Early life stages of Northern shrimp (Pandalus borealis) are sensitive to fish feed containing the anti-parasitic drug diflubenzuron

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ABSTRACT

Increasing use of fish feed containing the chitin synthesis inhibiting anti-parasitic drug diflubenzuron (DFB) in salmon aquaculture has raised concerns over its impact on coastal ecosystems. Larvae of Northern shrimp (Pandalus borealis) were exposed to DFB medicated feed under Control conditions (7.0 °C, pH 8.0) and under Ocean Acidification and Warming conditions (OAW, 9.5 °C and pH 7.6). Two weeks' exposure to DFB medicated feed caused significantly increased mortality. The effect of OAW and DFB on mortality of shrimp larvae was additive; 10% mortality in Control, 35% in OAW, 66% in DFB and 92% in OAW + DFB. In OAW + DFB feeding and swimming activity were reduced for stage II larvae and none of the surviving larvae developed to stage IV. Two genes involved in feeding (GAPDH and PRLP) and one gene involved in moulting (DD9B) were significantly downregulated in larvae exposed to OAW + DFB relative to the Control. Due to a shorter intermoult period under OAW conditions, the OAW + DFB larvae were exposed throughout two instead of one critical pre-moult period. This may explain the more serious sub-lethal effects for OAW + DFB than DFB larvae. A single day exposure at 4 days after hatching did not affect DFB larvae, but high mortality was observed for OAW + DFB larvae, possibly because they were exposed closer to moulting. High mortality of shrimp larvae exposed to DFB medicated feed, indicates that the use of DFB in salmon aquaculture is a threat to crustacean zooplankton.

1. Introduction

The continued growth of marine aquaculture production has presented the industry with environmental and production challenges, one of which is the ectoparasitic salmon louse (Lepeophtheirus salmonis) (Torrissen et al., 2013). A common method to control populations of lice within farm cages is treatment by various pharmaceuticals (Lillicrap et al., 2015). The use of benzoylurea-type insecticides, which act as chitin synthesis inhibitors (CSIs) has increased due to development of resistance in salmon lice to other types of medicine such as emamectin benzoate (Folkehelseinstituttet, 2015; Jones et al., 2013). Salmon are treated with medicated feed containing CSIs to remove moulting stages of salmon lice (Torrissen et al., 2013). The salmon lice absorb CSIs from salmon blood and mucus, and die during moulting. CSIs enter the coastal environment from waste feed and fish faeces (Langford et al., 2014; Samuelsen et al., 2015). The increasing use of chemicals in aquaculture is raising concerns over the survival and well-being of populations of economically important crustaceans such as shrimp, lobsters and crabs, as well as other ecologically important crustaceans which represent key elements of many marine food chains (Langford et al., 2014; Macken et al., 2015; Samuelsen et al., 2014). The two main CSIs used in medicated feed are diflubenzuron (DFB) and teflubenzuron (TFB). Since 2009 a total of 24 567 kg DFB (41 million kg medicated feed), and 14 981 kg TFB (7 million kg medicated feed) have been used as anti-parasitic medicine in Norwegian salmon farms. It has been known for decades that crustacean larvae are very sensitive to DFB (Christiansen et al., 1978; Eisler, 1992). In 1980 Christiansen & Costlow warned about the high toxicity of DFB for crustacean larvae (Christiansen and Costlow, 1980) and a freshwater field experiment showed an immediate decline of crustaceans following treatment with DFB (Dimilin®) (Ali and Mulla, 1978). Elevated levels of DFB have recently been documented in the tissues of shrimp and other crustaceans living in areas where DFB medicated feed is used (Langford et al., 2014). During treatment of farmed salmon, planktonic crustaceans may

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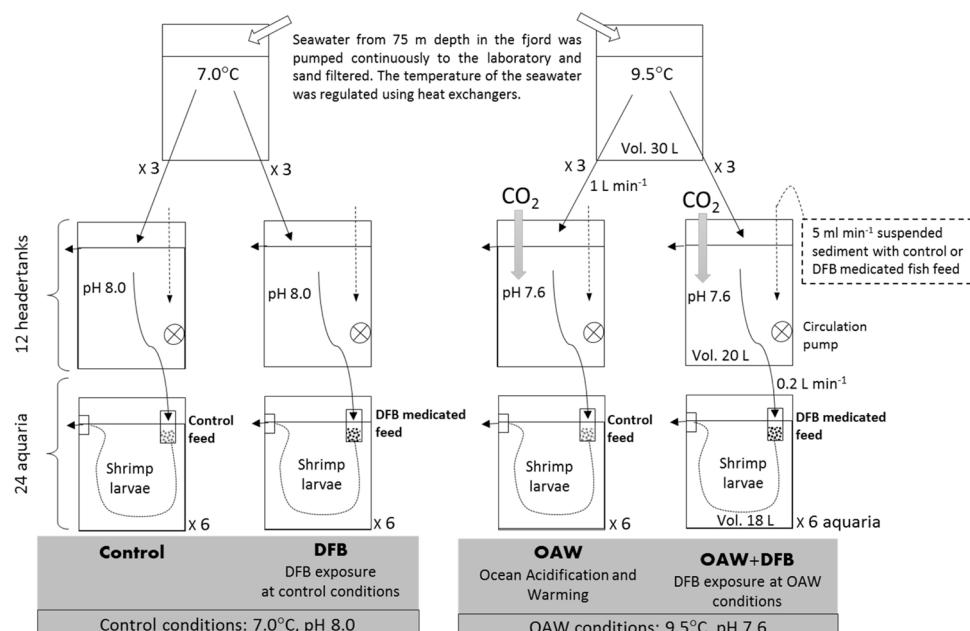
Fig. 1. Experimental set-up for *P. borealis* larvae exposed to DFB medicated feed under Control and Ocean Acidification and Warming conditions (OAW). Six replicate aquaria were used for each treatment in Exp. 1 and four replicates in Exp. 2. Suspended sediment was only added in Exp. 1.

be exposed to suspended particles of medicated feed and dissolved DFB. The question is whether crustaceans exposed to DFB medicated feed can accumulate lethal concentrations of the compound. In the present study, laboratory experiments have been performed to investigate whether exposure to the commercially available medicated feed Releeze vet. (EWOS), containing DFB as the active ingredient, has a negative impact on planktonic larvae of the Northern shrimp *Pandalus borealis* Krøyer, 1838, an ecologically and commercially important species (Bergström, 2000). High mortality has been documented for adult *P. borealis* exposed to DFB medicated feed (Bechmann et al., 2017), but to our knowledge there are no published papers on the effects of DFB medicated feed on crustacean zooplankton. The primary objective in this study was to investigate whether exposure to DFB medicated feed could cause negative effects on survival, development, feeding, swimming, respiration and gene expression in *P. borealis* larvae. Effects observed in *P. borealis* larvae will also have relevance for predicting effects of exposure to DFB medicated feed in other crustacean zooplankton, although there may be differences in species sensitivity. *P. borealis* larvae undergo a 2–3 month planktonic development period characterized by five planktonic zoea stages (Ouellet and Allard, 2006; Rasmussen and Aschan, 2011; Shumway et al., 1985). The first three larval stages are concentrated in the upper 25 m of the water column (Chabot and Ouellet, 2005; Ouellet and Lefaivre, 1994). Early life stage development is a crucial period in the life of all organisms and is considered to be particularly vulnerable to changes in environmental conditions and toxicant exposure (Przeslawski et al., 2015).

According to climate models, the temperature of the oceans will increase by 2–4 °C (ocean warming) and pH will decrease by a further 0.3–0.5 units (ocean acidification) by the year 2100 (IPCC, 2014). The importance of research on multiple drivers, such as environmentally hazardous substances, climate change and ocean acidification is emphasized by the scientific community (Breitburg et al., 2015; Hooper et al., 2013; Moe et al., 2013; Nikinmaa, 2013). Responding to several drivers simultaneously can result in considerable energy expenditures for an organism by involving multiple molecular and cellular signalling pathways (i.e. modes of action) (Breitburg et al., 2015). Therefore, the secondary objective in this study was to investigate the combined effects of DFB medicated feed (a toxic driver) and ocean acidification and warming (global drivers).

2. Materials and methods

Shrimp larvae were exposed to small (< 1 mm) particles of DFB medicated feed under Control and OAW conditions in two experiments performed in a flow through exposure system. In Exp. 1, the effects of two weeks' exposure to DFB on survival, development, feeding rate, swimming activity, metabolic rate and gene expression for shrimp larvae were investigated. In Exp. 2 the effect on survival of shrimp larvae exposed for one day to DFB was studied.

2.1. Collection of shrimp

Shrimp (*P. borealis)* were collected from Hillefjord (North of Åmøy Rogaland County, Norway; 59° 04′ 00″ N, 5° 45′ 00′ E) using a shrimp bottom trawl on 10th February 2014 (for Exp. 1) and 13th March 2015 (for Exp. 2). Trawling lasted 20 min at 100 m depth. To avoid damage to the shrimp from the trawl net, a barrel (1 m × 1 m) was secured to the bottom of the trawl. Shrimp were sorted by hand and transferred to 50 L tanks that were regularly oxygenated to maintain > 80% oxygen saturation. The shrimp were transported to the laboratory within 2 h of capture. Upon arrival at the laboratory, shrimp were randomly divided between several large tanks (vol. 500 L) for acclimation. Ovigerous shrimp used for producing larvae for Exp. 1 (2014) and Exp. 2 (2015) were transferred to the exposure system after seven and three days, respectively. Each tank was continuously supplied with sea water directly pumped from 75 m depth in the fjord close to the laboratory facilities. The sea water was sand-filtered prior to use in the experimental system. Shrimp were fed raw fish or pellets of fish feed ad libitum during the acclimation period before starting the experiments.

2.2. Environmental scenarios

The shrimp larvae were exposed to DFB at the temperature and pH level found in Hillefjord around the time shrimp were collected (Control conditions: pH_{NBS} 8.0, 7.0 °C), and at the predicted elevated temperature and reduced pH for the year 2100 according to the IPCC (IPCC, 2014) (OAW conditions: pH_{NBS} 7.6, 9.5 °C). Fig. 1 illustrates the experimental design and exposure system. The shrimp were kept in a continuous flow system, where temperature was regulated using heat exchangers. In the OAW treatment the desired pCO_2 equilibration was

achieved via manipulating pH using the method by (Widdicombe and Needham, 2007). A detailed description of the exposure system can be found in (Arnberg et al., 2013), and an overview of the test conditions (temperature, pH, oxygen, salinity, carbonate system parameters) is presented in Supplementary Table 1.

2.3. Exp. 1. Shrimp larvae exposed for two weeks to DFB

2.3.1. Collection of larvae

Ovigerous shrimp were transferred to 48 glass aquaria (vol. 18 L, flow 0.2 L min^{-1}, one shrimp *per* aquarium) and kept under Control and OAW conditions for two weeks. Twenty-four aquaria were used for acquiring larvae to start the experiment, in addition to collecting larvae for genomics, whilst 24 were used for exposing the larvae to experimental conditions. The experiment had six replicate aquaria for each of the four treatments; Control, DFB, OAW, OAW + DFB (Fig. 1). There was an average of 187 larvae *per* aquarium. The larvae in each aquarium were monitored from hatching (less than 24 h old) until they reached stage IV zoea. The shrimp larvae were fed freshly hatched *Artemia salina* nauplii and algae *(Thalassiosira weisslogi* 1200™, Microalgae, Vigra, Norway) as described in Arnberg et al. (2013).

2.3.2. DFB exposure

The exposure was designed to simulate the presence of DFB [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)-urea] medicated fish feed and resuspended contaminated sediment particles in the water during the medication period for salmon. Larvae in DFB and OAW + DFB were exposed for two weeks starting one day after hatching. Two weeks is the maximum treatment period with DFB medicated feed recommended for salmon farms (Felleskatalogen, 2015). The larvae were exposed to the medicated fish feed (pellet size 3.5 mm) EWOS Releeze vet. containing 0.6 g DFB kg^{-1} (EWOS, Bergen, Norway). A similar type and size of unmedicated fish feed (Opal 50 40A 500, EWOS, Bergen, Norway) was used for the control treatments. Pellets (2 g) were placed in a cylinder (height 50 mm, diameter 20 mm) with 1 mm mesh at the bottom. The water flow into the aquaria went through the cylinders. Hence, the shrimp larvae were only in contact with suspended particles of feed smaller than 1 mm and dissolved DFB from the feed. The fish feed was replaced with fresh feed daily for two weeks. In addition to the fish feed in the cylinders, shrimp larvae were exposed to a low concentration of suspended sediment particles containing clean or medicated fish feed. The contribution of DFB from the sediment particles entering the aquaria with the inlet of water was low compared to the contribution from the DFB medicated feed placed in the cylinder in each aquarium. A stock solution of sediment with 8% fish feed (by dry weight) was continuously pumped into the header tanks giving a concentration of 0.5 mg particles L^{-1}. The sediment slurry with fish feed was prepared in two 100 L polyethylene tanks, one with control feed (without DFB) and one with DFB medicated feed. Stirrers with stainless steel propellers were mounted at the top of the tanks, and kept the sediment in suspension. A peristaltic pump was used to deliver a continuous flow of the sediment suspension to each header tank (5 mL min^{-1}, Fig. 1) where it was diluted with 1 L min^{-1} of seawater. A circulation pump in the header tanks kept the particles in suspension. Water from the header tanks entered the aquaria by gravitation (0.2 L min^{-1}). Particles of feed and sediment were removed from the bottom of the aquaria by siphoning when larvae were taken out for stage determination.

2.3.3. Survival and development

Survival of larvae in Control and DFB was followed until 27 days post-hatch (dph), when the Control larvae reached stage IV, and survival of OAW and OAW + DFB larvae were followed until 19 dph when the OAW larvae reached stage IV. All larvae were siphoned out of the aquaria and into glass bowls to record survival and developmental stage at 6, 9, 13, 17, 19 and 27 dph according to (Arnberg et al., 2013; Bechmann et al., 2011). After stage determination, larvae were

returned to their aquaria.

The determination of swimming, feeding and respiration rates was performed with larvae of the same developmental stage from all treatments. Since development is faster under OAW conditions, the tests were performed on different dph under the two environmental scenarios.

2.3.4. Feeding experiment

Feeding rate, number of *Artemia* nauplii (prey) consumed *per* individual shrimp larvae *per* unit hour, was investigated as described in (Arnberg et al., 2013). Tests with stage II larvae were performed at 14 dph for larvae from Control and DFB, and at 10 dph for larvae from OAW and OAW + DFB. Tests with stage IV larvae were performed at 30 dph for larvae from Control and DFB. Since no stage IV larvae were available from OAW + DFB, feeding rate for stage IV larvae from OAW was compared to feeding rate for stage III larvae from OAW + DFB at 22 dph.

2.3.5. Determination of metabolic rates

Respiration rate (nM O$_2$ h^{-1} DW) for stage IV larvae was tested 30 dph for Control and DFB, and 22 dph for OAW. None of the larvae exposed to OAW + DFB developed to stage IV, hence respiration rate was not measured for this treatment. To estimate metabolic rate, the rate of oxygen consumption was measured as described in (Arnberg, 2016) and (Chabot and Ouellet, 2005). Background respiration was between 1 and 4% of the total larval respiration. These background rates were then used to correct the measured larval respiration values. In each test eight shrimp larvae were placed in 50 mL glass bottle respirometers with the appropriate treatment header tank water used in the experiment and sealed with the airtight stoppers, and then placed in temperature controlled rooms to maintain a constant temperature. Tests were done with 3 replicate batches of 8 larvae from 5 Control aquaria, 3 DFB aquaria and 6 OAW aquaria. Duration of the incubation was 20 h. The respiration rate was determined in temperature controlled rooms; at 7.0 °C for the Control and DFB larvae, and at 9.5 °C for the OAW larvae.

2.3.6. Swimming tests

Early larval stages of shrimp larvae exhibit positive phototaxy and this response to light is crucial in nature to put them in proximity to their food and enhance their chances of successful growth and development. A simple behavioural assay was used in this study to record the ability of larvae to respond to light following their exposure to the various treatments. Swimming activity tests using surviving mobile shrimp larvae at stages I, II and IV were performed. Positive phototaxy in larvae was measured by recording their movements in proximity to a white light source. Two infrared light emitting diodes (LED) were aligned with phototransistors with each pair set at 90° across the width of a clear glass tube, 16 cm in length and 15 mm diameter. A white light LED was positioned directly above these beams to attract active swimming larvae. As larvae repeatedly swam towards the light and fell back from the surface of the water, they passed through the infrared light beams causing a change in the output voltage of the paired phototransistors. These changes were recorded using a data logger (NI USB – 6009, National Instruments, Austin, TX, USA). Data were downloaded and processed using Microsoft Excel. Regular changes in voltage during the 1 h recording period for each treatment tank provided evidence of activity close to the light. A test vessel was prepared from each replicate treatment tank, each containing between two and five mobile larvae (Stage I: 5 larvae, Stage II: 3 larvae and Stage IV: 2 larvae). The relatively low numbers of larvae used for the activity assessments in each treatment replicate allowed for direct comparison of swimming intensity among the treatments that may have been masked with the use of greater numbers. Test vessels were filled with water from the relevant treatment tank and then suspended within 5 L plastic tanks containing seawater at the relevant exposure temperature. Light

responsive tests with larval stages I, II and IV were performed 6, 14 and 30 dph, respectively, for Control and DFB, and 3, 10 and 22 dph, respectively, for OAW. Since none of the larvae developed to stage IV in OAW + DFB, only stage I and II larvae were tested at 3 and 10 dph. The difference in timing was due to faster larval development in the OAW conditions. All tests were conducted in a constant low light environment. Phototactic swimming responses in larvae are presented as mean total beam breaks per hour taken from replicate tanks of each of the treatments.

2.3.7. Reference transcriptome

Total RNA was extracted from the heads (including eyes and brain) of flash frozen adult female shrimp from each of the treatments (Control – including two recent moults, DFB, OAW and OAW + DFB) as described in Seear et al. (2010). These shrimp had been fed control or medicated feed ad libitum for two weeks. The RNA was DNased with a TURBO DNA-free kit (Life Technologies, Paisley, UK), purified with RNeasy columns (Qiagen GmbH, Hilden, Germany) and eluted into nuclease and protease free water (Fisher Scientific, Loughborough, UK). RNA quantity and quality were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). RNA was pooled equally from four adult shrimp from each of the four treatments and 25 μg submitted to GATC Biotech (Konstanz, Germany) for normalised cDNA library construction and sequencing on a Pacific Biosciences RS II instrument (C2 chemistry) using seven SMRT cells each with 1×180 min movie run times. The resulting subreads.fasta were processed to generate the reference transcriptome as described in the Supplementary Materials.

2.3.8. Shrimp larvae gene expression

Larvae from Control, DFB and OAW + DFB were sampled for analysis of gene expression just before moulting to stage II (i.e. 9 dph for Control and DFB and 6 dph for OAW + DFB). The larvae were exposed to medicated feed as explained above. Unfortunately, there were no OAW larvae available for genomics. The expression of five transcripts selected from the reference transcriptome based on their known moulting, metabolism, stress, and motor activity was compared between Control and DFB stage I/II larvae and between Control and OAW + DFB stage I/II larvae using quantitative PCR (qPCR), as described in the Supplementary Material. These transcripts had significant sequence homology to genes encoding for the proteins glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme; troponin C, which is part of the troponin complex essential for contraction in skeletal and cardiac muscle; peroxiredoxin, an antioxidant enzyme; pancreatic lipase-related protein (PLRP) which has lipase activity (Lowe, 2000) and DD9B, known to be upregulated during pre- and post-moult in crustaceans (Seear et al., 2010; Watanabe et al., 2000), has a chitin binding domain, and is likely to play a role in the calcification of the crustacean exoskeleton (Watanabe et al., 2000).

2.4. Exp. 2. Shrimp larvae exposed for one day to DFB

Ovigerous shrimp were transferred from the holding tanks to the same exposure system as described above for Exp. 1 (Fig. 1). After six days, batches of shrimp larvae from each female were collected and the experiment was started. Four replicate aquaria with an average of 141 larvae were assigned to four treatments; Control, DFB, OAW and OAW + DFB. The larvae in DFB were exposed to medicated feed for 24 h twice, starting 4 and 16 dph, and the larvae in OAW + DFB were exposed for 24 h once, starting 4 dph. All the larvae in the 16 aquaria were siphoned out of the aquaria and into glass bowls (as described above) to record survival at 11, 22 and 33 dph.

2.5. Chemical analyses of DFB

The total concentration of DFB (total amount of DFB attached to

feed particles and dissolved in the water) was analysed in water samples from the shrimp larvae aquaria in Exp. 1. Water samples were siphoned from three aquaria from each treatment 4 and 24 h after placing the cylinder with fish feed in the aquaria. The samples were extracted by solid phase extraction using Oasis HLB and eluted with methanol before evaporation to near dryness under nitrogen and reconstituted with ACN/water. The tissue concentration of DFB in one pooled sample of stage I shrimp larvae kept under the same conditions as in Control and DFB (Exp. 1) for 8 days was analysed. Fresh control and DFB feed pellets were also analysed. Approximately 4 g of pellets were homogenized, of which 0.3 g was used for the analysis. All samples were analysed by liquid chromatography coupled to mass spectrometry (Waters UPLC, Zevo G2S QTOF mass spectrometer) operating in negative ESI mode. Liquid chromatography was performed on an Acquity BEH C18 column (1.7 μm, 100×2.1 mm) (Waters, MA, USA). The methods and validation of the determination of DFB have been described in Langford et al. (2014).

2.6. Statistical treatment

The effect of DFB, OAW, and their interaction on the variables measured in this study was analysed using General Linear Model (GLM) tests, two-way ANOVA, or a Wilcoxon Rank Sum test (Bhattacharyya and Johnson, 1977). Data tested with ANOVA met the assumption for normality of distribution, tested by the Kolmogorov-Smirnov test, and variances were homogeneous using the Levene's test. In the cases where normality or homogeneity criteria were not met, a Wilcoxon Rank Sum test was used to analyse the effects of DFB and climate on the biological parameters. All analyses were conducted using SPSS® v22 (IBM, Chicago, USA).

3. Results

3.1. DFB exposure

The concentration of DFB in the Releeze pellets was 0.7 g kg^{-1}, slightly higher than the value given by the producer (0.6 g kg^{-1}). The pellets appeared to be intact when removed from the cylinders, but more particles were observed in the aquaria during than after the exposure period, indicating that small particles of fish feed entered the aquaria. The mean total concentration of DFB in the aquaria, including the particles, was 1.5 μg L^{-1}. The values include dissolved DFB and DFB attached to particles in the water (i.e. feed particles, *Artemia* nauplii, algae). There was no significant difference in DFB concentration between DFB and OAW + DFB ($p > 0.05$), but the concentration was 41% lower 24 h after adding the pellets to the cylinders compared to 4 h ($p = 0.015$, Supplementary Fig. 1). The tissue concentration of DFB in the pooled sample of exposed shrimp larvae was 180 ng g^{-1} WW. The concentration in the pooled sample of control larvae was below the limit of detection for the analytical method (0.8 ng g^{-1} WW).

3.2. Survival

3.2.1. Exp. 1. two-week exposure to DFB medicated feed

Two weeks' exposure to DFB medicated feed caused a significant increase in mortality, and the effect of OAW and DFB on mortality of shrimp larvae was additive; 10% mortality in Control, 35% in OAW, 66% in DFB and 92% in OAW + DFB (Fig. 2, Supplementary Table 2). There was a similar increase in the mortality of shrimp larvae exposed to DFB medicated feed under Control and OAW conditions; 56 percent points higher mortality of DFB than Control larvae, and 57 percent points higher mortality of OAW + DFB than OAW larvae at 19 dph. Mortality was significantly increased in DFB compared to Control from 13 dph ($p = 0.004$), and in OAW + DFB compared to both OAW and Control from 6 dph ($p_{max} = 0.041$). Mortality of shrimp larvae was significantly higher in OAW than in Control from 9 to 19 dph
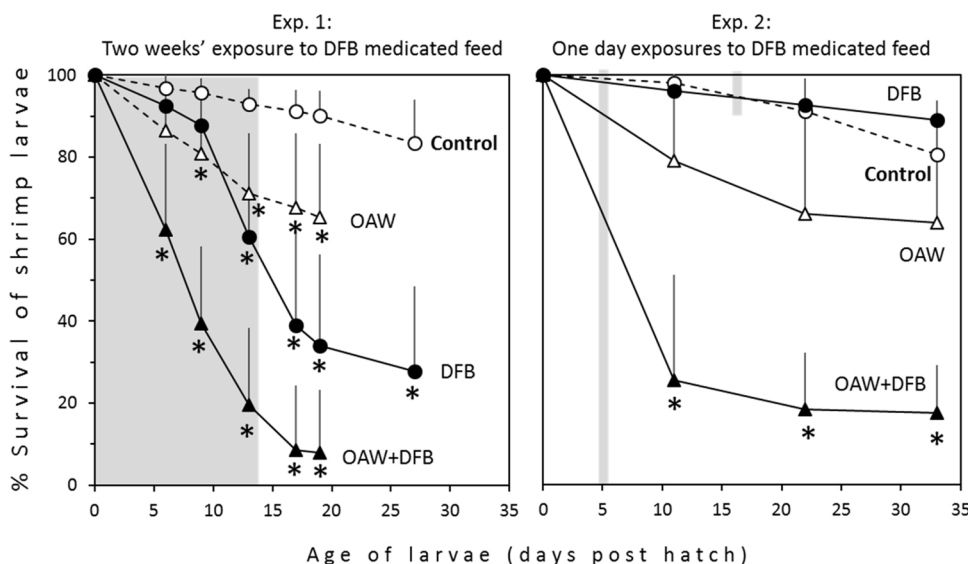
Fig. 2. Mean percent survival (+SD) for *P. borealis* larvae exposed to DFB medicated feed. For each of the four treatments (Control, DFB, OAW and OAW + DFB) there were 6 replicate aquaria with an average of 187 larvae in Exp. 1, and 4 replicate aquaria with an average of 141 larvae in Exp. 2. * denotes significantly lower survival than the Control (p < 0.05). The grey areas indicate the exposure periods to DFB medicated feed.

(F_{max} = 12.97, p < 0.002). When the OAW larvae reached stage IV at 19 dph, mortality was 25 percent points higher than in the Control. The effect of OAW and DFB on mortality of shrimp larvae was additive, with no significant interaction of DFB and OAW at 19 dph ($F_{3,23}$ = 0.005, p = 0.942).

3.2.2. Exp. 2. one day exposure to DFB medicated feed

Survival for larvae in Control and OAW was similar in Exp. 1 and Exp. 2 (Fig. 2, Supplementary Table 2 and 3). One day exposure at 4 dph and 16 dph did not reduce survival of DFB larvae (p > 0.05). One day exposure at 4 dph, however, resulted in approximately 50% lower survival in OAW + DFB than in OAW (p = 0.029). There was significantly lower survival in OAW + DFB than in Control (p = 0.014) at all sampling days. At the end of the experiment, 33 dph, the mean mortality was 19% in Control, 36% in OAW, 11% in DFB and 82% in OAW + DFB. The OAW + DFB larvae were exposed closer to their first moult than the DFB larvae due to the shorter intermoult period under OAW conditions. The plan with the second exposure day 16 dph was to expose the DFB larvae just before the second moult, but many empty moults were observed indicating that the larvae had moulted one day earlier than expected. Therefore, neither of the two exposure days for DFB larvae occurred as close to moulting as the 4 dph exposure for OAW + DFB larvae.

3.3. Larval development

Exposure to DFB medicated feed caused slower development of larvae (Fig. 3). Significantly less DFB than Control larvae had developed to stage III at 17 dph (p = 0.004), but there was no significant difference in the percentage of stage IV larvae at 27 dph (p = 0.126). A stronger effect of DFB was observed on development time under OAW than Control conditions. Significantly less OAW + DFB than OAW larvae had developed to stage II at 6 dph (p = 0.026) and to stage III at 13 dph (p = 0.002), and none of the OAW + DFB larvae developed to stage IV. At 19 dph 70% of the OAW larvae had reached stage IV, but 77% of the surviving OAW + DFB larvae were still stage II (Fig. 3). The mean values in Fig. 3 are based on results from aquaria with surviving larvae. At 17 dph, all the larvae in two of the six replicate aquaria exposed to OAW + DFB had died, and two days later there were only three of six replicate aquaria with surviving larvae left. Larval development time was reduced in OAW compared to Control ($F_{3,20}$ = 6.75 p = 0.019). The intermoult period was approximately 9 days in Control and 6 days in OAW. Therefore, Control larvae moulted to stage II, III and IV at 9, 17 and 27 dph, and OAW larvae moulted at 6, 13 and 19

dph.

3.4. Swimming tests

Stage I shrimp larvae from all treatment tanks swam actively towards the light source (Fig. 4A), with DFB exposed larvae showing significantly higher swimming intensity than the Control group (p = 0.018). Swimming activity for stage II larvae was at a similar intensity for Control, DFB and OAW groups, although there was a marked significant decrease in activity in the OAW + DFB treatment group when compared against the Control group (T test, p = 0.0016) (Fig. 4B). For stage IV larvae (Fig. 4C) activity levels were similar among Control, DFB and OAW groups, but no larvae in the OAW + DFB treatment group reached stage IV.

3.5. Feeding rate

Each shrimp larvae from Control consumed on average two *Artemia* h^{-1} when they were at stage II, and twice as many when they reached stage IV (Fig. 5). Reduced feeding rate was observed for stage II larvae from OAW + DFB compared to OAW (p < 0.001), but not for larvae from DFB (p = 0.247) (Fig. 5). Feeding rate for stage II larvae was similar for larvae from Control and OAW ($F_{3,44}$ = 3.51, p = 0.068). There was a significant interaction between OAW and DFB ($F_{3,44}$ = 4.93, p = 0.032), reducing the feeding for stage II larvae in the OAW + DFB treatment, but not in the DFB treatment. In general, stage IV larvae had a higher feeding rate than stage II larvae (p < 0.001), regardless of exposure. There was no significant effect of DFB on feeding rate at the end of the experiment when the Control larvae had reached stage IV (p = 0.739). There was a tendency to increased feeding for stage IV larvae from OAW compared to Control (p = 0.059).

3.6. Respiration rate

Stage IV larvae had a mean oxygen consumption rate of 56.8, 51.7 and 61.46 nM O_2 h^{-1} DW in Control, DFB and OAW, respectively, with no significant difference between the treatments ($F_{max1,31}$ p = 1.11, p_{max} = 0.300). None of the larvae developed to stage IV in OAW + DFB, hence there was no data on respiration rate from this treatment.
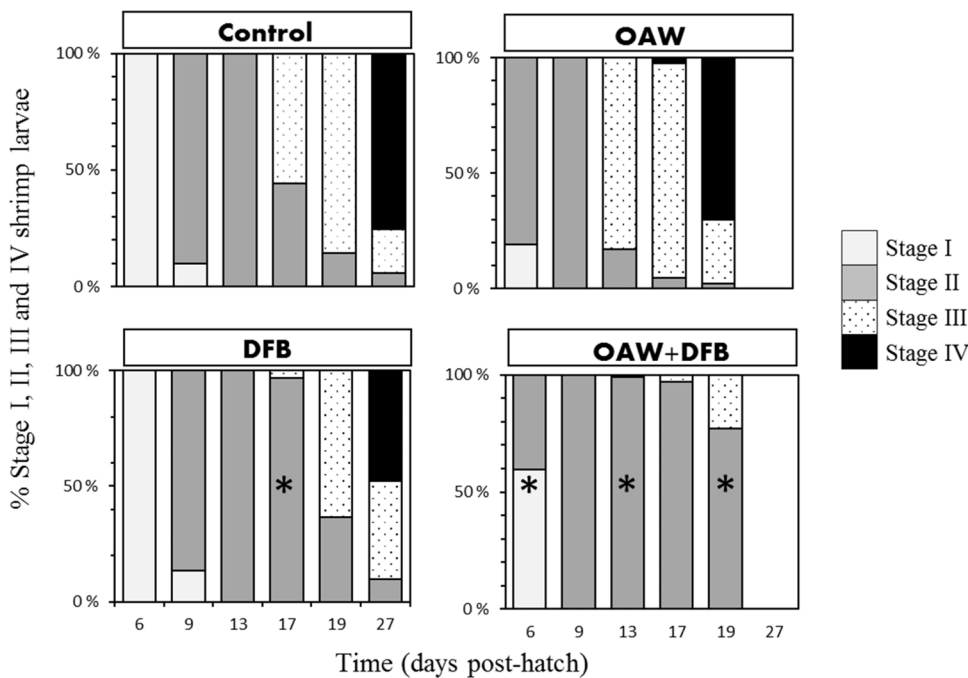
Fig. 3. Mean percentage of stage I–IV larvae with time for *P. borealis* exposed to DFB medicated feed the first two weeks after hatching (Exp. 1) compared to unexposed shrimp (n = 6 replicate batches of larvae per treatment). * denotes significantly slower development for larvae exposed to DFB relative to Control, or larvae exposed to OAW + DFB relative to OAW (p < 0.05).
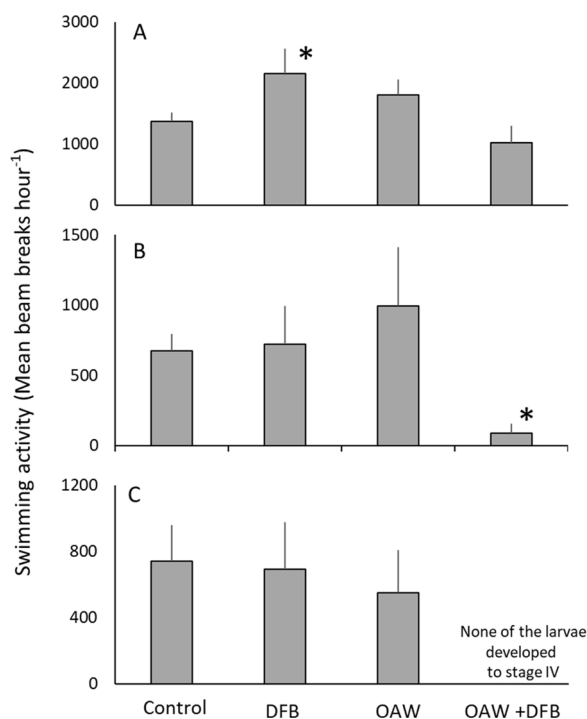

Fig. 4. Phototactic swimming activity in *P. borealis* larvae as mean beam breaks hour^{-1} (+ SE), from Control, DFB, OAW and OAW + DFB in Exp. 1. (A) Stage I, (B) Stage II and (C) Stage IV larvae. * denotes significant difference to Control group (p < 0.05).

Fig. 5. Feeding rate for stage II and stage IV *P. borealis* larvae from Exp. 1 (mean + SD). None of the larvae in the OAW + DFB treatment developed to stage IV, hence stage III larvae were tested at the end of the experiment. Number of *Artemia* consumed per shrimp larvae per hour was significantly different in groups with different letters above the columns (p < 0.001).

3.7. Genomics

3.7.1. Reference transcriptome

Following filtering of low quality sequence, 2.9 Gb of raw sequence was generated from the PacBio sequencing of a *P. borealis* head tissue library, with each PacBio sequence read containing multiple subreads (Supplementary Table 4). De novo assembly of the 2.5 million subreads (deposited in the NCBI Short Read Archive under BioProject ID PRJNA368982) resulted in over 19 000 contigs consisting of seven or more subreads, with an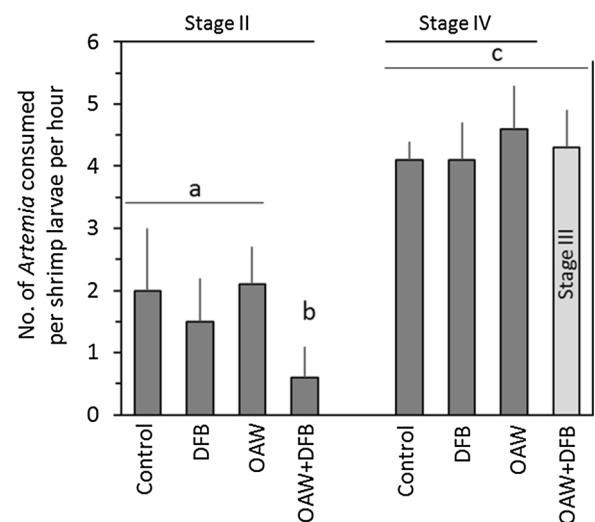 average length of 762 bp (Supplementary Table 4). Searches against the NCBI nr database revealed significant matches (E value < 10^{-5}) for over 10 600 of the contigs. These contigs were further annotated with Gene Ontology terms (Supplementary Fig. 2) and mapped to 54 KEGG Pathways, with the top three being oxidative phosphorylation, glutathione metabolism and porphyrin and chlorophyll metabolism.

3.7.2. Quantitative PCR

To investigate the effect of exposing shrimp larvae to DFB on gene expression, qPCR was performed on five genes selected from the reference transcriptome based on their known or proposed involvement in a number of physiological processes. *Peroxiredoxin* was the only gene significantly downregulated (−1.94 fold, p < 0.05, Fig. 6) in larvae from the DFB treatment (Fig. 6). In larvae from OAW + DFB, however, *GAPDH* (−4.75 fold, p < 0.01), *DD9 B* (−2.29 fold, p < 0.01) and
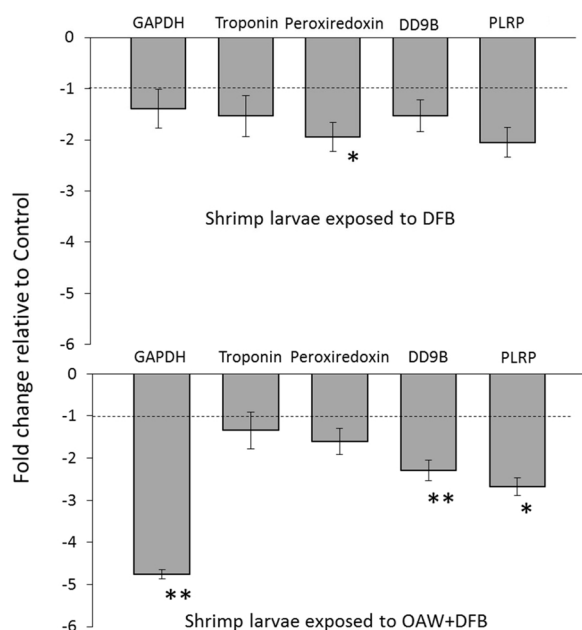
Fig. 6. Gene expression (average fold change ± SE, n = 6) in *P. borealis* larvae from DFB (top) and OAW + DFB (bottom) relative to larvae from Control. *GAPDH*: *Glyceraldehyde 3-phosphate dehydrogenase*. *PLRP*: *Pancreatic Lipase-Related Protein 2*. Asterisks indicate significant results * = p < 0.05, ** = p < 0.01.

PLRP (−2.67 fold, p < 0.05) were all significantly down regulated relative to larvae from Control (Fig. 6).

4. Discussion

4.1. Relevance of exposure

Exposing planktonic larvae to small particles of medicated feed simulates the situation in the field when salmon are treated with Releeze for two weeks. Small particles of feed will enter the water and become available for planktonic organisms such as shrimp larvae, even if salmon farms manage to keep the spillage of feed low. The feed spill from Norwegian salmon farms is estimated to be approximately 6–7% of the total amount of feed used (Torrissen et al., 2016). Small particles of feed (dust) are transported far away from the farm (Torrissen et al., 2016), and this type of dust from medicated feed in addition to small particles dissolving from the feed is a potential risk for planktonic crustaceans. Modelling predicts that organic effluents from salmon farms may be dispersed over large areas of fjord systems, although most of the organic effluents are dispersed to near-field sites (< 500 m from the release point) (Bannister et al., 2016). A recent field study demonstrated that organic particles containing the chitin synthesis inhibitor teflubenzuron can be transported more than 1 km away from a salmon farm (Samuelsen et al., 2015), indicating that zooplankton could be exposed to flubenzuron particles far away from the treated farm. Few studies have been performed monitoring the concentrations of DFB in the water during treatment with medicated feed, except (Langford et al., 2014) who detected 0.3 µg L^{-1} DFB in water samples. The value reported in (Langford et al., 2014) was lower than the concentration measured in Exp. 1 (1.5 µg L^{-1}), but the concentrations of DFB found in the organic material from sediment traps placed under a fish farm during medication were much higher (up to 259 µg g^{-1} DFB) (Selvik et al., 2002). The total concentration of DFB in particles and water was analysed in Exp. 1, hence the measured concentration could be relevant for the field. The DFB concentration in the aquaria following the addition of medicated feed was higher after 1 h than 24 h. This shows that more particulate and dissolved DFB leaked from the pellets during the first hour in seawater than later, and that DFB was

continuously removed from the aquaria with the outflow of water. DFB has a log K_{ow} of 3.8 and tends to remain bound to sediment and organic materials in the environment (Burridge et al., 2010), therefore DFB was most likely bound to organic material in the aquaria (Releeze, sediment, algae, *Artemia*). The most likely explanation for the high tissue concentration of DFB in exposed shrimp larvae is that the larvae fed on small particles of medicated feed, although other types of food (*Artemia* nauplii and algae) were available.

4.2. Effects of DFB medicated feed

4.2.1. Mortality

High mortality has been documented for crab larvae (*Rhithropanopeus harrisii*) exposed to 1–10 µg L^{-1} DFB (Christiansen et al., 1978), for early life stages of the copepod *Tisbe battagliai* exposed to 0.032 µg L^{-1} (Macken et al., 2015) and for several other crustaceans exposed to DFB within this range of concentrations as summarized by (Eisler, 1992). All the earlier studies on effects of DFB were done with dissolved DFB which is relevant for testing effects of the insecticide Dimilin®, and with species that thrive at temperatures around 20 °C. The high mortality (> 50%) of *P. borealis* larvae exposed for two weeks to DFB medicated feed in Exp. 1 shows that DFB in medicated feed is available for shrimp larvae. The total concentration of dissolved and particle bound DFB in the present study was similar to the concentrations of dissolved DFB affecting crustaceans in earlier studies (references above). One day exposure to DFB medicated feed 4 dph caused high mortality in OAW + DFB, where moulting occurred right after the exposure (6 dph), but no mortality in DFB where larvae moulted 9 dph. Due to the shorter intermoult period under OAW conditions the OAW + DFB larvae had less time to excrete accumulated DFB before they moulted, than the DFB larvae. Therefore, the tissue concentration of DFB may have been higher in the OAW + DFB larvae just before moulting, which is a critical period for the larvae. Shorter time to excrete DFB and/or higher sensitivity just before moulting could explain why only larvae from OAW + DFB were affected by one day exposure 4 dph. Exposure to DFB caused higher mortality of crab larvae *(Rhithropanopeus harrisii)* when exposed late rather than early in the intermoult period (Christiansen et al., 1978). Further experiments are needed to investigate if exposure to DFB the day before moulting at 7.0 °C causes the same increase in mortality as for the OAW + DFB larvae. In the field, larvae will moult on different days and a percentage of the population can potentially be exposed right before moulting when crustaceans are most sensitive to DFB exposure. The effects of exposing different percentages of a population to DFB on the long-term abundance is currently being investigated with an age-structured population model (S.J. Moe, pers. comm.). In future studies the minimum exposure time needed to induce increased mortality for different developmental stages of *P. borealis* should be investigated, including the effects of multiple pulsed exposures.

4.2.2. Development time

In OAW + DFB none of the larvae reached stage IV, indicating that none of the larvae would survive until settlement. In DFB, less serious effects were observed on development time for the surviving larvae. Due to the shorter intermoult period for shrimp larvae at OAW than at Control conditions (present study, (Arnberg et al., 2013)), the DFB larvae moulted once and the OAW + DFB twice during the two-week exposure period in Exp. 1. Exposure through two instead of one critical pre-moult period may explain why none of the OAW + DFB larvae reached stage IV, although the few surviving DFB larvae succeeded. Higher uptake rate of DFB under OAW conditions could also explain the more serious effect on larval development in the OAW + DFB treatment compared to the DFB treatment (Camp and Buchwalter, 2016; Noyes et al., 2009). Previous studies did not report any effect of DFB on development time of crustacean larvae. Exposure to DFB had no effect on duration of crab larvae development (Christiansen et al., 1978) nor

development time of the copepod *Tisbe battagliai* (Macken et al., 2015).

4.2.3. Swimming

Swimming activity in stage I larvae was similar for all treatments, indicating that DFB had no immediate debilitating toxic effects on the mobility of the tested shrimp larvae. Following the first moult, stage II larvae were found to be active close to the light source in all test groups with the exception of the OAW + DFB treated larvae. These showed a marked significant reduction in activity compared with the Control group. Reduced swimming activity in nature will reduce the ability of larvae to capture food and develop successfully. In stage IV larvae, swimming activity was similar in all treatments apart from OAW + DFB, where none of the larvae had reached stage IV at the end of the experiment. Previous investigations studying the effects of insecticides on crustacean larvae have reported changes in behaviour. Reduced swimming activity was observed for crab larvae (*Rhithropanopeus harrisii*) exposed to 1–10 µg L^{-1} DFB (Christiansen et al., 1978), with reduced positive phototaxis observed for larvae of this species exposed to 0.1 µg L^{-1} DFB (Forward and Costlow, 1978). Similar results have been reported for grass shrimp (*Palaemonetes pugio*) larvae exposed to 0.5 µg L^{-1} DFB as embryos (Wilson et al., 1985). Reduction in swimming activity observed in the stage II OAW + DFB larvae may be related to general physiological toxicity resulting from DFB exposure, though the possibility remains that incomplete or suppressed moulting, or indeed the presence of post-moult skeletal deformities could lead to the observed changes.

4.2.4. Feeding rate

Shrimp larvae consumed around 2–4 *Artemia* nauplii h^{-1} larvae^{-1} confirming the results in (Arnberg et al., 2013). In the present experiment, the feeding rate was similar for shrimp from Control and OAW, but (Arnberg et al., 2013) documented higher feeding rate for shrimp larvae exposed to OAW than Control. Significantly reduced feeding rate was observed for stage II larvae from OAW + DFB, but not in larvae from DFB. The documented reduced swimming activity for stage II larvae from OAW + DFB could explain the reduced feeding rate, as the larvae need to be able to swim to catch *Artemia* nauplii in the feeding test. Reduced feeding rate and inability to exhibit positive phototaxis has also been documented for DFB exposed cladocerans (Eisler, 1992). Larvae that eat less may not get sufficient energy to moult, hence effect on swimming and feeding could lead to indirect effects on moulting success. The negative effect on larval development in OAW + DFB, however, is most likely a direct effect of the chitin synthesis inhibitor on moulting.

4.2.5. Gene expression

Three gene transcripts were significantly downregulated in OAW + DFB larvae relative to Control larvae (*GAPDH*, *PLRP* and *DD9B*), while only one gene transcript was significantly downregulated in DFB larvae (*peroxiredoxin*). No OAW larvae were available for the qPCR experiment, so the changes in gene expression could have been solely due to the effect of OAW. In addition, this study looked at gene transcription but post-transcriptional and post-translational modifications can also alter protein abundance. The effects of DFB under OAW conditions observed at the transcript level are, however, supported up by the feeding rate and development time observations above. The glycolysis gene (*GAPDH*) and lipase gene (*PLRP*), are both involved during feeding, and were significantly downregulated only in OAW + DFB larvae which agrees with the reduced feeding only in OAW + DFB, but not DFB larvae as described above. The significant down regulation of the moulting gene (*DD9B*) in OAW + DFB larvae relative to Control is in line with the observation that only 23% of OAW + DFB exposed larvae developed beyond stage II, since the larvae need to moult in order to develop to the next stage. While DFB may not inhibit the expression of *DD9B* directly, it is likely that as a consequence of DFB inhibiting chitin synthesis, a range of moulting genes

including *DD9B* are also affected. Similar gene expression results were reported in a similar study, where (Bauer et al., 2013) exposed lobster larvae to the agricultural pesticide endosulfan, an endocrine disruptor that has been shown to inhibit moulting in both insects (Sharma et al., 2011) and crustaceans (Zou and Fingerman, 1997). As in this study (Bauer et al., 2013) found that cuticle genes and antioxidant genes were downregulated in endosulfan-exposed larvae. In another gene expression study (Olsvik et al., 2015) investigated the effects of teflubenzuron in juvenile lobster and showed that a number of moulting genes and antioxidant genes were upregulated. The differences between (Olsvik et al., 2015) and (Bauer et al., 2013) and the present study, may be due to different experimental setups. For example, Olsvik et al. used claw tissue from juvenile lobster while Bauer et al. and this study used whole larvae. Additionally, the upregulated moulting genes in the Olsvik et al. study did not include cuticle genes, and mainly consisted of chitin synthesis and moult regulatory genes that comprise different components of the moult cycle (Kuballa et al., 2007; Seear et al., 2010; Song et al., 2017).

4.3. Effects of OAW

OAW larvae had 25 percent points higher mortality than control larvae. Most of the OAW larvae did, however, survive and higher survival has been documented for shrimp larvae exposed to the same OAW scenario in earlier experiments (Arnberg, 2016; Arnberg et al., 2013). Climate change may lead to mismatches between the reproductive cycles of marine organisms and their planktonic food (Durant et al., 2013; Durant et al., 2007; Edwards and Richardson, 2004; Parmesan, 2006; Poloczanska et al., 2013). The effects of elevated temperature on the phenology of *P. borealis* could increase the risk for trophic mismatch for shrimp larvae in the future ocean (Koeller et al., 2009). Different populations of *P. borealis* have adapted to local temperatures and bloom timing, matching egg hatching to food availability under average conditions (Koeller et al., 2009). This strategy is vulnerable to interannual oceanographic variability and long-term climatic changes. In the present study, *P. borealis* larvae exposed to OAW had significantly faster development (33% shorter intermoult period) than Control larvae. A similar response to OAW has been observed in earlier studies with shrimp larvae (Arnberg, 2016; Arnberg et al., 2013). Exposure to ocean acidification alone only caused a small delay in development (Arnberg et al., 2013; Bechmann et al., 2011), but exposure to elevated temperature had the same effect as OAW (Arnberg et al., 2013). More frequent moulting at elevated temperature has been documented for all life stages of *P. borealis,* in addition to faster larval developmental for eggs and embryos (Brillon et al., 2005; Nunes, 1984; Shumway et al., 1985). Shorter intermoult period for female shrimp and faster oogenesis could affect spawning time and hence hatching time (Nunes, 1984; Shumway et al., 1985). Even if *P. borealis* may have enough phenotypic plasticity to adapt physiologically to future OAW conditions, the question is, if the plasticity in spawning and hatching time can mitigate the risk for trophic mismatch for the *P. borealis* larvae in the future ocean. The importance of both these effects (future OAW conditions and trophic mismatch) for long-term population abundance will be investigate using a population model and a range of environmental scenarios (J. S. Moe, pers. com).

4.4. Combined effect of DFB medicated feed and OAW

Future climate conditions are expected to influence the toxicological consequences of exposure to chemicals (Moe et al., 2013; Noyes and Lema, 2015; Noyes et al., 2009). Even if shrimp may adapt to future OAW conditions, faster larval development at higher temperature may indirectly increase the sensitivity of early life stages to toxic drivers like DFB. Thermal adaptation may not mitigate the increased toxicity of pesticides at higher temperatures (Dinh Van et al., 2014). Moreover, adaptation to changed climate could potentially make the population

less capable of adapting to toxicant stress (climate-induced toxicant sensitivity; (Hooper et al., 2013; Moe et al., 2013)).

There was an additive effect of OAW and DFB on mortality of shrimp larvae. An additive effect of global and toxic drivers has also been documented in growth measurements of shrimp larvae exposed to OAW and oil pollution (Arnberg, 2016). Early life stage development is considered to be particularly vulnerable to environmental drivers and toxicant exposure (Przeslawski et al., 2015). Exposure to DFB medicated feed caused high mortality of adult *P. borealis*, but the combined effects of DFB and OAW was less than additive for adult *P. borealis* exposed to the same treatments as the shrimp larvae in the present study (Bechmann et al., 2017). The negative effects documented in *P. borealis* exposed to DFB medicated feed, indicate that the use of DFB as an anti-parasitic drug is a threat to crustaceans living in areas with salmon farms.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2018.02.021.

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