EFFECTIVENESS OF TEFLUBENZURON FOR TREATING SEA LICE ON ATLANTIC SALMON: RANDOMIZED CONTROL CLINICAL TRIAL, HISTORICAL CONTROL CLINICAL TRIAL, AND AN ASSESSMENT OF OUTCOME MEASUREMENTS AND SAMPLING METHODS

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Patrick Jon Campbell

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University of Prince Edward Island

Charlottetown, PEI

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ABSTRACT

Sea lice, an external parasite of fish in sea water, are widely considered to be the most important parasitic disease affecting Atlantic salmon in sea cage sites. In New Brunwick's Bay of Fundy it has been estimated that the lice caused about $20 million in damage in 1995 due to salmon mortalities and reduction in the quality of harvested fish.

A double-blind, randomized clinical trial was performed to investigate the effectiveness of teflubenzuron to control sea lice (*Lepeophtheirus salmonis*) on cage reared Atlantic salmon (*Salmo salar*). The teflubenzuron was administered in the feed at a dosage of 10 mg/kg biomass/day for seven days. A total of forty sea cages from three commercial cage sites in Atlantic Canada were used in this Good Clinical Practice (GCP) trial. Cages were matched by site, cage size, and mean pre-treatment lice counts. One cage from each matched pair was randomly allocated into the medicated group, and one cage to the control group. During the feeding and post-treatment sampling, site investigators and site workers were blind to the treatment allocation of each cage. Post-treatment lice counts were performed at one and two weeks after the end of treatment. Chalimus stages in medicated cages were significantly lower than in control cages at one week(79% reduction, $p<0.001$), and at two weeks (53% reduction, $p<0.001$). Mobile (pre-adult and adult) stages were also significantly reduced in medicated cages at one week(69% reduction, $p<0.01$), and at two weeks (40% reduction, $p<0.01$). These reductions may underestimate the full potential of teflubenzuron, due to the low parasite levels experienced during the summer of 1996, and due to recruitment from the untreated cages. A historical control clinical trial was also performed at one site using teflubenzuron administered in the feed at a dosage of 10 mg/kg biomass/day for seven days. This study involved the treatment of all nine cages on site. Six of the cages were pre-market size salmon and three cages contained smolt. At one week after the end of treatment chalimus stages were reduced by 92% ($p<0.05$) and mobile stages were reduced by 74% ($p<0.05$) from pre-treatment levels. Two weeks after the end of treatment chalimus stages were reduced by 41% ($p<0.05$) and mobiles were reduced by 61% from pre-treatment levels. Three weeks after the end of treatment chalimus stages were still reduced 36% ($p<0.05$) from pre-treatment levels, but mobile stages had increased by this time.

During and after these studies the opportunity was taken to assess some of the outcome measurements and sampling methods used. A comparison between feed-and-dip sampling and crowd-and-dip sampling showed no systematic difference between the two methods for lice counts or weights. An anesthetic bath was shown to significantly reduce the number of mobile lice by about 13% (paired t-test, $p<0.01$) on sampled fish. There was a high level of agreement between two different lice counters, although a small but statistically significant difference was observed in larval lice counts. Lice counts on fish which were lethally sampled were found to be consistently lower than the ante-mortem counts.

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LIST OF ABBREVIATIONS USED IN THE TEXT

cm	centimeter
CF	condition factor
°C	degrees Celsius
1st	first
g	gram
GCP	Good Clinical Practice
HDPE	high density polyethylene
kg	kilogram
l	liter
lng	length
m	meter
mg	milligram
mg/kg	milligrams per kilogram
mg/l	milligrams per liter
mm	millimeter
mo	month
n	number of observations
#	number
NADA	New Animal Drug Application
NBDFA	New Brunswick Department of Fisheries and Aquaculture
p	probability of type I error
ppb	parts per billion
QA	quality assurance
r	correlation coefficient
SCUBA	self contained underwater breathing apparatus
2nd	second
sd	standard deviation
SEM	standard error of the mean
SOP	standard operating procedure
3rd	third
TMS	tricane methanosulfanate
wt	weight
YC	year class

1.0 INTRODUCTION

1.1 Atlantic salmon production cycle

Salmonids have been cultured in fresh water for many years. However, the growing of salmonids in sea cages is relatively new. Commercial farming of Atlantic salmon began during the 1960's when marine enclosures were first used in Norway. This soon led the way for a rapidly growing Atlantic salmon aquaculture industry in Norway in the 1970's (1).

Atlantic salmon have been raised commercially in the Bay of Fundy since 1979. The total harvest that first year, from a single farm, was about 6 metric tonnes and worth about $40,000. By the 1990's it had grown to become a major industry in the area. In 1997, there were 76 sites which produced over 18,000 metric tonnes of fish valued at almost $140 million (2). Secondary economic benefits to the local economy from associated industries such as smolt production, value added products (smoked salmon), fish-health services, manufacture and sale of fish feed and sea cages, nets, etc., likely account for more than double this value (1).

In Atlantic Canada, it takes a salmon 32 to 44 mo to grow from an egg to market weight (approximately 3.5 - 4.5 kg). The production cycle mimics and enhances the natural life cycle of salmon. The main differences are in the egg incubation and fry stages where water is maintained at a higher temperature than the fish would experience in nature. This is done to increase the rate of

growth, and therefore compress the amount of time between egg and smolt stages (1) and to produce larger smolts. The main steps in Atlantic salmon culture are: 1) collection and fertilization of eggs, 2) incubation and hatching (which takes approximately 2 mo), 3) grow out to produce 75 - 150 g smolt which are transferred to sea cages, and 4) grow out to harvest (3). Smolts are produced in freshwater over a 16 mo period, while growth from smolt to harvest (or to broodstock) occurs in salt water over 15 - 27 mo, depending upon harvest strategies.

1.1.1 Freshwater phase

Salmon are spawned in the fall, usually October - December in Atlantic Canada. The broodstock are generally four year old fish which have been kept from harvest solely for this purpose. Artificial spawning is accomplished by stripping the eggs using pressure on the abdomen of anaesthetised female fish and milt from an un-anaesthetised male fish(1). Female salmon (i.e. hens) are considered "ripe" if the eggs are easily stripped. Eggs and milt are collected at the sea cage site, then transported to the hatchery for fertilization and incubation. The eggs and milt are mixed together to allow for fertilization, then put into freshwater. Water enters the fertilized eggs and hardens them, making them less vulnerable to damage while at the same time preventing the entry of additional sperm (1). Once the eggs are "water-hardened" they are placed in shallow trays for incubation.

The fertilized eggs are incubated for about two months (450 - 500 degree

days) before hatching, depending on water temperature (4). In nature, at temperatures close to 0°C for much of the winter, the eggs may hatch in March to May. In a production hatchery, eggs are usually incubated at temperatures up to 8 to 10°C which results in hatching in December or January (1).

The newly hatched sac-fry, or alevins, use nourishment from the yolk sac for about two months. As the yolk sac is depleted and internalized, fry swim to the surface (swim-up), and fill their swim bladders by gulping air at the surface. After achieving buoyancy, they begin to feed. The juvenile fish are referred to as "fry" during their first summer, then "parr" until they are large enough and physiologically adapted to go to sea, when they are called "smolts" (1). This is usually achieved at about 18 mo (designated S1 smolts) after the eggs were originally collected. Fish that are transferred from the hatchery at 12, 24, and 30 mo post egg collection are referred to as S0 or S0 ½ , S1 ½, and S2 smolts respectively.

1.1.2 Salt water rearing

The ultimate goal of the salt water phase is to feed the fish until they are large enough to harvest. Obviously, management of the fish involves much more than just feeding. Other management considerations include inventory determinations, grading, removal of dead and moribund fish, disease monitoring and treatments, harvesting, and equipment maintenance.

Terminology

In New Brunswick, S1 and S2 smolts are transferred to sea cages from mid-April to June , S0 and S1½ smolts (i.e. "fall smolts") in November. Ideally the transfer is done when the temperature of the freshwater in which the smolts have been raised is close to the temperature of the sea water. Once in sea cages, they require at least 15 mo to reach market size (3.5 - 4.5 kg). Once at sea, the fish are usually designated by their "year class" or YC. For example: fish transferred to sea cages in May of 1998 are called the 1998 year class, May of 1999 are the 1999 YC. Fish that are transferred in the fall of 1998 would usually be called "fall 1998 YC". In other parts of the world, the industry often refers to the year class as the year of hatching. A 1998 YC in seawater in New Brunswick, transferred as an S1, would be called a 1997 YC if using hatch year designation. Fish that are in their first summer in sea cages are often called "post-smolts" or sometimes simply "smolts". After they have been at sea for one year they are typically called "pre-market" fish.

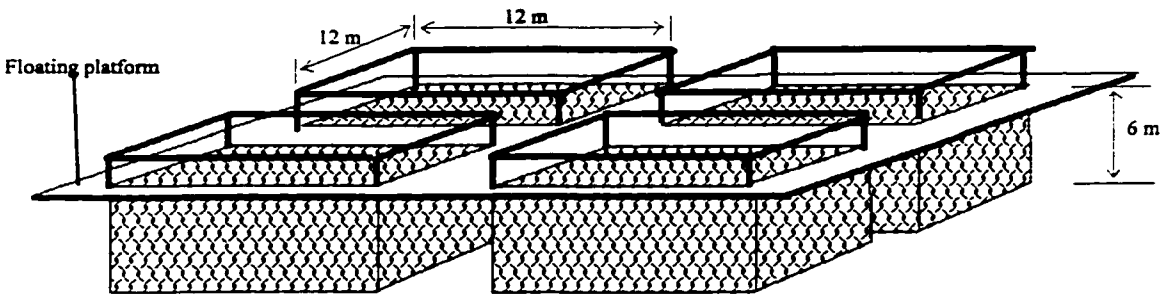
Sea cages

Sea cages, alternatively called "net pens", are simply net enclosures which confine the fish. The mesh size of the net is determined by the fish size. It should be small enough to keep all of the fish in, but large enough to allow a maximum of water exchange (1). The net is supported by a rigid, or semi-rigid floating frame structure. There is also usually a stronger, larger-mesh net

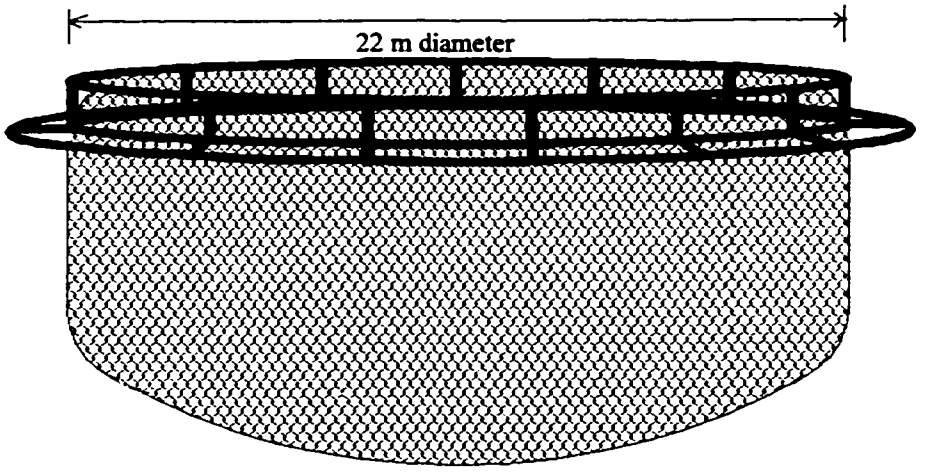
surrounding the first net and separated from it by 0.5 to 2 m to deter predators (mainly seals in eastern Canada) (1).

There are many sizes and styles of sea cage frames (see Figure 1). The most popular cages consist of square steel or high density polyethylene (HDPE) frame platforms which usually consist of several cages in a large raft. These nets are usually 12 by 12 m or 15 by 15 m and about 6 m deep. Another common style is the round "polar circle" which is constructed of HDPE tubes. They are generally 70 m in circumference, 22 metres in diameter and 6-8 metres deep, although other sizes (50, 60, and 100 m) are occasionally used. Depth of net is determined by many factors, including bottom topography, number of fish, etc., and other areas may use deeper nets. The steel cages have the advantage of a walkway around each cage. The polar circles have the advantage of larger water volume with capacity for greater numbers of fish and a corresponding advantage of "economy of scale". However they are more difficult to manipulate or to access fish for sampling.

The most common maintenance performed on the cages is periodic cleaning to remove fouling organisms from nets, the presence of which restricts water flow through the cage. This cleaning can be done in place with a pressure washer (either underwater or by pulling part of the net out of the water) since the majority of fouling occurs in the top two meters. Alternatively the net can be exchanged for a clean one, and the fouled net is cleaned elsewhere which prevents fouled water exposure to the fish.



Raft of square cages



Polar circle

Figure 1. Schematic drawings of two popular styles of sea cages. Above is a representation of a raft of square cages. Below is a polar circle. In both styles the net extends up to the hand rail. Predator nets are not shown. Tidal currents can create distortions in the hanging structure of the nets.

Management practices

The feed used for salmon in sea cages is usually a commercially prepared dry pellet. However, in Atlantic Canada many farmers also use moist feed, which appears more palatable, particularly for recently transferred fish (1). Feeding is performed by hand or with mechanical feeders. The goal with either method is to disperse the feed over as much of the surface of the cage as possible so that as many fish as possible will have access to it. Feedings are usually done twice a day, or sometimes three times per day for younger fish. Most sites feed fish to satiation at each feeding. However, some farms use mechanical feeders which feed a predetermined amount at each feeding, an amount calculated by multiplying the biomass by the recommended feeding rate at the current water temperature.

Weight samples are commonly performed as a way to monitor growth and to plan harvests. They are also essential for determining cage biomass necessary for medication dosage calculations. Either the crowd-and-dip or the feed-and-dip method is usually employed for these samples. The number of fish sampled for weighing is determined by the farmer's need for precision and logistical constraints. Twenty to 50 fish are common sample sizes.

Determination of the number of fish in a sea cage often poses a significant challenge. Most farms know how many fish were put into each cage because the smolt were individually counted (by personnel or electronically) at the hatchery at the time of transfer. Farm records of the number of mortalities collected during mort dives are then used to calculate the total population size. The farm can

verify the number of fish in a cage only during grading and at harvest. Inaccurate records are not detected until harvest in many instances.

Grading (or size sorting) is done to a limited degree by many seawater grow-out farms. The purpose of grading is to reduce the density of fish in a cage, to make fish size more uniform, or to selectively harvest the largest fish in a cage. Smolt will commonly be stocked in a cage at approximately twice the desired number of market sized fish. The population of fish in cages stocked in this manner will be "split" approximately half way through the growing cycle, as extra cages become available. Some farms may divide by size of fish (the larger 50% of the fish into one cage and the smaller 50% into another cage), other farms divide only by number of fish (50% into each of two cages). Grading performed at harvest can be accomplished by selectively dipping out the largest fish, by using the crowd-and-dip method, and leaving the smaller ones. Alternatively, if fish are brought up to a harvest vessel or platform, upon visual inspection the smaller fish can be directed to another cage, and the larger ones directed to the harvest line.

Health monitoring practices

The collection and removal of mortalities is usually performed once per week during times of low mortality rates, but can increase to daily during periods of suspected problems. It is accomplished by a diver (using SCUBA) who retrieves the dead and moribund fish from the bottom of each cage. Moribund fish which are seen swimming near the surface and sufficiently slow to be caught are usually removed with a dip net. Elevated mortality rates detected on the "mort

dive" are used as a primary indicator of health problems in a cage (5).

Disease investigations are usually initiated because of an increase in mortalities, or because of a decrease in feed consumption (5). In these cases, moribund and freshly dead fish are collected, examined, and sampled for further diagnostic testing. Moribund fish caught at the surface are often selected because they are the fish which are most likely to demonstrate the presence of a disease or pathogen, and because they are easy to catch. However, surface-catchable fish do not necessarily reveal the true disease pattern in a cage (6). The monitoring of changes in the prevalence of a pathogen in apparently healthy populations is less frequently employed due to the high cost and perceived lack of need. The only exception may be in research situations or possibly lice monitoring.

Sampling for the purpose of monitoring sea lice populations is routinely performed by dip-netting fish out of the cage, anaesthetising them, and counting the lice. The fish are generally captured by luring them within reach with feed, then dipping them out. This practice is called feed-and-dip sampling and is done because it is the most convenient way to collect small numbers of fish. Five to ten fish are sampled per cage when this method is used routinely. Alternatively, the fish can be crowded to one side of the cage by either pulling up the net, or by using a seine net to capture a large proportion of the fish in a cage. The fish are then dipped out of the crowded area. This is referred to as crowd-and-dip sampling (7). As this sampling procedure is more amenable to larger sample sizes, twenty fish are commonly sampled for lice counts.

For production fish, disease treatments are virtually always performed at the cage level. The therapeutant can be administered orally, such as antibiotics or in-feed sea lice treatments, by mixing the drug of choice into the feed at the mill. The other treatment option commonly used is a medicated bath, which is used for some sea lice treatments. Injectable vaccines (or medications) are administered prior to smolts being transferred to seawater and are rarely used once the fish are in salt water.

1.2 Sea Lice

"Sea lice" is a generic term for external copepodid crustacean parasites of fish in sea water and are widely considered as the most important parasitic disease affecting Atlantic salmon in sea cage sites (8, 9). They belong to the Phylum Crustacea, the class Copepoda (10), order Caligoida (11), suborder Siphonostomatoida, and the family Caligidae (12).

There are two species of lice which are commonly found on salmon in the Bay of Fundy, *Lepeophtheirus salmonis* (this species is often called the salmon louse) and *Caligus elongatus* (13). *Caligus elongatus* can be found on over 80 different species of fish (8). Although *C. elongatus* is quite prevalent, infection intensities have remained relatively low in the Bay of Fundy (13) and have rarely caused sufficient impact to warrant control measures. However, *C. elongatus* infestations have caused significant impacts on salmon in Scotland and Sweden (14). In contrast, *L. salmonis* is found mainly on salmonids (8) and has caused

considerable damage at salmon farms in the Bay of Fundy, as in other areas of the world.

Norwegian salmon farms, in the 1960s, were the first to experience disease outbreaks due to sea lice , followed by Scottish farms in the mid-1970s (8). Both *C. elongatus* and *L. salmonis* have been observed on cultured salmon in the Bay of Fundy at least since 1987, with infestation levels and prevalence apparently remaining consistently low up to 1994 (13). Sea lice first became a major problem in the Bay of Fundy in the fall of 1994 when *L. salmonis* populations increased to levels which caused considerable damage. During this time, infestation levels on untreated market-size fish increased from an average of 2 - 3 lice per fish in August to 117 lice per fish by late October (based on an average of six sites) (13). The majority of cage sites had salmon with infection levels sufficient to cause damage to fish and result in large scale downgrades of product for marketing and, in some cases, cause mortalities (13). It has been estimated that lice caused about $20 million in lost revenue due to damage in 1995 (15). Since the initial losses in 1994, the ability to manage lice burdens has improved with better access to treatments and better general health management. However, sea lice management continues to require attention and refinement.

1.2.1 Life cycle and biology

The life cycles of *L. salmonis* and *C. elongatus* are very similar (14). However, as *L. salmonis* is the primary parasite in the Bay of Fundy which causes production losses, it will be the focus of discussion. *L. salmonis* has ten stages in its life cycle (see Figure 2): two free-swimming nauplius stages, one free-swimming infective copepodid stage, four attached chalimus stages, two pre-adult stages, and an adult stage (16). As with other crustaceans, they molt during growth and shed their exoskeleton between each molting stage.

Upon hatching, there are two free-swimming nauplii stages (nauplius I and II) each about 0.5 mm in length (17). The next stage is the free-swimming copepodid stage which is about 0.77 mm in length (17). It seeks out and settles on the host fish. Once settled, the copepodid produces and attaches a "frontal filament" which anchors the larva to the host until the first pre-adult stage (9). The parasite then develops through four larval stages, called chalimus I (average length 1.2 mm) through chalimus IV (average length 2.8 mm) (16). Sexual differences, in shape and size, are evident by the fourth chalimus stage (18). The fourth chalimus stage is the earliest at which *Lepeophtheirus* species can be grossly distinguished from *Caligus* species (16).

Upon molting to the first pre-adult stage, the lice are no longer attached to the fish, but are mobile and move freely over the surface of the fish except during molting at which time a temporary frontal filament attaches the lice to the fish in the post-chalimus stages (19). For obvious reasons, pre-adult and adult lice are

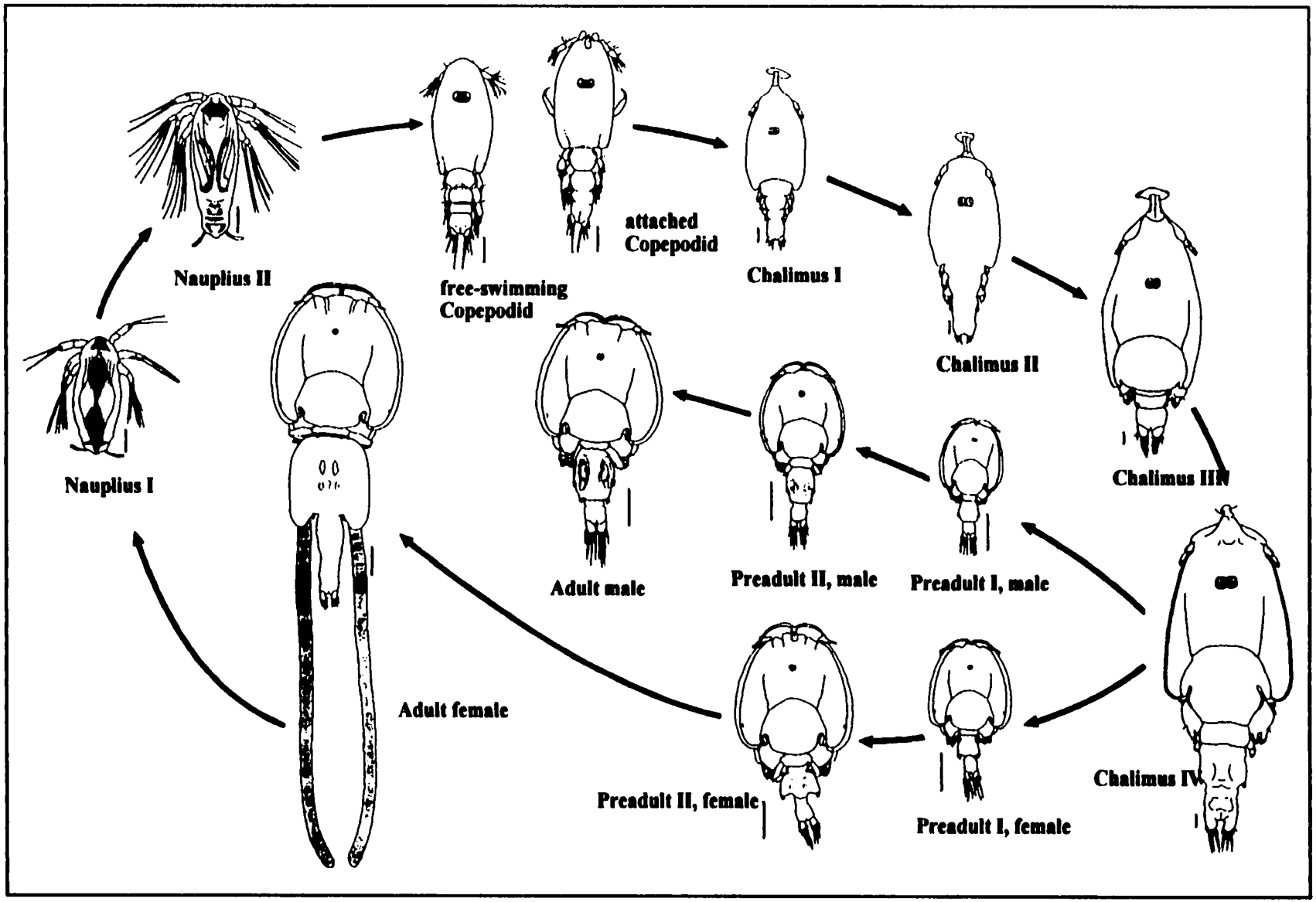


Figure 2. Life cycle of *Lepeophtheirus salmonis*. Scale bars: nauplius - chalimus = 0.1 mm, preadult - adult = 1 mm. (18)

often called "mobile" stages. Pre-adult females are larger than males in both the first (average lengths of 3.7 mm versus 2.9 mm) and second pre-adult stages (5.4 mm versus 4.27 mm) (16). The second pre-adult stage is followed by a single adult stage (average female length 9.96 mm, male 5.40 mm) (16).

Mating in *L. salmonis* begins with the formation of a precopulatory pair, in which a male mounts a suitable female and remains attached to her. Precopula usually occurs between adult males and pre-adult II females and lasts for about 4 days, after which time the female molts. Copulation occurs about a day after the molt. Egg production begins nine days later (19). Adult females with egg strings are called gravid females, or gravids. Hogans and Trudeau (20) report an average of almost 200 eggs per female, whereas Jackson and Minchin (21) report finding over 700 eggs per female on farmed salmon.

The rate of development, generation time, and reproductive output are all temperature dependent (22). Wootten et al (14) observed a generation time of about 6 weeks at 9 - 12 ^{0}C. Johnson and Albright (23) estimated a generation time of 7.5 - 8 weeks at 10^{0} C. The approximate time spent at each stage at 10^{0} C is: egg and nauplii stages - 1.5 weeks, larval stages (copepodid thru chalimus 4) - 3.5 weeks, pre-adult stages - 2 to 3 weeks (with males reaching maturity earlier than females) (23). Development of the free-swimming stages seems to be rarely successful below 3 ^{0}C (13) and infestation intensities (number of lice per fish) tend to increase with increasing temperatures due to greater reproductive and development rates (13,24).

1.2.2 Pathology

The primary damage caused by sea lice results from the feeding activity of pre-adults and adults on the skin of the fish (14). The larval stages will feed on the host's mucus and epithelium and cause localized areas of damage with limited impact (8). However, the mobile stages will feed on the host's mucus, epithelium, subcutaneous tissue, and blood and are able to cause serious damage to the fish. As few as five adult lice have been known to cause significant pathology on newly introduced smolts (14). The mobile lice seem to concentrate their feeding activity, and subsequent damage, to the dorsal surface of the head, between the dorsal and adipose fins, and ventrally caudal to the anal fin (25). Infested salmon initially show grey patches on the back and head where sea lice have been feeding (14). Fish with this discoloration have been called "white-heads". More severe damage can cause sub-epidermal hemorrhages (14), or even gross bleeding, resulting in "red-heads". Damage from sea lice feeding can get so severe that the skull can become exposed (13, 14). Loss of the protective epithelial layers can put the host under severe osmotic stress as well as opening a portal of entry for pathogens and secondary infections. Mortalities probably result from osmoregulatory failure (14).

Sea lice have also been implicated as possible vectors of such infectious agents as Infectious Salmon Anemia virus (26) and *Aeromonas salmonicida* (27). They have also been associated with outbreaks of vibriosis at some Scottish sea cage sites, although it was not clear whether the lice were attacking fish debilitated with the disease, or whether the parasites caused damage allowing a

secondary *Vibrio* infection (14).

Economic costs of sea lice infestations go well beyond fish mortalities. Other costs include: the costs of prevention and treatment (28), lost growth, increased feed conversion ratio, secondary infections, and morphological damage resulting in down grades at market (29). Feed conversions are less than optimal so it takes longer to grow a fish to the same size (increased labor costs) and it takes more feed (increased feed costs). Fish may be harvested at a smaller size than they would usually, which results in not only less product to sell, but also marketing fish in a lower priced size range.

1.2.3 Treatment

There are several treatments available for controlling sea lice on salmon. Each has advantages and disadvantages which may apply to different farming or production situations.

Tarpaulin enclosures

Bath treatments are administered to fish by immersing the fish in a treatment solution for a specified period of time. This is usually accomplished in sea cage situations by putting a tarpaulin around and under the cage to prevent the treatment chemical from dispersing into the surrounding water during the treatment. To minimize the amount of chemical needed to treat the cage, the cage volume is reduced as much as possible by pulling up the net and sometimes

employing a cork line.

The water volume within the enclosed area is optimally reduced to minimize the amount of chemical used while subjecting the fish to as little handling stress as possible. Oxygen is usually added to the cage via diffusers (i.e., air stones) during the treatment and dissolved oxygen monitoring is advisable. The oxygen diffusers also function to mix the treatment solution. After the required treatment duration, the tarpaulin is released. If the fish show signs of distress due to low oxygen or treatment toxicity, the treatment is discontinued immediately by releasing the tarpaulin. Upon release the treatment solution is allowed to disperse in the surrounding water. This presents environmental concerns for areas surrounding treated cages.

The possibility of chemical exposure to humans during bath treatments poses a risk for the people administering the treatments (30). Extreme care must be taken in handling these chemicals. Specialized training and precautions including face masks, respirators, and splash resistant clothing are advisable, particularly for the more dangerous chemicals.

Bath treatments are also relatively equipment- and labour-intensive. Specialized equipment includes oxygen cylinders and diffusers, oxygen monitors, chemical handling equipment, protective clothing and gear, a tarpaulin and boom and winch to lift it (particularly for the very large tarpaulins required for 70m circular cages). It takes several workers to get the tarpaulin in place and the cage volume reduced, then mix and apply the chemical, and monitor oxygen. It may require up to 5 person-hours to treat a single cage.

Pesticides for bath treatments

Various organophosphates have been the most commonly used bath treatments over the past 30 years in Europe. All are cholinesterase inhibitors and act by interfering with neuromuscular transmission. Cholinesterase inhibition is not confined to the parasite, the host and the applicators can also be affected. Salmon are only able to tolerate 4 times the treatment concentration of dichlorvos, and 5 times the azamethiphos target treatment concentration. Only mobile stages of lice are susceptible to organophosphates. Resistance to organophosphates has been observed in lice in Europe (31).

Azamethiphos[1] is the organophosphate compound approved for use in Canada. Reports of effectiveness range from 85 - 98.3% removal of mobile stages in susceptible lice populations (32, 33). In New Brunswick it costs about $600 for enough azamethiphos to treat a 70 m diameter circular cage of fish. This is in addition to labour and equipment costs.

Pyrethrins and pyrethroids (synthetic compounds similar in chemical structure to pyrethrin) are also used as bath treatments. Cypermethrin is a pyrethroid which is effective against both chalimus and mobile stages of lice (34). Its acts by causing paralysis through maintaining sodium channels in an open state (35). Although used in the U.S.A. under an Investigational New Animal Drug permit, cypermethrin formulations are not available for sea lice control in Canada. Pyrethrin was used briefly in trials but variable success has resulted in

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[1]Salmosan, Ciba-Geigy Canada, Mississauga, Ontario

New Brunswick salmon farmers abandoning its use.

The first bath treatments used in New Brunswick were in December of 1994, after permission was granted for emergency treatments using hydrogen peroxide under the trade name Salartect (36). It is effective against mobile stages by causing them to fall off of the fish, possibly due to the formation of oxygen emboli within the lice (37). It is considered environmentally friendly because the compound degrades to hydrogen and oxygen (37). However, the huge volumes required to treat cages, the expense (about $500/cage treatment), and fish mortalities experienced when treating at higher temperatures (>13 ^{0}C) (37) has reduced its role in lice management.

Oral Treatments

Oral sea lice treatments are mixed with the feed and fed to the fish as a medicated feed at a prescribed dosage and length of time. They have the advantage of no additional labor or equipment costs to treat the fish and are safer for workers to administer. Also, since the medication is taken internally by the fish, it is more efficiently targeted to the lice, rather than disbursed into the water column (i.e. the dosage is based on the fish biomass, rather than the total volume of water in which the fish are held during a bath treatment). However, the excreted drug and drug metabolites could be concentrated in the sediment under the cages, rather than being washed away with the water. Currently there are two classes of oral treatments being used: avermectins (ivermectin and emamectin) and chitin inhibitors (diflubenzuron and teflubenzuron).

Ivermectin is effective against all stages of lice found on the fish (38). However, it is toxic to salmon even if administered at doses less than double the therapeutic dose, or even at low doses administered too frequently (38). This toxicity appears to be because of a poorly developed blood-brain barrier in salmon (39). It is subject to long withdrawal periods (1000 degree days or 180 days) due to slow clearance and the fact that the manufacturer does not support its use. Ivermectin is not licensed for use in fish in any country (40, 41). In Canada it can only be used as an "extra-label" product under veterinary prescription. Consequently, the withdrawal period is a conservative estimate of required time for residue depletion at water temperatures commonly experienced in the Bay of Fundy. The long withdrawal period restricts its use past the first summer in sea water after smolt transfer. However, it is inexpensive to use (about $150 per treatment), in part due to the lack of registration costs.

There are also concerns with the effects of ivermectin on the biota of sediments under and near cage sites. Since ivermectin is primarily excreted unmetabolized in the feces (39) and appears to bind tightly to sediments, there is the potential for it to accumulate in sediments near fish farms (42). Also, Davies et al. (43) suggests a half-life of greater than 100 days in marine sediments. It has been demonstrated that ivermectin can adversely affect invertebrate species which may comprise the benthic community (44).

Emamectin has been used experimentally. Like ivermectin it appears to be effective against all developmental stages present on fish (45, 46 - unpublished observations). It appears to be less toxic to some fish and non-target species

(45). Information on environmental persistence or withdrawal periods is not available.

Chitin-inhibitors are in the process of gaining regulatory approval and have been used experimentally. Diflubenzuron (47) and teflubenzuron (48) kill lice by interfering with the synthesis of the chitinous exoskeleton. After exposure to these compounds, lice have defective exoskeleton formation and will die following a molt. Consequently, these products are effective against all stages of lice which molt (chalimus and pre-adult). The louse eggs may also be damaged (49).

They affect chitin synthesis, and therefore are virtually non-toxic to vertebrates (42). Lufenuron (a chitin synthesis inhibitor) has been on the market as an oral treatment for fleas on dogs and cats for several years (50).

The amount of drug required is dependent on the weight of the fish, therefore the cost per treatment increases as the size of fish increases. Teflubenzuron is expected to cost about $100 per treatment in a cage of smolt, and up to $2000 for a cage of pre-market size fish. Because of the short half-life in salmon, withdrawal periods are expected to be short (47).

1.3 Conclusions

Sea lice present an important challenge to salmon farmers. The treatments which are currently being used have limitations and concerns regarding their safety and environmental impact. New treatments are required to better manage sea lice. Regulatory approval for new products depends on rigorous trials to demonstrate effectiveness and safety under farm production

conditions.

Studies presented in this thesis tested the effectiveness of teflubenzuron for controlling sea lice (*Lepeophtheirus salmonis*) on cage reared Atlantic salmon (*Salmo salar*). Effectiveness was assessed through a randomized control clinical trial, and an historical control clinical trial. Pharmacokinetic properties of teflubenzuron were not available and were not discussed.

During and after these trials the opportunity was taken to assess the following outcome measurements and sampling methods:

-Feed-and-dip and crowd-and-dip sampling methods were compared to test if there was a relative selection bias between them for both weights and lice counts.

-Lethal sampling and anesthetic use were both tested to see if either introduced a systematic bias in lice count data.

-The ability to obtain repeatable lice count data with different lice counters was tested by comparing lice counts from two counters.

-Utilizing the clinical trial data sets, sample size calculations were performed to demonstrate sample size requirements for obtaining weight and lice count data at various precision levels.

2.0 CLINICAL TRIALS IN AQUACULTURE

2.1 Introduction

Clinical trials are controlled, randomized, prospective experiments which compare the effectiveness of treatments, or prophylactic procedures, and are performed in a clinical setting. They are essential for assessing the value of treatments under real production conditions. The dynamics of the host, pathogen, and environment can produce both subtle and dramatic differences when comparing laboratory and field studies (51). In most instances, the well designed and conducted clinical trial will provide the most valid and useful information about treatment effectiveness under clinical or field conditions (52, 53).

Veterinarians need clinical trials to ensure that they are able to give sound advice and guidance to producers when selecting effective products (54). Regulatory agencies require evidence of safety and effectiveness before they will grant approval (55, 56), and clinical trials are the best way to provide this information.

When generating clinical trial data that are intended to be submitted to regulatory authorities, Good Clinical Practice (GCP) should be followed (57). GCP is "a standard for the design, conduct, performance, monitoring, auditing, recording, analyses, and reporting of clinical trials that provides assurance that the data and reported results are credible and accurate" (57). GCP is essentially

a set of accounting and record keeping principles for documentation of what is done during a trial, and ensures that it is done in such a way that studies can be reconstructed at any time afterwards. GCP was developed to address concerns about the safety of the products being tested. It was first applied to clinical trials of products intended for human use, and more recently, to trials of products intended for animal use.

Compared to human medicine, there are relatively few examples in the literature of veterinary clinical trials that have been carried out to GCP standards (58). There are several apparent reasons for the lack of published reports. GCP trials tend to be cumbersome, expensive, and labour intensive to conduct (53), and they are relatively new to veterinary medicine, and particularly new to aquaculture. Also, the primary purpose of trials that have been conducted to GCP standards has been to satisfy regulatory requirements for product approval applications, and therefore sponsoring companies simply less incentive to take the additional step of publishing the results.

GCP clinical trials will likely become an increasingly important component of clinical research because of demands by government regulatory agencies for validation of studies of product safety and effectiveness. The costs associated with the entire chemotherapeutant development and approval process can exceed $250 million from discovery to approval (59). Sponsors need to ensure that studies conducted in support of a registration application are: 1) performed in such a way that regulatory agencies will have assurance that trial data are credible, and 2) done correctly the first time to avoid wasting time and resources.

This chapter will outline considerations unique to aquaculture clinical trials. GCP requirements will also be discussed with particular emphasis to practical implications in aquaculture.

2.2 General principles of clinical trial design

There are three important components of any clinical trial: choosing an outcome measure, preventing bias, and establishing the role of chance (60).

One of the first considerations in designing a clinical trial is to clearly state the primary focus and what outcome will be measured to address that question. One or two measures of outcome which are clinically important and subject to objective measurement should be chosen (60).

A bias is any factor other than the treatment which "causes" a difference between groups (60). Biases can be introduced in the selection of study subjects, in the outcome measurements, or by confounding factors (61). Significant biases can lead to inaccurate or even false conclusions as to treatment effectiveness. Matching, blinding and randomization are three methods which should be used whenever possible to minimize the effects of bias.

The role of chance can not be eliminated in any study. However, one can determine the probability of making conclusions based on chance (60). Type I errors (incorrectly concluding that a product does work) and type II errors (incorrectly concluding that a product does not work) both need to be avoided. One way to avoid these is by utilizing sample size and power calculations prior to conducting a trial.

2.3 Considerations and challenges unique to aquaculture

Clinical trials are fraught with difficulties regardless of the species or therapy of interest. Nevertheless, the results of clinical trials are often critical in determining how a product will work in the production settings in which they will be used. Whereas laboratory trials are conducted on a much smaller scale than field trials and allow for greater control by the investigator, aquaculture presents unique challenges in the design and conduct of valid clinical trials.

Many of the challenges faced while conducting clinical research in aquaculture are attributable to the nature of the production systems: ie. large numbers of animals live in water where they are not readily visible or accessible. Direct observation or clinical evaluation of fish which are in an aquatic environment is difficult, and the large numbers of fish makes examination of all individuals impractical. The tagging of individuals is prohibitively expensive, time consuming, stressful to the fish, and therefore inefficient, particularly when the objective is to determine treatment differences applied at the group (cage, pen, tank, or pond) level (62). Both the need for capture, and the large numbers of individual animals, necessitates the need for a formalized selection and sampling process to minimize bias.

Examination of individual fish requires capture, removal of the animal from water, and anesthesia. This introduces time as an important consideration because the assessment requires a minimum amount of examination time. Since it takes up to ten minutes for the fish to become anaesthetised, staggering groups for anesthetic induction is necessary. Data collection under such conditions often

requires a coordinated "assembly line" approach with one person capturing the fish, one anesthetising them and monitoring fish in the anesthetic bath, one or more making observations and one person recording the data. There may also be others who are crowding fish in the next cage while one cage is being examined.

In addition, fish are maintained in a containment system, usually either tanks or net pens. Fish within a tank or pen share a common environment and feeding regimen so group effects must be addressed in the trial design. The experimental unit is usually at the group level. Group effects must be considered when allocating and administering treatments, sampling, and assessing statistics.

Some aquaculture production facilities are land based and easily accessed. However, most salmon grow-out sites are located in seawater, and many are located in remote areas. The potential impact of delays in visiting the study site due to equipment failures or inclement weather must be addressed prior to the start of a study.

2.3.1 Challenges due to the production setting

Performing research in a production setting presents many challenges that can threaten the success of the trial. The success of a trial depends on the commitment of everyone involved, which obviously includes the fish farmer. To justify participating fully, the farmer must believe that the success of the trial is in the best interest of the business. Communication between researchers and farm management is very important. Farmers should be fully aware of any risks

involved, especially if there will be groups of his fish that will not receive treatment. Details regarding what happens and who is responsible for losses should be established before the trial gets underway. Early stopping rules such as withdrawal from the study due to unexpected disease or harvest require full discussion and agreement before the study starts.

The owner of the farm is not the only person who needs to be familiar with the trial protocol. The importance of the auxiliary personnel who work with the fish is often underestimated (51). Most farms will usually have a site manager and several site workers who will actually be the individuals participating in the trial. It is imperative that all of the site workers are informed, or else a shift change or work rotation could result in a set of well meaning, but un-informed workers participating in the study. Communication with and understanding by the site workers is extremely important in getting compliance with a protocol. Not only do they have to know what to do, they have to be convinced that it is necessary to do it correctly.

A farm which is to be part of a clinical trial must be chosen based on more than just a willingness to participate. The facilities also need to be examined. The location, the availability of a work platform, and the presence of sufficient numbers of cages or tanks with similar characteristics are important factors.

Record keeping practices at a candidate farm should be assessed. These records can answer whether or not the disease of interest has historically been present and therefore the probability that it will occur during the study. They could also show if another disease problem, which could compromise the trial,

has had a history of occurring. The quality of the records may also give an indication of how well study records may be kept. If a farm already keeps clear, detailed, complete records on items such as feeding, mortalities, and treatments, it should be relatively easy to adapt current practices for a clinical trial. On the other hand, if farm records are obviously not kept or are not a concern of farm management and staff, this may signal a future difficulty with protocol compliance. The fewer changes that are made on the farm to accommodate a trial, the more likely results will be generated.

2.3.2 Treatment and dosing considerations

Clinical trials in non-aquaculture settings usually involve treatments applied to individual animals. It is generally a simple matter of calculating the correct dosage and administering such products by injection or orally. The dosage is based on the individual's weight, or a standard dose is used for all animals in a given size range. There is generally confidence that each study individual receives the correct dosage of the product being investigated.

Aquaculture treatments are usually applied at the group level (by cage, tank, or pond). Whether the medication is oral or administered as a bath treatment, all dosing calculations and administration apply to the group, not the individual.

Dosage of in-feed treatments are usually calculated based on the weight of the fish, as it would be for terrestrial based animals. However the total biomass of the group is used for the dosage calculation. The group biomass must be

based on the average weight of fish in the group, and the farmer's estimate of how many fish are in the group. During administration of in-feed treatments, one has to aim for an average dose. Some fish are more aggressive than others during any one feeding and will eat more of the medicated feed, some will eat less. One risk associated with this was demonstrated with preliminary studies using trichlorfon as an oral treatment for sea lice (63). During these studies it was apparently not uncommon for some of the fish to receive an overdose. Consequently, some in-feed treatments should be spread over multiple feedings. Hopefully by the end of the entire treatment period, all individuals will have had a chance to receive an appropriate dose.

Bath treatments are administered based on a specified concentration of chemical in contact with the fish for a specified period of time. Dosing calculations are based on the volume of the water in which the fish are contained. In a tank this may be calculated quite precisely. However, in a cage that has been surrounded by a tarpaulin, the calculation must be based on estimated dimensions. Prior to treatment, the volume of the net enclosure must be reduced and then surrounded by an impermeable tarpaulin. The shape is difficult to characterize, and consequently volume is difficult to calculate. An added difficulty is that the shape can vary from cage to cage due to water currents pushing the tarpaulin. Another consideration with group treatments is that it is a relatively inefficient system for testing a product. It is economically inefficient because a very large number of individuals must be treated, while only a relatively small proportion are examined. It is statistically inefficient because a relatively small

sample is used to estimate effects in a large group and therefore sampling error is a concern. Treatment administration is often labour intensive. Feeding all of the cages on a site may take several people several hours twice a day. Bath treatments require a great deal of cage preparation prior to treatment. For these reasons, group treatments can be relatively more expensive to study than individually applied treatments.

2.3.3 Outcome measurements and sampling

A successful clinical trial depends on having measurable outcome(s) to compare between the treatment groups. These outcomes can be related to production parameters such as growth rate or feed conversion, or they can be a measure of a disease parameter such as mortality rate, lice burdens, or disease prevalence. The aquaculture industry tends to be well behind other agricultural industries in the continuous monitoring of production performance parameters, due in part to the difficulty in performing incremental observations before harvest (51). This presents an inherent problem for investigators trying to detect subtle effects in a production setting (51).

2.4 Statistical considerations

The statistical aspects of a trial are a very important part of the planning process and they should be carefully considered during the trial design. It is useful to consult a statistician during protocol development rather than waiting

until the analysis stage. Some specific considerations are described below.

2.4.1 Unit of Concern

In determining the sample size requirements of a trial, the investigator must first identify the smallest independent grouping of test subjects that can be assessed. For many clinical trials involving terrestrial species, the individual is the unit of concern, or experimental unit. In aquaculture, the pen, cage, or tank of fish is usually the unit of concern. There are two main reasons for this: 1) management techniques such as feeding, treatments, health assessments, and harvesting are usually done by pen, and 2) the huge numbers of individuals makes the task of identifying and finding specific individuals virtually impossible.

When the unit of concern is a group of fish, there are two ways to approach the analysis. The first, more conservative, approach is to simply look at the mean value for the group. Another approach is to utilize a method which combines individual and group effects.

2.4.2 Sample sizes

Once the experimental unit has been selected, the number of units needed to evaluate the efficacy and safety of the product being studied must be estimated. It is intuitively obvious that the more subjects that are in a study, the more confidence there is that observed differences are not due to random variation (64). Estimating the necessary sample size for the experimental units

should be based on: 1) variability between units (pens or cages), 2) precision of estimate required, 3) confidence, 4) power, and 5) logistical considerations (65).

The measure of variability between units used in these calculations is the standard deviation (square root of the average squared deviation from the mean) (66). The precision of the estimate required is an estimate of a treatment effect or the minimum difference between two treatments that the investigator wishes to detect. Confidence is the probability that a statistically significant difference is not due to chance. Power is the probability of not committing a Type II error, i.e. the probability of finding a statistically significant difference when one exists. Logistical considerations include cost, time considerations, and ability to fit into farm production practices.

One formula used to calculate the sample size for an outcome variable with a continuous distribution being measured (65) is:

$$n = 2 \left[\frac{(Z_\alpha - Z_\beta)S}{(X_e - X_c)} \right]^2$$

n = sample size in each group

Z_α = value of Z corresponding to confidence (1.96 for 95% confidence)

Z_β = value of Z corresponding to power (-0.84 for 80% power)

S = estimate of the standard deviation of the outcome parameter

X_e = estimate of mean of outcome in exposed (treated) group

X_c = estimate of mean of outcome in unexposed (control) group

Calculating the necessary number of experimental units may not be the only sample size calculation. If all of the animals within each unit can not be sampled, then the number of individuals to be sampled from each unit must be calculated. This is based on much the same factors: 1) variability within a group (between individuals), 2) precision of estimate required, 3) confidence, 4) logistics. Power is not part of the calculation because the goal is to determine an average for an outcome parameter within the group, not to detect a difference.

The formula is (65):

$n = 4S^2/L^2$

n = sample size

4 is the approximate square of the Z value (1.96) corresponding to 95% confidence

S = standard deviation of the parameter being measured

L = maximum allowable difference between the estimate and the true average value

2.5 Controlling bias

Ideally, the only difference between the treatment group and the control group, chosen to represent a population of interest, is the treatment (66). A bias would be any systematic introduction of differences or distortion of differences between groups. There are many possible sources of bias in a clinical trial. It can be introduced during selection of the study population, treatment allocation, selection of individuals for examination, outcome assessments, and/or final

analysis. Consequently, it is important to recognize that although bias can never be completely controlled, efforts to limit and define bias must be continuous (67).

Following is a description of three commonly used methods that help control bias in clinical trials: matching, randomization, and blinding.

2.5.1 Matching

One way to help ensure similarity between treatment groups, and control for potential confounding factors is to use matching (61). In prospective studies such as clinical trials, matching is done by selecting research units which are similar in respect to potential confounding variable(s), then the matched units are allocated to treatment and control groups (65). Potentially confounding variables which are used for matching could be any risk factor(s) which could have an effect on the outcome of choice. Matching will also often lead to a more statistically efficient analysis than will selection by random sampling (61). This statistical efficiency is gained if matching is successful because the two groups to be compared should be more similar to each other than they would be if units are randomly allocated. This similarity serves to decrease background variation, with the result that any observed differences between groups should be due to the treatment effect under study.

The following is one example of how matching can be used where cage size and lice load are identified as potential confounders. If a site used in a trial consisted of 20 study cages and six of these cages were 15 m square and 12 of the cages were 12 m square, then the cages should be allocated to medicated

and control groups after being matched based on cage size and closest average number of lice per fish. Matching based on cage size ensures that equal numbers of cages within each cage size are in each treatment group. Matching based on lice counts ensures that lice numbers are similar between treatment groups prior to starting the experiment. Without matching, it is possible that the two groups could differ in pre-trial lice burdens considerably before treatment. If all of the cages with lower lice levels happen to be allocated to one group, and all the cages with high lice levels to the other group, a treatment effect would be either more difficult to detect or over-estimated. Post-treatment differences between matched groups will more likely be due to the treatment effect under study rather than differences in pre-treatment lice levels or cage size effects.

2.5.2 Randomization

Matching can help reduce bias caused by a few known variables (such as pre-treatment lice levels or cage size). However, to reduce the effects of other, usually unknown, extraneous factors, a formal randomization procedure is desirable for the allocation of experimental units into treatment groups (65). Randomization does not guarantee that groups will be the same with respect to known and unknown factors, but rather that the probability distribution of all possible outcomes of allocation is available (65).

Randomization can be achieved by flipping a coin, drawing numbers from a hat, through use of random numbers tables, or by using a random number generator (65).

Selection of sampling units from the experimental units (i.e. sampling fish from study cages) should also be subject to randomization whenever possible. In many non-aquaculture clinical trials, it is fairly easy to produce a randomized list of individuals which can be subjected to probability based selection for outcome measurements. In aquaculture, since it is usually impossible to identify and select specific individuals from each cage, random sampling is more difficult. Therefore non-probability based methods are usually used. A common practice in the salmon aquaculture industry is to throw out some feed and dip fish as they come up to eat, called feed-and-dip sampling. An alternative method is to crowd the fish using a seine, then dip them out, called crowd-and-dip sampling. It is not truly random since not all fish will be caught in the seine. Also, the person dipping may inadvertently select fish with certain characteristics, hence this method may be subject to a selection bias (7).

2.5.3 Blinding

Blinding refers to the components of the study design that prevent awareness of the specific treatment allocation by some or all individuals involved in the trial (68). The use of blind techniques prevents bias from a variety of sources (52). It prevents bias from the investigator during outcome assessment by removing any expectations due to treatment. Clients should be blinded to remove the possibility of preferential treatment for one group or the other. Knowledge of treatment allocation by the client could influence compliance, ancillary care and attention, or reporting of side-effects (68).

2.6 Requirements of Good Clinical Practice

The most common purpose of conducting trials to GCP standards is to satisfy regulatory agency requirements for product approval applications. Contact with the appropriate government agencies should be made before a trial commences in order to ensure the proposed protocol meets regulatory requirements.

The philosophy of GCP is really quite simple. Uelner (69) states that the basics of quality systems, such as GCP and Good Laboratory Practices (GLP), are the following: 1) state what will be done, 2) do what is stated, 3) record what is done, 4) verify the results, and 5) act on the differences.

Accomplishing these basic goals takes the commitment and coordination of a well trained team and complete documentation throughout the trial. It also requires the ability to adapt protocols to the particular situations encountered in production fish farms. A thorough knowledge of all production events enables investigators to anticipate most of the problems and plan for contingencies.

2.6.1 Personnel

The group of people needed to complete a clinical trial will, of course, vary with circumstances. However, in general there will be three key positions in the clinical trial team. They are: the investigator(s) conducting the research, a study monitor representing the sponsor, and a quality assurance person or team.

Investigator

The principle investigator, also called the study director, is the person responsible for the conduct of the trial. If there is a team of individuals needed to conduct the trial, the investigator is the responsible leader of the team (57). This individual assures that the protocol is approved and followed, and that the study complies with GCP standards (69). The study director takes final responsibility for the interpretation, analysis, documentation, and reporting of study results (70).

Study Monitor

The monitor is the person "who represents the sponsor in overseeing the investigator's implementation of a protocol and progress of the study" (71). The monitor acts as the main communication link between the sponsor and the investigator, and is usually employed by the sponsor. The extent and nature of the monitor's functions are determined by the sponsor and may depend on the needs of the study (57).

Quality Assurance Personnel

The quality assurance team (often simply called QA) has a critical part in the conduct of a GCP study. Their role is to inspect operational phases and audit study documentation for the purpose of assuring that the facilities, equipment, personnel, methods, practices, records, and controls used for a study conform with applicable rules and regulations (70). They also verify that the final report

provides an accurate account of the methods used, that the resulting data are accurate and complete, and that the conclusions truly reflect the raw data (72).

Prior to the initiation of a trial, QA reviews the protocol, Standard Operating Procedures (SOPs - described below) used by the research team, and all data collection sheets. They then offer suggestions for changes to clarify what will happen during the study. They ensure that contingency plans are in place for such events as early stopping, weather cancellations, and other uncontrollable circumstances.

During the study, QA inspects the study site(s) and makes observations during data collection to ensure that the protocol is being followed, and that data are being recorded as specified in the protocol.

Prior to reviewing the final report, QA reviews all records to ensure that data were properly recorded, and corrections were properly documented. QA also examines data entry for errors, accuracy of data analysis, and documentation of any protocol deviations. They ensure that the data support the conclusions.

2.6.2 Documentation

Documentation is of primary importance in a GCP trial. It must be possible to reconstruct a GCP study at any time afterward, through the complete and thorough documentation of the study. The protocol is the documentation of how the trial is to be conducted. Raw data consists of documentation of all information gathered during the trial. The study report contains the summary and

analysis of the data, as well as the conclusions drawn from the analysis.

Protocol

The study protocol is a detailed written plan for the study. It should describe the objective(s), design, methodology, statistical considerations, and organization of a trial (57). GCP requires that it must be in place and approved (signed) by all parties before a trial begins. The date that the protocol is signed by the investigator is considered the study initiation date (71). All amendments and deviations to the protocol are considered part of the protocol (71), and are to be maintained and archived with the protocol (69).

An amendment is any change made to the existing protocol that affects how the study is to be done. It is made after the protocol has been approved, but before that part of the study is actually performed. The amendment identifies what was changed, when it was changed, the reason(s) for the change, and the approval (signature and date) of the study director and monitor. For example, an approved protocol may state that 100 fish will be sampled from each cage, but before the study begins it is realized that this sample size will be impossible to accomplish due to time constraints. If a sample size of 50 fish is possible and will not compromise the study, an amendment would be written to state that 50 fish will be sampled from each cage rather than 100 fish.

If a protocol was not followed, this must also be documented, and noted as a protocol deviation. The deviation must describe in detail what was done differently from the protocol, the expected impact of the deviation on the study,

and acknowledgment (signature and date) by the study director and monitor. For example, a protocol states that 50 fish will be sampled from each cage, but due to a counting error only 45 were sampled from a particular cage. A protocol deviation must be written to explain what happened (sample size of 45 rather than 50 from cage X), why it happened (the investigator erred), and if it is expected to affect the study. The deviation is then included with the protocol.

Standard Operating Procedures

Standard Operating Procedures (SOP's) are specific instructions for the use and maintenance of instrumentation and equipment, or general procedures. They contain information that is very important to the quality of data, but is not directly relevant to a particular study and so is not included in a protocol. For example, a protocol would have information about what instrument is used in a study, but there would be a SOP to describe the operation, maintenance, and quality control procedures for that instrument. SOPs are not considered part of the study protocol, although they may be referred to in a protocol. They are considered part of the operation of a laboratory or test facility. They function to ensure consistency (73) and data quality (69). Many laboratories not only have SOPs for instrumentation, equipment, and analytical procedures, but also for data collection and recording, archiving, protocol development, and final report development. It is also possible to write SOPs on the preparation of SOPs (70). Any amendments or deviations to SOPs are to be treated as amendments and deviations to the protocol. Amendments are to be authorized by the study

director and kept with the original SOP. SOP deviations are to be documented the same way as protocol deviations.

All relevant SOPs should be immediately available to anyone who needs to refer to them. Also, a historical file of all SOPs with all revisions should be archived (69).

Raw Data

Raw data refers to any laboratory notebooks, worksheets, records, memoranda, notes or exact copies thereof, that are the result of original observations and activities of a study and are necessary for the reconstruction and evaluation of the report for a particular study (69). Raw data may include, but are not limited to, photographic materials, computer printouts, magnetic, electronic, or optical media, information recorded from automated instruments, and hand recorded data sheets (71).

According to Uelner (69) there are five characteristics of high quality data: 1) accuracy, 2) immediacy, 3) legibility, 4) durability, and 5) attributability. Data should be recorded promptly and directly with ink. They must be signed and dated by the person recording the data. All data should be clearly labelled and identified.

To correct already recorded data, the wrong entry must be lined out once, so that it is not obscured, then the correct entry made. The correction must be initialled, dated, and a reason specified for the correction.

All completed data sheets must be inspected and verified by another

person as understandable and complete. This verification should be done by someone not directly involved in the collection of the data he/she is inspecting. Verification of each data sheet is indicated with signature and date on each data sheet.

Computerized records are treated the same way. It must be documented who is responsible for data entry. Verification of data entry must be made by another person, ideally someone not directly involved in the collection of the data. This can be done by using printouts of the data set. The verifier's signature and date are included on each sheet, as well as any errors found. The person responsible for data entry can then correct any errors in the electronic file. Documentation of the corrections can be made on the same printouts, with signature and date.

It is advisable to have pre-designed data sheets for any information that will be recorded during a trial. Not only should observations of outcome measurements be recorded, but also records about such things as medication receipt and use, dosing calculations, equipment maintenance records, sample collection, sample handling and storage records, animal care and feeding records. The qualifications of all people involved in the study should also be included in the record. Regarding this information, it is important to record what was done, who did it, and when it was done.

Reports

The study is not considered complete until a final report is signed and

dated by the author(s) (71) or the study director (69). This date is considered the study completion date (71).

The report should include sufficient detail to allow reconstruction or replication of the study. This would include but not be limited to: names and addresses of each investigator and each facility where the study was performed; initiation and completion dates; objectives of the study; the materials and methods used to conduct the study; the results of the study; name of the monitor and any subcontractors stating the nature and extent of their participation; and a statement that the final study report is a complete and accurate representation of all study observations (71). This statement is usually prepared by the quality assurance personnel and is called the Quality Assurance Statement (69). The materials and methods section should include: a copy of the protocol with all amendments and deviations; a complete description of methods used; the number, species, breed or stock, sex, weight, age, and, when applicable, source of supply, physiological state, disease state, or other pertinent pathological findings of the animals used; animal identification procedures, and final disposition records for each animal; the investigational drug identified by name, identity, strength, purity, composition, quantity, and batch or code mark (to the extent known by the investigator); a description of the dosing regimen, including dose, rate, route, and duration of administration; a description of the transformations, calculations, or operations performed on the data, and any statistical methods employed to analyse the data (71).

The results of the study should include: a summary and analysis of the

data and the conclusions drawn from the analyses; a description of all adverse drug experiences observed during the study; a description of all circumstances that could have affected the quality or integrity of the data, specifying the time frame and extent of their occurrence; and the location of all study documentation (71).

The study report can be corrected or added to after the study completion date in the form of an amendment by the study director. The amendment should include what has been changed, how it has been changed, why it has been changed, and signed and dated (69).

Document Retention

As the purpose of conducting GCP studies is often to provide documentation in support of regulatory approval of products under investigation, documentation should be available to the regulatory agency in the event an audit is conducted. In the United States, the original documents should be promptly made available upon request for an FDA inspection (71). In Canada, the Food and Drugs Act (74) states that documentation be made available within 30 days of request.

All study documentation generated as a result of the study should be retained (71, 74). This includes all reports, raw data, documentation, records, protocols, and correspondence, personnel records, equipment maintenance and calibration records (69). It should be stored in a location secured against theft, loss, damage (69), deterioration, destruction, tampering, or vandalism (71). The

documentation should be in a location for orderly storage and expedient retrieval (71), or in a manner that enables an audit to be conducted (74).

The duration that records need to be retained depends on what is done with the results of the study. All documentation should be retained for two years following approval of a New Animal Drug Application (NADA) in the U.S. (71), or five years after submission of the NADA to the agency, which ever is longer (69). Samples and specimens need to be retained only until QA verification is complete (69), or as long as the quality of the preparation affords evaluation (71).

2.7 Summary

Examples of aquaculture clinical trials, particularly those done to GCP standards, are rare in the literature. This is likely because of the logistical and cost constraints of working in the aquaculture production setting. However, as will be demonstrated in the following chapter, GCP trials are possible at fish farms. The shortage of available chemotherapeutants for aquaculture use requires more companies willing to sponsor drug applications and conduct well designed clinical trials as part of the drug registration process.

3. RANDOMIZED CONTROL CLINICAL TRIAL TO INVESTIGATE THE EFFECTIVENESS OF TEFLUBENZURON TO TREAT SEA LICE ON ATLANTIC SALMON

3.1 Introduction

Lepeophtheirus salmonis is an ectoparasitic copepod which has become a serious problem in recent years for salmon producers in Atlantic Canada, particularly in New Brunswick. Significant economic losses have occurred due to infestations. In 1995, it is estimated that sea lice infestations caused about $20 million in losses to the New Brunswick industry. These losses were primarily due to increased mortality, down grading of carcass quality, and costs of treatments (15).

The current treatment options available in Canada are limited. Bath treatments with organophosphates (eg. azamethiphos) or hydrogen peroxide are labour intensive and pose a potential health risk to the applicators. Also, these treatments are generally only effective against the mobile stages of lice (33, 37). Ivermectin[2] has recently been the only oral treatment available, although it is not registered for food fish in any jurisdiction (41). Due to its narrow safety margin, its "off-label" prescription use, slow residue depletion rates and subsequent extended withdrawal time, ivermectin usage is often limited to use in fish at least one summer from harvest (i.e. first summer in sea cages). Emamectin, another

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[2]Ivomec®, Merck and Co., Inc., Rahway, NJ

oral formulation in the avermectin family, is being used experimentally (46, 75).

Teflubenzuron is an orally administered chitin synthesis inhibitor. In preliminary Norwegian studies, it has been shown to be effective against all stages of sea lice that undergo a molt, including the larval and pre-adult stages (49). Teflubenzuron is one of a group of compounds called the acylureas (benzophenylureas). These compounds act to disrupt the synthesis of chitin, a polysaccharide of particular importance to arthropods (76). Chitin is a long-chain polymer consisting of β-1,4-linked N-acetylglucosamine which, in the arthropod cuticle, is bound to a protein to form a glycoprotein (77). The acylurea compounds disrupt chitin formation. Therefore these compounds affect those periods of the life cycle (larval and pre-adult stages in sea lice) where chitin is being formed and where its incorrect or insufficient production can lead to malformations of the exoskeleton; however, the precise mechanism is uncertain (76).

Sea lice on the fish during treatment with teflubenzuron are affected by the compound. The chalimus and pre-adult stages are most susceptible, and morphologic examination of exposed lice show damage to the cuticle (49). Since acylurea compounds affect physiological processes that do not occur in vertebrates, they are considered to have a very high margin of safety (76). Orally administered chitin synthesis inhibitors are currently available for other species to control parasites. Lufenuron is used in dogs and cats as a treatment for fleas (50).

The objective of this study was to assess the effectiveness of

teflubenzuron in the reduction of sea lice (*Lepeophtheirus salmonis*) burdens on Atlantic salmon nearing market size.

3.2 Materials and Methods

This study was a double blind randomized clinical trial done to Good Clinical Practice (GCP) standards. It was conducted on three commercial sea cage sites during the period of August 6 to September 12, 1996. Medicated and control cages were matched by site, cage size, and average number of lice per fish prior to allocation to control for the confounding effects of initial lice burdens.

3.2.1 Site Selection Criteria

The clinical trial involved a total of 40 cages from three separate commercial seawater net pen sites in New Brunswick. Sites were required to meet the following basic criteria before acceptance into the trial:

1. Minimum of four cages with Atlantic salmon of the 1995 year class which were of similar weight and contained in cages with similar dimensions.
2. Owners and managers willing to accommodate the study design, particularly maintaining blindness of treatment allocation and delaying other lice treatments until lice counts were obtained.
3. Record keeping and farm husbandry practices permitted the necessary data collection for the trial (eg. regular lice counts and fish weight samples).

4. All sites within reasonable proximity to each other to permit same day travel between sites during the trial.

5. A history of lice problems in previous years.

All sites were initially selected by the project supervisor from the sponsoring company (the study monitor) and visited by the investigators for interviews with the owners and managers. All three of the sites were situated close to Lime Kiln Bay, St. George, NB. Each site is described in more detail in section 3.3.

3.2.2 Trial Initialization

After the sites were identified for the study, they were visited every week beginning in early June, when lice burdens had the potential to increase. A sample of fish was examined for lice numbers and the decision to begin the trial or not was based on these weekly lice counts. The pre-determined threshold for initiating the study at each site was originally set at an average of 10 lice (including all stages) per fish. However, as the lice numbers were unexpectedly low in the study area during 1996, it was decided in consultation with the study monitor, that five total lice would be sufficient to initiate the trial. Once the pre-trial assessment indicated a sufficient lice load, the pre-treatment sampling was performed and all treatment allocations were based on this sample.

3.2.3 Early Withdrawal Criteria

During the study, each farm manager was encouraged to visually evaluate

fish within each pen for evidence of skin damage due to lice and examine the lice count data. Each manager was unaware of the treatment allocation of each pen and, therefore, was given the opportunity to critically evaluate lice infestations with similar attention to both medicated and control groups. Should the lice levels exceed the threshold for the manager's confidence that other available treatments (i.e. organophospate or hydrogen peroxide baths) would be able to control lice problems, then the farmer was free to selectively remove cages from the study for resumption of other control measures. The farm management was requested to provide 48 hours notice of withdrawal from the study so that a final lice count could be attempted by the investigators. Any action (eg. withdrawal) directed at one cage would result in its corresponding matched cage receiving similar actions.

Farm management was also free to remove cages from the study due to other unanticipated management considerations such as, removal due to other concurrent disease problems requiring treatment or slaughter incompatible with the study design. In such a case, the study protocol required removal of both the affected cage(s) and the matched cage(s). The possibility of withdrawal delays due to medication was covered by having the intervention decisions made without knowledge of treatment allocation and then unmasking the selected cages to determine the need for withdrawal time. Again, it was requested that farm management provide 48 hours notice to permit a final lice count sample to occur.

3.2.4 Treatment Allocation and Blinding

Once lice counts reached the level required to initiate a trial at a site, fish were sampled from all cages to determine pre-treatment lice infestation levels and to estimate the fish weight and biomass. Within each site, individual cages were assigned a letter designation and matched pairs of cages were identified based on cage size (absolute match), then on mean sea lice counts (closest value match). A list of matched pairs was supplied to the sponsoring company for random allocation (using a random numbers table) of one cage from each matched pair into the medicated group and one cage into the control group. The result was that 50% of the cages at each site were assigned to the medicated group and 50% of the cages at each site were assigned to the control group, and that each group had comparable lice levels.

Since the site workers and the site investigators did not know which cages belonged to the medicated group or which belonged to the control group, all of the treatment feed bags, both medicated and control, were identical except for a label which indicated the site and cage designation.

3.2.5 Treatment

There were two treatments, medicated diet and control diet. The medication was administered at a dosage of 10 mg of active ingredient/kg biomass/day. The concentration of the teflubenzuron in the medicated feed was 2.0 kg/tonne. The medicated and matched control feeds were fed at a rate of 0.5% biomass per day to achieve the required dosage. The fish received the

treatment for a period of seven days. This treatment regimen was determined by a series of dose titration studies previously performed by the sponsoring company. These earlier studies examined dosages of 2-200 mg/kg of biomass and treatment periods of 3-14 days (78).

Moore-Clark (St. Andrews, NB) supplied medicated and control study feed to the sites. The feed was delivered in plastic bags which were labelled with site number and cage letter identification, weight of feed in the bag, and the number of bags that cage was to receive each day. An example bag label is provided in Appendix A. Each cage was labelled with a sign indicating site number, cage letter, number of bags of study feed to be administered per day, and the total weight of study feed for each day. An example cage label is provided in Appendix B.

The fish were fed to satiation in each of two feedings per day. If the predetermined amount of study feed was insufficient to satiate the fish within a cage for the first feeding, non-study feed was supplemented. If the fish did not eat all of the study diet during the first feeding, the remaining study feed was fed at the second feeding, prior to any non-study feed.

Non-study feed was the normal diet which the fish usually were fed. Both study and non-study feed consisted of 9.0 mm Moore-Clark™ extruded pellets. Site 2 was the exception; the non-study diet at Site 2 was Shur-Gain™ 8.0 mm Salmon Ration.

3.2.6 Sampling

Pre-treatment samples were taken from all cages at the beginning of each trial to determine pre-treatment lice infestation levels and to obtain the mean fish weight for biomass estimates. To estimate the biomass within a pen, a total of 100 fish per cage were sampled and weighed during this first sample period. Cage biomass was estimated using weight estimates from these pre-treatment samples and the inventory estimates available from farm records. Lice counts were performed on a minimum of 25 fish per pen, 50 fish whenever logistically possible. All cages received study feed for seven days, then each cage was sampled twice to determine post-treatment lice levels: once at 6-7 days after the end of treatment, and again 13-14 days after the end of treatment.

The number of fish to be sampled from each pen was determined by calculations using the formula: $n=4s^2/L^2$ (65) where n = sample size, 4 is the approximate value of the square of Z = 1.96 which indicates 95% confidence in the estimate, s = standard deviation, and L = allowable error (precision of the estimate).

Estimates of standard deviations were obtained from New Brunswick Department of Fisheries and Aquaculture lice count data from the summer of 1995. Data from nine cages with relatively high lice numbers and nine cages with low lice numbers were examined. In the high lice level cages, the mean(μ) was 38 lice per fish, and the standard deviation was 16 (~0.4 μ). Assuming that the estimate is to be within 20% of the mean(+/- 0.2 μ):

$n=4s^2/L^2$

$n=4(0.4\ \mu)^2/(0.2\ \mu)^2 = 16$ fish per cage

With a sample size of 16, if the sample mean were 40, there would be 95% confidence that the true mean was between 32 and 48 lice per fish.

In the cages with low lice burdens, the mean and the standard deviation were roughly equal at 1.8 lice per fish. Using the same formula, a sample size of 25 fish per cage would give a 95% confidence interval of +/- 40% (0.4u). There would be 95% confidence that the true mean would be between 1.2 and 2.8 if the estimate from the sample was 2.0.

Based on these calculations a sample size of 25 was set as the minimum acceptable sample size. For greater precision, a sample size of 50 would be used when possible.

Sampling Protocol

Sampling was done by crowding the fish and then capturing a small number of fish with a dip net, a method referred to as "crowd-and-dip" sampling. Crowding was accomplished by either seining or by reducing the net size within the cage. Two to five fish were dip netted at a time. Fish from every fourth dip net were sampled to help reduce selection bias. The fish from the un-sampled dip-nets were placed into the post-sample section of the pen, ie. the non-crowded portion of the pen, as were the sampled fish after evaluation. Non-replacement sampling was performed to reduce the stress of crowding and capture on the fish. The farm management at Site 1 objected to using every fourth dip-net. Consequently, consecutive dip nets were used at that particular site.

Anesthetic

Sampled fish were anaesthetized in a 50-100mg/l tricaine methanosulfanate[3] (TMS™) bath prior to the measurements. A plastic container measuring approximately 1m X 1m X 1m, approximately half filled with water, was used as an anesthetic bath. Approximate amounts of anesthetic were used by estimating the volume of the water and adding TMS™ powder measured by volume. Anesthetic concentrations were adjusted by adding more anesthetic powder or more water to the bath, depending on whether the anesthetic solution seemed too weak or strong. The anesthetic solution was judged to be too weak if after an appropriate amount of time spent in the anesthetic bath the fish remained overly active during examination attempts. The solution was judged to be too strong if fish reached an anesthetic plane more quickly than usual or if they seemed to be too "deep" (ie. very little movement during examination or recovery was slow).

The water in the anesthetic bath was changed as needed, usually after one or sometimes two cages. Temperature and dissolved oxygen were not routinely monitored, and no aeration was provided. Anesthetic baths were not tested for sea lice which had fallen off. A post-anesthetic recovery tank was used when available.

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[3]TMS™, Syndel International Inc., Vancouver, BC

Sampling Measurements

The following measurements were made on each fish sampled.

1) Fork length (length of the fish as measured from the rostral tip of head to the base of the fork in the tail) was measured to the nearest 1.0 cm using a measuring tape mounted in a PVC pipe which had been cut in half longitudinally.

2) Weight was measured to the nearest 10 g using an Accu-Weigh DSY-1000 scale[4].

3) a total body count of all copepodids

4) a total body count of all stage 1&2 chalimus (grouped as one count)

5) a total body count of all stage 3&4 chalimus (grouped as one count)

Copepodid and chalimus stages were classified based on relative size.

6) a total body count of all pre-adults

7) a total body count of all non-gravid adult lice

8) a total body count of all gravid females

9) a subjective score of sea lice damage

0=no damage

1=increased mucus

2=small (<2cm^2) areas of superficial damage

[4]Yamato Corporation, Colorado Springs, CO

3=large (>2cm^2) areas of superficial damage

4=small (<2cm^2) areas of deep damage (through the epidermis)

5=large (>2cm^2) areas of deep damage

Lice count procedure

Two of the investigators were trained as lice counters. To help ensure consistency, only these two people performed lice counts during the study.

After weight and length measurements were taken, the anesthetised fish were placed on a tray for the lice count procedure. The person counting lice (the counter) made consecutive longitudinal visual scans starting at the ventral midline of the fish, up one side, over the dorsum, and back to the ventral midline. As lice were found and identified, the counter told the person recording the data (the recorder) the stage of development and number of lice seen. The recorder entered the weight and length of the fish, and the lice counts. As the counter told the recorder what stage and how many lice were found, the recorder made tick marks on the data sheet in the appropriate space (see sampling data sheet in Appendix C). When the counter was finished with the fish, the recorder counted the number of tick marks in each space. This number was written and circled within the same space on the data sheet.

3.2.7 Statistical Analysis

All data were entered into a Quattro Pro[5] spreadsheet then transferred to Stata[6] statistical software package for analysis. Graphs were generated using either Quattro Pro or Sigma Plot[7].

Since data evaluation involved comparison of the two treatment groups, proper analysis required knowing which cages were in each group. Investigators were therefore unblinded at this point and given the treatment allocation information held by the sponsor.

The average number and standard deviation of total (all stages) lice per fish were calculated for each cage, and for each treatment group for each sampling period. This was done by site and overall (all sites combined).

The overall average number and standard deviation of individual stages of lice per fish was calculated by sampling period. The average number of lice per fish within each treatment group was calculated for each site by stage and sampling period.

Assessment of lice count differences between medicated and control groups at each sampling period was the primary analysis of interest. Overall significance was assessed using random effects linear regression with 'cage' as a random effect to adjust for clustering within cages, and 'site' as a fixed effect. For the purposes of this significance testing, the dependent variable was a log

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[5] Corel Corporation Limited, Ottawa, ON

[6] Stata Corporation, College Station, TX

[7] Jandel Corporation, San Rafael, CA (now owned by SPSS, Chicago, IL)

transformed lice count {log(count + 1)} to ensure that the data were approximately normally distributed. The general model for these regression analyses were:

$$y_{(i,j)} = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_{3(j)} + u_{(j)} + e_{(i,j)}$$

$y_{(i,j)}$ = log((lice count on i^{th} fish in j^{th} cage) +1)

α = intercept

x_1 = dummy variable for site = 2

x_2 = dummy variable for site = 3

$x_{3(j)}$ = treatment applied to j^{th} cage

β_1, β_2, β_3 = coefficients for fixed effects

$u_{(j)}$ = random effect of j^{th} cage

$e_{(i,j)}$ = residual error

Separate analyses were performed for: copepodids, chalimus 1 & 2, chalimus 3 & 4, pre-adult, adult, all chalimus (1&2 plus 3 & 4), mobiles (pre-adult & adult), and total (all stages) at each time period (pre-treatment, 1^{st} post-treatment, 2^{nd} post-treatment).

Lice counts in medicated cages were also calculated as a percent of lice counts in control cages (mean medicated/mean control X 100), and graphed for chalimus 1 & 2, chalimus 3 & 4, pre-adult, and adult stages at each site and overall. For the calculation of the reduction in lice levels, medicated cages and control cages were compared at each post-treatment sampling period. Percent reductions in the medicated group as compared to the control group ((1 - mean lice burden in medicated/mean lice burden in control) X 100) were calculated for each stage and sampling period where a significant difference was found.

Weight (kg), length (cm), and condition factor ($wt/lng^3*100,000$) data were also compared between the treatment groups using random effects linear regression with 'cage' as a random effect to adjust for clustering within cages, and 'site' as a fixed effect.

Damage scores were compared between treatment groups by a Chi-square test. The comparison was based on a damage (score $\geq$ 1)/no damage (score = 0) classification.

The percent reduction observed in each medicated cage as compared to total number of lice in the matched control cages was tested to see if percent reduction was a function of lice numbers. This relationship was evaluated by linear regression analysis with percent reduction (in each medicated cage as compared to its match at each post-treatment sampling period) as the dependent variable and total number of lice (in each control cage at each post-treatment sampling period) as the independent variable.

3.2.8 Lethal Sampling

Lethal sampling to collect tissue samples for ivermectin and teflubenzuron residue analysis was done at Site 3. Ivermectin residues were a concern because most fish of this year class received ivermectin treatments during the previous fall and if residues had been present at sufficient levels, they may have affected study results. This also allowed investigators to verify life-cycle identification criteria by careful examination of lice with a magnifying glass. The lethal sample consisted of ten fish from each of six cages during the pre-

treatment and first post-treatment sampling periods, and ten fish from each of 4 cages during the second post-treatment period. The six cages were selected by drawing numbers from a hat during the pre-treatment sampling period. Three matched pairs were selected by drawing numbers from a hat during the first post-treatment sampling period. The same cages were then sampled at the second post-treatment sampling period, with the exception of the cages that had been removed from the study.

The euthanised fish were a sub-sample of the original twenty five fish sample from each cage, and were the first ten fish to have lice counts performed on them. The fish, euthanased by an overdose in a TMS™ anesthetic bath (200 mg/l), were put in plastic bags and kept on ice until processing immediately upon return to shore from the day's sampling. Lice counts were performed with the aid of a magnifying glass. Muscle and liver samples were placed in whirl packs and kept on ice until placed in a freezer for storage until the analyses could be done.

Liver samples were delivered to the New Brunswick Department of Fisheries and Aquaculture for ivermectin residue analysis. Although both muscle and liver were collected for ivermectin analysis, due to NBDFA limitations in the number of samples which could be processed, a sub-sample of livers, in pools of 2 or 4 livers each, were analysed. Liver was chosen because it would contain a much higher concentration of the drug than muscle (79). Muscle and liver samples were delivered to the sponsor for teflubenzuron analysis.

3.2.9 Environmental measurements

Dissolved oxygen and water temperature were measured each day that treatment feed was fed, in each trial cage, at a depth of about 2 m. A YSI model 55[8] dissolved oxygen meter was used to make the measurements. A tide table was also included with the data.

3.2.10 Description of procedures and events at each of the study sites

Full communication of events occurred between the investigators, the sponsor, and the farmers while the study was being carried out. The following section describes the details of the studies at each of the three sites.

Site 1

Site 1 was located in Lime Kiln Bay. There were twenty-eight 12 m square steel cages on site at the time of the study. Fourteen of these contained pre-market fish and were included in the trial. The rest of the cages held smolt, brood stock, or were empty. A diagram of the study cages can be seen in Appendix D.

Site 1 Specific Feeding Procedures

Treatment feed was stored in the normal feed storage location for the site, a building on shore. The feed bags were organised on seven pallets so that each pallet had all of the treatment feed for each day of feeding at this site.

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[8] YSI Incorporated, Yellow Springs, OH

To reduce the risk of errors, the normal routine of the farm was accommodated in the administration of the study feed. This site utilized automatic mechanical feeders, with one permanently positioned at each cage. Each unit was filled with the appropriate amount of feed before each feeding, then operated until empty at feeding time. The simultaneous operation of all fourteen feeders was supervised by one site worker. The mechanical feeders at the site were normally filled every afternoon for the evening feeding. Then, immediately after the evening feeding, they were filled for the next morning. To avoid having the treatment feed sit in the feeders overnight, administration of treatment feed was initiated with the evening feeding.

As was standard procedure at this site, fish were fed at a rate of 0.9% biomass per day (normal feed plus treatment feed). Fish at this site were fed to biomass instead of to satiation because the feed was put into the feeders before it was administered; then the feeders were operated until empty.

Each afternoon during the treatment period, one pallet of treatment feed was lifted onto a truck and delivered to the wharf to be lifted onto a boat. One or two site workers and a research assistant then delivered the feed to the cages where feed was put directly from the boat into empty mechanical feeders at each cage. A label on each feeder identified the cage letter and proper amount of treatment feed. The research assistant inspected the labels and counted the bags after they were emptied into each feeder to ensure that the correct number were delivered. The bags were then discarded. The research assistant collected dissolved oxygen and temperature data while waiting for feeding time, and

remained on site to observe the entire feeding.

Description of Events at Site 1

Pre-treatment sampling was done on August 6 and 7, 1996 with lice counts performed on 50 fish per cage and weights measured on 100 fish per cage. One cage had weights measured on 99 fish. Lice counts were done on 50 fish, rather than 25, because it was logistically possible; also because the lice numbers were still relatively low, and the larger sample size provided a more precise estimate of lice numbers. Fish were sampled by pulling up the net to reduce cage volume and fish were dipped out according to the protocol.

Since all of the cages were the same size, matching of cages was based on nearest lice counts. A list of these matched pairs was supplied to the sponsor for randomization into medicated and control groups.

It was originally expected that feeding could begin the day after pre-treatment sampling. However the feed plant required more time to manufacture and deliver the feed. The feed was delivered late on the second day after pre-treatment sampling (August 8) with one pallet of feed for each cage. Upon inspection by site investigators and the study's quality assurance inspector early the next day (August 9) it was found that each pallet was appropriately labelled for the cage it was intended, but that each also had a label on it which said either 'medicated' or 'base diet'. The investigators left the storage facility while the quality assurance inspector removed those labels. Two bags of feed were found to be torn and some of the feed spilled. These two bags were returned to the

sponsor and replaced with intact bags of the appropriate feed.

A routine mortality dive following the pre-treatment sample and prior to administration of experimental feed was done at the site. A total of 71 dead fish (44,700 fish at risk for one week - representing a 0.02% daily mortality rate) were found by the diver in the study cages. This was much higher than normal mortality levels in these cages (usually about 0.003% per day). The mortalities were evenly distributed throughout the cages. The investigators were not present to examine the fish due to sampling commitments at a different site that day. Since the mortalities were found shortly after the sampling was done and mortality levels returned to normal the following week, it was possible that the deaths occurred as a result of stress due to sampling. The cause of death was unknown, but considered not to interfere with the study.

The study feed was administered for seven consecutive days (August 9 through 15). Post-treatment sampling was done on all cages at six days after the treatment period (August 21) and on all cages except two (cages H and E) at 13 days after the treatment period (August 28). A matched pair of cages (H and E) were removed from the study early and had the second post-treatment sample done at 11 days after the end of treatment (August 26) because site management needed to harvest a cage and did not wish to wait until the final sampling scheduled for day 13 (August 28). Once unmasked, the farm was informed by the sponsor that Cage H was the control cage and therefore eligible for subsequent harvest.

Site 2

Site 2 was located near Lime Kiln Bay in Bliss Harbour. There were ten 70 m polar circle cages at the site at the time of the trial. All cages held pre-market fish and were initially included in the trial. A diagram of the site is presented in Appendix D.

Site 2 Specific Feeding Procedures

Experimental feed was stored in the normal feed storage location at the site, a building on the site. Feeding was normally performed with two mobile mechanical feeders which moved from cage to cage. It was decided to have one of the feeders for medicated feed and the other for control feed so that any drug residues in a feeder could not affect non-medicated cages. This was accomplished by obtaining a list of the two treatment groups from Moore-Clark without identifying matched cages or treatment allocation. One feeder was assigned to feed one list of cages and the other feeder was assigned the other list.

Each morning the site workers would move the feed bags for their list of cages for that day from the building to the mobile feeder. Upon arrival at each cage, the worker would empty bags for that cage into the feeder, then feed it to the fish. Not all bags were emptied into the feeder at once in case the fish did not eat all of the treatment feed at this feeding. The site workers would feed the allotted feed, or as much as the fish in the cage would eat, before going to the next cage. After feeding the treatment feed, the site worker would return to the

storage building to get normal feed if any cages required it, and to store any uneaten study feed until the next feeding.

The research assistant would accompany one of the feeders to observe and count feed bags. For each cage, on each day, one label was removed from a bag and the total of empty bags was recorded on this label, as well as date and time. This label was saved for future reference and the rest of the bags were discarded. Upon return to the storage building the research assistant would perform the same task with the bags from the other feeder. Dissolved oxygen and temperature readings were taken by the research assistant during feeding.

This site normally used feed from a company other than the sponsor. Non-study feed at this site was Shur-Gain 8.0 mm Salmon Ration.

Description of Events at Site 2

Pre-treatment sampling was done on August 19 and 20. Fish were sampled by seining the cage and using every fourth dip-net of fish for the sample. Lice counts were performed on 50 fish per cage and weights measured on 100 fish. Since all of the cages were the same size, matching of cages was based on nearest lice counts. A list of these matched pairs was supplied to the sponsor for randomization into medicated and control groups.

The fish in cage B were experiencing high mortalities of an unknown cause which began before the trial started. The Monitor decided to make sure that cage B was not a medicated cage so that drug residues would not be a problem if site management decided to harvest the cage. Site management subsequently did

decide to harvest the cage and the matched pair were removed from the study. The removal of this pair, in combination with the use of mechanical feeders for groups of cages, effectively unmasked the treatment allocation to the site workers.

Feed was delivered on the day after pre-treatment sampling was completed (August 21) and treatment began on the following day (August 22).

On day two of treatment (August 23) one cage (cage J) mistakenly received 12 bags of study feed rather than the prescribed 11 bags due to a miscount by a site worker. It then received 10 bags of feed on day three (August 24). On days three and four (August 24 and 25) amounts of non-study feed were not recorded due to a misunderstanding by site workers. On day seven (August 28) one cage (cage A) received 11 bags of feed rather than the prescribed 10. As this was the last day of treatment, when the site worker discovered 11 instead of 10 bags remaining for this cage, he assumed that it was due to a miscount earlier in the week. It was later determined to be a surplus bag from the feed mill.

Damage scores were not recorded on the mortality records on day one of treatment (August 22) or on day seven (August 28). Also, one cage (cage B) was included on the mortality sheet even though it had been removed from the study.

Post-treatment sampling was done at seven (September 4) and at 14 days (September 11) after the end of the treatment period. During the day seven sample, lice counts were performed on 50 fish per cage. The site manager decided after this first post-treatment sample that he would bath treat all cages as soon as possible because of the high lice levels. He was unable to treat them

before day 14, permitting another sample. Due to fewer personnel available, and to ensure that all cages could be sampled in the available time, counts were obtained on 25 fish per cage for this second post-treatment sample.

Site 3

Site 3 was located near Lime Kiln Bay in Bliss Harbour. A total of 18 square steel cages (twelve 12 metre and six 15 metre) containing pre-market salmon were included in the trial. There were also approximately six circular cages at the site containing smolts, brood stock, and some pre-market fish. A diagram of the study cages can be seen in Appendix D.

Site 3 Specific Feeding Procedures

Treatment feed was stored in the normal feed storage location at the site, on pallets next to the cages. Each cage had one pallet with all of its treatment feed on it. Large canvas covers were used to protect the feed bags from weather and birds.

At feeding time the bags were removed from the pallets. The feed was fed directly from the bags or from a bin into which the bags were emptied. There was one bin at each cage and it was tied to the cage. Feed scoops were provided so that there was one scoop for each cage. The scoops were labelled so they would stay with only one cage.

Feed consumption data sheets (see a sample daily feed consumption sheet in Appendix C) were kept on clip-boards which were tied to each feed bin.

Upon arrival with the site workers each morning, the research assistant would complete his portion of the daily feed consumption data sheets as they were distributed to the cages. At the same time, the sheets from the day before were collected.

After distribution of the sheets each day, the research assistant would collect one label from each cage. Date, time, and the number of empty treatment bags for the cage were recorded on the label. This label was saved for future reference and the rest of the bags were discarded. Dissolved oxygen and temperature measurements were taken after the bag count was completed.

Description of Events at Site 3

The study at Site 3 was scheduled to begin in the same week as Site 1. Due to low lice levels at this site, the start was delayed 2 weeks until lice numbers were higher.

Pre-treatment sampling was done on August 22 and 23. Samples were obtained by seining the cage and using every fourth dip-net of fish for the sample. Initially, lice counts were performed on 50 fish per cage, and weights measured on 100 fish per cage. Due to the high lice numbers, this sample size became too time consuming to be practical. After two cages had been sampled (A and B), it was decided to count lice on only 25 fish per cage. The larger sample sizes in these cages were included when generating cage averages and analysis. One cage (A) had weights measured on 99 fish because of a miscount by the recorder. Three cages (E, F, and J) had weights measured on 99 fish and lice

counts measured on 24 fish because of a miscount. During the pre-treatment sampling, a measuring tape was used to measure length, instead of a graduated PVC pipe, which had been lost overboard. The graduated PVC pipe was recovered before the next sampling period.

The cages were matched based on cage size, then on nearest lice counts. A list of these matched pairs was supplied to the sponsor for randomization into treatment and control groups.

The treatment feed was delivered to the site on the day pre-treatment sampling was completed (evening of August 23) and the following day (morning of August 24). The site workers put the feed on pallets next to each cage. Feeding of experimental feed began the day after pre-treatment sampling was completed (August 24).

It had been intended that the Quality Assurance person would inspect the first feeding at this site. However, due to scheduling difficulties, a later feeding was inspected.

During the treatment period, all cages received the prescribed amount of treatment feed with the following exceptions and difficulties:

-On day two of treatment (August 25) it was discovered that the feed for cage C had been placed next to cage D, and the feed for cage D next to cage C. It was assumed that each cage received the feed assigned to the other cage on the morning of day one.

-On day two it appears from the feed records that three cages (E, F, and N) received regular feed prior to treatment feed during the evening feeding.

This was a deviation from the protocol which stated that treatment feed was to be administered first.

-On day five (August 28) it was discovered that a bag of feed labelled for one cage (R) was mistakenly fed to another cage (Q). Subsequently, the feed consumption sheets show that the first cage (R) was one bag short on the last day of feeding.

-On day seven (August 30) one cage (O) received 7 bags rather than the prescribed 6 bags.

-On day seven the site workers did not record regular feed amounts for 15 of the 18 cages. The other three cages only had morning amounts of regular feed recorded.

The management at the site was very concerned about the build up of lice numbers. The first post-treatment sample was not scheduled to be done until days six and seven after treatment (September 5 and 6). Site investigators agreed to sample some cages on the afternoon of day three (September 2) to obtain an estimate of lice numbers for site management. One matched pair was chosen for the sample (cages A and B). They had average total lice counts of 14.8 and 66.4 respectively. The site manager decided that he would begin treating as soon as possible. On day four after treatment (September 3) three matched pairs of cages were sampled (pairs M-R, O-P, N-Q). These three pairs were chosen because they were located separately from the others, and there was only time to sample a few cages (the weather did not allow for sampling until afternoon).

On day five (September 4) three cages (P, Q, and R) were given a bath treatment with azamethiphos. The treatments were done during flood tide, when the current flows away from the rest of the cages. Site investigators were sampling at another site and were not able to observe the treatments or to sample more cages at this site. The bath treated cages and their matched cages were removed from the study as per the study protocol.

The remaining cages were sampled on day six (September 5). However, because of the lower than expected number of lice noted during this sampling, no bath treatments were done on any of the remaining 12 cages. The second post-treatment sample was done on day 13 (September 12).

3.2.11 Trial Schedule

The clinical trial was delayed approximately 6 weeks due to low lice levels at the study sites. This delay did not adversely affect the study outcome, but it did necessitate a much longer and more demanding preliminary sampling period. It also caused the studies to be more "overlapped". Figure 3 shows time lines for each site. Table I shows all dates of trial sampling periods and the sample sizes used.

Figure 3. Time lines showing the clinical trial sampling and treatment schedule at the three sites.

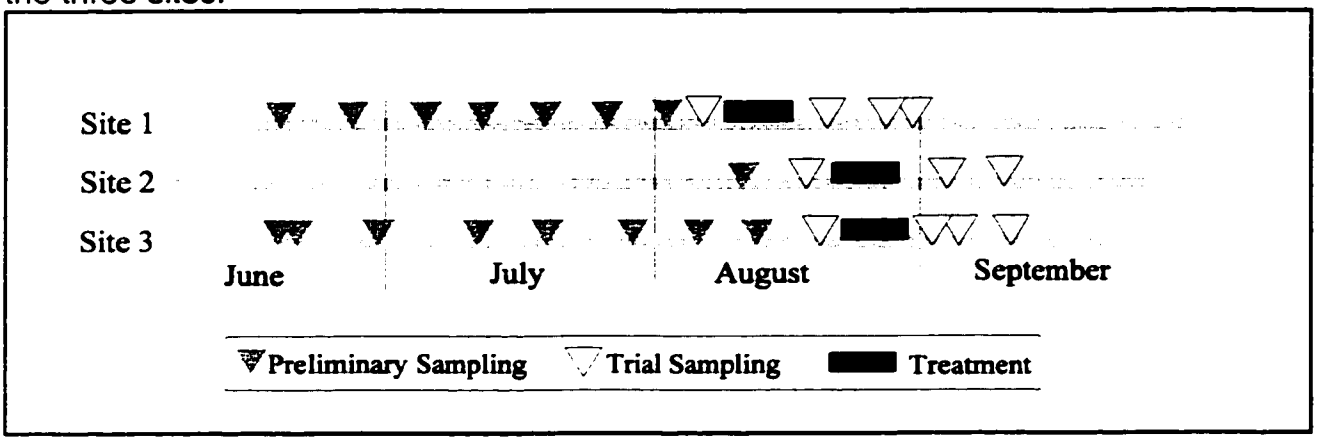

Table I. Sampling dates, treatment dates, and sample sizes for the clinical trial at all three sites

	Pre-treatment Sample	# of cages	# of fish per cage		Treatment Dates 1st	Post-treatment Sample	# of fish per cage	2nd Post-treatment Sample	# of fish per cage
			Wt	Lice counts					
Site 1	Aug. 6 and 7	14	100 [1]	50	Aug. 9 to 15	Aug. 21	50	Aug. 26 and 28	50
Site 2	Aug.19 and 20	10 [2]	100	50	Aug. 22 to 28	Sept. 4	50	Sept. 11	25
Site 3	Aug. 22 and 23	18	100 [3]	254	Aug. 24 to 30	Sept. 3, 5, 6	25	Sept. 12	25

[1] one cage had 99 fish weighed

[2] all ten cages were sampled before treatment, however one cage pair was removed prior to the post-treatment sampling periods

[3] four cages had 99 fish weighed

[4] three cages had lice counts on 24 fish, one cage had lice counts on 49 fish, one cage had lice counts on 50 fish

3.4 Results

3.4.1 Lice Counts

The three study sites were combined for the analysis to assess the effectiveness of teflubenzuron. Differences between sites in overall lice levels were controlled by including 'site' as a fixed effect in the regression analysis models. The clustering of fish within a cage (i.e., fish within a cage more likely to have similar lice counts than fish in different cages) was controlled by including 'cage' as a random effect in the model.

Table II shows the average number of lice per fish in each treatment group at each sampling period. Site specific lice counts can be found in Appendix E. Pre-treatment levels were included to show that there were no significant differences between treatment groups before treatment. The observed significance levels were derived from the random effects linear regression analysis. Percent reductions have been calculated where significant differences were found between treatment groups. The means shown are the averages of the non-adjusted (ie. not log transformed) cage means. The effect of medication was highly significant (as indicated by p-values<0.05) for all chalimus and pre-adults at the both post-treatment sampling periods. The effect on adults was statistically significant at the first post-treatment sampling period but not at the second. Graphs of lice stage distributions are shown in Appendix F. Graphs showing medicated as a percent of control average counts for each site are in Appendix G.

A comparison between percent reduction and total number of lice indicated

that as the average number of lice increased, the percent reduction increased. Linear regression analysis showed a significant relationship (p-value=0.003, r^2 = 0.31).

Table II. Comparison between medicated and control groups at each sampling period. Lice counts, weight, length, and condition factor data shown are all averages of the arithmetic cage means within each group. Standard deviations are indicated by brackets.

	Pre-treatment[1]			1st Post-treatment[1]				2nd Post-treatment[2]			
	Medicated	Control	p-value[6]	Medicated	Control	p-value[6]	reduction [3]	Medicated	Control	p-value[6]	reduction [3]
Copepodids	2.1 (2.0)	2.1 (1.9)	0.985	0.9 (0.5)	1.2 (0.6)	0.140		1.4 (1.6)	1.0 (1.2)	0.219	
Chalimus 1&2	1.8 (1.6)	1.9 (1.4)	0.368	**1.1 (1.2)**	**5.0 (4.2)**	**0.000**	**78%**	**1.1 (0.9)**	**2.4 (1.7)**	**0.000**	**54%**
Chalimus 3&4	0.6 (0.4)	0.6 (0.4)	0.991	**0.2 (0.2)**	**1.1 (0.9)**	**0.000**	**82%**	**0.3 (0.2)**	**0.5 (0.4)**	**0.001**	**40%**
Pre-Adult	3.5 (1.6)	3.0 (1.2)	0.340	**1.7 (2.0)**	**6.5 (7.3)**	**0.000**	**74%**	**0.5 (0.5)**	**0.9 (1.0)**	**0.002**	**44%**
Adult	0.6 (0.4)	0.6 (0.3)	0.757	**1.3 (1.4)**	**2.8 (3.7)**	**0.023**	**54%**	0.4 (0.6)	0.6 (0.7)	0.460	
Gravid	1.7 (0.8)	1.4 (0.6)	0.240	2.4 (1.3)	2.4 (1.4)	0.947		2.1 (1.5)	2.4 (1.8)	0.211	
All Chalimus	2.4 (1.9)	2.5 (1.6)	0.446	**1.3 (1.5)**	**6.1 (5.0)**	**0.000**	**79%**	**1.4 (0.9)**	**3.0 (1.9)**	**0.000**	**53%**
Mobiles[4]	4.1 (1.8)	3.6 (1.4)	0.353	**2.9 (3.2)**	**9.3 (10.8)**	**0.003**	**69%**	**0.9 (1.0)**	**1.5 (1.6)**	**0.038**	**40%**
Total	10.3 (5.0)	9.6 (4.5)	0.602	**7.6 (5.4)**	**19.0 (15.4)**	**0.000**	**60%**	**5.8 (4.2)**	**7.9 (5.1)**	**0.014**	**27%**
Weight (kg)	3.0 (0.4)	3.1 (0.3)	0.193	3.3 (0.6)	3.2 (0.5)	0.491		3.5 (0.5)	3.5 (0.4)	0.627	
Length (cm)	61.1 (1.7)	61.7 (1.9)	0.203	63.0 (2.3)	62.5 (2.7)	0.518		64.5 (2.0)	64.1 (2.4)	0.628	
Condition Factor[5]	1.32 (0.10)	1.30 (0.08)	0.319	1.30 (0.10)	1.29 (0.09)	0.866		1.29 (0.11)	1.30 (0.08)	0.820	

[1]Pre-treatment and 1st post-treatment calculations are based on 20 cages in each treatment group.

[2]2nd post-treatment calculations are based on 17 cages in each group

[3] "Reduction" is the % reduction seen in medicated cages as compared to control cages. It was calculated only where a statistically significant difference was detected.

[4] "Mobiles" includes pre-adult and adult stages.

[5] Condition Factor is $wt/lng^3 * 100000$

[6] p-values were the results of tests (random effects linear regression) on log transformed counts, consequently the proportional reduction was tested rather than absolute means

3.4.2 Damage scores

While there was a significant correlation between the number of lice and damage score ($r=0.33$, p-value< 0.01), Chi-square tests showed no difference in damage scores between medicated and control groups at each sampling period. The majority (68%) of the fish examined during the study had a damage score of "0" (no damage). Almost all of the rest (29%) had a damage score of "2" (small areas of superficial damage). Only 35 fish (0.8%) had damage scores of "3" or "4". There were no fish with a score of 5. Although not recorded, it seemed that a high proportion of the fish given a score of "1" (increased mucus) were dark in colour and appeared to be grilse (becoming sexually mature) (80). Table III shows the distribution of damage scores by treatment group at each sampling period.

Table III. Distribution of damage scores at each of the sampling periods during the clinical trial. Chi-square tests were performed on a no damage (score=0) versus damage (score $\geq$ 1) basis.

Pre-treatment

Damage score	0	1	2	3	4	5	total
control (n=20)	646	7	146	0	0	0	799
medicated (n=20)	657	13	127	0	0	0	797
total	1303	20	273	0	0	0	1596

Chi-square value with one degree of freedom = 0.67, p-value = 0.41

First post-treatment sample

Damage score	0	1	2	3	4	5	total
control (n=20)	481	12	271	9	2	0	775
medicated (n=20)	464	21	284	5	1	0	775
total	945	33	555	14	3	0	1550

Chi-square value with one degree of freedom = 0.78, p-value = 0.38

Second post-treatment sample

Damage scores	0	1	2	3	4	5	total
control (n=17)	342	15	229	14	0	0	600
medicated (n=17)	374	22	200	1	3	0	600
total	716	37	429	15	3	0	1200

Chi-square value with one degree of freedom = 3.55, p-value = 0.06

3.4.3 Lethal Sampling

Ivermectin Analysis

Ivermectin residues in the tested liver tissue averaged 2.4 ppb $\pm$ 1.6 sd. The analysis results by cage and sampling period can be seen in Table IV.

Lice counts on lethally sampled fish

The lice counts performed on the 160 euthanised fish were compared to the ante-mortem counts on the same fish. Interestingly, the euthanised fish "lost" an average of approximately 45% of the lice that were present when the fish were alive. The differences by stage are shown in Table V. The water which was in the bags with the euthanised fish was examined, but there was always debris (primarily regurgitated food) which made finding any lice, particularly chalimus stages, very difficult. Very few lice were found in the water or on the inside of the bags.

3.4.6 Environmental measurements

Temperature and dissolved oxygen readings were averaged by site and are presented in Table VI.

Table IV. Results of ivermectin analysis on liver tissue from lethally sampled fish during the clinical trial at Site 3. "Medicated" and "control" refer to teflubenzuron treatment group.

Pre-Treatment sample (each sample = 2 pooled livers)		Pooled sample #1 Ivermectin tissue levels in ppb	Pooled sample #2 Ivermectin tissue levels in ppb
Cage F		0.96	0.79
Cage I		0	0
Cage J		0.62	0.51
Cage L		0.98	0.76
Cage M		1.2	0.97
Cage R		1.56	1.28
1st Post-treatment sample (each sample = 2 pooled livers)			
Cage F	control	3.76	3.42
Cage G	medicated	4.15	4.09
Cage J	medicated	4.55	3.98
Cage L	control	4.85	4.96
Cage M	medicated	3.72	3.05
Cage R	control	3.3	3.46
2nd Post-treatment sample (each sample = 4 pooled livers)			
Cage F	control	3.94	
Cage G	medicated	2.33	
Cage J	medicated	1.56	
Cage L	control	2.48	

Table V. Average number of lice per fish on lethally sampled fish. (n=160 fish)

	Cop	Chal 1&2	Chal 3&4	Pre-adult	Adult	Gravid	Total
Pre-euthanasia	1.5	1.8	0.6	1.8	0.5	0.9	7.1
Post-euthanasia	0.7	1.1	0.3	0.9	0.5	0.4	3.9
% Loss	56	37	50	50	0	52	45

Table VI. Dissolved oxygen and temperature measurements averaged by site.

Site	Dissolved Oxygen (mg/l)		Temperature (°C)	
	mean (s.d.)	min - max	mean (s.d.)	min - max
1	7.5 (0.5)	6.54 - 8.87	12.3 (0.4)	11.7 - 13.1
2	8.1 (0.3)	7.42 - 8.80	12.0 (0.1)	11.8 - 12.3
3	7.0 (0.4)	6.10 - 7.83	12.1 (0.2)	11.7 - 12.3

3.5 Discussion

The effect of medication was most evident in the stages of lice which molt, namely the chalimus and pre-adult stages. At one week after the end of treatment there was a 79% reduction for chalimus stages and a 69% reduction for mobile (pre-adult and adult) stages in medicated as compared to controlled cages. This was lower than results reported by Ritchie (49) who found up to 95% effectiveness against chalimus and pre-adult stages with teflubenzuron treatments in sea cages, though the time after treatment was not reported. The effect of treatment was reduced by 14 days after the end of medication, but it was still significant with 53% and 40% reductions in chalimus and mobiles respectively as compared to control cages.

Reductions seen in this study may well underestimate the potential effect of teflubenzuron treatment. The control cages may have provided a ready supply of lice to transfer to the medicated cages. Ritchie (81) demonstrated that mobile stages can transfer between fish in the same cage as well as between fish in different cages. Increased recruitment rates of mobile lice due to neighbouring untreated cages should not occur under normal circumstances where all cages at a site would be treated.

The relatively low levels of lice infestations may have also contributed to an underestimate of effect simply because the range between medicated and control groups was so narrow. Had pre-treatment lice levels been higher, the observed magnitude of decrease in the medicated group may have been larger. This was

supported by the findings that, within matched pairs, as the average number of lice increased, the percent reduction increased.

There is also the possibility that the sampling interval may not have been ideal for detecting maximum reductions. While pharmacokinetic information is not available, the expected withdrawal time is short (47) which would indicate that the drug may not be active against lice for long after treatment. It may be possible that some copepodids could have attached and possibly molted once between the end of the treatment period and the first post-treatment sampling period.

The effect of the treatment on the adult lice was possibly more than would have been expected. Since the adults do not moult, it would not be expected that a chitin-inhibitor would have any noticeable effect on them. However, during the treatment period, some of the female adults may have matured into gravid females (developed egg strings), while the maturation of pre-adults into adults would have been reduced by the drug. This would have resulted in a reduction in the number of adults.

Teflubenzuron was more effective against the chalimus stages than the mobile stages. This is in contrast to the currently available bath treatments. Azamethiphos, an organophosphate bath treatment, which is the primary compound used for sea lice infestations in Canada, is more effective on the adult and mobile stages than the chalimus stages (32, 33). Hydrogen peroxide, also a bath treatment, is only effective against the mobile stages (82).

Three pairs of cages from Site 3 were removed after the first post-treatment sampling period due to high lice levels. Bath treatments (azamethiphos) were then administered to one cage from each pair. The primary impact of this on the study was a subsequent reduction in the number of cages available for the second post-treatment sampling period. This reduction in sample size may have reduced the power to detect a difference between medicated and control cages. Another potential impact on the study is that the bath treatments performed on these cages may possibly have affected lice populations in other cages at the site.

It can be seen from Table II that lice counts dropped between the first and second post-treatment sampling periods. This reduction occurred in all cages at Site 2 and Site 3. The reason was unknown. The only unusual events that occurred during that interval was the bath treatment of the 3 cages at Site 3, and a very large rainfall. It is possible that chemical treatment drift from the treated cages at this site (or neighbouring sites) adversely affected the lice in these cages. However, the treatments at this site were reportedly done while the tidal currents were moving away from the other cages. Even if the currents had been directed towards the other cages, the chemical should have been diluted and in contact with the fish for a much shorter time than required for a standard treatment. Therefore these bath treatments should not have affected the study cages. Also, it would be very unexpected that the rainfall could have so dramatically reduced the number of lice. Brocklebank (83) reports that incessant

rains will remove lice from salmon; however this rain lasted less than two days. Although other farmers in the area did not observe similar reductions (anecdotal reports), there was no on-going lice recording system to monitor changes. Since this apparent reduction occurred in all study cages, it may have reduced the power of the study to detect a difference between medicated and control cages but should not have otherwise affected results.

The lice counts on the lethally sampled fish permitted a detailed assessment of identification criteria for life-cycle stages on live fish. As a result of this assessment, it was concluded that identifications had been correct. It appears that all stages of lice were subject to the post mortem loss except adults. Whether these losses were as a result of the euthanasia bath, or possibly because of the time delay between euthanasia and examination is unknown. Bristow and Berland (84) also determined that lethal sampling (no anesthetic) followed by storage on ice prior to examination for external parasites was not a suitable method for accurate counts.

The ivermectin levels found in the lethally sampled fish were probably higher than would be expected in non-treated fish, but still well below what would be found in recently treated fish. The reason for the apparent increase from pre-treatment sampling period to the post-treatment sampling periods is not clear. The feed was not tested. Ivermectin treated fish have ivermectin residues in the liver which are much higher (459 ppb $\pm$ 103 SEM, with a half life of 98 days) (79) than the levels found in these fish (2.4 ppb $\pm$ 1.6 sd). Based on this, and the fact

that these fish had received no ivermectin treatments since the year before, it appears unlikely that the ivermectin levels were high enough to have affected lice burdens.

It was hoped that the damage scores would provide a useful measure of the effect of lice on the fish, and indirectly, a measure of treatment effect. However, the scoring system used during this trial proved not to be very useful for this purpose. The damage score of 1 (increased mucus) proved difficult to assess because it was very subjective, and may have been more of an indication of sexual maturity (grilse), rather than damage from lice.

There were several aspects of this trial that make it unique. It is the first example of an aquaculture clinical trial performed to Good Clinical Practice (GCP) standards in North America. As explained in Chapter 2, GCP provides a mechanism for validation of what was done during the trial.

The mechanisms and strategies used to help control bias were well beyond what is usually done in aquaculture trials. Unfortunately, blinding, matching, randomization, and efforts to control selection bias during sampling are all rarely used in aquaculture field trials. Negative controls are not often used in sea lice treatment studies because of the risk to untreated fish. In such studies the pre-treatment lice counts serve as a control and are compared to post-treatment lice levels.

The blinding of investigators and farm management to the treatment group was considered important for removing subjective biases in several aspects of the

study. Farm managers were allowed to alter the trial end-point for each cage by requesting the removal of any cage with either unacceptably high lice numbers or potential for skin damage. It was important that this subjective evaluation be performed without the bias of extra attention or anxiety about a cage being part of the non-medicated control group. As well, blinding prevented any other management differences between the two groups of fish which could possibly alter the lice numbers. Blinding investigators was important because lice counts could be subject to some unintentional and uncontrollable counting bias if someone was expecting a change due to the use of a treatment.

Sample size estimations were performed during the design phase of this study. Unfortunately, sample size calculations are rarely reported in other aquaculture studies. The standard number of fish sampled per cage seems to be about ten fish per cage in most sea lice studies. Based on the sample size calculations (see Methods) for this trial a sample size of ten fish per cage may be inadequate for a precise estimate of cage lice levels.

The ratio of 1:1, medicated to control cages, in this study was considered essential for several reasons. For a given sample size, having equal numbers in each group can often enhance precision (61). Also, in order to best utilize matching as a strategy for controlling bias, there had to be equal numbers of cages for each group. Finally, equal numbers of cages in each group can help to maintain a blinded study. This was highlighted at Site 2 when one cage had to be removed due to disease problems, and the treatment allocation was subsequently

revealed to site workers. The unexpected drop in lice levels found during the second post-treatment sampling period in all cages at Site 2 and Site 3 demonstrated the necessity for adequate numbers of control pens. Had this trial depended on before-treatment versus after-treatment lice counts, these reductions would have been incorrectly attributed to the medication.

Cage pairs were matched based on mean total lice counts and cage size, and then randomly allocated into medicated and control groups. Through this matching and randomization process it was assumed that the two treatment groups would not differ from each other in characteristics other than the treatment and that any unknown confounding factors would be balanced into both groups. The two groups had no significant difference (random effects linear regression, $p>0.05$) in weight, length, or condition factor (Table II). Also, no significant differences were found between the two groups before treatment in any of the lice stages (random effects linear regression, $p>0.05$).

This clinical trial demonstrated that teflubenzuron is effective for reducing sea lice on salmon. It also demonstrated that randomized, controlled, double-blind clinical trials can be performed in a commercial aquaculture setting.

4. HISTORICAL CONTROL CLINICAL TRIAL TO ASSESS THE EFFECTIVENESS OF TEFLUBENZURON TO TREAT SEA LICE ON ATLANTIC SALMON

4.1 Introduction

Teflubenzuron is an orally administered chitin synthesis inhibitor. In preliminary studies, it has been shown to be effective against all stages of lice that undergo a molt, including the larval and pre-adult stages (49). The clinical trial reported in Chapter 3 showed that teflubenzuron reduced chalimus burdens by about 80% and mobile stages by about 70% in medicated cages.

Teflubenzuron is likely to be used as a sea lice treatment for all cages at a site simultaneously. In the randomized clinical trial (Chapter 3), however, half of the cages at the site were medicated and half were negative controls. It has been demonstrated that mobile stages of *L. salmonis* can transfer between fish, and between sea cages within a site (81). Consequently, it is feasible that untreated cages in the clinical trial provided a reservoir of lice, some of which may have moved to the treated cages, thus reducing the apparent treatment effect.

The objective of this study was to monitor the effectiveness of teflubenzuron after a 7 day course of treatment under conditions of routine use and exposure rates. All cages were treated so as to assess the impact of treatment under conditions similar to the intended use of the product. Post-treatment lice counts were compared to pre-treatment lice counts. Although the

use of historical controls is not usually a preferred method in clinical trials, this design allowed for assessment with natural recruitment from the environment without the potential interference caused by recruitment from non-treated cages at the same site.

4.2 Materials and Methods

All cages at one seawater Atlantic salmon site were treated with teflubenzuron and lice were counted before and multiple times after treatment. The study began on July 8, 1996, and ended on August 8, 1996.

4.2.1 Site Selection Criteria

This was an trial involving nine cages from one commercial sea cage site of Atlantic salmon in the Bay of Fundy, NB. There were six cages of 1995 year class fish (pre-markets) and three cages of 1996 year class (smolt) fish. Prior to acceptance into the study, the site was required to meet the following basic criteria:

1. Minimum of four cages with Atlantic salmon of the 1995 year class which were of similar weight and contained in cages with similar dimensions.
2. Owners and managers willing to accommodate the study design and delay other lice treatments until lice counts were obtained.
3. Record keeping and farm husbandry practices permitted the

necessary data collection for the trial (eg. regular lice counts and fish weight samples).

4. A history of lice problems in previous years.

The site was selected by the project supervisor (the study monitor) from the sponsoring company and visited by the investigators for interviews with the owners and managers to determine the appropriateness of the site for the trial.

4.2.2 Trial Initialization

This study was done in conjunction with an eco-monitoring study which was conducted by the sponsor as a separate project. The initiation of the eco-monitoring study was dictated by the expected molting time of lobsters and not by the most optimal monitoring period for effectiveness of teflubenzuron on control of sea lice. Thus, the start date was dictated by other influences not directly related to this study.

4.2.3 Early Withdrawal Criteria

During the study, the farm management was encouraged to examine fish within each pen for evidence of skin damage due to lice and to evaluate the lice count data for evidence of unacceptably high risk for future lice damage. Should the lice levels exceed the threshold for confidence that other available treatments (i.e. organophospate or hydrogen peroxide baths) would still be able to control lice problems, then the farmer was free to select cages for removal from the

study. Farm management was also free to remove cages from the study due to unanticipated management considerations incompatible with the study design, such as other concurrent disease problems requiring treatment or slaughter. The farm management was requested to provide 48 hour notice of impending alternate treatments for lice or early withdrawal so that a final lice count could be attempted by the investigators.

4.2.4 Treatment

Teflubenzuron was administered at a dosage of 10 mg/kg biomass/day as a medicated feed. The concentration of the teflubenzuron in the medicated feed was 2.0 kg/tonne. The feeding rate for the medicated feed was set at a rate of 0.5% biomass per day. The fish received the treatment for a period of seven consecutive days. This treatment regime was determined by a series of dose titration studies performed by the sponsor. These earlier studies examined dosages of 2-200 mg/kg of biomass and treatment periods of 3-14 days.

Moore-Clark (St. Andrews, NB) supplied medicated feed to the site. The study feed was delivered in plastic bags which were labelled with site and cage identification, weight of feed in the bag, and the number of bags that each cage was to receive daily (see Appendix A). Each cage had a label with the same information (see Appendix B).

The fish were fed to satiation twice daily. If the predetermined amount of medicated feed (study feed) was insufficient to satiate the fish within a cage for

the first feeding, non-study feed was supplemented. If the fish did not eat all of the study diet during the first feeding, the remaining study feed was fed at the second feeding, prior to any non-study feed. Non-study feed was the normal diet the fish were usually fed. For the pre-market fish this was a 9.0 mm Moore-Clark pellet which was also the base diet for the experimental feed. The smolt at this site were normally fed a moist feed, as decided by the owners.

4.2.5 Sampling

Pre-treatment samples were taken from all cages at the beginning of the trial to determine pre-treatment lice infestation levels and to obtain the mean fish weight for biomass and dosage estimates. To accurately estimate the biomass within a pen, a total of 100 fish from each pen were sampled and weighed during this first sample period. Cage biomass was estimated using the weights from these pre-treatment samples and the inventory estimates available from farm records. Lice counts were performed on a minimum of 25 fish per pen. All cages received medicated feed for seven days, then each cage was sampled three times to determine post-treatment lice levels: at 7 days after the end of treatment, at 14 days after the end of treatment, and again at 23 days after the end of treatment.

Sampling Protocol

Sampling was done by crowding the fish and then capturing a small

number of fish with a dip net, a method referred to as "crowd-and-dip". Crowding was accomplished by seining approximately 2000 fish within the cage. Two to five fish were dip netted at a time from the seine crowded group for sample measurements. Fish from every fourth dip net were sampled to reduce selection bias by introducing a systematic sampling scheme. The fish from the un-sampled dip-nets were placed into the post-sample section of the pen. Non-replacement sampling was performed to reduce the stress of crowding and capture on the fish, and to avoid sampling the same fish more than once. A minimum of 25 fish were sampled from each cage.

Anesthetic

Sampled fish were anaesthetized in a 50-100mg/l triciane methanosulfanate[9] (TMS™) bath prior to the measurements. A plastic container measuring approximately 1m X 1m X 1m, approximately half filled with water, was used as an anesthetic bath. Dosing was approximated by estimating the volume of the water and adding TMS™ powder measured by volume. The dosage was then adjusted based on anesthetic effect.

The water in the anesthetic bath was changed as needed, usually after one or two cages. Temperature and dissolved oxygen were not routinely monitored, and no aeration was necessary, due to the frequent changing of anesthetic water.

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[9] TMS™, Syndel International Inc., Vancouver, BC

Sampling Measurements

The following measurements were made on each fish sampled.

1) fork length was measured to the nearest 1.0 cm. Using a measuring tape in a PVC pipe cut in half longitudinally.

2) weight was measured to the nearest 10 g using an Accu-Weigh DSY-1000[10] scale.

3) a total body count of all copepodids

4) a total body count of all stage 1&2 chalimus (grouped as one count)

5) a total body count of all stage 3&4 chalimus (grouped as one count)

6) a total body count of all pre-adults

7) a total body count of all non-gravid adult lice

8) a total body count of all gravid females

9) a subjective score of sea lice damage

0=no damage

1=increased mucus

2=small (<2cm^2) areas of superficial damage

3=large (>2cm^2) areas of superficial damage

4=small (<2cm^2) areas of deep damage

5=large (>2cm^2) areas of deep damage

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[10] Yamoto Corporation, Colorado springs, CO

Lice count procedure

Two of the investigators were experienced lice counters. To help ensure consistency, only these two people performed lice counts during the study.

The anaesthetized fish were placed on a tray after weight and length measurements were taken. The person counting the lice (the counter) started at the ventral midline of the fish and worked around the fish (ventral midline, lateral, dorsal, other lateral, and back to the ventral midline again) with consecutive longitudinal scans. As lice were found and identified, the counter told the person recording the data (the recorder) the stage of development and number of lice seen.

The recorder recorded the weight and length of the fish, and the lice counts. As the counter told the recorder what stage and how many lice were found, the recorder made tick marks on the data sheet in the appropriate space (see sample data sheet in Appendix C). When the counter was finished with the fish, the recorder counted the number of tick marks in each space. This number was written and circled within the appropriate space on the data sheet.

4.2.6 Environmental measurements

Dissolved oxygen and water temperature were measured at a depth between 2.5 - 3 m each day that treatment feed was fed, in each trial cage, using a YSI model 55[11] dissolved oxygen meter.

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[11] YSI Incorporated, Yellow Springs, OH

4.2.7 Data analysis

All data were entered into Quattro Pro[12] spreadsheet and then transferred to Stata[13] statistical software package for analysis.

Assessment of lice count differences between pre-treatment and each post-treatment sampling period was the primary analysis of interest. Descriptive statistics of lice counts were generated. Average numbers of lice per fish were calculated for each lice stage at each sample period, and for each cage at each sample period. Cage means were averaged to obtain sample period means and standard deviations. Percent reduction from pre-treatment lice levels ((Overall cage means post-treatment/overall cage means pre-treatment X 100) -100) was calculated and reported where a statistically significant difference ($p<0.05$) was found.

Significance was assessed using random effects linear regression with 'cage' as a random effect to adjust for clustering within cages. Including 'cage' also accounts for the lack of independence between sampling periods. For the purposes of this analysis, the dependent variable was a log transformed lice count {log(count + 1)} to ensure that the data were approximately normally distributed.

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[12] Corel Corporation Limited, Ottawa, ON

[13] Stata Corporation, College Station, TX

The general model for these regression analyses were:

$$y_{(i,j)} = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + u_{(j)} + e_{(i,j)}$$

y = log((lice count on i^{th} fish in the j^{th} cage)+1)

α = intercept

x_1 = dummy variable for 1^{st} post-treatment sampling period

x_2 = dummy variable for 2^{nd} post-treatment sampling period

x_3 = dummy variable for 3^{rd} post-treatment sampling period

β_1, β_2, β_3 = coefficients for sampling periods

$u_{(j)}$ = random effect of the j^{th} cage

$e_{(i,j)}$ = residual error

Analyses were performed for: copepodids, chalimus 1 & 2, chalimus 3 & 4, pre-adults, adults, gravids, all chalimus (1&2 plus 3&4), mobiles (pre-adult plus adults), and total lice (all stages). Reported mean values are non-adjusted (ie. not log transformed) averages of cage means.

4.2.8 Description of events during the study

Full communication of events occurred between the investigators, the sponsor, and the farmers while the study was being carried out.

Site description

The study site was located near Deer Island, NB. There were nine cages at the site, all were 70 m circumference circular cages. Six of the cages held pre-

market fish (1995 year class) and three cages held smolts (1996 year class). The cages with pre-market fish contained 18,000 to 20,000 fish each, and the smolt cages contained 35,000 to 40,000 fish each. A diagram of the site is shown in Figure 4.

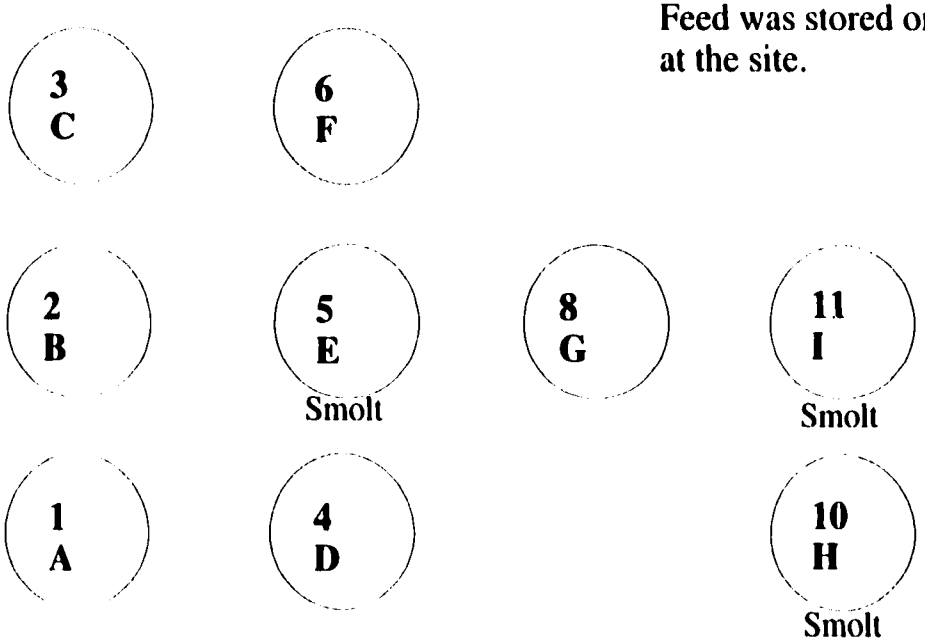
Figure 4

Historical Control Trial Site Diagram

Numbers = Farm's cage labels
Letters = Investigator's cage labels

All cages were 70 m (circumference) circles.

All cages were medicated.
Feed was stored on a barge anchored at the site.



Feeding procedures

Medicated feed was stored in the usual feed storage location for the site, a barge located near the cages.

Each morning the site workers would normally remove feed bags from the feed barge then transport the feed by boat to each cage for feeding. This procedure was repeated for the afternoon feeding. However, due to circumstances beyond the control of investigators, the feed was not delivered all at one time. On the first three days of treatment, the feed was picked up by the site workers on shore, rather than the barge, as it was delivered to the site in the morning. The research assistant observed the feedings, counted feed bags, and obtained dissolved oxygen and temperature measurements during the feedings.

Description of Events

This study was not expected to begin until late in the summer (August). However, it was initiated in early July to coincide with an eco-monitoring study which was also being conducted at this site. The eco-monitoring study began at this time to coincide with the expected molting time of lobsters (one of the species being assessed for unintended effects). Neither the methods, nor results, of the eco-monitoring study were available to the investigators in this study. Due to this unanticipated early start, quality assurance personnel were not yet available to perform necessary quality assurance procedures. Due to a lack of QA, this study was not done to GCP standards. However, all procedures were done according

to the protocol with minor exceptions as noted.

The study began with a pre-treatment sample on July 8. Fish were sampled by seining approximately 2000 fish and then using every 4th dip net of fish for measurements. Lice counts were performed on 25 fish per cage and weights were taken on a total of 100 fish per cage. Exceptions to this were that one cage (A) had weights measured on 98 fish, and another cage (H) had weights measured on 97 fish.

Medicated feed was administered to all cages on the site for seven consecutive days (July 10 to 16 inclusive). Site workers reported no observable adverse reactions to the feed.

On day one of treatment (July 10), enough feed for that day was delivered in the morning. On this day, one cage (C) received 8 bags rather than the prescribed 9.

On day two of treatment (July 11), enough feed for that day was delivered in the morning. On this day one cage (C) received 10 bags to make up for the previous day's shortage. It was noted that the fish in two cages (A and D) ate little of the feed offered on the morning of day two. There was no obvious reason for this decreased appetite. However, both groups had eaten very well the previous night and resumed full appetite by the afternoon of day two.

On day three (July 12), enough feed for that day was delivered in the morning. The remainder of the study feed was delivered the afternoon of day three.

Post-treatment sampling was done 7, 14, and 23 days after the end of treatment. Lice counts were performed on 25 fish per cage except that on day seven after the end of treatment (July 23), lice counts were performed on 50 fish in three cages (E, H, and I). These extra lice counts were included in the cage averages and analysis.

4.2.9 Trial Schedule

The trial began earlier than expected due to the needs of the concurrent eco-monitoring study. All trial dates, and sample sizes used are indicated in Table VII. The time-line for the study is depicted graphically Figure 5.

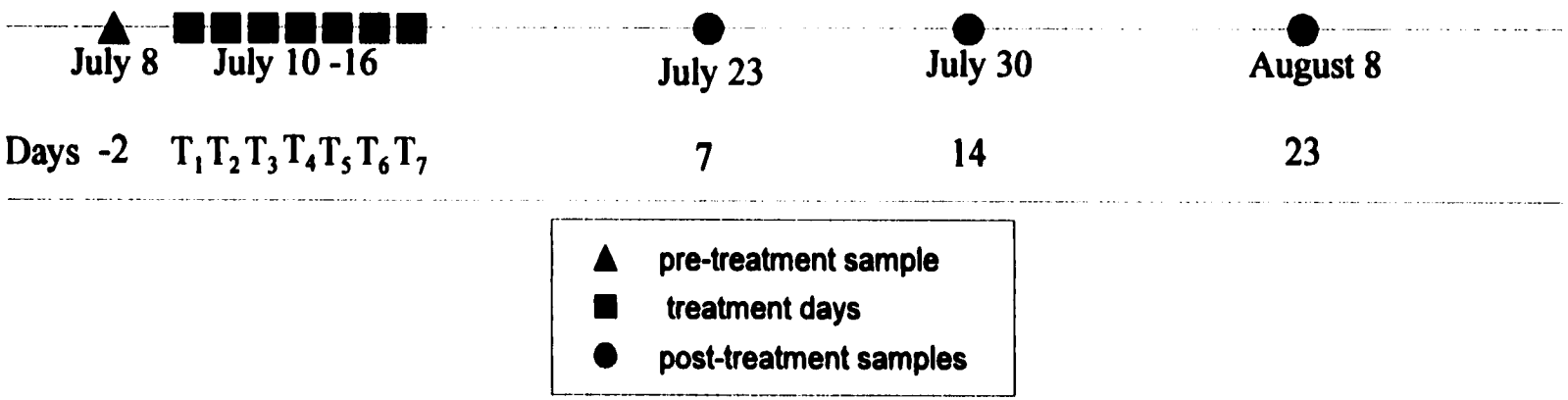
Table VII. Sampling dates, treatment dates, and sample sizes for the historical control clinical trial

Pre-treatment Sample	# of fish		Treatment Dates	1st Post-treatment sample	# of fish	2nd Post-treatment sample	# of fish	3rd Post-treatment sample	# of fish
	Wt	Lice counts							
July 8	100 [1]	25	July 10-16	July 23	25 [2]	July 30	25	Aug. 8	25

[1] cages A had weights measured on 98 fish and cage H had weights measured on 97 fish

[2] cages E, H, and I had lice counts and weight measures performed on 50 fish

Figure 5. Time-line showing the sampling and treatment schedule for the historical control clinical trial. The number of days before and after the treatment period on which sampling took place are indicated.



4.4 Results

4.4.1 Environmental measurements

Dissolved oxygen and temperature readings were taken once per day in each cage, during the seven days that experimental feed was administered. The temperature averaged 11.6^{0}C (standard deviation = 0.7), with a minimum of 10.0^{0}C and a maximum of 12.7^{0}C. Dissolved oxygen levels averaged 8.6 mg/l (standard deviation 0.7), with a minimum of 6.8 mg/l and a maximum of 11.05 mg/l.

4.4.3 Lice counts

The greatest effect of treatment was seen in the chalimus, pre-adult, and adult stages. At 7 days after the end of treatment there was a 92% reduction in chalimus stages and a 74% reduction in mobile (pre-adult and adult) stages when compared to pre-treatment levels. The total effect was reduced by 14 days after the end of treatment, but still evident with 41% and 61% reductions in chalimus and mobiles respectively. All treatment effects appeared to be very much reduced by 23 days after the end of treatment. Chalimus stages were still 36% reduced from pre-treatment levels, however, mobile stages had increased to 169% of pre-treatment levels. Copepodids were significantly reduced at all post-treatment sampling periods. Percent reduction in lice numbers by stage and sampling period are listed in Table VIII. Increases rather than reductions in lice numbers are indicated by (+). Graphs summarizing data in Table VIII are

available in Appendix H. The average(sd) number of lice (all stages) per fish for each of the cages is shown in Table IX.

Table VIII. Comparisons of lice counts at each sampling period during the historical control clinical trial. Data shown are averages of the cage means (n = 9 cages).

	Pre-treatment sampling period	1st Post-treatment sampling period			2nd Post-treatment sampling period			3rd Post-treatment sampling period		
	lice/fish (sd)	lice/fish (sd)	p-value [1]	reduction [2]	lice/fish (sd)	p-value [1]	reduction [2]	lice/fish (sd)	p-value [1]	reduction [2]
Copepodids	0.9 (0.5)	0.5 (0.3)	0.000	49	0.2 (0.2)	0.000	77	0.3 (0.1)	0.000	69
Chalimus 1&2	1.7 (0.8)	0.1 (0.1)	0.000	95	1.0 (0.5)	0.000	44	0.9 (0.3)	0.000	48
Chalimus 3&4	0.3 (0.2)	0.1 (0.05)	0.000	76	0.2 (0.1)	0.068		0.4 (0.2)	0.128	
Pre-Adult	1.6 (0.7)	0.4 (0.2)	0.000	73	0.7 (0.4)	0.000	56	4.2 (2.5)	0.000	(+)169
Adult	0.3 (0.2)	0.1 (0.1)	0.000	75	0.04 (0.1)	0.000	86	0.2 (0.1)	0.000	50
Gravid	0.3 (0.6)	0.3 (0.4)	0.374		0.2 (0.3)	0.320		0.3 (0.4)	0.273	
All Chalimus	2.0 (0.9)	0.2 (0.1)	0.000	92	1.2 (0.5)	0.000	41	1.3 (0.4)	0.000	36
Mobiles [3]	1.9 (0.7)	0.5 (0.4)	0.000	74	0.7 (0.4)	0.000	61	4.4 (2.5)	0.000	(+)132
Total	5.1 (1.7)	1.4 (0.8)	0.000	72	2.3 (1.1)	0.000	54	6.2 (3.1)	0.032	(+)22

[1] p-values were the results of tests (random effects linear regression) on log transformed counts, consequently the proportional reduction was tested rather than absolute means

[2] "Reduction" is the % reduction seen after treatment as compared to before treatment. It has only been calculated where a statistically significant difference has been detected ($p<0.05$). (+) indicates an increase in average lice numbers instead of a reduction.

[3] "Mobiles" includes pre-adult and adult stages.

Table IX. Cage mean lice counts (sd) at each sampling period during the historical control clinical trial.

pre-markets

Cage Letter	Pre-treatment sampling period (N=25 fish)	1st post-treatment sampling period (N=25 fish)	2nd post-treatment sampling period (N=25 fish)	3rd post-treatment sampling period (N=25 fish)
A	4.7 (2.6)	1.5 (1.4)	3.4 (2.5)	7.7 (3.2)
B	4.2 (2.5)	2.0 (1.4)	3.7 (3.1)	9.5 (8.1)
C	6.6 (2.8)	2.2 (2.4)	3.6 (2.8)	9.2 (4.5)
D	5.2 (3.7)	1.1 (0.9)	1.8 (1.1)	5.9 (2.9)
F	7.4 (4.5)	3.0 (4.8)	2.9 (1.7)	10.0 (3.8)
G	7.3 (3.0)	1.2 (1.2)	2.0 (1.4)	6.4 (3.7)
Mean (N=6 cages)	5.9 (1.4)	1.8 (0.7)	2.9 (0.8)	8.1 (1.7)

smolts

Cage Letter	Pre-treatment sampling period (N=25 fish)	1st post-treatment sampling period (N=50 fish)	2nd post-treatment sampling period (N=25 fish)	3rd post-treatment sampling period (N=25 fish)
E	3.8 (1.8)	1.0 (1.3)	1.4 (1.5)	2.7 (1.9)
H	2.9 (2.5)	0.4 (0.7)	1.0 (1.9)	2.0 (1.8)
I	3.9 (2.4)	0.5 (0.7)	1.2 (1.1)	2.9 (1.7)
Mean (N=3 cages)	3.5 (0.6)	0.7 (0.3)	1.2 (0.2)	2.6 (0.4)

4.5 Discussion

The effect of treatment was most evident in the stages of lice which molt. Chalimus stages were reduced by 92%, mobile stages were reduced by 74%, and all stages combined were reduced by 72% at one week after treatment. Though similar, this is somewhat higher than reductions seen in the clinical trial, which showed 79%, 69%, and 60% reductions respectively.

Copepodids were significantly reduced at each of the post-treatment sampling periods (49, 77, and 69% reductions). Direct effects to this stage would be unexpected because this stage has not yet molted after attaching to the fish. The reduction in copepodids may be because of adverse effects to the eggs and/or nauplius stages produced by gravid females which were on the medicated fish, resulting in fewer infective copepodids available for recruitment. In contrast, there was no effect observed in the copepodids during the randomized clinical trial. It is likely that gravid females in the non-medicated cages in that trial were able to produce a steady supply of infective copepodids to the rest of the site. This supports previous observations that egg strings on gravid females were damaged after teflubenzuron treatment (49).

This treatment compares favourably with other treatments available in North America. O'Halloran and Hogans (32) reported that azamethiphos reduced mobile stages by 98%, and chalimus stages by 68%. However, Roth et al. (33) reported reductions of over 85% for mobile stages, and no effect on chalimus, in

lice susceptible to azamethiphos. Hydrogen peroxide has been reported to remove 44 to 92% of mobile lice, depending on stage and sex of the lice (82).

While caution must be exercised when comparing the results of this study with the randomized clinical trial in Chapter 3 because of differences in methods and possible site effects, there are some questions that arise when looking at these two studies. It should be noted that these studies measured percent reductions in completely different ways. This study measured reductions post-treatment as compared to pre-treatment. The randomized clinical trial (Chapter 3) measured reductions in the medicated group as compared to the control group.

While not calculated for the clinical trial, it is possible to calculate percent reductions in medicated cages post-treatment as compared to pre-treatment. Table II in Chapter 3 shows that before treatment the medicated group had 10.3 lice per fish. At one week after treatment, they had 7.6 lice per fish. This was only a 26% reduction from pre-treatment levels. Using the same method, chalimus were reduced by 46%, and mobiles by 29%. These reductions in the randomized trial appear to be very different from this study. This trial seems to show that recruitment from untreated cages may very well have influenced the findings in the randomized trial. Alternatively, it may have been the result of better conditions for the lice to reproduce and infest salmon during the randomized trial which was conducted later in the summer.

As there were no negative control groups in the historical control trial, it was not possible to determine the protective effects of teflubenzuron at this site.

The observed increases in lice burdens at 23 days post-treatment likely would have been much greater if the fish had not been treated. Also, the lack of negative controls does not allow for a determination of how much of the reduction could have been due to unknown factors, such as occurred at Sites 2 and 3 in the randomized trial.

It was apparent that many pre-adult lice suddenly appeared before the third post-treatment sampling period (see Table VIII). Pre-adults increased from 0.7 per fish at the 2nd post-treatment sampling to 4.2 per fish at the 3rd post-treatment sampling. The source of these pre-adults is not clear. There were insufficient larval stages observed at the previous sampling period to account for this increase. The previous sampling period was only nine days prior, and it is highly unlikely that this was enough time for larval lice to attach as copepodids and grow to the pre-adult stage during that time. According to Johnson and Albright (23) it would take approximately 26 days, on average, for copepodids to mature to the first pre-adult stage at 10 ^{0} C . Grimnes and Jakobsen (85) found a development time of 14 days for lice to grow from the chalimus 1 and chalimus 2 stages to the first pre-adult stage at 9 - 10 ^{0} C . Although temperatures were not recorded during this stage of the study, Department of Fisheries and Oceans monitoring in nearby St. Andrews showed an average temperature of 12.5 ^{0} C for the month of July and 13.1 ^{0} C for August (86). While these temperatures are admittedly higher than 10 ^{0} C, it is unlikely that they were sufficient to hasten the development of the lice enough to account for the observed increase. At 10 ^{0} C

the chalimus 3 and 4 stages would have time to develop to the pre-adult stages (23). Even if the assumption is made that at 13^0 C all of the chalimus stages had sufficient time to develop, there was only a total of less than an average of 1.5 larval lice per fish observed at the previous sampling .

It is possible that the methods used did not allow for a complete count of all larval stages present. It has been demonstrated that significant proportions of copepodids can be found on the gills and in the buccal cavity (9, 23). If there were chalimus in the gills and buccal cavity, these lice would not have been counted. Subsequently, they would only be detected once they matured to the pre-adult stage and moved out of the gill and buccal cavity. This could account for some of sudden increase in pre-adults.

It is also possible that these lice came from outside of the site. It has been demonstrated that *Lepeophtheirus sp.* can transfer between fish in the same cage, and between fish in different cages (81). It has not been demonstrated how far pre-adults can travel to transfer between hosts. In this study the next closest site was approximately 500 metres away.

At the third post-treatment sampling period lice numbers had recovered to pre-treatment levels. This was 30 days after the treatment began. This would indicate that treatment with teflubenzuron should be repeated at about every 30 days, during the summer, to maintain control over the lice populations. This is the same treatment interval which is recommended when using the chitin synthesis inhibitor lufenuron to control fleas on dogs and cats (87).

This trial demonstrated that teflubenzuron is effective for treating sea lice on salmon. It supported previous observations (14, 88) that treating an entire site at once will probably have a greater impact on a site's overall lice load than treating cages individually. It also supports the possibility that lice control strategies which are based on coordinated lice control efforts within a geographic area may also have a greater benefit than individual farm strategies.

5.0 AN ASSESSMENT OF OUTCOME MEASUREMENTS AND SAMPLING METHODS IN AQUACULTURE

5.1 Introduction

The validity of data collection methods used in population based research must be established to ensure confidence in the resulting information. In an aquaculture setting, this is true whether the data are being gathered for research, or for production and/or health monitoring. Unfortunately, statistical evaluations of sampling methods routinely used on fish farms have been rarely performed (89).

In the Bay of Fundy salmon farming industry, health management decisions and policies at the farm-level are often based upon inadequate or invalid information, often depending on reference to anecdotal accounts of general trends. Many management decisions can have significant economic impacts on the farm, such as disease treatment decisions or harvest projections.

The goal of this study was to examine some aspects of data collection as it relates to weight samples and sea lice counts in sea cage salmon.

Fish weights are one of the most commonly measured production parameters on fish farms. Periodic weight samples can help producers track growth rates and feeding rates, and measures of productivity which are also indirect measures of fish health. These samples are also needed to calculate feed conversions and to plan harvests. With so much useful information that can

be gained from weight samples, it is important that they are done in a manner that produces meaningful data and in such a way that comparisons between cages and sites as well as temporal trends can be examined.

Lice counts are generally performed to help the farmers monitor and predict trends in lice levels and to make treatment decisions. Precise estimates of lice burdens are also necessary in studies of treatment effectiveness. The validity of various counting methods or how different methods might impact lice management on the farm is relatively unknown.

On many farms, a commonly practised method to collect fish for sampling is to offer fish a small amount of food and using a dip net, capture some of the fish that come up to eat. This is referred to as "feed and dip" sampling. While this is probably the most convenient method to collect some fish, it is not random in a formal sense. The feed and dip method may bias toward fish that are hungry, willing to come to the surface, the first ones to arrive, or the least able to escape the net once caught. Although this method of sampling is not done by a formal random process, it is hoped that the fish selected are representative of the population of fish in the cage. Staff often try to enhance the representative qualities by purposefully rejecting extremes of size.

One possible way to avoid this potentially biased sampling method is to seine the cage, or pull up the cage net, and dip fish from within the crowded area. This method is referred to as "crowd and dip" sampling. While this method is not completely random either, it does not have the same obvious potential for bias of the feed and dip method. These two methods were compared to see if a relative

systematic bias could be detected for weights or for lice counts.

In addition to the sampling method used, lice counts may be affected by a number of factors including: anesthetic protocol, variability between counters, the use of live versus euthanized fish, and the sample size employed. The objectives of this aspect of the study were to: 1) compare differences in weights and lice counts between feed-and-dip sampling and crowd-and-dip sampling, 2) measure the effect of an anesthetic bath on lice counts, 3) compare different lice counters, 4) compare anesthetized live fish to euthanized fish for lice counts, 5) calculate sample sizes for weight and lice sampling using our data set as a source of variability estimates.

5.2 Materials and Methods

The overall evaluations of the effects of sampling and data collection procedures on fish weights and lice counts were performed by carrying out a series of separate but related smaller studies. Each of these is described in more detail below.

All statistical analyses were performed using Stata[14] statistical software package. Details of statistical procedures used are included in the description of each specific study. Corel[15] Quattro Pro, Corel Presentations, or Sigma Plot[16]

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[14]Stata Corporation, College Station, Texas

[15]Corel Corporation Limited, Ottawa, Ontario

[16]Jandel Corporation, San Rafael, California (now owned by SPSS, Chicago, Illinios)

were used to generate graphs and charts.

5.2.1 Feed-and-dip versus crowd-and-dip sampling

A feed-and-dip weight sample was performed on 5 to 10 fish in each of 16 cages of pre-market size fish followed by a crowd-and-dip sample on each cage. Since there were usually more fish sampled by the crowd-and-dip method, the first 5 to 10 fish (however many had been sampled by the feed and dip method in that cage) were used for the comparison. In other words, if ten fish were sampled by feed-and-dip, then the first ten sampled by crowd-and-dip were used in the comparison. All fish were anesthetized with TMS prior to examination, and they were returned to the cage immediately after the data was recorded. The cage average weights and lice counts for each method were compared by a paired t-test. A correlation coefficient was calculated, and the cage means obtained for each sampling method were plotted against each other.

5.2.2 Effect of anesthetic on lice counts

A comparison was made between anesthesia bathed fish and water bathed fish to see if the anesthetic significantly reduced the number of lice on the fish for counting. Thirty-eight fish were used for this study, 19 in each group. The fish were pre-market size fish (2 to 3 kg). The fish were euthanized by a blow to the head, then tagged so that they could be identified. Lice counts were performed on each fish, then the fish were placed in either an anesthetic bath (50-100 mg/l TMS) or a water bath. The fish were left in the bath for 2-3 minutes

(normal time to anesthesia) then lice counts were performed again. Counted lice were classified into larval and mobile stages (mobiles included pre-adults, adults, gravid females). The mean number of lice per fish was compared before and after the baths using a paired t-test. Due to limitations in the number of people available, blinding was not possible.

5.2.3 Differences between counters

A comparison was made between two people counting lice on the same fish to see if their lice counts differed significantly. Counts were compared on 110 fish. Two fish were anesthetized at a time in a 50-100 mg/l TMS anesthetic bath. Both counters then counted lice on a fish, each with their own assistant recording the counts. The counters then traded fish and counted the lice on the other fish. The counts were done this way so that one half of the fish were examined by each counter first, thus controlling for effects due to loss of lice between the two counts. The counters did not know the other counter's results. Frequency distributions of differences between counters were generated and graphed. Counter 1 versus Counter 2 plots were generated, and correlation coefficients were calculated. Paired t-tests were used to assess differences between counters.

5.2.4 Lice counts on live versus lethally sampled fish

Ten fish were sampled from each of 16 cages of pre-market size fish. The fish were first anesthetized in a TMS (50-100 mg/l) anesthetic bath and lice

counts were performed. Then the fish were euthanized by overdose in a TMS (~200 mg/l) bath. The fish were then placed into a plastic bag (10 fish per bag), and stored on ice until investigators returned to shore that day. The time between euthanasia and performance of post-mortem counts was recorded.

Comparisons were made between cage average counts pre- and post-euthanasia. Cage averages were compared for each stage (copepodid, chalimus 1 & 2, chalimus 3 & 4, pre-adult, adult, gravid) as well as total lice. Statistical significance was assessed using paired t-tests (cage mean from the ten fish ante-mortem versus cage mean from the ten fish post-mortem).

Linear regression was used to test if the time elapsed between euthanasia and post-mortem counts was related to the percentage of lice lost. Percentage of lice lost in each cage was the dependent variable and time was the independent variable.

5.2.5 Sample size calculations

When deciding how many fish to sample from a cage, a balance must be reached between how many fish are needed to get a good estimate of average weight or lice number, and how many fish there is time and resources to examine. The number of fish needed to get an estimate of the cage average can be calculated if three things are known: 1) estimated variability within a cage (standard deviation), 2) required precision of the estimate, 3) required confidence level of the estimate. The calculation is based on the equation (65):

$$n=4s^2/L^2$$

Where n is the number of fish to be sampled, 4 is the approximate square of Z = 1.96 which indicates 95% confidence in the estimate, 's' is the expected standard deviation in our sample (a measure of variability), and L is the required precision of the estimate.

To estimate the standard deviation for the weight sample calculation, the weight data from 185 samples from 40 cages of pre-market size salmon were examined. These cages were located on four farms in the Bay of Fundy. Weight sample data had been collected throughout the summer as part of a clinical trial testing an in-feed lice control product. As many cages as possible were included to examine variability from as wide an average weight range as possible. Standard deviation estimates were also gathered from the weight data from 12 samples from 3 cages of smolts at one site which had also been part of the lice control study.

In order to obtain estimates of standard deviation for lice counts, lice count data from 57 cages were examined. These data were from all control cages in the sea lice trial.

Both lice counts and weight samples were performed on 25 to 50 fish per cage. Fish were sampled by crowding fish within the cage with a seine and dipping out fish for examination.

The sample size estimations were performed by three different approaches. The first set of estimates were based on the assumption that variability remained constant. For this method a reasonable estimate of variability was chosen based on the mean and median standard deviations from all of the

cages. This estimate of standard deviation was then used in the sample size calculation.

The second set of estimates were based on the fact that as lice counts or weights increased, variability increased. The relationship between standard deviation and the number of lice or weight of the fish was defined by regression analysis. The standard deviation, as a function of the mean, was then used in the sample size formula. For example, if it was found that s (standard deviation) = 0.5μ (population mean), then:

$$n = 4s^2 / L^2$$

$$= 4(0.5\mu)^2 / L^2$$

Sample size estimates were then generated across a range of required precision levels and population means.

The third set of estimates defined the required precision as a function of the mean, i.e. required precision was defined as a percentage of the mean rather than an absolute number. For example, if one wants the estimate to be within 10% of the mean ($L = 0.1\mu$), then:

$$n = 4(0.5\mu)^2 / L^2$$

$$= 4(0.5\mu)^2 / (0.1\mu)^2$$

The resulting sample size would always yield a mean within 10% of the population mean. Sample size estimates were generated across a range of precision levels.

5.3 Results

5.3.1 Feed-and-dip versus crowd-and-dip sampling

Mean cage weights, based on samples of five to ten fish, for each cage by each sampling method are listed in Table X. The average weight measured by the crowd-and-dip method was larger in 11 of the 16 cages. This would seem to indicate that the crowd-and-dip method favoured selection of larger fish than the feed-and-dip method. In three of these cages the difference in estimated cage mean weight was over 500 g per fish. However, a paired t-test on the cage averages showed that the mean difference was not statistically significant (p-value= 0.17). The average weights from each of the two methods plotted against each other for each of the 16 cages in Figure 6. The correlation coefficient was moderate (r=0.71) between the two sampling methods.

In 10 of the 16 cages, the fish collected by the feed-and-dip method had fewer lice than the fish collected by the crowd-and-dip method (Table XI). However, the fish sampled by feed-and-dip averaged only about 0.7 lice less than the crowd-and-dip fish. A paired t-test on the cage averages of larval stages, mobile stages, and total lice (all stages) showed that the difference between the two methods was not statistically significant (p-value >0.05). There was also no statistically significant difference when each stage of lice was compared individually. Mean totals from each method plotted against each other for each cage are shown in Figure 7. The correlation coefficient for the two methods was 0.94.

Table X. Mean cage weights (kg) and standard deviations based on five to ten fish samples as measured by the feed-and-dip and crowd-and-dip methods in each of the 16 cages sampled.

Cage	(A) Feed-and-dip mean weight (sd)	(B) Crowd-and-dip mean weight (sd)	(A-B)
1	2.99 (1.19)	3.94 (0.44)	-0.95
2	2.94 (0.55)	2.89 (0.33)	0.05
3	3.24 (0.65)	3.00 (0.71)	0.24
4	3.20 (0.57)	3.70 (0.78)	-0.5
5	3.71 (0.47)	3.31 (1.17)	0.4
6	2.77 (0.14)	3.16 (1.04)	-0.39
7	3.35 (0.50)	2.96 (0.65)	0.39
8	3.37 (0.53)	3.50 (1.15)	-0.13
9	3.69 (1.03)	3.37(0.39)	0.32
10	3.07 (0.56)	3.44 (1.41)	-0.37
11	1.88 (0.62)	1.91(0.15)	-0.03
12	3.00 (0.79)	3.88 (0.45)	-0.88
13	2.70 (0.74)	2.87 (0.28)	-0.17
14	2.31 (0.42)	2.36 (0.54)	-0.05
15	3.77 (0.64)	3.86 (0.41)	-0.09
16	2.86 (1.02)	2.99 (0.56)	-0.13
AVG	3.05 (0.50)	3.2 (0.55)	-0.15

Figure 6. Average weights from each sampling method plotted against each other for each of the cages sampled. Diagonal line shows where points would be if there was perfect agreement between methods. Points above the diagonal line indicate average weights were heavier for feed-and-dip, below indicate crowd-and-dip heavier.

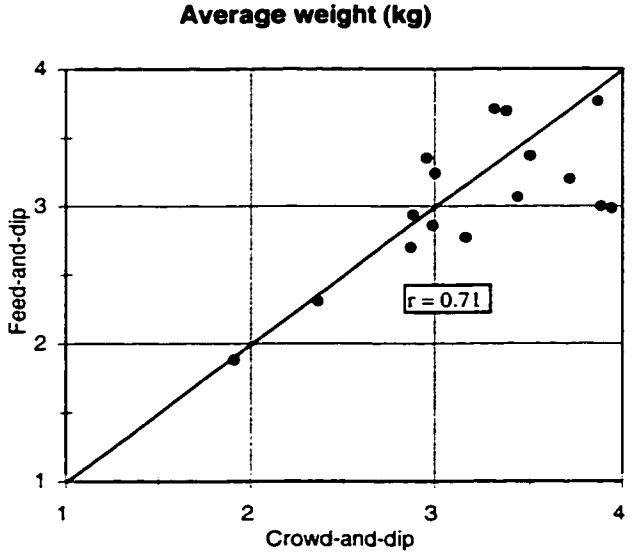
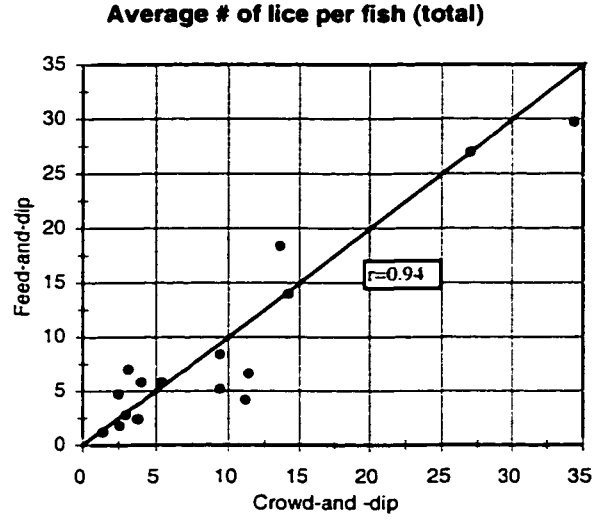


Table XI. Mean number of larval, mobile, gravid and total lice per fish (standard deviation) as measured by the feed-and-dip and crowd-and-dip methods in each of the 16 cages sampled. "Larval" includes copepodids, and all chalimus stages. "Mobile" includes pre-adult and adult stages. "Total" includes all stages found on the fish.

Cage	Feed-and-dip average # of lice per fish (sd)				Crowd-and-dip average # of lice per fish (sd)			
	larval	mobile	gravids	total	larval	mobile	gravids	total
1	0.4 (0.9)	1.6 (1.1)	2.2(0.8)	4.2 (0.8)	6.0 (8.0)	1.8 (1.1)	3.4(0.9)	11.2 (7.0)
2	16.0 (5.3)	9.2 (3.7)	1.8(0.8)	27.0 (7.9)	18.4 (7.3)	6.0 (3.5)	2.6(1.5)	27.0 (5.2)
3	8.8 (3.8)	3.4 (1.3)	1.8(1.3)	14.0 (4.8)	7.6 (4.8)	4.6 (2.1)	2.0(0.7)	14.2 (5.0)
4	3.6 (4.9)	1.2 (1.3)	0.4(0.5)	5.2 (6.0)	7.0 (6.5)	1.6 (2.5)	0.8(0.8)	9.4 (9.5)
5	0.2 (0.4)	0.8 (0.8)	1.8(1.3)	2.8 (1.1)	0.8 (0.8)	0.2 (0.4)	2.0(1.2)	3.0 (1.9)
6	3.0 (1.9)	3.6 (1.7)	0.4(0.5)	7.0 (1.6)	1.8 (2.5)	4.0 (0.5)	1.0(0.7)	3.2 (2.8)
7	4.8 (5.4)	0.6 (0.5)	0.4(0.5)	5.8 (5.8)	3.8 (3.0)	1.0 (1.2)	0.6(0.5)	5.4 (4.4)
8	5.8 (1.9)	1.6 (0.5)	1.0(1.0)	8.4 (1.1)	8.0 (4.0)	0.8 (0.8)	0.6(0.5)	9.4 (4.8)
9	1.4(2.2)	0.2 (0.4)	0.8(1.1)	2.4 (2.2)	2.8 (3.6)	0.2 (0.4)	0.8(0.8)	3.8 (3.8)
10	0.8 (1.3)	0.2 (0.4)	0.8(0.8)	1.8 (0.8)	1.6 (0.9)	0.2 (0.4)	0.8(0.8)	2.6 (1.8)
11	6.4 (1.7)	0 (0.0)	0.2(0.4)	6.6 (1.5)	9.8 (0.8)	0.4 (0.9)	1.2(1.3)	11.4 (1.3)
12	5.2 (5.1)	0.2 (0.4)	0.4(0.5)	5.8 (4.8)	3.4 (1.3)	0.2 (0.4)	0.4(0.5)	4.0 (1.6)
13	1.2 (0.4)	0 (0.0)	0(0)	1.2 (0.4)	0.6 (0.9)	0 (0.0)	0.8(1.3)	1.4 (1.5)
14	3.6 (1.8)	10.7 (4.9)	4.1(1.3)	18.4 (5.3)	2.8 (1.2)	8.4 (3.8)	2.4(1.6)	13.6 (3.4)
15	9.2 (3.4)	17.4 (5.8)	3.1(1.9)	29.7 (7.8)	7.8 (4.8)	21.5 (11.9)	5.1(3.2)	34.4 (13.7)
16	2.8 (2.1)	1.8 (1.5)	0.3(0.5)	5.8 (3.2)	1.5 (1.3)	0.8 (1.0)	0.3(0.5)	2.5 (1.0)
Avg.	4.6 (4.1)	3.3 (4.9)	1.1(1.2)	9.1 (8.7)	5.2 (4.6)	3.0 (5.5)	1.5(1.3)	9.8 (9.3)

Figure 7. Average total lice counts obtained by each method plotted against each other for each cage. Diagonal line shows where points would be if there was perfect agreement between methods.



5.3.2 Effect of anesthetic on lice counts

The 19 control fish had an average of 118 lice per fish (all lice stages) before and after the water bath. The 19 fish exposed to the anesthetic had an average of 120 lice before and 116 after the anesthetic bath. There was no statistically significant difference (paired t-test, p-value > 0.05) between the before and after counts in the control group. The anesthetic bath group did not show any significant difference (paired t-test, p-value > 0.05) in the larval stages or for total lice. However, there was a significant decrease (paired t-test, p-value < 0.01) after the anesthetic bath when comparing the mobile stages (mobile stages included all pre-adult, adult, and gravid lice). Before the anesthetic bath the fish averaged 39.5 mobile lice each, while after the bath the average was 33.9 mobile lice each (a reduction of about 13%). Average larval, mobile, and total lice per fish for both groups are shown in Table XII.

Table XII. Average number of lice per fish before and after exposure to anesthetic bath or water bath for two minutes. Each mean is based on a sample of 19 fish. The only significant (paired t-test) change in lice numbers as a result of a bath was found in mobile stages on fish exposed to the anesthetic bath.

	Number of larval lice per fish mean (s.d.) min-max	Number of mobile lice per fish mean (s.d.) min-max	Number of total lice per fish mean (s.d.) min-max
Control (water bath)			
Before bath	73.9 (41.6) 23-198	44.4 (25.8) 18-118	118.3 (51.8) 44-239
After bath	73.4 (41.3) 21-190	45.0 (28.9) 12-136	118.4 (53.8) 41-223
Difference	0.5 , p-value=0.63	0.6 , p-value=0.75	0.1 , p-value=0.98
Anesthetic bath			
Before bath	80.2 (71.9) 19-312	39.5 (27.7) 5-103	119.7 (94.7) 24-415
After bath	82.5 (78.8) 21-330	33.9 (22.6) 5-79	116.4 (97.3) 26-409
Difference	2.3 , p-value=0.41	5.6 , p-value=0.002	3.3 , p-value=0.21

5.3.3 Differences between counters

There was a high level of agreement between the two counters as can be seen in Table XIII. Counter 1 had an average of 9.5 total lice per fish and Counter 2 had a total of 8.7. The frequency distribution of differences between the two counters is shown in Figure 8A. In 27 of the 110 fish (25%) there was no difference in the total number of lice counted. In 62 of the fish (56%) the difference between counters was one or less. Most of the differences seen were in the larval stages of lice as can be seen in Figure 8B. A paired t-test comparing the mean counts showed that the differences between counters was not statistically different when looking at the mobile stages ($p > 0.05$). However the differences in larval stages and total lice (all stages) were statistically significant ($p < 0.01$). Counter 1 versus Counter 2 plots of larval and total lice are shown in Figures 9A and 9B respectively. Correlation coefficients were 0.93 for the larval stages and 0.95 for all stages combined.

Table XIII. Comparison of lice counts between two people counting the lice on the same fish. These means are based on 110 fish.

	Larval stages		Mobile Stages		Total	
	Mean (sd)	min-max	Mean (sd)	min-max	Mean (sd)	min-max
Counter 1	6.7 (6.9)	0-29	2.8 (3.0)	0-17	9.5 (8.8)	0-42
Counter 2	6.0 (6.5)	0-29	2.7 (3.0)	0-16	8.7 (8.5)	0-43
difference	0.7, p-value<0.01		0.1, p-value>0.05		0.8, p-value<0.01	

Figure 8A. Frequency distribution of the differences between counters for the total number of lice on the fish (all stages of lice). Each bar represents the proportion of fish with the indicated difference. (n=110 fish)

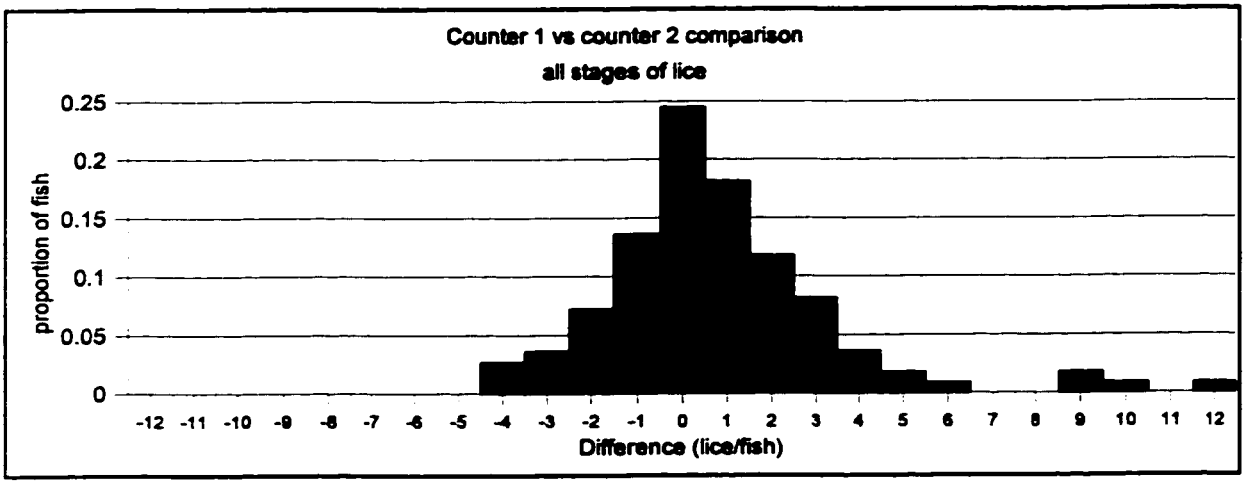


Figure 8B. Frequency distribution of the differences between counters for the larval and mobile stages of lice . Each bar represents the proportion of fish with the indicated difference. (n=110 fish)

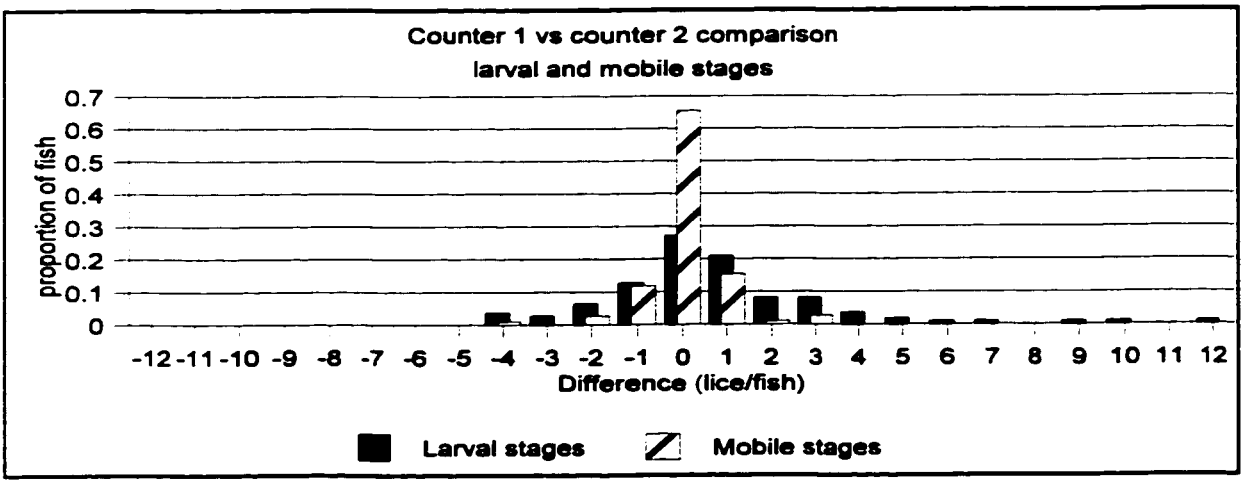


Figure 9A. Counter 1 versus Counter 2 larval lice counts, each point denotes one fish. Diagonal line shows where points would be if there was perfect agreement between methods. Each point represents one fish.

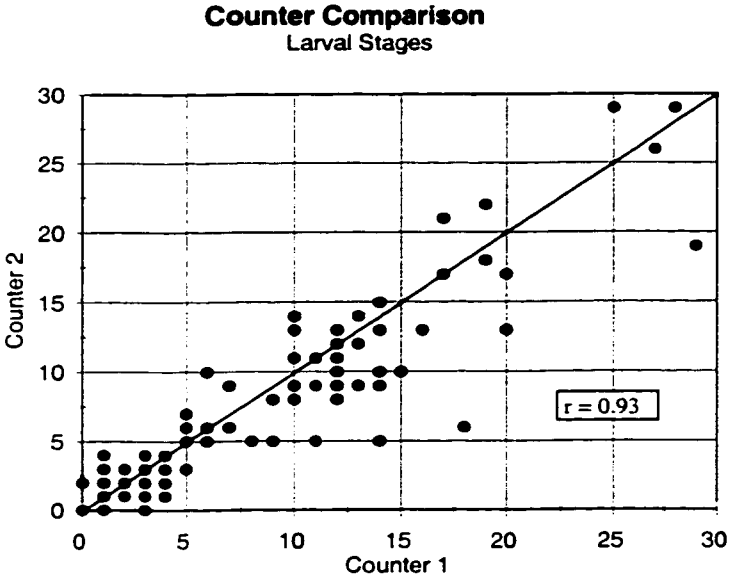
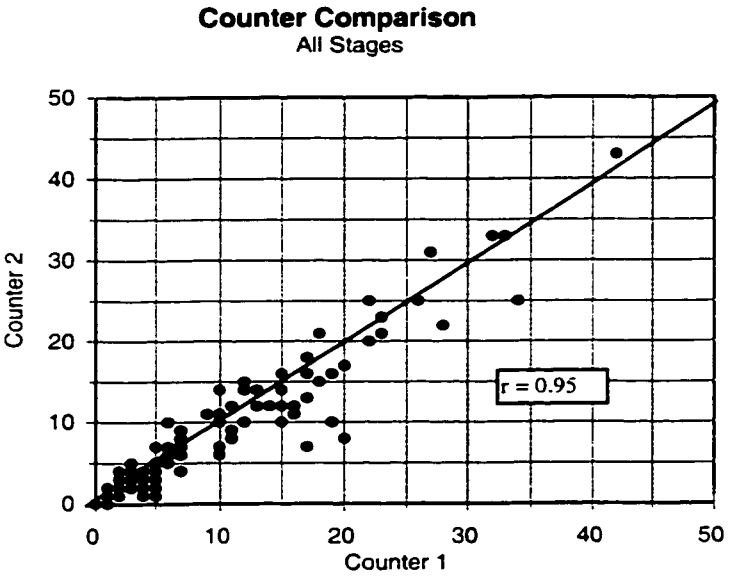


Figure 9B. Counter 1 versus Counter 2 total lice counts (all stages). Diagonal line shows where points would be if there was perfect agreement between methods. Each point represents one fish.



5.3.4 Lice counts on live versus lethally sampled fish

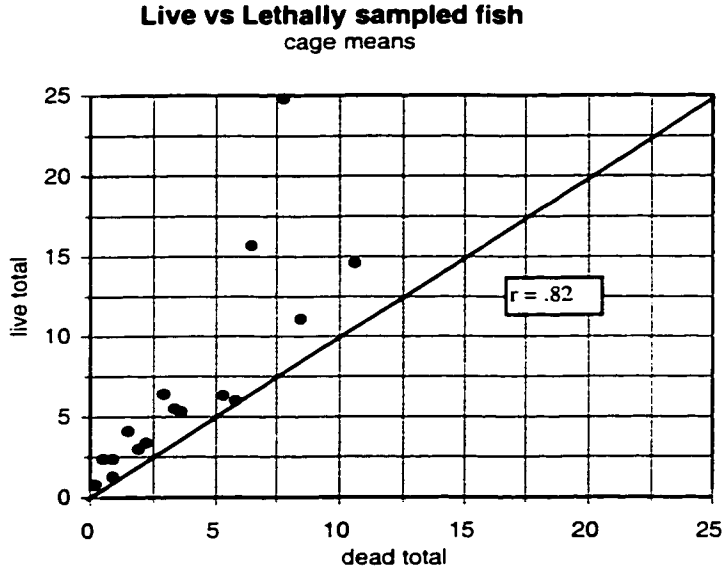
The overall average counts by stage are shown in Table XIV. The euthanised fish lost an average of approximately 45% of the lice that were present when the fish were alive. Statistically significant losses were observed in the copepodid, chalimus 1 & 2, gravid, and total (all) stages. The small amount of water which was in the bags with the euthanised fish was examined, but there was always debris (primarily regurgitated food) which made finding any lice, particularly larval stages, very difficult. Very few lice were recovered from this water or from the inside of the bags. A plot of counts for the live versus euthanised cage averages is shown in Figure 10.

The time between pre-euthanasia and post-euthanasia counts ranged from 0.5 to 10 hours, with a average of about 3.5 hours. While there was a weak correlation ($r=0.31$) between the amount of time elapsed and percentage of lice lost, regression analysis did not show a significant relationship (p-value = 0.25).

Table XIV. Average number of lice per fish on lethally sampled fish before and after euthanasia. Mean (standard deviation) and minimum - maximum shown for cage averages. Percent of lice lost calculated where a significant difference between pre- and post-euthanasia was detected (paired t-test, p-value < 0.05). (N = 16 cages).

	pre-euthanasia		post-euthanasia		% loss
Copepodid	1.5 (2.0)	0.1 - 7.4	0.7 (1.2)	0 - 4.7	56
Chalimus 1 & 2	1.8 (1.3)	0.1 - 4.6	1.1 (0.8)	0 - 2.6	37
Chalimus 3 & 4	0.6 (0.6)	0 - 2.6	0.3 (0.2)	0 - 0.6	
Pre-adult	1.8 (3.1)	0 - 12.4	0.9 (0.9)	0 - 2.5	
Adult	0.5 (0.7)	0 - 2.7	0.5 (0.7)	0 - 2.1	
Gravid	0.9 (0.6)	0 - 2.2	0.4 (0.3)	0 - 1.1	52
Larval stages	3.8 (3.1)	0.4 - 11	2.0 (1.8)	0.2 - 7.2	47
Mobile stages	3.2 (4.1)	0 - 16.8	1.9 (1.8)	0 - 4.7	
Total	7.1 (6.5)	0.8 - 24.8	3.9 (3.2)	0.2 - 10.6	45

Figure 10. Plot showing cage average counts ante-mortem versus post-mortem. There was a correlation coefficient of 0.82. Diagonal line shows where points would be if there was perfect agreement between methods.



5.3.5 Sample size calculations

Weights

The average fish weight per cage, based on 25 to 50 fish sampled per cage, in the 185 cages of pre-market sized fish was 3.0 kg, ranging from 1.3 kg to 4.6 kg. The average standard deviation was 0.66 kg , with a range of 0.35 to 1.34, with a median of 0.65 kg. In the smolt cages, average weights ranged from 0.13 kg to 0.27 kg with a mean of 0.2 kg. The mean and median standard deviations were both 0.03 kg, with a range of 0.02 to 0.05kg.

Assuming a constant level of variation (as measured by standard deviation), sample size estimates were generated for a range of required precision levels. The standard deviation estimates used for these calculations was 0.66 kg for pre-market fish and 0.03 kg for smolts.

The calculated sample size for a range of precision levels based on a 95% confidence level is shown in Table XV . The precision level represents the maximum deviation from the true cage mean that is acceptable 95% of the time.

For example, if one wanted to know within 200 g the average weight of fish in a cage of pre-market size fish, at least 43 fish would need to be sampled from that cage. The average weight as determined with that sample size would be within 0.2 kg of the true average weight of fish in the cage 95% of the time (assuming random sampling). If one wanted to know the average weight in a cage of smolt to within 10 g, at least 143 fish would need to be sampled from a cage.

Table XV. Calculated sample sizes required to have 95% confidence that the average weight estimated from a sample is within the required level of precision of the actual average weight of all fish in the cage.

Required precision (kg)	0.001	0.005	0.01	0.02	0.1	0.2	0.3	0.4	0.5
pre-market				4312	172	43	19	11	7
smolt	3564	143	36	9					

Using the data from the 185 cages of pre-market fish, a plot of average weight versus standard deviation showed that as weight increased so did the standard deviation. The plot and a regression line defining the relationship is shown in Figure 11A. The regression equation is:

s (standard deviation) = 0.21 + 0.15 weight ($p<0.01$, $r^2 = 0.32$)

Utilizing this equation, sample size estimates could be calculated for a variety of precision levels across a range of weights.

The results of these calculations are shown in Figure 11B. As average weight in a cage increased, the required sample size increased within a set level of precision. Also, as the required precision increased, the sample size increased. For example, if sampling a cage of fish that average 3 kg, a sample size of 25 would yield an estimate within 0.2 kg of the true average. To obtain an estimate within 0.1 kg of the true average, a sample size of 100 fish would be required.

The required precision can also be expressed as a function of the weight, i.e., the desired estimate is to be within a certain percentage of the true average. As required precision increases, sample size should increase as shown in Figure 12. For example, a sample size of 50 should always give an estimate within about 4% of the actual average weight of fish in a cage.

Figure 11A. Relationship between average weight of fish in a cage and the variability (standard deviation) observed in 185 cage-samples of pre-market salmon.

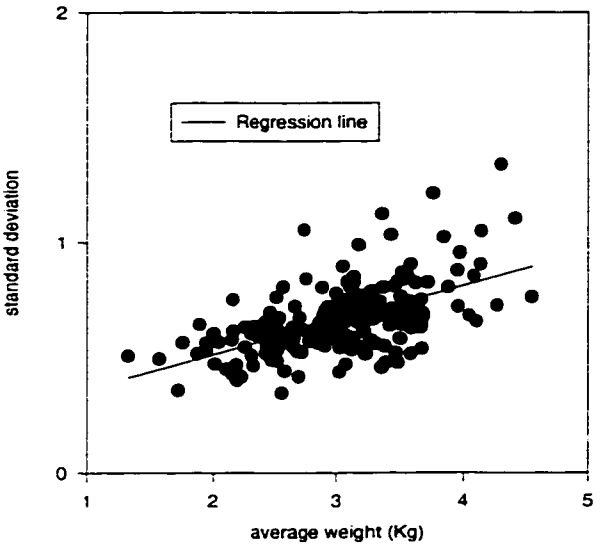


Figure 11B. Sample size estimations for weights based on the assumption that standard deviation is a function of average weight. Estimations presented are for pre-market salmon (2 to 4 kg). Precision levels within 0.1, 0.2, and 0.5 kg of actual cage average are shown.

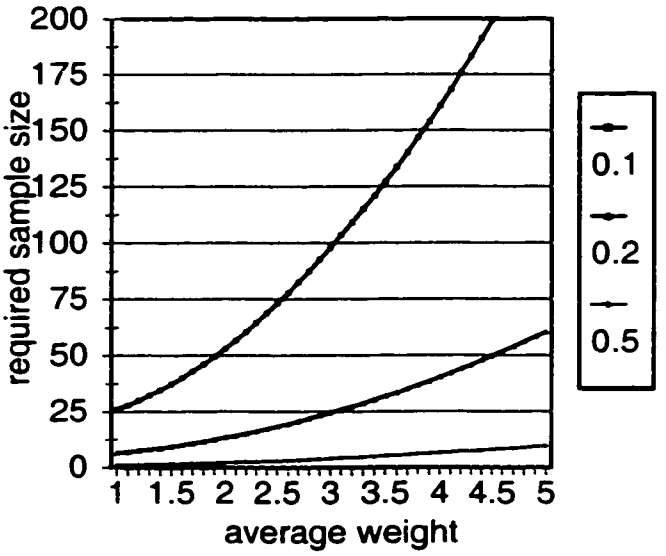
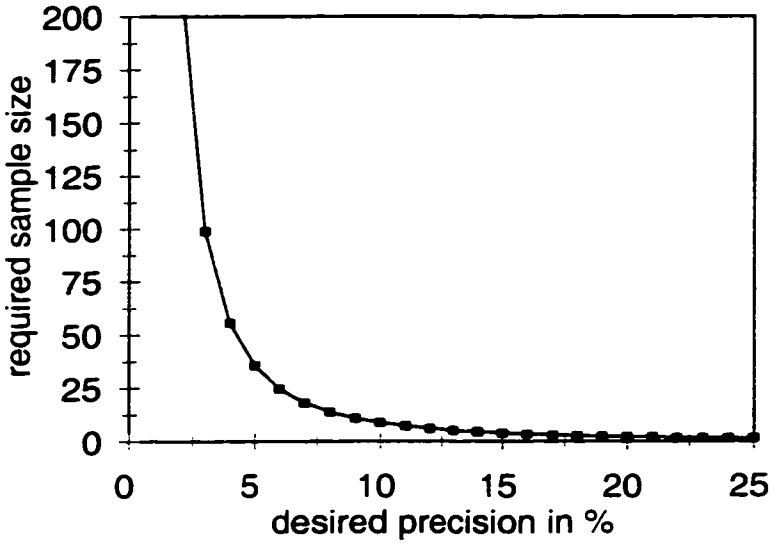


Figure 12. Sample size estimations for weights based on the assumption that variability is a function (s = 0.21 + 0.15 weight) of weight, and that required precision is a function (%) of weight.

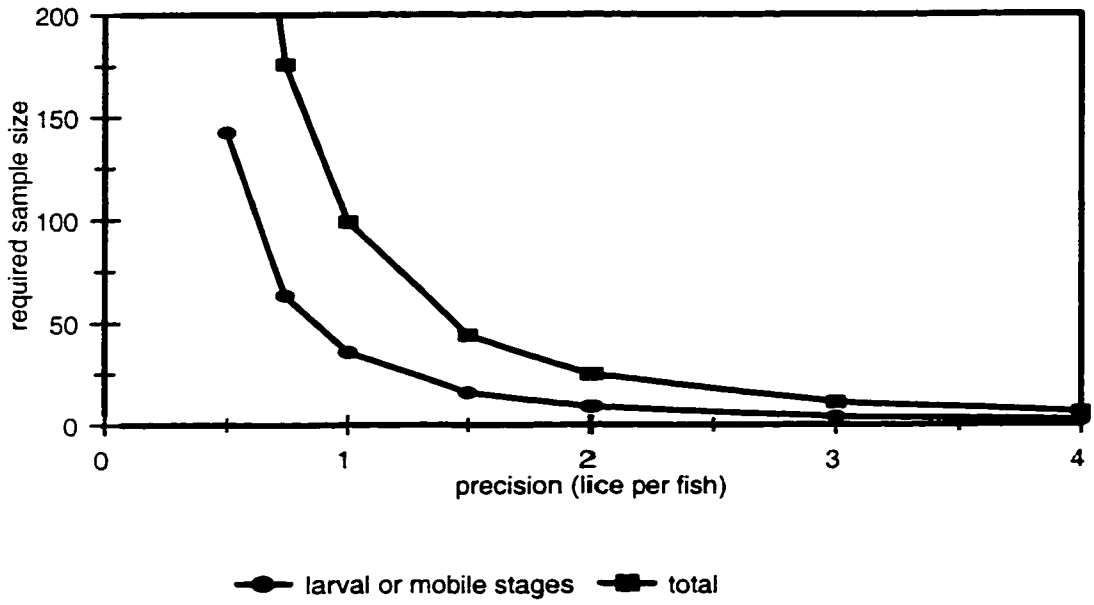


Lice counts

The 57 cages of pre-market fish averaged a total of about 12 lice per fish (all stages). On average the fish had 5 larval stages, 5 mobiles, and 2 gravid lice each. The cage level standard deviations for total lice ranged from 0.9 to 18.5, with a mean of 5.3 and median of 4.1 lice per fish. Standard deviations for larval stages (copepodid and all chalimus) ranged from 0.8 to 10.4 with a mean of 3.2 and median 2.6. Standard deviations for mobile stages (pre-adult and adult lice including gravid females) had a range of 0.6 to 11.9, the mean was 3.4, median of 2.5.

The sample size calculations were first performed with the assumption that variability was constant. Based on the above mean and median data, 5 lice per fish was chosen as a reasonable estimate of variability (standard deviation) for total lice. Three lice per fish was the estimate used for both the larval stages and the mobile stages. Calculated sample sizes, assuming a constant variation, for a range of precision levels based on a 95% confidence level are shown in Figure 13. For example, if the observed mean for total number of lice per fish is to be within 1 lice per fish of the true mean, then a sample size of 99 is required.

Figure 13. Sample sizes required to have 95% confidence that resulting mean will be within the desired number of lice per fish of the true mean.



Regression analysis of variability (standard deviation) and mean lice numbers showed that as lice levels increase, so does the variation. The regression equation for total lice (μ=population mean, i.e., average number of lice per fish) was: $s = 0.81 + 0.36\mu$ ($p<0.01$, $r^2 = 0.89$) (see figure 14A). The relationship for mobile stages was very similar with the equation: $s=0.83 + 0.38\mu$ ($p<0.01$, $r^2 = 0.94$). The relationship between number of larval stages per fish and standard deviation was: $s=0.63 + 0.49\mu$ ($p<0.01$, $r^2 = 0.81$).

Based on the relationship between mean number of lice and variability, estimates of sample size requirements were determined for total lice (all stages). The relationship between required precision, average number of lice per fish, and required sample size is shown in Figure 14B . As the average number of lice per fish increased, sample size must increase to maintain the desired level of precision. Also, as more precision is required, sample size must increase. For example, if a cage of fish had an average of about 10 lice per fish, and an estimate was required that was within 1 lice per fish, a sample size of about 55 fish would have to be taken. If the required precision only needed to be within 2 lice per fish, then a sample size of 13 would be sufficient in this cage. However, if there was an average of 40 lice per fish, then an estimate that would be within 2 lice per fish would require a sample size of about 200 fish.

The required precision can also be expressed as a function of mean lice number, i.e., the estimate must be within a certain percentage of the true mean. These calculations were performed based on the previously stated relationship between variability and average number of lice per fish ($s=0.36\mu$ for total lice).

The required sample size versus the desired precision as a percentage of the average number of lice is shown in Figure 15. Calculations were performed for total lice, mobiles, and larval stages. As the desired precision range decreased the required sample size increased. For example, if an estimate was required that was within 5% of the mean, a sample size of 200 fish was needed. If the estimate was required to be within 10% of the true mean, a sample size of 50 was needed. The number of lice per fish did not affect the sample size requirements in this model.

Figure 14A. Relationship between average number of lice (all stages) per fish and variability (standard deviation) observed in 57 cages of pre-market salmon. Each point represents one cage (25 to 50 fish were sampled per cage).

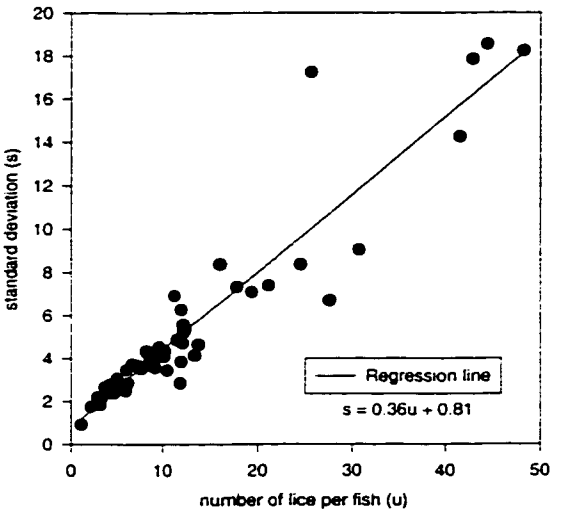


Figure 14B. Sample size estimations based on the assumption that standard deviation is a function of average number of lice (all stages) per fish when required precision is within 0.5, 1, 2, 3,and 5 lice per fish

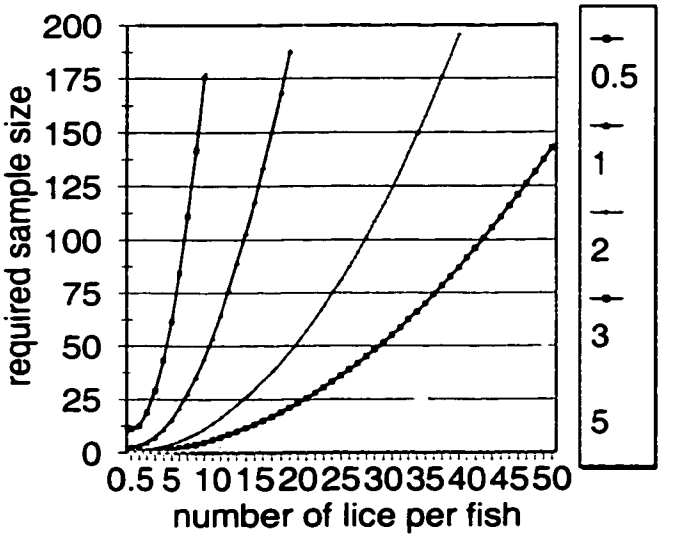
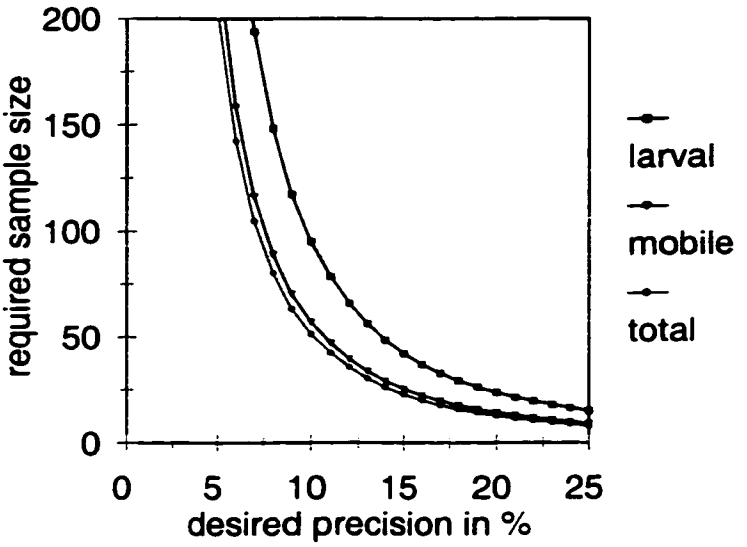


Figure 15. Sample size requirements when desired precision is a function (%) of average number of lice. Examples: A sample size of 50 fish will yield an estimate within 10% of the true average with 95% confidence. A sample size of 200 fish will yield an average within 5% of the true mean.



5.4 Discussion

5.4.1 Feed-and-dip versus crowd-and-dip sampling

No statistically significant differences were found between feed-and-dip versus crowd-and-dip sampling methods for either weight or lice count measurements. Fish sampled by the feed-and-dip method would be expected to be more aggressive, presumably larger, fish. Hammell (7) found that with a crowd-and-dip sample, fish tended to be larger than with a systematic random sample in a cage. It may be possible that both the feed-and-dip and crowd-and-dip methods express a bias towards larger fish in sea cage sampling. If that is true, it could be one possible explanation for the fact that no difference between methods was found in this study. Thorburn (89) found no difference in crowded samples from random sampling in small tanks with relatively small fish.

Another possible reason that no difference was identified could be due to limitations of this study. It must be acknowledged that the small sample size, 5 to 10 fish per method in only 16 cages, may not have had sufficient power to detect a difference. Based on Section 5.3.5 (sample size calculations), the sample size utilized for this assessment will yield an average weight that is only within about 0.5 kg of the cage average, and an average lice count within about 4 lice of the cage average. The differences between the two methods in most of the 16 cages were less than these. In only three cages, the difference between feed-and-dip weights and crowd-and-dip weights was greater than 0.5 kg. The average difference between methods for lice counts was only 0.7 lice per fish. Had the number of fish per method been increased and the number of cages sampled

been increased, a difference between the methods may have been found.

For logistical reasons the crowd-and-dip method offers an important advantage over the feed-and-dip method. When using the feed-and-dip method it was often very challenging and time consuming to get the 5 to 10 fish desired, especially if the fish had eaten recently. Also, as the sample size tables show, 5 to 10 fish did not offer a very precise estimate of cage level parameters. With the crowd-and-dip method it was much easier to obtain a large sample.

5.4.2 Effect of anesthetic on lice counts

A TMS anesthetic bath was shown to significantly reduce the number of mobile lice by about 13% on sampled fish. There was no observed effect on the attached larval stages. The effect on mobiles was similar to that found by Jackson and Minchin (90), who reported a mean sampling efficiency of 89.4% for pre-adult and adult *L. salmonis* based on lice numbers from water filtered from bins. Water had been filtered from both water bath bins and anesthetic (benzocaine) bath bins, but it was not specified which were used for the efficiency calculation. Hogans and Trudeau (20) examined some fish before and after anesthesia (0.1% phenoxyethanol) to determine the proportion of parasites removed by the action of the anesthetic. Unfortunately, specific results were not reported. Hogans and Trudeau (20) and also Jackson and Minchin (21) reported filtering anesthetic baths for the purpose of counting detached lice. Although it would have been interesting to compare the effect of the different anesthetics on lice counts, it was not possible based on information supplied.

5.4.3 Comparison between counters

There was a high level of agreement between the two lice counters. The only significant difference occurred in the evaluation of larval stages of lice. This was not surprising considering that these stages are small and difficult to see whereas the mobile stages are easier to detect. Even though the differences were statistically significant, it is debatable whether or not they should be considered clinically important (6.7 versus 6.0 larval lice per fish).

It is likely that with increasing numbers of lice, agreement between counters would be reduced. The more lice there are, the higher the chances of error.

This comparison was performed near the end of the sampling season during which both counters had gained considerable experience. These results indicated that, with training, different counters can yield consistent, repeatable data.

5.4.4 Lethal sampling

Lice counts on fish which were lethally sampled were found to be consistently lower than the ante-mortem counts. The reason for this apparent loss of lice was not clear. It could have been due to the time spent in the euthanasia bath, or the time spent on ice. Jackson and Minchin (90) report that killed fish had fewer pre-adult and adult lice than fish that had been anesthetized. Bristow and Berland (84) also found that lethal sampling and storage on ice was not a suitable sampling method.

Lethal sampling is a fairly common practice for sea lice studies. This study indicated that if lethal sampling is to be employed, lice should be collected or counted immediately. Any delay may result in a loss of lice and a subsequent underestimation of infestation levels. Methods which involve lethal sampling should also be carefully tested prior to a study to examine the potential for losses.

5.4.5 Sample size estimations

The sample size calculations highlighted that the required sample size was entirely dependant on the objectives of the sampling. Weight sampling used to detect a subtle difference in feed efficiency requires a very large sample size. If the sampling goal is to decide whether or not to treat for sea lice, 5 to 10 fish may be all that is required to determine if levels exceed the threshold set for treatment.

These results showed that when planning a trial with the goal of detecting a difference, or if sampling is done as part of a monitoring program, it is important to consider that larger fish and fish with higher lice burdens will need larger sample sizes in order to maintain precision.

5.5 Conclusions

The results of this study do not show a systematic bias between the crowd-and-dip and feed-and-dip methods. TMS was found to have a small but significant effect on mobile sea lice. Lethal sampling was shown to consistently result in reduced lice counts during this field study. Different counters were able to produce repeatable, consistent lice count data, especially when considering the

mobile stages. The sample size calculations showed that as the desired precision increases, the sample size must increase. It was also shown that as mean weight or lice count increased, variability increased, and therefore the required sample size must increase to maintain a constant level of precision.

6.0 GENERAL DISCUSSION

6.1 Effectiveness of teflubenzuron

The randomized clinical trial showed that teflubenzuron is effective for treating sea lice on salmon. At one week after the end of treatment there was a 79% reduction for chalimus stages and a 69% reduction for mobile (pre-adult and adult) stages in medicated cages as compared to control cages. The effect of treatment was reduced by 14 days after the end of medication, but still evident with 53% and 40% reductions in chalimus and mobiles respectively.

Without the use of blinded controls in this trial, the relatively low lice levels and the unexpected fluctuations would have rendered the trial useless as a measure of effectiveness. Thus the extra time and money used to perform a randomized double-blind study may have actually saved time and money in the long run by producing useful and meaningful data.

The historical control clinical trial suggests that the treatment effect found in the randomized clinical trial may have underestimated teflubenzuron's potential effectiveness when used in practice. Considering only the medicated cages in the randomized clinical trial, there was only a 26% reduction in all stages of lice between pre-treatment levels and the levels one week after the end of treatment. In contrast, the historical control trial showed that all stages of lice were reduced by 72% over the same time period. While caution must be exercised when comparing the results of the two studies because of differences in methods and possible site effects, the differences indicate that recruitment from untreated

cages in the randomized clinical trial may have led to an underestimate of treatment effect.

Due to the fact that the mode of action of teflubenzuron involves inhibition of chitin synthesis, the effect of medication was most evident in the stages of lice which undergo a molt. There was a 79% reduction in chalimus stages and a 69% reduction in mobile (pre-adult and adult) stages during the randomized clinical trial period, and a 92% reduction in chalimus stages and a 74% reduction in mobile stages seen in the historical control trial. The historical control trial also demonstrated a significant reduction in copepodids during the post-treatment period. There was no reduction in the numbers of gravid females, so the reduction in copepodids may indicate that treatment could affect the viability of eggs.

While there were significant reductions in most stages, the treatment still left a sizable population of lice, predominately adults and gravid females, on the fish population in medicated cages. This indicates that teflubenzuron may be most useful as a component of a sea lice management program which could include an initial bath treatment to remove mature lice, then periodic teflubenzuron treatments to keep lice levels low, and the lice population immature. This is especially important considering that as the lice mature, the stress caused by them increases (91). The combination of a chitin synthesis inhibitor (lufenuron) with an adulticide (pyrethrin) has been shown to be very successful in the control of fleas on dogs and cats (50). It is also likely that repeated teflubenzuron treatments over time would eventually shown reductions

in the adults and gravid females due to a combination of natural attrition and reduced recruitment from immature stages.

These trials evaluated the short term effectiveness of teflubenzuron after one treatment. It is suggested that future studies examine the effectiveness of periodic teflubenzuron treatments for controlling lice during an entire season, either as the only treatment or in combination with an initial adulticide treatment.

6.2 Assessment of outcome measurements

There was no systematic bias found between feed-and-dip versus crowd-and-dip sampling methods for either weight or lice count measurements. While the lack of a difference may have been due to low power in the comparison study, the findings do indicate that if any bias was present, it was probably subtle. Had there been any bias attributable to sampling method during the clinical trial, it would have been a non-differentiating bias (i.e. a bias which would not cause or increase a difference between groups) since all cages were sampled by the same method throughout the trial.

An anesthetic bath was shown to significantly reduce the number of mobile lice on sampled fish. While it has generally been accepted that this probably occurs, little previous work had been reported to quantify the effect. Although it was not examined, differences in the effects of various anesthetic agents on lice counts may exist. Perhaps one anesthetic is superior to others in minimizing the effect on lice counts.

There was a high level of agreement between the two lice counters. These

results indicate that, with training, different counters can yield consistent, repeatable data.

Lice counts on fish which were lethally sampled were found to be consistently lower than the ante-mortem counts. Since lethal sampling is a common practice, care should be exercised when using this method. More work needs to be done to determine the cause of these reductions, but in the meantime, lice counts from lethally sampled fish should be interpreted with caution.

The sample size calculations showed that as mean weight or lice count increases, variability increases, and therefore the required sample size must increase to maintain a constant level of precision.

6.3 Conclusions

The combined efforts of this work demonstrate that randomized, controlled, double blinded clinical trials performed to GCP standards are feasible and sometimes may be necessary in a commercial aquaculture setting to provide credible and accurate conclusions from the data generated. GCP standards are essential to assure the credibility of the data generated especially if the work is subject to regulatory review. The randomized and controlled aspects of the protocol increased the likelihood of the clinical trial being successfully completed in conditions where unforseen circumstances like weather and other disease factors could easily sabotage the trial.

Clinical trials which are performed as part of the drug application process

are a mixed blessing for researchers. While they provide a tremendous opportunity to perform quality research, working within the needs and concerns of the corporate sponsor can sometimes be a challenge. Confidentiality is a prime consideration when new products are being evaluated. Consequently, the sponsors of such studies may not be willing, or able, to share results of previous or coincident work being done on the same treatment. An added difficulty is that because the treatment is new, there is probably scant information in the literature about the treatment. A lack of information about the pharmacokinetic properties of teflubenzuron in fish is an example of this difficulty encountered during these studies.

More study needs to be done on the validity and limitations of certain sampling methods in field conditions as this work has shown that results can be skewed depending on the methods used. More solid evidence of the effects of different sampling methods would give investigators more information to choose appropriate methods which would yield the best results.

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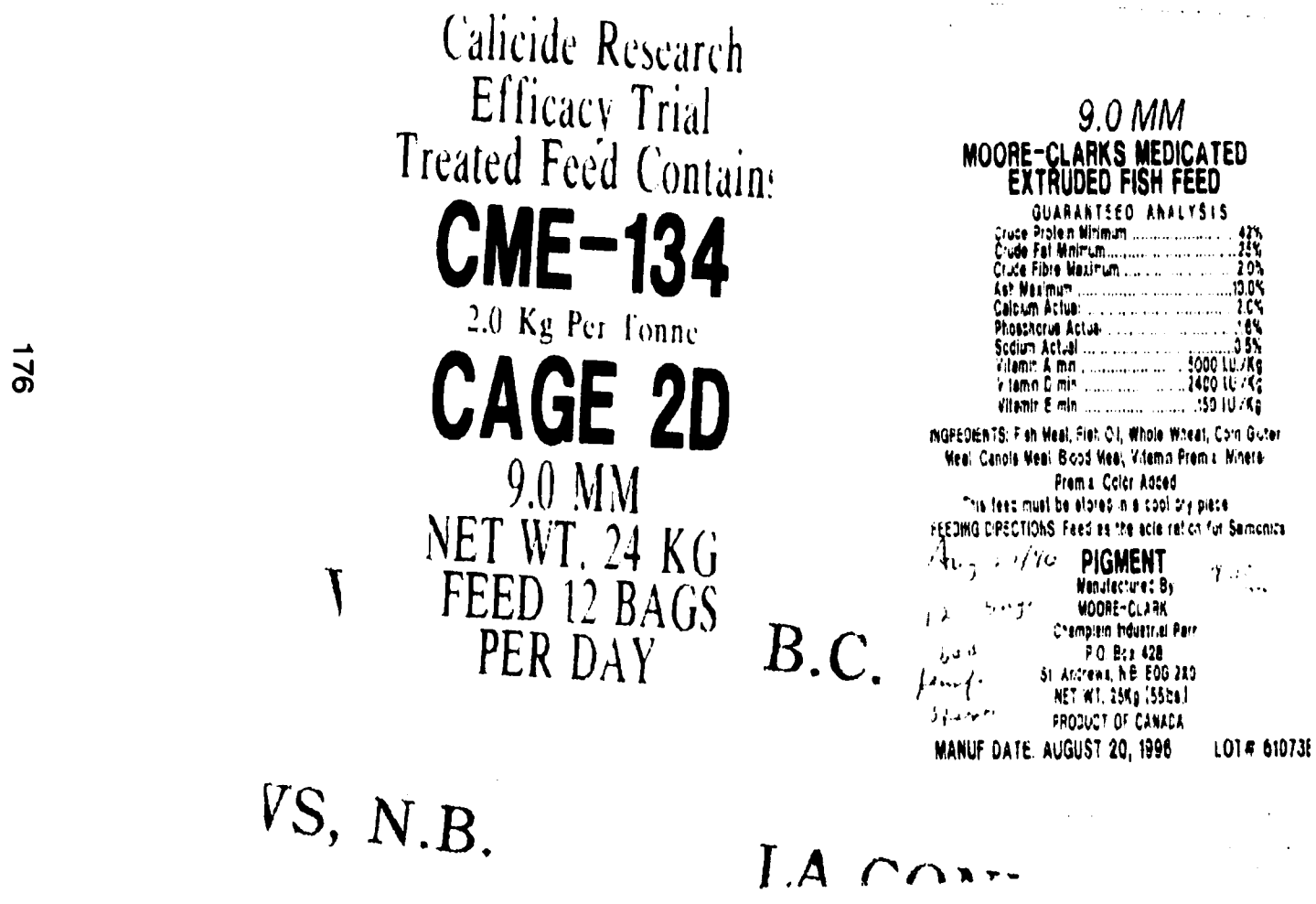
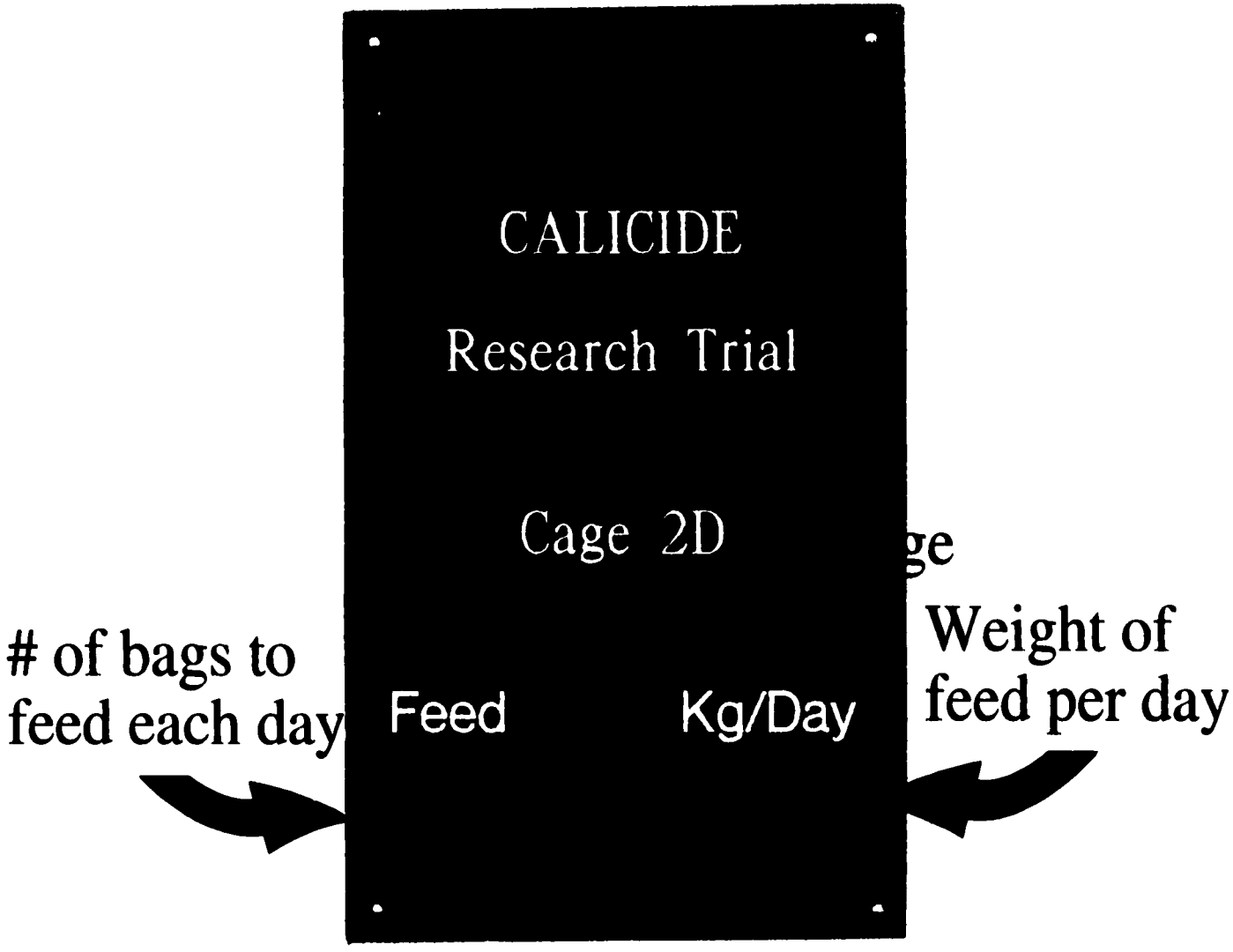
Appendix A - Example of a bag label



Appendix B - Example of a cage label



Appendix C - Data collection sheets

Calicide Clinical Trial (1996)

Note: Use Ink or dark pencil
Initial and Date all changes

Sampling Data Sheet

Date: _____ Time:_____ Lice counts by:_____

Site/Owner:_____Cage #:_____ Wt. Samples by:_____

Fish	Weight (kg)	Length (cm)	Copepodids	Chalimus 1 & 2	Chalimus 3 & 4	Pre-Adult	Adults	Gravid Females	Damage Score
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
25									

Damage score: **0**=normal, **1**=increased mucus, **2**=small area of superficial damage, **3**=large area of superficial damage, **4**=small area of deep damage, **5**=large area of deep damage

Data recorded by:_____

Read and understood by:

_____

Calicide Clinical Trial (1996)

Daily Feed Consumption
Note: Use Ink Or Dark Pencil Only

To be filled out by Research Assistant only.

Date:_____ Cage:_____
Site Location:_____

Treatment Feed Allocation
Feed letter:_____
Amount of feed:_____
Date/Time removed from storage:_____
Research Assistant Signature:_____

To be filled out by Site Worker only.
Date:_____ Cage:_____

Treatment feed

Amount fed	Time Fed	Site Worker's Initials
_____	_____	_____
_____	_____	_____
_____	_____	_____

Regular Feed

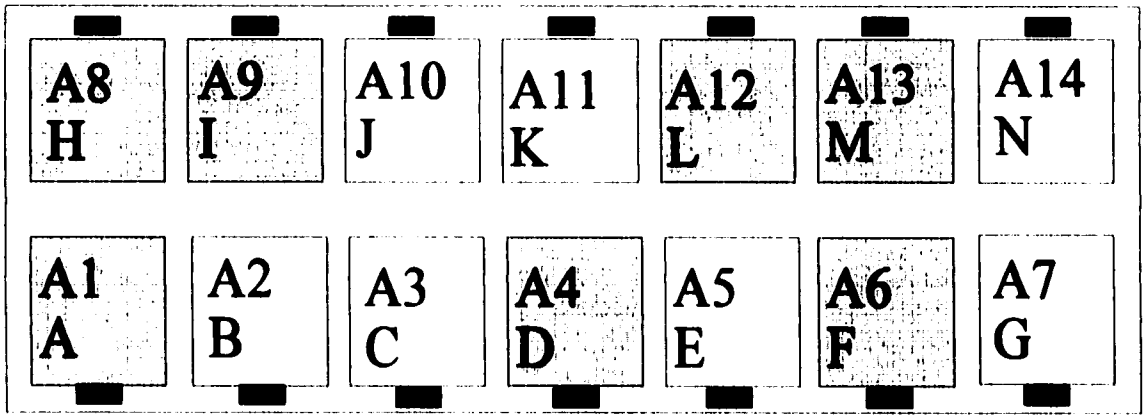
Amount fed	Time Fed	Site Worker's Initials
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

_____
Read and Understood by:

_____

Site 1

A1-A14 = Farm's cage labels
A-N = investigator's cage labels



Feed was stored in a warehouse on shore.

All cages - 12 meters square.

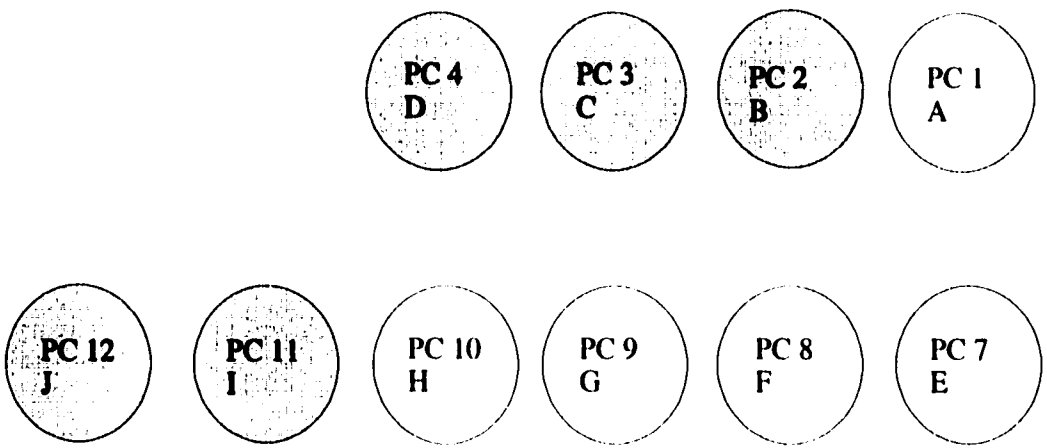
Mechanical feeders

Matched Pairs

control	medicated
M	N
D	K
F	G
H	E
A	B
L	J
I	C

Site 2

PC 1 - PC 12 = Farm's cage labels
A-J = investigator's labels



[redacted]

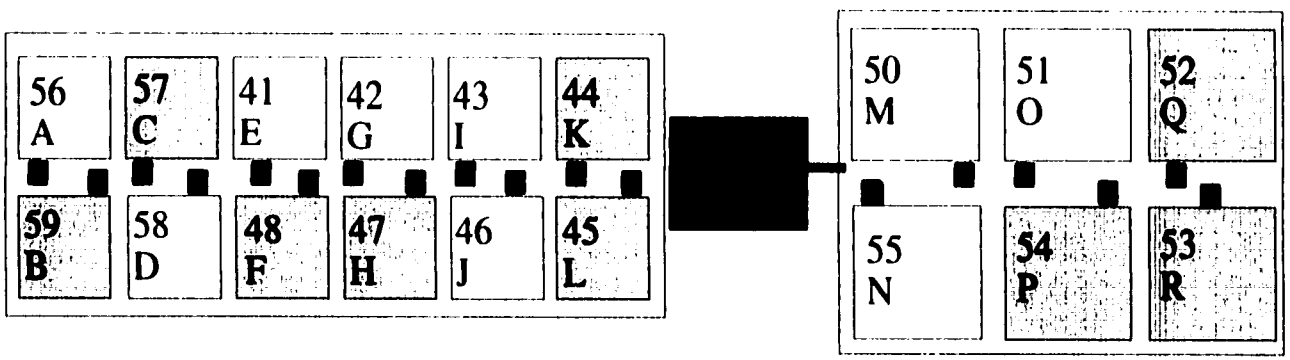
All cages - 70 meter
(circumference) circles

Matched Pairs

control	medicated
C	A
D	F
J	E
I	H
B	G -pair removed

Site 3

Numbers = Farm's cage labels
Letters = Investigator's cage labels



■ Feed Storage pallets
The feed designated for each cage was stored on the pallet next to the cage

Cages A - L - 12 meters square

Cages M - R - 15 meters square

Matched Pairs

control	medicated
B	A
C	I
H	D
K	E
F	J
L	G
R	M
P	O
Q	N

Appendix E - Cage average lice counts at each site

Site 1 - Cage mean lice counts
Total number of lice per fish at pre-treatment and each post-treatment sampling period in each cage at Site 1. Mean (standard deviation) is shown.

Matched Cage Pairs	Treatment allocation	pre-treatment N=50 fish	1st post-treatment N=50 fish	2nd post-treatment N=50 fish
A	control	5.0 (2.7)	5.1 (3.0)	6.3 (2.8)
B	medicated	5.8 (2.9)	3.2 (2.1)	6.1 (3.9)
D	control	3.0 (2.0)	3.4 (2.2)	4.3 (2.8)
K	medicated	3.2 (1.9)	3.1 (1.8)	6.3 (4.2)
F	control	7.7 (3.5)	10.5 (3.4)	16.1 (8.4)
G	medicated	9.7 (4.1)	8.8 (5.3)	13.6 (5.4)
H	control	6.9 (3.7)	7.4 (3.6)	8.6 (3.7)
E	medicated	7.1 (3.4)	5.3 (3.3)	6.3 (3.3)
I	control	4.0 (2.5)	4.4 (2.7)	8.3 (4.3)
C	medicated	3.8 (2.3)	3.0 (2.1)	5.9 (3.2)
L	control	4.7 (2.4)	6.1 (3.5)	8.5 (4.2)
J	medicated	4.7 (2.9)	3.5 (2.2)	7.5 (4.0)
M	control	9.6 (4.5)	12.1 (3.8)	19.4 (7.1)
N	medicated	9.3 (4.5)	7.8 (3.9)	16.3 (11.2)
Overall control mean N=7 cages		5.9 (2.3)	7.0 (3.2)	10.2 (5.5)
Overall medicated mean N=7 cages		6.2 (2.5)	5.0 (2.4)	8.8 (4.3)

Site 2 - Cage mean lice counts

Total number of lice per fish at pre-treatment and each post-treatment sampling period in each cage at Site 2. Mean (standard deviation) is shown.

Matched Cage Pairs	treatment allocation	pre-treatment N=50 fish	1st post-treatment N=50 fish	2nd post-treatment N=25 fish
C	control	9.2 (3.6)	30.7 (9.0)	10.2 (4.1)
A	medicated	9.2 (4.9)	11.4 (7.0)	4.6 (2.2)
D	control	10.3 (4.3)	42.9 (17.8)	11.6 (4.9)
F	medicated	11.2 (3.9)	13.5 (7.5)	6.2 (3.4)
J	control	12.3 (5.2)	48.3 (18.2)	11.3 (6.9)
E	medicated	13.3 (5.5)	22.3 (13.0)	6.6 (2.7)
I	control	9.5 (4.1)	44.4 (18.5)	12.2 (4.7)
H	medicated	9.9 (3.6)	18.5 (8.8)	8.3 (5.8)
Overall control mean N=4 cages		10.3 (1.4)	41.6(7.6)	11.3 (0.9)
Overall medicated mean N=4 cages		10.9 (1.8)	16.4 (4.9)	6.4 (1.5)

Site 3 - Cage mean lice counts

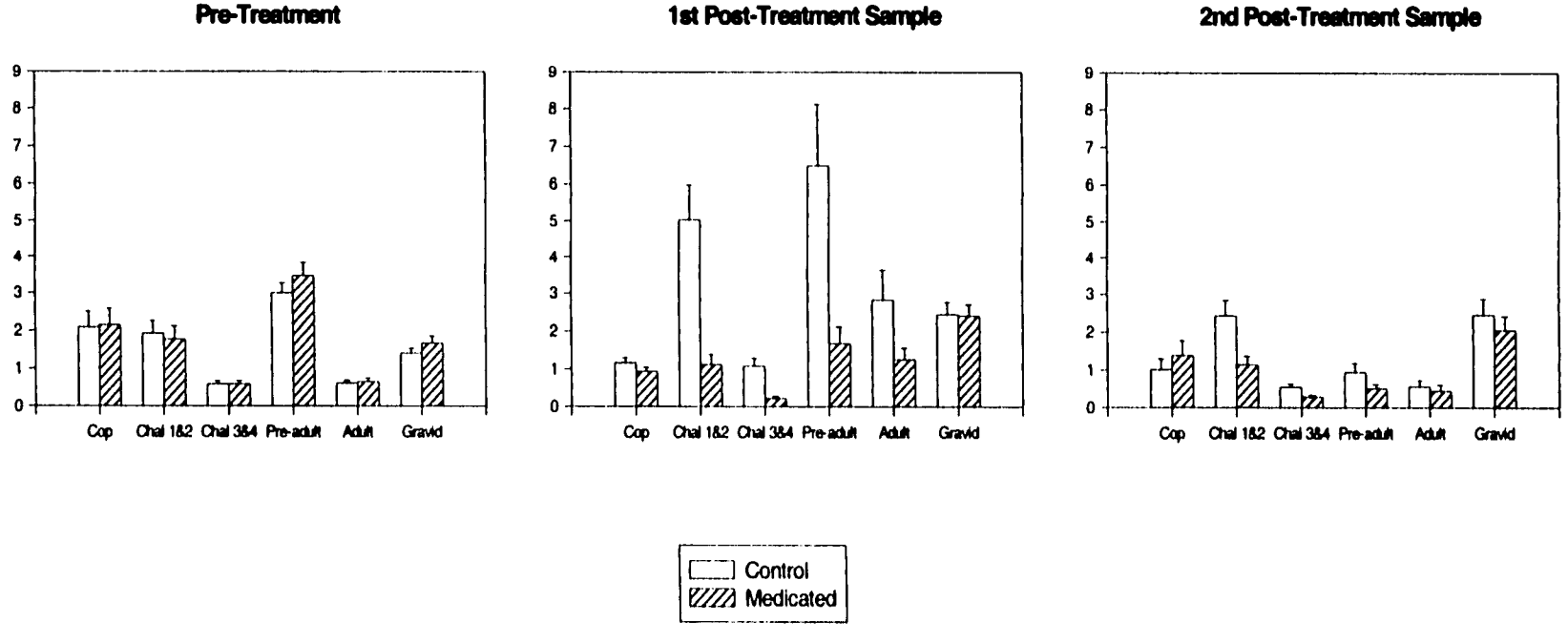
Total number of lice per fish at pre-treatment and each post-treatment sampling period at Site 3. Mean (standard deviation) is shown.

Matched Cage Pairs	treatment allocation	pre-treatment N=25 fish	1st post-treatment N=25 fish	2nd post-treatment N=25 fish
B	control	21.1 (7.4) [1]	26.1 (8.5)	5.0 (3.1)
A	medicated	24.0 (9.6) [2]	6.6 (4.4)	3.0 (1.6)
C	control	12.1 (6.3)	15.3 (5.7)	3.2 (1.8)
I	medicated	13.5 (7.3)	3.2 (2.5)	1.7 (1.4)
H	control	9.5 (4.4)	9.4 (4.2)	2.8 (2.2)
D	medicated	10.2 (4.2)	9.8 (7.3)	2.4 (2.4)
K	control	12.0 (2.8)	13.5 (4.1)	3.7 (2.6)
E	medicated	10.9 (6.2) [3]	3.3 (2.0)	1.0 (1.6)
F	control	6.0 (2.5) [3]	4.3 (2.4)	1.1 (0.9)
J	medicated	6.3 (3.3) [3]	3.2 (4.4)	1.2 (1.4)
L	control	6.7 (3.7)	5.0 (2.8)	2.1 (1.7)
G	medicated	8.5 (2.7)	4.3 (4.4)	1.5 (1.4)
R	control	12.5 (5.3)	25.6 (17.2)	-
M	medicated	16.2 (7.1)	6.9 (4.1)	-
P	control	12.3 (5.6)	27.6 (6.7)	-
O	medicated	10.7 (4.1)	5.4 (2.8)	-
Q	control	17.8 (7.3)	41.5 (14.2)	-
N	medicated	18.0 (6.3)	9.1 (4.0)	-
Overall control mean		12.2 (4.8) N=9 cages	18.7 (12.4) N=9 cages	3.0 (1.4) N=6 cages
Overall medicated mean		13.1 (5.5) N=9 cages	5.7 (2.5) N=9 cages	1.8 (0.8) N=6 cages

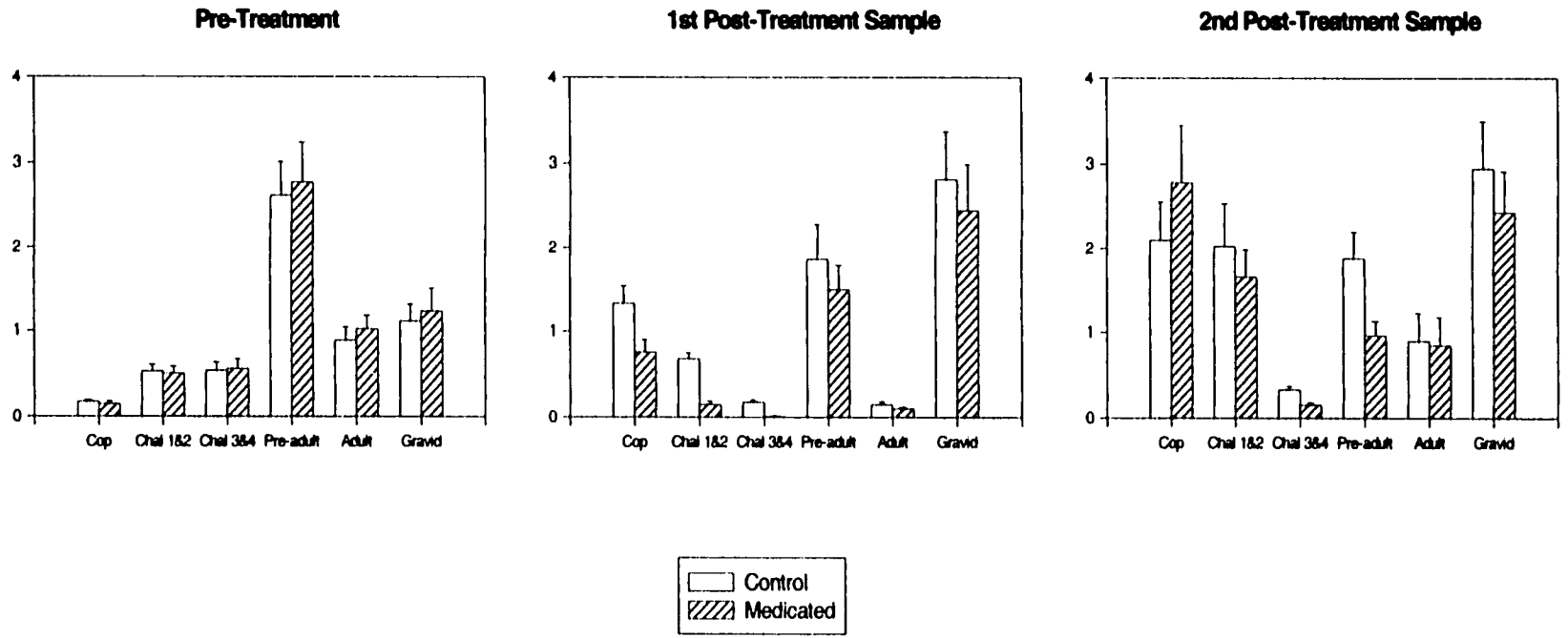
[1] N=50 fish, [2] N=49 fish, [3] N=24 fish

Appendix F - Lice stage distribution graphs - overall and for each site.

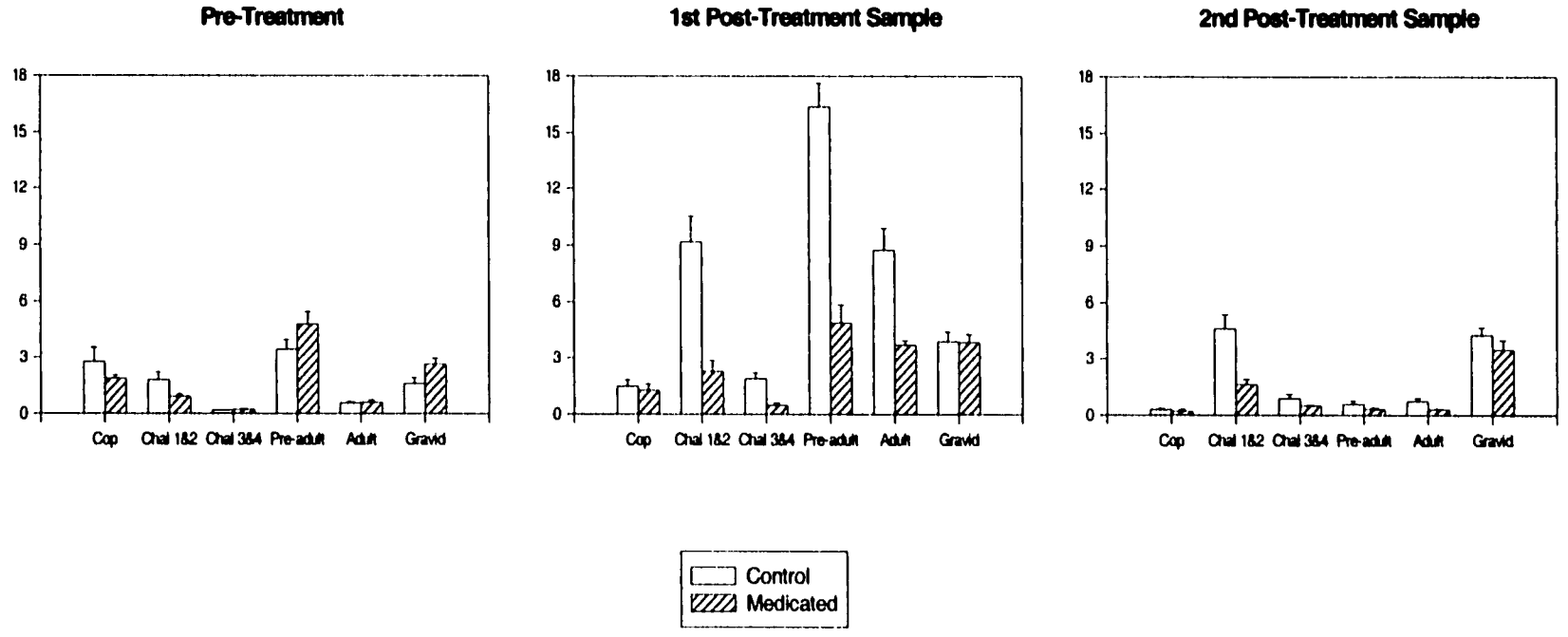
Overall - Average number of lice per fish (+ standard error) by stage and treatment group at each sampling period during the clinical trial. There were 20 cages in each treatment group at the pre- and 1st post-treatment sampling periods. There were 17 cages in each group at the 2nd post-treatment sampling period.



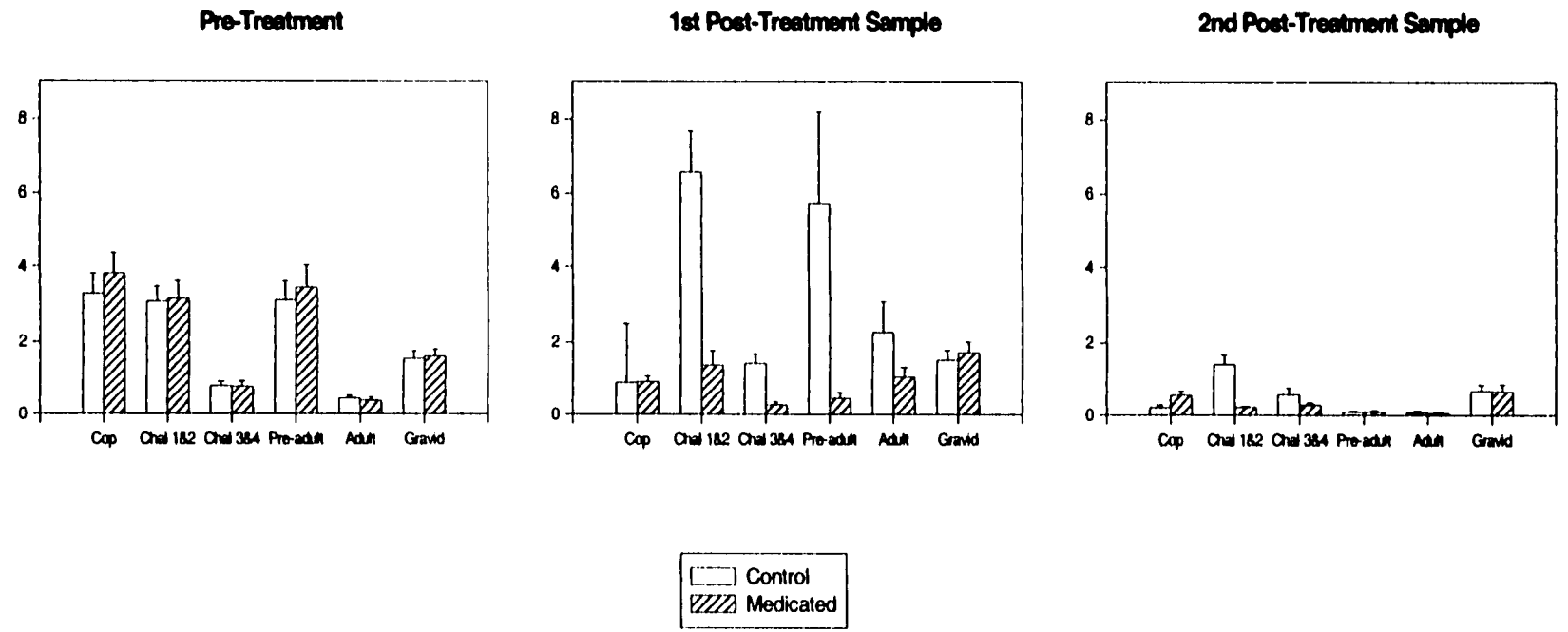
Site 1 - Average number of lice per fish (+ standard error) by stage and treatment group at each sampling period during the clinical trial. There were seven cages in each treatment group.



Site 2 - Average number of lice per fish (+ standard error) by stage and treatment group at each sampling period during the clinical trial. There were four cages in each treatment group at Site 2.

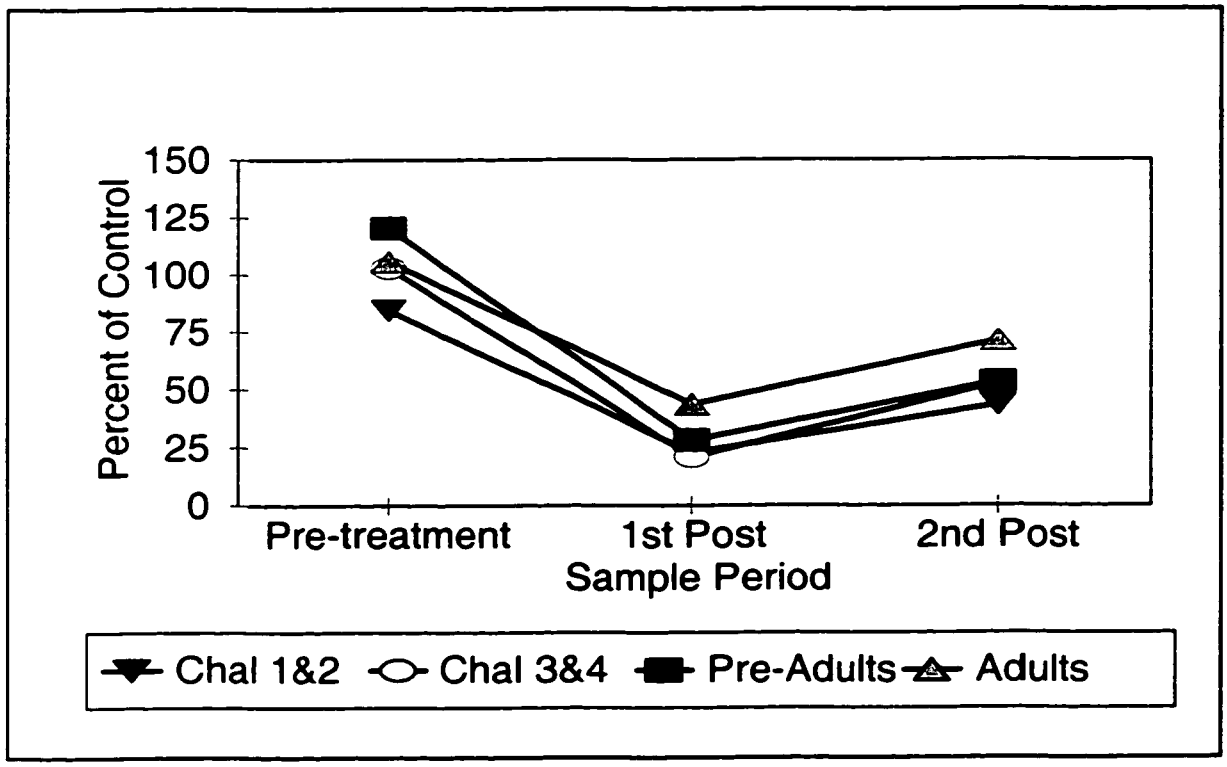


Site 3 - Average number of lice per fish (+ standard error) by stage and treatment group at each sampling period during the clinical trial. There were nine cages in each treatment group at the pre-treatment and first post-treatment sampling periods. There were six cages in each group at the second post-treatment sampling period.

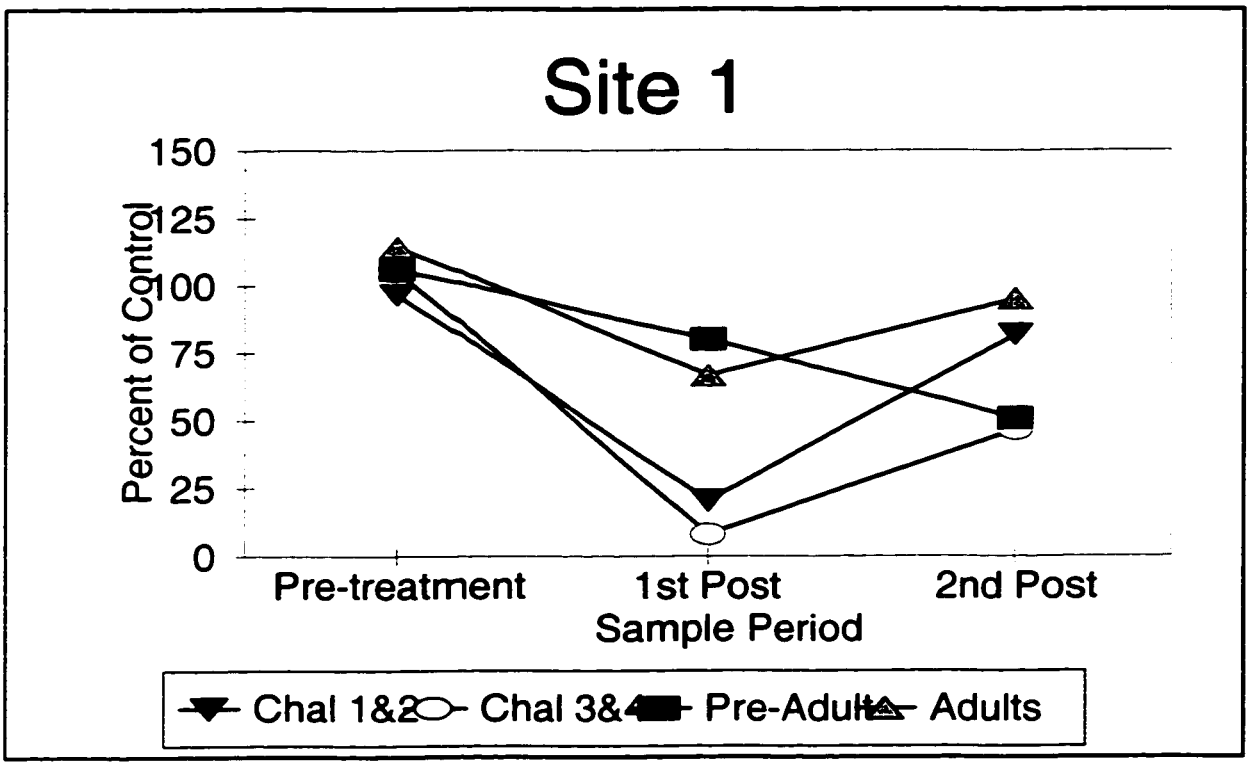


Appendix G - Lice counts in medicated cages as a percent of lice counts in control cages. Overall and by site.

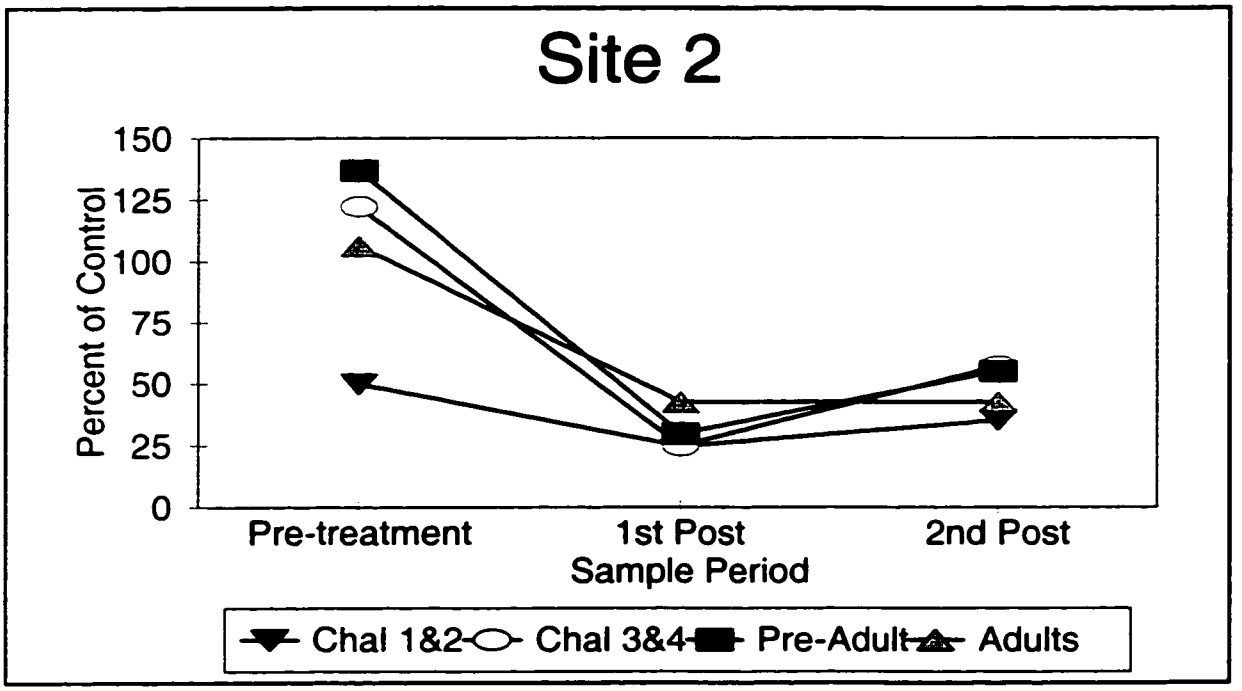
Overall - Lice counts in medicated cages as a percent of lice counts in control cages. There were 20 cages in each treatment group at the pre- and 1st post-treatment sampling periods. There were 17 cages in each group at the 2nd post-treatment sampling period.



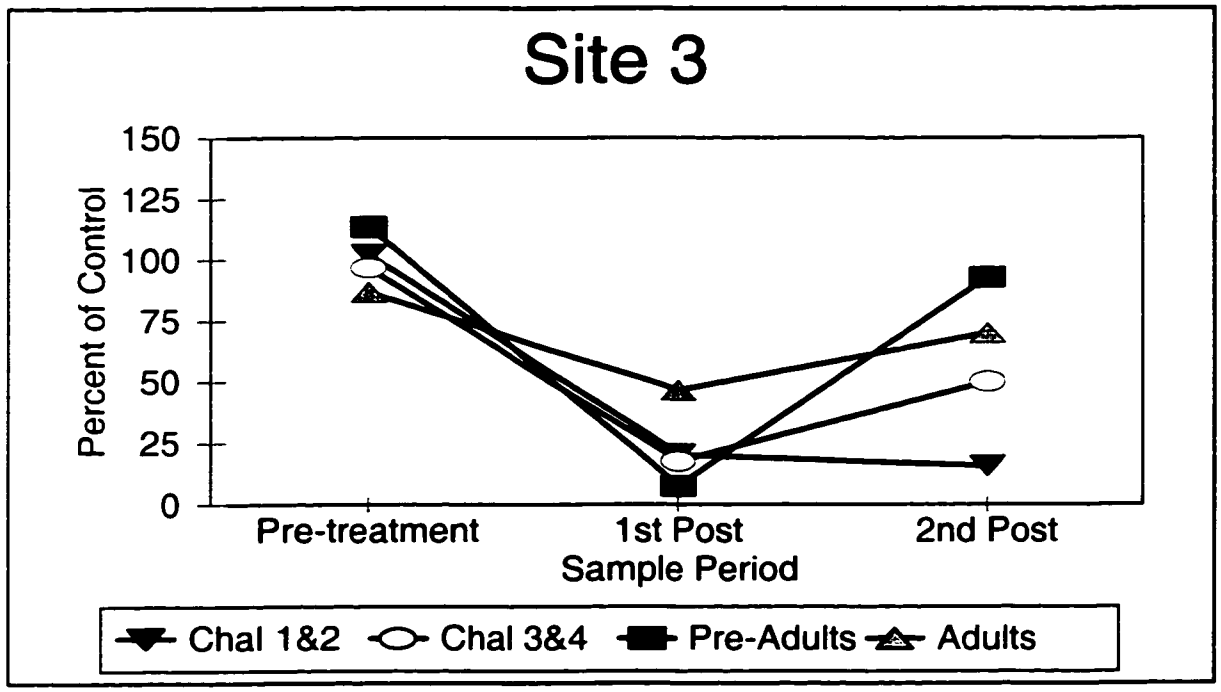
Site 1 - Lice counts in medicated cages as a percent of control.
Average lice counts for chalimus 1&2, chalimus 3&4, pre-adult, and adult stages in all medicated cages as a percent of lice counts in all control cages at Site 1.



Site 2 -Lice counts in medicated cages as a percent of control.
Average lice counts for chalimus 1&2, chalimus 3&4, pre-adult, and adult stages in all medicated cages as a percent of lice counts in all control cages at Site 2.

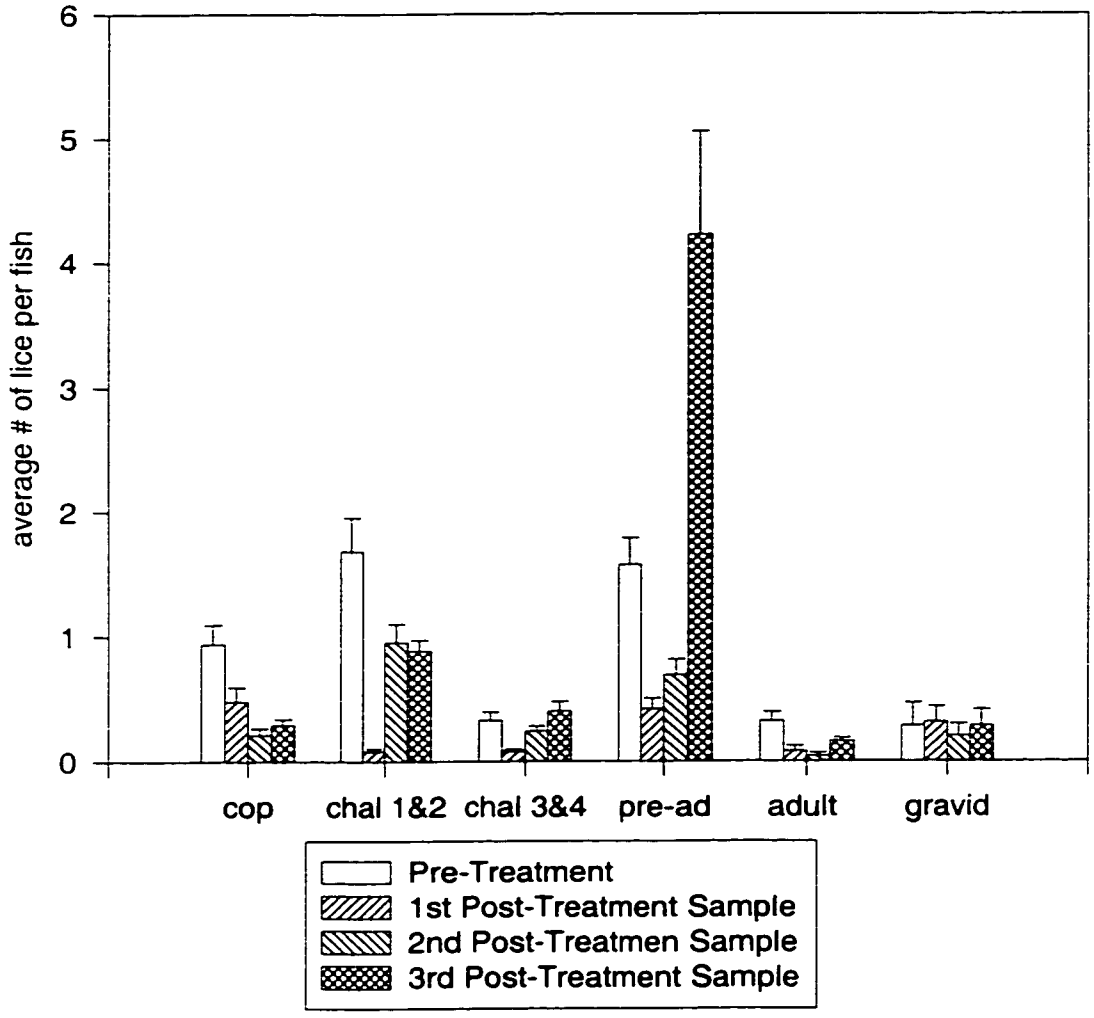


Site 3 -Lice counts in medicated cages as a percent of control.
Average lice counts for chalimus 1&2, chalimus 3&4, pre-adult, and adult stages in all medicated cages as a percent of lice counts in all control cages at Site 3.



Appendix H - Graphical summaries of treatment effects observed in the historical control clinical trial.

Average number ± SEM of lice per fish by stage and sampling period during the historical control clinical trial (n = 9 cages).



Average number ± SEM of chalimus and mobile stages per fish at each sampling period during the historical control clinical trial (n = 9 cages).