Aquaculture 282 (2008) 7–12

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Sensitivity assessment of *Caligus rogercresseyi* to emamectin benzoate in Chile

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ARTICLE INFO

Article history:
Received 28 November 2007
Received in revised form 28 April 2008
Accepted 11 June 2008

Keywords:
Sea lice
Caligus rogercresseyi
Lepeophtheirus mugiloidis
Resistance
Emamectin benzoate

ABSTRACT

The ectoparasitic copepod *Caligus rogercresseyi*, as distributed in the south of Chile, is the most important parasite of farmed salmonids because of the substantial, negative effects on survival, growth and increased susceptibility to infections, and this results in severe economic effects in seawater aquaculture.
The sensitivity of *C. rogercresseyi* to emamectin benzoate was studied in 18 salmon farms belonging to four large aquaculture companies in Chile. Emamectin benzoate is the only medicinal product allowed by the Chilean official authority for *Caligus* control since 2000. Sensitivity values, recorded as EC_{50} (the emamectin concentration immobilizing 50% of the test subjects) in adult *C. rogercresseyi* were between 57 and 203 micrograms per liter (ppb) in the survey carried out in the summer season, and between 202 and 870 ppb for the winter season survey. The EC_{50} control value, obtained from naïve *Lepeophtheirus mugiloidis* parasites, was 34 ppb. The results strongly indicate loss of sensitivity of *C. rogercresseyi* to emamectin benzoate at all sites analyzed.

1. Introduction

Sea lice are marine ectoparasitic copepods common on cultured and wild marine finfish, worldwide distributed in the marine environment. In the Northern hemisphere, *Lepeophtheirus salmonis* is the most important parasite for wild salmon and the salmon industry (Pike and Wadsworth, 1999; Rae, 2002, Costello, 2006). Resistance towards organophosphates (Jones et al., 1992) and pyrethroids (Sevatdal et al., 2005b) has been demonstrated for this parasite in Norway, Scotland and Ireland. In Chile, *Caligus rogercresseyi* is the dominant species in farmed salmonids. Its natural hosts are a wide variety of marine wild fish, which are attracted to the salmon farms by the salmon's food (Carvajal et al., 1998; Bravo, 2003). Even though the list of copepods described for marine wild fish in Chile is long (Stuardo and Fagetti, 1961; Fagetti and Stuardo, 1961), only *Caligus teres* (Reyes and Bravo, 1983) and later *C. rogercresseyi* (Boxshall and Bravo, 2000) have been recorded as problematic copepod parasites for farmed salmon in Chile.

Sea lice and their control have been one of the main problems for the Chilean salmon industry since 1982, when *C. teres* infesting Coho salmon was recorded for the first time (Reyes and Bravo, 1983). Since then, sea lice have been controlled by the use of antiparasitic agents. In the beginning, the salmon were treated with the organophosphates metrifonate (Neguvon™) and dichlorvos (Nuvan™) applied by bath. However, these agents were only effective against the adult stages, without affecting the chalimus stages. Alternative treatments (onion, garlic) had no effect. Ivermectin was introduced in Chile at the end of the 1980s and soon gained popularity because of its easy application through the feed and the superior efficacy, as it controlled both adult and chalimus stages of the parasite. Ivermectin was replaced by emamectin benzoate at the end of 1990s, a medicinal product with high efficacy against all developmental stages of *L. salmonis* (Stone et al., 1999, 2000). In 2000 emamectin benzoate was approved by the Chilean official medicinal authority as the only chemotherapeutant allowed to be used in the control of sea lice.

Emamectin benzoate [(4″*R*)-4″-deoxy-4″-(methylamino)avermectin B_1 benzoate] is a macrocyclic lactone developed for the control of insect pests (Mushtaq et al., 1996). It is a semi-synthetic derivative of the avermectins produced by *Streptomyces avermitilis* (Roberts and Hutson, 1999). In nematodes and arthropods, avermectins modulate specific glutamate-gated anion channels in synapses and muscle cells (Roberts and Hutson, 1999), thereby increasing the influx of chloride ions. This hyperpolarizes the cell, and prevents depolarization of the neuromuscular endplate beyond the threshold level (Davies and Rodger, 2000). Emamectin benzoate thus has neurotoxic properties, and it is most effective in arthropods following ingestion (Roberts and Hutson, 1999).

Four pharmaceutical laboratories in Chile, including Schering-Plough who developed the product, supply premixes containing emamectin benzoate. At the beginning, the treatments with 50 μg of active ingredient per kilogram biomass daily for 7 days gave a protection for almost 10 weeks at sites with little exposure to wind and current, and over 12 weeks at exposed sites (Bravo, 2003). However, since the end of 2005, several fish farmers in Chile recorded a loss of efficacy.

There are several reports of development of resistance in *L. salmonis* against medicinal products used for its control (Denholm et al., 2002;

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doi:10.1016/j.aquaculture.2008.06.011

Sevatdal and Horsberg, 2003). Considering this situation and the fact that for seven years only one product was authorized to control sea lice in Chile, without an integrated parasite management plan, a survey was carried out in Region X in the south of Chile (Region of Lakes; 42° L.S.) to evaluate the sensitivity of *C. rogercresseyi* to emamectin benzoate. In Region X, 84% of the Chilean salmon production takes place. This represents an important economic activity for the region and the country, with revenues of US$ 2.207 billion, which corresponds to 5% of the total revenue generated by exports from Chile.

2. Materials and methods

To evaluate the level of sensitivity of *C. rogercresseyi* to emamectin benzoate, the bioassay methodology developed by Sevatdal and Horsberg (2003) for testing *L. salmonis* sensitivity to pyrethroids in Norway, was implemented for *C. rogercresseyi*. Bioassays were applied in two surveys carried out in the study period. The first survey was conducted in the summer season, between November 2006 and January 2007. Adult parasites of both sexes were collected from 18 salmon farms from four areas between Puerto Montt and the southern end of the Chiloe Island (Fig. 1). The farms were grouped according to whether or not they were at wind and/or current exposed locations. The second survey was carried out in the winter season, between May and July 2007, in seven of the 18 salmon farms surveyed the first time. The number of sites included in this survey was lower, due to bad weather conditions, fallowing periods implemented in the sites in the area of Puerto Montt, and an outbreak of infectious salmon anemia (ISA) in the Chiloe Island in June 2007.

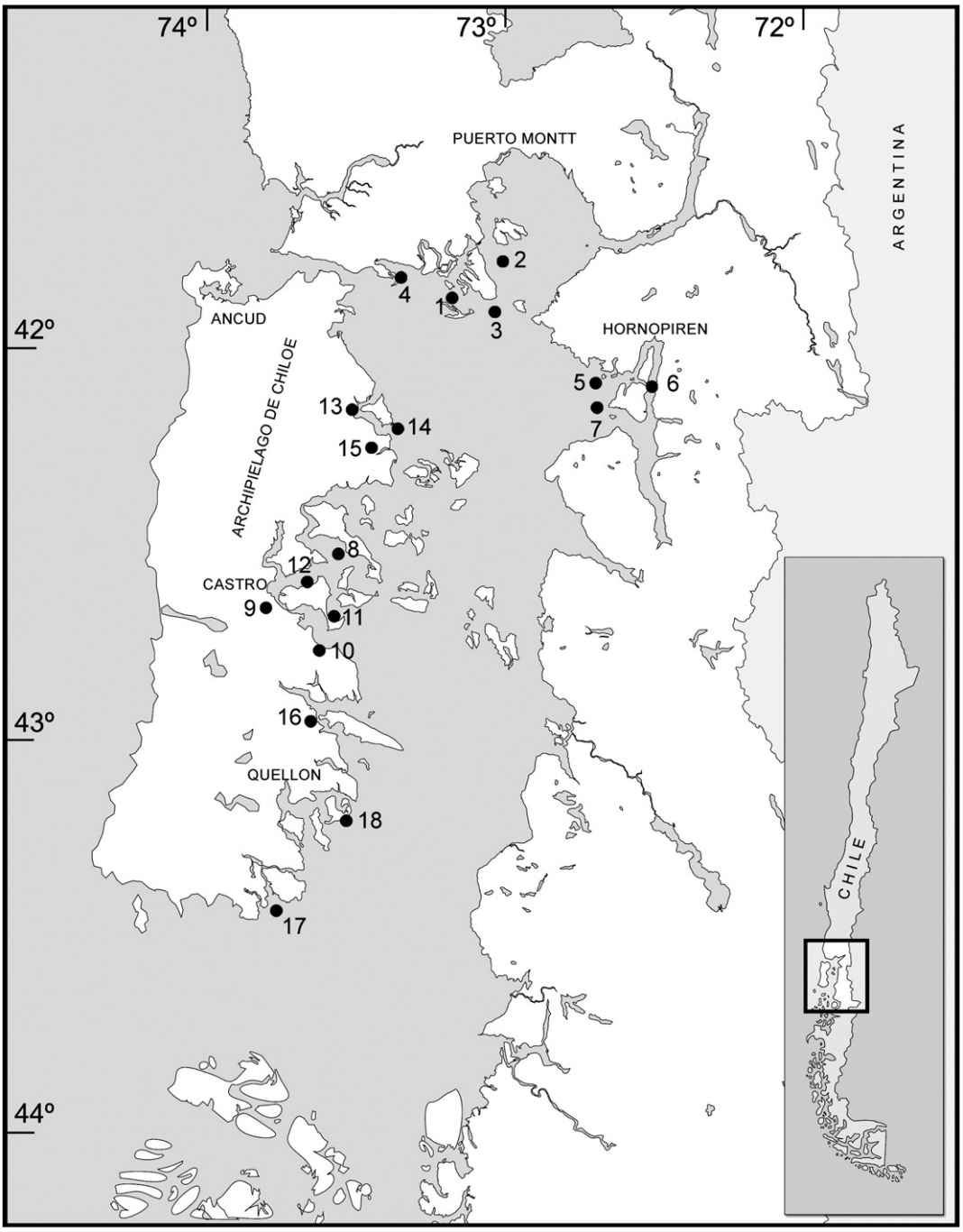

Fig. 1. Locations of sites.

Table 1
Sensitivity values (EC_{50}) of *C. rogercresseyi* towards emamectin benzoate (EMB) in 18 salmon farms in the south of Chile, recorded in the summer survey (November 2006–January 2007)

Area	Species	Farm	Operation years	Exposure level	EC_{50} (ppb) average (95% CI)	EMB-treatments	Prevalence (%) average (range)	Infestation level average (range)
Puerto Montt	AS	C1	5	N/E	166 (126–246)	12	89 (58–100)	14 (2–58)
	AS	C2	12	E	151 (109–246)	6	94 (68–100)	13 (3–33)
	AS	C3	4	N/E	157 (136–191)	7	99 (91–100)	18 (7–30)
	AS	C4	6	E	156 (123–208)	5	100 (100)	20 (8–30)
Hornopiren	AS	C5	10	N/E	168 (132–219)	7	91 (60–100)	4 (2–10)
	AS	C6	2	N/E	109 (20–239)	5	75 (51–100)	3 (2–9)
	RBT	C7	9	E	113 (86–151)	1	53 (18–100)	5 (2–8)
Ancud–Castro	AS	C8	1	E	177[a]	6	97 (87–100)	30 (4–149)
	AS	C9	4	N/E	103 (65–118)	6	95 (51–100)	13 (2–29)
	AS	C10	15	N/E	203 (156–262)	11	90 (18–100)	12 (1–26)
	AS	C11	18	N/E	135 (89–306)	8	96 (62–100)	11 (2–36)
	AS	C12	17	N/E	141 (18–307)	12	97 (69–100)	15 (3–51)
	AS	C13	12	N/E	176 (136–308)	13	100 (100)	26 (5–69)
	AS	C14	12	N/E	101 (83–122)	9	97 (71–100)	14 (3–48)
	AS	C15	5	E	149[a]	6	100 (100)	28 (8–64)
Quellón	AS	C16	8	N/E	57 (28–73)	4	89 (71–100)	9 (3–26)
	AS	C17	10	N/E	138 (119–160)	8	96 (60–100)	41 (4–147)
	RBT	C18	18	N/E	164 (113–312)	5	91 (60–100)	12 (2–49)

The prevalence, infestation levels and numbers of treatments were recorded for the period March 2006–March 2007.
AS = Atlantic salmon; RBT = rainbow trout. E = Exposed site (to wind and currents); N/E = non-exposed site.
[a] The confidence intervals could not be determined.

Parasites were carefully removed from fish anesthetized with benzocaine, transported live in cooled seawater, in containers supplied with aeration, to the laboratory at the Aquaculture Institute of the Universidad Austral de Chile in Puerto Montt, where the bioassays were carried out.

Seven groups of parasites from each site were exposed to different concentrations of emamectin benzoate (Sigma-Aldrich Corp., St. Louis, MO, USA). The concentrations used were 0, 20, 40, 80, 120, 160 and 320 micrograms per liter (ppb), dissolved in filtered seawater with a salinity of 3.1%. The solutions were prepared in Petri dishes using a protocol adapted in Canada (Burka, personal communication, 2006) from Sevatdal and Horsberg (2003). In each dish (20 ml), ten adult copepods, five of each sex, were placed. They were exposed for 24 h at 12 °C with a photoperiod of 12 h of light and 12 h of darkness.

The response of the lice to the varying emamectin benzoate dilutions was evaluated at 24 h after start of exposure. The response criteria were as proposed by Sevatdal and Horsberg (2003): 1) dead: no movements in extremities, gut or other organs; 2) moribund: not capable of attaching to a surface of Petri dish using the flat body as a "sucking disc". Movements of extremities or internal organs could still be observed; 3) live: attached to the walls of the Petri dish or active swimming behavior.

The EC_{50} value, i.e. the concentration that immobilized 50% of the target organism (moribund+dead), was used in the evaluation of sensitivity. The data from the bioassays were analyzed by probit analysis using the software POLO PC (LeOra Software, POLO-PC, 1987), and the 95% confidence limits were estimated. Analysis of variance and the Multiple Range Test, (LSD, Fisher) were used for estimating differences in sensitivities between exposed and non-exposed sites, years of operation and areas. A matched pairs *t*-test on logarithmically transformed values was used to evaluate the differences in sensitivity between seasons. The sensitivity data had to be logarithmically transformed before this analysis, as the data obtained in the winter survey did not comply with a normal distribution.

As it was impossible to get *C. rogercresseyi* not exposed to emamectin benzoate during the period of the study, the EC_{50} control value was obtained from *Lepeophtheirus mugiloidis*, collected from the wild marine fish *Elegipnos maclovinus* in the area of Puerto Montt. Considering that *L. mugiloidis* does not parasite salmon species, it was highly improbable that it had been exposed to emamectin benzoate. The resistance ratio was calculated according to Jones et al. (1992) using the formula:

$$\text{Recorded } EC_{50}(C.\ rogercresseyi)/\text{Control level } EC_{50}(L.\ mugiloidis).$$

In addition, the prevalence of *C. rogercresseyi* infestations was analyzed for each site between March 2006 and March 2007, and the number of treatments applied during this period was recorded. The average infestation intensity on each site was recorded by counting the number of parasites on the fish. Random samples of 15 fish per

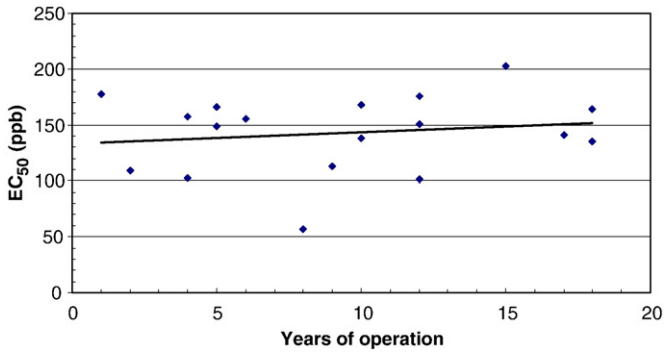

Fig. 2. Average emamectin sensitivities in *C. rogercresseyi*, recorded as EC_{50}, plotted against the years of operation in the 18 salmon farms included in the study (summer survey). No significant effects of years of operation ($R^2=0.025$, $p=0.530$).

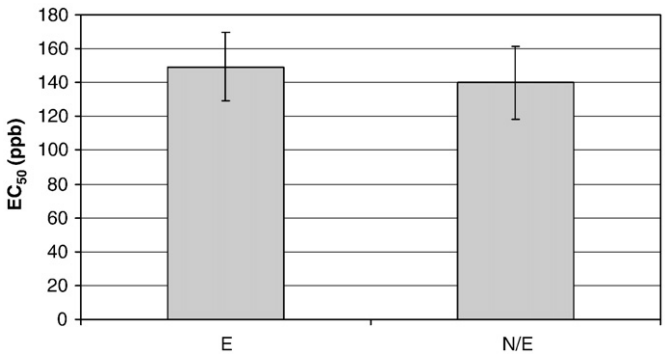

Fig. 3. Average sensitivities (±95% confidence intervals, summer survey) of *C. rogercresseyi* to emamectin benzoate, grouped according to the level of exposure of the sites. E = Sites exposed to wind and currents; N/E = non-exposed sites. No significant differences between exposure levels ($p=0.626$).

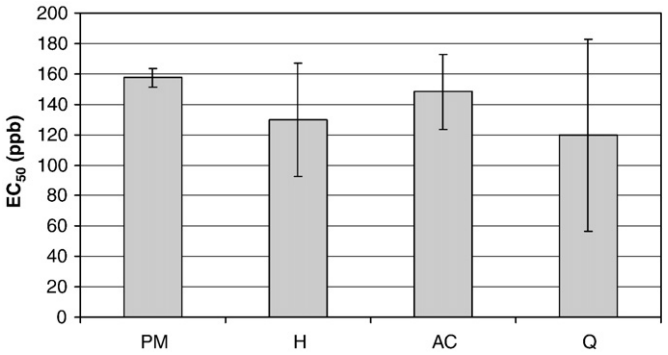

Fig. 4. Average sensitivities (±95% confidence intervals, summer survey) in *C. rogercresseyi* to emamectin benzoate in the areas where the 18 sites are located in Region X. PM = Puerto Montt; H = Hornopiren; AC = Ancud–Castro; Q = Quellón. No significant differences between areas (p=0.493).

cage were collected from three selected cages per site every two weeks. The same cages were sampled throughout the study period.

3. Results

In the summer survey, all EC_{50} values were over 100 ppb with exception of farm C16 (EC_{50}: 57 ppb), where an emamectin treatment was in progress at the time the parasites were collected (Table 1). The highest EC_{50} value, 203 ppb, was recorded in farm C10 located in the area of Ancud–Castro where the level of infestation and treatment intensities were more severe compared with the other sites.

No significant differences in sensitivities to emamectin benzoate were detected as a function of years of operation (Fig. 2, p=0.530), between sites with different exposure levels to wind and currents (Fig. 3, p=0.626), or between the four areas included in the study (Fig. 4, p=0.493).

The EC_{50} value obtained for *L. mugiloidis* not exposed to emamectin benzoate, was 34.2±10.4 ppb, which is 4.2 times less than the average EC_{50} value for *C. rogercresseyi* collected from the salmon farms in the summer survey (142 ppb). The resistance ratio obtained for the 18 farms analyzed ranged from 1.7 (C16) to 5.9 (C10), as shown in Fig. 5.

The lowest prevalence was recorded in farm C7 (53%) located in the area of Hornopiren where only one treatment with emamectin benzoate was applied during the study period. The farms in the other three areas showed prevalence values between 89 and 100% (Table 1). The lowest infection intensities were also recorded in the area of Hornopiren, with values between 3 and 5 parasites per fish. Farm C13, located in the area of Ancud–Castro, showed the highest number of treatments with emamectin benzoate in the period of study (13), a prevalence of 100%, and an average infection intensity of 28 parasites per fish.

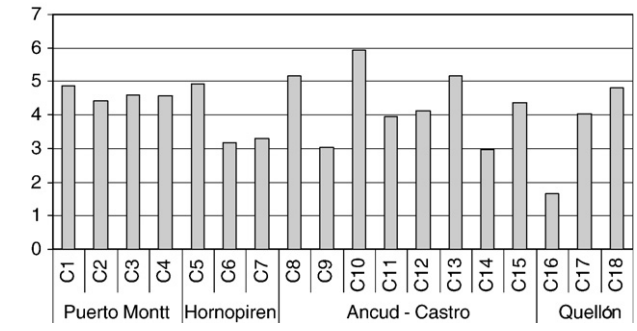

Fig. 5. The resistance ratio (EC_{50} recorded/EC_{50} controls, summer survey) in *C. rogercresseyi* to emamectin benzoate in the 18 salmon farms sampled in the study. Naïve *L. mugiloidis* served as controls.

Table 2
Sensitivity values (EC_{50}) and resistance ratio of *C. rogercresseyi* towards emamectin benzoate (EMB) in the winter survey (May–July 2007)

Area	Species	Site	EC_{50} (ppb) average (95% CI)	Resistance ratio
Hornopiren	AS	C5	202 (122–858)	6.4
Ancud–Castro	AS	C8	222[a]	6.5
	AS	C9	870[a]	25.5
	AS	C10	231 (156–696)	6.8
	AS	C12	426[a]	12.5
	AS	C13	282 (208–423)	8.2
Quellón	RBT	C18	350[a]	10.2

AS = Atlantic salmon; RBT = rainbow trout.
[a] The confidence intervals could not be determined.

The EC_{50} values obtained for the winter survey ranged between 202 and 870 ppb (Table 2). The highest EC_{50} value was recorded in farm C9, located in the area of Ancud–Castro. The resistance ratio obtained for these 7 farms ranged from 6.4 to 25.5 (Table 2).

The matched pairs test demonstrated that the EC_{50} values recorded in the winter seasons was significantly higher than the corresponding values recorded in the summer (p=0.039) (Fig. 6).

4. Discussion

The values of sensitivity obtained in *C. rogercresseyi* in this study were substantially higher than the values reported from Canada and Norway (Burka and Sevatdal, personal communications, 2006). The results obtained *in vitro* were in accordance with the efficacy observed in the field, where the emamectin benzoate did not give the same protection as recorded in the first years of application of the treatment (Bravo, 2003). The numbers of treatments recorded at the different sites were between 1 and 13 per farm in the study period (Table 1). The lowest number of treatments was recorded in farm C7, located in Hornopiren, an area highly influenced by freshwater. The highest number of treatments was recorded in the area of Ancud–Castro (Chiloe Island), where treatments were applied between 6 and 13 times per site, and where the impact of the infestation with sea lice was severe as seen from the values recorded for prevalence and intensity of infestation.

The results obtained through the summer survey showed no significant differences in emamectin sensitivities between *C. rogercresseyi* collected from the four areas of sampling or between parasites collected from sites with different levels of exposure to wind and currents. There were also no significant correlation between the numbers of years the site had been in operation and the sensitivity of the parasites. The resistance ratio for all farms was lower than 6.

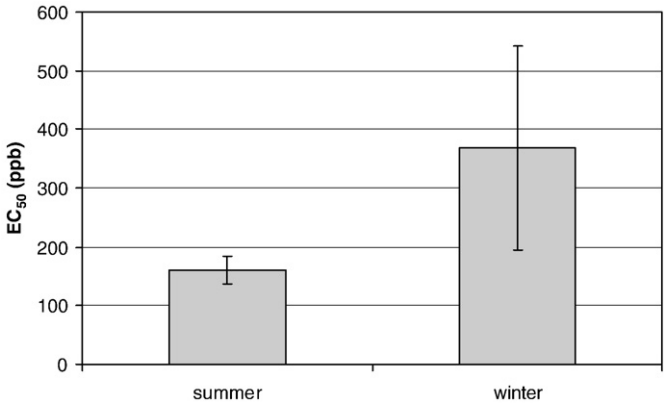

Fig. 6. Average sensitivities (±95% confidence intervals) of *C. rogercresseyi* to emamectin benzoate recorded at the same seven sites in summer 2006/2007 and winter 2007. The difference is significant (p=0.039).

However, in the winter survey, the EC_{50} values for *C. rogercresseyi* towards emamectin benzoate had increased further and the resistance ratio exceeded 6 in all seven farms where samples were collected, and 10 in three farms located at Chiloe Island. The increase in EC_{50} values in all seven farms could point to a seasonal variation in emamectin sensitivities in *C. rogercresseyi*. However, the water temperature in the Chiloe area only varies between 9 and 15 °C through the seasons, indicating that the drop in sensitivity may just as well have been caused by a further selection of resistant strains of the parasite in the area.

The results suggest that the apparent loss in sensitivity recorded in *C. rogercresseyi* from Region X was not dependent of the geographical location or how many years it had been in operation. Thus we can infer that the main cause of the loss of sensitivity in *C. rogercresseyi* has been the exclusive use of emamectin benzoate to control sea lice in Chile for more than seven years. In addition, another avermectin (ivermectin) was applied for approximately ten years during the 1990s, thus favoring selection for resistance towards this group of medicinal products for more than 15 years in the Chilean aquaculture. In contrast to the practice in other countries such as Norway and Scotland, no alternation between medicinal products of different classes and with different modes of action has been practiced in Chile.

These sensitivity studies were performed in 2006 and 2007, when clinical failures of emamectin treatments were evident in the whole Region X. This made it impossible to obtain samples of *C. rogercresseyi* from fish not exposed to avermectins. Thus, the sensitivity in naïve parasites of this species could not be established. To get an indication of the normal sensitivity towards emamectin, samples of *L. mugiloidis* were collected from wild fish in the area and subjected to the same bioassays. These parasites are of approximately the same size as the *C. rogercresseyi*, and the EC_{50} value obtained for *L. mugiloidis* (34.2 ppb) was close to the value reported for sensitive *L. salmonis* in Canada (21 ppb; Burka, personal communication 2006) and Norway (15.7–29.7 ppb; Sevatdal, personal communication 2006), which indicated that they were unlikely to have been exposed to emamectin. As the *L. mugiloidis* showed approximately the same sensitivity towards emamectin as *L. salmonis* in Norway and Canada, it is likely that naïve *C. rogercresseyi* also will have sensitivity in the same range. However, this remains to be confirmed. It is therefore not possible to conclude with absolute certainty that the sensitivity values presented here confirm a suspected resistance towards emamectin.

As clinical failures may be attributed to a low concentration of the active ingredient in the premix, samples of the products were obtained from each of the four laboratories supplying the Chilean aquaculture industry with emamectin premixes. These samples were analyzed for their content of emamectin benzoate in a Canadian laboratory, and demonstrated an emamectin content of 85–117% of the declared concentration (data not shown). Thus, it is not likely that products with too low concentrations of the active ingredient caused the clinical failures of the treatments in 2006 and 2007.

Emamectin benzoate has been used extensively in Chile, in doses higher than the recommended dose and with extended treatment periods. Still, the clinical effect has been declining. The emamectin benzoate has most likely just killed the susceptible parasites and allowed the reproduction of the resistance copepods, rendering the infestations uncontrollable by this treatment in the summer of 2007. Resistance develops through genetic selection, an evolutionary process (Kunz and Kemp, 1994), stimulated by exposure of sea lice to sub-lethal doses of a treatment agent. The traditional definition of resistance is a strain capable of surviving a dose of a control agent lethal to the vast majority of individuals in a normal population (ffrench-Constant and Roush, 1990), and seems valid for the current situation in Chile.

The current study does not give any indication of the mechanisms involved in the development of reduced emamectin sensitivity. In general, mechanisms can be kinetic (decreased accumulation of the active substance, caused by e.g. increased activity of P-glucoproteins, Tribble et al., 2007), metabolic (increased activity of unspecific esterases, oxidases or other metabolic enzymes, Sevatdal et al., 2000a) or mutations resulting in altered target sites for the compound (Fallang et al., 2005). The mechanisms will have to be demonstrated through separate studies.

The current study also demonstrated that the reduction of sensitivity was uniform within the whole waterbody east of the Chiloe Island (Fig. 1). Thus, the Chilean population of *C. rogercresseyi* in the Chiloe area seems to be uniform with a rapid exchange of genetic material. This is not surprising, as 84% of the Chilean production of Atlantic salmon takes place in this 300 km region. The rapid distribution of reduced sensitivity towards emamectin benzoate in Chile is in contrast to the situation with reduced *L. salmonis* sensitivity towards pyrethroids in Norway, Scotland and Ireland. In these countries, reduced sensitivity to pyrethroids was detected in geographically isolated places, but disappeared again after fallowing of the sites (Sevatdal et al., 2005b), most likely because the distances to the next fish farms were larger and the selection pressure was lower. However, as these incidents occurred with different classes of chemicals, the situations are not directly comparable.

In conclusion, the current study strongly indicated reduced sensitivity of *C. rogercresseyi* to emamectin benzoate in the 18 sites from where the parasites were collected in Region X. The situation most likely developed due to a unilateral use of avermectins in the region for a period of 15 years. An integrated parasite management plan where alternative chemical and non-chemical control options are utilized is needed in Chile in order to combat the *C. rogercresseyi* problem.

Acknowledgments

This study was carried out as a part of the Project Fondef DOI1255, with financial support by Conicyt. The authors thank Claudia Lagos for her help in the processing data, Francisca Erranz, Pier Bassaletti, Veronica Pozo and Melinka Mancilla who collected the samples in the field and for the help with the bioassay work.

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