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A Study of the Salinity Tolerances of Three Native Species of Minnows (Pimephales Promelas, Culaea Inconstans and Fundulus Diaphanus) Occurring in Northeastern North Dakota

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A STUDY OF THE SALINITY TOLERANCES OF THREE NATIVE SPECIES OF MINNOWS (*PIMEPHALES PROMELAS*, *CULAEA INCONSTANS* AND *FUNDULUS DIAPHANUS*) OCCURRING IN NORTHEASTERN NORTH DAKOTA

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Chairman

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INTRODUCTION

Laboratory experimentation has established tolerance limits of selected species of fish (Doudoroff and Katz, 1950; Brett, 1944, 1946, 1956; Fry and Hart, 1948). Requirements found in the laboratory represent only a small set of conditions prevailing in the total environment of the fish. Conditions tolerated by a species in the laboratory may not be the same in the natural environment. Therefore, it is important to use the information gathered under artificial conditions with discretion and restraint.

Previously, the salinity tolerances have not been studied for native species of fish taken from North Dakota waters. Water bodies in the vicinity of Grand Forks, North Dakota, differ greatly in salinity concentrations, and the local distribution of different species of fish have appeared to be related to salinity.

A laboratory investigation was made of the salinity tolerances of the three species of fish: the fathead minnow, Pimephales promelas Rafinesque; the brook stickleback, Culaea inconstans (Kirtland); and the killifish, Fundulus diaphanus (Le Sueur). These species of fish appeared to have overlapping distributions in two areas near Grand Forks, North Dakota.

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REVIEW OF THE LITERATURE

Tolerance studies of fish to various factors of their natural environment (thermal requirements, dissolved oxygen concentration, the toxicity of pH, carbon dioxide, and alkalinity) and to various organic and inorganic chemicals have been conducted for many years. Important contributions to the study of the thermal requirements of fish have been made by Brett (1941, 1944, 1956), Doudoroff (1942, 1945), Fry, Brett and Clawson (1942), Hart (1947), Loeb and Wasteneys (1912), and Sumner and Doudoroff (1938). Acclimation of fish to short-term temperature changes was demonstrated by these studies. Acclimation to an increased temperature within the physiological range of a species occurred in 2 to 5 days. At low temperatures, acclimation is usually complete in about three weeks. Doudoroff (1957) stated that the acclimation rate to a particular temperature depended on the thermal history of the fish. Fry et al., (1942) found that the goldfish (Carassius aurutas) increased its upper lethal temperature by 1°C for every 3°C rise in acclimation temperature up to 36.5°C. Loeb and Wasteneys (1912) and Doudoroff (1945), working with the genus Fundulus, observed similar temperature changes to those exhibited by the goldfish. Brett (1956) experimented with the upper and lower lethal temperatures in 23 species of fish. He found Pimephales promelas to be eurythermal with an upper lethal temperature of 33.2°C and a lower lethal temperature of

10.5°C when acclimated to 30°C.

Oxygen requirements of fish under varying conditions of temperature, pH, carbon dioxide and a variety of toxicants have been studied by Black, Fry and Black (1954), Clausen (1936), Fry, Black and Black (1947), Wiebe, McGavock, Fuller and Marcus (1934), Fry and Hart (1948), Doudoroff (1957) and other. Wells (1935) found than Fundulus parivipinnis exhibited differential oxygen uptake due to the size of the fish. Fry and Hart (1948) disagreed with Well's findings, and stated the divergences in the oxygen uptake curves could be eliminated if plotted on semi-logarithmic paper, instead of at constant rate intervals. Fry and Hart (1948) found that the rate of oxygen uptake increased as the temperature increased, until approximately 30°C, when the rate either stabilized or decreased slightly in the goldfish.

Moore (1942) studied the asphyxial levels of oxygen for several species of fish under winter and summer conditions. Moore lowered the fish to predetermined levels in live boxes in the lake being studied. Fundulus diaphanus, under winter conditions of 4°C was able to survive at 2 ppm dissolved oxygen. Moore concluded from the summer tests that the minimum amount of oxygen required per day a 15°C to 26°C was 3.5 to 5.0 ppm for warm water fish. The proposed values for a minimum

concentration of dissolved oxygen seemed erroneous because fish were subjected to rapidly changing conditions not normally encountered, resulting in high mortality of test fish. Dissolved oxygen concentrations ranged from 3.5-5.0 ppm in a narrow thermocline. Increased mortality was found when fish were placed below the thermocline without acclimation to low levels of dissolved oxygen and low temperatures.

Packard (1905) observed that Fundulus heteroclitus and Fundulus majalis survived for 5 hours and 65 minutes, respectively, in water almost completely devoid of oxygen. Wilding (1939), working with Pimephales notatus, observed that the asphyxial level of the fish at 20.5°C-26.0°C was at an average dissolved oxygen concentration of 2.23 ppm for 10.5 hours. At 7°-12°C the fish lived a period of 179 hours with the asphyxial level at 1.75 ppm dissolved oxygen. From his work he concluded that there was no correlation between carbon dioxide content and asphyxiation of the fish.

Doudoroff (1957) stated that free carbon dioxide was more important than pH values since it can be added to water without changing the pH concentration and may be most detrimental in strongly buffered systems. Free carbon dioxide rarely occurs above 20 ppm even in polluted waters. Concentrations of 50-100 ppm carbon dioxide will be fatal after long periods of exposure (specific examples of species were not given) Doudoroff (1957).

Fry et al., (1947) found that goldfish acclimated to higher temperatures showed increasing resistance to carbon dioxide, and that carbon dioxide did not impair the ability of the goldfish to extract oxygen from the test solution at increased temperatures.

In general, the amount of dissolved oxygen which is satisfactory in maintaining a species of fish has been reported to be 3.0 ppm and greater. Fish sensitive to low oxygen concentrations may die if the dissolved oxygen is at 3.0 ppm, but this is usually under very adverse conditions. Mortality above 3.0 ppm dissolved oxygen is not to be frequently expected (Doudoroff, 1957).

Osmoregulation in fresh-water fish is controlled by the kidneys and the gills, although the gills are primarily respiratory organs. The contribution of the gills in the process of osmoregulation have been studied extensively as the sites of active chloride secretion in fish. Smith (1930) was the first to postulate the concept that sodium chloride was actively secrted into the surrounding media against a concentration gradient.

Keys and Willmer (1932) found cells in the gills of the common eel and various species of fish which underwent a morphological change in response to various salt concentrations. Further cytological evidence for the functioning of chloride secreting cells was found by Copeland (1948) and Burns and Copeland (1950)

Their investigations indicated that chloride cells may be modified mucous cells. The cells were located near the afferent artery and were shown to respond to changes in salinity. After exposure to sea water the vesicles of the cells gave positive chloride tests as measured by cytological examinations of the gill tissue. In tap water, the cell vesicles showed a negative chloride test.

Burns and Copeland (1950) also reported the presence of chloride secreting cells in the epithelium of the oral cavity. The number of cells present in the opercular region suggested that this region may be important in osmoregulation. Verification of this factor is unknown at the present time.

Black (1948) observed that F. heteroclitus lost a measurable amount of salt when it was transferred from salt water to fresh water. Salt loss was probably a diuretic response to shock and handling, rather than an adaptation to fresh water. A 4 per cent gain in fish weight was measured 6 hours after placing it in fresh water. An increase in weight was not uncommon. Weight increases occurred due to natural osmotic changes resulting from placing the fish in a hypotonic medium. Black further noted that the fishes' weight stabilized after a period of time and then declined. The subsequent stabilization and the loss of weight by fish in the hypotonic medium was caused by a kidney response function-

ing to maintain water balance by excreting copius amounts of urine.

Meyer (1948) reported that carp lost an appreciable amount of chloride during the first 20 minuets they were placed in tap water. the rate of loss decreased during the next 6 hours after which the reabsorption of chloride began from the surrounding media. Chloride loss and gain was shown to be in the gill. Meyer had collected all urine and fecal material by placing a rubber bag around the fish leaving only the gill area exposed as the regulator of chloride loss and reabsorption.

Kidney function in fresh-water fish and the factors which govern reaction to hypotonic and hypertonic osmotic concentrations have been investigated by Grafflin (1937, 1938). Grafflin found that the kidney absorbed fluorescein dye at a faster rate from sea water than fresh water. The more rapid uptake of flourescein dye and its presence within the kidney tubules demonstrate that fish in a hypertonic media must drink large amounts of water in order to remain in osmotic equlibrium, with the kidneys functioning in maintaining water balance. Flourescein dye in the fresh-water media was not actively taken up, and the minute amounts present in the kidney tubules were reported to be from small amounts of water inadvertently swallowed by the fish.

The possibility of hormonal control of osmoregulation

has been studied by Gorbman and Berg (1955). In F. heteroclitus, there was a greater activity of the thyroid in hypotonic rather than hypertonic media, suggesting that the thyroid gland may be involved in osmoregulation. Thyroid function may be important in the tolerance limits of various species of fish, but the significance of their work has not been proven or disproven by comparative tests.

Toxicity of sodium chloride according to Ellis (1937) is the limit of osmotic pressure tolerated by a fish. The pressure suggested as limiting by Ellis is 6 atmospheres, which is equal to approximately 7,000 ppm sodium chloride. However, it is known that fresh-water fish have survived at 8,000-9,000 ppm sodium chloride which is greater than the osmotic pressure of the blood of fresh-water fish (Doudoroff and Katz, 1953).

Clemens and Jones (1954) studied the toxicity of brine water effluents from oil wells and sodium chloride on P. promelas and Fundulus kansae. The computed TL_m (median tolerance limit) for P. promelas was 8,954 ppm and 8,361 ppm sodium chloride at 23°C and 28°C respectively. For F. kansae acclimated to natural salinity conditions (6,329 ppm chloride), the TL_m was 24,649 ppm sodium chloride and in the unacclimated test it was 16,000 ppm sodium chloride at 16°C.

Bioassay methods have been standardized in recent years (Doudoroff, et al., 1951) and outlined in the American

Public Health Association manual (1960). Bioassays have proven to be a useful experimental procedure in establishing toxicity levels of industrial wastes (hot water, oils, chemicals etc.) on various species of fish. Despite the voluminous literature on bioassay tests, few attempts have been made to establish comparative differences between species based on resistance or vulnerability (intolerance) to various toxicants. The lack of comparative information has been caused by a failure to standardize the choice of species to be used in bioassays. In most cases, the only criteria used was the availability of the fish in the locality of the test center. However, bioassays have not been used for distribution studies of fish, although the method of testing has been proven to be a valuable experimental tool.

Doudoroff et al., (1951) pointed out that laboratory adapted fish must not be highly resistant to severe water conditions. Families of fishes thought to represent good test animals included the following: Centrarchidae (basses, sunfishes and crappies), Cyprinidae (true minnows excluding carp and goldfish), Salmonidae (trout, chars, and salmon), and the Catostomidae (suckers). The more common minnows endorsed by Henderson and Tarzwell (1957) as usable species included the fathead minnow, P. promelas, and the bluntnosed minnow, P. notatus. Other fishes which have been listed include the stoneroller, Campostoma

anomalum; creek chub, Semotilus atromaculatus; the shiners, Notropis sp.; and Notemigonius crysoleucas the golden shiner.

GENERAL MORPHOLOGY, HABITAT AND DISTRIBUTION OF THE SPECIES

Pimephales promelas Rafinesque

The fathead minnow is small, the adults usually 1.6-3.0 inches in length (Trautaman, 1957; Scott, 1955). Males are easily recognized during the breeding season (May to August) by the presence of nuptial tubercles on the snout. Females lack tubercles, but the body shape and morphological characteristics are similar except for the breeding colors (Harlan and Speaker, 1956; Eddy and Surber, 1947). The color of P. promelas varies from olive to black on the dorsal surface, while the abdomen remains white in color. A lateral line is incomplete and is most prominant near the anterior portion of the fish. Dorsal fin rays number 8-9, and the anterior fin ray is short and thickened.

The fathead minnow is widely distributed throughout the United States and Canada. Habitats vary from muddy to clear brooks and streams, ponds, rivers to small and large lakes (Forbes and Richardson, 1903; Scott, 1955; and Hubbs and Lagler, 1958). P. promelas is

tolerant of wide ranges of hydrogen ion concentration and turbidity (Trautman, 1957).

P. promelas ranges in the United States from Maine, west to the Susquehanna River system of New York, Ohio, Pennsylvania, and then southwest to Kansas and northern Oklahoma. In Canada P. promelas is found from the Prairie Provinces of western Canada, east through the drainages of Hudson Bay to the St. Lawrence drainages of Quebec (Hubbs and Lagler, 1958; Scott, 1955: Eddy and Surber, 1947). P. promelas has a very extensive distribution in Iowa (Harlan and Speaker, 1956) and in South Dakota (Bailey and Allum, 1962).

In North Dakota, P. promelas is very common in the tributaries of the Red River and in many coulees and fresh water lakes scattered throughout the state. The fish tends to avoid fast running water in favor of sluggish current in streams (Copes, 1964 personal communication; Hankinson, 1929; Bailey and Allum, 1962).

Fundulus diaphanus (Le Sueur)

The banded killifish is small, with a maximum length of 1.5-3.2 inches. The fish is easily recognized by conspicuous vertical bands and irridescent color on the sides (Scott, 1955; Trautman, 1957). The large eye is another distinguishing feature of the species. The dorsal fin contains 12 or 13 fin rays, and no lateral line is present in the species (Eddy and Surber, 1947;

Scott, 1955; Moore, 1957).

F. diaphanus inhabits clear to muddy waters of ponds, marshes and shallow lakes where there is an abundance of aquatic vegetation (Eddy and Surber, 1947; Trautman, 1957; Harlan and Speaker, 1956). Bottom types of the habitats are usually marl or mud and sometimes sand. The species is intolerant to turbidity and occurs in fresh to brackish water throughout its distribution (Scott, 1955; Trautman, 1957).

Distribution of the species includes the ranges of two subspecies: F. diaphanus diaphanus (Le Sueur), the eastern banded killifish and F. diaphanus menona Jordan and Copeland, the western banded killifish. The species ranges from eastern North and South Dakota, to Minnesota, Iowa, Wisconsin, Michigan, Illinois, Indiana, Pennsylvania and New York. In Canada, it is found from Quebec through the Maritime Provinces to Newfoundland (Hubbs and Lagler, 1958; Eddy and Surber, 1947; Trautman, 1957; Scott, 1955; and Moore, 1957).

In North and South Dakota, the species inhabits shallow lakes and marshes but is scarce throughout most of the two states. Bailey and Allum (1962) reported finding the species in two of their 137 collection stations scattered over the entire state of South Dakota. In the Grand Forks area of North Dakota, the species is fairly common in fresh and salt water coulees and in some salt marshes and small lakes. In Iowa, the fish

are restricted to natural lakes in the northern portion of the state (Harlan and Speaker, 1956). F. diaphanus has been reported from shallow lakes and streams in southern Minnesota as well as the upper reaches of Minnesota and Mississippi River systems (Eddy and Surber, 1947).

Culaea inconstans (Kirtland)

The brook stickleback is easily recognized by the presence of five free spines anterior to the dorsal fin. The species is small, ranging from 1.5-2.7 inches in length in the adult (Scott, 1955; Trautman, 1957). Color varies from dark green or brown to black on the dorsal surface which grades into lighter pigmentation on the abdomen (Eddy and Surber, 1947). The skin of C. inconstans is smooth and scaleless (Harlan and Speaker, 1956).

C. inconstans inhabits springs, ponds, small or large lakes, streams and brooks. Inhabited waters are usually rich in aquatic vegetation from which the fish gather material to build intricate nests during the breeding season (Harlan and Speaker, 1956). The fish is tolerant of wide ranges of hydrogen ion concentration but has a low tolerance to turbidity (Trautman, 1957). However, in North Dakota, C. inconstans was found in highly turbid waters indicating the species was tolerant to turbidity.

The range of the species in Canada extends from

British Columbia east to New Brunswick. In the United States, the species is found from Montana, east to the Great Lakes and Maine, west and south to the Ohio, Illinois, Mississippi and Missouri River systems (Hubbs and Lagler, 1958; Eddy and Surber, 1947; Trautman, 1957; Scott, 1955). In North Dakota, Minnesota, and Iowa the species is widely distributed in creeks, ponds, sluggish streams and springs (Eddy and Surber, 1947; Harlan and Speaker, 1957; Bailey and Allum, 1962; Hankinson, 1929).

THE EXPERIMENTAL DESIGN

All individuals tested were compared in a quantal response design formulated by Cole (1962) for use in toleration tests between various organisms. The actual design of the experiment was a closed sequential test. In the test, pairs of individuals were compared on the basis of an "all or none" response. The individuals were classified as "living or dead". The format and the theoretical mechanics of the test design were described by Bross (1952) and Fisher (1952). Specific characteristics of the statistical design can be found in Appendix I.

The sequential design was chosen because of a strong statistical significance when gross differences

between organisms were considered. The test pointed out important differences between the three species with a minimum amount of experimentation. This factor was extremely important when only small numbers of animals could be obtained. The design eliminated the need for computing the complicated probit analysis and the laborious task of checking the differences concluded from the probit.

MATERIALS, METHODS AND THEIR USES

Source, Collection and Transport of Fish

The accessibility and numbers of fish which could be collected at one time were the principle criteria used in selecting collecting sites. However, large numbers of the test species are located in the rivers and streams of North Dakota.

Pimephales promelas occurs in the English Coulee as it flows through the campus of the University of North Dakota. English Coulee has a watershed of approximately 13 miles. It originates 10 miles southwest of the University of North Dakota, Grand Forks, North Dakota. The average depth of the coulee is 4 feet, and the bottom sediments are comprised of mud and decayed organic matter. The chloride concentration of the water was 600 ppm.

Fundulus diaphanus and Culaea inconstans were

collected from Kelly's National Wildlife Refuge located 10 miles west and 4 miles north of Grand Forks, North Dakota in Blooming township (T154N, R52W, Sec's. 15, 22, 27). Kelly's Slough has a watershed of seven square miles before draining into the Turtle River, a tributary of the Red River. Kelly's Slough is primarily a large open body of water (approximately 150 acres), rich in submergent vegetation. Kelly's Slough has an average depth of 2.5 feet. The bottom sediments are comprised of gravel near the shoreline, with a muddy ooze making up the rest of the bottom area. Chloride concentrations were found to be 2500 ppm.

Periodic collections of the three native species were made on 8 dates (June 9, 17, 20; July 3 and 21; October 25 and 30; and November 6, 1964) from English Coulee and Kelly's Slough.

In English Coulee, a minnow seine (25 x 4 feet with 1/4 inch mesh) was used exclusively in collecting P. promelas. Concentrations of fish were large in early June, October and November. During late June and July, P. promelas were very scarce. Approximately 375 to 600 P. promelas were taken with two seine hauls in early June, October and November. From mid-June through July, only enough fish (15-20) were taken at one time to supply the needs of one experimental run.

Kelly's Slough was seined with good results during June. During July, October and November, seining was

impossible because of the large amount of submergent vegetation in the slough. To combat this problem, five (24 x 10 inch) conical plastic minnow traps were used to take F. diaphanus and C. inconstans. The traps were staked out parallel to the shoreline with a 10 yard interval between each trap. Traps were weighted down with lead wheel weights or rocks and suspended by wire at the desired depth. Fish were removed daily. C. inconstans was easily trapped, but F. diaphanus was not taken in traps in large numbers. During October and November, F. diaphanus was found in rocky cravasses of the spillway which leaves the slough.

After the fish were collected, they were placed in a large plastic tub containing six to eight gallons of water. Care was taken to see that fish were not overcrowded. To reduce water disturbance during transport, a two gallon plastic bucket was inverted into the plastic tub. Transportation back to the laboratory was made as quickly as possible.

Transportation effects occurred primarily in hot weather when normal temperatures could not be maintained in the carrying container. High rates of mortality occurred in all species when the water temperature was above 33°C. When mortality was high, entire lots of fish were discarded. It was then necessary to make several trips to the collecting sites to obtain enough fish for testing purposes. Ice was added to the carrying

container to keep the water temperature below the critical limits. Mortality decreased to less than 2 per cent when fewer fish were taken and when ice was added to the water.

Laboratory Techniques

Upon arrival at the laboratory the fish were sorted according to species, size and breeding condition. According to Doudoroff, et al., (1951) and the A.P.H.A. manual (1960), the largest fish in the experimental tests should not be greater than 1.5 times the smallest. Fish which were kept were approximately equal in size within the same species. Gravid females and large males were eliminated in order to reduce the possible changes in tolerance limits due to breeding conditions. Fish were then transferred to aquaria that served as holding and acclimation tanks. The capacity of the aquaria varied from 15 to 30 gallons. Fish were held in tap water that had been aerated in a 200 gallon galvanized steel tank. The chlorine content of the stored water became negligible after aeration for 24 hours.

The fish were allowed to adjust to the laboratory situation for three to four days. During this time they were observed closely to determine the effects of shock from handling, transportation, temperature, and excitability. Fish that suffered or responded to the above stresses usually died by the end of the observation period.

Mortality over the first few days was always between 1 and 3 per cent for P. promelas and approximately 1 per cent for C. inconstans, while no mortality was recorded for F. diaphanus. After the first few days the number of deaths seemed to stabilize and only incidental deaths occurred. On some occasions fin rot was found in P. promelas but not in the other two species. The disease was halted by adding liquid terramycin to water in the aquaria as described by Irwin (1959).

According to Doudoroff, et al., (1951), feeding should be at regular intervals during the period of acclimation in the laboratory, but not prior to testing by several days. The procedure was followed with all the individuals being fed live Daphnia pulex and D. magna in the summer and dried D. pulex in October and November.

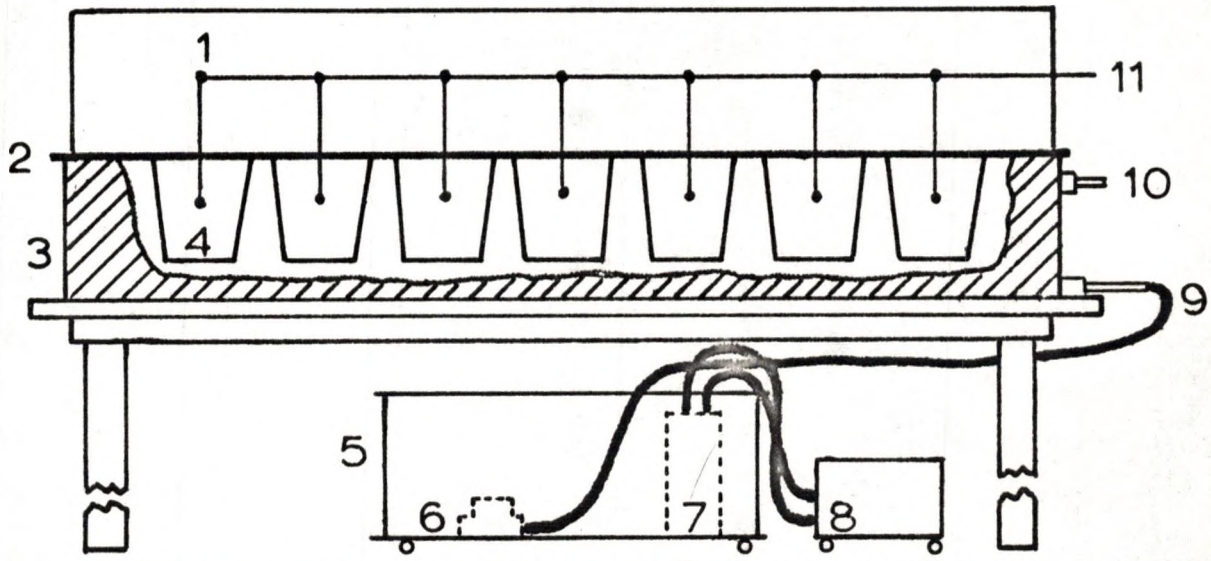
Acclimation and Water Quality

Acclimation of the fish to the laboratory conditions and temperatures was accomplished over a 7 to 12 day period. To reduce the length of acclimation time and possible losses due to laboratory conditions, fish were collected from natural environments when the water temperatures were at, or close to the selected test temperatures of 4°, 20° and 30°C. Fish collected in June were taken from 18° to 21°C water and tested at 20°C. Fish taken in July came from 27° to 30°C water

and were tested at 30°C. Fish collected in October and November came from 4°C to 6°C water and were tested at 4°C in the laboratory. Test temperatures were thought to be representative of extreme environmental temperatures encountered by the fish during all seasons of the year.

Acclimation temperatures were maintained in the laboratory in the following ways. The laboratory temperature maintained the 20 ± 1°C temperature. Aquasco 15 watt aquaria heaters were used to raise the temperature to 30 ± 1°C. The 4°C temperature was maintained by placing plastic buckets, each with a different species, into a refrigerator with a temperature of 4 ± 1°C. In all instances, air lines, with breaker stones, individually controlled by valves, led to the acclimation tanks from an air compressor.

Temperature control during the tolerance tests was accomplished by using a Blue-M Constant-Flow portable cooling unit. Water was circulated through the bioassay trough (12 x 28 x 90 inches) and the cooling tank by a submersible pump (Figure 1). The method was adequate to maintain a control of ± 0.5°C during testing periods. Plastic two gallon buckets were used as test containers because of the low thermal conduction of plastic to the surrounding water surfaces. Seven test containers were suspended in the bioassay trough by cutting circular holes in a 3/8 inch sheet of plywood which covered the entire surface area of the trough. Each test container



Cut-away side view. Scale: 3/4 inch = 1.25 feet

Figure 1. Bioassay test equipment.
(1) Individual control valve and oxygen line to test container.
(2) Plywood top.
(3) Galvanized steel trough.
(4) Plastic test container.
(5) Cooling tank.
(6) Submersible pump.
(7) Cooling coil.
(8) Power unit of cooler.
(9) Water inlet to trough.
(10) Water outlet of trough.
(11) Aeration line to oxygen cylinder.

was filled with seven quarts of distilled water. The individual containers were covered with a sheet of plate glass to reduce the amount of heat exchange and evaporative loss. Oxygen was used to aerate the bioassay containers.

The dissolved oxygen content of the various test solutions was measured by the Alsterberg (Azide) Modification of the Winkler method. Procedures for the test were taken from the A.P.H.A. manual (1960).

Salinity concentrations of all test solutions were made by using distilled water for the 0 ppm concentration, since the chloride content was less than 2 ppm, or by the addition of sodium chloride to the distilled water to make up the desired concentrations, The sequential test concentrations of 0 ppm and 10,000 ppm sodium chloride were chosen for two reasons. First, distilled water was known to cause an inward movement of water through the gills and oral membranes of the fish. This would cause an increased work load on the kidneys to maintain water balance within the individuals being tested. By subjecting the three species to a very hypotonic medium, I wanted to establish which species was more successful in maintaining its water balance by kidney function alone. It was further hoped that the results of the tests might have some relationship to the more successful species in the salinity tests. Secondly, the high salt concentration of 10,000 ppm sodium chloride was selected

because it was almost twice the sodium chloride concentration of 5,900 ppm prescribed by Burnstock (1958) for a physiological saline solution for fresh-water fish. By testing fish in the 10,000 ppm sodium chloride, results of the species tolerance to the increased concentration would be more valid than testing various concentrations equal to or greater than the environmental chloride concentrations in the two study areas.

Hydrogen ion concentrations were measured in each test container before and after each test with a Beckman Pocket pH Meter. The distilled water from the still had low pH values (6.5-6.9), and sodium bicarbonate was added to the water to raise the pH to the envrionmental ranges (7.4-8.3) of the species.

Selection of Fish for Testing

The selection of fish to be tested in the experimental design was random. A dip-net was passed through a sample of the population in the aquaria. Each individual was assumed to have an equal and independent chance of being selected. Sorting the fish in the early stages assured that any individual selected could be used in the experimental procedure. The physical condition of the fish used in the study was based on the standardized bioassay criteria set forth by Doudoroff et al., (1951). Duration of the tests in the sequential desigh lasted until one individual of the pair died. A total of seven fish

for each of the three species were tested at one time.

In addition, the bioassay tests were all run for a period of 48 hours. A series of experimental tests at various concentrations of sodium chloride were conducted to reduce the range between the upper lethal and lower non-lethal concentrations. The bracketing of the concentrations continued until they were separated by 1,000-2,500 ppm sodium chloride. The number of individuals surviving in the final tests were recorded and a straight-line graphical plot of the TL_m values were used to substantiate the results obtained from the sequential tests in the salt solution as to which species was more tolerant at various test temperatures.

Records were kept on all fish in the experimental tests. Data included: species name; temperature of the environmental and test waters; salinity concentrations; time at the beginning and end of the tests; sex determinations, and weights and lengths of the fish. Death of an individual during the test was chosen as the time when the fish ceased all opercal, pelvic and pectoral movement.

RESULTS

Sequential Tests

Table 1 shows the results of the sequential tests when the tolerances of the three species were compared. F. diaphanus and C. inconstans were consistently more tolerant of varied salinity conditions than P. promelas except at 20°C in distilled water. P. promelas and C. inconstans showed no differences when tested at 30° in distilled water, but both lost to (i.e. were less tolerant than) F. diaphanus under the same conditions. When F. diaphanus was compared to C. inconstans, F. diaphanus won in all but one test where salt concentrations were high, but lost repeatedly in the distilled water tests except at 30°C. At 4°C in high salt concentration, F. diaphanus and C. inconstans tolerated the condition for 144 hours without a single death occurring in either species. For this reason, both species were concluded to be equal in their tolerance for high sodium chloride concentrations at this particular temperature. Sex and size of the individuals were tested and had no influence on the outcome of the sequential tests.

The oxygen concentrations during the various tests are shown in Table 2. The fish were examined for anoxia due to low oxygen tensions and high carbon dioxide concentrations in the test solutions. In no case did the general characteristics of anoxia observed by other

workers occur. The characteristics of anoxia that were checked for, included hemmorhaging in the caudal fin and lip region. The oxygen concentrations were low enough to prevent oxygen poisoning from supersaturation of the blood, which occurs at high dissolved oxygen concentrations (Doudoroff, 1957).

The range of pH values before and after the tests are given in Table 3. Changes that took place were not great enough to cause the hydrogen ion concentration to be shifted above or below the levels encountered in the natural habitat. The small changes were attributed to respiration and waste products of the fish.

Bioassay Results

A test period of 48 hours was chosen to establish TL_m values to sodium chloride because of the evidence reported by Douglas (1961). He stated that the highest percentages of death will occur in the first 24 hour period with little or no changes at 48, 72, and 96 hours.

Figures 2 (4°C), 3 (20°C), and 4 (30°C) show the per cent survival when plotted against time in hours. Graphical results indicated a decrease in per cent survival with an increase in temperature for a test solution of 0 ppm sodium chloride for C. inconstans and F. diaphanus. Pimephales promelas showed a sharp increase in survival from 4°C to 20°C but decreased rapidly at 30°C. At 10,000 ppm sodium chloride, the

Table 1. Results of sequential testing.

Test and Winner / Species	4°C (4)	4°C (0)	20°C (4)	20°C (0)	30°C (4)	30°C (0)
P. promelas (1) vs. F. diaphanus (2)	(2) TR=7:0	(2) TR=7:0	(2) TR=7:0	(1) TR=11:2	(2) TR=7:0	(2) TR=9:1
P.promelas vs. C.inconstans (3)	(3) TR=7:0	(3) TR=7:0	(3) TR=7:0	(1) TR=11:2	(3) TR=7:0	Equal TR=6:7
F.diaphanus vs. C. inconstans	Equal TR=0:0*	(3) TR=14:3	(2) TR=7:0	(3) TR=7:0	(2) TR=7:0	(2) TR=9:1

(0) = 0 ppm sodium chloride in distilled water.
(4) = 10,000 ppm sodium chloride in distilled water.
TR = Test ratio of the number of times species A was better than species B.
*= TR = 0:0 means no death at the end of 144 hours.

Table 2. Oxygen concentrations at various salinity values

°C	Chloride concentration in ppm			
	0	10,000	16,000	20,000
	Dissolved oxygen in ppm			
4	12.9	11.8	11.1	10.1
20	9.2	8.1	7.7	7.2
30	7.5	6.7	6.6	6.0

Table 3. Range of pH values before (B) and after (A) testing.

4°C				20°C				30°C			
0 ppm		10,000 ppm		0 ppm		10,000 ppm		0 ppm		10,000 ppm	
B	A	B	A	B	A	B	A	B	A	B	A
7.6-7.8	7.6-7.8	7.7-7.8	7.7-7.9	7.7-7.9	7.6-7.8	7.4-7.9	7.3-7.7	7.7-7.9	7.5-7.8	7.6-7.9	7.5-7.6

Table 4. Mean survival time in hours and the percent survival (%) at the end of each sequential test.

	4°C				20°C				30°C			
	(0)		(4)		(0)		(4)		(0)		(4)	
	hours	%	hours	%	hours	%	hours	%	hours	%	hours	%
P.p.	15.75	0	33.37	0	89.9	84	2.62	0	4.25	0	3.50	0
C.i.	95.60	85	144.00	100	61.0	0	42.16	0	3.92	0	5.11	0
P.l.	35.25	26	144.00	100	45.8	0	55.00	100	5.66	77	6.00	100

(0)=0 ppm sodium chloride.
(4)=10,000 ppm sodium chloride.

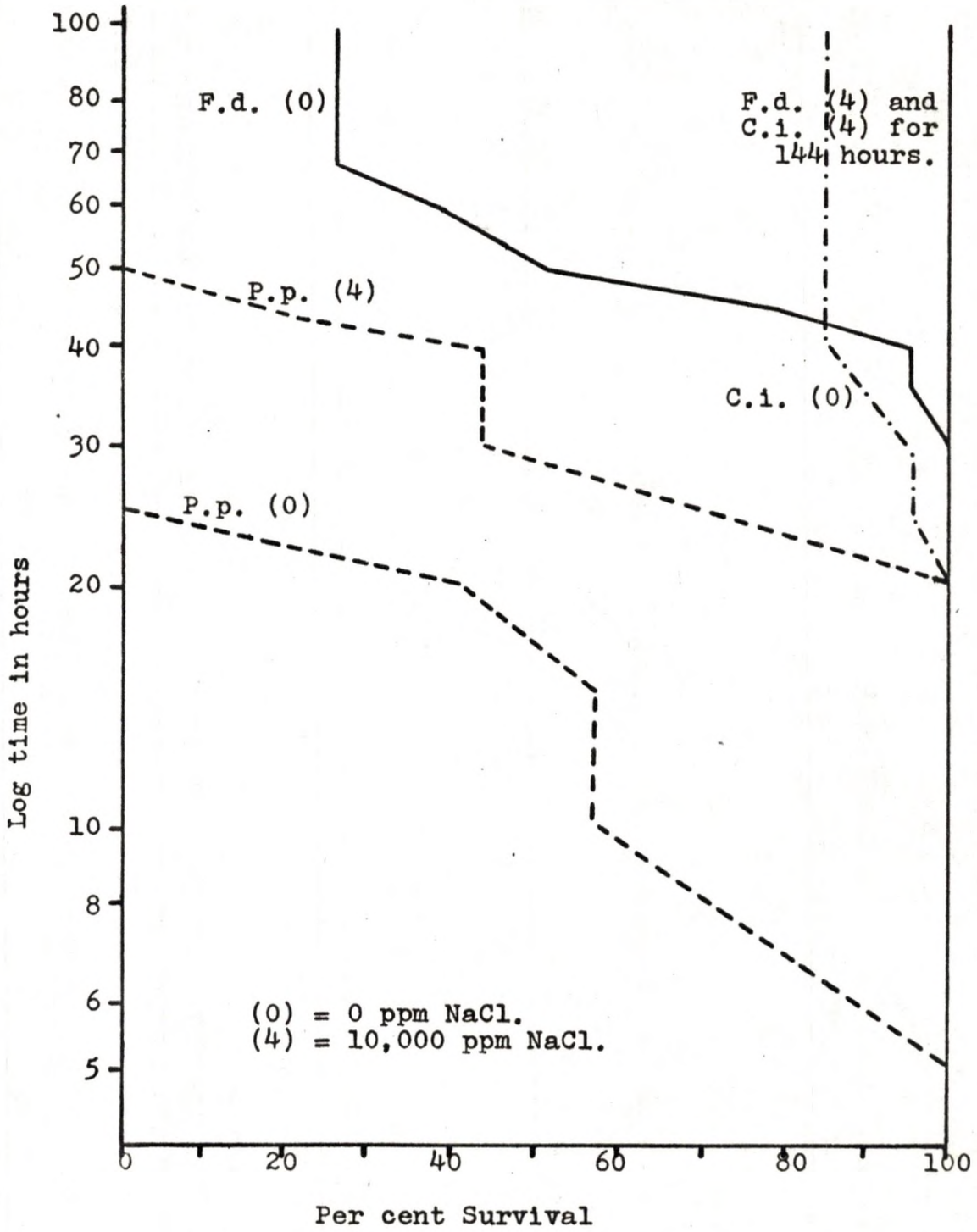


Figure 1. Per cent survival for each species at 4 ± 0.5°C during the sequential tests.

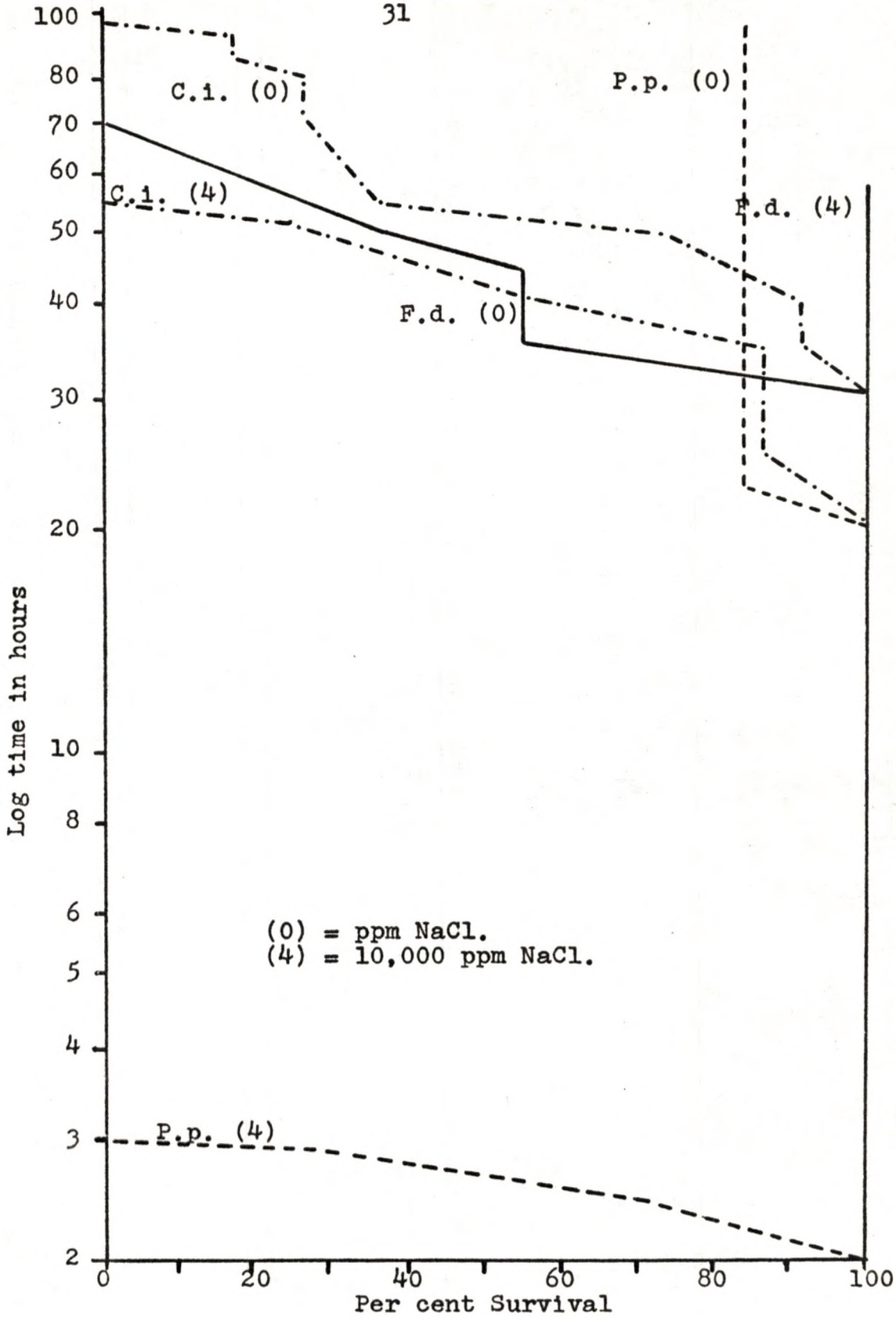


Figure 3. Per cent survival for each species at 20 ± 0.5°C during the sequential tests.

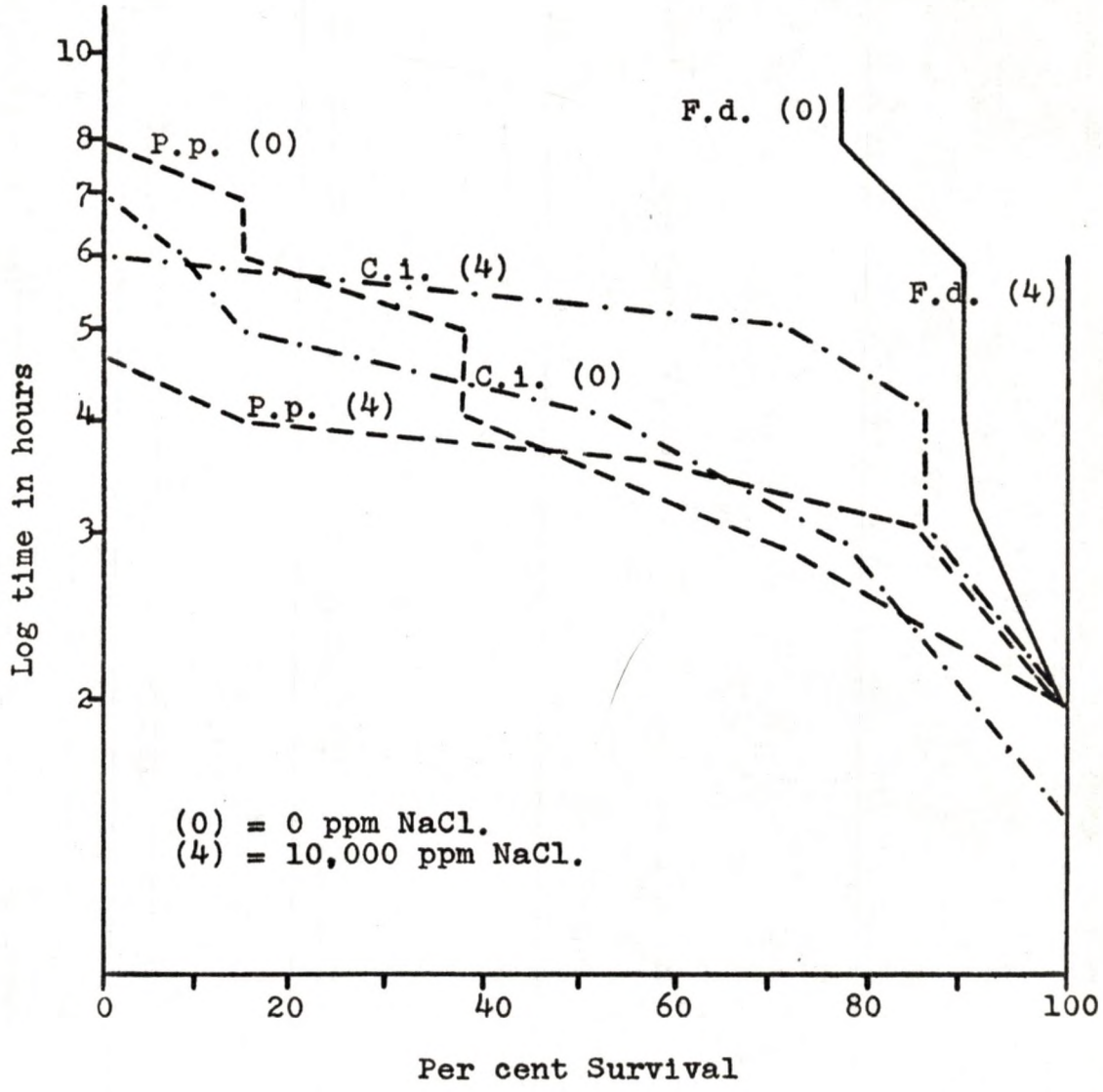


Figure 4. Per cent survival for each species at 30 ± 0.5°C during the sequential tests.

per cent survival again decreased with a rise in the test temperatures for P. promelas and C. inconstans. Fundulus diaphanus always showed a 100 per cent survival in the salt concentration until the end of the sequential tests. Computed mean survival times and per cent survival are shown in Table 4.

The results of the 48 hour bioassay tests are shown in Figure 5. The TL_m values were computed by the straight-line graphical plot method. The TL_m values indicated that each species can be ranked within certain concentration values which do not overlap for each individual test temperature. When the values are grouped together for all test temperatures, they still remain segregated except for one value. The concentration was 8,200 ppm sodium chloride, which equals the TL_m for C. inconstans at 30°C and P. promelas at 4°C. The TL_m values for the various test temperatures can be found in Table 5.

Even though only five fish were used at various concentrations instead of the usual 10 or 20 to establish TL_m values, the concentrations given in Table 5 were valid estimations of TL_m values for each species. The validity was based on the premise that the concentrations were bracketed with only 1,000-2,500 ppm sodium chloride separating the upper lethal and the lower survival concentrations before the TL_m values were computed. By bracketing the concentrations, the amount of error in computing the TL_m for each species was reduced. The TL_m

values tested in Table 5 do not represent absolute concentrations, but rather, estimations which suffice for most practical purposes.

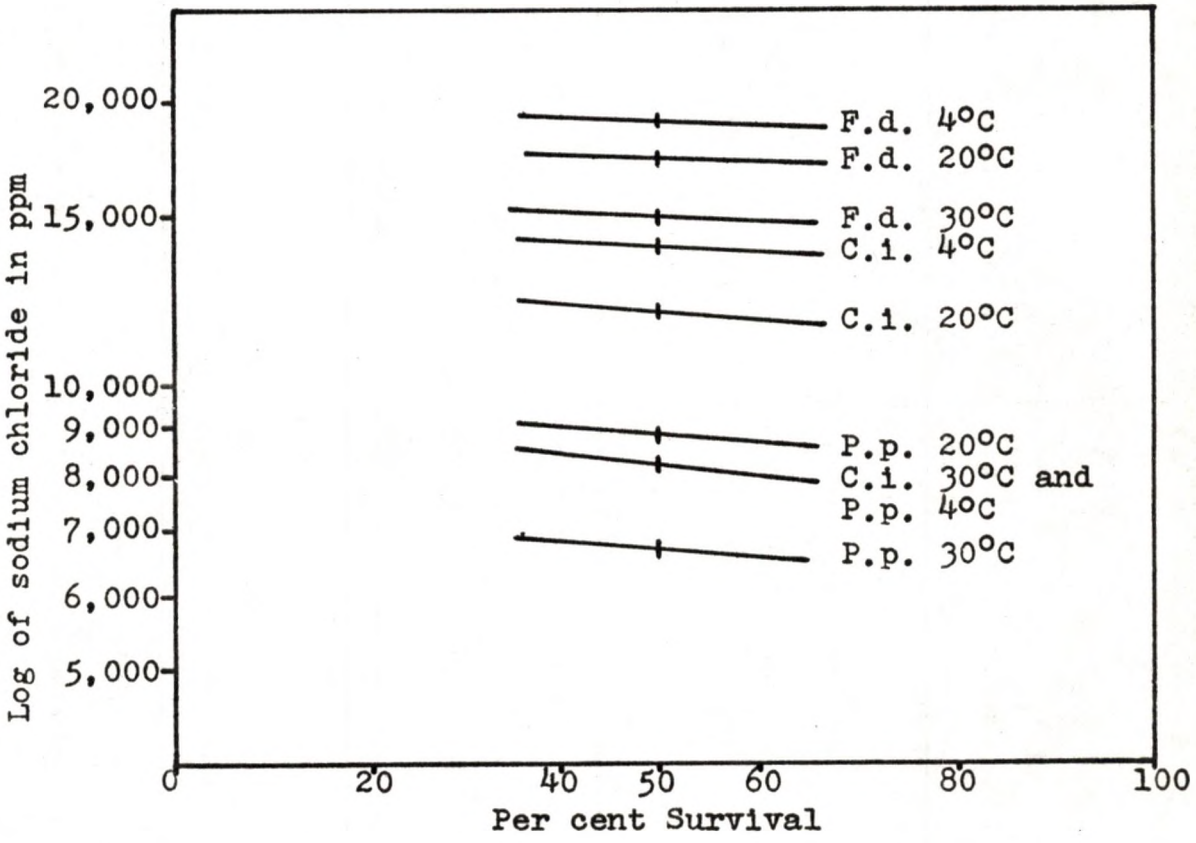


Figure 5. Straight-line graphical plot to establish 48 hour TL_m values for the three species of fish.

Table 5. TL_m (median tolerance) values for each species given in ppm sodium chloride for each test temperature.

Species	4°C	20°C	30°C
F. diaphanus	18,800	17,200	14,800
C. inconstans	13,900	11,900	8,200
P. promelas	8,200	8,700	6,700

DISCUSSION AND CONCLUSIONS

In general, the tolerance of F. diaphanus to high sodium chloride concentrations was far superior to that of C. inconstans or P. promelas. F. diaphanus was more tolerant in all the sequential tests in sodium chloride concentrations except at 4°C. At 4°C, F. diaphanus and C. inconstans were concluded to be equal in their tolerance to sodium chloride. The results of the 48 hour bioassay tests substantiated the results established in the sequential tests. The TL_m (median tolerance limit) for F. diaphanus was in close relationship to TL_m established for F. kansae by Clemens and Jones (1954). F. kansae exhibited a TL_m of 16,000 ppm sodium chloride when tested at 16°C. F. diaphanus tested at 20°C had a TL_m of 17,200 ppm. Results obtained by Loeb and Wasteneys (1912), working with Fundulus sp. (unidentified), showed no correlation with the present results. Loeb and Wasteneys found that at a concentration of 2,252 ppm sodium chloride, the fish would live an indefinite period of time. At increasing concentrations the fish died in a matter of minutes. The difference between the recent findings and Loeb and Wasteney's work was probably caused by species differences more than any other factor.

The ability of the genus Fundulus to adapt and survive in fresh or highly saline waters appeared to be related

to the chloride secreting cells in the gills and oral epithelium as described by Copeland (1948) and Burns and Copeland (1950). The inability of F. diaphanus to survive for extended periods of time in distilled water was evident (Table 4). However, F. diaphanus was able to live longer than P. promelas except at 20°C in distilled water, when P. promelas was more successful. When compared to C. inconstans, F. diaphanus "lost" in all the sequential comparisons with the exception of 30°C when it was more successful than C. inconstans. The ability to survive in distilled water at 30°C longer than the other two species, indicated a greater tolerance to high temperatures with a corresponding increase in metabolism and work load on the kidneys. The data further indicated a certain threshold level of salt must be present before F. diaphanus was capable of surviving for a great length of time, especially at higher temperatures.

F. diaphanus kidneys were able to withstand a tremendous water load, but without salt, F. diaphanus became the second most tolerant species to distilled water. C. inconstans was the most tolerant, with P. promelas being the least tolerant.

Culaea inconstans had an intermediate tolerance to salt concentrations. Tolerance levels were greatly affected by an increase in temperature. Toleration levels decreased rapidly with an increase in temperature (Figure 5

and Table 5). These results indicate that there is a greater interaction between salt and temperatures in C. inconstans than in the other two species. The presence or lack of salt at 30°C in the sequential tests caused an increased stress on the fish, increasing the rate of mortality. High temperatures probably succeeded in breaking down the osmoregulatory mechanism within the fish. The decrease in toleration was evident in the results of the TL_m values for 30°C (Figure 5). The enzymatic systems in the fish were probably the most adversely affected by an increase in temperature and the strain of the hypotonic and the hypertonic media.

The ability of Culaea inconstans to survive at low temperatures in high salt concentration indicated that there was a strong osmoregulation system capable of functioning at lowered metabolic rates. The presence or lack of chloride secreting cells or their function in controlling chloride excretion in C. inconstans cannot be assessed at this time. Cytological evidence of gill and oral tissues is needed to substantiate salt regulation in the species.

The discrepancy between mean time of death of C. inconstans for the sequential test at 20°C in 10,000 ppm sodium chloride solution and the bioassay test at 20°C can be attributed to individual variability within the species and small sample size. Some individuals lived longer than 48 hours, but several died between 20-30

hours. These factors account for the low mean time of death for C. inconstans (Table 4).

Pimephales promelas was the least tolerant species in either the hypotonic or hypertonic solutions. The only time P. promelas was more successful was at 20°C in distilled water. P. promelas lived longer in distilled water at 20° and 30° than in various sodium chloride concentrations at the same temperatures. At 4°C, it survived longer in the salt solution. Both P. promelas and C. inconstans showed equal tolerance to distilled water at 30°C. The ability of P. promelas to survive in the hypotonic rather than hypertonic media indicated that the species has an adequate mechanism for regulating and maintaining water balance. Assuming chloride cells were present, the intolerance to salt may have been caused by the inability of chloride cells to regulate chloride excretion. Chloride secreting cells have been found in many species of fish, but cytological examination of gill tissue from P. promelas has not been conducted to prove or disprove their presence in the species.

P. promelas exhibited optimum tolerance to distilled water and sodium chloride at 20°C. Computed TL_m was 8,700 ppm sodium chloride. The TL_m value at 20°C is similar to the TL_m of 8,954 ppm sodium chloride for P. promelas at 23°C (Clemens and Jones, 1954). A possible explanation for the optimum tolerance is that the species' osmoregulatory system was most efficient at 20°C. Higher tem-

peratures resulted in a more rapid breakdown of the osmoregulatory system than low temperatures. A more accurate understanding of the interacting osmoregulatory factors will be known when the function of enzymatic control of osmoregulation in various species of fish has been established.

Dissolved oxygen concentrations at various levels of salinity in both the sequential and bioassay test can be found in Table 2. The possibility that oxygen was the cause of death instead of osmotic factors seems to be purely negative from the results of the test. First, the asphyxial level established by Moore (1942) for F. diaphanus was 2 ppm dissolved oxygen at 4°C. P. notatus, a closely related species to P. promelas, exhibited an asphyxial level of 2.25 ppm dissolved oxygen at 20.5°-26.0°C. At 7°-12°C, the asphyxial level was 1.75 ppm dissolved oxygen (Wilding, 1939). Secondly, a comparison of TL_m values (Table 5) and dissolved oxygen concentrations (Table 2) indicated that the lack of osmoregulation was the cause of death in the test fish.

F. diaphanus was able to survive at high salt concentrations. In distilled water, the oxygen concentrations were greater by 2 ppm, but F. diaphanus failed to survive in distilled water for extended periods of time. This indicated that oxygen was not limiting, but rather the species was unable to regulate its water balance in distilled water.

The 48 hour TL_m values at 20°C for C. inconstans were higher than the salt concentration in the sequential test. A decrease of 0.4 ppm dissolved oxygen was noted between 10,000 and 16,000 ppm sodium chloride at 20°C. The dissolved oxygen difference between 0 ppm and 10,000 ppm sodium chloride was 0.8 ppm at 30°C. C. inconstans showed a longer mean survival time in saline solution than in distilled water. The TL_m value of 8,200 ppm sodium chloride at 30°C indicated the species was unable to maintain its osmoregulation.

Pimephales promelas is known to withstand low dissolved oxygen concentrations. Fish were able to survive longer at 20°C in distilled water when oxygen concentrations were 3.7 ppm less than at 4°C. The fish was able to withstand 10,000 ppm sodium chloride for a longer period of time at 4°C than at 20°C. This indicated failure of the fish to maintain osmoregulation or the inability to excrete salts with increasing temperatures. At 30°C the fish were able to live longer in distilled water than in sodium chloride. The dissolved oxygen difference was found to be 0.8 ppm less in the 10,000 ppm sodium chloride solution. The change in oxygen concentration did not seem to be great enough to be the cause of death. The TL_m value of 6,700 ppm sodium chloride at 30°C was a sharp decline from the TL_m value of 8,700 ppm at 20°C. The change in oxygen concentration between 0 ppm and 6,700 ppm sodium chloride was even less than the change

between 0 ppm and 10,000ppm sodium chloride at 30°C. Death was attributed purely to osmotic conditions and was in no way related to the dissolved oxygen content of the test solution.

The total chloride content of English Coulee was found to be 600 ppm. P. promelas was the dominant species and C. inconstans was subordinate. F. diaphanus was never collected or reported being present in the coulee. The dominance (atypically larger population) of P. promelas in the coulee can possibly be explained from the results of the 48 hour bioassays and the sequential tests. P. promelas recorded lower TL_m values (Table 5) than C. inconstans or F. diaphanus in sodium chloride. In the sequential tests in distilled water the species always lived longer than in saline water except at 4°C (Table 4).

The occurrence of P. promelas in Kelly's Slough (2,500 ppm chloride) indicated that the species had an available access route via the drainage system of the slougg from the Turtle River during high water. P. promelas was never collected after July, 1964. The disappearance of the fish (assuming they died) indicated some factors were acting against P. promelas inhabiting the slough. Possible suggestions for the absence of the species are based on field observations during 1964 and the spring of 1965. Young of the year P. promelas were never collected from Kelly's Slough. The lack of young fish may have been caused by a high salt conentration acting as an

inhibitor to fertilization. Assuming P. promelas was able to breed, the possibility of the adults and young of the year fish surviving the winter was remote since the slough freezes to the bottom except for a narrow mud-water interface zone. F. diaphanus and C. inconstans survived the winter and were present in the slough before the ice disappeared. A possible explanation for their ability to survive, is that they could exist in the mud-water interface zone by lowering their metabolic and respiratory functions while P. promelas was not able to do so. An accurate statement as to the eventual disappearance of P. promelas at various times of the year cannot be made on the basis of the concluded research. A more plausible explanation would be that other interrelated factors such as water chemistry, temperature and physical factors inhibited P. promelas from living in Kelly's Slough.

The absence of F. diaphanus in waters with low salt concentration in northeastern North Dakota suggests that other water quality factors are more important than the lack of salinity. F. diaphanus in the area around Grand Forks, North Dakota was found only in highly saline waters of clear sloughs or coulees. In western Minnesota the species was found in both low to high salinity areas. In northeastern North Dakota, fresh water bodies (English Coulee, rivers and streams) were highly turbid except near the headwaters. F. diaphanus

was never found in turbid waters of the tributaries of the Red River or in the approaches of the headwaters. Turbidity may have acted as a barrier for the species starting upstream. The species is known to be intolerant to turbidity (Trautman, 1957). F. diaphanus seems to be limited by the availability of water bodies low in turbidity (Kelly's Slough and Stewart's Slough) and not primarily by salinity concentrations.

Salinity concentration undoubtedly plays some part in the distribution of F. diaphanus. Dilution of moderately saline waters by periodic flooding would possibly be detrimental to the species. This assumption is based on the results of the tolerance tests. F. diaphanus showed that a certain salinity threshold must be present before the species was able to survive. The direct relationship between salts and the distribution of the species was not shown by the salinity tolerance tests, but highly saline waters appear to favor the distribution of F. diaphanus.

Culaea inconstans was found to be intermediate in tolerance to sodium chloride in the sequential and bioassay tests. C. inconstans was also the overlapping species in the distribution pattern of the three species in northeastern North Dakota.

Although Kelly's Sough was higher in total chlorides than English Coulee, Kelly's Slough supported a larger population of C. inconstans than did the coulee. The

presence of the species in both areas indicated that the species was capable of surviving over a wide range of salt concentrations.

The smaller numbers of individuals in English Coulee may be related to failure of the osmoregulatory system at high temperatures exhibited in the tolerance tests by the species. Above 20°C, the species shoed a rapid decline in ability to withstand salt or distilled water. This may be related to the species' natural habitat preference to colder waters of spring fed brooks, streams and lakes.

C. inconstans was found by Copes (1964, personal communication) in the upper waters of the Turtle and Forest Rivers where colder water and vegetation were present. The temperatures of English Coulee rose rapidly during low water and remained high, because vegetation was lacking to provide shade and cooler water temperatures at lower depths. A lack of vegetation for the species to build nests and competition with P. promelas during the breeding season may have been other factors contributing to fewer numbers of individuals. Another possibility is that turbidity in the coulee may have been a limiting factor in reducing the number of individuals, even though the species was more tolerant of turbidity than F. diaphanus.

Kelly' Slough seemed to provide a more suitable habitat for C. inconstans than English Coulee for several

reasons. First, the slough was not turbid. Secondly, and probably most important, there was adequate vegetation to provide breeding and hiding places for the fish and lower water temperature by shading the bottom area. The role salinity played in C. inconstans distribution was not concluded from the investigation.

SUMMARY

1. The sequential test design was shown to be a suitable bioassay technique for establishing salinity tolerances between different species of fish.
2. The study established that F. diaphanus was more tolerant to high sodium chloride concentrations than C. inconstans or P. promelas. In distilled water, F. diaphanus was found to be intermediate in tolerance in comparison with the other two species.
3. C. inconstans was found to be more tolerant than P. promelas to sodium chloride concentrations at all the various test temperatures. C. inconstans was more tolerant of distilled water than F. diaphanus at 4°C and 20°C, and was equal in tolerance with P. promelas at 30°C.
4. P. promelas exhibited longer survival times in distilled water at 20°C and 30°C than in the sodium chloride concentrations. P. promelas was found to have the greatest tolerance to distilled water and sodium chloride solutions at 20°C.
5. Significant differences in the species' ability to withstand varying salinity concentrations were demonstrated. Distribution of the species was concluded to be governed in part by salinity. However, field observations of unmeasured variables indicated that turbidity and adequate vegetation may be more important in the distribution of the species in northeastern North Dakota.

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APPENDIX I

The statistical test design

ERROR RISKS

In the sequential design test a series of small paired experiments were conducted until one of three decision regions was entered. The various regions entered were: A>B (species A was more tolerant than species B), A = B (species A equals species B), or A<B (species B was more tolerant than species A). When a decision area was entered, the testing procedure was ended since a conclusion had been reached on the basis of "small" tests. In all cases the error risks involved were quite small. The Type I error (rejecting the null hypothesis when indeed true) was fixed at the 5% level of significance. The null hypothesis, when indeed true, was rejected 5% of the time. The Type II error (not rejecting the null hypothesis when it was false) increased somewhat when there were small differences between organisms. However, when probability values were relatively large, the risk of accepting the null hypothesis when the alternative hypothesis is true was usually less than 1% in this design.

USE OF THE DESIGN

Appendix Figure 1 shows the sequential test design pattern and the various conclusion regions and their boundaries. In using this test, a series of small steps were taken. Before each comparison the experimenter

formulated a null hypothesis for one of the various alternatives. Individuals were subjected to a particular toleration stress and the results were recorded on the sequential pattern. If A>B, a small x was placed in the square above the starting point in the lower left hand corner. Formulation of the next null hypothesis was made on the basis of the previous test and comparisons between organisms were carried out again. If A<B, a small x was placed in the square to the right of the last square that was marked. This sequence of events took place until one of the three decision areas was entered. The minimum number of tests made to fall into one of the three regions was 7; the maximum number of possible tests was 41. In most cases, 41 trials were not necessary and usually 2/3 of the maximum number placed the decision into one of the three regions.

CHARACTERISTICS

The characteristics formulated by Cole (1962) are shown in Appendix Table 1. When comparing two individuals, there was a probability (P) of a given result of the test. Various (P) values are given in Appendix Table 1. When (P) = 0.4, the correct decision of A<B will occur 14.61% of the time. The possibility of A>B when it is not, will only occur in 0.36 % of the cases. In most instances the observer will make the Type II error in not denoting small differences between individuals.

At (P) = 0.4, the Type II error will occur 85.33% of the time. As the value of (P) increases the Type II error decreases and the Type I error becomes less significant i.e. (P) = 0.9, and the power of the test increases.

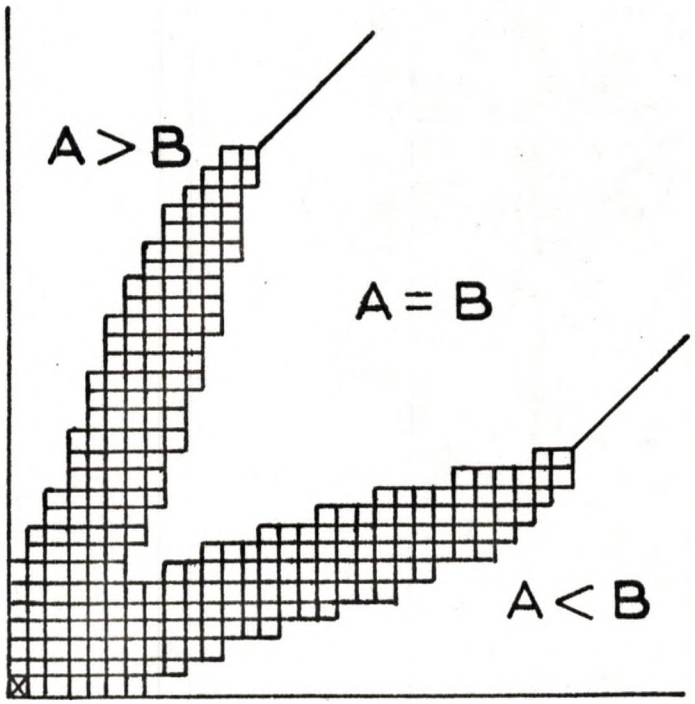

Figure 1. Sequential test pattern
(Taken from Cole, 1962)

Table 1. Characteristics of the sequential sampling design. Numbers in parentheses are median path lengths: the number of comparisons that will complete the experiment 50% of the time. (Taken from Cole, 1962).

Probability of "A" winning (P)	Percentage Falling in Each Decision Area			Weighted average median path
	Upper	Middle	Lower	
0.1.....	----	0.1009 (13)	99.8991 (10)	10
.2.....	.0016 (7)	8.0787 (13)	91.9197 (13)	13
.3.....	.0357 (10)	47.6093 (15)	52.3542 (13)	14
.4.....	.3650 (10)	85.3348 (15)	14.6125 (13)	15
.5.....	2.5399 (10)	94.9202 (15)	2.5399 (10)	15
.6.....	14.6125 (13)	85.3348 (15)	.3650 (10)	15
.7.....	52.3542 (13)	47.6093 (15)	.0357 (10)	14
.8.....	91.9197 (13)	8.0787 (13)	.0016 (7)	13
.9.....	99.8991 (10)	0.1009 (13)	----	10