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Objective:

To develop and test feed formulations and strategies that substantially enhance the palatability of Romet-30 to rainbow trout.

Executive Summary:

An innovative model system was developed for evaluating palatability improvements by carefully observing and quantifying feeding rate, behavior, and growth of rainbow trout in replicate tanks. The data from three consecutive experiments provided promising indications for palatability improvement and support for the technical approach taken by ProFishent. In Experiment 1, we examined the effects of Romet-30 concentration and feeding rate on feed palatability. We found that well acclimated, healthy rainbow trout tolerated moderate to high concentrations of Romet-30 when appetite was robust. To properly mimic the reduced appetite which is typically observed of infected fish needing antibiotic treatment, we first provided the trout with a carefully calculated level of control feed (without Romet) each morning. The test feeds were then delivered to assess relative palatability. For statistical comparisons, we evaluated growth, counted the number of rejected pellets on the bottom of each tank, and recorded the time required for total feed consumption. As an added criterion, we also noted the number of times the fish were observed directly rejecting (spitting out) pellets. This measure proved to be particularly informative because these pellets were often ingested a second time by another fish, meaning these data would be lost in a protocol that only counted pellets remaining at the bottom of a tank after a predetermined feeding period. A new palatability index rating system (0-4, lowest to highest palatability) was developed from the data obtained in Experiment 1.

In Experiments 2 and 3, we discovered important differences in palatability among our antibiotic-free control, Romet-30 control, and experimental diets. Specifically, the addition of fish oil to the surface of the feed reduced its palatability, while hydrolyzed fish protein concentrate significantly increased the acceptance of the medicated feed. We suggest that trout feed manufacturers reduce or eliminate oil additions to the surface of the feed containing Romet. In our experiments, 2% fish oil and 1% lecithin in the feed matrix were sufficient to allow proper pelleting. If a problem of excessive fines or low pellet integrity is known to occur without surface oil, the addition of an alginate-based top coating and/or extra pellet binder (like lignon sulfonate) should solve the problem. The addition of hydrolyzed fish protein concentrate powder should improve the palatability of the medicated ration. Additional gains in feed uptake could also be attained by adjusting the feeding schedule as described in the “Recommendations” section of this report.

Research Premise:

Diseased fish often exhibit reduced appetites as a result of bacterial infections and can benefit from increased feed palatability through maintenance of pharmacologically active levels of antibiotic, improved energy levels, and higher water quality. Appetite stimulants, masking flavors, or bitter taste barriers have a strong potential to benefit fish health and extend the market potential of Romet®.

Introduction:

Romet® is a broad-spectrum antibiotic that blocks the dihydrofolates and tetrahydrofolates needed to produce RNA purines, thus inhibiting bacterial reproduction (Fraser 1991, Blair 1999). When aquaculturists are confronted with a bacterial disease outbreak, a rapid response is generally critical to effective treatment. Unfortunately, this class of potentiated antibiotics has a bitter taste that can cause feed rejection after only two days in the required five-day feeding regimen, resulting in incomplete bactericidal activity and potential selection for antibiotic resistance. Cultured fish frequently demonstrate lower appetites due to bacterial infections and can benefit from increased feed palatability through maintenance of pharmacologically active levels of antibiotic, as well as through associated improved energy levels and water quality (Powell 2000).

We took a three-pronged approach to maximize our opportunity for success. The palatability of feeds containing Romet® was improved in three ways:

1) changing the constituents, or proportions of constituents, of the feed formulation
2) top-dressing the feed with a flavor-enhancing coating
3) developing a new combination of constituents and/or top-dress feed coatings

Feed formulation improvements often involve shifts in the proportions of ingredients, or the addition of one or more new materials that would be combined with the antibiotic at the feed mill. These approaches are preferred by feed mill operators because they do not require any changes in their machinery or manufacturing procedures. Many types of flavor enhancers were considered for inclusion for each test feed, including dried squid powder, fish meal (various sources), flavor enhancing amino acids, monosodium glutamate, blood meal, animal by-products, blood worms, krill, and/or various fish and shrimp oils.

Liquid or sticky powder coatings (top-dress method) were also tested. These are more versatile then formulation changes because these coatings can be applied at the feed mill (prior to bagging) or by the farmer, either in response to, or in anticipation of, medicated feed rejection. Top-dressed additives on the surface of feed can quickly trigger enhanced feeding by releasing water-soluble scents that stimulate the appetites of fish or shrimp. Surface coatings can also be designed to seal in disagreeable odors to further promote increased feed intake.

The selection of the top three additives for direct addition to the feed composition and top-dress testing was based on previous research data, availability, cost considerations, and input from Rangen scientists and Alpharma Incorporated. To promote flexibility for second-

generation improvements and maximize innovation, the results of each of the first two experiments described below were analyzed and used to design advanced feed formulations for a follow-up experiment. Trends in feeding behavior, comparisons among treatments, and the identification of mechanisms for changes in palatability were incorporated into the development of the each experimental feeds containing Romet®.

Methods:

Production of test feeds and additives

The laboratory-scale feed milling equipment at the Rangen Research Center was used to prepare experimental pelleted feeds using a non-proprietary open formula (modified Abernathy trout and salmon diet) so that the results could be published, if desired, at a later date (figure 1). All basic ingredients were purchased from the Rangen, Inc. feed mill with the assistance of Rangen's Nutritionist, Mr. David Brock. Dry ingredients were first mixed together using a Hobart mixer capable of holding 6 kg of dry material. Base dry mixes (in common with all feeds) were split into aliquots where the final unique dry ingredients (Romet-30, etc.) and wet ingredients (lecithin, fish oil, water) are combined. The fish oil used was tested for oxidation by the analytical laboratory at Rangen Inc. Feed pellets were made in a laboratory-scale California Pellet Mill (3/32" diameter, 4-6 mm length). The pellets were then dried overnight on metal screens, processed for fines removal, top dressed (depending on the treatment), and stored at 4 ^{o}C.

Acquisition, acclimation, and husbandry of laboratory fish

Two successive lots of specific pathogen-free juvenile rainbow trout were obtained as eyed eggs from Troutlodge Inc., shipped to Rangen Inc., and moved to holding tanks when they reached 2-3 grams in size. Water quality was consistently high because the water comes directly from an adjacent spring at a constant temperature (15 ^{o} C), hardness (120 mg/liter as $CaCO_3$) and oxygen concentration (10 mg/liter). The photoperiod was set to 14 hours of light and 10 hours of darkness; slowly dimming incandescent bulbs provided non-stressful transitions from night to day times. Fish were maintained for 6-8 weeks on control feed in 500-L main stock tanks, then distributed into 20-L test tanks (20 fish/tank) in an isolated room and held for 8-9 days before the start of each experiment. Each test tank was white and had a 12-inch high (above the water line) circular collar with an open top to allow the observation of the feeding activity. Although each tank was equipped with a double (inner and outer) center stand pipe to facilitate waste elimination, immediately prior to each feeding session, all remaining feces were removed with a rubber-tipped glass tube and siphon hose to allow accurate feeding observations and counting of uneaten pellets. Test feeds were carefully weighed for each tank in polypropylene trays to the nearest 0.01 gram for maximum accuracy. Time of feed consumption was recorded to the nearest second using a hand-held laboratory stop-watch timer (VWR). Treatments were randomly assigned to numbered replicate tanks.

Adjusted Abernathy 2000 Trout and Salmon Open Formula Specifications						90d shelf life
Exp. 1	Fish Nutrition 2002, Halver and Hardy					
		Gram weight				
% total	**Ingredient**	8,000				
40.00	**Anchovy Fish Meal**	**3200**				
10.00	**Soybean meal (oil extracted)**	**800**				
13.00	**Wheat middlings, mill run shorts (remainder)**	**1040**				
5.00	**Wheat flour**	**400**				
2.50	**Blood meal**	**200**				
5.00	**Wheat germ meal (less than 1 mm)**	**400**				
10.00	**Feather meal**	**800**				
1.50	**Poultry-by-product meal**	**120**				
0.50	**Vitamin premix (Rangen hi potency blend)**	**40**				
0.58	**Choline chloride (60%)**	**46.4**				
0.20	**L-Ascorbyl-2-polyphosphate**	**16**				
0.10	**Trace mineral mixture**	**8**				
0.50	**Lignon sulfonate (hi potency binder)**	**40**				
				Romet-30 Dose		
			Cntl	Low	Mid	High
		Dry mix =	5 kg	1 kg	1 kg	1 kg
Romet-30 aliquots (0, 0.835, 1.67, & 3.33% after water addition)			0	**8.85**	**17.70**	**35.30**
0.13	**Calcium propionate (mold inhibitor)**	10	**6.25**	**1.25**	**1.25**	**1.25**
1.00	**Soybean lecithin**	80	**50**	**10**	**10**	**10**
2.00	**Fish oil (in mix)**	160	**100**	**20**	**20**	**20**
92.01	Total (before water & top dress oil)	7360				
6.00	**12% water addition (will be ~6% after drying)**	960	**600**	**120**	**120**	**120**
	During milling discard 500 g between doses					
			5 kg	0.5 kg	0.5 kg	0.5 kg
2.00	**2% Fish oil top dress after drying**	130	**100**	**10**	**10**	**10**
100.00	**Total**					

Figure 1. Abernathy formulation with the 4 Romet-30 concentrations tested (0, low, mid, & high). Note the additional Romet-30 included to compensate for the further dilution caused by the water and fish oil (added in subsequent pelleting and top-dressing steps in production).

Palatability testing – Experiment 1

The palatability testing was compartmentalized into three experiments performed sequentially to allow improvements and design innovations to occur throughout the study. The purpose of Experiment 1 was to determine the effect of feeding level on palatability, identify an optimum feeding strategy, and develop a new index of palatability. The first experiment was a 5-day exposure test using Romet-30 at 3 concentrations with 2 replicate tanks of 20 fish per tank. To achieve the FDA-registered active ingredient concentration (50 mg of ormetoprim and sulfadimethoxine per kg of fish) in all three antibiotic groups, the feeds were provided to fish at 3 proportionally different amounts each day. The feeding levels were evaluated by including a control group for each Romet concentration. On days 2-

5, one replicate from each of the 0.5 & 1.0 % feeding rate treatments was given additional control feed to bring the total feed provided up to the 2.0% rate.

Experiment 1 **Romet-30 Inclusion Concentration Test (12 tanks)**
(6 treatments, 20 fish per tank, 2 tanks per treatment)

Negative control fed at 0.5% body weight per day
Negative control fed at 1.0% body weight per day
Negative control fed at 2.0% body weight per day

3.34% Romet-30 fed at 0.5% body weight per day
1.67% Romet-30 fed at 1.0% body weight per day
0.56% Romet-30 fed at 2.0% body weight per day

Figure 2. Experiment 1 design.

Palatability testing – Experiment 2

After 9 days of acclimation to the test tanks, Experiment 2 rainbow trout were monitored for a total of 10 days. Fish were given feeds containing Romet for the first 8 days (3 days beyond the required therapeutic feeding regimen) to properly assess the full impact of the feed stimulants on feeding behavior and growth. Control feed was given for the final 2 days to assess potential toxicity or appetite loss due to antibiotic accumulation. This step was designed to help determine whether the fish quickly regained their normal feeding behavior, or whether they exhibited a toxic response and continued to reject feed independent of its palatability.

An important modification to the feeding protocol was made to Experiment 2. To properly mimic the reduced appetite which is typically observed in fish needing antibiotic treatment, the trout were provided with a carefully calculated level of control feed (without Romet) each morning. The test feed (with or without Romet) was then delivered approximately 2 hours later to assess its relative palatability.

Experiment 2 **Romet-30 Palatability Enhancement Test (26 tanks)**

5 treatments, 20 fish per tank, 5-6 tanks per treatment

Feeding levels: 1.5% pre-test control, followed by a 1% test feed containing 1.67% Romet-30.

Negative control feed (without fish oil top dress)
Romet-30 antibiotic only control (without top dress)
Romet-30 + 6 % fish oil top dress
Romet-30 + 6 % hydrolyzed fish protein concentrate (without top dress)
Romet-30 + 6% Betafin palatability enhancer (without top dress)

Figure 3. Experiment 2 design.

Feeds were hand fed with a timer that was started when the first part of the ration was added to each tank. The entire feed ration was delivered to the tank steadily over the first 10-15 seconds. Several aspects of feeding behavior were recorded each day: the number of times pellet rejection by spitting was observed (starting 10 seconds after the start of the feeding), a count of the pellets remaining on the bottom of the tank after 90 seconds, the total time of feed consumption (up to 120 seconds maximum), and the palatability index (0-4 as defined below).

Improved Palatability Index

0 = Unpalatable, avoidance of food (>50%), spitting observed
1 = Partial avoidance (1-50%) and spitting of food
2 = Spitting of food (2 or more times), slow feeding, complete consumption in less than 90 seconds
3 = No spitting, moderate rate of consumption (30-60 seconds)
4 = Aggressive feeding, rapid rate of consumption (30 seconds or less)

Palatability testing – Experiment 3

The purpose of Experiment 3 was to design a second generation of feed treatments that optimizes the palatability of Romet to fish and takes the regulatory, economic, and labor concerns of aquaculturalists into consideration. The results of Experiment 2 prompted some additional research with marine kelp-derived alginates as potential non-lipid top-dress coatings. We adjusted formulation proportions, tested new alginate ingredients, and combined new feed treatments with ones previously evaluated. After investigating the properties of more than 50 alginate derivatives, we selected four semi-purified non-toxic alginate materials (Kelset, Keltone, Kelgin, and K3B426) for testing. Five short-term experiments were run to evaluate the effects of these alginates as either modifications of the Abernathy formulation, or as a top-dress coating on trout and salmon pellets. Particular

emphasis was placed on identifying workable inclusion rates, the effects of alginates on pellet agglutination upon drying, and possible benefits to pellet cohesiveness in water.

Hydrolyzed fish protein concentrate (used in Experiment 2) was selected for further testing in Experiment 3 as an additive with good potential to improve feed palatability. Squid-flavored powder (American Dehydrated Foods, Inc.) was identified as a promising flavor enhancer that could be used in combination with either fish oil or liquid alginate to promote feed uptake. The complete experimental design is summarized below.

Experiment 3 **Romet-30 Palatability Enhancement Test (25 tanks)**
5 treatments, 20 fish per tank, 5 tanks per treatment
Feeding levels: 1.5% pre-test control, followed by a 1% test feed containing 1.67% Romet-30.

Negative control feed with fish oil top dress
Romet-30 + 6% fish oil top dress (positive control)
Romet-30 + 6% fish oil top dress + squid flavor powder top dress
Romet-30 + hydrolyzed fish protein concentrate (in formulation) + fish oil top dress + squid flavor powder top dress
Romet-30 + hydrolyzed fish protein concentrate (in formulation) + alginate top dress + squid flavor powder top dress

Figure 4. Experiment 3 design.

As was done in Experiment 2, on days 1-6, the test feed (with or without Romet) was delivered approximately 2 hours after the morning appetite-suppressing control feed. However, following the observation of somewhat reduced appetites in Experiment 3, the morning control feed was withheld from the fish on days 7 and 8 to evaluate the effect of improved appetite on the feeding response to each of the five feed treatments. On days 9-10, the rainbow trout were fed control feeds both in the morning and again 2 hours later (similar to the protocol used in Experiment 2).

Growth comparison, data compilation, and statistical analysis

All fish from each tank were collected and weighed at several time points to evaluate potential growth suppression. Fish weights were recorded at the start of each experiment, at day 5, and at day 10 (Experiments 2 & 3). To ensure accuracy, a three-step procedure was used. First, the fish were poured into a bucket containing a large net. Second, they were lifted out of the water and transferred to another net where the remaining excess water was removed by blotting on a dry paper towel. Finally, they were released gently into a tared container of water on a calibrated scale. Total biomass for each tank was recorded to the nearest 0.1 gram. During this process, each tank was scrubbed clean and refilled prior to the

addition of the fish. At the termination of each experiment, all recorded observations were entered on computer. The data were tallied and statistical comparisons made among specific feed treatments relative to the Romet-only and negative controls. Percent data was normalized using an arcsine square root transformation (Zar, 1980). Statistical comparisons were performed using a one-way analysis of variance (ANOVA) with StatView software, version 5.01 (SAS, 1998). If the ANOVA calculated p value was less than 0.05, then a least squares difference (LSD) multiple range test was used to identify statistical differences among individual treatment groups.

Results:

Feed Formulation and Experiment 1

The Abernathy feeds, with and without Romet-30, consistently met expectations for production quality (pellet integrity, etc.) using a California pellet mill (figure 1). The final moisture content ranged between 6 and 8 percent after drying overnight. The peroxide test of the fish oil batch we used revealed an average value of only 1.70. Research in this area suggests that values of 6 and above are common in herring oil (Hung et al. 1980). Our low value is probably the result of the common practice of fish oil producers to add a small amount of food grade ethoxyquin as an antioxidant preservative. Approximately 7-9 days were needed to acclimate the rainbow trout to the smaller, white, palatability experiment tanks. Twenty fish per tank proved to be an optimum number for tracking spitting and feeding behavior over the 2-minute observation period used in these experiments.

Experiment 1 revealed that healthy growing trout have sufficient appetite to consume any of the concentrations tested over the five-day period. It was also determined that the use of a pre-treatment ration of control feed (at 1.5% body weight) could mimic the reduced appetite of sick fish. This ensured that the less palatable feeds containing Romet would be rejected to some degree, permitting differentiation among treatment groups. Also, weight gain tended to be greater in fish fed control feeds compared to fish fed Romet without palatability enhancers. The results of this experiment were used to generate the innovative Palatability Index (described in the methods section) and a new model test system that can be applied to a variety of questions relating to the palatability of fish feed additives.

Experiment 2

As expected from anecdotal evidence and Experiment 1 data, all of the feed formulations were completely consumed the first day. However, uneaten pellets were observed in multiple replicate tanks in all of the Romet feed groups on day 2, with a peak rejection occurring on day 3 (figure 5). Interestingly, feed rejection was greatest, and most consistently observed, in fish fed the fish oil top-dressed formulation. None of the other feeds in this experiment had any additional oil included. Of the feeds containing Romet, the ration containing 6% hydrolyzed fish protein concentrate (HFPC) was rejected the least. Fewer pellets remained after feeding in this group ($p < 0.05$) compared to fish fed Romet with an oil top dress (figure 6). The addition of 6% Betafin did not appear to influence the

palatability in either a positive or negative direction. A comparison of the times to total feed consumption revealed a similar pattern for the five test diets (figure 7).

The fish in the HFPC treatment group finished the daily ration significantly faster than the Romet controls. Particularly striking was the average number of times fish were observed expelling pellets (spitting) after initial consumption. Romet plus HFPC resulted in an average of only 1.1 expelled pellets, while Romet plus Betafin, Romet plus fish oil, and the anchovy control provoked 4.2, 5.8 and 6.6 mean expulsions, respectively (figure 8). The statistical analysis of the average palatability index values supported the hypothesis that the HFPC ration was the most palatable Romet-containing feed, with an average palatability index of 3.1 compared to 1.9 for the Romet control feed (figure 9). A comparison of percent weight gains was less conclusive (figure 10).

Experiment 3

The tests of the various alginate formulations resulted in the selection of a 1% solution of Keltone HV in water, combined with 2% squid flavoring. This mixture was coated on to the surface of the pellets in place of fish oil to make a thin hydrophilic barrier. The proper concentration and choice of alginates was paramount in producing a pellet with good cohesiveness without creating substantial pellet aggregates in the drying process.

An examination of pellet rejection over the course of the feeding experiment revealed that the fish rejected a portion of the feed as early as the first day (figure 11). The counts of pellets rejected by the fish in Experiment 3 were consistently higher in all of the test groups compared to the counts in Experiment 2. Again, the maximum rejection occurred on day 3. The Experiment 3 peak was higher than that of Experiment 2 (131 vs. 81 pellets). Time to consumption comparisons did not differentiate any of the test groups because the rations containing Romet in Experiment 3 were rarely consumed before the end of the 2-minute observation period (data not shown).

The average number of expelled pellets (spitting) was significantly higher in the positive control (14.6 pellets per feeding) compared to any of the other Romet feeds with palatability enhancers. The most palatable feed containing Romet appeared to be the alginate- plus-squid, top-dressed feed containing HFPC, with a significantly lower average of 6.7 pellets expelled during feeding (figure 12). The high variation in mean pellet expulsion among tanks on days 7 and 8 may have prevented any statistical discrimination of the means (figure 13). There were no significant differences among the Romet-containing treatment groups in palatability index value (figure 14), although a greater numbers of replicates may have permitted the detection of superior formulations. The difference between the growth of the Romet controls and the growth of any other group was not statistically significant (figure 15).

Discussion:

We successfully developed a model test system to evaluate palatability enhancers in rainbow trout. Under normal conditions, Romet-30 milled into the feed at the recommended concentration (1.67%) is palatable to healthy trout when given at the registered dose of 1% body weight per day (50 mg active ingredient/kg fish for 5 days). Fish fed twice as much at half the concentration, or half as much at twice the normal concentration, also completely consumed the medicated feeds. However, since fish infected with bacteria often exhibit reduced feeding, we decided to develop a methodology that would mimic this situation as part of the experimental model. This lower appetite was simulated by providing additional control feed (1.5 % body weight) each morning prior to dispensing the experimental Romet feeds. This practice was reasonable because the negative control fish (not receiving Romet) ate the control feeds rapidly and completely. Experiment 1 also revealed that the fish grew consistently about 2.5% per day. This growth rate was used to recalculate the feeding ration for each individual tank each day. Such a procedure is not often used on production farms, so it is possible that our test fish received more antibiotic feed than might be typically used at commercial facilities.

The Experiment 2 results clearly suggest that the addition of fish oil to the surface of feed containing Romet-30 significantly reduces its palatability. One explanation for this effect may be that these antibiotic compounds are very lipid soluble and are dissolving into the oil, thereby increasing the bitter flavor of these feeds. The low peroxide value and the freshness of the oil (taken directly from the feed mill only days before pelleting) are strong indications that the oil itself was palatable to the trout. The addition of 6% HFPC appeared to substantially enhance the palatability of feeds containing Romet without oil top dressing.

The addition of squid flavoring (with or without HFPC) in Experiment 3 did not sufficiently overcome the negative effects of adding fish oil to the surface of Romet feeds. The alginate coating with squid and HFPC additives showed the most improvement in this test. The results also indicate that the strain of rainbow trout may influence the feeding response to rations containing Romet. The fact that the fish groups tested in Experiment 2 and Experiment 3 both averaged 19 grams at the start of each trial suggests that size or stage of development was not a factor in this comparison.

Recommendations:

Our results suggest the following procedures for use in rainbow trout (with possible applicability to other species) to reduce or eliminate problems of reduced palatability of feeds containing Romet:

1. Start the feeding of antibiotic as soon as possible, while the fish still have a reasonable appetite. Taking the fish off feed one or two days, while the decision whether to treat is being made, could also benefit appetite and water quality.
2. Reduce or eliminate oil additions to the surface of the feed. In our experiments, 2% fish oil combined with 1% lecithin in the feed matrix worked well. If excessive fines or low pellet integrity is known to occur without surface oil, the addition of an

alginate-based top coating and/or extra pellet binder (like lignon sulfonate) should solve the problem.

3. The addition of 6% hydrolyzed fish protein concentrate powder should improve the palatability of the medicated ration. Higher inclusion percentages may be even more effective.
4. With the permission of a veterinarian or certified fish pathologist, the standard feeding schedule (5 consecutive days) could be altered to maximize the intake of feed containing Romet. Feeding the medicated feed on days 1, 2, 4, 6, and 8 would provide a full 5 days of Romet with the possibility of significantly greater consumption rates. General feeding activity and growth requirements would determine whether control or no feed should be given on days 3, 5, and 7. Significant rejection of Romet-containing feed on treatment days may require cessation of all feeding on the feed-optional days. Younger, faster growing fish held at warmer water temperatures may need to be fed more often. Fish maintained in cooler, slower growth conditions, or fish at later life stages, may be best served by withholding feed on the optional days.

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References:

Blair, M. 1999. Major groups of antimicrobial drugs. In "Judicious Use of Antimicrobial Compounds in Aquaculture", Western Fish Disease Pharmacology, AFS, Twin Falls, ID.

Fraser, CM. 1991. The Merck Veterinary Manual, 7th edition. Merck & Co., Rahway, NJ.

Hardy, RW and Barrows. 2002. Diet formulation and manufacture. In, "Fish Nutrition, Third Edition," Eds JE. Halver and RW Hardy, Academic Press. 506-601.

Hung, S, Cho, CH, and Slinger, SJ. 1980. Can. J. Fish Aquat. Sci. 37: 1248-1253.

Powell, DP. 2000. Common diseases and treatment. In "The Laboratory Fish", Academic Press, London, UK, ed. Ostrander, GK, pp. 79-92.

SAS Institute Inc. 1998. StatView, Ver 5.01, Cary, NC, USA.

Zar, JH. 1980. Biostatistical Analysis. Prentice-Hall Press, Englewood Cliffs, NJ, pp. 151-161.