

Negative impacts of the sea lice prophylactic emamectin benzoate on the survival of hatchery released salmon smolts in rivers

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Running title: Effect of emamectin on freshwater survival of salmon smolt

Abstract

Emamectin benzoate (EB) is a prophylactic pharmaceutical used to protect Atlantic salmon (*Salmo salar*) smolts migrating out of rivers and into the ocean against sea lice parasites. Randomized control trials comparing the marine survival of smolts treated with EB to a control group is used to calculate the fraction of marine mortality attributable to sea lice parasitism. However, it is assumed that there is no baseline difference in survival induced by the application of EB treatment. We used a combined laboratory and field study approach to investigate the potential impacts of EB treatment on behaviour and survival of hatchery-reared Atlantic salmon in western Norway. In aquaria experiments, EB-treated salmon smolts did not differ significantly in exploratory behaviour. Fish from treated groups responded similarly to simulated predator attack with spontaneous escape and elevated gill beat rate. Three rivers in the Osterfjord system of western Norway were selected for field experiments, Dale, Modalen, and Vosso. Dale River smolts were treated with intraperitoneal EB injections and had lower probability of detection in a wolf trap downstream of the release site than control smolts. Salmon smolts raised in the Vosso River hatchery were treated with EB delivered in their food and were detected on PIT antennas at the rivermouth at lower rates than control fish, but only when released at downstream sites. Calculation of risk ratios suggested that the bias in mortality caused by treatment with EB decreased the estimated survival of treated fish from an expected 18% to 46%, reducing the observable negative impact of sea lice on Atlantic salmon smolts. The results suggest that estimates of the fraction of mortality attributable to sea lice may be underestimated due to lower baseline survival of treated fish caused by treatment and bring urgent attention towards a potential systematic underestimation of the impacts of sea lice on wild salmon.

Keywords: migration; PIT telemetry; behaviour; mortality; Salmonidae; smolt

Introduction

Introduction of farmed Atlantic salmon (*Salmo salar*) into the coastal seas has disrupted many natural ecological processes (Gross 1998). The captivity of high densities of farmed Atlantic salmon along the coast has facilitated a higher carrying capacity of sea lice, which parasitize wild salmonids that also depend on coastal habitats for their migration. Sea lice are naturally occurring marine crustaceans. Species of sea lice are ectoparasites that spreads from hosts through a pelagic stage and can attach to farmed and wild fish. After it attaches, it will undergo several molting stages (Hamre et al. 2013) and mature as a mobile adult that, at critical density, can yield severe physiological impacts on its hosts, especially osmoregulatory failure (Grimnes and Jakobsen 1996). A prominent environmental impact is the amplification and spread of parasites from fish farms in areas and at times when salmonid smolts migrate to sea (Vollset et al. 2016). The smolt life stage is most vulnerable because the animal is small, has its pelagic migration route near fish farms that are hotspots for sea lice, and is undergoing a stressful transition between fresh- and saltwater. Survival of smolts can define the strength of that entire year class (Friedland et al. 2000; Thorstad et al. 2012). Consequently, the effect of sea lice on wild salmonids has become one of the most prominent environmental issues related to salmonid fish farming in the northern hemisphere (Vollset et al. 2017).

To study the relative impact of lice on post-smolt, prophylaxis against sea lice has been explored as a method to protect smolts against attachment of sea lice. The compound emamectin benzoate (EB; Gargan et al. 2012; Jackson et al. 2013) and an unknown compound named EX (Skilbrei et al. 2013, 2015) have been tested. Emamectin benzoate is an avermectin chemical derived from bacterial (*Streptomyces avermitili*) production initially developed as a crop pesticide (Leibee et al. 1995). EB is now widely administered to Atlantic salmon via

integration with feed (BurrIDGE et al. 2010). Stone et al. (1999) suggested $0.05 \text{ mg kg}^{-1} \text{ fish day}^{-1}$ for seven consecutive days confers resistance to all life stages of the sea lice parasite (Stone et al. 2000). Despite significantly reducing sea lice parasitism, EB is not 100% effective (Stone et al. 1999) and effectiveness is decreasing as lice are developing resistance to the drug (Aaen et al. 2015). Investigating the clinical effects of emamectin benzoate on Atlantic salmon has been accomplished by conducting randomized control trials (RCT) with Atlantic salmon produced in hatcheries. In RCT studies, paired groups (control and treated) are released as post-smolts and recaptured as adults as they return to spawn. The pharmacological effects on the salmon during the smolt life history phase and the benefits are measured by comparing the relative returns of treated and untreated salmon. Long-term studies have confirmed that prophylactic treatment yields higher rates of adult returns to spawning rivers (Skilbrei et al. 2015; Vollset et al. 2016). RCT studies have been used as important documentation and rationale for the implementation of a management system that regulates the allowable biomass in fish farms in different regions of Norway. Management decisions are predicated on the estimated parasite-induced mortality due to parasites from fish farms (Vollset et al. 2017).

Better survival of treated smolts compared to controls is expected to reveal the fraction of smolt mortality attributable to lice parasitism; however, if baseline survival of treated smolts is artificially deflated because treatment with EB affects survival, then mortality attributable to lice will be underestimated. Sufficient attention has not been given to the potential for direct negative impacts of treatment on fish during the smolt period. Indeed, the physiological effects of EB are poorly documented. Stone et al. (2002) found no physiological impacts on adult farmed salmon, yet there is no documentation on the effects of EB on salmon smolts, and in general no documentation of the effect of EB in the wild in areas void of parasites. Circumstantial evidence for a negative treatment effect were presented by Vollset et

al. (2016), which indicated that in groups where the general survival of the cohort was high, the treatment effect was significantly negative. Corroborating reports revealed that anti lice treatment had a negative effect when the infestation pressure of sea lice has been low.

Developing evidence for the effects of prophylaxis *in situ* requires information about the fate of treated and untreated fish, which can be extremely challenging once the fish has entered the marine environment. Given that smolts migrating in freshwater do not encounter sea lice, the mechanism of action for the pharmaceutical should not yield any benefit for survival but offers an opportunity to investigate differences in baseline behaviour and survival prior to entering the marine environment. Analyzing the effect of EB in freshwater also has the added benefit that the fish have not encountered any parasitic lice, making it possible to separate the effect of treatment and parasitism. Playing an important role in the management of wild Atlantic salmon populations and farmed salmon operations, it is crucial that data derived from randomized control trials be representative of the impact of salmon lice. However, we hypothesized that negative effects of treatment on freshwater survival, before salmon reach the ocean where they are vulnerable to sea lice parasites, may affect survival estimates and bias interpretations of data derived from RCTs such that the impact of sea lice is calculated to be lower than the true value. To test the hypothesis, we established a combined laboratory and field study to investigate the behaviour and survival of treated and untreated Atlantic salmon smolts in freshwater. Results from the laboratory behavioural trials and field survival experiments were designed to evaluate the validity of comparing sea survival between treated and untreated smolts by determining whether treatment itself biases the smolt survival already in freshwater and thereby the estimation of the fraction of mortality attributable to sea lice parasitism.

Methods

Study System

Research was carried out on Atlantic salmon, a species of cultural and economic importance in Norway and throughout the coastal north Atlantic. Atlantic salmon emerge in springtime from eggs laid in gravel nests and feed on insects for multiple years before most undergo a physiological and morphological transition termed smoltification to prepare them for migration to the ocean. Smolts migrate in groups downstream to the ocean where they transit from coastal areas to the open ocean where they are vulnerable to marine predators and pathogens (i.e. sea lice). Salmon may remain in the ocean for one winter and return at a relatively small size or remain for multiple years and grow larger. Maturing Atlantic salmon return to their home rivers (~90% philopatry) from their feeding grounds along coastal banks in the North Sea, North Atlantic Ocean, and Arctic Ocean during the spring and summer (May-September) and migrate up freshwater to spawn in the autumn (October-December).

In western Norway, the Osterfjord system north of the city of Bergen was once a dominant salmon producing system with six major rivers producing fish (Figure 1). Acidification of freshwater, hydropower exploitation altering flows, and introduction of farmed Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) in the region resulted in most rivers drastically declining in their salmon yields. The salmon population in the largest river in the region, Vosso, collapsed in the late 1980s and is undergoing restoration efforts including a hatchery that produces smolts from the national gene bank line. Acidification of the nearby Modalen River drastically reduced the salmon population there and it is now continuously limed and stocked with salmon from the Vosso River strain. Most hatchery smolts are not released directly into the river because of poor returns but are instead towed out

through the fjords and released in saltwater, a strategy that yields higher returns of the fish, which are all individually tagged several months prior to release. Smolts are also produced at the Dale River hatchery as a compensation for habitat lost due to hydropower exploitation in the river. RCT experiments in Dale and Vosso have been used for two decades to calculate the fraction of marine mortality attributable to sea lice parasitism.

Laboratory Experiment

Smolt Provenance

On the 15th of May, seven days after treatment, 14 one-year old hatchery produced Atlantic salmon smolts that had been treated orally with emamectin benzoate (mean length and weight \pm SE; 17.43 ± 0.58 cm, 44.47 ± 3.80 g) and 14 untreated (17.38 ± 0.41 cm, 45.42 ± 0.68 g) smolts were transported from the Vosso River hatchery to the experimental facilities at the Department of Biological Sciences, University of Bergen. The smolts were housed in separate 180 L tanks ($60 \times 60 \times 60$ cm) at natural photoperiod at ambient temperatures (8°C). Water was continuously renewed to maintain oxygen saturation $>95\%$. For five weeks after treatment groups were established (EB- treated and control), fish were fed Ewos fish feed (1% body weight $^{-1}$ day $^{-1}$) until 20 June when behavioral experiments were conducted. After the experiments, fish were sacrificed using an overdose of anesthetics (buffered 2 g l^{-1} MS-222).

Two behavioural assays were used to determine if EB affected smolt behaviours that are likely to be associated with post-release survival. Each individual was observed one time in each of the assays. Half of the fish from each treatment group were tested for exploratory behavior in the first assay in an open field arena and afterwards for response to a simulated predator attack in a glass aquarium when shelter opportunities were present. The other half

from each group were tested in the opposite order. The data from the untreated fish were used in comparison with wild caught individuals (not included here) in Salvanes (2017). In the laboratory study, we used the data in a new context to compare behaviours with unpublished data from EB treated individuals tested during the same experimental days.

Experimental Design

Open-Field Trials

Expressed behaviour depend on how an individual trade-off the benefit against cost of exploration (Renner 1990). Risk can be reduced by avoiding dangerous places, by changing activity pattern if a threat is encountered, or a prey can reduce visibility by freezing. Here we tested exploratory behaviour using classical open-field trials (Walsh and Cummins 1976). The test arena was a circular white plastic tank with diameter = 60 cm and height = 40 cm with water depth 11 cm. Light levels were constant during the trials, no light fell directly on the test arena, and no shadows occurred. The base of the tank was marked with a line 10 cm from the perimeter of the tank to separate central tank area from the fringe. This allowed standardizing observations of test fish use of the peripheral and central areas, and it allowed sufficient space for the size of our test fish (17 cm) to stay in the peripheral fringe area. Occupancy of peripheral areas near walls is used as an index of timidity (Walsh and Cummins 1976). Individuals that are explorative tend to use the central tank area most and move a large proportion of the time, and move more often between fringe and central area, while those that are less explorative will use the fringe area more and be immobile (freeze) more often. A video camera was mounted above the tank. To avoid disturbing the test fish the test tank, light and camera were behind a black curtain. The water was replaced between each fish trial. The

trials commenced by releasing the test fish carefully into the center of the arena. Behavior was recorded for 10 min. Videos were analyzed to determine the number of crossings of the line between the center and the fringe (activity measure 1), the proportion of time fish were moving (activity measure 2) or frozen, and the proportion of time in the center and in the fringe. To standardize the observations, all data used are from observations taken during the time 3 to 4 minutes after the test fish entered the open field arena. Hence, the initial 3 minutes fish were in the test arena was an acclimation period. The data were used to examine activity and exploratory behaviour.

Anti-predator behaviour

Anti-predator behavior was studied by testing the response of individual fish to simulated predator attacks in glass aquaria ($40 \times 29 \times 23.5$ cm) with water depth 18 cm at temperature 8.5 °C and $>90\%$ oxygen saturation. A flowerpot (9 cm diameter in the entrance and 6 cm in the back) was placed in one corner to represent a shelter opportunity for the test fish. Black plastic covered three sides of the aquarium. A camera was positioned in the front of the aquarium at 1.5 m distance. The aquarium and camera were behind black curtains to avoid disturbance of the test fish. The water was replaced between each trial and fish.

The trials commenced by releasing the fish into the aquarium with three minutes for acclimation. Then basic behavior was video recorded for 2 min, after which the test fish was exposed to a simulated predator attack from above. Its behavior was continued to be video recorded five more minutes. The simulated predator attack consisted of a grey and orange water tap (resembling a bird's head) that was lowered remotely, but rapidly, into the center of the aquarium by releasing and pulling the string mounted to the tap to mimic avian predators, which are common predators on freshwater fish (Gallagher et al. 2016). To examine whether

and how the juvenile salmon responded physiologically and behaviourally to a threat, videos were analyzed to examine whether the test fish exhibited an immediate flight response, whether they instantaneously went to shelter after the attack, and time spent associated with shelter before the first minute after attack. The gill beat rate (beats min^{-1}) was quantified and used as an index for respiration rate and oxygen supply under stress (Metcalf et al. 1995). The proportion of time individuals were associated with or used the shelter (closer than one body length from the shelter) was quantified.

Data Analysis

Exploratory behavior was quantified by monitoring individual behaviour in an open field arena. The number of times the test fish crossed the line between the fringe and the center of the tank was used as a proxy for activity and analyzed using a generalised linear model with a Poisson distribution. The proportion of time an individual spent in the tank center was used as a proxy for exploratory behavior and analyzed by logistic regression using the *glm* function and binomial family. The proportion of time frozen was analyzed using *glm* function, but simplified to 1 if the proportion was greater than 0.50 or 0 if less than 0.50. Each model had treatment (control or EB), individual length, and the interaction of treatment and length as fixed effects.

Four observations from the anti-predator behaviour were collected. First, the spontaneous flight response to a simulated predator attack (yes=1, no=0) was recorded as a binomial variable and modeled with logistic regression. Next, the change in ventilation (gill beats min^{-1}) from before (baseline) to after the simulated predator attack was tested using linear mixed effects modeling assuming normal distribution with a random intercept for individual using the *lme* function in the nlme package (Pinheiro et al. 2019). Fixed effects

were treatment (control or EB-treated) and time point (before or after attack), with the interaction between the two included to determine whether the change after predator encounter differed between control and EB-treated animals.

Field Experiments

Provenance of smolts

Dale River

The Dale River is a regulated river in western Norway (60.577856, 5.773372) home to both anadromous Atlantic salmon and sea trout. The Dale hatchery is approximately 3 km upstream of the confluence of the river with Dale Embayment, an elongated brackish estuary that connects with the Osterfjord and drains westward to the North Sea. A total of 800 smolts produced in Dale hatchery were used in the river release (L_T avg. = 185 ± 19 mm SD). The fish were randomly divided into four groups: two EB-treated groups and two control groups. The EB-treated fish received intraperitoneal (IP) injection with either 50 or 100 μ l (200 μ g EB/ml polypropylene glycol). The control fish received either an IP injection with 100 μ l polypropylene glycol or no treatment at all. Fifty fish of each group were released on May 8, 2019 at different sites in the river (a total of 200 fish per site, 50% treated). Two sites are located in the anadromous residual flow section and two are located above the anadromous part of Dale River, the uppermost site upstream of a waterfall.

Vosso River

The Vosso River (60.639044, 5.952015) is a large river in western Norway at the easternmost point of the Osterfjord. The Vosso River is separated into three systems, the uppermost Stranda River, the middle Vosso River, and the lower Bolstad River. The Voss hatchery produces Atlantic salmon smolts approximately 35 km upriver from the confluence of Vosso with the Bolstadfjord. There are two anadromous lakes in Vosso, the upper lake Vangst Lake separating the Stranda and Vosso rivers and the lower Evanger Lake between the Vosso and Bolstad rivers (Barlaup et al. 2018; Figure 1). Smolts (N = 995) were acquired from the national gene bank line of Vosso River Atlantic salmon produced at the Voss hatchery (L_T avg. = 145 ± 11 mm SD). Smolts were separated into eight tanks control and treatment groups with the treatment group fed pellets impregnated with emamectin benzoate. On May 9, 2019, 499 tagged smolts were released above Lake Evanger (50% treated) and 496 were released below the lake (50% treated).

Modalen River

The Modalen River is located to the north of Vosso and discharges into the Osterfjord at 60.815647, 5.800170. The river is regulated by a hydropower dam and has experienced acidification that has greatly reduced the population of Atlantic salmon and sea trout to the point that hatchery production from the Voss hatchery is relied upon to rebuild the population in combination with lime treatment. All smolts released in Modalen (N = 2249) were produced in the Vosso hatchery from the gene bank line of the Vosso River smolts (L_T avg. = 146 ± 11 mm SD), the exact same strain as described above but housed in separate tanks. Stocking fish produced at the Voss hatchery is the established protocol for restoration of the salmon population in the Modalen River after acidification extirpated the native strain. On

May 2, 2019, 2256 smolts were released at three sites in the Modalen River, 1551 at upstream sites (49% treated) and 698 downstream (52% treated; Figure 1).

Experimental Design

PIT Tagging Procedure

Atlantic salmon smolts were tagged according to procedures described in Barlaup et al. (2018). Fish were anaesthetized in Finquel Vet (tricaine methanesulfonate; 100 mg L⁻¹ in a sodium hydrogen carbonate buffered solution), measured, and cut with a surgical scalpel in the ventral abdomen to insert a 23-mm half-duplex PIT tag (diameter = 3.85 mm, weight = 0.6 g). The Norwegian Animal Welfare Committee (nr. 8485 and 10627) approved the study.

Recapture

Tagged Atlantic salmon smolts migrating in the Dale River were recaptured in a wolf trap near the rivermouth where Dale discharges into the estuary and connects with the Osterfjord (Figure 1). Wolf traps generate hydraulic head with a weir installed in the river and a porous trap that sifts large particles including fish and funnels them into a holding chamber (Wolf 1951). The wolf trap in Dale is operational in the springtime during the smolt run and was active from April 17 to July 1, 2019. The trap was attended daily to scan and release captured fish downstream.

Floating PIT antennas were placed in the lower section of Vosso (Bolstad River), and Modalen River, approximately 250-850 m before the river runs into the estuary (i.e., Bolstadfjord, Mofjord, respectively; Figure 1). The antenna setup consisted of four boat-

shaped styrofoam bodies each with an antenna in the bottom part of the floating device. The diameter of the actual antenna was ~115 cm with three loops of 4 mm² of copper wire connected to a half-duplex reader (Oregon RFID, Portland, Oregon, USA). The four antennas were suspended from a bridge and placed close to one another to float near the middle of the main current. The reading range was 40-80 cm for 23-mm tags with an electric field covering approximately 5 m². However, the actual reading range throughout the study period was at times, especially in between maintenance, reduced to half or even a third due to equipment failure. The antenna placement was based on the assumption that smolts typically migrate close to the surface in the thalweg of the river (Thorstad et al. 2012). All floating antennas were installed within 19 days in April 2019. During the entire study period, the antennas at each location were maintained at least fortnightly. In Vosso, the four floating antenna boats were installed on April 11, 2019 and taken out again on September 17, 2019. Out of these 160 days, there were two days of power outage and four days of substantially reduced detection efficiency in May. In Modalen River, the floating antennas were operating from April 29, 2019 until July 30, 2019. During these 92 days, we encountered technical issues preventing tag detection on four days in May, and two to six days of impaired detection efficiency in July. We do not anticipate that technical difficulties affected our results unless treated and untreated smolts have unequal detection responses to these outages, which we have no reason to suspect.

Data Analysis

Likelihood of detection/ recapture

We cannot directly estimate survival in this study so we rely on detection as a proximate estimate of survival with relative values within rivers providing valid comparisons, but not between rivers. For the Dale River, recapture in the wolf trap is considered a detection and in the Modalen and Vosso Rivers we considered registration on the PIT antenna a detection. Multivariable modelling of detection was difficult because a large number of zeros in the response (i.e. many fish undetected) and overall poor goodness of fit, therefore we initially conducted χ^2 goodness-of-fit tests comparing by river and release site (upstream or downstream) to test whether the proportion of untreated fish differed from the proportion of treated fish that were detected. For Dale where there were three treatments, we split the data so that each treatment was compared to the control values in an independent χ^2 test, yielding six tests for Dale (three treatments, upstream and downstream) and two for each of Vosso and Modalen (upstream and downstream).

Although logistic regression models did not have consistently good fits to the data, we present the results of these models. The three groups were not effective replicates because the Vosso hatchery fish (released in Vosso and Modalen) were treated with EB in their food whereas the Dale fish were treated with an injection of 50 μL EB, 100 μL EB, or 100 μL control fluid, along with a control group. Therefore we constructed a separate logistic regression model for Dale with treatment, release site (upstream or downstream), the interaction between treatment and release site, and individual length with the *lrm* function in the *rms* package (Harrell 2019). We compared the logistic model with and without an interaction and proceeded with the model with a lower AIC value. A similar approach was used for Vosso and Modal fish together in the same model with length, treatment, release site, and the interaction and again compared this to a model without an interaction. Goodness-of-fit was assessed by the native test in *rms*, the Hosmer-Lemeshow-Le Cessie test (Harrell 2019).

Migration time

Migration time was analyzed by linear regression taking the recapture date at the wolf trap (Dale) or first detection of a fish at a floating PIT antenna (Vosso and Modal), which was used as an indication of when the individual was moving to the ocean. The same putative predictor variables were tested as for detection: release location, EB treatment, and individual length, only using fish known to have been detected or recaptured in the model. Recapture date was considered as a numeric (Julian date) value.

Results

Behaviour Experiment

Explorative behavior did not differ in EB-treated and control smolts; both groups remained in the central area of the open field test arena for similar proportion of time (control: 0.17 ± 0.23 SD; EB-treated 0.10 ± 0.07 SD). However, there was a non-significant trend that EB-treated spend lower proportion of time in the center and tend to stay more in the fringe than control fish did ($t = 1.86$, $P = 0.08$). Activity did not differ in control and EB-treated fish as they moved equally often between the fringe and the central area of the test tank (control: 7 ± 6 SD times; EB-treated: 7 ± 6 SD times; $z = -1.11$, $P = 0.27$). They were also immobile (frozen) for similar amount of time (control: 0.10 ± 0.23 SD; EB-treated: 0.17 ± 0.36 SD; $t = -0.74$, $P = 0.46$).

EB-treated and control fish expressed similar anti-predator behaviours after simulated predator attack. Both treatment groups showed similar spontaneous flight response ($z = -0.47$, $P = 0.65$) and there was no effect of length ($z = -0.78$, $P = 0.39$) or interaction between length

and treatment ($z = 0.37$, $P = 0.72$). Both EB-treated and control fish increased their gill beat rate after the predator attack ($t = 2.75$, $P = 0.01$), but the increase was similar between treatments ($t = 0.01$, $P = 0.99$), and there were no interaction of treatment with length.

Field Experiment

Likelihood of detection/recapture

In Vosso and Modalen, control group fish were detected significantly more frequently than EB treated counterparts when they were released at downstream sites (Modalen: $\chi^2 = 9.39$, $df = 1$, $P < 0.01$; Vosso: $\chi^2 = 6.83$, $df = 1$, $P = 0.01$). In Dale, however, there were no differences in detection between the untreated group and any of the three treated groups (50 mL EB: $\chi^2 = 2.07$, $df = 1$, $P = 0.15$; 100 mL Control: $\chi^2 = 0.72$, $df = 1$, $P = 0.40$; 100 mL EB: $\chi^2 = 1.62$, $df = 1$, $P = 0.20$). When smolts were released at upstream sites in Dale, Vosso, and Modal, there was no differences in detections between EB treated fish and the control group (Dale: all $\chi^2 < 2.44$, $df = 3$, all $P > 0.12$; Modalen: $\chi^2 < 0.01$, $df = 1$, $P = 1.00$; Vosso: $\chi^2 = 3.53$, $df = 1$, $P = 0.06$). Fish released at downstream sites were detected with higher frequency than those released upstream.

According to logistic regression, detection of Dale smolts in the Wolf trap was not a function of fish length ($z = 0.95$, $P = 0.34$). The odds ratio of survival for fish treated with 100 μ L EB compared to no treatment (control) was 0.62, a significant decrease ($z = -2.11$, $P = 0.04$); 100 μ L control fluid also appeared to decrease the odds of survival (odds ratio = 0.63) but the difference was not quite significant ($z = -1.87$, $P = 0.07$). Odds of survival also increased for fish released at downstream sites ($z = -9.12$, $P < 0.01$; Figure 2; refer to site locations in Figure 1).

In Vosso and Modalen, longer fish had better odds of detection for each additional mm length ($z = 2.69$, $P = 0.01$). Similar to Dale, there was an effect of release location, with smolts released downstream having greater odds of detection (2.57) than those released at upstream sites ($z = -5.47$, $P < 0.01$). Treatment with EB also decreased odds of detection (0.84) relative to untreated fish ($z = -4.07$, $P < 0.01$). There was evidence of an interaction between release site and treatment in the Vosso and Modalen model ($z = 2.22$, $P = 0.03$) such that release site had a larger effect on detection of control than on treated fish. Fish released in Vosso had better detection odds (1.76, $z = 4.24$, $P < 0.01$) than fish in Modalen.

Migration timing

Migration timing in the river Dale was clearly different between salmon released at downstream sites compared to above the waterfall ($t = 5.03$, $P < 0.01$). There was also a significant effect of length wherein longer smolts migrated earlier ($t = -6.70$, $P < 0.01$). However, none of the treatment or sham injections had significantly different timing of detection (all $|t| > 1.01$, all $P > 0.31$). Salmon smolts released at downstream were detected on the PIT antenna May 27 ± 20 d (untreated) and June 4 ± 25 d (EB treated feed) on average. Those released at the upstream site, Tverrelva, June 26 ± 24 d and June 28 ± 27 d for control and EB treated fish. Fish released in Modalen were detected earlier than those in Vosso ($z = -4.43$, $P < 0.01$) and upstream released fish were detected later than downstream released fish ($z = 3.49$, $P < 0.01$). Longer smolts were also detected earlier ($z = -2.24$, $P = 0.03$). However, EB treatment did not influence the timing of migration in Vosso and Modalen ($z = -0.10$, $P = 0.92$).

Discussion

Unnaturally high densities of sea lice around aquaculture facilities that spill over to wild populations of Atlantic salmon threaten the viability of wild salmon and accurate quantification of impacts is crucial to effective management (Vollset et al. 2017; Hersoug et al. 2019). The combined laboratory and field experiment demonstrates that the anti-parasitic drug emamectin benzoate (EB) may reduce the likelihood of salmon smolt survival during their freshwater migration. Treatment with EB reduced the rate at which smolts were detected, but only at downstream sites. The difference in survival suggests that there may be an underestimation of the impact of sea lice on salmon smolt survival if bias is not accounted for.

The negative side effects of treating Atlantic salmon smolts against lice is crucial to understand, yet only one other study has attempted to quantify them. Sivertsgård et al. (2007) conducted an artificial infestation study on salmon post-smolt using telemetry and compared survival of untreated fish to a group treated with substance EX (which, according to Pharmaq, has low toxicity, protects the fish for 16 weeks, and prevents chitin synthesis in sea lice). They found no difference between groups, but the total migration days through the array was only up to ~200 hours. Relatively few fish from each group were detected, making it unlikely to detect anything but an acute mortality effect. Ultimately, the salmon migration in that study was not negatively affected by the pharmaceutical. Our findings for emamectin benzoate are clearly different, albeit using different methodologies. Our investigation only focused on the river whereas Sivertsgård et al. (2007) investigated the movement rate through the fjord. Our finding that treatment against sea lice has side effects on smolts that result in lower detection rates, and ostensibly lower survival, are therefore novel and highly relevant to management applications of randomized control trials on the effects of sea lice on salmon populations.

The results from the aquaria experiments did not reveal any mechanisms that could explain the differences observed in the field. However, we did observe a near-significant difference between control and treatment animals with respect to the time spent at the fringe of the aquarium that could be a focal point of further research. Predation risk triggers a response from the stress axis in fish, in which catecholamines and glucocorticoids are released into the bloodstream, which contribute to tachycardia and accelerated ventilation rate (Johnsson et al. 2001). Such a physiological response is expensive and fish eventually must rest to recover metabolic substrates. Metcalfe et al. (1987) demonstrated altered foraging behaviour by Atlantic salmon following a predator encounter, suggesting a refuging response to manage predation risk. Consequently, we expected that if EB impacted the physiology of the fish we would observe longer recovery time after predation attacks.

The effect of EB in the field was significant in Vosso and Modal, where EB was administered through feed. However, the effect was not as evident in the study in Dale, where EB was administered using intraperitoneal injection (Glover et al. 2010). Smolts in Dale were larger, which may affect the response to vaccination. However, given the differences in treatment type it is not possible to conclude anything about size effects between systems. The injection experienced by smolts in Dale could impact fish by the mechanical action of puncturing the coelom to inject the fluid or via the direct impact of the fluid. There may be an interaction between treatment (control or EB) and volume of fluid, but a crossed design was not possible to determine whether the effect of injection or volume was more important because the volume could not be administered without an injection. Survival of fish treated with 50 μ L EB was not different from controls. The logistic regression and chi-square test yielded conflicting results for the difference between control and 100 μ L EB treated smolts released at downstream sites. Despite the greater intrusion needed to administer EB via injection, it may be preferred to control the dose by some practitioners (Skilbrei et al. 2008),

which can be more variable when it is administered in the fish's food as was done in Vosso and Modalen fish. Correspondingly, differences in baseline survival appeared less pronounced for the Dale fish treated by intraperitoneal injection compared to differences between orally treated and untreated fish from the Voss hatchery. This merits further investigation.

Likelihood of being detected leaving the river was strongly dependent on where the fish was released in the river, similar to results from Barlaup et al. (2018) and Flavio et al. (In Press). Differences were evident in all three rivers and suggest that mortality during the freshwater migration may be an important bottleneck for the survival of hatchery reared salmon post-smolts. Upstream release sites were separated by waterfalls in Dale and Modalen and by a lake in Vosso. Adult salmon can ascend waterfalls (Lennox et al. 2018) and presumably smolts are adapted to tumbling over them on their way to sea, but they may still be a bottleneck caused by the waterfall. Whether such mortality exists, and whether it is additive or compensatory, may be a good avenue for further research particularly in rivers where egg planting beyond the naturally anadromous reaches is used as a population recovery strategy. Smolts have also been observed to have high rates of mortality when transiting lakes (Honkanen et al. 2019; Kennedy et al. 2019). Releasing smolts at sites upstream of these putative bottlenecks for survival likely contributed to the significant differences in detection that we observed between groups released upstream and downstream.

We attempted several models to compare the detection rates of PIT tagged salmon migrating downriver. Survival analysis would have been an effective approach because it integrates both the time to event (i.e. detection) as well as the fate of the fish (detected or not). However, survival analysis assumes continuous monitoring, which we did not have, so we could not accurately assign a time to censor fish that were not detected. For example, if a fish was never detected it may have died anywhere from the date of release to the date when the wolf trap or PIT antennas were taken out of the river, without knowing the time correctly a

survival analysis would be inauthentic and fit poorly. Logistic regression generally has lower power than survival analysis to differentiate the probability of binary events, and indeed the logistic models we attempted to fit tended to have relatively poor fit of binned residuals. To be conservative, we presented the results of the Chi-squared tests, which we supported with logistic regression models.

It is difficult to confirm exactly how large the bias due to a negative effect of treatment is on the calculated parasite induced mortality reported in the literature. Our study only reports on mortality that occurs during the very first stages, and it is unknown whether the mortality would have increased if it would be possible to follow the group further into the sea. Average estimates of risk ratio of EB treatment in release group studies has been reported to be 1.18 (CI: 1.07-1.3; Vollset et al. 2016) in Norway which indicates that 18% more treated than untreated fish return as adults if they are treated with EB and released as smolts. The assumption that the added mortality in the untreated group is due to sea lice infestations during the early marine migration has been used to calculate mortality attributable to sea lice on the marine survival of Atlantic salmon. The estimates from Vosso indicate that the risk ratio of EB released in freshwater and recaptured as post-smolts as they migrate to the sea might be as low as 0.81 (Vosso and Modalen data). If the difference in baseline survival is applied to the release group studies used in the meta-analysis by Vollset et al. (2016), and we assume that mortality due to treatment with EB and sea lice is independent, the estimated risk ratio of treatment with EB would be $1.18/0.81 = 1.46$ (CI: 1.31- 1.6) if it would be possible to eliminate the negative effect of EB. Consequently, 46% rather than 18% more treated than untreated fish would have returned as adults if they did not suffer from sea lice mortality.

Conclusion

Study designs that appropriately can disentangle the effect of diseases on the survival of marine fish are rare. Paired randomized control trials using an anti-parasitic drug prophylactic to shield the control group of infestations with sea lice has been viewed as one of the most robust study designs that can give insights into the effect of a single factor given an otherwise extremely variable data. Such studies have been the backbone of numerous publications trying to parameterize the effect of sea lice on the marine survival of Atlantic salmon (Jackson et al. 2013; Skilbrei et al. 2015; Vollset et al. 2016). Several of the studies have pointed at the potential bias a negative effect of treatment would have on the estimation of parasite induced mortality (Gargan et al. 2012; Vollset et al. 2016; Bøhn et al. In Press), and a significant negative treatment effect has been associated with a generally high cohort survival (Vollset et al. 2016). Even so, no studies have to date attempted to study if EB has a negative effect on the likelihood of survival in the wild. Our study, although limited to the freshwater phase, is the first of its kind to document that there is a negative effect of EB treatment in the wild. The results therefore have significant implications for the use of EB as a tool for estimating the impacts of parasitic sea lice on marine survival of Atlantic salmon. Therefore, inferences drawn from comparisons between untreated and treated salmon with respect to the risk of lice-induced mortality and the decisions made by managers regarding the levels of lice and extent of mortality suffered by migrating salmon must account for bias in baseline survival.

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Figures

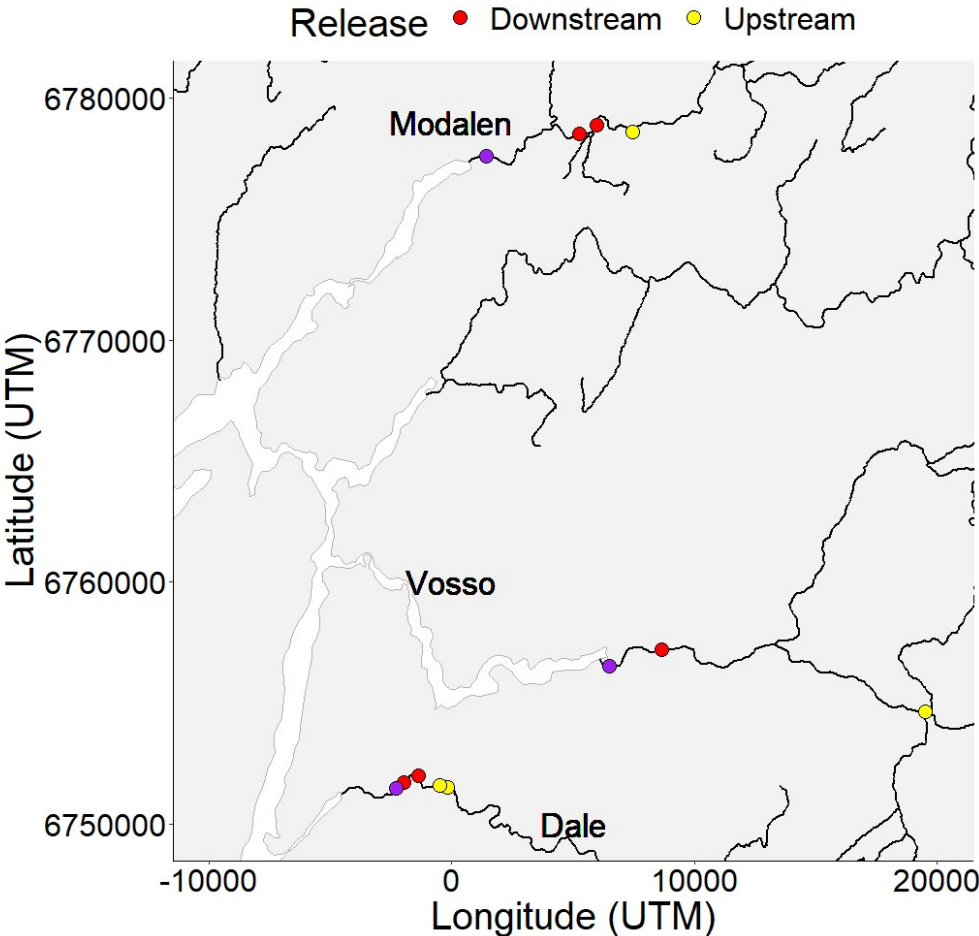


Figure 1. Map of release locations and detection sites for the three field sites in this study. Treated and untreated Atlantic salmon (*Salmo salar*) smolts were released at upstream (yellow) and downstream (red) sites and detected on floating PIT antennas (Vosso and Modalen, purple) or a wolf trap (Dale, purple).

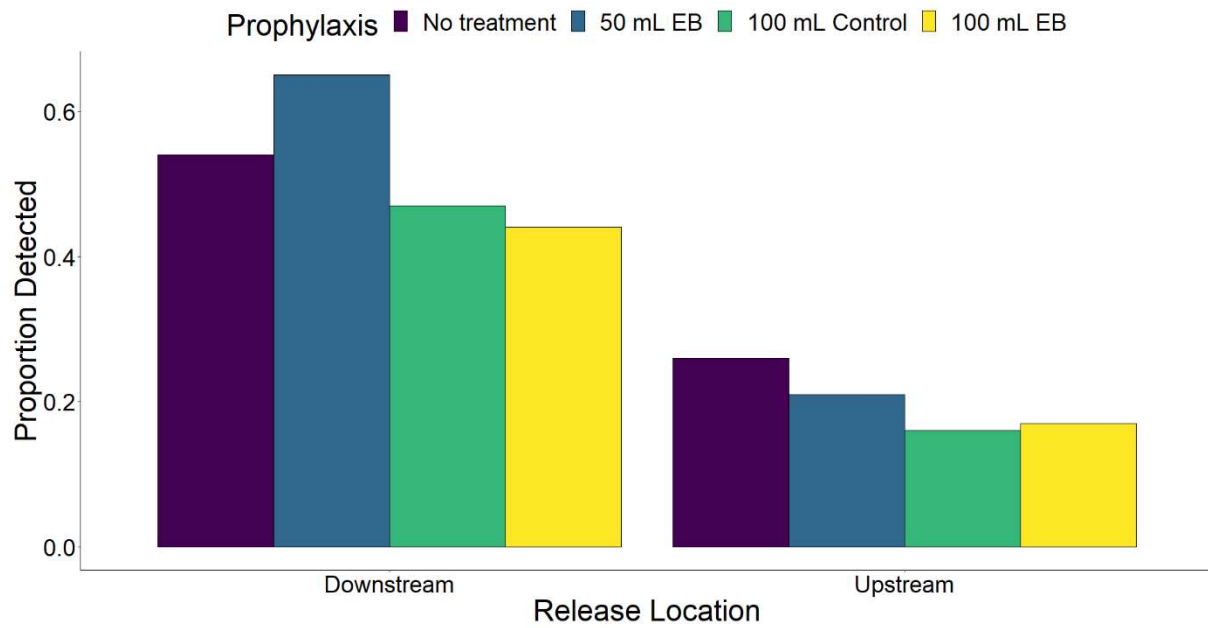


Figure 2. Proportion of Atlantic salmon (*Salmo salar*) smolts released at upstream or downstream locations in the Dale River, Norway and recaptured in a downstream wolf trap as a function of emamectin benzoate (EB) prophylaxis.

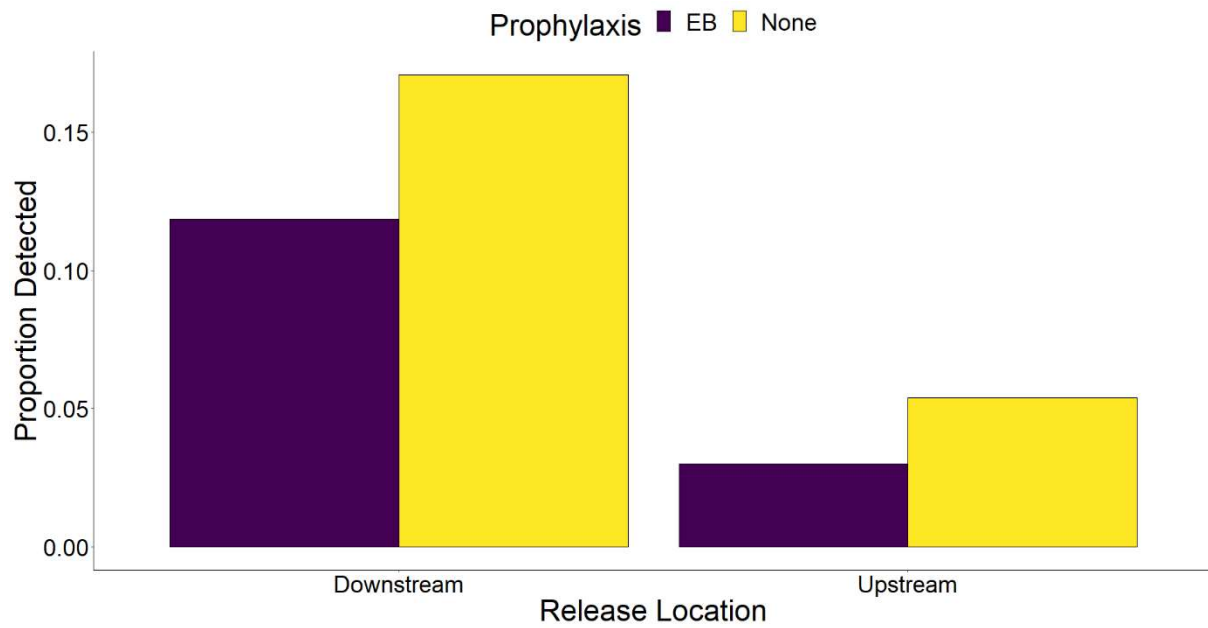


Figure 3. Proportion of Atlantic salmon (*Salmo salar*) smolts released at two locations (upstream, Vosso; downstream, Bolstad) in the Vosso River, Norway and detected at a floating PIT antenna near the confluence of the river with the sea as a function of emamectin benzoate (EB) prophylaxis.

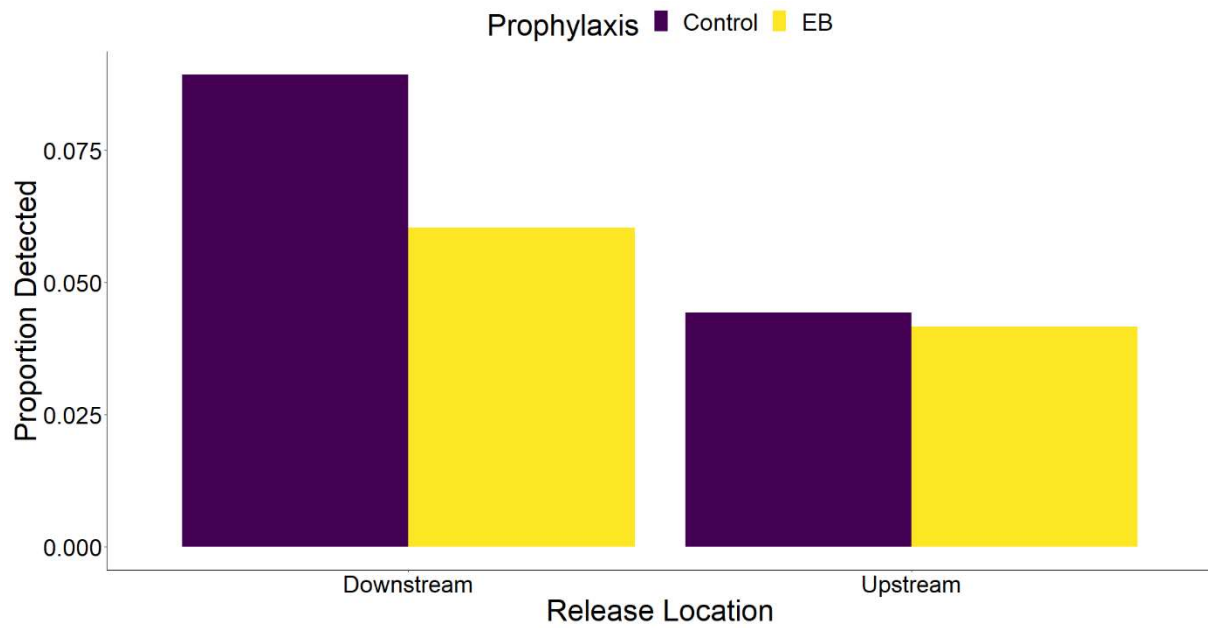


Figure 4. Proportion of Atlantic salmon (*Salmo salar*) smolts released at three locations in the Modalen River, Norway and detected at a floating PIT antenna as a function of emamectin benzoate (EB) prophylaxis.