The protective effect of the *Phe362Tyr* mutation in salmon lice' (*Lepeophtheirus salmonis*) AChE when exposed to full-scale azamethiphos bath treatments

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ABSTRACT

Organophosphates are applied for medicinal bath treatments of salmon lice (*Lepeophtheirus salmonis*) infested farmed salmonids. This chemical class remains important despite the development of resistant parasites, due to few available treatment options. The protective effect of the *Phe362Tyr* mutation in one of the acetylcholinesterase (AChE) genes of L. *salmonis* has previously been studied in small-scale treatments with the organophosphate azamethiphos. The current study was aimed at investigating the protective effect of this mutation in field treatments of commercial fish farms. In addition the effect of different methods of sampling on the occurrence of salmon lice with zero, one or two copies of *Phe362Tyr* (homozygote sensitive (SS), heterozygote (RS) and homozygote resistance (RR) respectively) were investigated.

Salmon lice were collected prior to and after azamethiphos treatments in four fish farms. The resistance genotypes were determined for each parasite. No SS lice were found in any of the farms post-treatment. Both RR and RS genotypes protected the salmon lice from the effect of azamethiphos, but the protective effect of RR genotype was greater than of RS. The study thereby emphasizes the strong selection pressure towards resistant parasites imposed by an azamethiphos treatment event in farms where resistant parasites are present.

The distribution of SS, RS and RR genotypes were not found to differ between lice from different net pens, sampled at different dates or picked off fish of different health statuses in a study with repeated sampling from a single farm.

1. Introduction

Salmon lice (*Lepeophtheirus salmonis*) are one of the biggest health threats in the aquaculture of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) in the Northern Hemisphere (Torrissen et al., 2013). The feeding behavior of these parasites poses a threat for the fish. Aggregations of lice on fish can cause wounds, which may lead to anemia, give rise to secondary infections and cause osmoregulatory problems. A heavy lice load may even be fatal to the fish (Wagner et al., 2008). The adult female parasites produce eggs that hatch into planktonic larvae (Johnson and Albright, 1991). The larvae are spread by the water current and parasites produced in one fish farm can thereby infest both fish in nearby farms and wild salmonids (Kristoffersen et al., 2018; Kristoffersen et al., 2014).

In most countries or regions farmers are subjected to regulations giving maximum thresholds for salmon lice abundance (Fisheries and Oceans Canada, 2016; Sernapesca, 2015; Ministry of Trade, Industry and Fisheries, 2012). These regulations are in place to preserve farmed fish health, control parasitic outbreaks and protect wild salmon stocks.

Norway is the world's largest producer of farmed Atlantic salmon and at the same time the host of many wild salmonid populations (http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en and https://lakseelver.no, accessed 08.08.18). In the case of Norway, salmon lice thresholds are set to maximum 0.2 adult females per fish during the wild smolt migration period and 0.5 adult females per fish the rest of the year (Ministry of Trade, Industry and Fisheries, 2012).

Traditionally, salmon lice control has been accomplished using medicinal treatments (Torrissen et al., 2013). The available medicines belong to a few chemical classes and nearly all of the substances applied have been on the market for several years. Reliance on a few chemical classes and the frequent treatments over many years, have resulted in resistant salmon lice. Many non-medicinal treatment options have been developed to control salmon lice, but so far medicinal treatments are still being use in salmonid production in all regions where salmon lice or other sea lice must be controlled (Aaen et al., 2015).

One of the chemical classes applied against salmon lice are the organophosphates, which have been used for bath treatments of

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salmonids since the 1970s (Brandal and Egidius, 1979). The organophosphate azamethiphos is applied in all salmonid producing countries in the Atlantic Ocean and in Chile, for treatment against various sea lice species (Aaen et al., 2015). Organophosphates inhibit the effect of acetylcholine esterase (AChE), leading to a buildup of acetylcholine in the synaptic cleft (Casida and Durkin, 2013). This hinders normal neural impulses and the lice are paralyzed and fall off the fish.

Resistance in L. *salmonis* towards organophosphates has been known since the 1990s in Europe (Jones et al., 1992). The resistance mechanism in L. *salmonis* towards organophosphates has been elucidated; it is caused by a *Phe362Tyr*-mutation in one of the two L. *salmonis* genes coding for AChE (Kaur et al., 2015a; Kaur et al., 2015b). A rapid molecular resistance test has been developed to detect this mutation. The *Phe362Tyr*-mutation has since been detected along the Norwegian coast and in all major salmonid producing areas in the Atlantic Ocean (Kaur et al., 2017; Kaur et al., 2016). Organophosphate resistance is also suspected in sea louse *Caligus rogercresseyi*, which is a common problem in Chilean salmon farms (Agusti et al., 2016; Marín et al., 2015). A mutation in one of the AChE genes of *C. rogercresseyi* is indicated to be involved in organophosphate resistance (Agusti-Ridaura et al., 2018).

Resistance towards most of the other chemical classes available for salmon lice treatments are also seen in all major salmonid producing regions in the world, recently including British Columbia, Canada (Messmer et al., 2018; Aaen et al., 2015). The use of azamethiphos has therefore not terminated, despite of possible resistance. Knowing the treatment effectiveness and thereby the effect on resistance selection from treatments of lice with zero (SS), one (RS) or two (RR) copies of the *Phe362Tyr*-mutation is important to avoid unsuccessful treatments, and to understand variations in treatment effectiveness to prolong the effectiveness of the chemical class for salmon lice treatments. Azamethiphos treatment efficacy, on SS, RS and RR lice, following laboratory treatments of salmonids infested with salmon lice has previously been studied (Jensen et al., 2017; Kaur et al., 2015b). Treatment effectiveness has also been studied after field treatments in commercial fish farms, but without knowledge of the drug resistance status of sea lice on the farms (Jimenez et al., 2018; Gautam et al., 2017).

The methods for sampling salmon lice for resistance testing have previously not been investigated. The choice of method is not regulated. Sampling could be performed relatively easily from available fish in the water surface of a net pen using a dip net to catch fish. It could also be performed in a more labor-intensive way, such as using a larger net to first crowd the fish and thereafter using a dip net to select individual fish, from different net pens ensuring a random selection of fish.

The aim of the current study was to examine the protective effect of the *Phe362Tyr*-mutation in full-scale azamethiphos treatments against salmon lice. An additional aim was to investigate if there were differences in the frequency of SS, RS and RR genotypes between lice sampled from the same farm, but from different net pens, at different sampling times or from fish of different health statuses.

2. Methods

2.1. Pre- and post-treatment sampling

Salmon lice were collected from four farms on the southwest coast of Norway. The farms were selected because they had already decided to treat their fish against salmon lice using azamethiphos. One net pen was sampled in two farms and two net pens at the other two farms. The same net pens were sampled pre- and post-treatment; March 2014 for farm A, March and April 2014 for farm B, January and February 2015 for farm C and February 2015 for Farm D. The salmon lice were collected from anesthetized fish, that were routinely selected for counting lice on the farms according to Norwegian regulations acting for 2014 and 2015. These regulations implied that a minimum of 10 fish should be randomly selected from the net pens (Ministry of Trade, Industry and

Table 1
Dates (dd.mm.yyyy) of treatment (tx), the number of genotyped salmon lice (N) pre- and post-treatment and the sampling time as number of days pre- and post-treatment, per farm and net pen. The farms are named by letters and the net pens by numbers. Abundances of pre-adult and adult mobile lice (Lice ab.) counted on a minimum of 10 fish, are given for the pre- and post-treatment days for the given net pens.

Farm and net pen	Tx date	N pre-tx.	Days pre-tx.	Lice ab. pre-tx.	N post-tx	Days post-tx.	Lice ab. post-tx.
A1	25.03.2014	59	7	5.65	58	6	2.31
B1	31.03.2014	58	13	3.85	58	7	0.71
C1	26.01.2015	56	3	1.9	26	7	0.22
C2	24.01.2015	59	1	1.9	55	9	0.51
D1	18.02.2015	60	14	3.35	60	7	0.3
D2	18.02.2015	58	14	3.6	59	7	0.3

Fisheries, 2012). The exact number of fish the lice were collected from is not known. The lice were fixed in 70% ethanol for later genotyping. Sampled lice were of pre-adult and adult stages of both sexes and the aim was to sample between 50 and 60 salmon lice at each net pen at each sampling time, although this was not obtained post-treatment in one of the net pens. Number of samples obtained, date of treatment and the sampling times are presented in Table 1.

2.2. Treatments

Treatments were conducted with Salmosan® vet containing 500 mg/g of the active substance azamethiphos. Nominal Salmosan® concentrations in the treated net pens were 0.25 ppm (g/m³ water) (0.125 ppm azamethiphos) in farms A, B and D and 0.3 ppm (0.15 ppm azamethiphos) in farm C. Exposure time of fish to Salmosan® was reported to be 60 min for farms A, B and D and 47 min for farm C. Water temperatures varied from 6.5 to 7.4 °C between the treatments. The number of fish in each treated net pen varied from approximately 42 000 to 174 000 (Table 2). All treatments were performed in non-permeable tarpaulins set up around net pens where the water volume had been reduced. Salmosan® was dissolved and distributed according to the summary of product characteristics (SPC) (http://www.vmd.defra.gov.uk/ProductInformationDatabase/, accessed 08.08.18). No other treatments (medicinal or non-medicinal) were performed against salmon lice from the date of the pre-treatment sampling till the date of the post-treatment sampling.

Treatment effectiveness (TE (%)) was estimated by counting all salmon lice in a minimum sample of 10 fish per net pen at according to the formula:

$$TE(\%) = \left(1 - \frac{Xt1}{Xt0}\right) * 100$$

where X is the mean count of all mobile stages of lice of both sexes, i.e. pre-adult and adult stages, and t0 and t1 are pre- and post-treatment

Table 2
Estimated treatment volume in m³, concentration of azamethiphos in g/m³ water, treatment time in minutes, treated number of fish, fish size in kg and treatment density in kg fish per m³ water for the various net pens.

Farm and net pen	Volume	Cons.	Treatm. time	No. Fish	Fish size	Treatm. Density
	m³	g/m³	min		kg	kg fish/m³
A1	18,000	0.125	60	130,000	4.82	34.8
B1	17,000	0.125	60	160,700	4.38	45.6
C1	7000	0.15	47	68,686	1.9	18.6
C2	16,000	0.15	47	173,988	4.76	51.8
D1	4300	0.125	60	41,860	4.3	41.9
D2	6500	0.125	60	89,286	2.8	38.5

respectively.

We also estimated the substance specific mortality of each genotype of salmon lice, which was expressed as:

$$\mu_1 \frac{SSt_0}{nt_0} + \mu_2 \frac{RSt_0}{nt_0} + \mu_3 \frac{RRt_0}{nt_0} = \frac{SSt_1}{nt_1} + \frac{RSt_1}{nt_1} + \frac{RRt_1}{nt_1}$$

where μ denotes mortality of the various genotypes, n is the total number of lice in the sample, and t0 and t1 are pre- and post-treatment respectively. Assuming 100% substance specific mortality of the SS genotype and two scenarios (S1 and S2) for substance specific mortality of the RR genotype, either 0% (Kaur et al., 2015b) or 19.1% (Jensen et al., 2017), the substance specific mortality of the RS genotype could be estimated by minimizing the difference between the expected and observed proportions of the various genotypes post treatment:

$$min\left((SSobs - SSexp)^2 + (RSobs - RSexp)^2 + (RRobs - RRexp)^2\right)$$

where obs denotes the observed proportion of the various genotypes and exp. denotes expected proportions of the various genotypes for given substance specific mortalities. The total substance specific effect is then given by:

$$\frac{(\mu_1 SS + \mu_2 RS + \mu_3 RR)}{nt_0}$$

2.3. Study of sampling methods

A second study was also conducted to look at variation in the level of salmon louse resistance to azamethiphos between net pens, over time and between fish swimming in the water surface with non-normal swimming behavior and the rest of the fish, within a farm. Salmon lice were sampled from four net pens in a farm (farm E) located on the west coast of Norway the 05.09.2016 (dd.mm.yyyy) and 12.09.2016, from fish (unknown numbers) that were routinely subjected to lice counting on the farm or removed from the net pen because they were regarded as non-healthy, and fixed in 70% ethanol for later genotyping. No medicinal treatments against salmon lice were applied at the farm in the period between the first and the last sampling. Both normal healthy fish, and apparently non-healthy fish (fish swimming in the water surface with non-normal swimming behavior), were sampled for lice. Altogether 451 lice of pre-adult and adult stages of both sexes were genotyped from this farm (Table 3).

2.4. Genotyping

Genotyping of the sampled salmon lice were performed by PatoGen Analyse AS using a TaqMan assay specific for detecting the *Phe*362*Tyr*-mutation. By combining TaqMan probes, each louse was classified as homozygous sensitive (SS), heterozygous (RS) or homozygous resistant (RR) according to Kaur et al. (2015b).

Table 3

The number of genotyped salmon lice per net pen, sampled from apparently non-healthy fish (fish swimming in the water surface with non-normal swimming behavior) and healthy fish (all other fish). The number of salmon lice samples on 05.09.2016 and 12.09.2016 respectively are provided in brackets.

	Number of salmon lice at the two sampling dates (05.12.16, 12.09.16)	
Net pen	Healthy fish	Apparently non-healthy fish
1	111 (54, 57)	3 (0, 3)
2	66 (36, 30)	35 (5, 30)
3	88 (55, 33)	29 (2, 27)
4	89 (59, 30)	30 (0, 30)

3. Results

3.1. Pre- and post-treatment sampling

A total of 350 and 316 salmon lice specimens were genotyped pre- and post-treatment, respectively, from 4 farms and 6 net pens (Table 1). The pre- and post-treatment proportions of the genotypes varied between the included net pens and farms, except for the proportion of SS lice post-treatment (Fig. 1). The general trend seen in all net pens were a complete extermination of SS lice post treatment and a that the proportion of RR lice compared to RS lice increased post-treatment compared to pre-treatment, indicating an increased treatment effectiveness on RS lice compared to RR lice.

The estimated substance specific mortality of the RS genotype for the six net pens, under the assumption of 100% mortality of the SS genotype and either 0% (scenario 1) or 19.1% (scenario 2) mortality of the RR genotype, are shown in Table 4.

Treatment effect varied from approximately 59% to 92% for the six treatment units (Table 4). The estimated substance specific effects were higher according to mortalities in scenario 2 compared to scenario 1, but generally lower than the treatment effects (Table 4).

3.2. Study of sampling methods

Table 5 summarizes the proportions of the various genotypes of all salmon lice sampled. The proportions are calculated for the categories; net pens, sampling dates and fish health status. Similar proportions of genotypes were found within each of these categories. There were no significant differences in chi2 tests of the distribution of genotypes for any of the compared categories of salmon lice specimens (net pens: d.f. = 6, chi2 = 1.91, p = 0.93; dates: d.f. = 2, chi2 = 1.11, p = 0.57; fish health: d.f. = 2, chi2 = 1.67, p = 0.43).

4. Discussion

The current study shows that the *Phe*362*Tyr*-mutation protects salmon lice from dying during full-scale bath-treatments with the organophosphate azamethiphos. This is in accordance with previous results from small-scale laboratory treatments (Jensen et al., 2017; Kaur et al., 2015b). The field treatment effectiveness on SS salmon lice was estimated to be 100%. The effectiveness on RS lice was calculated to be higher than on RR lice, under the assumption of either 0% or 19.1% treatment effectiveness on RR lice. These two figures were found in Kaur et al. (2015b) and Jensen et al. (2017) respectively. To calculate the exact treatment effectiveness on RS and RR lice, all affected and surviving lice from a group of fish should be sampled. However this is difficult under field conditions.

Under the assumption of 0% and 100% treatment effectiveness on RR and SS lice, respectively, mortality of the RS lice was calculated to be between 43% and 88% for the treated net pens. When keeping the assumption for SS lice while assuming a 19.1% treatment effectiveness on RR lice, the mortality of the RS lice was calculated to be between 54% and 91% (Table 4). In the two laboratory studies the same figure was 44% and 80%, Kaur et al. (2015b) and Jensen et al. (2017) respectively.

The observed variability in RS mortality seen in the current study could be explained by several different factors, such as exposure time, treatment concentration and sampling error when sampling from a heterogeneous population. The variability could also be caused by practical treatment differences, such as difficulties in exposing all fish to the same concentration throughout the entire treatment volume and for the whole treatment period. Uneven distribution in time and space (especially horizontally in the net pen) has been demonstrated using fluorescein in addition to the treatment chemotherapeutant (Høy and Oppedal, 2013). It is not possible to know the exact treatment concentration; the water volume in the flexible tarpaulin is affected by
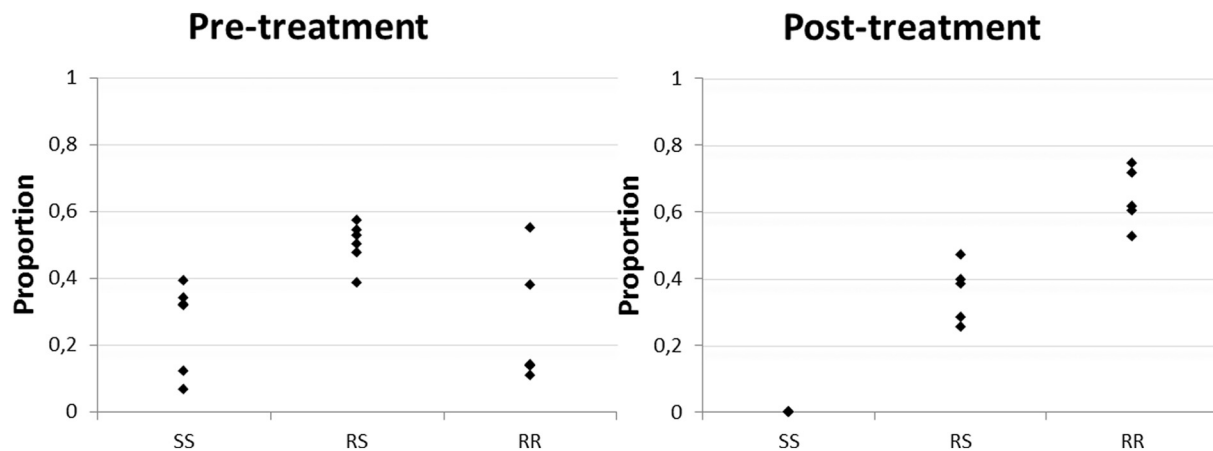
Fig. 1. Plot of salmon lice genotype proportions for azamethiphos resistance (homozygous sensitive (SS), heterozygous (RS) or homozygous resistant (RR)) in 6 net pens from four salmon farms, pre and post azamethiphos treatment.

Table 4

The number of lice with given genotypes (homozygous sensitive (SS), heterozygous (RS) or homozygous resistant (RR) for the *Phe362Tyr*-mutation) pre- and post-treatment (t_0 and t_1 respectively). μ_2 expresses the proportional substance specific treatment mortality of the RS genotype, given proportional mortalities of the SS and RR genotypes according to scenario 1 (S1) and scenario 2 (S2), respectively. The substance specific mortality of the SS genotype was set to 100% in both S1 and S2, while it was set to 0% and 19.1% for the RR genotype in S1 and S2 respectively. Substance effect (%) is the estimated percentage reduction of the louse population in the given net pen due to the substance specific effect of treatment for S1 and S2. Treatment effectiveness (%) is the percentage reduction in mean lice counts post-treatment compared to pre-treatment.

Farm and net pen	Pre-treatment			Post-treatment							
	SS_{t0}	RS_{t0}	RR_{t0}	SS_{t1}	RS_{t1}	RR_{t1}	μ_2S1	μ_2S2	Substance effect S1 (%)	Substance effect S2 (%)	Treatment effectiveness (%)
A1	4	23	33	0	17	43	0.43	0.54	23.1	37.8	59.1
B1	7	29	22	0	15	44	0.74	0.79	49.1	58.8	81.6
C1	18	32	6	0	10	16	0.88	0.91	82.4	86.2	88.4
C2	23	28	8	0	24	27	0.75	0.79	74.6	79.1	73.1
D1	20	31	8	0	23	35	0.83	0.86	77.5	81.7	91.0
D2	18	31	8	0	26	29	0.77	0.81	73.5	78.3	91.7

Table 5

Proportion of salmon lice with different genotypes (homozygous sensitive (SS), heterozygous (RS) or homozygous resistant (RR) for the *Phe362Tyr*-mutation) in farm E presented according to the level of three different factors: net pen, sampling date and fish health-status.

Unit	SS	RS	RR
Net pen 1	0.11	0.55	0.34
Net pen 2	0.08	0.57	0.35
Net pen 3	0.10	0.55	0.35
Net pen 4	0.12	0.50	0.38
05.09.2016	0.10	0.56	0.33
12.09.2016	0.10	0.52	0.38
Healthy	0.11	0.53	0.36
Apparently non-healthy	0.07	0.59	0.34

water currents and analysis of azamethiphos concentration requires laboratory facilities.

Another possible explanation to the observed variability could be that salmon lice which survived the treatment as attached stages and thereafter developed into pre-adults were sampled for the post-treatment analysis. This is due to the fact that attached stages of salmon lice are not affected by azamethiphos treatments (Roth et al., 1996). It is however assumed to be a minor issue in the current study; no SS-lice were detected post-treatment and these young pre-adults were to be expected to have a genetic make-up more similar to the pre-treatment status of the net pens, when SS-lice were found.

In studies of both L. *salmonis* and *C. rogercresseyi* increased azamethiphos exposure time in farm treatments has shown to increase field treatment effectiveness (Jimenez et al., 2018; Gautam et al., 2017). In these studies resistance levels of the salmon lice were not known and the effect of the potential interaction between resistance genotype and exposure time on treatment effectiveness could therefore not be elucidated. When comparing two laboratory treatment studies of L. *salmonis* with azamethiphos applied at two different exposure times, increased efficacy was seen after 60 min compared to 30 min exposure. This was valid both for RS and RR lice (Jensen et al., 2017; Kaur et al., 2015b). All SS parasites died in both these laboratory studies. In the current study the RS mortality was closer to the level of mortality seen in the laboratory study with 60 min exposure and closest under an assumption of 19.1% RR lice mortality compared to an assumption of 0% mortality. This coincides with the field exposure times being closer to 60 min (47 and 60 min) as applied in the study by Jensen et al. (2017). The field treatment effectiveness seen in the current study might also have been influenced by the increase in treatment dose (0.125 and 0.15 ppm azamethiphos) compared to the concentration applied in Jensen et al. (2017) and Kaur et al. (2015b) (0.1 ppm). The potential effect of these two different exposure times could not be investigated in the current study due to small sample size.

Uneven distribution of the chemotherapeutant throughout the water volume, suggested to be a possible cause of variable RS mortality in the present study, did not affect the SS mortality. This could be explained by an extreme sensitivity of SS lice to azamethiphos exposure. In a study by Roth et al. (1996), full treatment effectiveness was seen when azamethiphos was applied at a dose of 0.05 ppm on known sensitive populations of salmon lice (sensitivity was assessed in in-vitro bioassays prior to treatment). The same study showed that applying

azamethiphos at a dose of 0.2 ppm increased the treatment effectiveness in farms with resistant parasites compared to treatment using the dose currently approved in the SPC of Salmosan® vet (0.1 ppm azamethiphos). This strategy was however not able to bring treatment effectiveness to the level seen at the farm with sensitive parasites (Roth et al., 1996). If the azamethiphos resistance seen in the study by Roth et al. (1996) was caused by the presences of the *Phe*362*Tyr* mutation in the lice, the results indicate that treatment effectiveness of RR and/or RS lice are concentration dependent. Kaur et al. (2017) showed the presence of the *Phe*362*Tyr* mutation in salmon lice sampled in Scotland in 2002, in Norway in 1998 and in Canada in 1999. It is therefore possible that this mutation also was the cause of resistance in the early 1990s. In the current study the highest RS mortality was found in farm C which used the highest treatment concentration (0.15 ppm azamethiphos), despite this farm using the shortest exposure time (47 min). Again the small sample size prevents firm conclusions.

Under the assumptions of 0% or 19.1% RR mortality and 100% SS mortality, the overall anticipated substance specific treatment effectiveness could be calculated. For all but one net pen (C2), this figure was lower than the calculated treatment effectiveness based on pre- and post-treatment lice counts. The observed additional effect of treatment not explained by exposure to azamethiphos could be caused by non-substance specific effects, such as the mechanical effects that might cause salmon lice to be rubbed off the fish when the fish are crowded during a treatment. This effect has previously been indicated for fresh water well-boat treatments (Reynolds, 2015). The mechanical effects on the lice during the course of a well-boat treatment are however most likely greater than during a tarpaulin treatment, because of the pumping of fish in and out of the well. Another possible explanation is the sampling error that both estimation of genotype distribution and treatment effectiveness is subjected to, especially due to small sample sizes (Jimenez et al., 2012).

In the study by Roth et al. (1996) the farms with drug resistant parasites showed inter-net pen variability in treatment effectiveness. No significant inter-net pen difference in genotype frequency was found in the current study. Variability was non-significant between different sampling dates or between healthy and apparently non-healthy fish. Sampling was however performed in one, single farm, possibly more homogenous with regards to azamethiphos resistance than other farms, and on two dates with just one week between. The strong genotype-dependent mortality from azamethiphos treatments seen both in the present and in previous studies (Jensen et al., 2017; Kaur et al., 2015b), strongly indicate that if only single net pens on a farm have been treated with azamethiphos, post-treatment inter-net pen heterogeneity with regards to azamethiphos resistance is to be expected.

Knowing the protective effect of resistance genes in field treatments is important in order to give good estimates of treatment effectiveness. This knowledge can be further applied to optimize treatments and to avoid unsuccessful treatments. The results also provide important background information when trying to predict or hinder the development of resistance at a farm or in a region, for example by providing actual values for parameters of resistance in models by McEwan et al. (2015, 2016).

The protective effect of the *Phe*362*Tyr*-mutation in the AChE of salmon lice in laboratory treatments was also present in farm treatments, as expected from the evolutionary success this mutation has shown throughout the Atlantic Ocean (Kaur et al., 2017). This protective effect varied somewhat between treatment events. Since not all factors affecting this variation are known, the exact treatment effectiveness of a lice population containing RS and/or RR lice cannot be calculated pre-treatment. The effect of a single azamethiphos treatment on resistance selection can however be expected to be significant, since all the SS and more than half of the RS lice of the affected life stages will most likely will be killed by a treatment, thereby leaving few sensitive alleles for the upcoming generations of lice.

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