mix-1) was made of 10 mg teflubenzuron and 10 g glucose. The total amount of pellets per group was calculated to be approximately 1200 pellets ≈ 144 g pellets. The amount of teflubenzuron per pellet was calculated to be 0.44 µg in the high dose group, and 0.044 µg in the low group, which gives a total of 528 µg (high dose) and 52.8 µg (low dose) teflubenzuron to coat on 144 g pellets. A full description of how the two doses (528 µg and 52.8 µg) was prepared from the 1 mg/g mix can be obtained from Appendix 1. To lace the drug to the surface of the feeding pellets, cod-liver oil (Møllers, Orkla health) was used for both doses. The percentage of the concentration given in Figure 2.1.2 shows the amount of teflubenzuron in each pellet, compared to the daily amount given to farmed salmons (100%).

Figure 2.1.1: (A and B) the tanks and cages used in the feeding experiment at the Matre Research Station. (C) The carapace length of shrimps. The pictures are taken by Pål A. Olsvik at Matre Research Station.

At the end of the experiment at Matre April 4, 2016 the size of the pink shrimps was record as carapace length (CL) and total weight (g). The carapace length is the part of the shrimps that cover the back part of the head/breast piece (Figure 2.1.1). As the total length of shrimps is difficult to measure because of the tale/back part shape, CL is the most common way to measure length in crustaceans. Of the 52 pink shrimps originally used in this experiment, five shrimps were found dead in their cages at the end of the feeding trial. The remaining 47 shrimps had an average CL at 14 mm, and total weight of 2.39 g. Surviving shrimps after 46 days of feeding were examined for deformities. The shrimps were then killed by freezing in liquid nitrogen and stored at – 80°C at the molecular lab at NIFES. At NIFES, the shrimps were homogenized with mortar and pistil on dry ice to avoid damaging the RNA. All equipment used for homogenizing where cleaned with RNase zap™ (Sigma-Aldrich) to reduce contamination. After homogenizing, tissue samples were stored at – 80°C. Some tissue samples were aliquoted for chemical analysis in the Contaminants laboratory, and these samples were stored at -20°C.
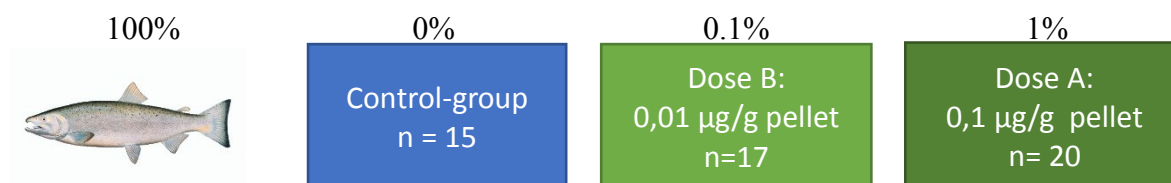
Figure 2.1.2: Concentration of teflubenzuron given in the feeding experiment at Matre Reseach Station, Institute of Marine Reseach, in Norway given as percentage of normal fish therapy dose given daily to salmons affected by lice. *N* gives the number of shrimps in each group.

Extra shrimps

Several additional pink shrimps were obtained from IMR September 8, 2016. Twenty of these shrimps, with an average CL of 15.56 mm and total weight of 3.095 g, were killed in liquid nitrogen and stored at - 80°C. The same homogenizing procedure was performed on these shrimps as for the pink shrimps from Matre Research Station. These shrimps were not a part of the feeding experiment, and the purpose with these shrimps was mainly for testing of new kits. However, as the controls from the feeding experiment contained teflubenzuron above LOQ, the additional shrimps were used as extra controls in all analyses. In this work, the additional shrimps are presented as extra controls.

2.2 Pharmacokinetic analysis

For the pharmacokinetic analysis, a method developed by NIFES for quantitative determination of diflubenzuron and teflubenzuron in seafood was used. Tissue was aliquoted from the whole animal, and the amount measured before starting this assay.

2.2.1 Chemicals

Teflubenzuron (analytical standard), diflubenzuron (analytical standard), diflubenzuron-d4 (analytical standard), acetonitrile, heptane, diethyl ether and acetone (all HPLC grade) were purchased from Sigma–Aldrich (Steinheim, Germany). Tetrahydrofuran (HPLC grade) and ammonium hydroxide (25%) (PA grade) were purchased from Merck (Darmstadt, Germany). The water used was purified with a Milli-Q water purification system from Millipore.

2.2.2 Stock solution and working solutions

Five stock solutions were already prepared before chemical analysis. To prepare the stock solutions and the working solutions, same procedure as describe in the work by Erdal (Erdal, 2012) was performed with just a few modification. In this work, five stock solutions were prepared, one for internal standard (IS), two for diflubenzuron (control and standard curve) and two for teflubenzuron (control and standard curve). From these stocks solutions, two working solutions was made, one for the controls and one for the standard curve. To prepare the stock solutions, 10.00 mg ± 0.04 mg of the substance was add to a vial and diluted with tetrahydrofuran to a concentration of 1 mg/mL. To prepare the working solutions, 50 µL of teflubenzuron and 50 µL diflubenzuron from each stock solutions were mixed before diluting with acetonitrile: water (1:1) to 10 mL, giving a concentration of 5 µg/mL. Thereafter 100 µL of the diluted solution was further diluted with acetonitrile: water (1:1) to 10 mL, giving a final concentration of 50 ng/mL. Diflubenzuron was only included in this analyse due to being a part of the original method.

2.2.3 Preparation/spiking of samples

Approximately 0.5 g (Appendix 2) was aliquoted from tissue samples and then transferred into 15 mL falcon-tubes, and stored at -20°C. In addition to the pink shrimp samples, 9 samples from the extra shrimps, 5 controls, a blank sample with matrix from shrimp and a blank sample without matrix were included. For all the samples, a 9-point calibration curve was set, marked

N_{1-9}. N_1-N_6 were spike with 20, 80,200,400,600 and 800 µL of standard solution (teflubenzuron / diflubenzuron 50 ng/mL), while N_7-N_9 were spike with 10, 20 and 30 µL of the mid-solution (5 µg/mL teflubenzuron / diflubenzuron) made during dilution preparation. For validation, one control (K_1) was spike at LOQ level (1 ng/g), while the rest of the controls were spike at 10 ng/g (K_2, 3 parallels) and 100 ng/g (K_3). Diflubenzuron-d4 was used as internal standard (IS), and stock solutions with diflubenzuron were included in this method. All the samples were added 200 µL IS (50 ng/mL diflubenzuron -d4 solution).

2.2.4 EXTRACTION AND DETERMINATION OF TEFLUBENZURON IN SHRIMPS

Teflubenzuron, diflubenzuron and IS were extracted from the sample matrixes by dissolving each sample in 5 mL acetone, before shaken on whirl mixer for 1 min. Afterward the samples were placed in ultrasonic bath for 10 min. and then centrifuged (Eppendorf centrifuge 5810 Hamburg, Germany), for 3 min. at 3220 g.

The supernatants were transferred to 10 mL centrifuge tubes, before steaming dry with nitrogen gas (cleanness $\geq$ 99.995) at 40°C. The dried samples were dissolve in 5 mL heptane and carefully shaken on whirl mixer before they were transferred to 20 mL Automated Solid Phase extraction (ASPEC) tubes. The samples were purified by solid phase extraction on ASPEC GX-274 (Gilson, Middleton, USA). At first, the silica-column was conditioned with 2.5 mL heptane prior to loading the sample. Afterwards, the column was washed with 3 mL of heptane before washing with 5 mL diethyl-ether/heptane (5:95) and 5 mL diethyl-ether/heptane (10:90). At the end, the analytes were eluted with 5 mL diethyl-ether/heptane (40:60) before evaporated to dryness using nitrogen gas. Thereafter the analytes were dissolved in 250 µL acetone nitrile /water (75:25). The samples were filtered through a 0.45µm syringe filter and transfer to a 2.0 mL HPLC vial before analysing with LC-MS/MS.

3.2.5 LC-MS/MS (QQQ)

LC-MS/MS is a selective and sensitive instrument using the benefits of liquid chromatography (LC) to separate the compounds, and triple quadrupole mass spectrometry (MS/MS) to detect and measure the concentration. When preforming LC, high pressure is used to force a solvent through a column containing a stationary phase. Throughout the column the molecules are separated by speed (retention time) due to their affinity to the stationary phase. Detection with mass-spectrometry (MS) on the other hand, uses mass-to-charge ratio of ionized molecule to

distinguish molecules. To give a mass spectrum, the molecules are first ionized, and then accelerated by an electronic or a magnetic field, before being separate by mass charge ratio (m/z)(Harris, 2010).

To analyse teflubenzuron, the LC-MS/MS was carried out using a HP 1200 LC-system (Hewlett-Packard, Waldbronn, Germany) couplet to an Agilent 6460 triple quadrupole mass spectrometer (Agilent technologies, Waldbronn, Germany). Masshunter software (Agilent Technologies) was used for data treatment. The injection volume was 2 μL, and a solution of acetonitrile (75%) and purified water (25%) was used as mobile phase with a flow rate of 0.4 mL/min at room temperature. The retention time for teflubenzuron was 0.83 min, and 0.55 min for IS. Agilent Jet Stream negative electrospray (AJS ESI) was used to ionize the liquid at the interface, and multiple reaction monitoring (MRM) was used to detect the analytes. The mass to charge ratio (m/z) was 379.0/339.0 for teflubenzuron quantifier transition, 379.0/195.9 for teflubenzuron qualifier transition and 313.0/ 293.1 for IS (Appendix 2). Furthermore, these parameters were used: drying gas temperature: 300°C; gas flow: 5 L/min; nebulizer pressure: 45 psi; sheet gas heater: 250°C; sheat gas flow: 11 L/min; Capillary voltage: 3500 V and charging voltage: 500 V. Level of quantitative (LOQ) was set to 0.2 ng/g. The method was linear (R= 0.97) up to 1000 ng/g, and the relative standard derivation was 20%.

2.3. GENE-EXPRESSION ANALYSES

For the gene-expression analyses, RNA was isolated from the shrimp tissue, and the quality and integrity were controlled, before the RNA was synthesised to cDNA. Two cDNA plates were prepared, and the primers were run at RT-qPCR. All potential primers were tested with One-Step PCR, followed by gel-electrophoresis. All materials and kits used in these analyses are listed in Table 2.3.1 and Table 2.3.2. Materials and kits used for the colorimetric analyses to measure content of protein carbonyl and malondialdehyde (MDA) in the tissue-samples are also included in the table.

Table 2.3.1: Materials used in the gene-expression analyses and the colorimetric analyses.

Chemical/reagens	Supplier/product no.	Method
Purified water from Milli-Q water system	Millipore	all
Chloroform	Sigma-Aldrich Cat. No 1731042	RNA-isolation
QIAzol Lysis Reagents	Qiagen cat. No 79306	RNA-isolation
RNase Zap™	Sigma-Aldrich no R2020	RNA-isolation
TagMan® reverse transcription reagens kit TaqMan RT buffer 10X 25mM magnesium chloride Deoxy NTPs RNase inhibitor Multiscribe reverse transcriptase (50U/μL)	Applied Biosystems Item no. N808 0234	RT-reaction
LightCycler® 480 SYBRGreen master	Roche Applied Science No. 4887352001	RT-qPCR preparation
OneStep RT-PCR kit 5x Qiagen One Step RT-PCR buffer 5x Q-solution dNTP mix	Qiagen No 210212	OneStep RT-PCR

Qiagen One Step RT-PCR Enzyme mix		
RNase inhibitor	Applied Biosystems (RT reaction kit) Item no N8080234	OneStep RT-PCR
TE buffer (1X) PH8	PanReac Applichem Item no A2575	Diluting primers
Utra Pure™Agarose	Invitrogen Item no. 0000419824	Electrophorese
TAE buffer 50 x	BioRad cat. No 161-0773	Electrophorese
Gel Red™ nucleic Acid Strain	Qiagen no 130175937	Electrophorese
Gel pilot loading gel x5	Qiagen no.142324491	Electrophorese
Gel pilot 50 bp ladder	Qiagen no 1036712	Electrophorese
<u>Protein carbonyl colorimetric Assay kit</u> 12M Hydrochloric acid 2,4-dintrophenylhydrazine (DNPH) 1mg/g trichloroacetic acid (TCA) Ethyl acetat Etanol Guanidine hydrochloride	Cayman Chemical No. 10005845 No. 10005846 No. 10005847 No. 10005850 No. 10005849 No. 10005848	Measure content of protein carbonyl
Phosphate buffered saline tablet	Sigma-Aldrich No. P4417	Protein carbonyl colorimetric Assay kit
1mM EDTA	Sigma-Aldrich Cat. No 03680	Protein carbonyl colorimetric Assay kit

Lipid peroxidation (MDA) assay kit	Sigma-Aldrich cat. no	Measure content of MDA
MDA lysis buffer		
TBA	MAK065A	
MDA standard	MAK085D	
BHT(100x)	MAK085E	
	MAK085C	
Glacial acetic acid	Merck No. 100063	Measure content of MDA

Table 2.3.2: Kits used in the gene-expression analyses and the colorimetric analyses.

Kit	Description	Supplier/product no.
EZ1 RNA universal tissue kit	Isolate RNA from tissue	Qiagen, no. 956034
RNA 6000 Nano LabChip Kit	Check integrity of RNA	Agilent Technologies
TaqMan® reverse transcription reagens kit	Make cDNA out of RNA	Applied Biosystems Item no. N808 0234
OneStep RT-PCR kit	Checking primers for qPCR	Qiagen cat. No 210212
Protein carbonyl colorimetric assay	Measure content of protein carbonyl	Cayman Chemical, product no 10005020
Lipid peroxidation(MDA) assay kit	Measure content of MDA	Sigma Aldrich cat. MAK085

2.3.1 RNA-ISOLATION

When analysing gene-expression, the first step is isolation of RNA from tissue. For mRNA analyses, it is of crucial importance to isolate high quality RNA, as leftovers of proteins and DNA can interfere with reagents or samples during preparation and measurements. For the pink shrimp tissue, a method for purification of RNA using an EZ1 Bio robot (Qiagen) and liquid-liquid extraction with guanidinium salt-phenol solution and chloroform was used. Due to the reaction with phenol and chloroform, a phase separation occurs as DNA and proteins fractionate into a phenol phase at the bottom of the vial, leaving RNA in an aqueous phase at top. Guanidium salts are added with phenol to minimize cleavage of nucleic acids during reaction (Zumbo, 2012). After separation, the RNA is further purified at the EZ1-robot, where the RNA is bound to magnetic beads during time run, and eluted by water at the end.

At first, 750 µL QIAzol lysis reagent and 4 pellets were added to a 2 mL tube suitable for homogenizing. The tissues (about 60-80 mg) were transfer to the tubes and homogenized at 6000 rpm, 3 x 15s (precellys 24, Bertin technologies), before incubating for 5 min. at room temperature. Afterwards, 150 µL chloroform was added. The samples were shaken for 15 s and incubated for another 2-3 min. before centrifuging for 15 min, 12 000 g at 4°C (Centrifuge 5415, Eppendorf, Hamburg, Germany). While the samples were centrifuged, DNase was thawed on ice, before 10 µL was add to each reagent cartridges to digest and remove DNA contamination in the samples. The elution tube (1.5 mL), sample tube (2 mL), disposable filter tips and disposable tip holders were prepared according to the protocol for EZ1 RNA universal tissue kit (Qiagen). The upper layer containing RNA was transfer to the sample tubes before running EZ1. The samples were put on ice afterwards.

2.3.2 QUALITY CHECK

The concentration and purity of RNA were measured with the NanoDrop® ND1000 (Termo Fisher Scientific, Wilmington, USA) according to the manufacturer's protocol. To check the integrity of the RNA, the Bioanalyzer 2100 and the RNA 6000 Nano LabChip Kit (Agilent Technologies, Santa Clara, USA) were used according to the manufacturer's protocol. All samples were run simultaneously (twelve per chip), chosen randomly from the samples from Matre and IMR.

2.3.3 RT-REACTION (REVERSE TRANSCRIPTASE REACTION)

For the synthesis of cDNA from RNA, TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA) was use, and the procedure was performed according to manufacturer's protocol to a final reaction volume of 50 µL. The RNA samples were thawed on ice before the needed amounts (2.40-26.49 µL) to make a 50 ng/µL (±5%) dilution with ddH$_2$O were transferred into new tubes and diluted.

A standard curve containing RNA from all samples was made as shown in Figure 2.3.3.1.

A 40 µL **B** 40 µL **C** 40 µL **D** 40 µL **E** 40 µL **F**

+ 40 µL ddH$_2$O in tube B-F

100 ng/µL   50 ng/µL   25 ng/µL   12.5 ng/µL   6.25 ng/µL   3.125 ng/µL
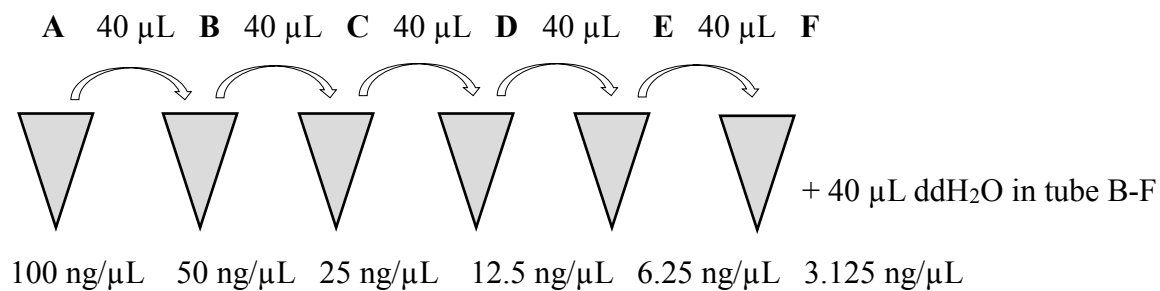
Figure 2.3.3.1: The diluted standard curve with the concentrations 100 ng/µL, 50 ng/µL, 25 ng/µL, 12.5 ng/µL, 6.25 ng/µL and 3.125 ng/µL. Tube B-E were first diluted with 40µL ddH$_2$O before transferring 40 µL of the mixture into the next tube. At the end, tube A-E had a total volume of 40 µL, while F had a total volume of 80 µL.

Two cDNA plates were prepared, with the standard curve run in triplicates (for both plates), and samples run in triplicates (plate 1) and duplicates (plate 2) on a 96 well reaction plate. Each cDNA plate also contained two controls, a non-amplification control (nac) without enzymes and no-control template (ntc) without RNA. The setup for both cDNA plates can be seen in Figure 2.3.3.2 and 2.3.3.3. The reverse transcriptase mix (RT mix) without enzymes was prepared according to the manufactures guideline (Appendix 3) and 40 µL was added to the nac well, before adding enzymes in the RT mix and distribute to the rest of the wells. In addition to the RT mix, 10 µL of diluted RNA was add to each well, except or the ntc and nac well. In the nac well, 10 µL of diluted RNA mix with similar concentration was add, and for the ntc well, the remaining 10 µL was fill with ddH$_2$0, given a total of 50 µL in each well. The plate was centrifuged at 50 g (centrifuge 5810, Eppendorf, Hamburg, Germany) for some seconds, and RT reaction was performed at the PCR machine, Gene Amp PCR System 9700 (Applied Biosystems, Foster City, USA) overnight following the program given in Appendix 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	std1	std1	std1	std2	std2	std2	std3	std3	std3	std4	std4	std4
	1000 ng	1000 ng	1000 ng	500ng	500ng	500ng	250ng	250ng	250ng	125ng	125ng	125ng
B	std5	std5	std5	std6	std6	std6	2016-529/1	2016-529/1	2016-529/1	2016-529/2	2016-529/2	2016-529/2
	63ng	63ng	63ng	31 ng	31 ng	31 ng	500ng	500ng	500ng	500ng	500ng	500ng
C	2016-529/3	2016-529/3	2016-529/3	2016-529/4	2016-529/4	2016-529/4	2016-529/5	2016-529/5	2016-529/5	2016-529/6	2016-529/6	2016-529/6
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng
D	2016-529/7	2016-529/7	2016-529/7	2016-529/8	2016-529/8	2016-529/8	2016-529/9	2016-529/9	2016-529/9	2016-529/10	2016-529/10	2016-529/10
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng
E	2016-529/11	2016-529/11	2016-529/11	2016-529/12	2016-529/12	2016-529/12	2016-529/13	2016-529/13	2016-529/13	2016-529/14	2016-529/14	2016-529/14
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng
F	2016-529/15	2016-529/15	2016-529/15	2016-529/16	2015-529/16	2016-529/16	2016-529/17	2016-529/17	2016-529/17	2016-529/18	2016-529/18	2016-529/18
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng
G	2016-529/19	2016-529/19	2016-529/19	2016-529/20	2016-529/20	2016-529/20	2016-529/21	2016-529/21	2016-529/21	2016-529/22	2016-529/22	2016-529/22
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng
H	2016-529/23	2016-529/23	2016-529/23	2016-529/24	2016-529/24	2016-529/24	2016-529/25	2016-529/25	2016-529/25		nac	ntc
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng			

Figure 2.3.3.2: c-DNA plate 1 with samples 1-25 (controls and low dose) run in triplicates. Nac is without enzyme, and ntc is without RNA

	1	2	3	4	5	6	7	8	9	10	11	12
A	std1	std1	std1	std2	std2	std2	std3	std3	std3	std4	std4	std4
	1000 ng	1000 ng	1000 ng	500ng	500ng	500ng	250ng	250ng	250ng	125ng	125ng	125ng
B	std5	std5	std5	std6	std6	std6	2016-529/26	2016-529/26	2016-529/27	2016-529/27	2016-529/28	2016-529/28
	63ng	63ng	63ng	31 ng	31 ng	31 ng	500ng	500ng	500ng	500ng	500ng	500ng
C	2016-529/29	2016-529/29	2016-529/30	2016-529/30	2016-529/31	2016-529/31	2016-529/32	2016-529/32	2016-529/33	2016-529/33	2016-529/35	2016-529/35
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng
D	2016-529/37	2016-529/37	2016-529/38	2016-529/38	2016-529/39	2016-529/39	2016-529/40	2016-529/40	2016-529/41	2016-529/41	2016-529/42	2016-529/42
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng
E	2016-529/43	2016-529/43	2016-529/45	2016-529/45			2016-529/47	2016-529/47	2016-529/48	2016-529/48	2016-529/50	2016-529/50
	500ng	500ng	500ng	500ng			500ng	500ng	500ng	500ng	500ng	500ng
F	2016-529/51	2016-529/51	2016-1415/1	2016-1415/1	2016-1415/2	2016-1415/2	2016-1415/3	2016-1415/3	2016-1415/4	2016-1415/4	2016-1415/5	2016-1415/5
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng
G	2016-1415/6	2016-1415/6	2016-1415/7	2016-1415/7	2016-1415/8	2016-1415/8	2016-1415/9	2016-1415/9	2016-529/46	2016-529/46	nac	ntc
	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng	500ng		
H												

Figure 2.3.3.3: c-DNA plate 2 with samples 26-33, 35,37-43,45-48,50-51 (low dose and high dose) + 1-9 (extra controls) run in duplicates. Nac is without enzyme, and ntc is without RNA

2.3.4 REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)

RT-qPCR is a method used to quantify small nucleic acids from a single or double stranded DNA template when comparing gene expression from different tissues. The principle of PCR is to synthesize a new strand complementary to the DNA template with a heat-stable polymerase. Two primers (with similar melting temperature) have to anneal each complimentary template before the polymerase can extend the primers into a new strand by using dNTPs (four nucleotides of DNA) as building blocks. The method is based on a heat cycle, as high temperature (95°C) is required to separate the strands, before temperature is lowered for the annealing of primers to occur. The elongation step (extension of primer) is often set to 60-72°C, depending on the method used. A fluorescent reporter is also required to bind to the products and report by fluorescence the amount of product produced during reaction (Kubista et al., 2006). In the procedure used for this thesis, SYBR Green dyes was use as a fluorescent reporter.

Moreover, 3 or more endogenous controls (reference gene) are often necessary to normalize for variation between samples and correct for errors in sample preparation (Derveaux et al., 2010). For teflubenzuron in shrimps *actb,uba52, ef1a and rpl13* were selected as potential reference genes and the program GeNorm v.3.5 was used to determine the stability of the reference gene and to calculate a normalization factor, M (Vandesompele et al., 2002)

In addition to the four references genes, 13 genes were quantified with RT-qPCR. Primer Express 2.0 software (Applied Biosystems,Foster City, USA) was used to select appropriate primers form the sequenced shrimp gene. Exon-exon borders were not considered when making the PCR primers due to lack of genomic information. A transcriptome made from whole-shrimp RNA (from both control and exposed shrimp) was sequenced and assembled with Illumina paired-end read (PE150) to search for target genes. The sequencing was prepared by using the NEBNext Ultra RNA library Prep Kit (Illumina, NEB, USA) according to the manufactory's guideline. Contigs (overlapping DNA sequences defining a region of the genome) were annotated to the seven databases NR, NT, Swiss-Prot, KEGG, COG, GO and InterPro by using a BLAST cut of 10^5. The unassembled singletons were annotated using ESTcan employing default settings (Iseli et al., 1999). Similarity to previously examined gene sequences from experiments with dose-response effects of teflubenzuron exposure in transcripts in juvenile lobster (Olsvik et al., 2015), were examined by using Geneious Software (Biomatters, Auckland, New Zealand).

At first, the cDNA plates were thawed on ice, before centrifuging at 800 g (centrifuge 5810, Eppendorf, Hamburg, Germany) for one min. and vortex at 1500 rpm for 5 minutes (MixMate PCR96, Eppendorf, Hamburg, Germany). A two-fold dilution (50:50) of cDNA was performed by Biomak®4000 pipetting robot (Beckman Coulter, USA), before making the SYBRGreen reaction mix (Appendix 3) with the gene specific primers (500 nM of each). Biomak®4000 pipetting robot was further used to distributing 8 µL of the master mix and 2 µL of cDNA to a Lightcycler® 480 multiwell plate (384 well plate). Afterwards, the plates were spun down at 1500 g for 2 min. before running on CFx384 touch™ Real-Time PCR Detection System (Bio-Rad, USA).

Table 2.3.4.1: Accession, gene name, abbreviation, amplicon size and PCR efficiencies for the PCR primers. The PCR efficiencies marked in red are too high, and these genes were excluded from further analysis.

Accession/contig name	Abbreviation	Gene name	Forward Primers	Reverse primer	Amplication size (bp)	PCR efficiency
CL10.Contig10_PAND_MONT	actb	Actin beta	GGGTCAGAAAGGACGCCTACA	TCTCCATGTCGTCCCAGTTG	110	2.102
Unigene5748_PAND_MONT	uba52	Ubiquitin A-52	CGACTTCGTGGGTGGGTGTCAGC	TTGGGTGAAGAGGAGCATA	103	2.194
CL497.Contig3_PAND_MONT	ef1A	Elongation Factor 1 alpha	AGACCGACGAACTGGCAAAG	GTCTCCACGCACATGGGTTT	105	2.360
Unigene19060_PAND_MONT	rpl13	Ribosomal protein L13	ATGTGCGCGTCGTTGGTTCACT	GGTGACGACAGGCCTAAGC	116	2.021
>c186887_g1:orf1 len=678 frame:-2	Mn-sod	Manganese superoxide dismutase	GGGATGGCTGGGATACAACA	GACCAGTGGTGGCTTCCAAA	86	2.172
>c190402_g1:orf1 len=441 frame:1	CuZn-sod	Copper-zinc superoxide dismutase	GGACGGTGAAGGGTCTATCG	TCGCTCCAGTGGGTTGTAATG	110	3.687
Unigene1425_PAND_MONT	cat	Catalase	CTACCAGCGAGATGGGCCTAT	CGACATCCATGGTGCAACAG	134	2.214
Unigene17524_PAND_MONT	gpx1	Se-glutathione peroxidase-1	GTATGTGCGTCCAGGGAACA	TCGCTCCTTCAGGTACTGGAA	106	2.202

Unigene ID	Symbol	Description	Forward	Reverse		
>Unigene12450_PAND_MONT	gpx4	Se-glutathione peroxidase-4	GGCCATACTTGCATCGTTG TG	TCCGTAGGCCTTTGGATTCA	112	2.035
Unigene27413_PAND_MONT	gclc	Glutamate-cysteine ligase catalytic subunit A	GCTGGGATGCGTGGATTTT	TCGTGGATGGCTCTGTGTGA	109	2.144
c204153_g4;orf1 len=1653 frame:-1	gss	Glutathione synthease	TCCCCCAGCATCCAGTATCA	GAAAGAGTTGCCCGACGTGTT	120	2.136
CL1199.Contig1_PAND_MONT	hsp70	Heat shock protein-70	TGCTGGTTGGGAGGTTCTACGA	TGCTGCGCCATATGCTACAG	119	2.208
Unigene28315_PAND_MONT	bclx	Bcl-2 related protein	TTGGAGTCGGATAGTGGCAA T	TCACGTAGGGGGTCAAAACC	140	2.318 = put as 2.
Unigene30129_PAND_MONT	cyp3a	Cytochrome p450 family3 subfamily A A	GGACCTCGAAGTTGTTGTTGG	TGACGGTTGGCATTTCAAAC	136	2.202
CL1964.Contig1_PAND_MONT	gstm3	Glutathione-S-tranferase Mu3	GTTCCGCCATTATGCGTCAT	GAGGGCGTGCAAATGTCATGT	125	2.119
>c191246_g1;orf1 len=1200 frame:3	chit1a	chitinase	AGGTCCCAAGCGGGAAGGT T	CCTCCTCCACCCACTTGAAA	121	2.17
Unigene9404_PAND_MONT	ctbs	Chitobiase	GCATCCCAGCGCGGAAAAGTT	CCCCGCTGCATCACTACAGT	137	2.166

2.3.5 ONE STEP RT-PCR

One Step RT-PCR is a method where the synthesis of cDNA from RNA and PCR occurs in same vial and is often used to test the integrity of potential primers with gel-electrophoresis, before RT-qPCR. Qiagen One Step RT-PCR kit was used in this work, and the method was performed according to the manufactures protocol. All primers were diluted with TE buffer to a concentration of 50 μM before starting this assay.

All the non-enzymatic reagents were mixed and 0.2 mL was added to new vials, before adding the enzymes and the RNA. The mix was centrifuged (MiniStar Silverline, VWR, Vienna, Austria), and the RT-PCR reaction was performed following the program given in Appendix 3, at Gene Amp PCR System 9700 (Applied Biosystems, Foster city, USA).

2.3.6 ELECTROPHORESIS WITH AGAROSE GEL

Electrophoresis can be used to separate different fragments of DNA by size. The principle of this method is that negatively charged DNA will move towards a positively charged electrode, and the speed is dependent on size of the DNA fragments. Larger fragments will move slower than small fragment. The resistance in the gel also affects the speed of fragments, and higher density forces the fragments to move slower. Density of gel and the choice of ladder (fragments with known length) is selected by PCR-product size (number of base pair in DNA). In this work, PCR products containing few base pair (100-150 bp) were use, and for this reason, a higher density agarose gel (2%) was prepared and a 50 bp ladder used.

When preparing a 2% agarose gel, 3-gram agarose powder was measured and transferred to a 500 mL Erlenmeyer flask. The powder was dissolved in 150 mL of 1xTE buffer under heat (microwave) and cooled before adding 15 μL Gel Red™ nucleic acid Strain. Gel Red™ nucleic acid Strain is added to visualize DNA under UV-light as it binds to the moving fragments. The agarose gel solution was poured into the casting vessel and bobbles were removed with the 15 well comb. The comb is used to form wells in the gel. The agarose gel had to congeal before adding a primer mixture of loading buffer (2 μL) and PCR product (8 μL) for each primer, to the wells of the gel. A 6 μL ladder was added to the first well. After running electrophoresis, the gel was visualized and photographed in G-box gel doc (Syngene, Cambridge, England).

2.4. OXIDATIVE STRESS ASSAY

2.4.1 PROTEIN CARBONYL COLORIMETRIC ASSAY KIT

A kit from Cayman Chemical, product no. 10005020 was used for colorimetric measurements of protein carbonyl content. Protein carbonyls are aldehydes or ketones formed at the side chains of proteins after attack from free oxygen radicals, indicating oxidative stress (Dalle-Donne et al., 2003). All reagents, except for the phosphate saline buffer solutions were obtained from supplier. The two solutions, phosphate saline buffer and phosphate buffer with 1mM EDTA were prepared at the lab. For the phosphate buffer with 1mM EDTA, pH was adjusted to 6.7 with diluted 2.5 M hydrochloric acid. For the centrifuge, Eppendorf centrifuge 5810 (Hamburg, Germany) was used.

For preparation, homogenized tissue (200-300 mg) was aliquoted on dry ice and rinsed in phosphate saline buffered solution. Afterwards 1 mL of cold phosphate buffer containing 1mM EDTA was added before the samples were homogenized at 6000 rpm, 3 x 15s (Precellys 24, Bertin technologies) and centrifuged for 15 min., 10.000 g, at 4°C. The supernatant was then transferred to another vial to measure the absorbance ratio (280/260 nm) using the Nanodrop ND1000 with the homogenizing buffer as blank. A ratio lower than 1 indicated contamination of nucleic acids.

At first 200 µL of sample was transferred to 2 mL plastic tube, one for sample tube (S) and one for control tube (C). Then 800 µL of DNPH was added to the sample tubes, and 800 µL 2.5 M HCl was added to the control tubes, before vortexing and incubating in the dark for one hour. Every 15 min. during incubation, the samples were vortexed. After incubation, 1 mL of 20% TCA was added to the samples, vortexed and placed on ice for 5 min. Afterwards, the samples were centrifuged for 10 min., 10 000 g at 4°C, and the supernatants were discarded. The next step was to resuspend the pellets in 1 mL of 10% TCA, before the samples were vortexed and set on ice for another 5 min. The samples were centrifuged for 10 min., 10 000 g at 4°C, and the supernatant was discarded once more. For the washing step, 1 mL of ethyl acetate 1:1 ethanol was used and these steps were repeated three times. At first, the ethyl acetate-ethanol mix was added to each sample, vortexed and centrifuged for 10 min., 10 000 g at 4°C before discarding the supernatant. The final step was to resuspend the protein pellet in 500 µL of guanidine hydrochloride, vortexed and centrifuged to remove any leftover debris. Then 220 µL of supernatant of the S tube was transferred to two wells of the 96 well plate, and 220 µL of supernatant of the C tube to another two wells of the 96 well plate. The absorbance (at 380 nm)

was measured at a Victor x5 (Perkin Elmer, Waltham, MA) spectrophotometer, before the concentration was calculated according to the manufactures guideline.

2.4.2 LIPID PEROXIDATION (MDA)

Malondialdehyd (MDA) is the main product of the reaction when lipids are oxidized, and the content of MDA can be measured as an indication of oxidative stress (Qiu et al., 2011). All the reagents, except from the glacial acetic acid, were supplied in the lipid peroxidation (MDA) kit from Sigma Aldrich, Catalogue no. MAK085. In this assay, MDA concentration is determinate by colorimetric analysis. For the centrifuge, Eppendorf centrifuge 5810 (Hamburg, Germany) was used.

At first, all the samples were thawed at room temperature. The TBA solution was made by reconstituting a bottle with TBA powder with 7.5 mL glacial acetic acid before bringing the volume to 25 mL with purified water. After that, the aliquoted tissue (about 10 mg) was put on ice and 300 µL of MDA lysis buffer with 3 µL of BHT (100x) was added before homogenizing at 6000 rpm, 2 x 15s (Precellys 24, Bertin technologies) Then the samples were centrifuged at 13 000 g for 10 min. and the pellets discharged. Meanwhile, the MDA standards for colorimetric detection were prepared by diluting 4.17 M MDA standard with 980 µL of water to make a 0.1 M MDA standard solution, of which 20 mL was further diluted with 980 µL to get a 0.2 mM standard. From the 0.2 mM solution 0, 2, 4, 6, 8 and 10 µL were transferred into separate micro centrifuge tubes, generating 0 (blank), 4, 8, 12, 16 and 20 nM standards by diluting with ddH2O to a final volume of 200 mL. Afterwards, 600 µL of the TBA solution was add to each vial containing standards and samples to form a MDA-TBA adduct. Then the samples were incubated at 95°C for 60 min. After incubation, the samples were placed on ice and cooled to room temperature for 10 min. From the reacting mixture, 200 µL was pipetted into a 96 well plate and analysed (532 nm) at a Victor x5 (Perkinelmer, Waltham, MA) spectrophotometer. The concentration was calculated according to the manufactures guideline.

3. RESULTS

3.1 STATISTICAL ANALYSIS

The statistical analyses for this thesis were performed in GraphPad Prism 7.02 software (GraphPad Software Inc, San Diego, CA, USA 2017). In GraphPad Prims 7.02 one-way analysis of variance (one-way ANOVA) with Holm-Sidak's multiple comparison test was used to compare the means of accumulated teflubenzuron, CL and weight in all treatment groups, and to compare the mean normalized expression (MNE) of the transcripts between the groups. In cases where the Bartlett and Brown Forsyth's test showed a significant difference (P <0.05), the data was log-transformed (Y=Log(Y)). The same statistical method was also used to compare the concentration of protein carbonyl and MDA between the groups. Correlation analyses were first performed in Statistica 13.1 (Dell Inc. Tulsa, OK, USA 2016), but as the correlation analyses performed in GraphPad included more data, this was the program used. Spearman's rank correlation analysis was used to look for significant correlations among the transcript, content of protein carbonyl, content of MDA, and accumulated teflubenzuron. Moreover, CL, total weight, deformities, speckled eyes, black dots and moulting were also included in correlation analyses. For the last four parameters, only the x-factor one or zero were given. As these are nominal data, Fisher's exact test was perform at Graphpad's webpage (GraphPad, 2007) to look for any significant difference among the three groups.

3.2 Morphological studies

After 46 days of feeding, the mean CL (mm) was 14.4 ±1.7, 13.4 ±1.8 and 14.1 ±1.9 for control, low dose and high dose respectively. The extra control shrimps had an average CL at 16.3 ±1.4. The mean body weight (g) was 2.6 ±0.9, 2.2±0.9 and 2.4±0.7 for the control, low dose and high dose, and 3.5±1.0 for the extra control shrimps, respectively. A significant difference in CL and weight were found between the extra control shrimps and other three treatments, but there was no significant difference among the initial controls, low and high dose.

Moreover, thirteen individuals, four from control and high dose and five from the low dose had gone through moulting during the feeding experiment. Deformities like speckled eyes, stiff and cracked walking legs were seen on shrimps receiving either low or high dose of teflubenzuron (Figure 3.2.1), and five shrimps from the high dose group were found dead. The dead shrimps were disintegrated, and could not be examined any further. Moreover, several shrimps, from both control, low dose and high dose had black spots on the exoskeleton (Figure 3.2.2). Results from Fishers exact test can be found in Table 3.2.3. As can been seen in this table, there were significant differences between the control group and the high dose group when comparing deformities, black dots and speckled eyes. Significant differences were also found between the control group and the low dose group for the first two parameters. When comparing mortality rate between the groups, a significant difference was found between the high dose group and the low dose group.

Figure 3.2.1: Shrimp from the low dose group with deformed walking legs.

Figure 3.2.2 shrimp from the low dose group with black spots on exoskeleton.

Table 3.2.3: Fishers exact test for comparing visible deformities, black dots and speckled eyes among the three treatment groups after 46 days of feeding with either none, a low dose (0.1% of normal fish treatment dose) or a high dose (1% of normal fish treatment dose) of teflubenzuron. Control: n=15, low dose: n=17, high dose n=15.

Parameters	Groups compared	P-value	Significant
Deformities	Control vs low dose	0.0192	Yes
	Control vs high dose	0.0020	Yes
	Low vs high dose	0.4765	No
Black dots	Control vs low dose	0.0029	Yes
	Control vs high dose	0.0421	Yes
	Low vs high dose	0.4905	No
Speckled eyes	Control vs low dose	0.2280	No
	Control vs high dose	0.0063	Yes
	Low vs high dose	0.1283	No
Mortality	Control vs low dose	Not available	
	Control vs high dose	0.0550	No
	Low vs high dose	0.0498	Yes

3.3 PHARMACOKINETIC ANALYSES/ ACCUMULATION OF TEFLUBENZURON

The concentration of teflubenzuron (ng/g w.w.) have been summarized in Table 3.3.1. As expected, the average concentration of teflubenzuron was higher in the high dose group compared to the other treatment groups. However, levels of teflubenzuron higher than LOQ were seen in the controls (2.0 ng/g w.w), indicating that the control had been exposed to teflubenzuron in some way. To exclude the possibility for error during analysing, same procedure was performed with four samples of the initial control shrimps, and ten samples of the extra control shrimp from IMR. The mean concentration of teflubenzuron in the extra shrimps were lower than the controls, but the concentration of the extra controls were still above LOQ (0.7 ng/g w.w). With the lowest concentration at LOQ level, these extra shrimps were better suited as controls. However, these additional controls were not included in the feeding experiment at Matre Research Station, and this will be discussed later.

Statistical analyses of accumulated teflubenzuron during the feeding experiment and the four different treatment groups showed a significant difference between high dose treatment and the three other treatment groups, extra control (P-value 0.02), control (P-value 0.02) and low dose (P-value 0.02) respectively. Moreover, no significant differences were found between accumulated teflubenzuron in control and extra control (P-value 0.99).

Table 3.3.1: Concentration of teflubenzuron in pink shrimps, measured at LC-MS/MS after 46 days of feeding with either none, a low dose (0.1% of normal fish treatment dose) or a high dose (1% of normal fish treatment dose) of teflubenzuron. The extra shrimps were not a part of the feeding experiment. Extra shrimps: n=10 Control: n=15, low dose: n=17 , high dose n=15

Group	Average teflubenzuron	Min. teflubenzuron	Max teflubenzuron
Control	2.0 ng/g w.w	1.1 ng/g w.w.	2.9 ng/g w.w
Low dose (B)	6.7 ng/g w.w	1.2 ng/g w.w	24 ng/g w.w
High dose (A)	70 ng/g w.w	4.7 ng/g w.w	369 ng/g w.w
Extra Control	0.7 ng/g w.w	0.2 ng/g w.w	1.1 ng/g w.w

3.4 GENE-EXPRESSION ANALYSES

To examine oxidation of protein, lipids and DNA (indirectly), genes indicating oxidative stress were analyzed at OneStep qPCR and RT-qPCR. For preparation, RNA had to be isolated and the quality validated at Bioanalyzer 2100 Expert (Section 2.3.1 and 2.3.2). At first the ratio of 260/280nm had to be analysed by NanoDrop®, to show if there was any leftover of protein or phenol in the samples. For pure RNA a ratio of ~ 2 is often accepted (ThermoScientific, 2010). A ratio lower than this indicates contamination. The 260/280 nm ratio was between 2.04-2.21 for all the samples. For the Bioanalyser 2100 Expert, eight samples were analysed and estimated a peak around 42 (Figure 3.4.1). The Bioanalyzer 6000 Nano LabChip Kit is developed for mammalian RNA, hence the RNA integrity number (RIN) for crustacean was not available for detection. This is because the RIN number give the ratio of the 18S rRNA and the 28S rRNA, while in crustaceans the 28S peak is partly or fully missing due to a "gap deletion" or a "hidden break" in the 28S rRNA (McCarthy et al., 2015). During analysing, heat-denaturing forces a splicing of the 28S rRNA in crustaceans into two fragments. Due to similar size of the new fragments and the 18S rRNA fragment, a single bond with a "degraded" 28S rRNA is formed at the 18S position on the electropherogram. With these two peaks at such close positions, no ratio between 28S rRNA and 18S position is available for detection, and the RIN number cannot be used to validate the integrity of the RNA. Other studies on arthropods have given similar explanation to the missing RIN number (McCarthy et al., 2015).
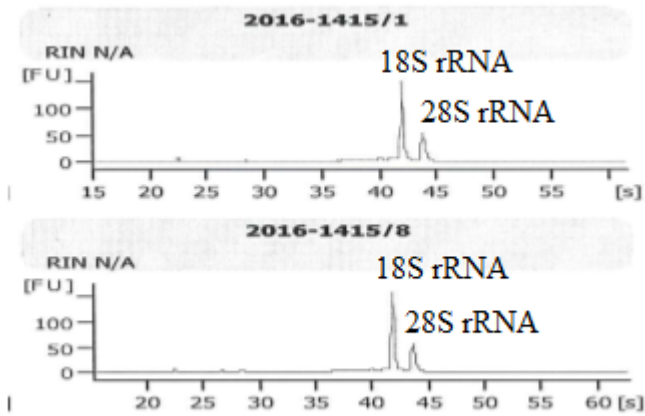
Figure 3.4.1: The electropherogram image for the isolated pink shrimp RNA. The two peaks (18S rRNA and 28rRNA) are too close for a RIN number to be calculated.

3.4.1 GEL-ELECTROPHORESIS

The RNA synthesized to cDNA though one-step PCR was validated with agarose gel-electrophorese. Figure 3.4.1.1 shows the gene size determination after separation. The bonds are not perfectly clear, but the measured sizes for each gene were easy to detect, and there were no double bonds among the genes. All the genes were between 50-200 bp roughly.
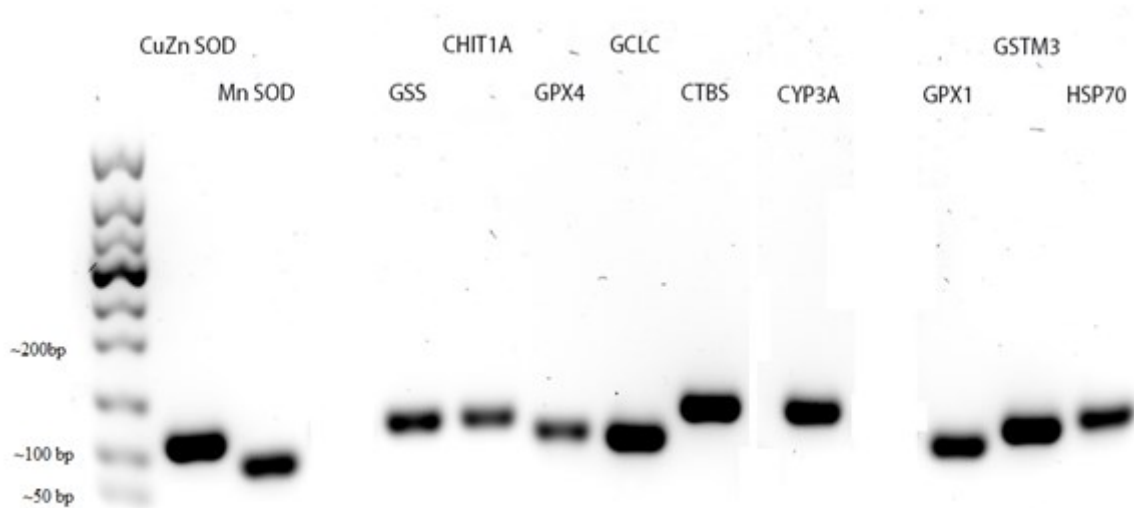
Figure 3.4.1.1: Image of the 12 target genes run on a 2.0% w/v agarose gel, with a 50 bp ladder.

3.4.2 GENE EXPRESSION AND OXIDATIVE STRESS

After running RT-qPCR, mean normalized expression (MNE) were determined for 12 genes respectively. None of the references genes were included as target gene. The efficacy for all genes was high (more than 2-fold higher in most cases) and shows the numbers of copied fraction of target gene in one PCR cycle. The threshold cycle value (CT), given for each gene, tells the measured amount of cycles needed before the fluorescence reaches a specific level for detection. The level correlates to the number of nucleic acid present in the samples (ThermoFisher, 2016). A mean CT value was calculated for each sample duplicate/triplicate and adjusted by the amplification efficiency before using geNorm v3.2 to determine MNE based upon *actb, rpl13* and *uba52* (M-value < 0.8).

Further statistical analysis was run with GraphPad Prism 7.02. The transcript *CuZn-sod* was excluded from further analysis due to too high PCR efficiency. In GraphPad, the adjusted CT sample value for each gene was analysed with ROUT's test to remove any outliers (GraphPad guide, 2017). Comparisons analyses were performed on the three treatment groups, and only one significant difference was found (*gpx4*). The transcript, encoding a protein responsible for degradation of hydrogen peroxide in the cells, showed a significant difference between the control and the high dose group (P value = 0.0003).

As for the additional control samples included in this study, seven gene transcripts gave statistically significant differences when comparing the means of the three treatment groups with the mean of the extra control (Figure 3.4.2.1). Of the seven gene transcripts, four transcripts (*Mn-sod, gpx4, gclc, gss*) were linked to the defence mechanism for oxidative stress, two transcripts (*cyp3a, gstm3*) were linked to detoxification of toxins within the cell, and one transcript (*ctbs*) was linked to the synthesis of chitin.

Correlation analysis was preform to calculate the association between two variables (correlation coefficient) between -1 and +1. The closer the correlation value is towards either -1 or +1, the stronger is the relationship between the variables (StatisticsSolutions, 2017). A correlation analysis among the transcript, content of protein carbonyl, content of MDA and accumulated teflubenzuron, gave a positive correlation between lipid peroxidation and the oxidative stress marker *gss* (ρ= 0.52) and the marker of chitin-synthesis *ctbs* (ρ= 0.30). A negative correlation was found between the lipid peroxidation and the stress induced protein *hsp70* (ρ= -0.35). In the protein carbonyl assay, a negative correlation was found with the oxidative stress marker *cat* (ρ= -0, 33) indicating a medium-strong association.
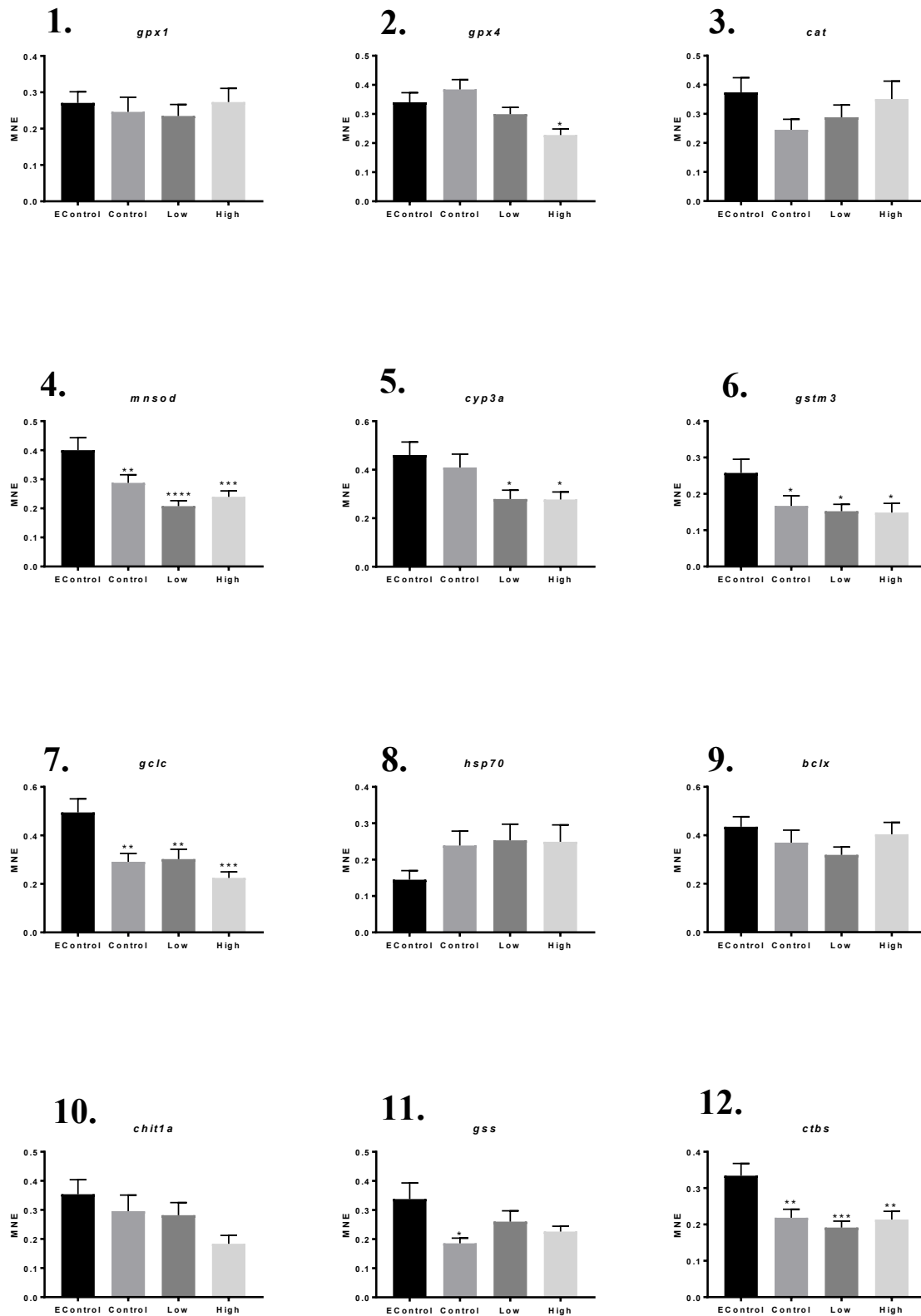
41

Figure 3.4.2.1: Dose-response effects of teflubenzuron exposure in transcripts regulating oxidative stress, detoxification and the synthesis of chitin in pink shrimps. (1) *gpx1*, (2) *gpx4*, (3) *cat*, (4) *Mn-sod*, (5) *cyp3a*, (6) *gstm3*, (7) *gclc*, (8) *hsp70*, (9) *bclx*, (10) *chit1a*, (11) *gss*, (12) *ctbs*. Control: *n*=15, low dose: *n*=17 , high dose *n*=15. Mean ± SEM.

Both data from the protein carbonyl assay and the lipid peroxidation assay were analysed with one-way ANOVA on GraphPad with Holm-Sidak's post-hoc test (Figure 3.4.2.2). Before analysing, outliers were removed with ROUT's test. A significant difference was found in concentration of MDA between the extra control and the three treatment groups from the feeding experiment. In the protein carbonyl assay, a significant difference was found between the high dose group and the low dose group. Accumulated teflubenzuron gave a significant negative correlation with *Mn-sod, gpx4, cyp3a, gclc* and *ctbs*, which was medium strong for *Mn-sod, gpx4,cyp3a* and *ctbs,* and weak for *gclc.*

The Spearman correlation analysis was also performed among all parameters; CL, weight, deformity, black dots, speckled eyes, moulting, concentration of teflubenzuron, protein carbonyl content, MDA content and the transcripts. In this correlation analyse, concentration of TFB was positively correlated to speckled eyes and deformity seen after the feeding-experiment, and negatively correlated to CL and total weight. All correlations mentioned were of medium-strong association. Moreover, moulting was negatively correlated to *ctbs* and positively correlated to *gclc, hsp70,cat* and *gpx1* (Appendix 5).
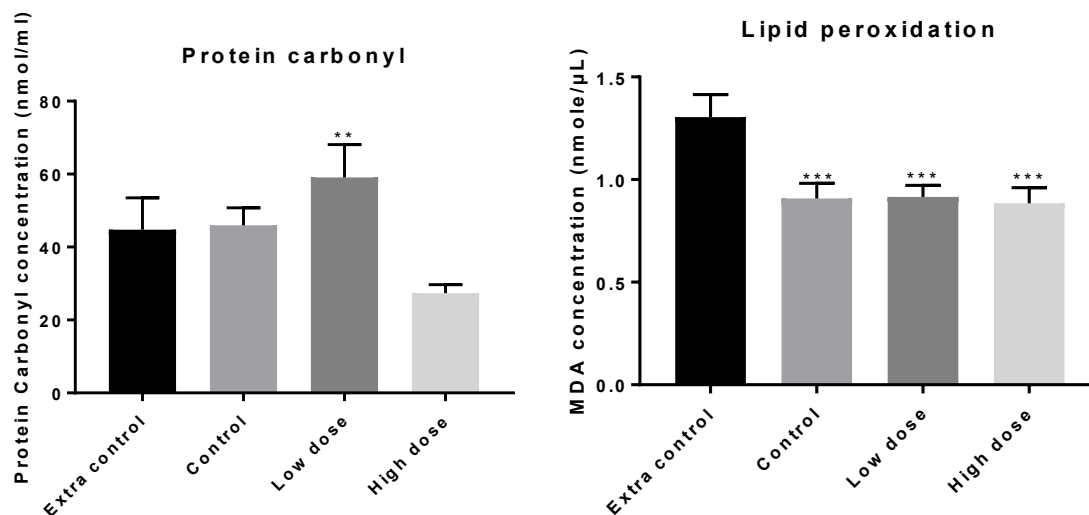
Figure 3.4.2.2: Content of protein carbonyl (PC) and lipid peroxidation (LP) in pink shrimp's tissue exposed to teflubenzuron for 46 days at two levels, a low dose (0.1% of normal fish treatment dose) and a high dose (1% of normal fish treatment dose). The extra controls were not a part of the feeding experiment. For PC, the significance is compared to the high dose group, whereas the significance in LP is compared to the extra controls. Extra shrimps PC: N=9, control PC: N=14, low dose PC : N=15, high dose PC: N=14. Extra shrimps LP: N=9, control LP: N=15, low dose LP: N=17, high dose LP: N=15. Mean ± SEM.

4. DISCUSSION

In the present work, pharmacokinetics and dose-response effects of teflubenzuron were examined in pink shrimps after 46 days of feeding with either none, a low dose or a high dose of teflubenzuron. Previous studies with teflubenzuron exposed to juvenile lobster showed a profound effect on transcriptional changes in gene-expressions connected to detoxification, stress and moulting (Olsvik et al., 2015) and toxicological effects like deformities and death (Samuelsen et al., 2014). However, pharmacokinetic and the toxicological effects of teflubenzuron in pink shrimp had not been investigated earlier.

4.1 THE FEEDING EXPERIMENTS

In the feeding experiment, the acclimation period was set to 14 days, to minimize the risk of mortality from stress when the shrimps were adapted to the new environment. Previous unpublished feeding experiments with shrimps, performed by IMR, resulted in high mortality among the controls within the first week. In contrast to the previous study, the cages used in this experiment were bigger and the acclimation time yielded no mortality among the shrimps. However, at the end of the feeding experiment five shrimps were found dead in their cages. High doses of teflubenzuron were assumed to be the cause of death, since all the dead shrimps were from the high dose group and statistical analyses with Fisher's exact test gave a significant difference when comparing cumulative mortality between high dose group and low dose group. However, when comparing mortality between high dose group and control group, no significant difference was recorded. As the numbers (n) of test-species in the groups varied from 15 in the control group, to 20 in the high dose groups, n of test-species may have influenced the results. The concentrations of teflubenzuron given in this experiment were based on a lethal-dose experiment performed by IMR. However, the concentration of teflubenzuron given in the low dose ($0.01\mu g/g$) was non-lethal to the shrimps in this experiment, and the dose was probably too low for the shrimps to respond strongly. Moreover, the medical pellets were only given two days a week for 46 days, in contrast to a 7-day medication period given to salmonids, where is reasonable to think that aquatic animals can be exposed daily during medication. When comparing with a study on mortality and deformities in European lobster juveniles exposed to teflubenzuron, doses of 20 mg/kg and 10 mg/kg (same as the daily dose given in marine salmonid farms) were given for 4 or 7 days depending on weight. After 3 months of monitoring, 38% (n=42) of the lobster died in the high dose group, and 41% (n =46) died in the low dose

group (Samuelsen et al., 2014). The high dose (20 mg/ kg) in the study by Samuelsen et al. (2014) was chosen from concentrations found in faecal material from medicated salmons. With the finding of such high concentrations in salmon faeces during medication, both doses of teflubenzuron given in this experiment were low in comparison, indicating that shrimps can be exposed to higher concentrations in nature. However, the accumulated levels of teflubenzuron varied greatly within the surviving shrimps from the high dose groups (4.7 ng/g w.w to 369 ng/g w.w). Uneaten pellets could explain some variation, as pellets might be lost from the cage during the feeding trial, or there might be a difference in appetites/ behaviour among the shrimps. A difference in behaviour could be due to sex or moulting status. As shrimps are usually protandric (change sex from male to female during their life cycle)(Wieland, 2004; Bauer, 2007), sex also might have an impact on size and age. The correlation analysis showed a negative significant association between concentration of teflubenzuron and weight, which might indicate that accumulated concentrations of teflubenzuron had a direct effect on growth by impacting moulting frequency or by redirecting energy from growth towards detoxification. There have been several toxicological studies on the effect of diflubenzuron in crustacean, where the drug has caused retardation of regeneration (Weis et al., 1992) and has had an effect on behaviour (Savitz et al., 1994). According to Weis et al. (1992) regeneration and moulting in crustaceans are often linked together and controlled by the neuroendocrine system, and that most studies reports an effect on both regeneration and moulting when studying the response to pollutants in crustacean (Weis et al., 1992). Moreover, the deep-water shrimp (*Pandalus borealis*) change sex in spring or in summer (Bauer, 2007), and as the feeding trial took place in the spring, the drug may have had an effect on the sex change from male to female. Differences in body fat content can also explain some variations, as the drug is highly lipophilic. Moreover, moulting can also lead to the elimination of some of the chemical when the old exoskeleton is shed. At the end of the feeding experiment pieces of the old exoskeleton was remained in some cages, and the old exoskeleton could have contained some teflubenzuron. Overall, it seems like there might be an individual difference in uptake and accumulation of teflubenzuron among the shrimps, and that the drug negatively affects the regeneration in shrimps. A difference in accumulated levels of teflubenzuron among individuals was also seen in the study of juvenile European lobster one day after the 7-days medication period ended (Samuelsen et al., 2015).

Furthermore, this experiment had some limitation, as age and exact time of moulting for each shrimp were unknown. In an extended experiment with larger *n*, variable age would have had less impact on the studied parameters. In this case, one cannot exclude natural reasons as a cause of death. Previous studies have indicated that adult crustaceans are more tolerant to toxicant than larval form (Roth et al., 1993), but with mature shrimps as test-species, size-dependent effects on tolerance were probably negligible.

At the end of the feeding experiment, four shrimps from the control group and the high dose group and five shrimps from the low dose group had gone through ecdysis (moulting). Pieces of the old exoskeleton were found in the cages, and for some, the newly developed exoskeleton were still soft. As shrimps often feed off their old skeleton, leftover of exoskeleton may indicate that the shrimps had newly been through moulting, especially in individuals with soft exoskeleton. Moreover, it is known from the literature that exposure of teflubenzuron prior to moulting negatively affects formation of a new exoskeleton, by inhibiting the synthesis of chitin. If the shrimps were recently past moulting, it is reasonable to think that shrimps from the high dose group would not be alive if the feeding experiment had continued for some more weeks. In addition, deformities were seen in all shrimps that had gone through moulting in the high dose group, confirming the toxicological effects of teflubenzuron in the moulting stage.

In the literature, deformities have been mentioned as an outcome of teflubenzuron exposure, and abnormal swimming have been reported from studies of crustacean exposed to diflubenzuron (Roth et al., 1993). As teflubenzuron in considered more potent than diflubenzuron, lower concentration of the chemical should in theory yield similar deformities. In this work, deformities like stiff and crocked legs were seen in individuals from both treatment groups. Walking leg and claw deformities have previously been reported in juveniles European lobster exposed to teflubenzuron in doses of 5% and 20% of the daily dose given to salmons (10 mg/kg) for 114 days (Olsvik et al., 2015), suggesting that teflubenzuron can cause deformities in crustaceans even when small doses of the chemical are given.

The black spots, seen in many individuals across the groups, seems to have derived from another source than teflubenzuron. The most reasonable explanation has been proposed in an article on black spotted shrimps, that claimed oxygen to be the cause of black spots (melanins), catalyzed by the enzyme tyrosinase (Fieger, 1951).

4.2 PHARMACOKINETIC ANALYSES/ ACCUMULATION OF TEFLUBENZURON

In the pharmacokinetics studies, concentrations above LOQ were found among the initial control shrimps (Figure 4.2.1). Therefore, these controls were reanalysed with the extra control, concentrations above LOQ were found in both groups, the controls and in the additional controls. Although the concentrations were 2.7-fold lower in the extra controls. As the analytical method is developed by NIFES and was performed with an analyst well known with the method, errors during sample preparation and extraction are unlikely. In addition, tissue from shrimps bought at Bryggen, in Bergen, was used as blank and they did not contain residues of teflubenzuron above LOQ. This indicated that the controls and the extra controls had been exposed to teflubenzuron. However, a possible explanation for exposure of teflubenzuron could be contamination during feeding at Matre Research Station. The pellets given to the shrimps were coated with a mixture of glucose, teflubenzuron and cod-liver oil, and by accident the same tweezer were used to feed all shrimps groups. If the shrimps were fed in correct order: controls, low dose and high dose, and the tweezer was properly cleaned in between, the risk of contamination should have been minimal. In this case, many people were involved in the feeding process, and therefore the possibility of incorrect feeding order or improperly cleaning of tools or hands during feeding should be considered. The tanks holding the extra controls had also previously been used for other feeding experiments with teflubenzuron, and due to high lipophility, leftovers of teflubenzuron could have been present in the walls of the tanks. This possibility is considered less likely, as the tanks had been properly cleaned between each feeding experiment. Moreover, as the chemical is in use as a treatment against salmon lice in salmonid farms, contamination in nature can not be excluded as a reason, even though the odds are minimal.
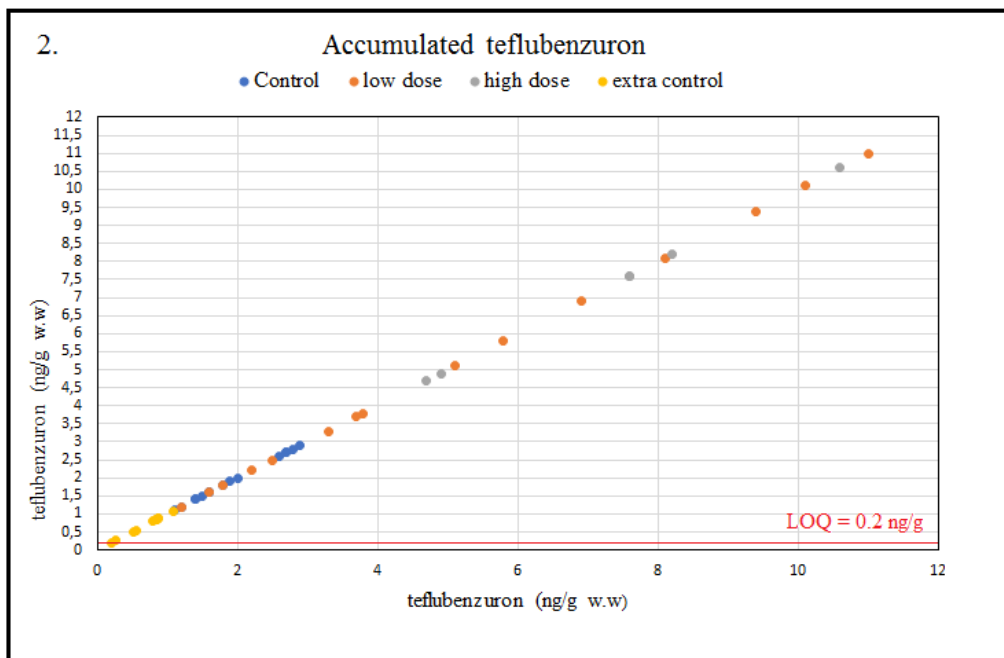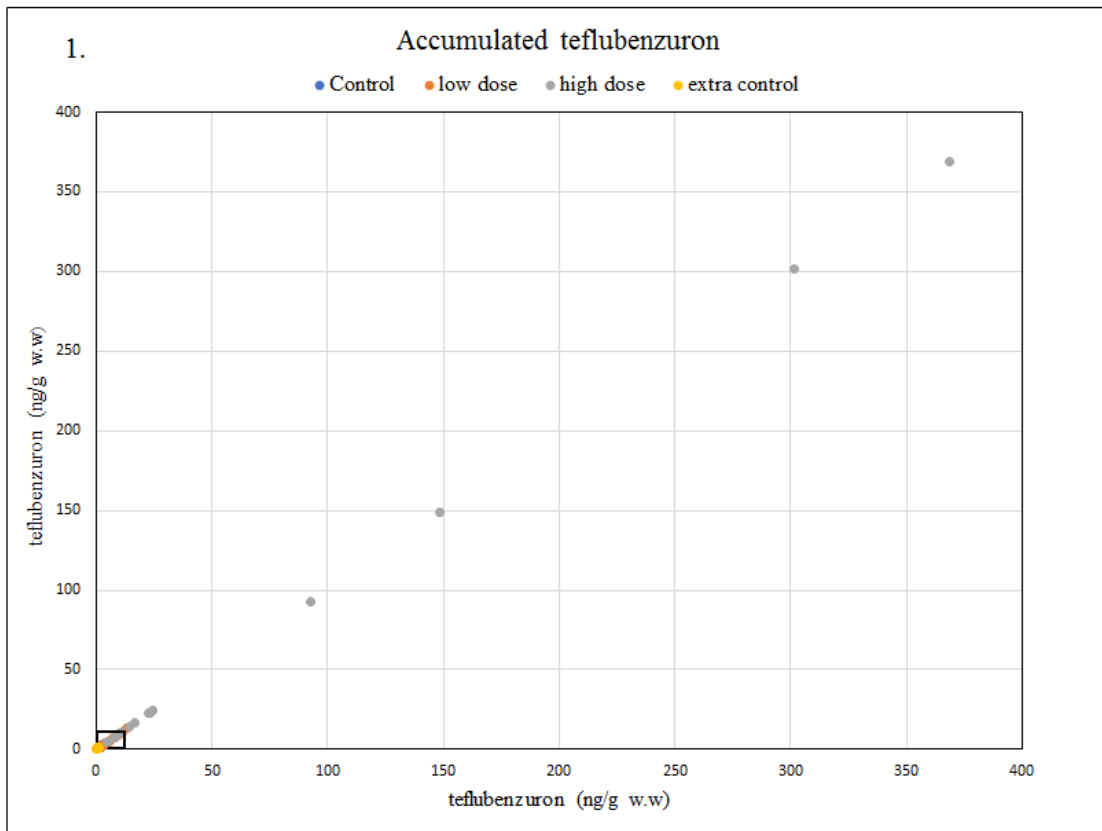
Figure 4.2.1: Concentration of teflubenzuron found in the shrimps from Matre Research Station and the extra control given from IMR. The picture below (2) is an enlarged section of the picture above (1), and the section is marked in the left corner of picture 1. The red line shows the LOQ limit.

4.3 TRANSCRIPTIONAL STUDIES OF TEFLUBENZURON IN SHRIMPS

Detection of teflubenzuron in the initial controls had no implications on pharmacokinetic analyses. For the molecular and biochemical studies on the other hand, it might have made a difference, and the extra shrimps were included in the statistical analyses, even though they contained teflubenzuron as well. However, there were some challenges to consider if the extra shrimps should be involved in these analyses. Due to fact that these shrimps were obtained from IMR months later and had been kept and fed in the lab for a longer period of time, they did not meet the criteria as proper controls for the 46-days feeding experiment. There was also a statistical difference in weight and CL between the extra control and the other groups, which can affect metabolic analyses. On the other hand, the extra shrimps were from the same catch, had been fed with the same feed pellets as the controls, and had lived in tanks with similar water temperature as the initial shrimps. With no ideal control group in this experiment, the extra shrimps were included in all sample analyses as they contained considerable less teflubenzuron that the original controls.

4.3.1 GENE-EXPRESSION ANALYSIS

For the gene-expression analysis, the integrity of potential PCR primers were tested with gel-electrophorese before running RT-qPCR. In addition to the twelve genes analyzed in this current work, gel-electrophorese indicated that the transcript *CuZn-sod* was usable for RT-qPCR. However, as Figure 3.3.5.1 shows, the PCR efficiency for *CuZn-sod* was far above upper acceptable limit (3.687, should be between 1.80-2.10). A high PCR efficiency for *CuZn-sod* could be explained by coamplication of nonspecific products like primer dimer during RT-qPCR, or by high levels of inhibitors in sample. Pipetting error when making the standard curve, could also lead to an increased PCR efficiency, but as the same standard curve was used for all the primers, this cannot explain the high efficiency alone (BioRad, 2006). However, pipetting error could been an explanation to why all the PCR-efficiencies were slightly higher than the desirable efficiency value. In addition, the RT-qPCR machine was newly approached by NIFES, and the calculated PCR efficacy in the new machine was higher compared to the old machine. This suggest that type of qPCR machine has an impact on estimated efficiency. Svec et al. (2015) also saw a significant difference in estimated efficacy between several q-PCR machines in their study, probably due instrument and software settings, and differences in algorithm used to detect CT-value.

Only one out of twelve genes responded significantly to exposure of teflubenzuron when comparing gene-expressions with the initial groups from the feeding experiment. The transcript (*gpx4*) was significantly lower expressed in the high exposure group compared to the control group. Although Figure 4.3.2.1 shows a decreased expression from the control group towards the high dose group, no significant differences were found between the low dose group and the controls. A decrease in expression of genes encoding GPX4, may suggest that teflubenzuron can cause a disruption of the antioxidative defence. However, with an unbalance in pro-oxidants/antioxidants one should expect a significant difference in expression of other ROS regulators like the SOD enzymes, seen in a study with pacific white shrimps exposed to ammonia (Liang et al., 2016), or CAT, suggested to play an important part of the antioxidative defence in invertebrates (Livingstone, 2001). In contrast to GPX*,* CAT showed a similar or a higher activity in invertebrates compared to vertebrates in the study (Livingstone et al., 1992). However, a difference in the antioxidative defence mechanism between aquatic species have been seen (Livingstone, 2001), and there is limited information about the antioxidative defence mechanism in shrimps. Moreover, levels of mRNA do not necessary reflects the activity of the protein, and a study by Liu et al. (2007) showed an increase activity of GPX 12 hours after injecting shrimps with the bacteria *V. alginolyticus* (Liu et al., 2007). A significant decrease in the high dose group for genes encode GPX-4, and non-significant expressions of genes encoding other ROS regulators (*mnsod, cat ,gclc, gss*), will only suggest a weak association between accumulated teflubenzuron and oxidative stress.
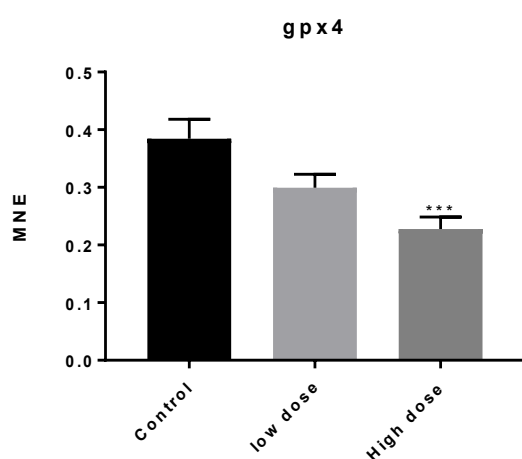

Figure 4.3.2.1: Dose-response effects of teflubenzuron in transcript *gpx4*. Mean ± SEM.

Nevertheless, when comparing the MNE of the extra controls with the other groups, the results suggest that detectable concentrations of teflubenzuron in the control group could be a reason why no-significant differences in expressions were measured. When comparing the mean of the extra control with the other treatment groups, a difference in transcripts involved in mechanisms connected to oxidative stress (*Mn-sod, gpx4,gclc, gss*), detoxification (*cyp3a,gstm3*) and chitin synthesis (*ctbs*) were found. For all transcripts, a significantly decrease in MNE was seen between the extra controls and the high dose group. This reduced level of expressions in the exposed groups were significant for most genes, with one exception (*gss*), which showed difference only between the extra controls and the initial controls. With a significant decreased expression in genes encoding CYP3A, GSTM3 and CTBS, the comparison with the extra controls, indicates that teflubenzuron might deactivate detoxification mechanism in cells, and that the drug affects the post-moulting phase by inactivation of CTBS. Previous studies have suggested that the cytochrome P450 enzymes (family 2 and 3 more specifically) are important regulators of detoxification processes in shrimps (James and Boyle, 1998), and a decrease in transcripts encoding CYP3A can be explained by a toxic effect of tefubenzuron impacting the cytochrome P450 system. Regulation of CTBS on the other hand, is influenced by stage of moulting, and results from a study by Zou and Fingerman (1999) showed a significant increase in CTBS activity in crabs prior to moulting (Zou and Fingerman, 1999). Several of the initial shrimps had gone through moulting at the end of the feeding experiment, which can explain a decreased activity of the CTBS enzymes for these three groups. Unfortunately, no data were available for moulting status in the extra shrimp group. An increase of CTBS activity in the extra controls may suggest that several of the extra shrimps were in a pre-moulting phase. In addition, the Spearman's rank correlation analysis also indicated a negatively correlation with *ctbs* and moulting, which suggest the expression of genes encoding CTBS increases when the shrimps are not moulting. This can further support the results from the study by Zou and Fingerman (1999), which indicated that CTBS activity increased at the pre moulting stage, and decreased at the post-moulting stage. Moreover, correlation analysis with accumulated teflubenzuron levels gave a significant relation with the transcripts linked to oxidative stress (*Mn-sod,gpx4 gclc*), detoxification (*cyp3a*) and chitin synthesis (*ctbs*), which all responded significantly different by including the extra controls. The correlation coefficients were negative for all transcripts.

When using whole animal tissue it is difficult to measure tissue-specific effects of teflubenzuron, as there may be known or unknown substances within the organism that interacts with the drug tested (Murphy, 1991). As the extra controls had been fed for a longer time, the content of minerals and fat in their diet can explain some differences in transcriptional levels between the groups. Moreover, in the The European Agency for the Evaluation of Medicinal products (EMEA) studies of salmon exposed to teflubenzuron, the highest concentrations of the drug were found in liver, gut and kidney (EMEA, 1999). When including all organs of the shrimp, expressions from many different types of cells are measured in one analyse. As the gene-expression varies between organs and tissue (Robalino et al., 2007), responses from sensitive cells may be obscured by stronger responses from less active and more abundant cell types, like muscle cells. It is known that the hepatopancreas plays a major role in detoxification of substances in shrimps, hence the amount of genes linked to detoxification might be greater in tissue from the hepatopancreas (Sreeram and Menon, 2005). Furthermore, studies in juvenile European lobster exposed to teflubenzuron showed an effect on transcription of genes linked to detoxification in claw tissue (Olsvik et al., 2015), while in an unpublished study, isolation of high quality RNA from leg-tissue in shrimps failed. Even though studies with teflubenzuron exposure to insect have shown an impact on a numerous tissues and cells, (Merzendorfer, 2013), it seems like the use of specific tissue cells, from organs like the hepatopancreas might be preferable in toxicological studies in shrimps. Furthermore, antioxidative activity is shown to vary with age, season and moulting (Livingstone, 2001). With a small n, lack of data on age and moulting can affect the results from the transcriptional studies and the colorimetric analyses of MDA levels and protein carbonyl concentration indicating oxidative stress. The two latter will be discussed next.

Oxidative stress can be induced by contaminates, and provoke DNA oxidation, lipid oxidation and protein oxidation. In this study, kit to measure content of protein carbonyl and content of MDA was used in addition to gene expression analysis, to look for signs of oxidative stress in the shrimps. Protein oxidation can lead to the formation of protein carbonyl, and the content of protein carbonyl were measured with the protein carbonyl colorimetric assay. The results gave a significant difference in content between high dose group and low dose group. Figure 3.3.2 shows a small, but non-significant increase in content of protein carbonyl from the controls to the low dose group, and a large significant decrease from the low dose group to the high dose group. As the content of protein carbonyl indicates oxidative stress, a higher content of protein carbonyl should be expected in the high dose group. However, it seems like the content of

protein carbonyl increases before it reaches a threshold and then decreases. This pattern could either be explained by a stronger activation of defence mechanism when the threshold limit is reached, or due to the breakdown of proteins. Correlation analysis gave a significant negative correlation between protein carbonyl and the oxidative stress regulator *cat*, which plays a part in catabolizing hydrogen peroxide into oxidized glutathione and water. Catabolizing hydrogen peroxide prevent the specie from attacking lipids or proteins, and a positive correlation between *cat* and content of protein carbonyl should be expected as one would think that higher content of protein carbonyl would induced an increased activation of CAT-enzymes.

Free oxygen radicals attacking polyunsaturated fatty acid generate the main product of lipid peroxidation, MDA. Concentration of MDA could therefore be an indication for oxidative stress, and were measured with the lipid peroxidation assay in this study. The measured MDA content showed a significant difference between the extra controls and the three treatment groups, but no significant difference was found between the initial controls and the treated groups. The mean MDA content between the initial treatment groups were similar, indicating that exposure of teflubenzuron did not affect lipid peroxidation in the shrimps. However, the higher levels of MDA in the extra controls was unexpected. One would assume that this level would be even lower than with the initial groups, as the additional controls contained considerable less teflubenzuron. This could be explained by the duration of feeding and likely reflect changed fatty acid composition of the animals kept longer in the lab. Correlation analysis on MDA content gave a significant connection with gene involved in stress regulation (*hsp70*), chitin synthesis (*ctbs*) and regulation of oxidative stress (*gss*). A correlation between expression of genes involved in the oxidative defence mechanism and lipid peroxidation justifies application of the colorimetric method of measuring MDA content in this work.

Correlation analyses with accumulated teflubenzuron did not show any association between the content of protein carbonyl and content of MDA. This may suggest that the applied doses of teflubenzuron did not induce detectable oxidative stress in the exposed shrimps. However, as the transcripts linked to oxidative stress (*Mn-sod, gpx-4, gclc*) were negatively correlated to accumulated teflubenzuron, and there was a significant difference in content of protein carbonyl between high dose groups and low dose group, the current study indicates that teflubenzuron negatively affected the shrimps and induced a weak oxidative stress.

8. CONCLUSION

This study shows that pink shrimps can accumulate quit high levels of teflubenzuron after 46 days of exposure. The accumulated levels of teflubenzuron in shrimps from the two exposed groups suggest that there are individual differences in uptake and accumulation of teflubenzuron, and that the drug has an impact on growth. This could possibly be linked to feed intake and moulting stage of shrimps at sampling. The findings from the morphological studies indicate that even small doses of teflubenzuron can cause deformities in shrimps, and that the highest dose of teflubenzuron ($0.1\mu g/g$) can lead to death.

For the molecular and biochemical part of the experiment, high doses of teflubenzuron seem to induce weak oxidative stress, even though correlation analyses with accumulated teflubenzuron did not give any significant association between content of MDA and content of protein carbonyl. Surprisingly, the control shrimps contained teflubenzuron above LOQ, rendering them sub-optimal for use as controls for the molecular and biochemical examinations.

7. FURTHER STUDIES

In the current work, detectable levels of teflubenzuron were found in the controls. This might have rendered the controls sub-optimal for the molecular and biochemical parameters measured in the shrimps. With available time, the experiment should be repeated ensuring the controls contain no teflubenzuron. Future studies should also focus on tissue-specific responses to teflubenzuron exposure, especially on the hepatopancreas as the main detoxifying organ in crustaceans. Moreover, there is still a need for more information about the impact on antiparasitic agents, like teflubenzuron on non-target species living close to the fish farms.

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APPENDIX 1

Weights:

Mean-weight shrimps: 4.36 ±1.17g

Mean-weight pellets: 0.12g

Doses:

1% of 10 mg/kg daily fish therapy dose.

10 mg/kg = 10 µg/g

1% of 10 µg/g = 0.1 µg/g, which gives a dose of 0.44 µg per pellet given to a shrimp of 4.4 g.

0.1% of 10 µg/g = 0.01 µg/g, which gives a dose of 0.044 µg per pellet given to a shrimp of 4.4 g.

1200 pellets have a weight of approximately 144 g

Dose 1 %. (Dose A)

0.44 µg per pellet x 1200 pellets= 528 µg teflubenzuron coated on 144 g pellets.

Preparations:

10 mg of teflubenzuron was mixed with 10 g glucose, which gave a concentration of 1 mg/g (1000 mg/g). Then 528 mg of the 1 mg/g-mix, was mixed with 2.5 g glucose and coated on 144 g pellets.

Dose 0.1% (Dose B)

0.044 µg per pellet x 1200 pellets= 52,8 µg teflubenzuron coated on 144 g pellets.

Preparations:

1 g from the 1mg/g-mix was mixed with 9 g glucose to a concentration of 100 µg/g. Then 528 mg of the 100 µg/g-mix was transferred and mixed with 2.5 glucose.

APPENDIX 2

TABLE 2A. SETUP FOR THE STANDARD CURVE IN THE PHARMACOKINETIC ANALYSIS

	Added 50 ng/mL (5 µg/mL*) standard (µL)	Concentration in the samples (ng/g)	Added IS 50 ng/mg (µl)	Location of controls
BUM			200	
BMM			200	
N1	20	1	200	K1
N2	80	4	200	
N3	200	10	200	K2 M1 M2 M3
N4	400	20	200	
N5	600	40	200	
N6	800	50	200	
N7	10*	50	200	
N8	20*	100	200	K3
N9	30*	150	200	

TABLE 2B. MASS TRANSITION AND INSTRUMENT SETTING

Compound	Transition (m/z)	Collision energy (V)	Fragmentor (V)	Cell accelerator (V)	Dwell time (ms)	Comment
Teflubenzuron	379.0 -> 339.0	4	100	4	80	Quantifier
	379.0 -> 195.9	18	100	4	80	Qualifier
Diflubenzuron-d4	313.0 -> 293.1	5	100	4	80	Internal standard
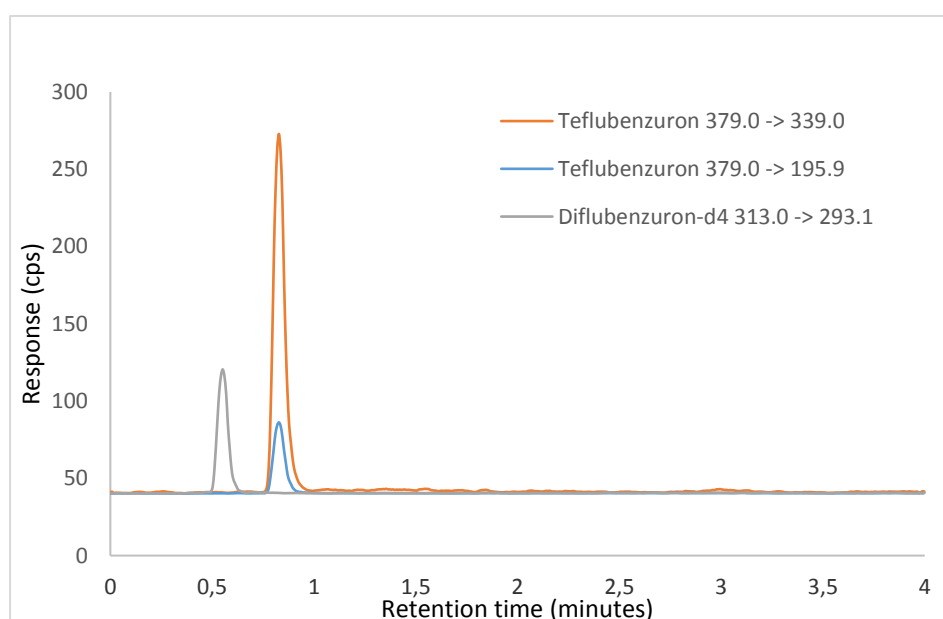
FIGURE 2C. RETENTION TIME FOR TEFLUBENZURON (QUANTIFIER AND QUALIFIER) AND IS

TABLE 2D. MEASURED CONCENTRATION OF TEFLUBENZURON IN RESPECT TO WEIGHT.

Shrimp no.	CL (mm)	Total weight (g)	Measured amount (g)	Teflubenzuron (ng/g w.w)	Notice
1	16.4	4.15	0.57	**1.4**	control
2	13.3	2.32	0.56	**1.1**	control
3	15.8	3.20	0.59	**1.9**	control
4	14.1	2.00	0.53	**2.9**	control
5	14.1	1.74	0.52	**2.6**	control
6	13.6	2.12	0.52	**2.8**	control
7	15.1	2.86	0.62	**1.6**	control
8	10.8	1.14	0.46	**2.7**	control
9	11.8	1.57	0.51	**1.5**	control
10	13.3	1.86	0.50	**2.0**	control
11	15.5	2.85	0.54	**2.7**	control
12	15.9	3.20	0.54	**1.4**	control
13	14.9	2.97	0.50	**1.2**	control
14	17.1	4.11	0.57	**1.8**	control
15	14.2	2.37	0.54	**1.6**	control
16	12.9	1.91	0.53	**2.2**	Low dose
17	14.6	2.65	0.53	**24**	Low dose
18	13.4	2.22	0.51	**8.1**	Low dose
19	15.7	3.48	0.68	**5.8**	Low dose
20	15.3	3.49	0.65	**1.8**	Low dose
21	11.5	1.42	0.52	**1.6**	Low dose
22	16.8	4.02	0.65	**1.2**	Low dose
23	10.0	1.10	0.40	**9.4**	Low dose
24	13.0	1.88	0.51	**3.7**	Low dose
25	16.2	3.22	0.61	**3.8**	Low dose
26	14.4	2.79	0.51	**3.3**	Low dose
27	13.4	2.01	0.53	**5.1**	Low dose
28	11.8	1.68	0.51	**13**	Low dose
29	12.6	1.52	0.52	**10**	Low dose
30	12.8	1.67	0.53	**11**	Low dose
31	12.2	1.52	0.50	**2.5**	Low dose
32	11.6	0.97	0.43	**6.9**	Low dose
33	13.4	2.26	0.52	**23**	High dose
35	13.0	1.94	0.52	**7.6**	High dose
37	10.8	1.15	0.49	**8.2**	High dose
38	13.8	2.20	0.52	**25**	High dose
39	15.1	2.47	0.50	**14**	High dose
40	14.8	2.79	0.53	**17**	High dose
41	15.5	3.16	0.53	**23**	High dose
42	10.0	0.88	0.35	**302**	High dose

43	14.8	2.86	0.5	**93**	High dose
45	15.3	3.05	0.52	**7.6**	High dose
46	16.8	3.43	0.60	**4.7**	High dose
47	16.3	3.24	0.51	**149**	High dose
48	12.9	1.86	0.54	**11**	High dose
50	14.4	2.29	0.51	**4.9**	High dose
51	15.1	2.78	0.56	**369**	High dose
1	18.3	5.0	0.49	**0.51**	Extra control
2	16.6	4.0	0.51	**0.80**	Extra control
3	14.1	5.0	0.49	**0.84**	Extra control
4	18.2	3.0	0.47	**0.88**	Extra control
5	16.5	3.0	0.48	**0.87**	Extra control
6	16.4	3.0	0.59	**0.85**	Extra control
7	14.6	3.0	0.48	**0.20**	Extra control
8	15	3.0	0.51	**0.26**	Extra control
9	16.1	3.0	0.48	**1.1**	Extra control
10	17.2	4.0	0.50	**0.55**	Extra control

APPENDIX 3

TABLE 3A. REVERSE TRANSCRIPTASE MIX

	Reagens	50 μL	μL added RT mix plate 1 (x 102)	μL added RT mix plate 2 (x 90)
Non-enzymatic reagents	ddH$_2$O	8.9	907.8	801
	10X TaqMan RT buffer	5.0	510	450
	25mM magnesium chloride	11.0	1122	990
	10mM deoxyNTPs mixture (2.5mm of each dNTP)	10.0	1020	900
	*50μM oligo d(T)$_{16}$	2.5	255	225
Enzymes	RNase inhibitor (20U/μL)	1.0	102	0
	Multiscribe Reverse Transcriptase (50U/μL)	1.67	170.34	150.3

TABLE 3B. REVERSE TRANSCRIPTASE REACTIONS TERMS

Steps	Incubation	RT	Reverse transcriptase inactivation	End
	HOLD	HOLD	HOLD	HOLD
Temperature (°C)	25	48	95	4
Time (min)	10	60	5	∞
Volume (μL)	50			

TABLE 3C. SYBR GREEN MASTER MIX

Reagents	Volum each sample (μL)	μL added mix plate 1	μL added mix plate 2
SYBR GREEN Master mix	5	575	505
ddH$_2$O	2.8	322	283
Primer fw (50μM)	0.1	11.5	10.1
Primer Rw (50μM)	0.1	11.5	10.1

FIGURE 3D. RT-QPCR REACTION PROGRAM

1 95.0 C for 5:00
2 95.0 C for 0:10
3 60.0 C for 0:10
Slow Ramp Rate to 2.2 C per second
4 72.0 C for 0:10
+ Plate Read
5 GOTO 2 , 44 more times
Melt Curve 65.0 to 97.0 C, increment 0.5 C.
for 0:01
6 Melt Curve 65.0 to 97.0 C, increment 0.5 C.
for 0:01
+ Plate Read
END
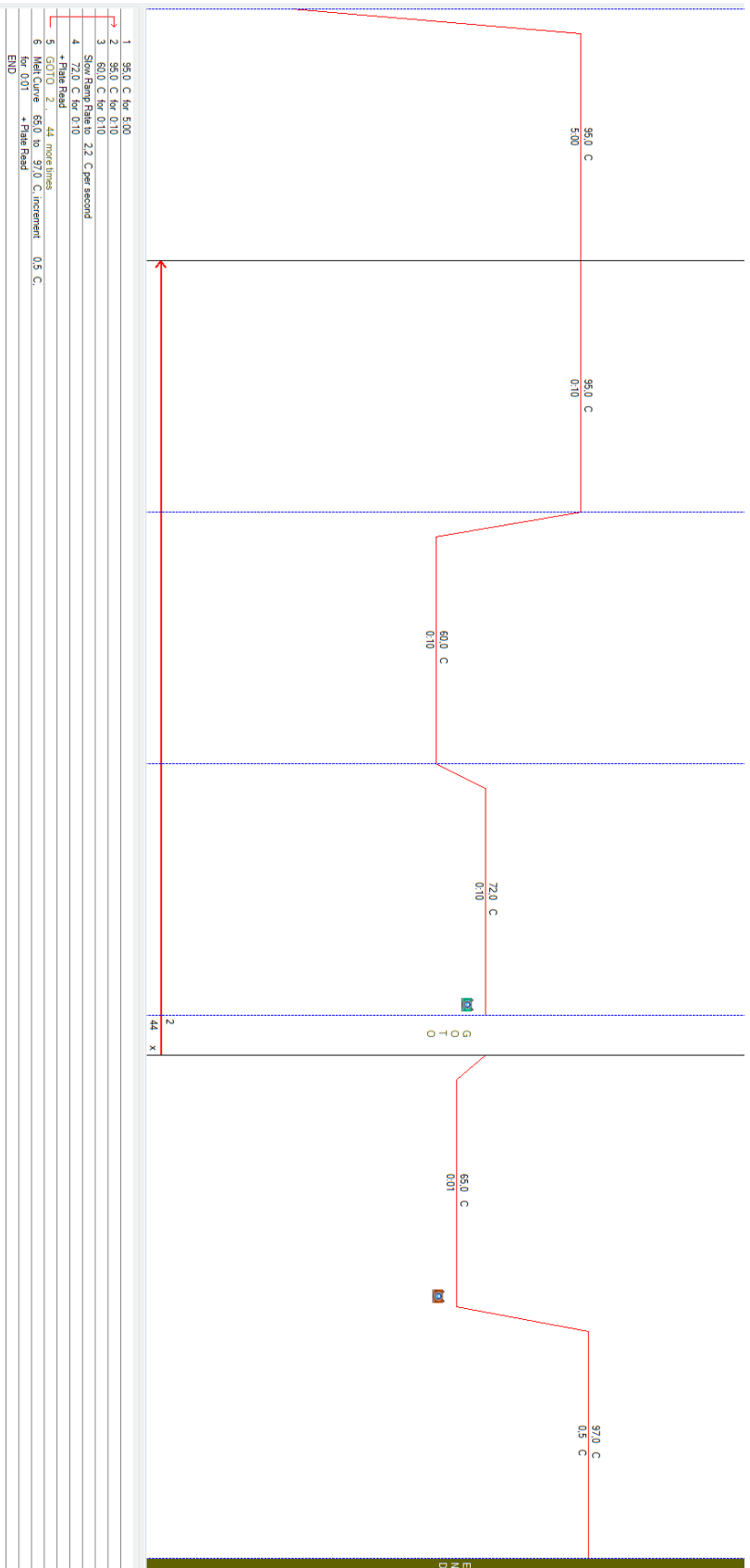
TABLE 3E. QIAGEN ONESTEP RT-PCR

Components	25µL rxn*	µL added	Final concentration
5 x QIAGEN OneStep RT-PVR buffer	5 µL	40	1x
Q solution	5 µL	40	5x
dNTP mix (10mmM of each dNTP)	1 µL	8	400 µM of each dNTP
Primer forward	0.3 µL		0.6 µM
Primer revers	0.3 µL		0.6 µM
RNase inhibitor	0.25 µL	2	
QIAGEN OneStep RT-PCR enzyme-mix	1 µL		
ddH$_2$O	11.35 µL	90.8	Up to 25 µl incl. template RNA
Template RNA	0.8 µL	6.4	Ca 0.5-1 µg RNA

Step	Time	Temperature	Comment
Reverse transcriptase	30 min	50°C	Can increase to 60°C
PCR activation	15 min	95° C	
3-step cycle: Denaturation	 45 sec (30-60)	 94° C	Number of cycles: 35 (35-40)
Annealing	45 sec (30-60)	60° C (50-68)	
Extension	1 min	72° C	Ca. 5°C under primers T$_m$
Final extension	10 min	72° C	Increase with 30-60 sec for product 1-2kb.

APPENDIX 4

CALCULATION OF THE CONCENTRATION OF CARBONYL IN THE SAMPLES FROM THE ABSORBANCE VALUE

CA = Average absorbance samples – average absorbance controls

Protein Carbonyl (nmol/ml) = $[(CA)/(*0.011 \ \mu M^{-1})](500 \ \mu l / 200 \mu l)$

TABLE 4A. CALCULATED CONCENTRATION OF PROTEIN CABONYL IN THE SAMPLES

Shrimp No.	Protein carbonyl (nmol/mL)	Notice	Shrimp No.	Protein carbonyl (nmol/mL)	Notice
1	24.2	Control	33	45.3	High dose
2	44.0	Control	35	20.9	High dose
3	40.1	Control	37	16.5	High dose
4	39.3	Control	38	37.4	High dose
5	88.9	Control	39	31.8	High dose
6	71.5	Control	40	21.6	High dose
7	44.4	Control	41	22.2	High dose
9	49.3	Control	43	20.5	High dose
10	52.2	Control	45	24.7	High dose
11	58.5	Control	46	29.0	High dose
12	31.7	Control	47	29.1	High dose
13	34.1	Control	48	-28.2	High dose
14	42.5	Control	50	29.2	High dose
15	23.3	Control	51	-80.4	High dose
16	27.9	Low dose	1	5.60	Extra control
17	27.1	Low dose	2	69.7	Extra control
18	23.1	Low dose	3	55.9	Extra control
19	30.8	Low dose	4	75.8	Extra control
20	37.6	Low dose	5	66.4	Extra control
21	99.6	Low dose	6	59.9	Extra control
22	135	Low dose	7	1.45	Extra control
24	103	Low dose	8	23.5	Extra control
25	-115	Low dose	9	27.5	Extra control
26	71.5	Low dose	10	61.7	Extra control
27	47.2	Low dose			
28	48.9	Low dose			
29	49.2	Low dose			
30	70.2	Low dose			
31	56.5	Low dose			

CALCULATION OF THE CONCENTRATION OF CARBONYL IN THE SAMPLES FROM THE
ABSORBANCE VALUE

Background = the blank MDA standard

Absorbance for each samples and standards = Measured aborbance – background

$(S_a - S_v) \times D$ = concentration of MDA (nmole/µL)

S_a = Amount of MDA in the unknown sample

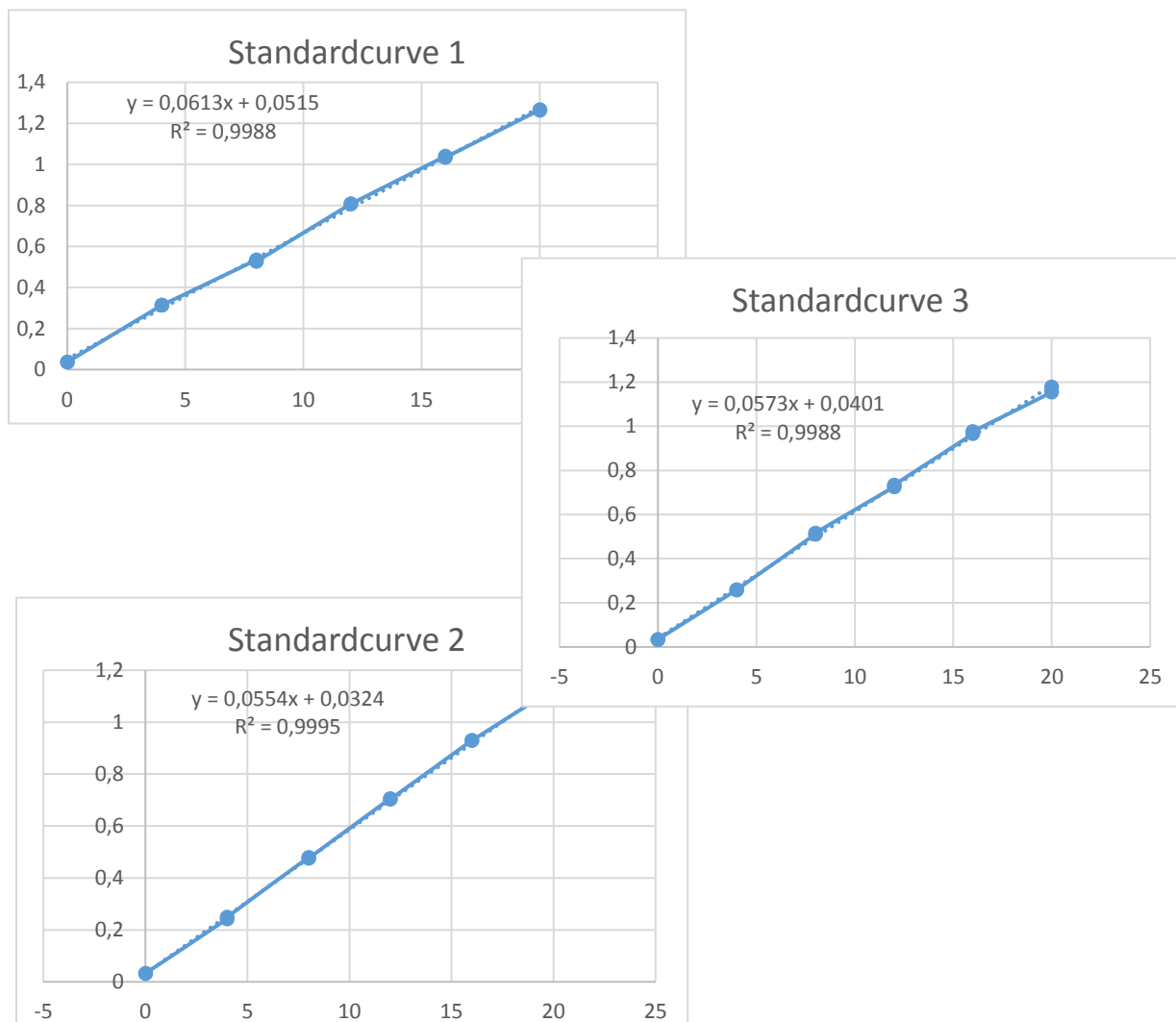S_v = sample volume added to wells (µL)

D = Dilution factor

FIGURE 4B. STANDARD CURVE 1, 2 AND 3

TABLE 4C. CALCULATED MDA-CONCENTRATIONS IN THE SAMPLES

Shrimp no.	MDA (nM/µL)	Notice	Shrimp no.	MDA (nM/µL)	Notice
1	0.75	Control	33	0.86	High dose
2	0.80	Control	35	1.6	High dose
3	0.81	Control	37	0.95	High dose
4	0.77	Control	38	0.85	High dose
5	1.2	Control	39	0.90	High dose
6	1.4	Control	40	0.97	High dose
7	1.3	Control	41	1.1	High dose
8	1.4	Control	42	1.1	High dose
9	0.71	Control	43	0.47	High dose
10	1.0	Control	45	1.0	High dose
11	1.0	Control	46	0.42	High dose
12	0.83	Control	47	0.98	High dose
13	0.75	Control	48	0.65	High dose
14	0.68	Control	50	0.55	High dose
15	0.32	Control	51	0.83	High dose
16	0.58	Low dose	1	1.7	Extra control
17	0.99	Low dose	2	0.71	Extra control
18	0.90	Low dose	3	1.2	Extra control
19	0.73	Low dose	4	1.3	Extra control
20	0.90	Low dose	5	1.7	Extra control
21	1.3	Low dose	6	1.5	Extra control
22	0.87	Low dose	7	0.94	Extra control
23	0.86	Low dose	8	1.3	Extra control
24	0.80	Low dose	9	1.5	Extra control
25	0.75	Low dose			
26	1.3	Low dose			
27	0.78	Low dose			
28	1.1	Low dose			
29	0.82	Low dose			
30	1.2	Low dose			
31	0.68	Low dose			

APPENDIX 5

TABLE 5A. CORRELATION ANALYSES. GREEN MARKS SIGNIFICANT CORRELATIONS.

	Group	CL	Weight	Deformities	Black dots	Speckled eyes	Moulting	TFB	Carbonyls	MDA
Group										
CL (mm)	**-0.30**									
Weight (g)	-0.26	**0.96**								
Deformities	**0.51**	**-0.29**	**-0.28**							
Black dots	0.05	-0.02	-0.02	-0.15						
Speckled eyes	**0.48**	-0.12	-0.13	0.11	0.10					
Moulting	0.16	0.02	0.01	-0.02	-0.20	0,08				
TFB (ng/g)	**0.87**	**-0.37**	**-0.37**	**0.46**	0.14	**0.39**	0.21			
Protein Carb.	-0.26	-0.06	-0.18	-0.09	0.26	-0.05	-0.04	-0.21		
MDA content	-0.24	0.00	-0.07	0.03	-0.12	-0.08	-0.20	-0.10	0.12	
Gpx1	-0.01	0.25	0.19	-0.12	0.21	0.12	**0.34**	0.03	-0.24	-0.14
Gpx4	**-0.48**	**0.40**	**0.35**	-0.24	0.04	-0.17	0.14	**-0.37**	0.05	-0.02
Cat	-0.01	**0.37**	**0.33**	-0.18	-0.16	0.05	**0.32**	0.06	**-0.33**	0.02
Mn sod	**-0.42**	**0.53**	**0.47**	**-0.33**	-0.08	-0.11	0.04	**-0.35**	0.04	0.11
Cyp 3A	**-0.36**	**0.42**	**0.45**	**-0.45**	0.20	-0.09	-0.24	**-0.39**	0.01	0.07
Gstm3	**-0.28**	**0.32**	**0.27**	-0.17	**-0.29**	-0.13	0.15	-0.13	-0.20	0.13
Gclc	**-0.42**	**0.36**	0.26	**-0.31**	-0.11	-0.14	0.21	**-0.27**	0.21	0.24
Hsp70	0.12	0.12	0.13	-0.03	0.02	-0.07	**0.47**	0.14	0.00	**-0.34**
Bclx	-0.09	0.23	0.19	-0.02	-0.07	0.11	**0.33**	-0.09	0.02	0.22
Chit1A	**-0.32**	**0.39**	**0.33**	**-0.29**	0.01	-0.15	0.07	-0.26	0.19	0.03
Gss	-0.04	0.04	0.00	-0.03	-0.02	-0.09	-0.08	0.08	0.08	**0.52**
Ctbs	**-0.36**	0.25	0.24	-0.26	0.15	0.00	**-0.42**	**-0.31**	0.02	**0.30**