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Mortality and deformities in European lobster (*Homarus gammarus*) juveniles exposed to the anti-parasitic drug teflubenzuron☆

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ABSTRACT

This study describes experiments carried out to examine effects of the antiparasitic drug teflubenzuron, used in delousing farmed salmon, on a non-target species, the European lobster (*Homarus gammarus*). Juvenile lobsters were fed two doses of teflubenzuron, 10 and 20 mg/kg successively for 7 days corresponding to a standard medication of the fish (10 mg/kg day) and twice the standard dose (20 mg/kg day). Monitoring lasted 3 months to include at least one moulting period for all individuals. Cumulative mortality was higher in all replicates given medicated feed compared with the control group. Mean cumulative mortality for each dosing was 41 ± 13% for 10 mg/kg and 38 ± 8% for 20 mg/kg, i.e. no difference. Drug residue was analysed in all juveniles that died, in addition to 12 juveniles at day 8 and the first 12 surviving lobsters. A decline in concentration of teflubenzuron from over 8000 ng/g (day 5) to 14 ng/g (day 70) was observed in the juveniles that died during the experiment. Twelve individuals that died contained 82 ng/g or less whereas the mean concentration in the first 12 lobsters that survived moulting was 152 ng/g. Following a single oral administration, the half-life of teflubenzuron in lobster was estimated to 3.4 days and the initial concentration (C_0) to 515 ng/g at time t_0. At the end of the study a considerable number of juvenile lobsters were observed with deformities in various organs; carapace, walking legs, cheliped, tail fan, abdomen and antenna. The occurrence of observed deformities varied from 0 to 15% in treated replicates and will most likely affect ability to locate and consume food (antenna, claw and walking legs), respiration (carapace) and ability to move/swim (walking legs, tail fan and abdomen). In total, the mortality and senescent damages were close to 50% in all replicates. Juveniles that survived medication without deformities however, moulted and increased in size at each moult equally well as the unmedicated controls.

1. Introduction

Salmon lice (*Lepeophtheirus salmonis*, Krøyer 1837) are small marine ectoparasites that feed on mucous, blood and skin on salmon and trout and if present in sufficient numbers, may cause mortality by osmotic stress and secondary infections by bacterial and viral pathogens. Infestation with sea lice is therefore considered a major problem for the Atlantic salmon (*Salmo salar*) industry in Europe and North America (Pike and Wadsworth, 1999; Costello, 2006; Burka et al., 2012). In addition to reducing the general welfare of the farmed fish, salmon lice cause a significant economic loss due to reduced growth, increased mortality, downgrading of flesh quality and the cost of delousing treatments (MacKinnon, 1997). Excessive numbers of adult lice in a salmon farm also increase the number of free swimming larvae in the surrounding water, hence generating a negative impact on wild populations of sea trout (*Salmo trutta*) and migrating wild post-smolts of Atlantic salmon (Wagner et al., 2008; Costello, 2009).

Several antiparasitic agents are currently in use for delousing farmed fish and the compounds are either dissolved in water and used for bath treatment or administered orally via the feed. In Norway, teflubenzuron [1-(3,5-dichloro-2,4-difluorophenyl)-3-(2,6-difluorobenzoyl)urea] and diflubenzuron [1-(4-chloro-phenyl)-3-(2,6-difluorobenzoyl)urea]) were frequently in use from late 1990s and until 2001. From 2002, emamectin and the pyrethroids, cypermethrin and deltamethrin were the drugs of choice but due to instances with reduced sensitivity for these drugs, flubenzurons were reintroduced in 2009. Consumption of teflubenzuron was 2028, 1018, 26 and 751 kg active compound in the years 2009–2012 respectively

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(Norwegian Institute of Public Health, Oslo, Norway, www.fhi.no). Flubenzurons are orally administered agents that act by interfering with the synthesis of chitin in the salmon lice. They are effective against all stages of sea lice that undergo moulting, including the larval and pre-adult stages (Branson et al., 2000; Ritchie et al., 2002; Campbell et al., 2006a,b). Teflubenzuron is administered via medicated pellets containing 2 g teflubenzuron per kg feed and the recommended dosing regimen is 10 mg/kg fish daily for 7 days. The excretion pathway of teflubenzuron in Atlantic salmon is mainly via the liver and bile to the intestine and the elimination is temperature dependant with half-lives in muscle between 2 and 6 days (Anon, 1999). Since the bioavailability in Atlantic salmon is low (approximately 10%) and the metabolism is minimal, most of the drug will be released from the fish as parent compound via faeces in the period of medication and immediately following a treatment (Anon, 1999). A preliminary study analysing faecal material from Atlantic salmon undergoing medication with teflubenzuron showed concentrations more than twice the initial concentration in the medicated feed (Samuelsen, unpublished data). Solubility of teflubenzuron in water is low (0.0094 mg/L at 20 °C), and the substance associate readily with particles rich in organic content (Marsella et al., 2000). It is therefore reasonable to believe that once pellets and faecal material containing teflubenzuron reach the marine sediment, the compound does not readily disappear. Based on results from a Scottish study, a half-life of teflubenzuron of 115 days in a marine sediment could be calculated (Langford, 2011). It has been shown that the organic waste in the form of excess pellets and faecal material from fish farms undergoing medication contains high concentrations of flubenzurons (Selvik et al., 2002). However, concentrations found in sediment samples collected in the vicinity of five investigated farms were much lower. The highest concentration was 40 mg/kg sediment (wet weight) (Selvik et al., 2002; Langford, 2011; Samuelsen, unpublished data). In contrast, using a remotely operated vehicle (ROV) pellets and large faecal particles were registered on the bottom in the vicinity of fish farms (Kutti, 2008). It is therefore reasonable to expect that the access to drug containing organic waste for non-target species will vary considerable even within a small area.

Since a major part of the teflubenzuron administered via fish feed will end up in the surrounding environment, there have been concerns about the possible toxic effects on non-target marine biota (Burridge et al., 2010; Eisler, 1992; Fisher and Hall, 1992; Haya et al., 2005). Since teflubenzuron reduces chitin synthesis, the drug is relatively non-toxic to marine species like fish, algae and molluscs, but is potentially highly toxic to any species that undergo moulting within their life cycle, including commercially important species like lobster, crabs and shrimps. A study of the effect of orally administered flubenzurons on non-target crustacean is, however, complicated by the fact that the investigation must include a moulting phase and the experimental set-up be designed to assure low mortality of non-medicated control groups. Furthermore, since it is likely that non-target crustaceans may ingest teflubenzuron from contaminated sediments, faeces, excess food pellets or by eating contaminated deposit feeders like polychaetes it is difficult to predict a dosing regimen that in full represents the condition under a fish farm. In order to have effect, a defined concentration of the drug must probably be present in an individual at a specific time period during the moulting cycle. The concentration range that induces mortality following moulting in marine non-target crustaceans is, however, not known. Furthermore, since teflubenzuron affects the formation of the new exoskeleton it is a possibility that non-fatal damage may occur. Copepod eggs (*Acartia tonsa*) exposed to diflubenzuron dissolved in water at 1 and 10 μg/L showed reduced hatching ability and those that hatched were abnormally shaped and failed to moult at the next stage of development (Tester and Costlow, 1981).

For drugs like teflubenzuron giving no immediate and acute effect, knowledge of the uptake and elimination kinetics in a specific species will provide important information in determining the period of time after an exposure where the drug is expected to have effect.

The aim of this study was to examine the effects of teflubenzuron on juvenile lobster when offered doses similar to the dose administered to the farmed fish and twice this dose corresponding to the consumption of drug containing faecal particles. Furthermore, the uptake and elimination of teflubenzuron in lobster was studied following a single oral administration.

2. Materials and methods

2.1. Animals and feed

This study was approved by the National Animal Research Authority (NARA) in Norway and has been carried out in accordance with *The Code of Ethics of the World Medical Association for animal experiments* http://europa.eu.int/scadplus/leg/en/s23000.htm.

A series of experiments were run to assess the effects of orally administered teflubenzuron on European lobster (*Homarus gammarus*). Juvenile stages of lobster were chosen as they moult more frequently than subadults and adults. The juveniles were produced at the Institute of Marine Research (IMR) field station at Parisvatnet located outside Bergen (60°37′75″ N, 4°48′11″ E) in late July/August 2010. Broodstock were collected from the surrounding area close by, in Øygarden. Sizes of the juveniles were recorded as carapace length (CL) measured as the distance from the posterior rim of the eye socket to the posterior edge of carapace and total length (TL) measured as the distance from the anterior tip of rostrum to the end of telson. CL and TL were recorded with a calliper to the closest 0.1 mm below. Wet body weight (BW) was recorded on a TE2101 Sartorius scale to the nearest 0.1 g.

Two doses of teflubenzuron were chosen, 10 and 20 mg/kg. The lower dose corresponds to the dose administered to the salmon whereas the high dose is similar to the concentration found in faecal material from salmon undergoing medication. The lobsters were fed special commercially produced pellets (3 mm) patented by *Norwegian Lobster Farm* (www.norwegian-lobster-farm.com/en/) and produced by *Nofima* (www.nofima.no/en). The pellets had an average weight of 50 ± 5 mg. The medicated feeds were customised to a 3 g individual and made by homogeneously mixing 60 or 120 mg teflubenzuron with 1 g glucose and using a few drops of herring oil, and by gentle shaking the drug was coated on two batches of 100 g pellets. Assuming even distribution, this corresponds to concentrations of 30 μg (low dose) or 60 μg (high dose) drug per pellet. Analysis of 10 pellets from each prepared dose revealed an average concentration of 36 ± 6 μg/pellets for low dose and 72 ± 13 μg/pellets for the high dose (analytic procedures described in Section 2.4.1) providing doses of 12 mg/kg (low dose) and 24 mg/kg (high dose) for a 3 g lobster.

2.2. Elimination of teflubenzuron

The purpose of this experiment was to assess the elimination rate of the drug from lobster and calculate the elimination half-life. The experiment was conducted at the IMR laboratory in Bergen, Norway in the period 13th February to 5th Mars in 2012. A total of 36 lobster juveniles with a mean weight of 4.3 ± 1.0 g were used. Juveniles were kept separately in 18 cm × 16 cm × 13 cm flow-through plastic containers stacked in two stands each housing a maximum of 25 containers (5 × 5). Seawater was obtained from 120 m depth close to the IMR-Bergen facility, heated to a temperature of 15.0 ± 0.5 °C and added to the experimental containers at a flow of about 1 L/min. The juveniles were starved for

3 days prior to administration of one high-dose pellet containing 72 ± 13 μg teflubenzuron. All 36 juveniles consumed their pellet and six individuals were randomly sampled at day 2, 4, 7, 11, 16 and 20 post medication and analysed for drug residues.

2.3. Simulation of a 7-day medication period–followed by monitoring for 3 months

The main focus of this experiment was to simulate a medication period of 7 days, similar to the prescribed medication regime at a salmon farm. The objective was to assess long-term effects over a 3-month period in mortality, growth and concentration of teflubenzuron in lobster juveniles. The experiment was conducted at the IMR field station at Parisvatnet outside Bergen, Norway in the period August 22 to November 23 in 2011, a total of 93 days. The experimental period was chosen to include at least one moulting for all individuals. Sea water was obtained from 25 m depth and the temperature in the tanks decreased during the experimental period from 14 to 9 °C. A total of 286 lobster juveniles were divided into three major groups, two fed medicated pellets (high and low dose) and one control group fed un-medicated feed. Each medicated group was divided into three replicates receiving low dose: C (n = 38), F (n = 38) and G (n = 48) and high dose: A (n = 38), D (n = 38) and E (n = 48). Number of juveniles in the control group was 38. Replicate F in low dose and control was run outdoor, while the other replicates were run indoor. A net covered the outdoor replicates. The juveniles were held separately in white PVC plastic compartments of 170 mL (7.0 cm × 3.5 cm × 7.0 cm). The bottom of the compartments was perforated with 2.5 mm diameter round holes to ensure water flow. Each replicate and the control consisted of 8 × 5 (or 10 × 5) compartments, termed as a tray, held together by plastic strips. Each tray was placed in separate 600 L (1 m × 1 m × 0.6 m) flow-through fibreglass tanks with a water flow of 10–15 L/min. Water level in the tanks was about 0.5 m and flow was set to 30–40 L/min.

Prior to the experiment, the lobsters were starved for 3 days. The medication period lasted from day 1 to 7 and the pellet given at day 7 was left for another 3 days before being removed if not consumed. Based on previous knowledge of feed consumption, lobsters with a weight of less than 2.0 g (n = 135 including the control) were fed every second day (in total 4 medicated pellets) whereas lobsters with a weight equal to or higher than 2.0 g (n = 151 including the control) were fed daily (in total 7 medicated pellets). Each individual was inspected daily during the medication period, and uneaten pellets registered. Mortality and moulting was recorded daily. At day 8, the day after the last medication, two individuals from each of the six medicated replicates were sampled and analysed for drug residues. Likewise, the two first juveniles in each medicated replicate that survived moulting were sampled and analysed for drug residues. Cumulative mortality per day was calculated subtracting sampling at day 8 and sampling of the first two juveniles surviving moulting in each replicate. Likewise, cumulative moulting was calculated subtracting sampled numbers, including mortality. At the end of the experiment (day 93), all survivors were checked for deformities and CL, TL and BW recorded. Juveniles with deformities were monitored over several moulting periods to study the permanency of the damage.

2.4. Analytical procedures

Teflubenzuron (analytical standard), diflubenzuron (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. Stock solutions of teflubenzuron and diflubenzuron were prepared at a concentration of 1 mg/mL in tetrahydrofuran and stored at 4 °C. Working standards were prepared by dilution of stock solutions with a mix of acetonitrile:water (50:50, v/v).

2.4.1. Concentrations in feed pellets

Each pellet was added to a 10 mL tube containing 2 mL acetonitrile, shaken for 10 min followed by transfer (1 mL) to an Eppendorf tube and centrifugation for 5 min at approximately 14,500 × g using a Biofuge A table centrifuge (Heraeus Sepatech, Osterode am Hartz, Germany). A calibration curve for teflubenzuron ranging from 5 to 40 μg/mL was made in triplicate by dilution of stock solution with acetonitrile:water (50:50, v/v). Analysis of five non-medicated pellets was included in order to confirm the absence of teflubenzuron. The samples were analysed by High-Performance Liquid Chromatography with ultraviolet detection (HPLC-UV) using the equipment and analytical procedure as described in Selvik et al. (2002).

2.4.2. Concentrations in lobster juveniles

Each individual lobster was weighed, placed in a 25 mL centrifuge tube and thoroughly crushed using a glass rod. Diflubenzuron (50 μL, 0.5 μg/mL) was added to the samples as internal standard. Acetone (5–10 mL depending on sample size) was added and the mixture was homogenised by a whirl mixer and placed in an ultrasonic bath (40 kHz) for 10 min before the samples were centrifuged for 3 min at 2500 × g (Eppendorf Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany). The resulting extract was transferred to a 10 mL centrifuge tube. To remove fat, heptane was added (1 mL) and the solution was shaken before centrifugation at 1250 × g for 2 min. From the resulting two-phase mixture the upper heptane phase was removed and discharged. This step was repeated once. The acetone extract was evaporated to dryness under nitrogen at 40 °C, the residue dissolved in heptane (5 mL) and cleaned-up by solid phase extraction using a GX-271 ASPEC (Automated Solid Phase Extraction) system from Gilson (Middleton, USA). The solid phase extraction column, Bond Elut Si, 500 mg, 3 mL (Agilent Technologies, Boeblingen, Germany) was conditioned with 2.5 mL heptane prior to loading of the sample. After loading, the column was washed with 3 mL of heptane, 5 mL heptane/diethyl ether (95:5, v/v) and 5 mL heptane/diethyl ether (90:10 v/v). The analyte and internal standard were eluted with 5 mL heptane/diethyl ether (60:40, v/v). The eluate was evaporated to dryness using nitrogen at 40 °C and re-dissolved in 250 μL of a solution of acetonitrile:water (40:60 v/v). The sample was filtered through a 0.45 μm syringe filter and was ready for analysis. For the calibration curves, blank samples were spiked with teflubenzuron at concentrations of 15–500 and 500–8000 ng/g and prepared as described above. The standard curves were prepared in triplicate.

The samples were analysed using a HP 1100 LC-system (Hewlett-Packard, Waldbronn, Germany) coupled to an Agilent MSD quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany). ChemStation software (Agilent Technologies, Waldbronn, Germany) was used for instrument control and data analysis. The analytical column used was a Shodex Asahipak ODP-50 4D 4.0 mm × 125 mm, 4 μm (Showa Denko, Munich, Germany). The injection volume was 10 μl. The mobile phase was a mixture of acetonitrile and aqueous ammonium hydroxide (75:25, v/v) at an isocratic flow rate of 0.7 mL/min at 25 °C. The retention time was 4.8 min for diflubenzuron and 5.3 min for teflubenzuron. The analyte and internal standard were analysed by negative electrospray ionisation (ESI) and detected as their deprotonated molecular anions by selected ion monitoring (SIM). The mass to charge (m/z) ratio was 378.9 (qual.) and 359.0 (quant.) for teflubenzuron and 309.0 (qual.) for diflubenzuron. The following experimental parameters were used: nebuliser pressure, 40 psig;

drying gas temperature, 350 °C; drying gas flow, 8 L/min; needle voltage, 3600 V; desolvating chamber temperature, 300 °C; fragmentor voltage, 70 V. The limit of detection (LOD) was determined to 5 ng/g and the method was linear ($r = 0.98$) over the ranges studied (15–500 ng/g, 500–8000 ng/g). Inter-run precision ranged from 7.5 to 10.0%.

2.5. Statistical analysis

For estimation of elimination half-life ($t_{½}\ \beta$) a linear regression analysis was made on logarithmically (ln) transformed drug concentrations versus time using the formula $t_{½} = \ln 2/k$, where k is the slope of the regression line. The initial concentration (C_o) was determined by extrapolation of the regression line to time zero (t_o). Analysis of variance (ANOVA) was made on sizes comparing replicates and treatments. To analyse differences between low and high dose concentrations, analysis of covariance (ANCOVA) was applied to log-transformed concentrations.

3. Results

3.1. Elimination of teflubenzuron

Following intake of one pellet with high dose teflubenzuron (72 μg) the concentrations in the lobsters decreased with time (Fig. 1). The highest concentration, 602 ng/g, was measured at the first sampling, i.e. 2 days after intake. The lowest concentration at that sampling was 215 ng/g giving an indication of the individual variation. From the data presented in Fig. 1 an elimination halflife ($t_{½}\ \beta$) of 3.4 days and an initial concentration (C_o) 515 ng/g at time t_o could be determined.

3.2. Simulation of a 7-day medication period-followed by monitoring for 3 months

3.2.1. Mortality

Overall 41% ($n = 46$) of the juveniles administered low dose of teflubenzuron died. Mortality was 30%, 39% and 56% in the different replicates. Mortality commenced with one juvenile at day 4 and of 15 juveniles that moulted during the medication period, 10 died immediately and one died when moulting for a second time at day 51 containing 15 ng/g teflubenzuron. The remaining five juveniles did not moult a second time and were still alive at the end of the experiment. Of the juveniles exposed to a high dose of teflubenzuron 38% died ($n = 42$) and mortality varied between 32%, 44% and 47% in the different replicates. The mortality commenced at day 5 (three juveniles) and off the seven individuals that moulted during the medication period six died. The only survivor also moulted

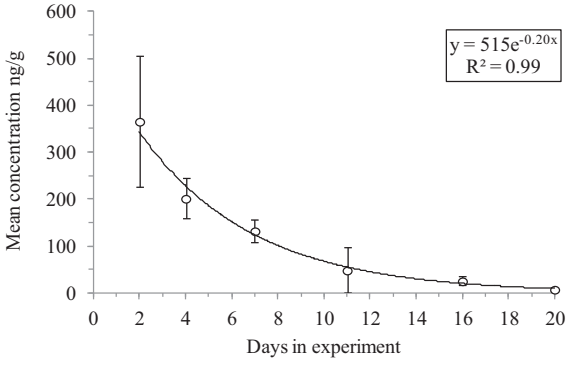

Fig. 1. Mean concentration of teflubenzuron ($n = 6$ at each sampling time) vs time profile in juvenile European lobster (*Homarus gammarus*) following a single oral administration of 72 μg teflubenzuron. Standard deviation are given as vertical bars.

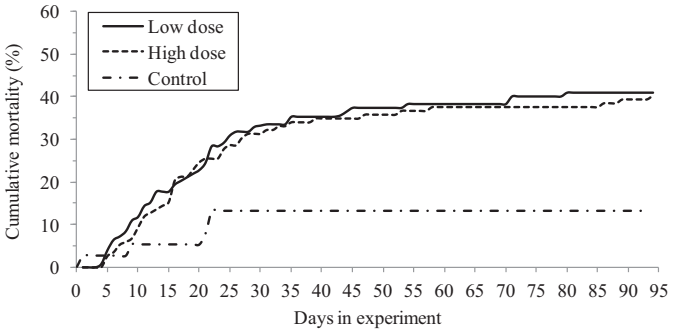

Fig. 2. Cumulative mortality (%) in European lobster (*Homarus gammarus*) juveniles given pellets with low or high dose of teflubenzuron from day 1 to 7 compared with control with no medication in the feed. The numbers for low and high doses are the means of three replicates.

successfully a second time at day 50 and was alive at the end of the experiment. For both low and high dose, more than 80% of the mortality had taken place before day 30. In the control group total mortality amounted to five individuals (13%), all died within the first 23 days. No difference was found in mean cumulative mortality comparing high- and low-dose treatments at the end of the study whereas both treatments had higher cumulative mortality compared to the control group (Fig. 2). Some of the juveniles died while in a moulting process as shown in Fig. 3.

Successful moulting after the last dose of medicine started at around day 30, in both treatments. Not all moults were however recorded as the juveniles can eat their exoskeleton in a few hours and moultings can therefore be missed. As a consequence, only 58% of the juveniles in the low-dose treatment were recorded to have moulted compared to 79% in the high-dose treatment and 72% in the control. However, in the end of the experiment, measurements of CL and TL showed that all surviving juveniles had moulted at least once but recordings of exact dates were missing. Four juveniles were recorded to have moulted twice.

3.2.2. Growth

When the experiment was initiated the mean juvenile size was 16.0 ± 2.1 mm CL; 45.4 ± 6.3 mm TL and 2.2 ± 0.9 g BW and no significant difference (ANOVA, $p > 0.05$) were registered in CL, TL or BW between replicates or treatment groups. In the experimental period the lobster increased in weight and size equally in all groups and replicates. At the end of the experiment (day 93) no significant difference were found in neither CL, TL or BW (Table 1) (ANOVA, $p > 0.05$).

3.2.3. Concentration of teflubenzuron in the juveniles

Due to variable appetite the prearranged dose was not consumed by all individuals during the medication period. However, as the number of pellets eaten by each juvenile was recorded it was

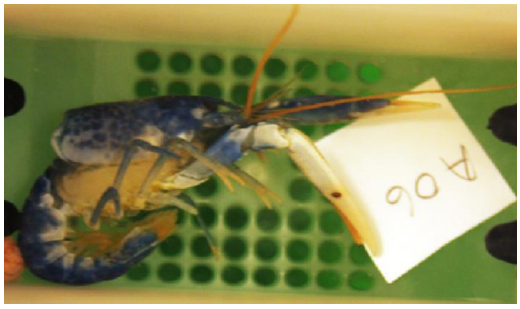

Fig. 3. European lobster juvenile (nr. A06) had consumed three pellets with high dose of teflubenzuron before it died during the moulting process, at day 7. The new exoskeleton is visible under the old one.

Table 1
Summary of carapace length (CL), total length (TL) and body weight (BW) of European lobster (*Homarus gammarus*) at day 93.

	Low dose	High dose	Control
Carapace length (mm)	18.6 ± 7.7	18.3 ± 2.3	18.0 ± 2.2
Total length (mm)	52.9 ± 5.7	52.7 ± 6.9	52.8 ± 6.5
Body weight (g)	3.7 ± 1.2	3.7 ± 1.6	3.6 ± 1.4
Number	66	68	32

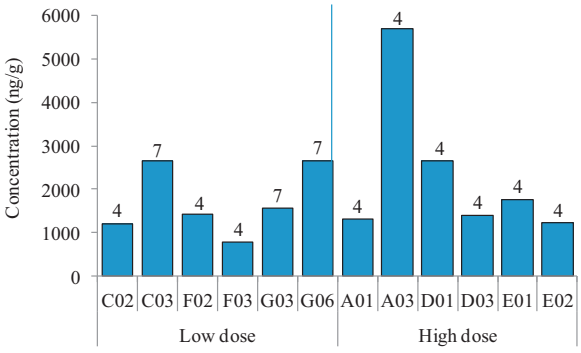

Fig. 4. Concentration of teflubenzuron (ng/g) at day 8 in juvenile European lobster (*Homarus gammarus*) that had consumed the prescribed number of pellets (marked above each column) with low or high dose of teflubenzuron from day 1 to 7.

possible to calculate the given dose for each individual. In the low-dose group the juveniles consumed on average 79 μg/g, compared with 173 μg/g in the high-dose group. All the 12 lobsters sampled one day after the end of medication (day 8) consumed the appointed number of pellets. The mean concentrations for low and high doses were 1715 ± 770 and 2338 ± 1727 ng/g, respectively (Fig. 4). However, since one individual (A03) in the high-dose treatment was a major contributor to the difference, the mean value for this group was reduced to 1666 ± 588 ng/g if A03 was excluded, giving no significant difference in mean concentration between low and high doses. The first six juveniles surviving low dose moulted from day 20 to 42, and those surviving high dose at day 23 (all six). The concentrations in these juveniles were on average 174 ± 103 ng/g and 130 ± 115 ng/g in low- and high-dose treatment, respectively. There was a large variation between the juveniles, e.g. at day 23 (high dose) the concentration varied from 26 to 351 ng/g.

A decline in concentration of teflubenzuron with time was observed in those juveniles that died during the experiment (Fig. 5). The highest concentrations were detected in two low-dosed juveniles with 8176 and 6843 ng/g, respectively. In the high-dose treatment, the highest concentration detected was 4450 ng/g. Overall, there were no significant differences in concentrations when comparing low and high-dosed juveniles (ANCOVA,

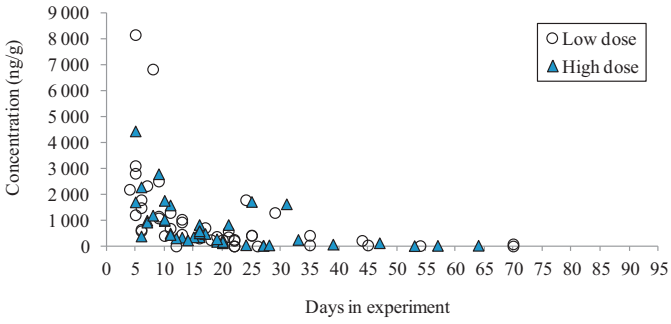

Fig. 5. Concentration of teflubenzuron (ng/g) in the juvenile European lobster (*Homarus gammarus*) that died during the experiment. Teflubenzuron was administered from day 1 to 7.

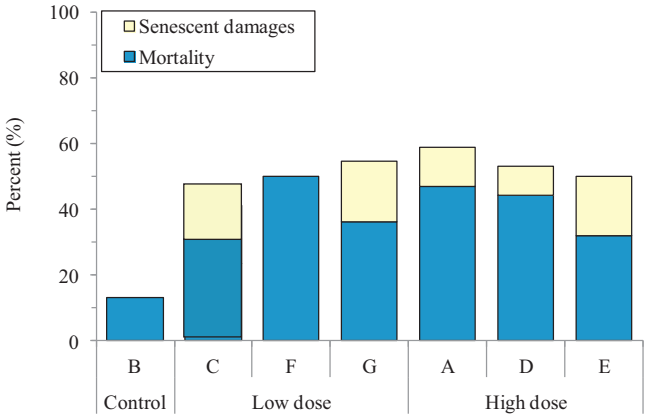

Fig. 6. Ratio of % cumulative mortality and senescent damages between replicates of European lobster (*Homarus gammarus*) juveniles at day 93 when the experiment was terminated.

$p = 0.178$). Mortality also occurred in individuals with low concentration of the drug, and even in individuals moulting later than 60 days after medication was ended. Eleven individuals that died contained 82 ng/g or less and the lowest concentration was 14 ng/g.

3.2.4. Deformities

When the experiment was terminated (day 93), the surviving juveniles were inspected for deformities. Since deformities were absent among juveniles in the control group it was assumed that the deformities were senescent damages due to the exposure for teflubenzuron. In overall, 29 of the 126 surviving juveniles were deformed but no difference in number between low- and high-dose treatment was found ($n = 14$ and 15, respectively). Deformities varied between replicates from 0 to 15%, and the replicates with lowest mortality had highest number of survivors with senescent damages (Fig. 6). Deformities were found in the carapace, walking legs, cheliped, tail fan, abdomen and the antennas (Table 2 and Fig. 7b–d). Nine juveniles had developed multiple deformities and where one juvenile had three (swollen carapace, stiff walking legs, damages to the tail fan), while the other eight had two. The most common co-occurring deformities were damages to the tail fan and stiff walking legs (33%).

Affected juveniles were monitored over several moulting periods and stiff walking legs (Fig. 7b) were replaced in one moult whereas severe damages to the chelipeds (Fig. 7d) needed three moultings to appear normal. Damages to the tail fan seemed permanent as this was not repaired through moultings.

4. Discussion

The chitin synthesis inhibitor teflubenzuron is widely used as an orally administered agent against ectoparasitic salmon lice in marine salmon farming. Due to low bioavailability and metabolism in the fish and high affinity for organic particles a major part of the drug will end up in the surrounding environment associated with faeces or uneaten pellets. Concerns have been raised about possible effects of various antiparasitic drugs used in fish farming on non-target marine species. In this study, the effect of teflubenzuron was tested on European lobster juveniles. Two doses were used. One represented the dose in medicated feed prescribed for the salmon, low dose. The second, high dose, represented the higher concentration found in faeces of medicated salmon. The study showed that lobsters were affected, either by mortality or senescent damages as observed 3 months after medication, giving a total effect (mortality and senescent damages) of ingesting teflubenzuron close to 50%, independent of whether low or high dose was administered. The

Table 2
Classification of deformities found in juvenile stages of European lobster (*Homarus gammarus*) affected by teflubenzuron in the feed from day 1 to 7. Observations were made at day 93.

Organ affected	Category	Description	Occurrence (%)
Carapace	Puffy	Carapace puffy/swollen, or up folded on one side often leaving some parts of the gills exposed	15
2nd to 5th pereopod	Stiff/twisted	The joints were fused together as if over calcification made the joints grown together. The entire pereopod leg was like one stiff piece, sometimes "frozen" in an arbitrary/twisted position	20
1st pereopod/cheliped	Miss-shaped	Various shapes of the cheliped deviating from normal	15
Uropod	Damages	Damages to parts of the tail fan, or even lacking one or both of the tail fans	23
Abdomen	Stiff	Abnormal shape of the abdomen as if some of the segments were once broken and then grown back in a wrong shape	18
2nd antenna	Stiff	Segments of the antenna were fused, almost as if over calcification made the joints grown together. Felt "stiff" when touching. Difficult to observe when animal was out of water	10

Fig. 7. Senescent damage in juvenile European lobster (*Homarus gammarus*) observed at day 93 after multiple administration of the antiparasitic drug teflubenzuron from day 1 to 7. (a) Normal lobster with no senescent damages, (b) damages in the walking legs, (c) swollen carapace, (d) deformed cheliped.

lack of difference in mean cumulative mortality between high and low dosed groups is reflected in the lack of significant difference in mean concentrations when comparing dead juveniles from each dose and in the concentrations measured in 12 individuals after last medication.

Simulating a 7-day medication period does not necessarily mimic the natural conditions under a fish farm since lobsters in the wild have the possibility to choose from a variety of food sources. However, no preferences were found in a study giving European lobster juveniles the option to choose between medicated pellets (teflubenzuron), non-medicated pellets or *Artemia* sp. (Jelmert, unpublished data). In our study only drug-containing pellets were offered to the lobsters, and the dosing regimen could be regarded as a worst-case scenario. On the other hand, in controlled experiments it was shown that juveniles are able to collect and store food in e.g. substrate and/or shelter. If this is the case in the wild, the number of stored and consumed medicated pellets may even exceed what was administrated in this experiment. However, as one of the aims of the study was to gain information concerning which concentrations of teflubenzuron induced mortality in juvenile lobster, we found the experimental set-up legitimate to use.

Elimination halflife ($t_{½}$ β) of teflubenzuron after a single dose administration of 72 μg was 3.4 days at a temperature of 15 °C. No elimination data are available for teflubenzuron in crustaceans but in Atlantic salmon muscle, $t_{½}$ β values of 4.7 and 2.6 days, respectively, were found following a single oral administration of 10 mg/kg of teflubenzuron or multiple administrations of 10 mg/kg daily for 7 days (Anon, 1999). The salmon were held at 10 °C. At 6 °C the $t_{½}$ β value following a multiple-dose study was 3.8 days (Anon, 1999). The elimination in lobster juveniles was therefore found to be in the same range as in Atlantic salmon muscle. However, as the drug could be detected 60 days (approximately 18 half-lives) after medication in lobster, it is possible that teflubenzuron is distributed according to a two-compartment model and that, due to the experimental period of only 20 days, a second and slower elimination pathway was not revealed.

When comparing results from the single and multiple administrations in this study it is clear that multiple administrations caused a build up of the drug in the lobster body. However, the difference in mean concentrations after 8 days comparing low- with high-dose treatment was non-significant which may indicate lower palatability administering the high dose pellets and/or decreased absorption rate with increasing dose, processes that previously has been described for antibacterial agents in fish (Hustvedt et al., 1991; Rigos et al., 1999). Furthermore, lobsters this size will break up the pellet in smaller pieces while eating and the amount of spill is difficult to measure, which may account for the large individual variations in concentrations that was observed.

There are very few data available describing the mortal effect of flubenzurons, particularly on non-target marine crustaceans and after oral administration of the drug. While LC_{50} values have been established for a number of terrestrial insects and also some aquatic crustaceans when exposed to the drugs dissolved in water (summarised in Langford, 2011), studies involving the effect of orally administered drugs are sparse. Scottish Environmental Protection Agency (SEPA) have considered the ecotoxicity of teflubenzuron when used as an anti sea-lice agent in aquaculture and set an Environmental Quality Standard (EQS) value for teflubenzuron in marine sediment to 2 ng/g (dry weight). The defined EQS value was based on a No Observable Effect Concentration (NOEC) value for the amphipod *Corophium volutator* after a chronic exposure for 28 days and a safety margin of 10 (SEPA, 1999). Unfortunately no detailed information was given about the experimental set-up and findings, excluding a direct comparison of results.

This study showed that individuals survived moulting 3 weeks after the last medication and with a concentration of 350 ng/g teflubenzuron. In contrast, 11 individuals died with concentrations of 82 ng/g or less, the lowest 14 ng/g. This demonstrates the variation in sensitivity between individuals and made it difficult to determine a break point, where higher concentrations most likely would induce mortality and lower concentrations most likely would not. On the other hand, the study reveals the lower concentration range that induces mortality in lobster juvenile and consequently indicates the concentration range that might have effect on other non-target crustaceans, providing similar sensitivity. In two field studies the concentrations of teflubenzuron were measured in crustaceans trapped in the vicinity of fish farms during and shortly after medication (Langford, 2011; Samuelsen, unpublished results). Langford (2011) found minor residues of the drug (between 0.2 and 11.3 ng/g) in deepwater prawns (*Pandalus borealis*) collected by trawl 1–5 km away from the farms and a maximum of 185.7 ng/g in brown crab (*Cancer pagurus*) caught by traps at a distance of 100–300 m from the farms. In the study by Samuelsen (unpublished results) crustaceans were trapped within an area of 300 m from a commercial farm under treatment and drug residues were found in all examined species. The maximum concentrations were 200 ng/g in deepwater prawns, 319 ng/g in Norway lobster (*Nephrops norvegicus*), 393 ng/g in squat lobster (*Munida* sp.) and 865 ng/g in King crab (*Lithodes maja*) indicating that the concentrations of teflubenzuron in defined individuals were high enough to induce mortality if moulting was impending.

Lobster is covered by an exoskeleton that needs to be shed in order to grow. Since teflubenzuron reduces chitin synthesis, it is assumed that mortality in all the medicated individuals was connected with ecdysis. The exoskeleton is divided into three layers all consisting of chitin, protein, calcium and magnesium salts, phosphates and a few other components, but where the composition varies in the different layers (Bobelmann et al., 2007; Romano et al., 2007; Al-Sawalmih et al., 2008; Sachs et al., 2008; Fabritius et al., 2009; Kunkel et al., 2012). Moulting in lobster is a complex process and it might be that chitin is formed during a short period during the moulting process, and vulnerability is higher during this stage. The working mechanism of flubenzurons in blocking the chitin synthesis will affect formation of the new exoskeleton leading to a thinner and weaker structure. The cause of death may be disruption of blood vessels in the process of liberation from the old exoskeleton. No examination of the thickness and stratification of the exoskeleton were, however, performed in this study but an ongoing study aims at recording the changes that take place in the exoskeleton in lobster juveniles during a moulting period, and revealing how teflubenzuron is affecting the process.

This study is the first to report a considerable number of juvenile lobsters with different abnormalities following administration of teflubenzuron. Nevertheless a similar effect was reported already in 1981 on the copepod, *A. tonsa* by Tester and Costlow (1981) who found that the hatching viability was less than 50% after a 12-h exposure to 1 μg/L of diflubenzuron and less than 5% after 24-h exposure to 10 μg/L. Those that hatch were abnormally shaped and failed to moult at the next stage of development. In contrast, juvenile lobsters with abnormalities in our study were able to moult successfully and start regenerating the damaged parts. However, since the deformities most likely will affect the ability to locate and consume food (antenna, claw and walking legs), respiration (carapace) and ability to move/swim (walking legs, tail fan and abdomen) the possibility that they will survive in the wild is small.

Interestingly, our study revealed no negative effect on growth of juveniles that survived medication without deformities, compared with controls.

It has been shown that flubenzurons are present in the sediment and sediment dwelling organisms for an extended time after medication of a farm (Langford, 2011; Selvik et al., 2002, Samuelsen, unpublished results). Further research should therefore include

long-term exposure of juvenile lobsters to lower drug concentrations and furthermore to include other important crustacean like crabs, shrimps and crayfish. Such data are deemed necessary in order to evaluate the environmental effects of flubenzurons when used in aquaculture.

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