

A Statistical Analysis of the Distribution of a Larval Nematode (*Anisakis* sp.) in the Musculature of Chum Salmon (*Oncorhynchus keta*-Walbaum)

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The pepsin-HCl digestion technique is probably the best method of isolating *Anisakis* larvae from the musculature of chum salmon. Some losses can be expected due to breakage of the resistant cuticle of *Anisakis*, and can be estimated to be about 6% when counting the parasites with the unaided eye.

Comparisons of *Anisakis* infections of the dorsal and ventral musculature indicated that very few larvae are found in the dorsal portions (above the lateral line). Forty-seven per cent of the fish had no dorsal infections, and 90% of the fish had less than 10% of total infection in the dorsal musculature.

Comparisons of *Anisakis* infection ratios of the right and left sides below the lateral line showed some variation. Chi-square tests of the hypothesis that 50% of the infections occur in either side indicated no significant differences. There were indications that large total infection intensities displayed less variation.

Comparisons of *Anisakis* infections of the anterior and posterior portions of the lateral musculature indicated that the infections were greater in the anterior portions. Calculated 95% confidence limits showed 50.0 to 59.0% infection in the anterior portion compared to 41.0 to 50.0% in the posterior portion.

Analyses of the *Anisakis* distributions within the anterior portions of the lateral musculature were hindered by extreme variation in the counts. In general, an average of 60% of the anterior portion infections occurred in the upper two quadrants of musculature, and 40% in the lower two quadrants. Changes in distributional patterns were affected by total parasite density, rather than by age or geographical location.

For purposes of comparing infection rates in chum salmon, distributional patterns indicate that the minimum sample of musculature which should be taken from each fish is one side below the lateral line.

The one factor which probably contributes the greatest effect on distributional patterns of *Anisakis* within chum salmon musculature is the total intensity of infection (or population density of *Anisakis*) in each fish.

Investigations of North Pacific salmon have included a study of the parasites of chum salmon as possible indices of the continental origin of Asian and North American stocks. Samples of chum salmon of known

continental origin and samples of high seas catches were collected in 1955, 1956, and 1957. Over 30 species of metazoan parasites were recorded during the three sampling years. A larval nematode, *Anisakis* sp., showed some promise as an index of the continental origin of chum salmon.

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Briefly, larval nematodes of the genus *Anisakis* occur in the viscera and musculature of a number of marine fishes. Adult forms of *Anisakis* occur in marine mammals and sea birds. Salmon serve as intermediate hosts following the ingestion of infected planktonic invertebrate hosts. The larval *Anisakis* presumably penetrate the walls of the digestive tract and enter the coelom. Once in the coelom, they migrate through the peritoneal lining into the body musculature where they arrange themselves in tightly coiled lenticular cysts. It is reasonably certain that the larval nematodes persist in the musculature for at least a year and probably longer (Bishop and Margolis, 1955). Development within the musculature does not extend beyond the boring-tooth, larval stage; thus, the infection may be accumulative with the progression of time. Intensities of infection within age groups may vary from year to year due to changing ecological conditions during the marine residence of the salmon.

Preliminary examinations of samples of chum salmon musculature from the Asian and North American continents disclosed greater intensities of *Anisakis* infections in Asian stocks. It was theorized that statistical examinations of the quantitative intensities of infection within year classes might provide racial discriminates of high seas samples if Asian and North American criteria could be established.

Thus, the use of *Anisakis* as a racial discriminate of chum salmon was resolved to quantitative estimates of infection intensities, and it was necessary to establish uniform sampling and recovery techniques.

OBJECTIVE

Manual dissection of the musculature of whole fish for isolation of the parasites is time consuming, fatiguing, and subject to error. Stern *et al.* (1958) developed a procedure for isolation of *Anisakis* larvae by peptic digestion of the musculature. The procedure is rapid, comparatively simple, economical, and suitable for examination of large numbers of samples. The development of an expanded sampling program necessitated the investigation of additional methods for reducing time spent per sample.

The peptic digestion technique reduces the time and error involved in isolating the parasites from the musculature. Additional time could be saved if some particular portion of the musculature which displayed a consistent distribution of *Anisakis* could be utilized rather than the musculature in its entirety.

The distributions of *Anisakis* in the dorsal, ventral and lateral musculature were partially examined by Stern *et al.* (1958). Distributions of *Anisakis* in the anterior and posterior regions of the lateral musculature have been examined by the junior author.³ The combined results of these experiments and the following series of experiments, which were designed to determine the distributions of *Anisakis* within the anterior portion of one side of the fish, were examined to evaluate:

1. The distributional patterns of *Anisakis* within the musculature;
2. The consistency of non-random distributions if they exist;
3. The potentiality of various portions of the musculature as indices of infection intensities.

METHODS AND MATERIALS

Division of the Lateral Musculature

The examination of *Anisakis* distributions in the whole left and right sides was completed prior to the incorporation of the digestion technique. Although manual dissection is not favored and subject to error, the samples of musculature examined for this experiment were thoroughly searched and it is believed that all of the larvae were recovered.

Division of the Dorsal and Ventral Musculature

Stern (op. cit.) chose the lateral line as a division between the dorsal and ventral musculature. Thus, the dorsal counts were taken from that portion of the musculature which lies above the lateral line, and the

³ Uzmann, J. R., and Lander, R. H. 1958. Progress report, Studies on parasites of chum salmon. Pacific Salmon Investigations, U. S. Fish and Wildlife Service, Seattle, Washington, October 1958. 10 p. Ditto process.

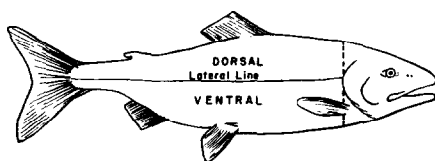


FIG. 1. The division of the whole musculature into dorsal and ventral portions. The lateral line divides the two portions.

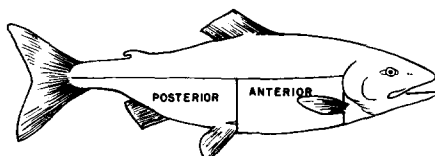


FIG. 2. The portions of musculature examined in the studies of *Anisakis* distributions within the lateral ventral musculature.

ventral counts from that portion which lies below the lateral line (Fig. 1).

Division of the Lateroventral Musculature

Uzmann (op. cit.) chose the anterior insertion of the pelvic fin as a division point between anterior and posterior portions of the lateral musculature below the lateral line (Fig. 2). In this experiment he chose either the right or the left side. The selection was based on the quality and condition of each side. The digestion method was used for isolation of *Anisakis* from the musculature.

Preparation of the Anterior Lateroventral Musculature Samples

The analyses of the distributions of *Anisakis* within the anterior fillet of the right side are based on the examination of 194 fish from two different geographical areas (Table I). The fish were numbered, measured, weighed, and sexed. Ages were determined from scale samples.

For this experiment, we selected a sample of the right ventral musculature, with definite topographical boundaries (shaded area—Fig. 3), as being the anterior sample of the musculature for all fish examined. Thus, each fish used was represented by a section of the musculature bounded dorsally by the lateral line, ventrally by the mid-line along the belly, anteriorly by the posterior insertion of the pectoral fin, posteriorly by

the anterior insertion of the pelvic fin, and interiorly by the right side of the vertebral column. The samples were assigned the same identification number as the whole fish and refrozen until needed. The division of the sample into four quadrants is described in the *Digestion Procedure*, outlined below.

The natural resistance of the cuticle of *Anisakis* to pepsin-HCl proteolysis permits successful isolation of the parasites from the musculature under controlled conditions. Optimum conditions for peptic digestion can be maintained in a thermo-regulated water bath with motor-driven stirring paddles.

Digestion Procedure and Division of the Anterior Lateroventral Musculature into Four Quadrants

The hot water bath of the digestion apparatus was pre-heated and thermostatically adjusted for a temperature of 50–52°C. A pepsin-HCl solution was prepared of 2.5 g of 1:10,000 pepsin added to a solution 8.3–8.5 m of 12 N HCl in 1 liter of warm water.

The samples were removed from frozen storage and thawed in tepid water. Pre-warming of the solution and flesh facilitates rapid digestion. Excess moisture was absorbed with paper towels and each sample was weighed to the nearest gram. The sub-

TABLE I
Samples Used in Quadrant Analyses

Year sampled	Area of collection	Age of fish	No. of samples	Area represented
1957	Ketchikan	4	50	S. E. Alaska
1957	Ketchikan	5	50	S. E. Alaska
1958	Skagit River	3	50	Puget Sound, Wash.
1958	Skagit River	4	44	Puget Sound, Wash.

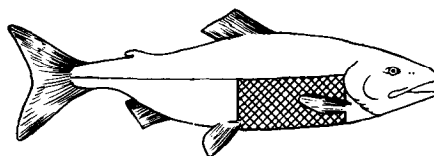


FIG. 3. The shaded area represents the sample taken from each fish for studies of *Anisakis* distributions within the anterior musculature.

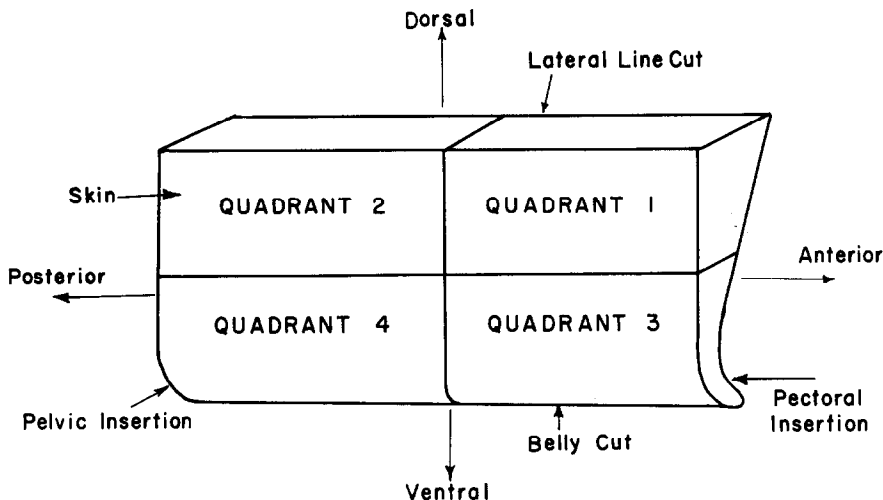


FIG. 4. Schematic drawing showing the position of the fillet in relation to the body and the equilateral quadrant relationships used in the studies of *Anisakis* distributions in the anterior portion of the ventral musculature.

peritoneal ribs were removed (the skin and bones are indigestible in pepsin-HCl), and each sample was cut into four quadrants by measuring the length and breadth along the skin-side surface, making each cut along the mid-point planes (Fig. 4). The skin was removed from each quadrant, and the flesh weight of each quadrant was recorded with previously obtained data.

The musculature of each quadrant was gently broken into smaller pieces and placed in individual glass beakers with an identification tag clamped to the rim (the quadrants were numbered by their relationships to other regions of the body). Five milliliters of pepsin-HCl solution per gram of flesh were added, and the beakers were placed in the hot water bath. The digestion apparatus used in these experiments (Fig. 5) holds eight 2-liter beakers or ten 1-liter beakers. The nylon agitators were run at moderate speeds (100–150 r.p.m.) for 45–55 minutes. The resultant mixtures were strained through a standard 80-mesh per square inch grading sieve. The sieve was washed several times to rinse away the fine suspension of digested flesh. Isolated parasites were transferred to a petri dish to which was added a 5–10% Formalin solution. When the dish was placed over a black background the parasites (which were usually tightly coiled) appeared opaque to white in color.

Counting Techniques

Each quadrant count was made twice. The first, or direct, count was made with the naked eye by one observer, counting only whole parasites or what appeared to be whole parasites. Fragments were not counted. The parasites (including all fragments) were transferred to labeled vials containing Formalin. The second or microscopic count was made by a different observer using a microscope and 60 magnification. The criterion established for enumeration by the microscopic technique was the presence of the head, including the labia. Thus, a fragment which might be composed of just the head with labia and a small portion of the pharyngeal region would be counted as a whole worm, while another fragment composed of the entire worm minus its head was not counted at all. Theoretically, this criterion would nullify the effects of fragmentation which might be found in nonmicroscopic enumerations, and present a true count which would be the standard for individual count comparisons, provided that no heads were lost during isolation and transfer.

Fragmentation Studies

Fragmentation studies were conducted on 50 three-year-old and 44 four-year-old chum salmon from the 1958 Skagit River late fall

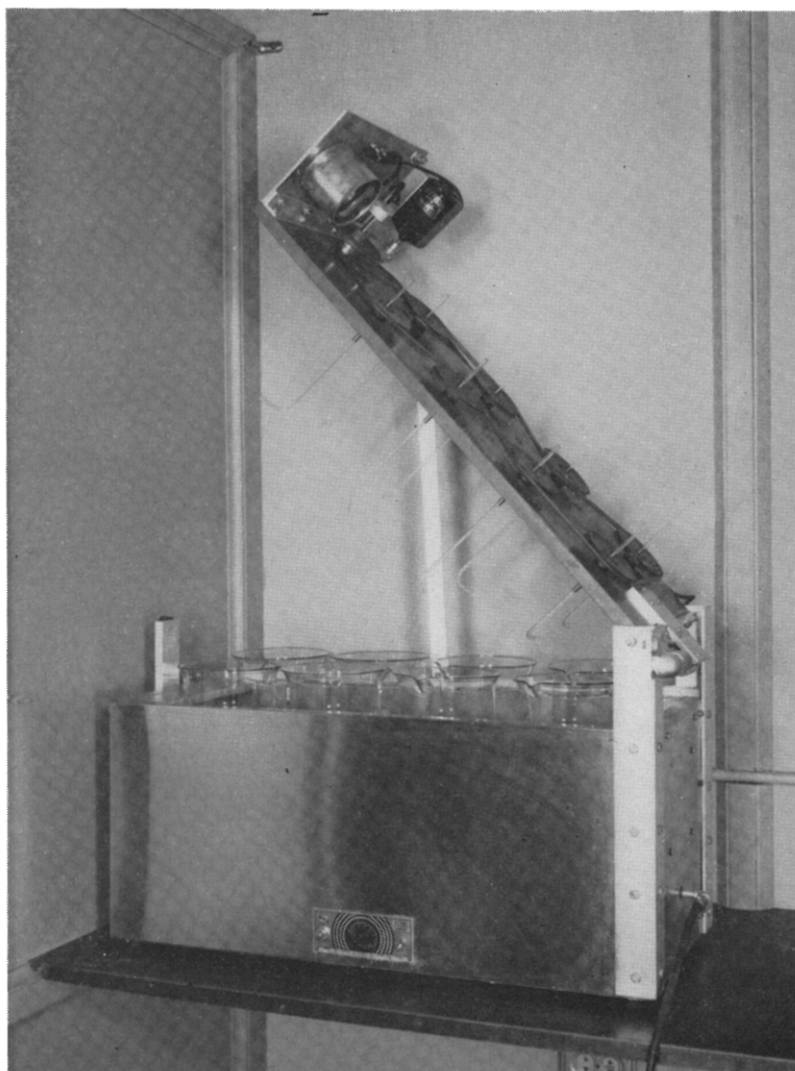


FIG. 5. Apparatus used for the isolation of *Anisakis* from the musculature

run as separate experiments to determine how parasite breakage affects the counts. These fish were taken in a commercial gill net at the mouth of the river in the late afternoon. The following morning they were placed in a cooler ($-1^{\circ}\text{C} \pm 1^{\circ}\text{C}$) at the laboratory. Samples of the anterior lateroventral musculature were taken during the next two days, tagged, and placed in frozen storage (-20°C). Prior to freezing, the largest percentage of *Anisakis* were probably still alive, although dormant, since they are known to withstand 0°C temperatures for over 72 hours in *Sebastes* sp. and *Clupea pallasii*

(Gustafson, 1953). Thus, these samples were subjected to only one freezing instead of two as in all other samples examined for this experiment. We believe that the *Anisakis* larvae extracted from these samples had less fragmentation and better appearance than any examined previously. Since both the fish and the parasites were in prime condition, the fragmentation in these samples should represent the minimal amounts which might be encountered in examination of other samples under present sampling programs.

When examined microscopically, all

worms and fragments were counted. If an entire worm were present, it was recorded on a standard form for individual fish-quadrant counts. If a head and some fraction of an attached body were observed, it was counted as a whole worm on the standard form and counted again on a separate tally sheet under the subheading "Heads without complete bodies." If a fragment was less than one-half of a full body coil, it was tallied under the subheading "Fragments." If a fragment had more than one-half of a full coil, but no head, it was tallied under the subheading "Bodies without heads."

ANALYSES OF ENUMERATION METHODOLOGY

Differences in Counting Techniques

A critical examination of the counting methodology was made in the experiments conducted on the four quadrant *Anisakis* distributions within the anterior musculature of the right side. Both methods involved counting of the same samples.

TABLE II

Changes in the Mean Counts of Anisakis in the Quadrant Analyses by Logarithmic Transformations

	Experiment number	Method A-eye B-scope	Number of fish (n)	Quadrant number			
				1	2	3	4
Total <i>Anisakis</i> counts	1	A	50	174	158	80	53
		B		165	157	74	56
	2	A	50	215	227	134	144
		B		195	196	111	125
	3	A	50	103	92	84	51
		B		99	86	74	49
	4	A	44	199	148	113	136
		B		184	144	112	128
Modified mean <i>Anisakis</i> counts (Barnes method of transformation)	1	A	50	4.40	4.26	2.60	2.05
		B		4.32	4.09	2.47	2.06
	2	A	50	5.14	5.59	3.50	3.59
		B		4.86	4.98	3.30	3.26
	3	A	50	3.07	2.86	2.65	1.97
		B		2.99	2.74	2.44	2.06
	4	A	44	5.56	4.35	3.60	3.50
		B		5.18	4.24	3.52	3.33

Examination of data tabulated from *Anisakis* counts of over 1500 samples of antero-lateral samples from widely scattered geographical locations has shown that *Anisakis* infections display a negative binomial distribution. Quantitative analysis of *Anisakis* in the case of stock discriminations of North Pacific chum salmon thus far has involved tests of mean counts. Any future studies of *Anisakis* infections in chum salmon by biological or technological services will, in all probability, be concerned mainly with mean counts. Therefore, it is appropriate to treat the two methods of enumeration as independent tests of the mean counts. Treatment of the mean counts still requires transformation, due to the negative binomial distribution. Since the standard deviation is roughly proportional to the mean, and large numbers of zero infections occur, the appropriate transformation is the logarithm of $(x + 1)$ (Barnes, 1952).

Table II indicates the typical changes of the mean counts after transforming back again by adding 1.15 times the transformed variance to the transformed mean before taking the anti-logarithm (Barnes, 1952). From the hypothesis that the difference of the transformed means of both methods is zero, *t*-tests at the 5% level (for *n*-2 degrees of freedom) show no difference in the two methods when tested for each of four quadrants for the four experiments.

Since the microscopic method consumes more time and requires additional equipment, it is evident that it is the least practical of the two for general analyses of *Anisakis* counts. The fragmentation studies of the Skagit River samples provide further evidence that this microscopic technique is impractical.

The Effects of Fragmentation

The criteria established for the fragmentation studies have been explained previously in the text. Table III shows the recorded counts of the two Skagit River samples examined.

It is obvious that a critical analysis of these counts is not axiomatic, or statistically reliable, but it is representative of the minimal fragmentation which could be expected

in any chum salmon sample using the present sampling techniques.

Our basic assumptions in analyzing the fragmentation of *Anisakis* were:

1. That the digestion technique would isolate all of the larvae;

2. That some breakage would occur due to agitation of the solution;

3. That some portions of larvae with broken cuticles would become lost during the isolation and transfer processes.

The question of determining the effects of these possibilities and their relationships to enumeration methodology rests on an estimate of the actual number of whole parasites originally present.

Table III shows the total number of whole worms in the three-year-old fish as 296. Additional "fragments," etc., are present and represent a certain unknown number of parasites which had lost partial body components during the isolation and transfer processes. The smallest components, the "fragments," could be from whole worms which were broken up but not lost, or they could be parts of whole worms which were lost. Since we had no way of telling which case they represented, we felt that including them in the treatment of the data was not justifiable.

TABLE III

Fragmentation of Anisakis as Observed in 94 Samples of 1958 Skagit River Chum Salmon

	Age 3	Age 4
Number of fish	50	44
Whole worms—by microscopic examination	296	535
"Heads without complete bodies"	12	32
Total count—by microscopic examination:	308	567
Total count—by direct observation:	330	596
Mean count—by microscopic examination:	6	13
Mean count—by direct observation:	7	14
"Bodies without heads"	52	93
"Fragments" (less than one-half full coil)	50	70

TABLE IV

Estimated Losses of Anisakis Due to Fragmentation

	Total number of <i>Anisakis</i> estimated to be present	Count by method	Discrepancy	Loss ratio in percent*
<i>Experiment No. 1</i>				
Age 3 fish				
Direct observation	348	330	18	5.2
Microscopic examination	348	308	40	11.5
<i>Experiment No. 2</i>				
Age 4 fish				
Direct observation	628	596	32	5.1
Microscopic examination	628	567	61	9.7

* Note the minor differences in the percentages of *Anisakis* missed between experiments for each method.

Although the "heads without bodies" cannot be definitely assigned to other body portions, it is not unreasonable to assume that they probably belonged to the "bodies without heads." Thus, for the 3-year-old fish, 12 additional parasites are accounted for, leaving 40 (52-12) bodies unaccounted for. These 40 "bodies without heads" must represent entire worms whose "heads" were lost during some phase of the laboratory procedures. For the 3-year-old fish then, we calculated $296 + 12 + 40 = 348$, the total number of parasites estimated to be present, discounting the "fragments." Parallel estimates of total counts for the 4-year-old fish were made (Table IV).

The greater percentage of parasites missed by the microscopic examinations was sufficient evidence to establish the support of using the direct observation of "naked eye" counts in tabulation of the results of the four quadrant distribution patterns as well as providing a minimum "loss ratio" due to fragmentation.

ANALYSES AND RESULTS

Distributions of Anisakis in the Dorsal and Ventral Musculature

Templeman, Squires and Fleming (1957) examined the distributions of various nema-

TABLE V
Intensities of Infection of Anisakis in the Dorsal and Ventral Portions of the Musculature (Revised from Stern et al., 1958)

Dorsal <i>Anisakis</i> counts	Ventral <i>Anisakis</i> counts	Total counts	
0	3	3	
1	6	7	
2	6	8	
1	10	11	
0	12	12	
3	9	12	
0	13	13	
0	14	14	
0	14	14	
1	13	14	
1	13	14	
0	15	15	
0	15	15	
1	15	16	
1	17	18	
0	20	20	
1	20	21	
2	20	22	
2	20	22	
0	22	22	
0	24	24	
0	25	25	
1	25	26	
2	28	30	
0	30	30	
2	32	34	
3	35	38	
0	50	50	
0	53	53	
2	68	70	
Totals	26	647	673
Means (N = 30)	0.9	21.6	22.4

todes in the musculature of cod, plaice and other ground fishes of the East Coast and concluded that the intensity of infection varied inversely as the distance from the coelom. Since nematode habits do not differ greatly, similar phenomena might be expected of *Anisakis* infections in salmon.

Stern *et al.* (1958) examined 30 chum salmon from scattered North Pacific sampling areas, using the digestion method. They chose the lateral line as the division point of the dorsal and ventral musculature.

Less than 4 % of the mean number of parasites occurred in the dorsal musculature, with 95 % confidence limits ranging from 1.3 % to 6.4 %. The percentages of dorsal infection for individual fish vary from zero to 25 %, but the extremely high percentages of dorsal infection occurred only in fish with low total intensities of infection (Table V).

Figure 6 shows the distribution of the percentages of dorsal infection. Ninety per cent of the fish had less than 10 % dorsal infections. Forty-seven per cent of the fish had no dorsal infections at all. The three fish which had dorsal infections greater than ten percent had very low total infections. There appears to be only fractional increases in intensities of infection in the dorsal musculature when the total intensities increase (Fig. 7), with no significance to the slope of the line. However, it is not known what the effects of extremely large total infection intensities may be, and in certain North Pacific areas, total counts of between 100 and 1000 *Anisakis* have been observed and could be expected. If we assume that *Anisakis* would take a path of least resistance, a

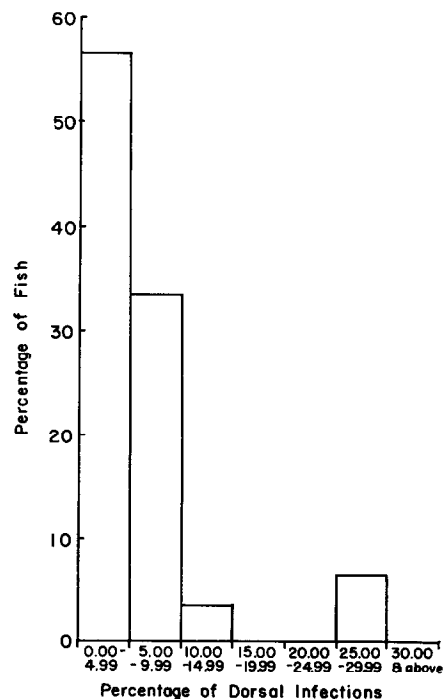


FIG. 6. The distribution of the percentages of *Anisakis* in the dorsal musculature from 30 chum salmon examined by Stern *et al.* (1958).

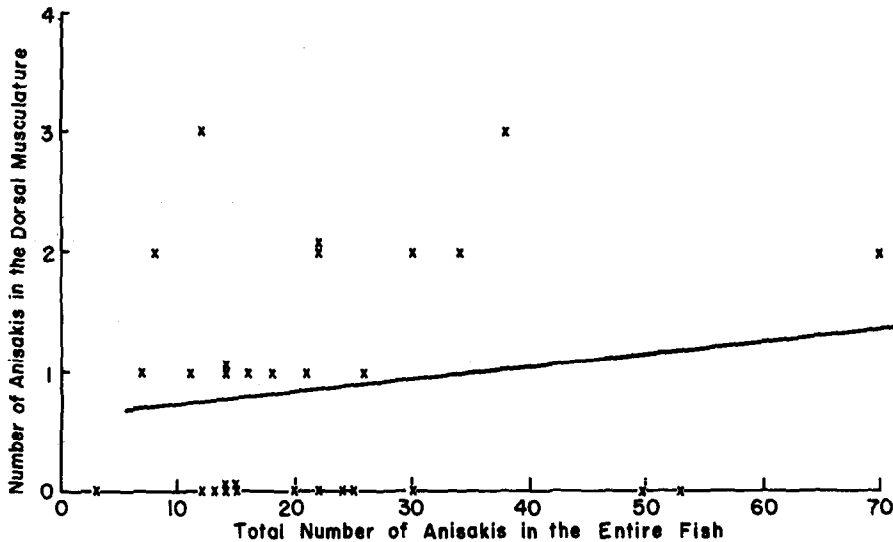


FIG. 7. Regression of the number of *Anisakis* larvae in the dorsal musculature (Y) on the total in fection in the entire fish (X).

lesser intensity of infection might be expected in the dorsal musculature due to the natural barrier formed by the position of the vertebral column and its closely associated organs, such as the kidneys and swim bladder.

Distributions of Anisakis in the Lateral Musculature

Comparisons of whole left side and whole right side infections are based on data obtained from 27 samples of chum salmon taken in 1955 from five widely scattered North Pacific areas. Isolation of the parasites was by the dissection method.

An average of 47.6% of the parasites occurred in the right side and 52.4% in the left side (Table VI). A preliminary chi-square test of the left side⁴ was made for the hypothesis that the side counts are independent of the total counts. The results of the test showed a probability (*P*) of independence greater than ($>$) 0.5 for 26 degrees of freedom (d.f.), which indicated that the total counts had no specific effect on the side counts.

Figure 8 shows the distributions of the percentages of *Anisakis* in the right and left sides. Note that there is considerable overlap

in the ranges of 40% to 60% infection between both sides.

Ninety-five per cent confidence limits were calculated for each side. For the right side the limit is 0.47 ± 0.08 or 0.39 to 0.55, and the limit for the left side is 0.53 ± 0.08 or 0.45 to 0.61. This extended overlap in the 0.50 range suggests that there may be no significant difference in expected ratios of 0.50 for each side.

Chi-square was used to test the difference between individual fish distributions for the hypothesis that 50% of the parasites occur in either side. Samples 1, 2, and 3 were deleted in order to maintain expected values equal to or greater than ($\geq$) 2.0 (Cochran, 1954). Of the 24 samples tested, 18 (or 75%) had higher left side counts, which might suggest some preference by *Anisakis* for the left side. In order to examine thoroughly the possibilities of excess and defect, deviations with a common sign, and discrepancies between positive and negative deviations, the sum, pooled and interaction chi-squares were calculated.

	D.F.	Chi-Square	<i>P</i>
Sum	24	25.28	>0.250
Pooled	1	2.28	>0.100
Interaction	23	23.00	>0.250

⁴ Either side may be tested, since the side counts are complements of the total counts.

TABLE VI
Intensities of Infection of Anisakis in the Right and Left Sides of the Musculature

Right side <i>Anisakis</i> counts	Left side <i>Anisakis</i> counts	Total counts
1	0	1
1	2	3
3	1	4
0	5	5
2	4	6
1	6	7
4	4	8
3	5	8
4	5	9
5	5	10
10	1	11
4	7	11
8	7	15
8	8	16
8	9	17
6	14	20
9	15	24
15	12	27
17	20	37
25	26	51
27	31	58
31	30	61
30	32	62
36	46	82
58	61	119
62	69	131
103	104	207
Totals	481	529
Means	17.8	19.6
(N = 27)		37.4

We accepted the 1:1 ratio hypothesis on the basis of the three chi-square tests shown above. In the sum tests, of seven samples with total counts (N) equal to or less than ($\leq$) 11, three contributed to the high value of chi-square. Only one contributed in the range $12 \leq N \leq 20$, and none where $N > 20$. Although 9 out of 11 samples where $N > 20$ had slightly higher left side counts, an additional chi-square test of the 1:1 ratio hypothesis for these 11 samples had a resulting chi-square with $P > 0.95$ for 11 d.f. This suggests that lightly infected fish will display random ratio distributions with wide ranges. As the total intensities of infection

increase, the distributions of the individual fish ratios will more approximate the expected 1:1 ratio.

Distributions of Anisakis within the Ventrolateral Musculature

Comparisons of the anterior and posterior portions of the lateral ventral musculature were made from 36 samples representing widely scattered areas of the North Pacific. The digestion method was used to isolate *Anisakis* from the musculature.

Table VII shows the individual counts and percentages of infection for the anterior and posterior portions. Twenty-three out of 36 (63.9%) fish had higher counts in the anterior portion.

Figure 9 shows the distributions of the percentages of infection in both portions. Sixty-nine per cent of the anterior portions had percentages of infection in the range 40.0% to 69.9%. This indicates that there is a strong possibility of *Anisakis* preference for the anterior portion of the musculature, which seems reasonable in view of its proximity to the coelomic region.

Since we were interested in the consistency of the ratio distributions within the muscula-

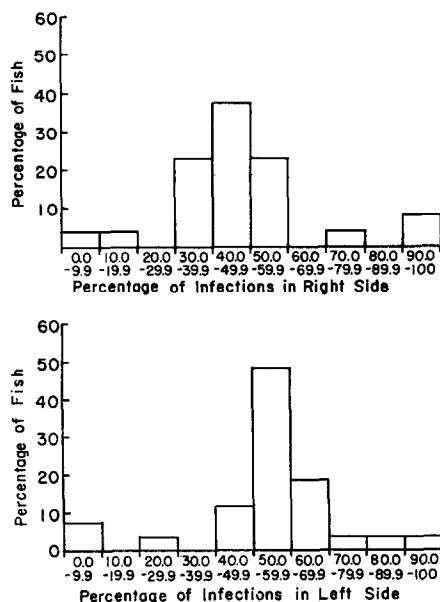


FIG. 8. The distributions of the percentages of *Anisakis* in the right and left sides of the musculature from 27 chum salmon

TABLE VII

Intensities of Infection of Anisakis in the Anterior and Posterior Portions of the Ventral Musculature of One Side of the Fish

Anterior <i>Anisakis</i> counts	Posterior <i>Anisakis</i> counts	Total counts
11	3	14
6	9	15
8	7	15
5	13	18
7	13	20
7	15	22
12	11	23
13	11	24
14	11	25
17	9	26
13	15	28
16	13	29
18	11	29
20	11	31
22	9	31
14	17	31
23	9	32
12	20	32
30	8	38
26	12	38
20	19	39
29	10	39
24	16	40
22	19	41
19	28	47
22	27	49
20	29	49
30	19	49
40	17	57
33	24	57
23	36	59
39	23	62
41	22	63
42	36	78
35	45	80
93	102	195
Totals	826	699
Means (N = 36)	22.94	19.42
		42.36

ture, we began our examinations of *Anisakis* distributions in the anterior and posterior portions of one side of the musculature by testing the hypothesis that 50% of the infections in one side occurred in either portion. The sum, pooled and interaction chi-squares were calculated.

	D.F.	Chi-Square	P
Sum	36	95.78	<0.005
Pooled	1	10.58	<0.005
Interaction	35	85.28	<0.005

We therefore rejected the 1:1 ratio hypothesis on the basis of the three chi-square tests shown above.

We felt that the possibility existed that a closer approximation to a 1:1 ratio hypothesis between the two portions of individual samples might be present at higher density ranges. The samples were split into two groups: Group A (18 samples) of low density, with $N \leq 32$, and Group B (18 samples) of higher density, with $N > 32$. A chi-square test of the 1:1 ratio hypothesis for Group A showed P for chi-square less than ($<$) 0.005 and for Group B showed P for chi-square < 0.005 . Thus, we must reject the 1:1 ratio hypothesis.

We then examined the hypothesis that the individual infections approximate the mean

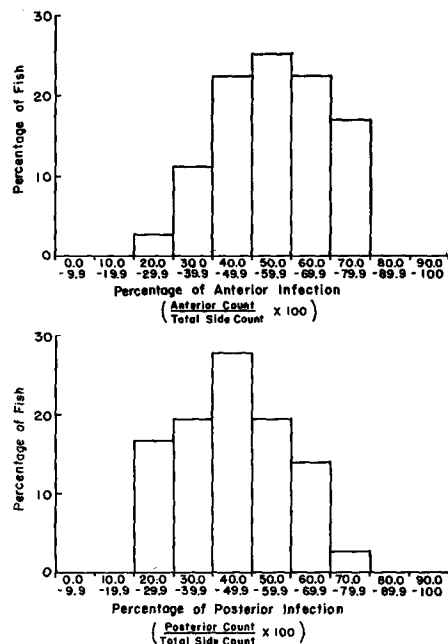


FIG. 9. The distributions of the percentages of *Anisakis* in the anterior and posterior portions of the lateral ventral musculature.

ratio of each portion for each density range. Here we found for Group A, which had a mean anterior portion infection ratio of 0.54, and a mean posterior portion infection ratio of 0.47, that P for chi-square < 0.010 . For Group B, which had a mean anterior portion infection ratio of 0.55 and a mean posterior portion infection ratio of 0.46, we found that P for chi-square < 0.005 . Thus, we must also reject the mean ratio hypothesis for the two density ranges. In this second hypothesis we have a closer approximation of what the expected ratios should be. If the differences between individual fish are so great as not to accept this hypothesis, then they are not likely to accept any other hypothetical ratios. For example, while the 95% confidence limits for the mean ratios of the anterior portions are 0.50 to 0.59, the probability of one anterior portion falling in this range is only 0.30.

Distributions of Anisakis within the Anterior Sample

The quadrant analyses. The general approach used in the previous analyses of *Anisakis* distributions within other portions of the musculature requires some modification in order to make comparative studies of the distributions within the anterior fillet or portion sample.

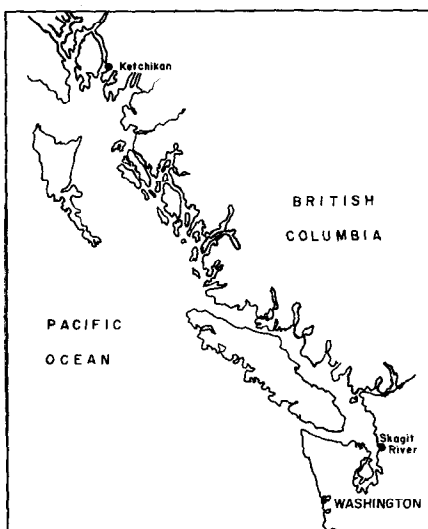


FIG. 10. Geographical locations from which samples were taken for anterior sample studies.

As the size of the musculature sampled is reduced, the quantities of parasites isolated are reduced. Although the areas from which the portion samples were taken are widely separated geographically (Fig. 10), the mean levels of infection for both areas are quite low, regardless of the age or size of the fish at maturity. Some of the anterior portion samples have zero infections for all four quadrants combined. It was felt that an anterior sample with a zero infection (regardless of what the infection in the remaining musculature might have been) could not be used in the analyses since the individual quadrant counts would be zero and thus would have no distributions to examine.

Analyses of *Anisakis* distributions of the larger portions of the musculature were comparisons of two independent structures; i.e., dorsal against ventral, left side against right side, etc. The percentage of infection of one portion was a direct complement of the other for any given fish. In the quadrant analyses, however, four separate components are being compared, rather than two. Thus, the percentages of infection in one or two quadrants may remain stable while the percentages of infection in the others may vary. For purposes of simplicity, quadrant infections used in the analyses of these four experiments are expressed as ratios; quadrant infection/total infection in the fillet. For example, quadrants 3 and 4 could maintain infection ratios of .25 each, while 1 and 2 vary from 0 to .50 each. Quadrant 4 (located in the posteroventral portion of the anterior portion sample) illustrates this phenomenon clearly. The graphs in Fig. 11, 12, 13, and 14 show the percentage distributions of the infection ratios for all four quadrants. While the first three quadrants exhibit distribution changes between ages and areas, quadrant 4 maintains a relatively stable slope. With the exception of quadrant 4, the distributional variation is extensive. Thus, the analysis of *Anisakis* distributions of the four quadrants resolves itself to two primary functions:

1. The differences between individual fish within groups;
2. The differences between groups of fish segregated by age and location at the time of capture.

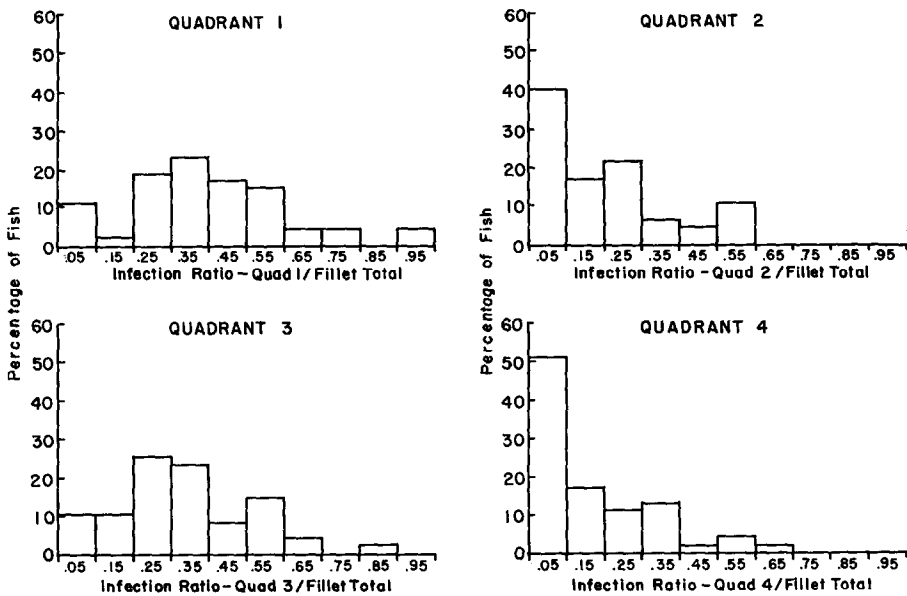


FIG. 11. Four quadrant experiment No. 1. 1957 Ketchikan 4-year-old chums. No. of fish = 47

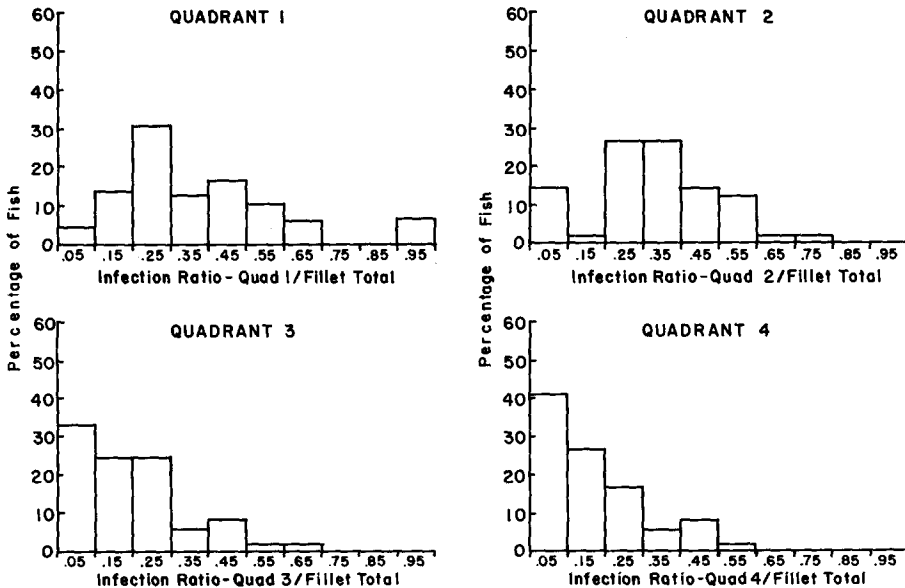


FIG. 12. Experiment No. 2. 1957 Ketchikan 5-year-old chums. No. of fish = 49

The intensity of infection is a related secondary function which must be taken into consideration when applying analytical methods.

We wished to use chi-square to test the homogeneity between individual fish infections for each quadrant; however, the low

counts per quadrant presented problems of low expectance values. It is possible to compute tables when expected numbers are small (Haldane, 1939), but not without considerable effort. Cochran (1954) suggests grouping in such a way as to have expectance values > 2 , and degrees of freedom < 30 in

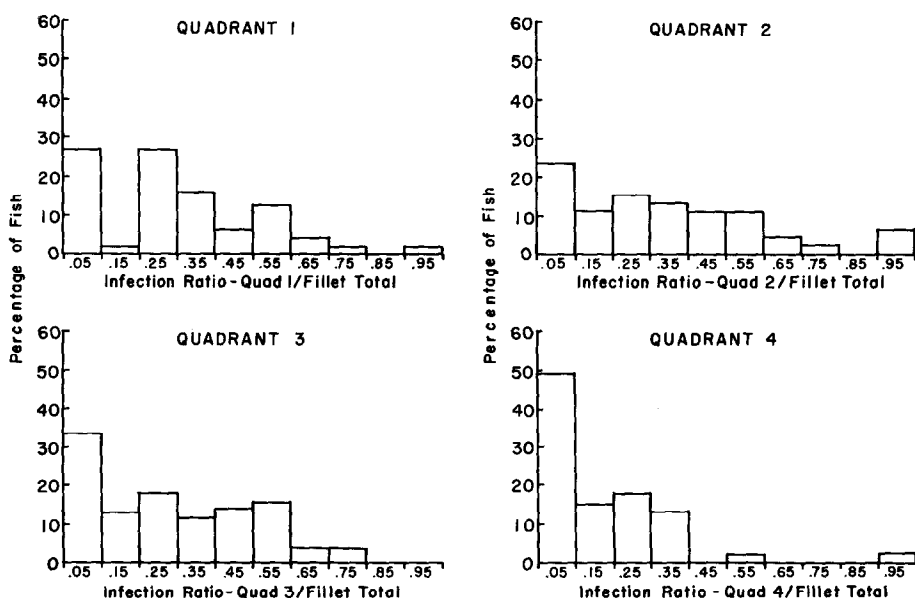


FIG. 13. Experiment No. 3. 1958 Skagit River 3-year-old chums. No. of fish = 45

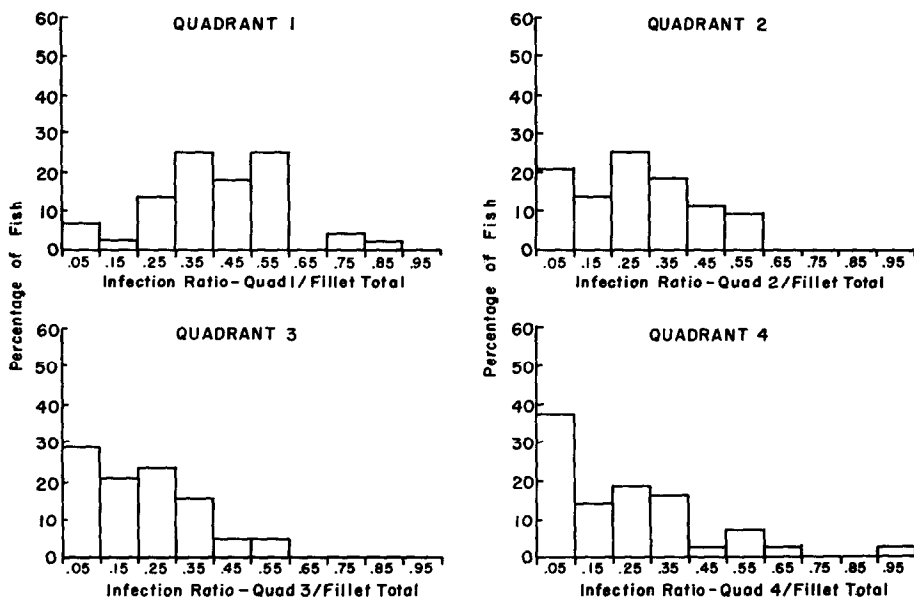


FIG. 14. Experiment No. 4. 1958 Skagit River 4-year-old chums. No. of fish = 43

order to maintain normal distributions of chi-square. In these experiments, grouping loses the sensitivity of chi-square in testing individual homogeneity, but gains the value of grouping by density in such a way as to test both the functions of individuality of

small grouped samples and the density of infection.

The first chi-square tests were of fish grouped by densities within ages and areas. The fish within each density range were divided randomly and arranged in groups

TABLE VIII

Results of Chi-Square Tests of Homogeneity between Quadrants within Ages, Areas and Densities for Estimated Proportions of Infection

Code	Area	Experiment number	Age	Density range	Number of groups	Number of fish/group	Chi-square	d.f.	P	Estimated proportion Quadrant number			
										1	2	3	4
A	Ketchikan	1	4	$0 < n \leq 14$	10	4	25.6	27	>0.50	.36	.33	.16	.15
B	Ketchikan	1	4	$n \geq 15$	3	2	6.1	6	>0.25	.40	.35	.18	.07
C	Ketchikan	2	5	$0 < n \leq 9$	8	3	11.8	21	>0.90	.34	.29	.20	.17
D	Ketchikan	2	5	$10 \leq n \leq 14$	7	2	17.7	18	>0.25	.34	.38	.17	.11
E	Ketchikan	2	5	$n \geq 15$	11	1	31.8	30	>0.10	.27	.30	.19	.25
F	Skagit River	3	3	$0 \leq n \leq 10$	9	4	20.5	24	>0.50	.34	.31	.23	.12
G	Skagit River	3	3	$n \geq 11$	4	2	7.7	9	>0.50	.28	.24	.28	.20
H	Skagit River	4	4	$0 \leq n \leq 10$	9	3	31.3	24	>0.10	.43	.23	.16	.18
I	Skagit River	4	4	$11 \leq n \leq 26$	5	2	16.3	12	>0.10	.33	.26	.30	.11
J	Skagit River	4	4	$27 \leq n \leq 99$	6	1	15.9	15	>0.25	.30	.27	.19	.24

such that the expected number of parasites from the sum of each group was > 2 , and that the number of groups within each density range did not exceed eleven in order to have thirty or less degrees of freedom.

The test was of the hypothesis that for each quadrant there is no difference between groups whose expected counts are based on the quadrant ratio means of that density range. Table VIII shows the results of the chi-square tests of this first hypothesis. The ratio means for each quadrant were carried out to five places (shown to two places in the table) and the expected values for each group were calculated from the quadrant ratio means for each density range. One sample in Experiment 4, Skagit River 4-year-olds with a total fillet count of 100, was dropped from the tests. We felt that this sample represented a separate, extremely high density range which did not fit in with the others. Chi-square tests of the remaining grouped samples all have values of $P > .10$, for the various ratio means. The results of these first tests showed no significant differences. Thus, for grouped fish within these densities, ages, and areas, it is possible to make some prediction of the quadrant infections per group based on the mean ratio of infection for each quadrant within a given density range. The estimated proportions of infection for each quadrant for each density range varied considerably. However,

quadrants 1 and 4 remained relatively stable in five out of the six low density ranges with mean infection ratios of 0.34 and 0.13, respectively. Since samples were combined into groups in eight out of the ten chi-square tests, homogeneity between individuals within ages and areas cannot be tested.

The second chi-square test was of the hypothesis that when calculated from the overall mean ratios for each quadrant, there is no difference in the estimated counts between ages, areas, or densities. To do this, we combined counts within density ranges (Table IX). The estimated proportions (or mean ratios) for each quadrant for all areas, ages, and densities combined were used to compute the expected quadrant counts for each density range. For the estimated proportions, the probability (P) that there is no difference in the estimated counts between ages, area, or densities is less ($<$) than 0.005 for 27 degrees of freedom. Thus, the second hypothesis does not hold true. However, 63% of the contributions to chi-square > 1.0 were attributed to the higher density ranges, a salient implication of the effect of density on group homogeneity.

To examine these implications, we developed two additional hypotheses, identical to the second except that we divided the densities and made two tests; one compared all ages, areas, and low densities to overall quadrant mean ratios for the low densities;

TABLE IX

Combined Counts within Density Ranges Used for the Chi-Square Test of Age, Area, and Densities vs. Quadrant Distributions

Code	Quadrant				Totals
	1	2	3	4	
	Obs.	Obs.	Obs.	Obs.	
A	98	91	45	41	275
B	67	59	31	12	169
C	49	42	28	24	143
D	55	61	27	18	161
E	111	125	78	102	416
F	60	54	41	21	176
G	40	34	40	29	143
H	65	35	25	28	153
I	53	42	48	17	160
J	63	57	39	50	209
	661	600	402	342	2,005

Estimated pro- 0.33 0.30 0.20 0.17
portions
(mean ra-
tios)

the other compared all ages, areas, and higher densities to overall quadrant mean ratios for the higher densities. Determination of the dividing point of low and higher densities was placed near 15. The groups from the previous tests were rearranged and separated into the two categories as shown in Table X. The two resulting chi-square tests based on the mean ratios of each category show $P < .005$ (for 12 degrees of freedom) for the higher densities, rejecting this hypothesis, and $P > .10$ (for 12 degrees of freedom) for the low densities, which is acceptable.

The conclusions which can be made from these last two tests are doubtful from the point of predictions of infections in the various quadrants due to the extreme variations encountered. However, we can conclude, with reasonable certainty, that density of infection can influence a sufficient number of individual distributions to the extent that they affect entire groups, more so than differences in age, or geographical origin of the fish samples.

The extreme variation of infections en-

countered in these quadrant studies leads us to believe that only weak generalizations can be made. From the data arranged in Table XI, we conclude that there is no strong

TABLE X

Combined Counts within Density Ranges Used for the Chi-Square Tests of Age, Area and High and Low Densities vs. Quadrant Distributions

	Code	Quadrant				Total
		1	2	3	4	
		Obs.	Obs.	Obs.	Obs.	
Low densities	A	98	91	45	41	275
	C	49	42	28	24	143
	D	55	61	27	18	161
	F	60	54	41	21	176
	H	65	35	25	28	153
	Totals	327	283	166	132	908
	Mean ratios	0.36	0.31	0.18	0.15	
High densities	B	67	59	31	12	169
	E	111	125	78	102	416
	G	40	34	40	29	143
	I	53	42	48	17	160
	J	63	57	39	50	209
	Totals	334	317	236	210	1,097
	Mean ratios	0.30	0.29	0.22	0.02	

TABLE XI

Mean Percentages of Anisakis Infections in the Quadrants of the Anterior Portion Samples Grouped by Ages, Areas and Densities (coded)

	Code	Mean percentage of infection Quadrants					
		1	2	1 & 2 comb.	3	4	3 & 4 comb.
Low densities	F	34.1	30.7	64.8	23.3	11.9	35.2
	A	35.6	33.1	68.7	16.4	14.9	31.3
	H	42.5	22.9	65.4	16.3	18.3	34.6
	C	34.3	29.4	63.7	19.6	16.8	36.4
High densities	D	34.2	37.9	72.1	16.8	11.1	27.9
	G	28.0	23.8	51.8	28.0	20.2	48.2
	I	33.1	26.2	59.3	30.0	10.6	40.6
	B	30.1	27.3	57.4	18.7	23.9	42.6
	E	26.7	30.0	56.7	18.8	24.5	43.3

indication that the distributions of *Anisakis* are affected by age, or by the geographical area from which the samples came. However, there is a strong indication of distribution changes with changes in the total parasite population.

As the total number of parasites increases, the proportion of parasites in the upper and lower quadrants is more nearly equal. The factors affecting this change in distributions with increasing densities are not understood, since greater quantities of musculature appear in the upper two quadrants. As the total parasite density increases, then, the population of parasites per cubic unit of the lower two quadrants increases at a greater rate than that of the upper two quadrants.

Examinations of possible relationships between individual quadrant counts and physical characteristics of the fish, such as sex, length, body weight, and the weight of the flesh examined showed extreme variation. Thus, if any one particular factor contributes to the distributions of *Anisakis* in the quadrants, it appears to be the total population of *Anisakis* itself. Whether changes in the physicochemical character of the musculature become causative agents in the control of *Anisakis* distributions with increasing intensities is not known.

CONCLUSIONS

The information on *Anisakis* distributions given in this study is not complete nor sufficiently extensive to cover all situations which might be encountered. However, from the data examined to date, there are certain basic, useful conclusions concerning *Anisakis* distributions in chum salmon, which are:

1. From the samples of dorsal and ventral distributions, and the samples of lateral distributions which we have examined, there is no indication that changing distributions are a function of age or geographical location.

2. From the evidence available on the distributions within the various portions of the lateral musculature, there is no indication that distributional differences are caused by differences in age, geographical location, or morphological characters of the fish.

3. There is conclusive evidence that the distributions are affected by changes in the total intensity of infection. These conclusions are based on what we call low and higher infections, such as those used in the tests. It remains to be seen what effect extremely large infections have on distributional patterns.

4. If a sampling program were arranged which necessitated the examination and comparison of infection rates of individual fish, the variation of the parasite distributions within the musculature would require the examination of the entire musculature below the lateral line. The loss of parasites in the dorsal musculature would have little effect when making comparisons of individual infection rates in chum salmon.

5. If a sampling program were arranged such that the samples could be grouped and comparisons of infection rates were based on mean counts, the distributional evidence indicates that examination of the musculature from one side below the lateral line would be sufficient. The group means for one side should closely approximate 50 percent of the group total infections in the musculature below the lateral line.

6. The use of samples of musculature smaller than one side below the lateral line appears to be extremely limited. The difficulties of accurate analysis of data from smaller samples whose distributions are affected by the parasite densities would warrant rejection of the use of such samples for comparative studies.

7. In general, the variation of the distributions within the musculature sampled becomes more extreme between individual fish as the size of the musculature sampled decreases, and must be given thorough consideration when comparative quantitative studies are to be made.

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REFERENCES

- BARNES, H. 1952. The use of transformations in marine biological statistics. *Journal du Conseil Exploration Mer* 18(1), 61-71.

- BISHOP, Y. M. M., AND MARGOLIS, L. 1955. A statistical examination of *Anisakis* larvae (Nematoda) in herring (*Clupea pallasii*) of the British Columbia coast. *Journal of the Fisheries Research Board of Canada* **12**(4), 571-592.
- COCHRAN, W. G. 1954. Some methods for strengthening the common chi-square tests. *Biometrics* **10**(4), 417-451.
- GUSTAFSON, P. V. 1953. The effect of freezing on encysted *Anisakis* larvae. *Journal of Parasitology* **39**(6), 585-588.
- HALDANE, J. B. S. 1939. The mean and variance of chi-square when used as a test of homogeneity when expectations are small. *Biometrika* **31**, 346-355.
- STERN, J. A., CHAKRAVARTI, D., UZMANN, J. R., AND HESSELHOLT, M. N. 1958. Rapid counting of nematoda in salmon by peptic digestion. U. S. Fish and Wildlife Service, Special Scientific Report-Fisheries No. 255. 5 p.
- TEMPLEMAN, W., SQUIRES, H. J., AND FLEMING, A. M. 1957. Nematodes in the fillets of cod and other fishes in Newfoundland and neighboring areas. *Journal of the Fisheries Research Board of Canada* **14**(6), 831-887.