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Sensitivity assessment in the progeny of *Caligus rogercresseyi* to emamectin benzoate

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Abstract

The loss of sensitivity to emamectin benzoate recorded in *Caligus rogercresseyi* in the Region X in Chile (41º46'S72º56'W; 43º07'S73º38'W) has been attributed to the exclusive use of this product to control sea lice for more than seven years. In order to evaluate the sensitivity through successive generations of lice, a study was carried out between August 2006 and April 2007. Seven successive generations from a presumably resistant population of *C. rogercresseyi* were cultivated in the laboratory without the selection pressure of the antiparasitic drug. Results of the bioassay applied to the progenies showed that there was no recovery of sensitivity over the seven inbred generations studied. This may point to a long-standing resistance problem against emamectin benzoate.

Introduction

Caligus rogercresseyi is the most problematic parasite for salmonids reared in seawater in Chile because the serious economic effect for the salmon industry. Sea lice infestations on salmonids in marine aquaculture were reported for the first time in Chile in 1982 (Reyes and Bravo, 1983), and since then, several chemotherapeutants have been used for their control. Metriphonate (Neguvon™) was the first product used to control *Caligus teres* between 1981 and 1985, replaced by another organophosphate, dichlorvos (Nuvan™) between 1985 and 2000, both applied by baths. Ivermectin administered in-feed was introduced in Chile at the end of the 1980s, but between 2000 and 2007 the only medicinal product authorized for this use in Chile was the avermectin emamectin benzoate (EMB), without alternative treatments available. *C.rogrecresseyi* was first recorded infecting Atlantic salmon (*Salmo salar*) in 1997 (Boxshall and Bravo, 2000) and since then the lice have almost exclusively been exposed to this treatment, which enhanced the selection pressure for resistance (Bravo *et al*., 2008).

Emamectin benzoate is a macrocyclic lactone, belonging to the avermectin class of insecticides. Following its introduction as an insecticide in horticulture in the late 1990s, resistance has been reported in several pests (Attique *et al*., 2006; Kang *et al*., 2006; Reyes *et al*., 2007). When EMB (Slice™) was introduced into the Chilean market in 1999, one standard treatment with the product (50 μg of active ingredient per kilogram biomass daily for 7 days) controlled the sea lice infestations on the salmon for at least five weeks in the summer and even longer in the winter (Bravo, 2003). Since early 2005, a notable loss of efficacy was

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noticed in several fish farms, corroborated in a study carried out between 2006 - 2007 where the sensitivity was monitored in 18 sites located in the Region X (41°46'S72°56'W; 43°07'S73°38'W). The bioassays applied to adult *C. rogercresseyi* collected from the different sites demonstrated resistance development in the lice towards EMB as the most plausible cause (Bravo *et al.*, 2008).

EMB is also widely used in Europe and North America (Denholm *et al.*, 2002), and until now it has been one of the most effective and valued chemotherapeutants administered against sea lice infections. The major advantage of EMB is that it can offer sustained periods of louse clearance, because of its effect on all parasitic stages of the sea lice (Stone *et al.*, 2000). Furthermore, as an in-feed treatment, it can be safely and effectively administered during adverse weather conditions, allowing coordinated actions within an area. However, treatment failures have also been reported form Ireland, Scotland and Norway. Several studies have been carried out in the last years to evaluate the sensitivity of sea lice to this product. The results have been based on epidemiological data (Lees *et al.*, 2008), biochemical data (Tribble *et al.*, 2007) and bioassays (Westcott *et al.*, 2008; Bravo *et al.*, 2008). Bioassays are valuable tools to detect decreased sensitivity towards a chemotherapeutant when the mechanism of resistance is unknown (Denholm *et al.*, 2002).

A drop in sensitivity does not need to have a long-standing impact on the control options for a parasite if the sensitivity is spontaneously reverted to normal in a few generations, or if there is a cost for the parasite to maintain the resistance.

The aim for the present study was to study whether or not the sensitivity towards EMB increased when successive generations of a resistant strain of *C.rogercresseyi* were cultivated under laboratory conditions in absence of selection pressure from the chemotherapeutant.

Materials and methods

In August of 2006, samples of *C. rogercresseyi* were collected from Atlantic salmon (*Salmo salar*) in a farm located in the area of Puerto Montt (41°46'S72°56'W), treated with EMB one month previously. Adult *Caligus* of both sexes were carefully removed from fish anaesthetized with benzocaine (10% in ethanol, 1ml/L), 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 cultivation and bioassays were carried out.

The sensitivity of the adult *Caligus* to EMB was evaluated using the bioassay methodology developed for the pyrethroid deltamethrin by Sevatdal and Horsberg (2003), and adapted for EMB in *C. rogercresseyi* (Bravo *et al.*, 2008). A stock solution was prepared by dissolving 5 mg of EMB (Sigma-Aldrich Corp., St. Louis, MO, USA) in 50 ml of methanol. A working solution was prepared by diluting 10 ml of the stock solution with 990 ml of filtered seawater at 31‰. Parasites were exposed to seven concentrations of EMB (0, 20, 40, 80, 120, 160 and 320 ppb), with its respective replicate. The solutions were prepared in Petri dishes using a protocol adapted in

Canada (Westcott *et al.*, 2008). In each dish (20 ml), ten adult *Caligus*, five of each sex, were placed. They were exposed for 24 hours at 12°C with a photoperiod of 12 h of light and 12 h of darkness in a chamber. The response of the lice to the varying EMB dilutions was evaluated at 24 hours after start of exposure.

The response criteria were the same proposed by Sevatdal & Horsberg (2003): 1) Dead: no movements in extremities, gut or other organs; 2) Moribund: not capable of attaching to a surface of the 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 data from the bioassays were analysed by probit analysis using the software POLO PC (LeOra Software Inc. Berkeley, California, USA). The concentrations immobilizing 50 % of the lice (EC_{50}) was calculated, and the 95 % confidence limits were estimated when possible.

The strain of *C. rogercresseyi* was inbred for seven generations. Egg strings at the point of hatch were carefully removed from females not used in the bioassay. The eggs strings were incubated in containers with filtered seawater at 12ºC with a photoperiod of 12 h light and 12 h of darkness. Once the copepodid stage had developed, approximately at 72 degree-days (°D), two rainbow trout (*Oncorhynchus mykiss*) weighing approximately 120 g and kept in tanks of 0.5 m^3 supplied with running seawater at ambient temperature and salinity, were infested with about 600 copepods per fish to produce subsequent generations. The adult stages were obtained at about 400°D. The temperature (°C) and salinity were recorded daily. When over 140 adult parasites of both sexes were available, the sensitivity of that generation was tested using the bioassay. The rest were used for production of the next generation of *C. rogercresseyi*. The Probit analysis comparing slope and intercepts of probit lines (Polo PC, LoOra Software Inc. Berkeley, Calefornia, USA) was used to compare the sensitivity to emamectin benzoate between sex and generations of *C. rogercresseyi*.

As it was impossible to collect *C. rogercresseyi* not exposed to EMB, the EC_{50} control used was the value obtained from the marine copepod *Lepeophtheirus mugiloidis* (34.2 ppt), collected from the wild marine fish *Elegipnos maclovinus* as also described in Bravo *et al.* (2008). Analyses carried out in an external laboratory revealed that the fish from which the parasites were collected did not contain residues of EMB. *L. mugiloidis* does not parasite salmonids fish and the EC_{50} value was similar to the EC_{50} values in sensitive sea lice reported for Canada (Westcott *et al.*, 2008) and Norway (Sevatdal, 2005). The resistance ratio was calculated according to Jones *et al.* (1992), using the EC_{50} values recorded for *L. mugiloidis* (34.2 ppb) as the control. The formula used was (EC_{50} *C. rogercressey* / EC_{50} *L. mugiloidis*).

Results

The sensitivity could only be evaluated for the generation number 0, 2, 3 and 7 as it was not possible to obtain a sufficient number of lice for bioassays from the other generations.

The study demonstrated EC_{50} values from 143.6 to 232.0 (Table 1). The values of EC_{50} recorded for the generations 0, 2 and 3

Table 1. Sensitivity values (EC_{50}) of inbred adult *C. rogercresseyi* towards emamectin benzoate trough the generations following collection of adult females from the field.

Generation	Days post collection	ºD	EC_{50} females	Upper 95%	Lower 95%	EC_{50} males	Upper 95%	Lower 95%	EC_{50} Pooled samples	Upper 95 %	Lower 95 %	Resistance ratio
G-0	site	0	169.0	330.7	112.7	156.1*			162.9	223.9	127.8	4.8
G-2	66	801	133.3*			152.2	266.3	126.5	143.6	209.9	116.1	4.2
G-3	95	1,204	139.6	321.4	85.3	172.7*			151.9	268.6	105.9	4.4
G-7	203	2,800	201.8	301.7	159.8	254.7	331.8	204	232.0	284.9	198.2	6.8

* Confidence intervals could not be calculated due to a suboptimal fit of data to the model

showed not significant differences between them ($p > 0.05$), showing differences with the highest value found for the 7th generation ($p < 0.05$). Trough the generations, males showed generally lower sensitivity to emamectin benzoate than females (Figure 1), but the difference was not statistically significant ($p > 0.05$). Due to a suboptimal fit of the data to the model it was not possible to calculate the 95 % confidence intervals for all groups of males and females (Table 1). The resistance ratio ranged between 4.2 in the second generation to 6.8 for the seventh generation.

Discussion

Results of EC_{50}-values obtained for the different generations (143.6 to 232 ppb), were similar to the values obtained through a survey carried out in the area of Puerto Montt between November 2006 and January 2007 (Bravo *et al.*, 2008). The EC_{50}-value obtained in the 7th generation was higher than the values recorded in the first generations which in some way could explain the persistent loss of sensitivity of *C. rogercressey* against the emamectin benzoate recorded in Chile.

The parasites used in the study were inbred over the seven generations without being exposed to a selection pressure of EMB. Thus, an increase in sensitivity towards EMB could not be expected if the reduced sensitivity was caused by one or more inherited mechanisms present in the majority of the original population. This was demonstrated as a probability, as the study demonstrated no increase in sensitivity against EMB over the generations. An increase in sensitivity might also have been expected if the resistance was linked to an up-regulation of physiological

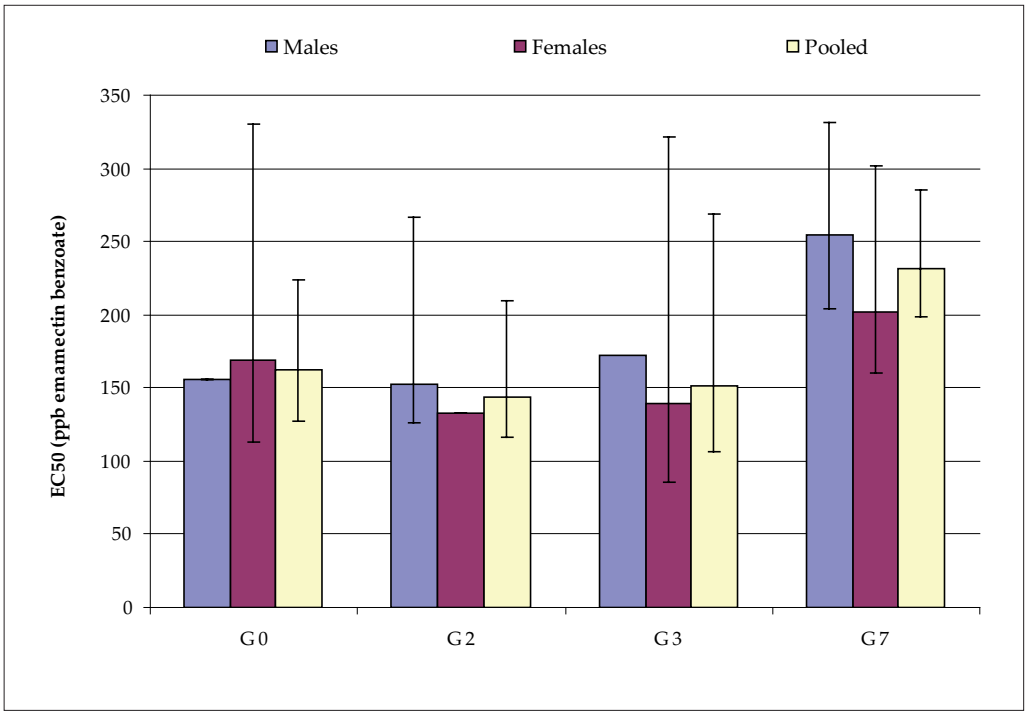

Figure 1. EC_{50} values obtained for each generation under study.

traits not needed in the absence of a selection pressure from EMB, e.g. an overproduction of EMB-metabolizing enzymes. The exact mechanisms must be demonstrated through separate studies.

A gradual loss of sensitivity is expected when several genes are involved, while single gene mutations frequently have an instant, major effect on the sensitivity (ffrench-Constant *et al.*, 2004). However, since no fully sensitive *C. rogercresseyi* parasites were available, the only indication of the magnitude of loss of sensitivity came from a study of a related louse, *L. mugiloidis* that does not parasitize salmonids. *E. maclovinus* is the naturals host for *L. mugiloidis* and also for *C. rogercresseyi* (Carvajal *et al.*, 1998; Bravo, 2003; Bravo *et al.*, 2008). The low sensitivity recorded in *C. rogercresseyi* towards EMB within the whole waterbody in the Region X, precluded the use of these lice as controls, even when collected from other fish species. This is due to the uniform reduction of sensitivity, attributed to the exclusive use of EMB to control sea lice in Chile for more than seven years (Bravo *et al.*, 2008).

In Scotland, it has been demonstrated that even when *L. salmonis* infestations were reduced after the application of emamectin benzoate, not all treatments were effective, with evidence of variation across geographical regions and a progressing reduction in efficacy during the period 2002-2006 (Lees *et al.*, 2008).

The sensitivity values recorded in Chile (EC_{50} of 103 - 203 ppb, Bravo *et al.* 2008) were considerably higher than the values reported for *L. salmonis* in Canada (25 - 118 ppb in the field, 21 ppb in the laboratory; Westcott *et al.*, 2008). Westcott *et al.* (2008) found a difference in sensitivity to EMB between males and females. We also found a small difference in sensitivity between sexes for *C. rogercresseyi*, where males showed somewhat lower sensitivity through the generations, however this difference was not statistically significant. This might be explained by similar size for both sexes of *C. rogercresseyi*, about 0.5 cm length, and similar weight (3.5 mg for females and 3.7 mg for males), in comparison with *L. salmonis* where the adult female has an average weight of approx. 25 mg, and the adult male approximately 5 mg.

The resistance ratio obtained in this study ranged between 4.2 and 6.8 similar to the values obtained in the survey carried out in the summer of 2007 (1.7- 5.9) (Bravo *et al.*, 2008). No continuous selections for even more resistant lice were made through this study, an approach that might have given an indication about the number of genes involved. The lice remained viable for the seven generations, but the study was not designed to demonstrate viability or breeding success in competition with fully sensitive lice. A fitness cost due to excessive production of metabolizing enzymes, over-expression of efflux pumps (Tribble *et al.*, 2007) or dysfunction of ion channels is possible, but must be determined in other studies.

In conclusion, reduced sensitivity against EMB was demonstrated to persist for at least seven generations of *C. rogercresseyi*. Thus, a reversal to normal sensitivity may not be expected unless the resistant populations are mixed with populations displaying normal sensitivity. Considering the rapid distribution of reduced sensitivity towards EMB in Chile, fully sensitive parasites are most likely uncommon in the Region X. Thus, a dilution effect is unlikely. These results should alert other salmon producing countries about the risk of a persistent resistance problem against EMB in other species of sea lice as well.

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