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Title: REPRODUCTIVE BEHAVIOR OF THE THREESPINE STICKLEBACK EXPOSED TO CHLORAMINES

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Mature freshwater threespine sticklebacks Gasterosteus aculeatus L. were continuously exposed to four different concentrations of inorganic chloramines, <1.0, 1.0, 4.3 and 11.4 μg/liter, and a control for 3.5 months. During this time their reproductive behavior was observed under laboratory conditions simulating their natural environment. There were no observed differences in behavior that could be attributed to exposure to the chloramines. Generally, normal reproductive behavior occurred in expected sequence and resulted in the production of three to five broods of young per three replications at each treatment level, although none of the young survived to the end of the test.

At the end of the test, standing crops of amphipod and isopod populations inhabiting the chambers were similar in control and treatment aquaria. The occurrence of several size classes indicated that reproduction was occurring at all treatment levels.

Early in the study, as compared to the controls, growth of periphyton on aquaria walls was delayed at all tested chloramine concen-

trations. By the end of the test, these differences were not so obvious, but estimates of standing crops reflected a trend of decreasing biomass with increasing levels of exposure to chloramines.

This experiment demonstrated the feasibility of simulating for purposes of behavioral studies the natural environment of the freshwater threespine stickleback.

Reproductive Behavior of the Threespine Stickleback Exposed to Chloramines

by

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I am grateful to the United States Environmental Protection Agency, especially Dr. Donald I. Mount, for making this training possible.

This study is dedicated to my wife, Phyllis, daughter, Amy, and son, Thomas.

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INTRODUCTION

Significant advances in the knowledge of fish behavior have resulted from laboratory studies of the threespine stickleback *Gasterosteus aculeatus* L.. Partially owing to such studies, derived methods of behavioral research have led to greater understanding of the general behavior patterns that occur in other species of fish. The threespine stickleback appears to be a useful animal for studies of the effects of pollutants on behavior both because of the extensive descriptive information available on its reproductive behavior and because of it adapting well to laboratory conditions. Its reproductive behavior consists of definite phases: migration, claiming a territory, nest building, courtship, and parental care of the young. Each phase is characterized by the appearance at high frequency of various activities that form a functional unit, with some overlapping of activities but at lower frequency. Detailed ethological analysis of these stages was initiated by Tinbergen and Pelkwijk in 1934 at Leiden University, Netherlands. This work has been continued by several investigators (Van den Assem, 1967; Guiton, 1960; Van Iersel, 1953). Life history information is also available (McPhail, 1969; Hagen, 1967; Greenbank and Nelson, 1959), this making possible laboratory simulation of many aspects of the stickleback's natural environment. Creation of a series of similar laboratory ecosystems permits bioassays yielding information on the direct and indirect effects of toxicants on the behavior of these fish.

Information is limited on the mechanisms of toxicant induced changes in the behavior of fish and other aquatic organisms. Behavior is a very complicated phenomenon through which an organism is capable of adjusting its feeding, reproduction, and other functions to its environment. Many aspects of behavior have been studied by ethologists. In general, the ethologist begins with a behavioral sequence and analyzes it down to the level of its parts. The aspects of behavior usually considered are causation, ontogeny, function or survival value, and evolution. Causal ethological analysis employs the "black box" approach, the box being the machinery inside the animal through which behavior or output (glandular and muscular activity) is produced, under the influence of sensory stimuli or the input. By observing the output in detail under experimentally manipulated conditions, the ethologist can learn to predict the presence of mechanisms in the black box and define them on a basis of the function they fulfill (Baerends, 1971). So long as toxicants act as inputs resulting in changes in behavior, an ethological approach can be used to determine both the direct effects of a pollutant on a fish and indirect effects caused by changes in its environment, such as altered food availability. The research here reported utilized this approach to examine the response of the threespine stickleback to long-term sublethal exposure to inorganic chloramines.

Disinfection of effluents from wastewater treatment plants and treatment of municiple water supplies with chlorine, for the protection of public health, are the major sources of chloramines in aquatic environments. Other sources of chloramines in aquatic ecosystems are industries that use chlorine as an antifouling agent in cooling water systems. Tsai (1973), in comparative studies of fish diversity above and below 149 wastewater treatment plants, related reductions in diversity to low concentrations of total residual chlorine. He concluded that if all species were to be protected in areas immediately below outfalls, no detectable concentration of total residual chlorine should be permitted. Brungs (1973) reviewed the literature on the effects of chlorine on aquatic life and concluded that in waters continuously receiving treated wastes residual chlorine should not exceed 10 µg/liter for the protection of more resistant species only, or should not exceed 2 µg/liter for the protection of most species. He also cites an unpublished report by Arthur (1972) who studied the effects of a chlorinated secondary treatment plant effluent containing only domestic waste on the survival and reproduction of the amphipod Gammarus pseudolimnaeus and the cladoceran Daphnia magna. Amphipod reproduction was reduced at chloramine concentrations above 12 µg/liter. The cladocerns died at 14 µg/liter and normal reproduction occurred only at 3 µg/liter and below. In an earlier study Arthur and Eaton (1971) found that fathead minnow reproduction was impaired at 42.8 µg/liter chloramine concentration and no effects were seen at 16.5 µg/liter chloramine concentration.

The objective of this study was to develop and maintain in the laboratory a series of aquatic ecosystems to determine any apparent sublethal effects of residual chlorine exposure on the reproductive behavior of the threespine stickleback. An ecosystem can be defined as biological community together with the chemical conditions and the physical resources of its location (Warren, 1971). A successful laboratory ecosystem would be one that can assimulate introduced light and organic matter by means of a food web providing for fish and other organisms of interest. In this study, the community consisted of organisms both passively and directly introduced into simulated natural conditions. These systems received not only light but also organic matter much in the same manner as would a woodland stream, but in controlled amounts.

METHODS AND MATERIALS

The experimental aquaria measured 46 centimeters wide by 41 centimeters deep by 122 centimeters long (Figure 1) and were covered on three sides with sheets of black plastic. Water depth was 24.8 centimeters. A 7.6 centimeters layer of crushed gravel, approximately 1.3 centimeters in diameter, was used as a basic substrate in each chamber. Across the short axis, rows of gravel projecting about 5 centimeters above the basic substrate were used to divide each aquarium into three equal areas sufficient in size to permit the establishment of at least one male threespine stickleback territory per area (Van den Assem, 1967). Within each division, a circular area, 23 centimeters in diameter, of coarse dark brown sand was introduced for possible use as nest sites by the fish. Presoaked grass roots were provided for use as nest building material. Five species of aquatic plants (several of each) were placed in each aquarium. One 15 watt Daylight fluorescent tube, 45.7 centimeters long, was suspended 49 centimeters above the surface of the water in each aquarium. A laboratory photoperiod schedule corresponding to the natural light conditions at Corvallis, Oregon was followed. Photoperiod adjustments were made weekly.

Sand filtered creek water was delivered to a headbox which fed a diluter system (Chadwick et al., 1973) designed to deliver water having four concentrations of residual chlorine and a control. The continuous exchange flow was maintained at a rate of 400 ml/minute in each of three replicates of each treatment level and control. The laboratory temperature regime (Figure 2) followed the seasonal but not the daily

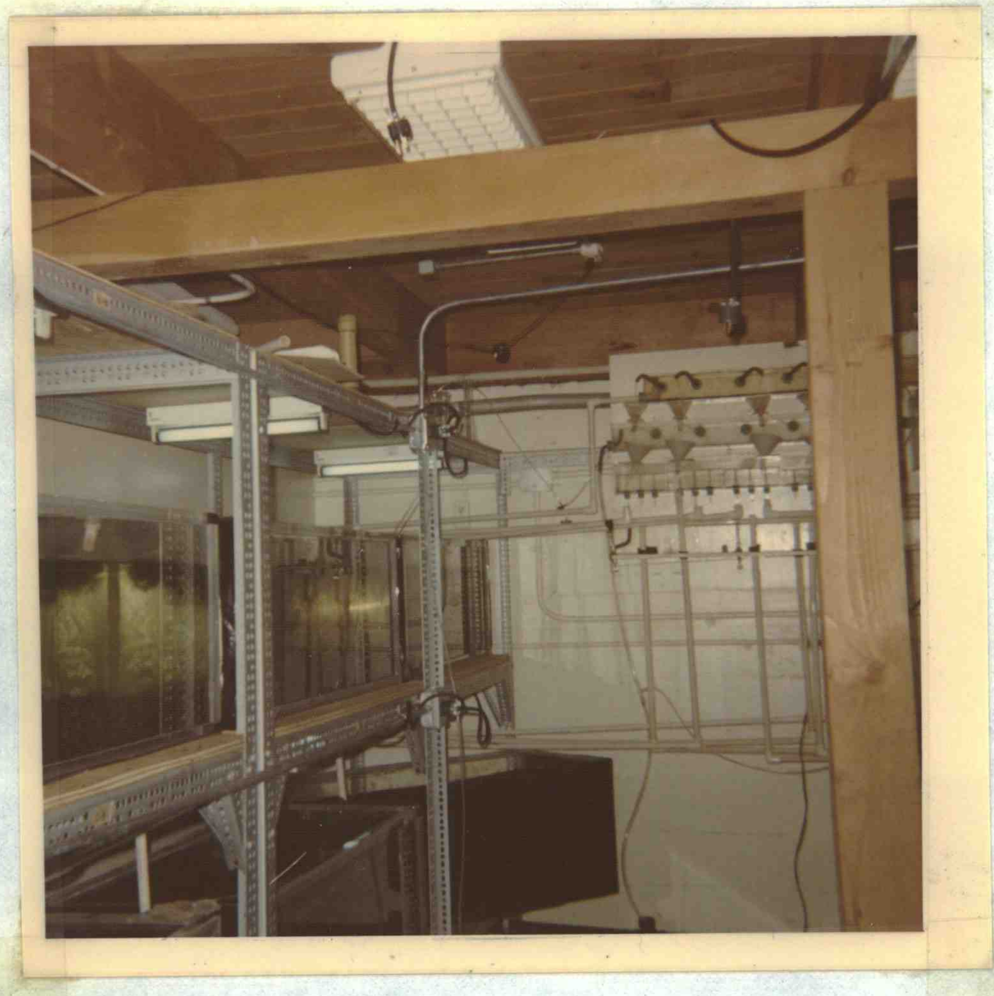

Figure 1. Experimental aquaria used in this study.

regime of the natural habitat of the fish. This was accomplished by means of a thermostatically controlled emersion heater in a headbox. Water temperatures were measured six days a week in the aquaria. Chemical characteristics of the water were determined with methods described by the American Public Health Association et al. (1965). These ranged from 45 to 87 mg/liter in total akalinity (as C_aCO_3), 48 to 88 mg/liter in total hardness (as C_aCO_3), 10 to 11 mg/liter in dissolved oxygen, and 7.1 to 7.8 in pH.

Chloramines were formed by combining solutions of ammonium chloride and sodium hypochlorite in a molar ratio of chlorine to ammonia of 0.7/1.0. This maintained a small excess of ammonia in solution and reduced the possibility of free chlorine residual in the test water. To maintain residual chlorine concentrations, component solutions were held in enclosed glass tanks and mixed together in a constant flow system. The mixture flowed into a changer allowing a retention time of about two hours for the formation of chloramines. Total residual chloramines were measured by an amperometric titration method similar to that described by the American Public Health Association et al. (1965). The range of concentrations were chosen to provide ranges slightly higher than those recommended not to be exceeded in order to protect aquatic life by Brungs (1973). Measurements were made on samples siphoned from similar positions in all aquaria. These measurements were made at least six times a week and alternated between the replicated aquaria at any one treatment level. Previous unpublished studies[1/] in which the above chloramine formation and detection methods were employed,

1/ Larson, G. L., personal communication, methods used in studies at the Pacific Cooperative Water Pollution Laboratories, Oregon State University, Corvallis, Oregon.

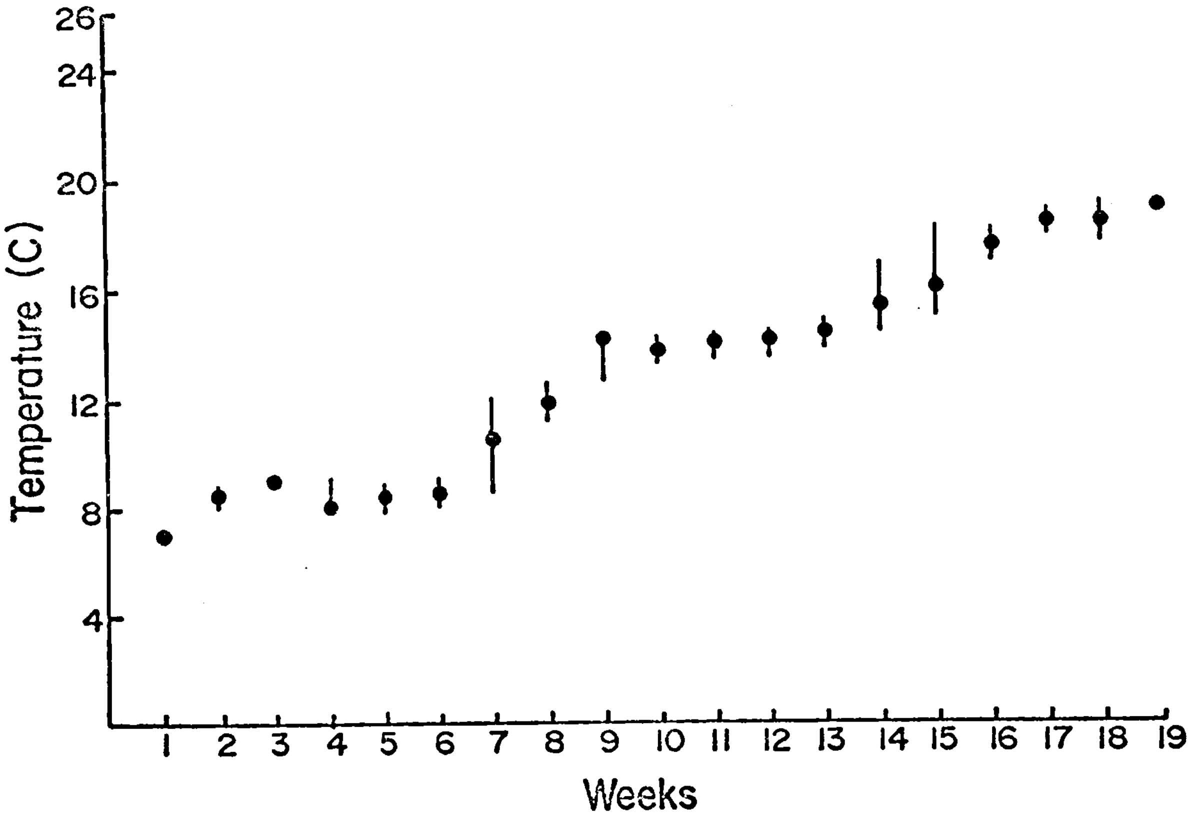

Figure 2. The experimental water temperature regime (weekly means and ranges) from March 4, 1974 to July 23, 1974.

demonstrated monchloramine to predominate and dichloramine to be present up to 22 percent of the total chloramines. The means ± 1 standard deviation of measured total chloramines and the ranges, in μg/liter at each treatment level, in this 4.5 month study were 0.0 ± 0 (control), 0.0 ± 0 (undetectable, less than 1.0 μg/liter), 1.0 ± 0.9 (0.0 to 2.0), 4.3 ± 1.9 (0.0 to 8.0), and 11.4 ± 4.0 (0.0 to 22.0). For simplicity, mean total chloramine concentrations will be used in the following discussion. The wide variations in daily chloramine concentrations were due to high chloramine demands caused by high turbidity in the test water following several days of rain and subsequent high run-off into the creek that was the laboratory source of dilution water. This happened most frequently in the early part of the study.

The freshwater threespine sticklebacks used were collected from Gray Creek (part of the Willamette River system) located on the Finley National Wildlife Refuge near Corvallis, Oregon. They were of a partially plated (6-10), unkeeled (leiurus) form. The male nuptial color was later found to be a dark black, similar to those described by McPhail (1969) from the Chehalis River system in western Washington. Fish collections were made between December 10, 1973 and January 15, 1974. After collection, the fish were held in an outdoor tank provided with flowing creek water at ambient water temperatures. This fish stock was fed live amphipods and earthworms.

On March 4, 1974, nine fish were placed in each test aquarium. 2/ These fish ranged in weight from 0.35 to 1.67 grams. Earlier dissection

2/ One aquarium having a concentration of < 1.0 ug/liter total chloramines broke on March 20, 1974 and was repaired and restocked with nine fish averaging 0.57 grams in weight.

of a large sample had demonstrated that fish within this range contained developing gonads, but sexual characteristics were not evident at the time of stocking. The fish were fed chopped earthworms or Tubifix sp. At least 15 grams of food was divided evenly among the aquaria daily. In an attempt to develop a self-sustaining population of food organisms for the sticklebacks, amphipods (Crangonyx sp.) were placed in each aquarium daily for the first week and thereafter once weekly until May 12, 1974, when the last introduction was made. The amphipods were from an outdoor rearing tank. This culture was composed predominately of amphipods, but isopods and chironomids were also present. Periodically, 5-10 grams of presoaked alfalfa leaves [3/] and straw up to about 5 centimeters in length were placed in each chamber. The leaves were used to provide nutrition for the amphipods and the straw was used to provide nest building material for the fish.

Terminology and descriptions of the threespine stickleback reproductive cycle by Van den Assem (1967) and Van Iersel (1953) were used as a normal behavior standard. The sequential behavioral phases and selected related activities (Appendix 1) were recorded for the fish in each chamber. Observations were begun on April 20, 1974. Observations on fish in each aquarium were made for ten minutes one day each week for the first three weeks. Thereafter, observation periods for the controls and fish at the 4.3 and 11.4 μg/liter concentrations were increased to six each week. Tests at the <1.0 and 1.0 μg/liter con-

3/ Liss, W. J., personal communication, maintained amphipod populations using alfalfa as nutrient source at the Pacific Cooperative Water Pollution Laboratories, Oregon State University, Corvallis, Oregon.

centrations were maintained on an observation schedule of at least once a week because of time limitations. During these observation periods, the frequency of occurrence of attacks (ending in biting) and fanning bouts initiated by the territorial fish nearest the aquaria outlets were recorded. On June 27, 1974, the behavioral observations were ended and the fish from all of the aquaria were individually weighed. The fish from two of the three replicates at each concentration and control were preserved in a 10 percent formalin solution. The fish from the third aquarium at each treatment level were placed back into their respective aquaria: each such aquarium received eight fish having a combined weight of from 10.08 to 10.64 grams. Introduction of food was discontinued at this time. After 26 days, these fish were reweighed to determine if the laboratory ecosystems could sustain the fish populations. During this period, introduction of alfalfa into the aquaria was continued.

Estimates of the periphyton standing crops in the aquaria from which the fish had been removed were made by scraping (on July 2, 1974) a 15.2 x 120.3 centimeter section of one wall of each aquarium.[4/] These samples were preserved in a 5 percent formalin solution. Later the samples were filtered through a preweighed oven dried W and R Balston #1 filter paper, dried in an oven at 70 C, removed, and then allowed to stand in the laboratory for 2 hours before reweighing. All weights were taken on an analytical balance accurate to 0.1 milligram. The gravel and sand substrates in these aquaria were removed by hand.

4/ One sample from a <1.0 μg/liter aquarium was accidentally lost.

Macro-invertebrates were sampled from the benthic sediments by siphoning and straining the remaining contents through a 130 micron mesh plankton net. The materials collected were preserved in a 10 percent formalin solution. A subsample, 5 percent by weight after removing excess liquid by squeezing lightly, was picked of macro-invertebrates, which were then sorted and counted to estimate the standing crops in each aquarium.

RESULTS AND INTERPRETATION

Generally, the reproductive behavior phase sequences observed in control sticklebacks and those exposed to chloramines were similar to the behavioral standard (Appendix 1). This is demonstrated in Table 1 by the apparent behavioral sequences of the fish. Fighting behaviors associated with the establishment and maintenance of territories were obvious in all aquaria on March 19, 1974. Thereafter, each fish appeared to occupy and defend a territory. The bottom territories were usually occupied by the larger fish, which were mainly males but sometimes females. The males were identified by their nest building activities and black nuptial colors, the females by their robust appearance and behavioral responses to courting males. The other fish in the aquaria appeared to have territories in the overlying water columns. These territories were evident when the fish could be clearly identified because they were the smallest or largest in an aquarium; such a fish could be found defending continuously the same general area. The dates on which nest building, courtship, and parental behaviors were first observed in the aquaria were quite variable (Table 1). Nest building behaviors were observed in all 15 aquaria while courtship behaviors were observed in 12. The initiation of parental activity was hard to pinpoint except after spawning was actually observed, which was only four times. According to Van Iersel (1953), male fanning activity occurs most frequently throughout the parental phase of the reproductive cycle but is not restricted to this phase. It also occurs at the end of the nest

Table 1. The 1974 dates on which fighting, nest building, courtship and migratory behaviors and larvae were first observed in the controls and aquaria exposed to chloramines.

Behavior	Mean total chloramine concentrations														
	Control			<1 μg/liter			1 μg/liter			4.3 μg/liter			11.4 μg/liter		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Fighting	3-19[1/]	3-19	3-19	3-19	3-19	3-19	3-19	3-19	3-19	3-19	3-19	3-19	3-19	3-19	3-19
Nest building	4-16	4-19	4-20	4-26	4-16	4-16	4-8	3-22	4-23	4-8	4-8	4-14	4-14	4-8	4-12
Courtship	6-4	--	5-8	--	5-30	5-3	--	4-26	5-3	4-18	4-10	5-8	5-10	4-22	5-10
Fanning	5-3	5-3	4-26	5-10	5-3	4-20	4-26	5-3	5-3	4-18	4-19	5-27	5-10	4-20	4-26
Migratory	--	--	--	--	--	--	--	6-8	6-24	--	--	--	--	6-12	6-4
Larvae first seen	5-11	5-22	5-22	5-22	--	6-4	5-22	5-9	5-20	5-15	5-9	5-15	5-25	5-22	5-9

1/ The first day on which all of the aquaria were observed.

building phase and during the courtship phase. This behavior was observed in all aquaria during the nest building and parental phases and during the courtship phase in the 12 aquaria where this behavior was observed.

Successful reproduction occurred in the control and at all chloramine concentrations. Young sticklebacks were produced in 14 of the 15 aquaria (Table 2). Up to four nests were present at one time in some of the aquaria. As many as five fish, some of them females, were observed defending bottom territories in several aquaria. But young were observed in only one to two nests per aquarium, and none of these survived to the end of the experiment. The larval and young fish were seen in and around the nests for one day in some nests and up to 14 days in others. In 11 out of the 15 aquaria, an additional one or two males were observed to have completed the reproductive cycle, but no young were observed in or around their nests. It is highly probable that young were produced but lost to cannibalism by the parent males or other fish in the aquaria. Parental care has been reported to last for up to two weeks following embryo hatching; after this the young appear to be treated as food organisms (Van Iersel, 1953). Such cannibalism could well account for the failure of young to survive to the end of the experiment. Robbing nests of embryoes is not uncommon where competition for nesting space is high (Van den Assem, 1967), as was the case in an aquarium of the <1.0 µg/liter chloramine concentration where no young were observed. This aquarium contained seven males and two females throughout the test and was the only aquarium where nest robbing was actually observed, although this was only once. This type

Table 2. The mean of the measured total chloramine concentrations, minimum and maximum number of nests present at any one time, total number of broods produced, identifiable sex composition of initial nine fish stocked, mean, initial and final fish weights in wet grams, and the number of fish surviving to the end of the experiment.

Mean total chloramine concentration in μg/liter	Replicates	Number of bottom territories		Maximum number of nests present	Number of broods produced	Identifiable sex composition		Mean fish weight (grams)		Number of fish surviving
		Minimum	Maximum			Males	Females	Initial	Final	
0.0 (control)	A	3	5	4	2	5	2	0.93	1.66	5
	B	3	5	1	1	1	8	0.83	1.29	9
	C	2	5	2	2	4	4	0.87	1.26	8[1/]
0.0 (<1 μg)	A[2/]	3	5	2	2	3	5	0.57	1.18	7
	B	3	5	2	0	7	2	0.82	1.37	9
	C	2	3	3	1	5	3	0.88	1.33	8[1/]
1.0	A	2	3	2	1	4	4	0.82	1.66	3
	B	2	3	2	2	3	4	0.95	1.29	7
	C	1	3	2	1	3	5	0.83	1.31	8[1/]
4.3	A	3	3	3	1	4	3	1.04	1.85	3[1/]
	B	2	5	2	2	4	4	0.97	1.32	7
	C	3	5	3	1	3	5	0.81	1.21	8[1/]
11.4	A	3	3	3	1	7	2	0.78	1.43	7
	B	3	5	3	1	5	4	0.82	1.28	9
	C	2	5	2	2	4	5	0.87	1.26	8[1/]

1/ These fish were returned to the aquaria after weighing to determine if they could be sustained by the invertebrate populations.

2/ Early in the study this aquarium broke and was restocked.

of behavior probably was a factor in brood production in the other aquaria. Possible lack of food suitable for the young could have contributed to their mortality, although some young were observed apparently feeding.

After completion of the parental cycle, some males did not enter another reproductive cycle; after a lapse of time, others began another cycle. During the period before another cycle began, some fish exhibited evidence of migratory behavior. This consisted of persistent swimming up and down against the glass wall near the water inlet and complete disregard of other attacking fish. Such behavior was observed in two aquaria at the 1.0 and 11.4 µg/liter concentrations (Table 1). At times, some of these fish would return to their former territories, defend them awhile and then return to this migratory-like behavior. Exposure to chloramines was apprently not involved in this behavior: it was not observed at the intermediate concentration (4.3 µg/liter) and has been previously observed (Van Iersel, 1953).

No differences in the data on fanning bout and attack behavior of the sticklebacks could be attributed to exposure to chloramines in three territories where the reproductive cycles of males occurred approximately in phase: in one control aquarium and two aquaria at the 11.4 µg/liter concentration. In the other cases the data was not directly compared because of temporal differences in behavioral sequences. But expected trends of increased fanning prior to embryo hatching followed by cessation of fanning when larvae were present occurred in 12 out of the 15 territories where data on this behavior was collected (one territory in each aquarium). Fanning was observed in three of the other four instances. In these three, males appeared to have reached the courtship phase of reproduction, one male successfully courted and apparently spawned (in the aquarium at <1 μg/liter where no young were observed) and the other males (at the 4.3 μg/liter chloramine level) probably did not receive a clutch of eggs. This was evident in one territory because the females died prior to or during the male's courtship activities. The fourth territory was occupied by a female (Appendix 2).

Long-term exposure to chloramine concentrations up to 11.4 μg/liter had no apparent effect on survival of adult threespine sticklebacks. Of the 27 fish tested at each treatment level, 22 survived in the controls and 18 to 24 at each level of chloramine exposure. In several aquaria, including a control, only males, three to five in number, survived to the end of the test (Table 2). Generally, in aquaria where mortality occurred, fish had been infected with fungus and attacked frequently by other fish prior to their death. Thus, death was probably caused by infections resulting in part from injury and exhaustion caused by the aggressive behavior of other fish. Van Iersel (1953) states that inferior males often are chased until they die; this is especially so in tanks having inadequate cover for the fish.

Of the several plant species placed in each aquarium, only *Ceratophyllum sp.* survived. The poor plant survival was probably due to environmental conditions prevailing at the time of transplanting. Substrate conditions, photoperiods, and cold water temperatures were not conducive to growth and survival of the plants. The Ceratophyllum sp. did not appear to grow, so on May 21, 1974, additional plants of this species were placed in each chamber in an attempt to provide more shelter for the young sticklebacks. Small amounts of Lemna sp. were also introduced at the same time and did grow and reproduce at all treatment levels.

Growths of periphyton were observed in the control chambers on April 12 and 20, 1974, but such growths were not obvious in the aquaria receiving chloramines. By May 4, 1974, differences in periphyton densities were clearly apparent between treatment levels. The control aquaria had a very heavy growth, the <1.0 μg/liter treatment aquaria had only light growth, the aquaria at the 1.0 and 4.3 μg/liter concentrations contained even less periphyton, and none was apparent at the 11.4 μg/liter concentration. During the following weeks as water temperatures increased, periphyton colonization was apparent in all chambers. The periphyton biomasses appeared to increase and decrease in a cyclic manner making comparison between treatments difficult. Estimates of periphyton standing crops at the end of the experiment indicate some effect of the chloramines on periphyton growth (Figure 3).

Estimates of amphipod and isopod standing crops at the end of the experiment are presented in Table 3. Amphipod standing crops in chloramine treated aquaria were similar to those in the controls, with the exception of the two aquaria at <1.0 μg/liter, which had been handled differently.[5/] In all aquaria amphipod reproduction was indicated by

5/ One aquarium had broken and was restocked; the other was sampled in different manner than the rest.

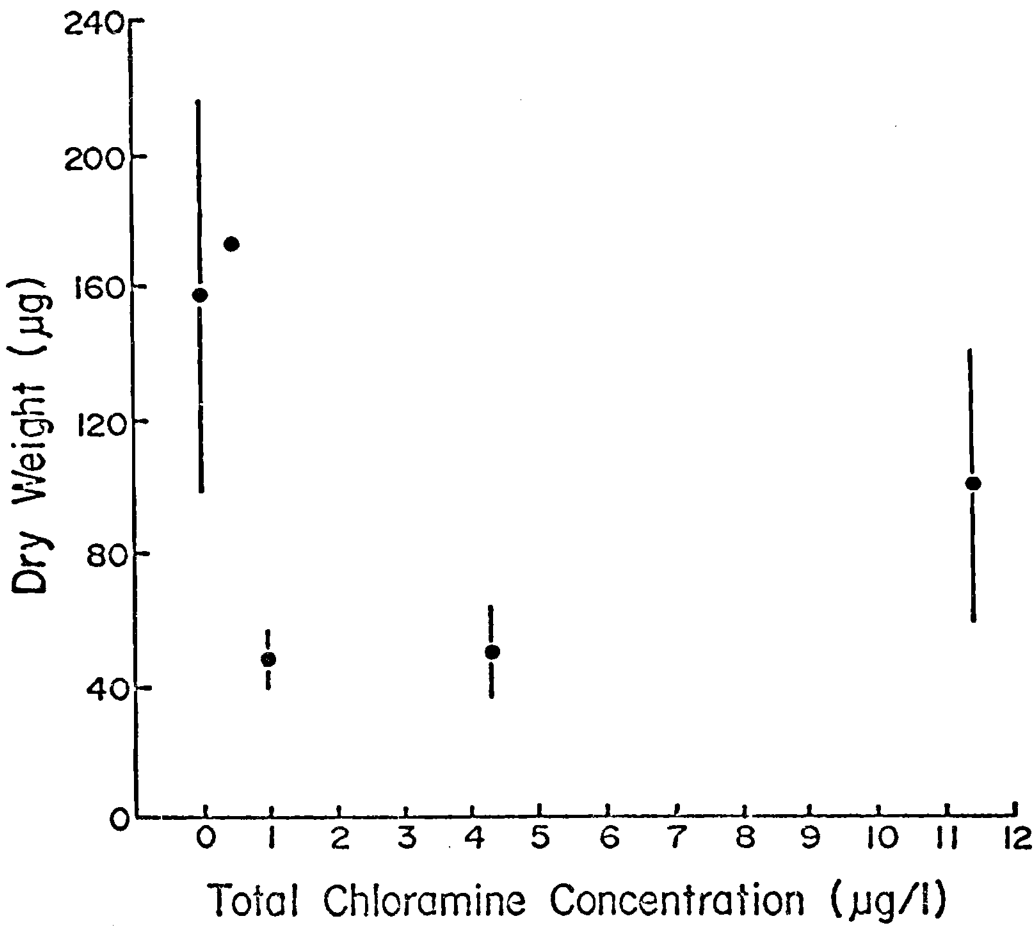

Figure 3. The mean and range of dry weights of periphyton, scraped from 15.2 x 120.3 centimeter sections of the aquaria walls. Two of the three aquaria at each experimental treatment level were sampled but one sample at the <1 µg/liter chloramine level was accidentally lost.

the presence of very small and early instar developmental stages. Estimates of isopod standing crops are more variable but also indicate that isopod reproduction was occuring at all chloramine concentrations. Several other species of invertebrates and very small salamander larvae were present in low numbers in the samples. These organisms were probably introduced into the aquaria as early larval instars with the plants or with the amphipods, as were the isopods and some chironomids. But some could have been introduced through the dilution water supply.

On July 17, 1974, water samples were taken from within the substrate of one aquarium at the 11.4 μg/liter level of chloramines as well as from the overlying water. The samples of water drawn from the substrate contained 0 and 3 μg/liter chloramines, while the two from the overlying water contained 9 and 10 μg/liter. Although one sample contained 3 μg/liter, which very well could have been due to faulty siphoning technique, the absence of chloramines in the other sample indicated that water exchange in the substrate was minimal and probably that a high chloramine demand existed there. It is quite possible that organisms within the substrate were not exposed to measurable amounts of chloramines.

The fish that were returned to the aquaria for 26 days to feed only on the available invertebrates lost weight. The combined weights of the fish populations in the control and at the <1.0, 1.0, 4.3, 11.4 μg/liter concentrations of chloramines decreased by 19, 20, 16, 23, and 38 percent, respectively. The production rate of invertebrates apparently was insufficient to sustain the fish populations. The

Table 3. Estimated numerical density per aquarium for three size groups of amphipods and isopods at the end of the experiment.

Mean total chloramines (μg/liter)		Size groups >4mm	2-4mm	<2mm	Total
			Amphipods		
0.0 (control)	A	60	1220	1780	3060
	B	100	560	760	1320
0.0 (<1.0 μg/l)	A 1/	120	300	280	700
	B 2/	100	360	240	700
1.0	A	20	420	880	1320
	B	240	440	1160	1880
4.3	A	300	460	1020	1780
	B	160	340	860	1360
11.4	A	60	680	1320	2020
	B	300	1000	1500	2800
			Isopods		
0.0 (control)	A	100	280	20	400
	B	100	260	220	580
0.0 (<1.0 μg/l)	A	180	140	60	380
	B	40	100	0 3/	140
1.0	A	20	20	60	100
	B	180	180	120	380
4.3	A	260	220	60	540
	B	160	80	0 3/	240
11.4	A	200	220	40	460
	B	100	0 3/	40	140

1/ This aquarium had broken and was restocked.

2/ This aquarium was not sampled in the same manner as the rest.

3/ Size group present in total sample but absent from the subsample; probably less than 20 in number.

higher loss of weight at the 11.4 μg/liter concentration suggests a possible direct effect of the toxicant on the fish although possible effects on the production and availability of their food organisms cannot be entirely discounted.

DISCUSSION

Behavioral differences attributable to chloramine exposure apparently did not occur in the threespine stickleback. Migratory behavior was not observed when the fish were first placed in the aquaria and was probably due to this phase having been completed in the wild or in the holding tank. Initiation of the other behavioral phases was quite variable, but not unusually so in comparison to behavior in natural populations, where evidence of reproduction has been observed for some freshwater threespine stickleback populations from February to September (McPhail, 1969). Such a long reproductive season can result in diffent size and age classes maturing at different times. The initial size differences of the fish used in this experiment could well account for the observed variability.

Under the conditions of this experiment, the similarity of the number of broods produced at the different treatment levels supports the conclusion that long term exposure to chloramines at concentrations up to 11.4 µg/liter did not appreciably affect overall reproductive performance. Chloramines at the 11.4 µg/liter level did not appear to affect amphipod reproduction. The one time sampling of the water from within the substrate in an 11.4 µg/liter exposure aquarium indicated that organisms living in this area may not have been exposed to detectable levels of chloramines even though the fish in the overlying water were exposed. The only positive effect of exposure to chloramines noted in this study was the observed differences in periphyton growth on the exposed chamber walls which occurred early in the study. The standing crop measurements at the end of the test also reflected a trend of lower standing crops at total chloramine exposure levels of 1.0 μg/liter and higher. The evidence is sufficient to indicate further study is necessary to quantify such subtle effects of chloramine exposure, in order to predict the overall effect of low levels of chloramines on natural ecosystems.

The ability of the food web to support sticklebacks in these laboratory ecosystems was limited. The alfalfa based energy system appeared to be operating and providing the resources necessary for amphipod reproduction, although not enough were produced or available to sustain the fish population. The apparent reproduction of the amphipods within each chamber suggests that the creation of a system capable of supporting a reproducing threespine stickleback population is possible. This might be accomplished by using a late spring or early summer starting date. This would offer the advantages of long photoperiods and warmer water temperatures for plant and animal growth. A more natural gradual increase of light intensity in the morning and decrease in the evening might make the light sensitive amphipods more vulnerable to capture by the fish. Unwanted organism introduction could be minimized by using well water and aquarium reared plants and animals. By increasing the area suitable for amphipod production in relation to suitable fish habitat, more food could be made available to the fish. After a sustaining amphipod population is established, threespine stickleback sac larvae could be introduced and the fish biomass allowed to adjust to the capacity of the system to produce food organisms and shelter. If enough amphipods are available for optimum fish growth, the fish should reach a breeding size, 30 to 54 mm in standard length (Hagen, 1967), by the following spring. Natural populations have been reported to contain mature one year old fish (Greenbank and Nelson, 1959). Laboratory rearing of threespine sticklebacks from fertilized eggs to mature adults has been reported by McPhail (1967), but he did not state how long it took to reach maturity. Hagen (1967) reported rearing males to maturity in 18 weeks at water temperature ranging from 18 to 22 C. Females contained developing ovaries at the end of this time but did not ripen. The overall view gained from these studies suggests the possibility of laboratory ecosystem production of mature sticklebacks in year-long experiments.

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APPENDIX

Appendix I. Normal reproductive behavioral phase sequence of the threespine stickleback and selected associated behaviors.

1. Migrating behavior
    a. Fluttering

2. Settling (establishing territory)

3. Fighting behavior (while defending a territory)
    a. Charging
    b. Biting
    c. Spine erection
    d. Spine fighting
    e. Chasing
    f. Fleeing
    g. Threatening

4. The nest building phase (male)
    a. Sand digging
    b. Searching for material
    c. Bringing material
    d. Testing material
    e. Glueing
    f. Pushing
    g. Sucking
    h. Boring
    i. Bringing sand
    j. Creeping thru nest
    k. Fanning

5. The courtship phase
    a. Entry (female)
    b. Zig-zag (male)
    c. Headup posture (female)
    d. Leading (male)
    e. Following (female)
    f. Creeping (female)
    g. Quivering (male)
    h. Spawning (female)
    i. Female leaves
    j. Fertilizing (male)

6. Parental Phase (male)
    a. Fanning
    b. Pushing holes in nest
    c. Nest pulling
    d. Retrieving young

Appendix II. The total number of attacks and fanning bouts resulting from the ten minute observation periods of the sticklebacks occupying the territories nearest the aquarium outlets. Data taking was started on April 20, 1974 and ended on June 25, 1973.

Mean total chloramine concentration in μg/liter	Replicates	Total number of attacks	Total number of fanning bouts	Number of days observed for 10 minutes
0.0 (control)	A	78	92	42
	B	212		17[1/]
	C	249	51	42
0.0 (< 1 μg)	A	248	61	29
	B	66	42	19
	C	42	38	19
1.0	A	24	12	6[2/]
	B	117	44	19
	C	130	33	19
4.3	A	74	44	42
	B	205	156	42
	C	112	140	42
11.4	A	323	173	42
	B	168	78	42
	C	109	110	42

1/ This territory was occupied by a female. When this was evident, data collecting was discontinued.

2/ Early in the study all of the females died so data collecting was discontinued.