A Behavioral Bioassay Examining the Effects of Ethanol on Flagfish Reproduction

by

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A THESIS

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ABSTRACT

A behavioral bioassay method was developed to examine the effects of ethanol on the reproductive success and behavior of flagfish (Jordanella floridae). Reproductive behavior was divided into eight categories: egg tending; nesting; t-circling; chasing a faded female; spawning; guarding; chasing; and inattentive behavior. The dominant male in each tank was observed for ten minutes daily for five days before and after ethanol exposure. The data was examined to determine the frequency with which each behavior occurred, the percent of the Total Frequency that each behavior represented, the total time spent at each behavior, and the sequential order in which the behaviors occurred.

Total Frequency, the number of times the fish switched from one behavior to another, was reduced when the fish were exposed to concentrations of ethanol ranging from 0.5 to 3.0 g/liter. When behavior was analyzed with respect to the eight behavioral categories, it was found that no single behavior contributed to the decline but rather the number of times a fish engaged in each behavior was reduced. Accompanying this reduction in activity was an increase in inattentive behavior. When sequences of behavior were examined, no significant change occurred even at the higher ethanol concentrations.

Fewer eggs were recovered from adults exposed to 2.0

and 3.0 g/liter. Of the eggs produced, hatchability and larval survival exceeded 94% and 90%, respectively, at all concentrations tested. It was concluded that the reduction in spawning activities at concentrations of 1.5, 2.0, and 3.0 g/liter ethanol was due to the overall reduction in activity. It appears that the dominant male must maintain a minimal threshold of activity to successfully spawn.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to the following people who made this project possible.

I thank Dr. George Ozburn, Dr. Walter Momot, Dr. John Jamieson, Mr. Davy Jones, and Mr. Alasdair Smith for their technical and professional assistance.

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INTRODUCTION

In the natural environment, the survival of the organism is frequently related to its behavior; such as the ability to capture prey, to successfully spawn, and to avoid predation. Toxicants which modify patterns of behavior can reduce the chances for individual and specie survival by altering the ability of the fish to perform its normal activities. The organism has a limited capacity to compensate for and to adjust to stresses such as toxicants (Fry, 1947; Iverson and Gutherie, 1969). Behavioral toxicity studies are directed towards determining the limits of the stresses under which the organism is capable of normal performance.

But, most fish behavioral studies using a toxicant examine a single behavioral parameter such as avoidance, swimming ability, or learning. (A tabular review of selected literature on fish behavioral studies using toxicants is presented in Appendix A.) Since toxicants are known to affect different behavioral responses depending on concentration and time of exposure, and since the effects may not necessarily develop at equal rates (Kalant, et al., 1971), examining a single behavioral parameter may not be representative of the effects of that toxicant. In addition, studies measuring only one behavioral response, such as learning, may be only partially suitable in determining the effects of a toxicant since the ecological significance of that behavior may be uncertain (Sprague, 1971).

One area of behavior which is ecologically significant is reproductive behavior since a reduction in spawning success can ultimately lead to a collapse in the fish population. In addition, reproduction is not a single behavior, but rather a composite of several other behaviors: courtship; nesting; parental behavior; and fighting (Tinbergen, 1951). Disruption of any of these subcategories can result in unsuccessful spawning.

The purpose of this study was to examine the sublethal effects of a toxicant on flagfish reproductive behavior and success. Rather than examining a single behavior, this method was designed to examine the components of reproductive behavior. Each behavior was examined to determine if quantitative changes occurred in the time spent at each behavior or in the frequency with which each behavior occurred. In addition, the sequential order in which the behaviors occurred was examined to determine if the behavioral patterns were altered. Hatching success and larval survival were examined to determine if survival of the off-spring was affected by the toxicant.

MATERIALS & METHODS

This section has been divided into five subsections: I. Chemical Analysis; II. Behavior; III. Egg Production; IV. Egg Hatchability; and Larval Survival.

I. CHEMICAL ANALYSIS

Water Chemistry

Temperature, pH, and dissolved oxygen readings were taken daily using mercury thermometers, a Delta Scientific Dissolved Oxygen Meter, and a Radiometer pH M-64 Meter, respectively. In addition to these parameters, the Thunder Bay Regional Ministry of the Environment analyzed the water supply.

Ethanol Determinations

Ethanol concentration in each tank was determined using a modified Widmark method (Hallett, unpublished) as follows. Samples were collected daily from at least one tank at each concentration throughout the exposure period.

A 10 ml sample was taken from the center of each tank and filtered through a Whatman #1 filter into a 50 ml flask. Two 10 ml distilled water samples were also prepared to determine the volume of ammonia sulfate nedded to titrate the potassium dichromate solution. A 0.5 ml subsample of the filtered sample was placed into a 250 ml flask containing 10 ml potassium dichromate solution. These flasks were placed in an oven at 60±2° C for 2 hours. Samples were removed and cooled. To provide a sufficient volume to titrate, 100 ml distilled water was added to each sample. Since the addition of water to the potassium dichromate solution generated heat, the samples were cooled again. Samples were then titrated against the ammonium sulfonate as an indicator. When the sample turned clear, the titration was complete.

The reagents were prepared as follows:

Reducing solution:(FAS): 8.866 g ammonium ferrous sulfate was dissolved in distilled water. Then, 200 ml concentrated sulfuric acid was added and the volume was adjusted to 2 liters with distilled water.

Oxidizing solution: 1.7050 g potassium dichromate was dissolved in 100 ml distilled water. Then, 1750 ml 14 N sulfuric acid was added.

All glassware was cleaned in chromic acid solution, rinsed several times with hot water, and finally rinsed with distilled water.

Ethanol concentrations in the tanks were determined using the following calculations:

15 ml FAS = 0.002 g ethanol

1 ml FAS = 0.133 mg ethanol

mg of ethanol / ml = g of ethanol / liter

[(R) (0.133 mg EtOH / ml FAS)] / 0.5 ml sample

where R ml of FAS needed to titrate the blank minus ml FAS needed to titrate the sample.

II. BEHAVIOR STUDY

TEST FISH

Flagfish were obtained from the United States Environmental Protection Agency (U.S. E.P.A.), Duluth, Minnesota in December 1980. These fish were brought into spawning condition in preparation for egg collection. Eggs were collected during late January and early February 1981. The resulting progeny was used for subsequent experiments.

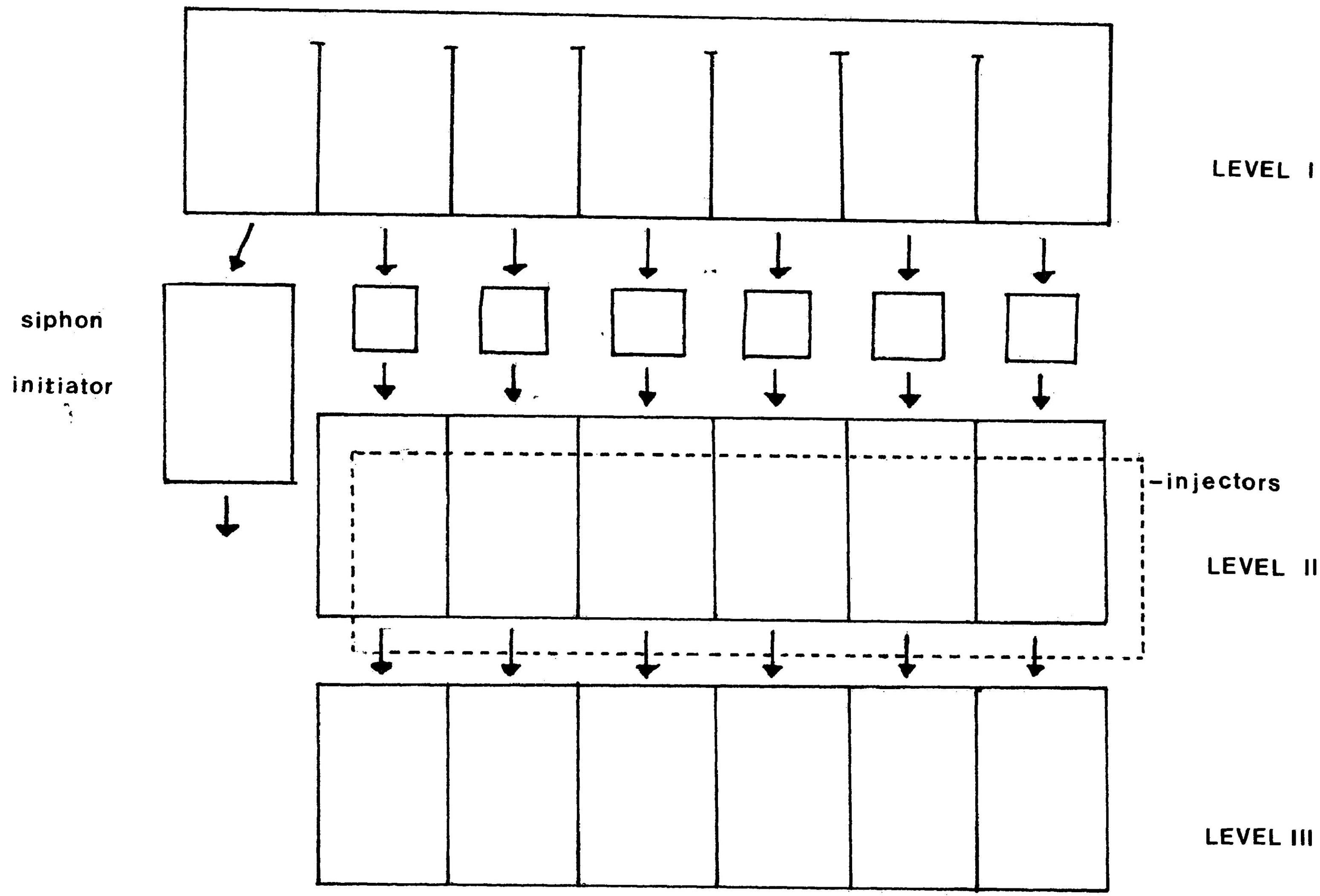
MATERIALS AND APPARATUS

Flow-through Diluter System

The equal volume diluter system used in this study has been described by DeFoe (1975). Modifications on this system have been described by Murphy (1978). The system consisted of three levels as shown in Figure 1. Level 1 filled with water from a header tank until all seven cells were full. When the seventh cell was full, it flooded into a siphon initiator cell in Level II causing a venturi vacuum which simultaneously released the water from the cells in Level I to the corresponding cells in Level II. As the siphon initiator cell filled, the float switch was triggered initiating the injector system which released ethanol into the Level II cells. The exhaust phase of each ethanol delivery permitted each syringe to refill from the ethanol reservior containing 99% ethanol.

The pneumatic injector system used to deliver the ethanol has been described by Smith, et al. (1977). Five injectors

Figure 1. Schematic illustrating equal volume diluter


LEVEL I
siphon
initiator
-injectors
LEVEL II
LEVEL III

were used, each calibrated to deliver a prescribed volume of ethanol. The remaining cell received dilution water only.

Standpipe siphons delivered the water from Level I to II, and from Level II to III. Mixing occurred at Level II and Level III. Each cell in Level III delivered an equal volume (500 ml) to four test tanks. Diluter filling was controlled by a 5 minute electrical timer providing 90% replacement in each test tank in approximately 12 hours.

Exposure Tanks

Twelve exposure tanks were used in this experiment: six were arranged to the left of the diluter system and six to the right. Each of the six treatment tanks was connected to a different cell on Level III of the diluter system. Each tank contained an inflow pipe, and outflow standpipe, an airstone, and 1/4 kw immersible heater. Exposure tanks were maintained at approximately 26°C with a constant photoperiod (LD 16:8).

Spawning Substrates

Spawning substrates consisted of a 10 cm X 15 cm inverted "V" shaped stainless steel frame wrapped tightly with green orlon yarn in parallel strands. The yarn was preboiled to remove excess dye.

Event Recorder

Fish behavior was recorded using a six channel event recorder. Each channel was assigned a behavior. Depressing a button produced a mark on the event recorder tape which indicated that a particular behavior had been initiated; releasing that button indicated the termination of that behavior. The event recorder was adjusted to produce 10mm of tape per second. The duration of each behavior was obtained by measuring the interval between button depression and release. This value was converted from millimeters to seconds.

PROCEDURE

Adult fish were fed previously thawed brine shrimp (Salina artemia) twice daily. All tanks were siphoned after feeding to remove excess food and wastes.

Flagfish were selectively culled in July 1981 to provide two males and three females in each tank. Two spawning substrates were placed at opposite ends of each exposure tank. Fish were allowed 4 days to habituate to their new environment and to establish dominance around the substrate area. All substrates were removed in the morning of the fourth day and the position of the substrate guarded by the dominant male was recorded. Twenty-four hours after removal, the substrate of the dominant male was replaced to its original location in each exposure tank. Food was then introduced into each tank. One-half hour later each tank was siphoned. Ten minutes after siphoning the behavior recording was initiated. This time was chosen because most spawning activity takes place immediately after feeding (Foster, et al., 1969)

The behavior of the dominant male in each tank was recorded daily for a 10 minute interval. To obtain baseline behavior data for each dominant fish, every tank was observed for 5 days prior to adding the ethanol. After the behavior was recorded on the fifth day, the substrates were removed. They were replaced 24 hours to coincide with the introduction of ethanol. No behavioral responses were recorded on the day the ethanol was introduced. The following day the routine of feeding, siphoning, and behavior recording was resumed. Behavior was again recorded for 5 consecutive days.

The behaviors were defined in such a way that the fish could engage in only one behavior at a time and at any point in time must be engaged in one of the behaviors. The behaviors observed were defined as follows:

Egg tending: the male is occupied with fanning or mouthing the eggs, or cleaning the substrate of debris and food. He is usually oriented 90 degrees to the substrate and is rapidly moving his pectoral fins.

Chasing a faded female: male chases, butts, or bites a female who has lost her coloration signifying her readiness to spawn.

Nesting: male maintains a position within 8 cm of the substrate but is not actively engaged in another behavior; most often all other fish are located at a distance from the substrate. The fins are not extended.

Spawning: male is actively spawning the female; the male presses the female against the substrate. Both are on their sides and rapidly undulating their caudal fins.

T-circling: the female has approached the male and orients herself perpendicular to the male. She may maintain this position for several seconds while the male attempts to parallel her side.

Chasing: the male aggressively approaches another fish with fins extended.

Guarding: the male extends all fins and assumes a position between the substrate and another fish.

Inattentive: the male does not assume any of the behaviors described above. For example, all the fish are gathered under the substrate and it is no longer apparent which male is dominant.

At the completion of this experiment, all tanks were scrubbed and disinfected with chlorine. The experiment was replicated 3 days later following the same procedure.

III. EGG PRODUCTION STUDY

Test Fish

Laboratory raised flagfish were used in this experiment. These fish were obtained as described in the Behavior Study section above.

MATERIALS AND APPARATUS

Exposure Tanks

The apparatus used in this study was the same as that previously described except that only six exposure tanks were used.

Flow-through Diluter System

The diluter system is described in the Behavior Study section above.

Spawning Substrates

The spawning substrates are described in the Behavior Study section above.

PROCEDURE

Fish were fed brine shrimp as described previously twice daily. Again, 1/2 hour after the morning feeding the tanks were siphoned to remove excess food and wastes.

Two males and three females were placed into each tank. One substrate was placed in each tank. Fish were allowed 4 days to establish dominance around the substrate area. All substrates were removed in the morning of the fourth day and replaced the following morning to coincide with the introduction of ethanol. Approximately 3 hours after feeding the substrates were removed and checked for the presence or absence of eggs. Eggs were brushed from the substrate by gently rubbing the fingers across the substrate. Clean substrates were returned to their respective tanks.

In addition, the presence or absence or absence of eggs was recorded on the behavior study tanks without removing the substrates.

IV. EGG HATCHABILITY STUDY

EGG SOURCE

Eggs collected from the Egg Production Study were used in this experiment.

MATERIALS AND APPARATUS

Exposure Tanks

The apparatus used in this study was the same as that previously described except that only six exposure tanks were used.

Flow-through Diluter System

The diluter system is described in the Behavior Study section above.

Rocker Arm Assembly

The rocker arm assembly has been described by Murphy (1978). Briefly, the rocker arm assembly consisted of a 12.7 mm aluminum rod suspended above the lower bank of tanks. Two 6.4 mm aluminum rods per tank were attached perpendicular to the former rod. These rods each had two points of attachment for the egg cups. The 12.7 mm rod was connected to a five rpm electric motor with cam which continuously raised and lowered the egg cups within the tank to facilitate oxygen and carbon dioxide gas exchange at the egg membrane surface.

Egg Cups

The egg incubating cups were constructed from 120 ml, 5 cm diameter, round, glass jars. The bottoms of the jars and the center of the bakelite caps were removed and replaced with stainless steel screen (No. 40 mesh). The screen was fastened with silicone sealant. A stainless steel hook was fastened to each cap providing a point of attachment to the rocker arm assembly.

PROCEDURE

Eggs collected from the Egg Production Study were placed into egg cups for incubation. No more than 50 eggs were placed into each cup. Egg cups were dipped in 4 ppm malachite green for 3 to 5 minutes immediately after collecting and daily thereafter until all had hatched. This was a preventitive measure to mitigate loss of eggs due to fungal infestation.

Egg cups were placed on the rocker arm assembly so that eggs were incubated in the same concentration of ethanol from which they were spawned. Eggs were examined daily and any dead eggs were removed.

V. LARVAL SURVIVAL STUDY

TEST FISH

Eggs were collected and incubated prior to ethanol introduction. Larvae not older than 2 day post-hatch were used in the Larval Survival Study.

MATERIALS AND APPARATUS

Exposure Tanks

The exposure tanks are the same as those used in the Egg Hatchability section above.

Flow-through Diluter System

The diluter system is described in the Behavior Study section above.

Larval Baskets

Larval baskets were constructed from winchester acid bottles. The tops and bottoms were removed leaving a 13 cm high container. Stainless steel screen (No. 40 mesh) was attached to the bottom. A 3 cm strip of screen was attached around the top circumference of the container to obtain additional height and water movement. Three 6 cm high glass vials were attached to the base of the basket which raised the basket off the bottom surface of the tank permitting water to pass through the lower screen in the basket. All attachments were adhered with silicone sealant.

PROCEDURE

Fifty newly hatched larvae were placed in each larval basket. Three baskets were randomly placed in each test tank so that 150 newly hatched larvae were exposed to the ethanol for 6 days. Larvae were fed newly hatched live brine shrimp once daily. Tanks were siphoned daily to remove excess food. Larval survival was recorded at the termination of the experiment.

RESULTS

This section has been divided into five subsections: I. Chemical Analysis Results; II. Behavior Study Results; III. Egg Production Results; IV. Egg Hatchability Results; and V. Larval Survival Results.

I. CHEMICAL ANALYSIS RESULTS

Water Chemistry

The results of the chemical analysis on the water supply is presented in Table 1 in Appendix B. The mean temperature, pH, and dissolved oxygen readings are presented in Tables 2 and 3 in Appendix B, for the first and second run, respectively. There was little fluctuation in any of these parameters and the values obtained are in the ranges of the U.S. E.P.A. recommended values for aquatic bioassays.

Ethanol Determinations

The mean ethanol concentrations for the first and second run are presented in Table 4 in Appendix B. These values are calculated from the mean daily concentration at each ethanol level. There was little fluctuation in the ethanol concentrations measured.

II. BEHAVIOR STUDY RESULTS

Qualitative Description of Behavior

During the week prior to alcohol addition, the behavior of the dominant male flagfish had two distinct behavioral patterns. One of these patterns involved t-circling, spawning, and chasing a faded female. The other behavior involved tending the eggs and guarding the substrate area. These behaviors varied in frequency and duration depending on the behaviors of the other fish in the tank and the readiness of the female to spawn.

In the first pattern of behavior, generally a female would approach the substrate in a passive manner either dropping slowly from above the substrate or slowly approaching from the opposite end of the tank. When a female approached the substrate in this way, her distinct coloration had faded. If no females approached the substrate to spawn, the male pursued females in the other end of the tank. The male then returned to the substrate and engaged in egg tending, nesting, and guarding. Frequently, following these forays, a faded female did approach the substrate, possibly in response to this activity.

If no other fish approached the spawning area, the male would attempt to parallel the faded female's side resulting in t-circling. The male frequently chased or butted a t-circling female. This produced one of two responses:

either the female would flee to the opposite end of the tank; or t-circling resumed and spawning occurred. Subsequent to spawning, the male chased the faded female from the substrate.

If another fish approached the substrate during spawning, the dominant male usually interrupted his courtship to chase the intruder away and, at times, chased the female he was engaged with. In instances where the dominant male continued spawning rather than chasing the intruders, the intruders canabalized the eggs on the substrate.

After the dominant male had spawned and cleared the substrate area of intruders, he resumed the second general pattern of behavior. This pattern involved an alternation of egg tending, nesting, guarding, and chasing.

<u>Effects of Ethanol on the Frequencies of Behaviors</u>

Data were pooled according to ethanol concentration so that four fish comprised the behavioral data at each exposure level. Week 1 represented the control period and Week 2 the ethanol exposure period. In this manner each fish was compared to its own control. This procedure minimizes individual behavioral differences.

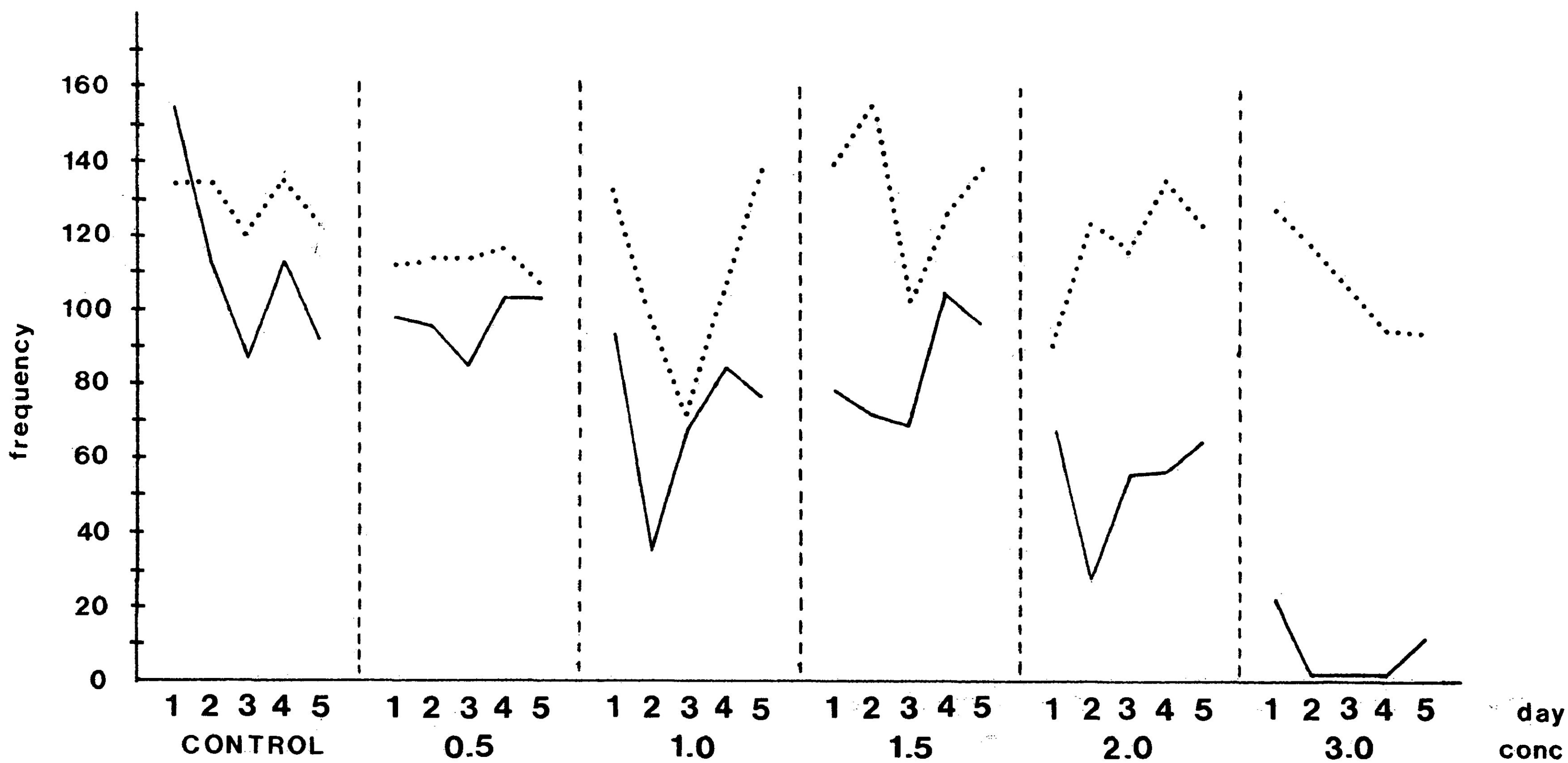
The number of times a fish switched from one behavior to another was represented by the Total Frequency. This parameter measured the activity level of the dominant male fish. The mean Total Frequency for each tank was determined for each level of ethanol used in the study. There was a general reduction in Total Frequency after the introduction of ethanol especially at 2.0 and 3.0 g/liter ethanol (Figure 2). A linear trend analysis was performed on the Total Frequency data and was significant at the $p<.05$ level, $F(1,18) = 14.26$, indicating that the tendency to engage in reproductive activity decreased as ethanol concentration increased from 1.0 through 3.0 g/liter.

No single behavior contributed to the reduction in Total Frequency, but rather the frequency of each behavior was reduced (see Figures 3 through 9). This reduction became more pronounced at the higher ethanol concentrations, 2.0 and 3.0 g/liter ethanol. There was one exception to this general pattern; at 0.5 g/liter ethanol frequency of chasing increased (Figure 9). The controls remained relatively constant for all behaviors except for a decline in the frequency of spawning, t-circling, and chasing a faded female. Linear trend analyses were conducted on the frequency data for each behavior (Table 1). Except egg tending, all behaviors were significant at the $p<.05$ level in the linear trend analysis, indicating that the frequency of these behaviors varied inversely with ethanol concentration.

<u>Effects of Ethanol on Percent of Total Frequency</u>

Since the frequency of each behavior had declined across concentrations the data was standardized in the form of percent of Total Frequency to determine whether the pattern and relative

Figure 2. Mean Total Frequency for all behaviors.
(... control period; — ethanol period)



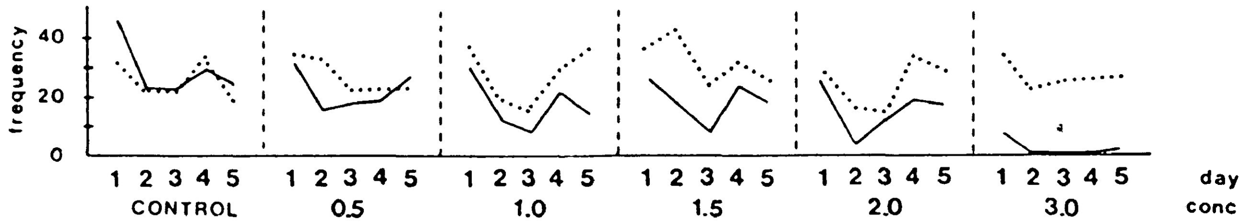
ALL BEHAVIORS

Figure 3. Frequency of nesting behavior
(... control; ___ ethanol)

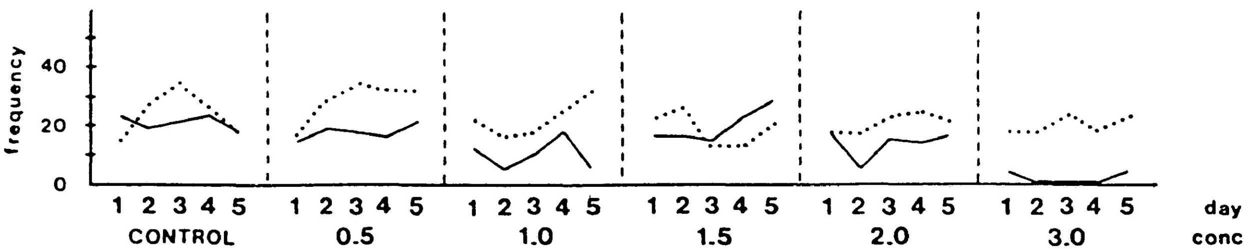
Figure 4. Frequency of egg tending behavior
(... control; ___ ethanol)

Figure 5. Frequency of t-circle behavior
(... control; ___ ethanol)

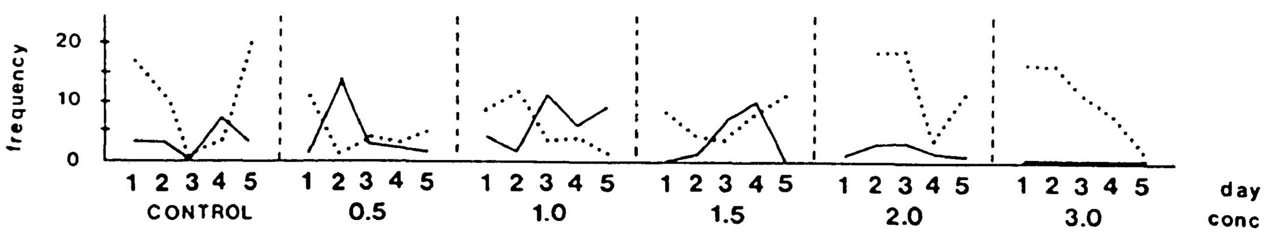
Figure 6. Frequency of spawn behavior
(... control; ___ ethanol)



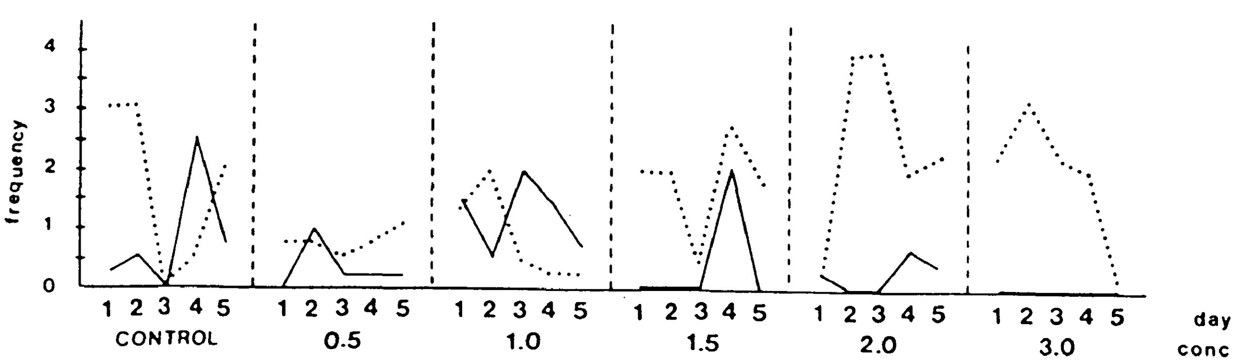
NESTING



EGG TENDING



T-CIRCLE

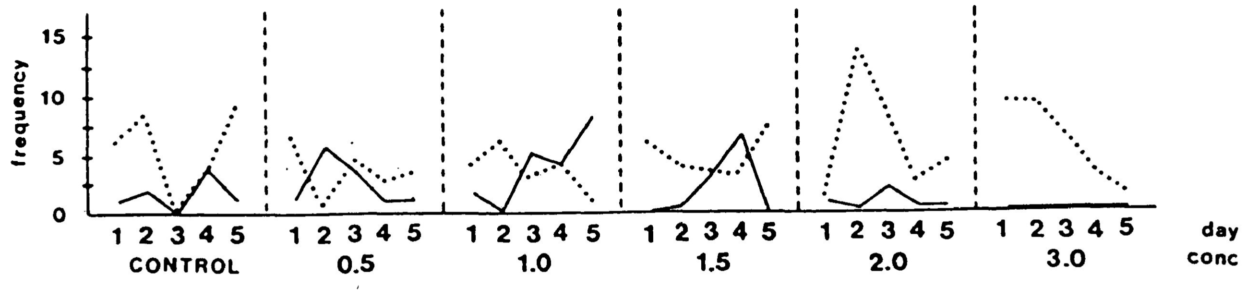


SPAWN

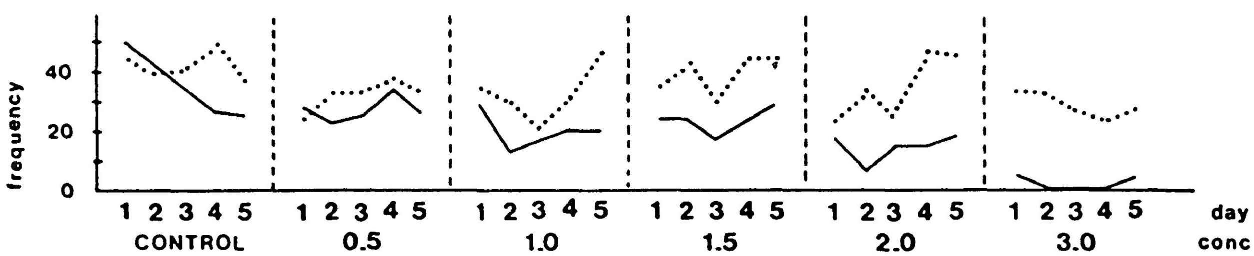
Figure 7. Frequency of chase faded female behavior
(... control; ___ ethanol)

Figure 8. Frequency of guard behavior
(... control; ___ ethanol)

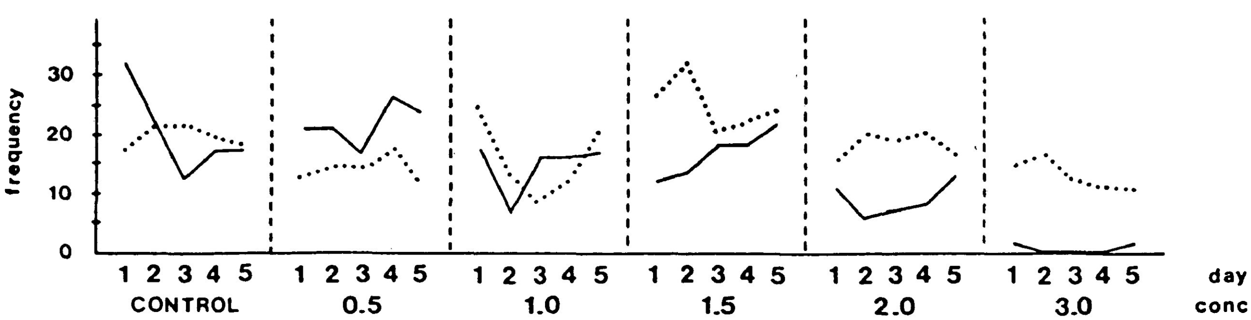
Figure 9. Frequency of chase behavior
(... control; ___ ethanol)



CHASE FADED FEMALE



GUARD



CHASE

Table 1. Linear trend analysis on the frequency data for each behavior.

Behavior	F-value
Egg Tending	1.26 (NS)*
Chase Faded Female	5.78
Nesting	16.80
Spawning	6.61
T-Circle	4.46
Chase	10.99
Guard	13.67
Inattentive	13.02
Total Frequency	14.26

*Not significant at the $p<.05$ level.

levels of behavior had changed. The data was converted using the following formula:

% Total Frequency =

(frequency of a behavior/Total Frequency) X 100

When the control data was examined for all tanks, it appeared that each behavior in the control situation made a consistent contribution to the frequency. For example, guarding contributed between 25 and 35% of the total behavior whereas chasing ranged from about 10 and 20% (Figures 10 through 16).

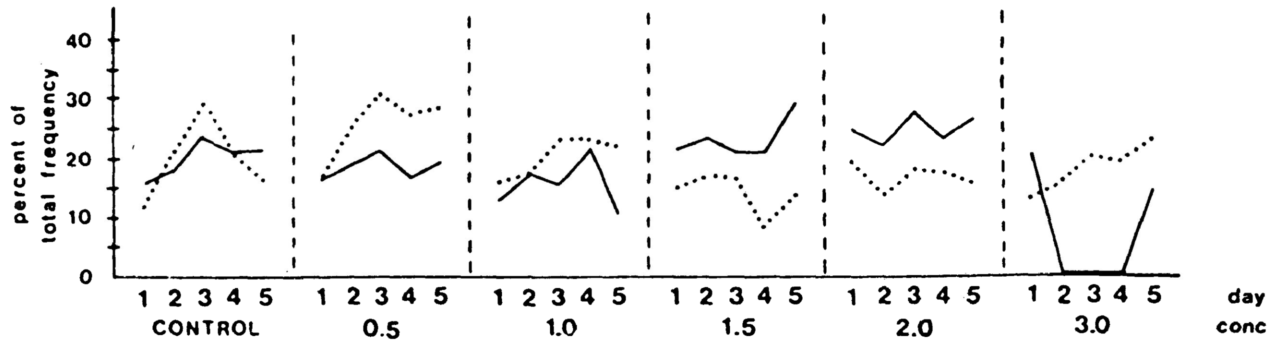
The percent of Total Frequency after ethanol exposure showed some fluctuations when compared to the control situation. The fish exposed to 0.5 g/liter ethanol and 1.0 g/liter ethanol showed an increase in the percent of Total Frequency for chasing behavior, and a decrease in egg tending and nesting behaviors. Fish exposed to 1.5 g/liter ethanol showed an increase in egg tending and a decrease in guarding and spawning. At 2.0 g/liter ethanol egg tending and nesting increased; t-circling, spawning, and chasing a faded female decreased; and chasing and guarding remained unchanged from the control situation. The behavior of fish exposed to 3.0 g/liter ethanol remained within the ranges of the control period for egg tending, nesting, guarding, and chasing on Days 1 and 5; but these fish were inattentive for the entire observation on Days 2, 3, and 4. T-circling, chasing a faded female, and spawning were eliminated.

Figure 10. Percent of Total Frequency for egg tending behavior (... control; ___ ethanol)

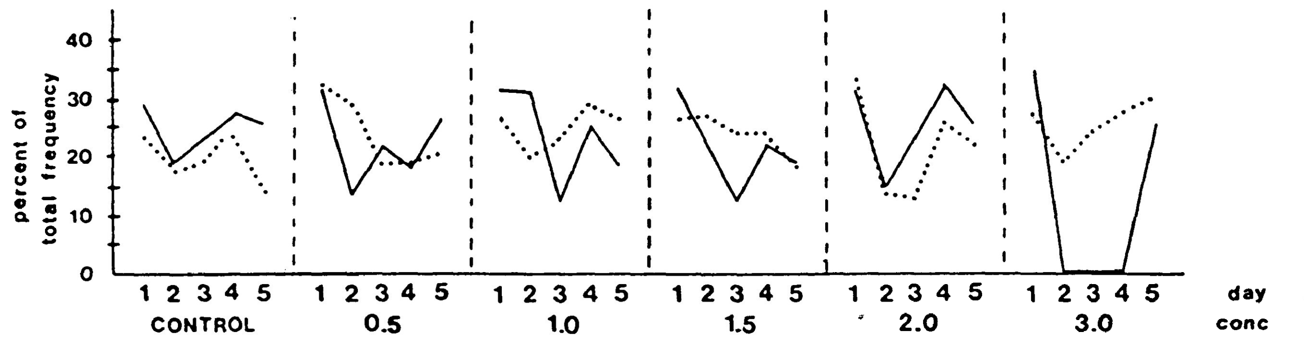
Figure 11. Percent of Total Frequency for nesting behavior (... control; ___ ethanol)

Figure 12. Percent of Total Frequency for t-circle behavior (... control; ___ ethanol)

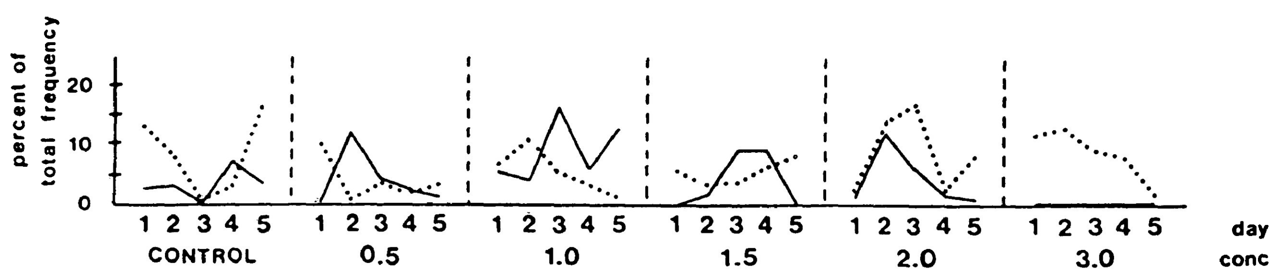
Figure 13. Percent of Total Frequency for spawn behavior (... control; ___ ethanol)



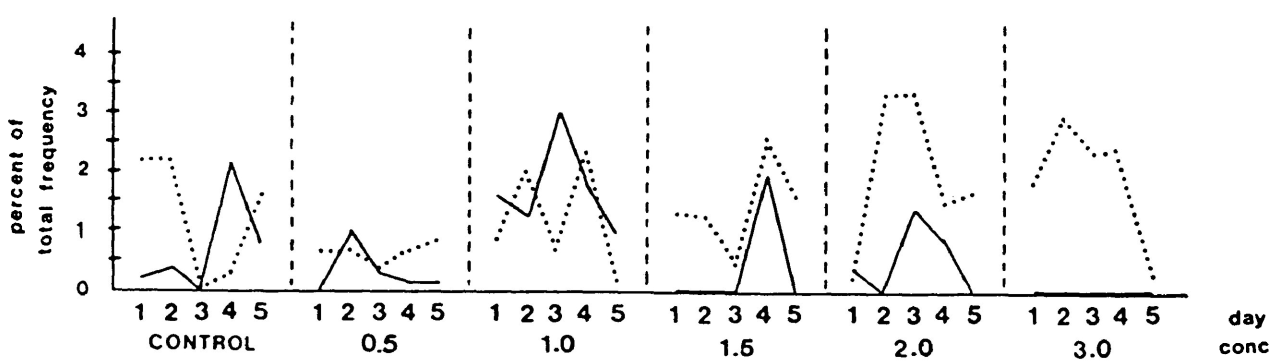
EGG TENDING



NESTING



T-CIRCLE

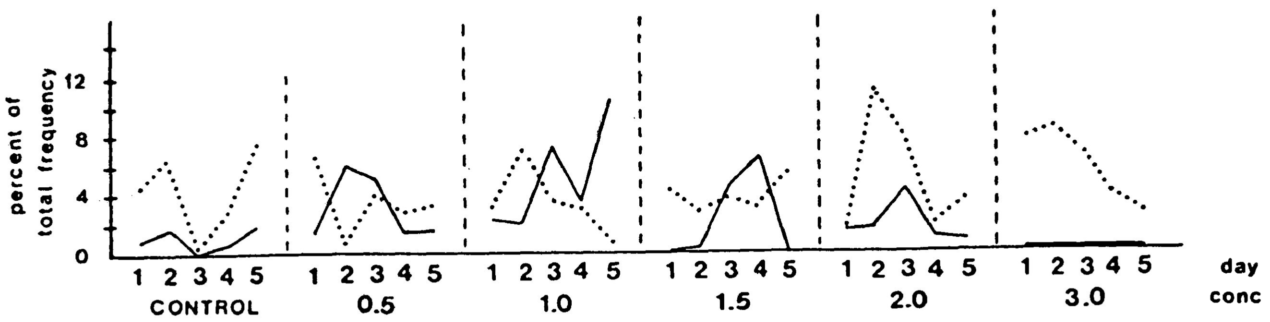


SPAWN

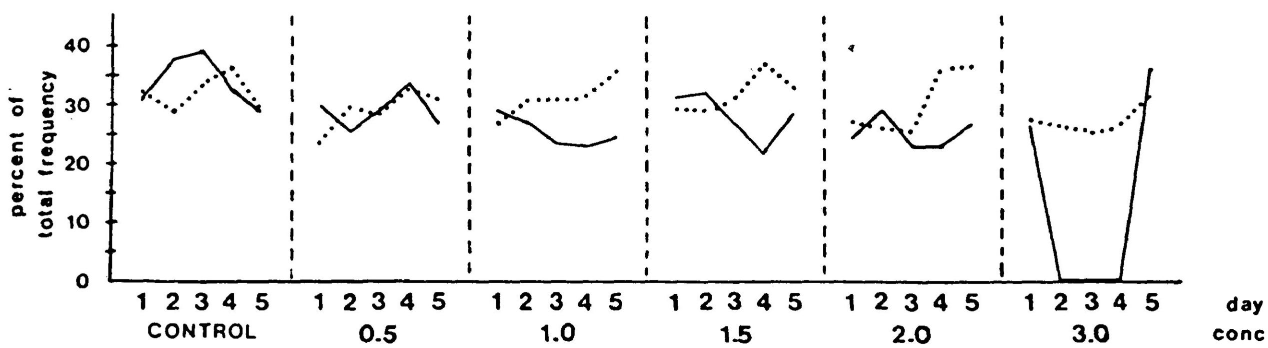
Figure 14. Percent of Total Frequency for chase faded female behavior (... control; ___ ethanol)

Figure 15. Percent of Total Frequency for guard behavior (... control; ___ ethanol)

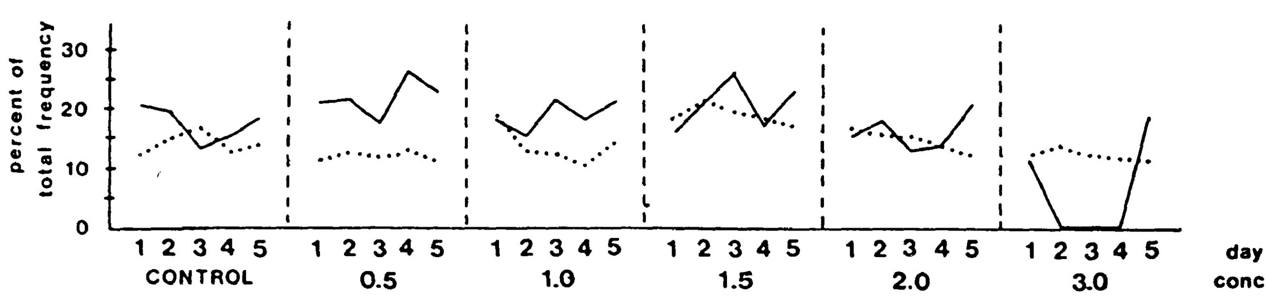
Figure 16. Percent of Total Frequency for chase behavior (... control; ___ ethanol)



CHASE FADED FEMALE



GUARD



CHASE

Table 2. One-way ANOVA on the percent of Total Frequency

Behavior	F-value
Egg Tending	3.37
Chase Faded Female	Ø.64(NS)*
Nesting	4.15
Spawning	1.75(NS)*
T-circle	Ø.86(NS)*
Chase	3.93
Guard	3.14

*Not significant at the p<.Ø5 level

A one-way ANOVA was performed on the difference scores between Week 1 and Week 2 across concentrations (Table 2). Egg tending, nesting, guarding, and chasing were significant at the $p < .05$ level, indicating that the relative levels of these behaviors were affected by ethanol concentration.

<u>Effects of Ethanol on Sequences of Behavior</u>

To examine the frequency with which each sequence of behavior occurred, a two dimensional transitional matrix was computed with the vertical axis representing the "preceding" behavior and the horizontal axis representing the behavior that "followed". Since the frequency data by definition is a shift from one behavior to another, no behavior can be followed by itself. Therefore the diagonal cells remain empty.

The frequency data obtained from all tanks in the control situation were pooled into one matrix and are presented as the upper values in Table 3. The lower values for each block represent the maximum-likelihood estimate if the behaviors were occurring randomly. These values were derived using the method described by Wagner (1970). A Chi square analysis was performed using the estimated matrix as the expected values and the control matrix as the observed values. The resulting Chi square value was very large, $x^2 = 13528$, and was significant, indicating that the behavioral sequences do not occur randomly.

Since the behavior was not random, another set of matrices

Table 3. Frequency data for sequences of behavior in the control situation (upper values) and the maximum-likelihood estimates if the behaviors were occurring randomly (lower values).
* denotes sequences that occur most frequently

	egg tending	chase faded female	nesting	spawn	t-circle	chasing	guarding	inatten.
egg tending	X	20 109.49	1183 * 789.14	0 34.35	36 180.89	468 * 463.74	1137 * 1266.57	2 1.48
chase faded female	50 119.11	X	147 156.27	16 6.90	324 35.82	47 91.83	75 250.81	2 .29
nesting	1670 * 862.09	81 156.93	X	10 49.95	181 259.25	625 * 664.63	1241 * 1815.26	2 2.12
spawn	3 38.07	35 6.93	38 49.95	X	83 11.45	23 29.35	34 80.17	0 .09
t-circle	28 200.90	418 36.57	101 263.56	177 11.64	X	115 154.88	252 423.02	0 .49
chasing	61 505.40	29 92.00	189 663.05	3 29.28	65 151.99	X	2160 * 1064.19	0 1.24
guarding	1247 * 1334.05	61 242.84	2015 * 1750.19	4 77.30	351 401.19	1156 * 1028.49	X	3 3.28
inatten.	2 1.57	1 .29	1 2.06	0 .09	1 .47	0 1.21	4 3.31	X

was developed so that the fish at each concentration acted as their own controls. The raw frequencies for each week were summed according to concentration. This frequency was then divided by the Total Frequency of the summary matrix and multiplied by 100 to obtain the percent of Total Frequency for each block. The data is presented in the form of percent of Total Frequency so that comparisons can be made across concentrations (Tables 4 through 9). The upper values in these matrices were the values obtained during the ethanol exposure period; the lower values were the control period data.

In the control situation 10 of the possible 56 sequences represented over 80% of the Total Frequency. These 10 sequences are noted with an asterik. When the sequences were examined across concentrations, all of the 10 sequences which represented most of the total acitivity are retained after ethanol exposure, even at the highest concentration. A one-way ANOVA was performed on the difference scores between the control period and the ethanol exposure period for these 10 sequences. The ethanol exposure did not significantly change the behavioral patterns; $F(5,54) = 1.33$, which was not significant at the $p < .05$ level.

Table 4. Percent of Total Frequency for sequences of behavior in controls during the ethanol exposure period (upper values) and during the control period (lower values).
* denotes sequences used in the ANOVA analysis

Table 5. Percent of Total Frequency for sequences of behavior in 0.5 g/liter ethanol during the ethanol exposure period (upper values) and during the control period (lower values).
* denotes sequences used in the ANOVA analysis

follows

precedes	egg tending	chase faded female	nesting	spawn	t-circle	chasing	guarding	inatten.
egg tending		0 .2	8.7* 6.6	0 0	.2 .1	3.0* 2.9	7.3* 9.7	0 0
chase faded femalo	.2 .2		.3 0	.1 0	.5 2.3	.4 .2	0 .6	0 0
nesting	11.3* 8.8	.2 .5		0 0	1.1 1.2	4.7* 2.7	8.0* 6.6	0 0
spawn	0 0	.2 .3	0 .2		.4 .4	.1 .1	0 .3	0 0
t-circle	.1 0	.8 2.8	.8 .7	.5 1.2		.3 .8	1.1 2.7	0 0
chasing	.6 .5	.1 .2	1.4 .8	0 0	.2 .7		15.5* 12.4	0 0
guarding	6.8* 9.9	.3 .3	14.2* 10.7	0 .1	1.0 3.5	9.4* 7.7		0 0
inatten.	0 0	0 0	0 0	0 0	0 0	0 0	0 0	

follows

precedes	egg tending	chase faded female	nesting	spawn	t-circle	chasing	guarding	inatten.
egg tending		0 .2	9.6* 10.8	0 0	0 .3	4.6* 4.9	4.3* 9.9	0 0
chase faded female	.5 .8		.4* .7	0 0	1.3 1.8	.3 0	.3 .1	0 0
nesting	8.5* 12.1	.5 .4		0 0	1.2 .9	6.1* 2.8	5.6* 7.4	0 0
spawn	0 0	.1 .3	0 .1		.2 .2	0 0	0 0	0 0
t-circle	0 .2	2.1 2.5	.6 .5	.4 .7		.9 .1	.4 .5	0 0
chasing	1.8 .5	.2 .1	1.5 .5	0 0	.4 .1		18.4* 11.5	0 0
guarding	8.0* 12.0	.3 .2	9.6* 10.4	0 0	1.1 1.1	10.3* 5.3		0 0
inatten.	0 0	0 0	0 0	0 0	0 0	0 0	0 .1	

Table 6. Percent of Total Frequency for sequences of behavior in 1.0 g/liter ethanol during the ethanol exposure period (upper values) and during the control period (lower values).
* denotes sequences used in the ANOVA analysis

Table 7. Percent of Total Frequency for sequences of behavior in 1.5 g/liter ethanol during the ethanol exposure period (upper values) and during the control period (lower values).
* denotes sequences used in the ANOVA analysis

follows

precedes	egg tending	chase faded female	nesting	spawn	t-circle	chasing	guarding	inatten.
egg tending		.1 .1	8.9* 9.5	0 0	.1 .4	4.4* 2.4	4.2* 7.6	0 0
chase faded female	.6 .3		.5 .6	.1 0	2.5 1.6	.6 .3	.4 .4	0 0
nesting	7.7* 10.5	.3 .2		.1 0	2.1 1.2	5.7* 4.4	5.1* 8.7	.3 0
spawn	0 0	.2 0	.3 .2		1.0 .4	.1 0	0 0	.3 0
t-circle	.3 .3	3.2 2.4	.9 .3	1.5 .7		1.5 .6	.6 1.1	0 0
chasing	1.4 .1	.5 0	1.1 1.1	0 0	.8 .1		15.7* 13.2	0 0
guarding	6.4* 8.8	.2 .8	10.0* 13.0	0 0	1.8 1.7	7.8* 6.7		.1 0
inatten.	0 0	0 0	.3 0	0 0	0 0	.2 0	.1 0	

follows

precedes	egg tending	chase faded female	nesting	spawn	t-circle	chasing	guarding	inatten.
egg tending		0 .2	11.6* 4.6	0 0	.2 .1	8.0* 4.3	4.0* 5.3	0 .1
chase faded female	.2 .2		.2 1.2	.1 .1	1.4 1.8	.4 .3	.1 .2	0 0
nesting	8.8* 8.9	.1 .6		0 .1	.7 1.2	5.5* 5.4	5.3* 7.8	.2 0
spawn	0 0	.2 .3	0 .5		.2 .4	0 .2	0 .2	0 0
t-circle	.2 .1	2.0 1.9	.4 .3	.3 1.4		1.1 .6	.2 1.2	0 0
chasing	.9 .2	.1 .2	.2 2.0	0 0	.9 .1		18.4* 17.0	0 0
guarding	12.7* 5.2	.1 .5	8.9* 14.6	0 0	.7 1.8	5.4* 8.6		.1 .1
inatten.	0 0	0 0	.1 0	.1 0	.1 0	0 0	.1 .1	

Table 8. Percent of Total Frequency for sequences of behavior in 2.0 g/liter ethanol during the ethanol exposure period (upper values) and during the control period (lower values).
* denotes sequences used in the ANOVA analysis

Table 9. Percent of Total Frequency for sequences of behavior in 3.0 g/liter ethanol during the ethanol exposure period (upper values) and during the control period (lower values).
* denotes sequences used in the ANOVA analysis

follows

precedes	egg tending	chase faded female	nesting	spawn	t-circle	chasing	guarding	inatt.
egg tending	X	0 .2 *	12.6 5.6	0 0	.4 .4	5.2 1.8 *	6.8 8.0 *	0 0
chase faded female	.3 .4	X	.5 .5	0 .5	.8 3.0	.2 .4	0 .9	0 0
nesting	15.8 8.1 *	.1 .3	X	.1 0	.5 .5	5.4 3.3 *	4.0 8.1 *	.5 0
spawn	0 0	.3 .2	.1 .2	X	.1 1.0	.1 .4	0 .4	0 0
t-circle	.1 .4	1.4 4.0	.2 .1	.4 1.5	X	.8 1.4	.7 1.9	0 0
chasing	.8 .4	0 .4	1.7 1.6	0 0	.8 1.1	X	13.5 11.9 *	0 0
guarding	8.1 6.8 *	0 2.4	10.8 10.4 *	0 0	1.0 3.2	5.1 8.3 *	X	.2 0
inatten.	0 0	0 0	.2 0	0 0	.1 0	0 0	.4 0	X

follows

precedes	egg tending	chase faded female	nesting	spawn	t-circle	chasing	guarding	inatten.
egg tending	X	0 .1 *	10.7 9.3	0 0	0 .2	2.3 2.7 *	4.6 5.9 *	0 0
chase faded female	0 .1	X	0 1.4	0 .2	0 3.0	0 .4	0 .9	0 0
nesting	13.8 11.2 *	0 1.3	X	0 .3	0 1.5	3.8 3.1 *	9.2 7.5 *	3.8 0
spawn	0 0	0 .1	0 .1	X	0 .9	0 .1	0 .5	0 0
t-circle	0 .2	0 3.7	0 1.6	0 1.2	X	0 1.0	0 1.5	0 0
chasing	0 .4	0 .1	2.3 .6	0 .1	0 .4	X	10.8 10.9 *	.8 0
guarding	3.8 6.2 *	0 .8	16.2 11.7 *	0 0	0 3.3	6.9 5.2 *	X	3.1 0
inatten.	0 0	0 0	2.3 0	0 0	0 0	1.5 0	3.8 0	X

Effects of Ethanol on Time Spent at Each Behavior

The mean time spent at each behavior per observation period was also calculated for each ethanol concentration (Figures 17 through 24). Egg tending, guarding, spawning, t-circling, and chasing a faded female all decreased in mean time as ethanol concentration increased. Nesting time increased during the second week in the control tanks and in concentrations of 0.5 and 1.0 g/liter ethanol, and decreased in the remainder of the ethanol concentrations. Chasing time increased at 0.5 g/liter, and decreased at 1.5, 2.0, and 3.0 g/liter ethanol. Mean time spent at inattentive behavior increased as the ethanol concentration increased.

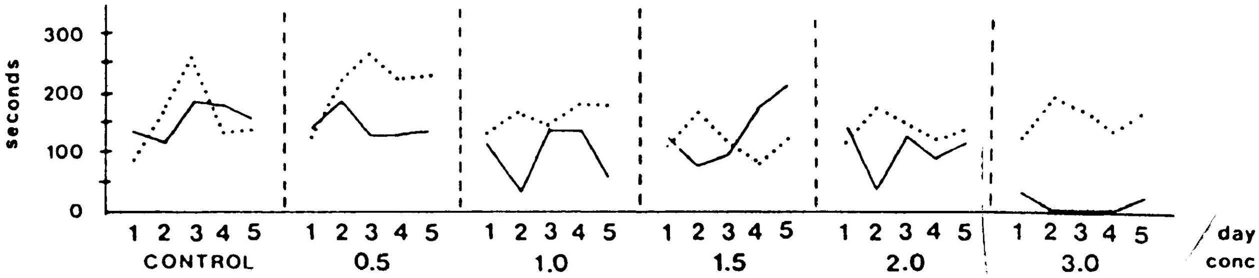
Linear trend analysis was performed on this data and is presented in Table 10. All behavior times except egg tending and t-circling were significant at the $p < .05$ level, indicating that time spent at these behaviors decreased as ethanol concentration increased.

Figure 17. Time spent at egg tending behavior
(... control; ___ ethanol)

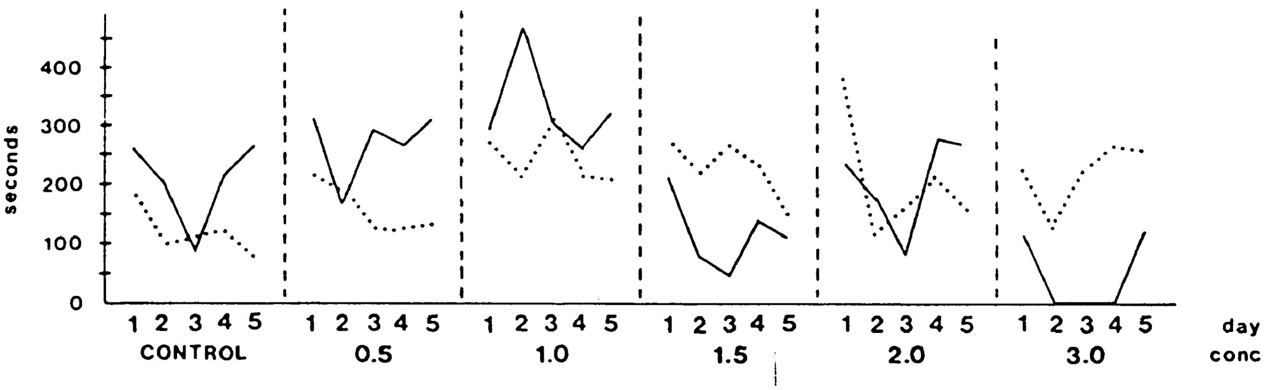
Figure 18. Time spent at nesting behavior
(... control; ___ ethanol)

Figure 19. Time spent at t-circle behavior
(... control; ___ ethanol)

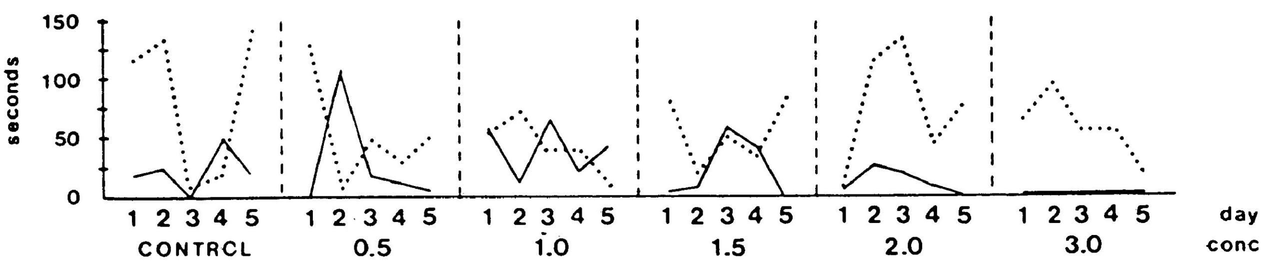
Figure 20. Time spent at spawn behavior
(... control; ___ ethanol)



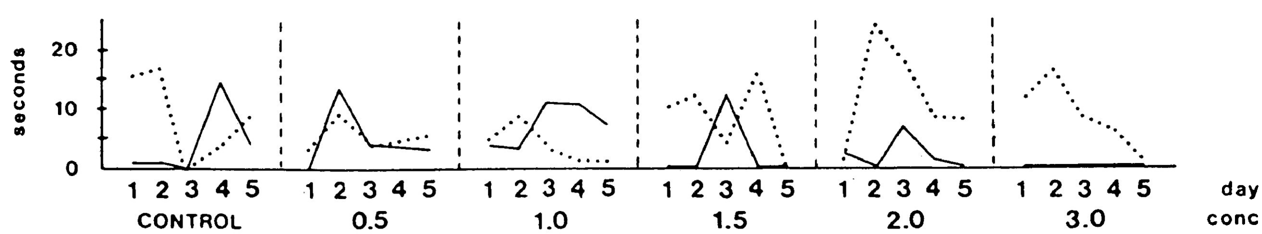
EGG TENDING



NESTING



T-CIRCLE



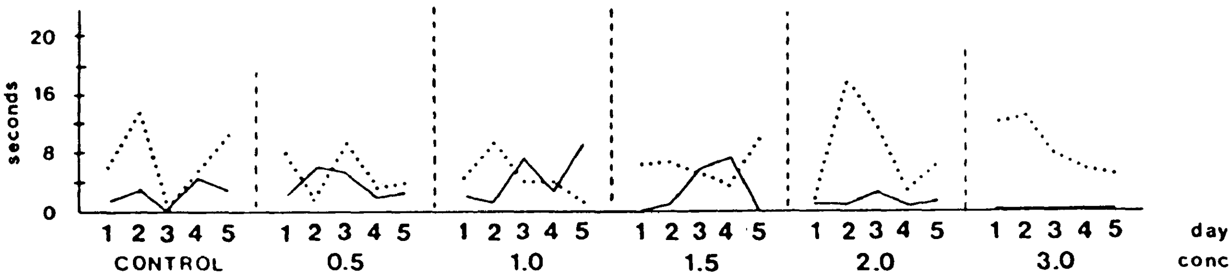
SPAWN

Figure 21. Time spent at chase faded female behavior (... control; ___ ethanol)

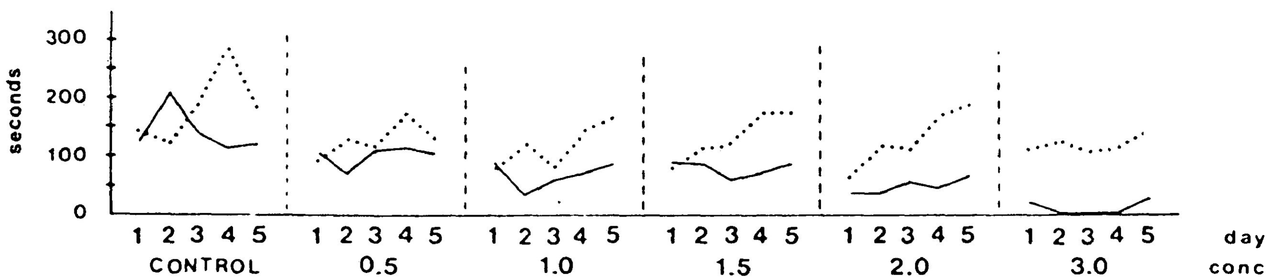
Figure 22. Time spent at guard behavior (... control; ___ ethanol)

Figure 23. Time spent at chase behavior (... control; ___ ethanol)

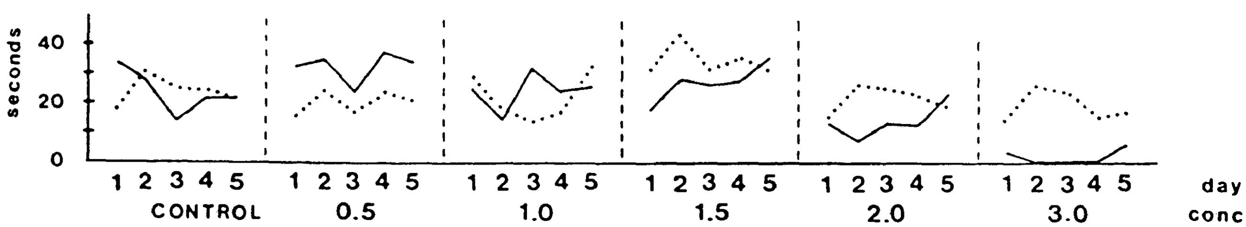
Figure 24. Time spent at inattentive behavior (... control; ___ ethanol)



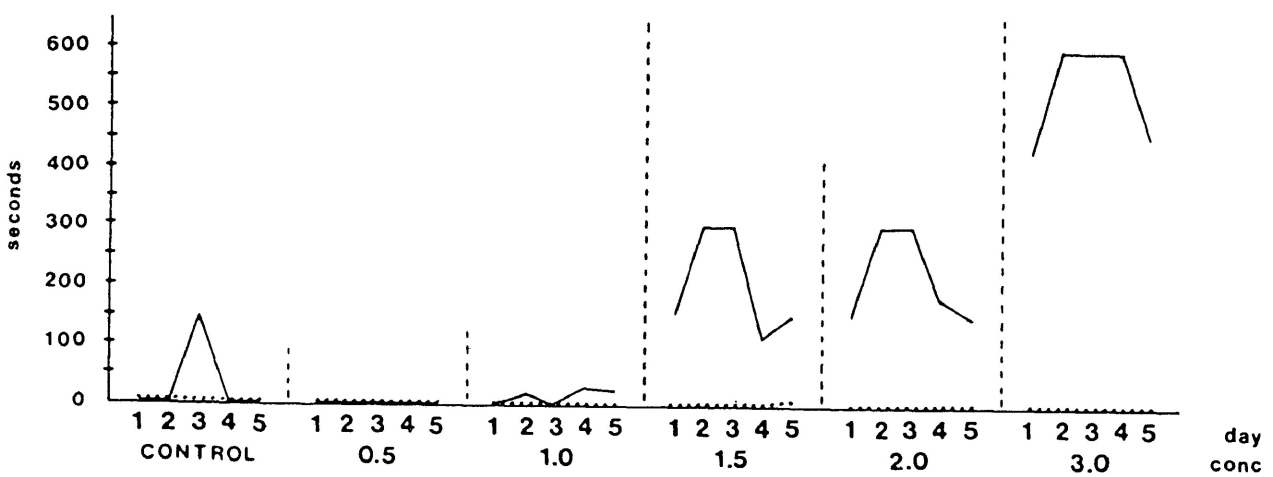
CHASE FADED FEMALE



GUARD



CHASE



INATTENTIVE

Table 1Ø. Linear trend analysis on time spent at each behavior.

Behavior	F-value
Egg Tending	3.76(NS)*
Chase Faded Female	4.89
Nesting	23.8Ø
Spawning	4.97
T-circle	1.2Ø(NS)*
Chase	9.34
Guard	25.47
Inattentive	53.79

*Not significant at the $p<.Ø5$ level.

III. EGG PRODUCTION RESULTS

A Chi square test performed on this data (x^2=22.22, df=5) indicated that egg production was reduced at the highest ethanol concentration, 3.0 g/liter ethanol (Table 11).

IV. EGG HATCHABILITY RESULTS

The percent of eggs hatching after incubation in ethanol concentrations ranging from control through 3.0 g/liter is presented in Table 12. Percent survival for all concentrations exceeded 94%. The results indicated that ethanol did not induce high embryo mortality at any of the concentrations tested.

V. LARVAL SURVIVAL RESULTS

The percent of larval survival across ethanol concentrations ranging from control through 3.0 g/liter is presented on Table 13. Percent survival for all concentrations exceeded 90%. These results indicated that ethanol was not lethal to the larvae at the concentrations tested.

Table 11. Presence or absence of eggs on the substrates during the ethanol exposure period

	Ethanol Concentration (g/liter)					
	Control	Ø.5	1.Ø	1.5	2.Ø	3.Ø
Eggs	1Ø	12	12	12	11	4
No Eggs	3	1	1	1	2	9

Table 12. Percent of eggs hatching across ethanol concentrations.

Conc. (g/liter)	Percent Survival	Number of Eggs Incubated	Number of Dead Eggs
Control	98.5	136	2
Ø.5	94.5	2ØØ	11
1.Ø	94.2	121	7
1.5	98.1	155	3
2.Ø	96.1	127*	5
3.Ø	1ØØ	2ØØ**	Ø

*95 eggs collected from Control tanks and incubated in 2.Ø g/l

**1ØØ eggs collected from Control tanks and incubated in 3.Ø g/l

Table 13. Percent of larval survival across ethanol concentrations

Conc. (g/liter)	Percent Survival	Number Larvae Exposed	Number Dead Larvae
Control	98.7	150	2
0.5	90.7	150	14
1.0	100	150	0
1.5	99.3	150	1
2.0	97.3	150	4
3.0	96.0	150	6

DISCUSSION

When the behavior during the control period was examined, each behavioral category contributed a relatively constant percent of the Total Frequency. It would seem that the behavior was consistent, although some variation among the males did occur. Slater (1981) discussed reasons for individual behavioral variations. He stated that "it may benefit animals to adopt different strategies depending on what others are doing." This phenomena was most pronounced when the subordinate male intruded into the substrate territory of the dominant male. A guard behavior was frequently sufficient to deter a passive intruder from the area, whereas a chase was necessary if the intruding male was more aggressive.

When the fish were exposed to concentrations of ethanol ranging from 0.5 to 3.0 g/liter ethanol for 5 days there was a decrease in the Total Frequency of behaviors which became more pronounced at the higher concentrations of ethanol. When the observed behaviors were divided into eight discrete categories no single behavioral category contributed to the reduction but rather the frequency of each category was reduced. Accompanying this reduction in activity was an increase in time spent at inattentive behavior which increased as ethanol concentration increased. Although the activity of the dominant male was reduced, there were no obvious signs of motor impairment in these fish.

When the Total Frequency was examined the only behavior without significant change was egg tending. The fish seem to maintain this behavior regardless of the ethanol. The time spent at egg tending was also not significantly different than the controls.

Time spent at t-circling did not differ from the control situation but the frequency of t-circling was reduced. Therefore, the dominant male spent more time at each bout of t-circling. The proportion of successful spawnings, as indicated in the sequences of t-circling to spawning, was the same as would be expected in the control situation, although it appeared that fewer eggs were produced at the higher ethanol concentrations. Since the subordinate fish canabalize the eggs on the substrate, egg counts would reflect the ability of the dominant male to guard the substrate and chase intruders, rather than the number of eggs produced. Since the frequency and the time spent at guarding and chasing behaviors were reduced after the ethanol exposure, subordinate fish were more likely to canabalize the eggs.

Peeke, et al. (1973) noticed a similar pattern when examining aggressive behavior in convict cichlids. These authors found with increasing concentrations of ethanol (0.7, 1.8, and 3.3 g/liter ethanol) there was a progressive decrease in the number of attacks against intruders.

When the percent of Total Frequency was calculated for sequences of behavior, it was found that these sequences did not occur randomly. Of the 56 possible sequences, 10 cumulatively contributed over 80% of the Total Frequency. If these sequences of behavior were drastically altered after ethanol exposure, it would seem likely to reduce the communication between the male and the females, causing an interruption or elimination of spawning. But, when examined across all ethanol concentrations tested, these 10 sequences were retained despite the reduction in Total Frequency. Therefore, the ethanol had not appreciably altered the pattern of behavior except to reduce the amount of activity.

This reduction in activity was accompanied by a reduction in spawning behavior. The reduction in spawning behavior found at the higher concentrations of ethanol (1.5, 2.0, and 3.0 g/liter) was not due to a lack of egg viability at these concentrations. It would seem likely that if the eggs were not surviving, the adults would no longer tend to the developing eggs or continue to spawn (Van Iersel, 1970). But, the egg hatchability study showed that the eggs were viable since over 94% hatched in all concentrations of ethanol tested. The ethanol, therefore, was affecting the behavior of the adults in such a way as to reduce reproductive behaviors regardless of egg viability. This effect can not be attributed to any one behavioral category, nor to the critical sequences of reproductive behavior, since these were not affected by the ethanol treatments. Rather, a reduction in total activity appears to be the controlling factor, a quantitative not qualitative effect. It appears that the dominant male must maintain a minimal amount of the activity to successfully spawn.

CONCLUSIONS

The methodology used in this study is representative of a type of behavioral design which could be performed to determine sublethal effects of toxicants on aquatic organisms. The method lends itself to chemicals that are known to affect the central nervous system and therefore, behavior. Rather than examining a single behavioral parameter, this method examines the components of flagfish reproductive behavior: egg tending; nesting; t-circling; chasing a faded female; spawning; guarding; and chasing. As demonstrated, the data can be examined for changes in Total Frequency, time spent at each behavior, and sequences of behavior. Behavioral alterations attributed to the toxicant can be examined with respect to effects which may develop at different rates depending on concentration and time of exposure, and therefore assist in determining safe levels of toxicants discharged into the aquatic system.

In this study sublethal ethanol concentrations did not affect survival of the eggs or larvae at any of the concentrations tested, yet parental behavior was altered in such a way that the fish at the highest concentration were no longer spawning. Although the ecological significance of some behavioral alterations may be ambiguous the impact of this result is certainly not. Since recruitment is inherent to the continuation of a species, this behavioral alteration would eventually result in a collapse of the fish population.

APPENDIX A.

A Review of Selected Literature on Fish Behavior

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Moroco steindachneri, Cyprinus carpio, Carassius auratus, Acheilognathus limbata, Tribolodon hakonensis	pH, copper, ammonia	avoidance	gradient tank, measured frequency of avoidance	copper and ammonia were attractive, most fish attracted to alkaline water, average range was pH 7.65 to 9.38	Ishio, 1965
Salmo salar	Dimilin-GI and carrier concns.	avoidance	olfactometer	repelled by flume, decreased time spent in flume, entry into flume could interfere with spawning migrations.	Granett, Morang, and Hatch, 1978
Oncorhynchus keta	sulfite mill effluent	vertical distribution vs. distance from effluent	field study, vertical cages with 6 one meter compartments suspended in water	significant relationship between water quality and fish distribution, controls showed definite preference for surface	McGreer and Vigers, 1980
Gasterosteus aculeatus	sulfite mill effluent	vertical distribution	field study, vertical cages with 6 one meter compartments suspended in water	inconsistent results	McGreer and Vigers, 1980
Salmo salar	bleached kraft mill effluent	avoidance	steep gradient trough	responses were vague; only showed definite avoidance at lethal concns.	Sprague, 1964

Species	Toxicant	Behavior measured	Methods	Effects	Authors
Phoxinus phoxinus	phenol	avoidance	avoidance tube; after time interval, flow reversed	showed no capacity for recognizing and avoiding any concn.	Jones, 1951
Phoxinus phoxinus	para-cresol	avoidance	avoidance tube; after time interval, flow reversed	avoided all concns. which produced loss of equilibrium in less than four minutes	Jones, 1951
Phoxinus phoxinus	ortho-cresol	avoidance	avoidance tube; after time interval, flow reversed	avoided high concn., but at greater dilutions, recognition declines rapidly	Jones, 1951
Salmo salar	copper-zinc	avoidance	sharp gradient trough	two metals together some potentiation	Sprague, 1964
Salmo gairdneri	zinc sulfate	avoidance	sharp gradient trough	as concns. of zinc increased, time spent in zinc solution decreased	Sprague, 1968b
Salmo gairdneri	zinc sulfate	avoidance	sharp gradient trough	avoided zinc	Sprague, 1964
Salmo gairdneri	phenol	avoidance	sharp gradient trough	did not avoid almost lethal concns., swam frantically but apparently could not discriminate	Sprague, 1964
Salmo gairdneri	chlorine	avoidance	sharp gradient trough	avoided concns. which would be lethal in 10 days, also avoided concn. which would be rapidly lethal but preferred intermediate concn.	Sprague, 1964

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Carassius auratus	copper and temperature	avoidance, time spent in compartment and number of entries	photoconductive cells in circular tank divided into compartments	fish avoid copper containing compartments both in frequency of entries and time spent; when copper is associated with a slight rise in temperature, fish are attracted to it	Kleerekoper, Waxman, and Matis, 1973
Rhinichthys atratulus	unchlorinated sewage effluent	avoidance	fish avoidance trough, measured time spent and number of avoidances	did not avoid, total time spent was more sensitive	Fava and Tsai, 1976
Rhinichthys atratulus	ammonia-nitrogen	avoidance	fish avoidance trough, measured time spent and number of avoidances	did not avoid, time spent was more sensitive	Fava and Tsai, 1976
Rhinichthys atratulus	chlorinated sewage effluent	avoidance	fish avoidance trough, measured time spent and number of avoidances	avoided	Fava and Tsai, 1976
Rhinichthys atratulus	chloramines	avoidance	fish avoidance trough, measured time spent and number of avoidances	avoided	Fava and Tsai, 1976
Rhinichthys atratulus	free chlorine	avoidance	fish avoidance trough, measured time spent and number of avoidances	avoided	Fava and Tsai, 1976

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Menidia menidia *Morone americana*	chlorine, temperature, salinity, and pH	avoidance	trough divided into quadrants	avoided chlorine; salinity was most important variable	Meldrim and Fava, 1977
Lagodon rhomboides	chlorine	avoidance	automated infra-red lights, rectangular tanks	most time spent in uncontaminated area	Cripe, 1979
Carassius auratus	mercury	avoidance	automatic system, records position and rate of movement	moves out of contaminated area twice as fast as it enters it	Scherer and Nowak, 1973
Lepomis gibbosus	ethanethiol	avoidance	trough divided into six compartments, gates between compartments raised and toxicant introduced	attraction	Summerfelt and Lewis, 1967
Lepomis gibbosus	A-chloro-acetophenone	avoidance	trough divided into six compartments, gates between compartments raised and toxicant introduced	avoidance	Summerfelt and Lewis, 1967
Cyprinodon variegatus	DDT	avoidance	Y-maze apparatus	avoided concn. near the 24 hour LC50; fish did not sense an increase in concn.	Hansen, 1969
Cyprinodon variegatus	Endrin	avoidance	Y-maze apparatus	avoided concn. near the 24 hour LC50; fish did not sense and increase in concn.	Hansen, 1969
Cyprinodon variegatus	Dursban	avoidance	Y-maze apparatus	avoided concn. near the 24 hour LC50; fish did not sense and increase in concn.	Hansen, 1969

Species	Toxicant	Behavior measured	Methods	Effects	Authors
Pygosteus pungitius	ethyl alcohol	avoidance	avoidance tube	avoidance	Jones, 1947
Pygosteus pungitius	chloroform	avoidance	avoidance tube	avoidance	Jones, 1947
Pygosteus pungitius	formalin	avoidance	avoidance tube	avoidance	Jones, 1947
Pygosteus pungitius	zinc sulfate	avoidance	avoidance tube	avoidance	Jones, 1947
Pygosteus pungitius	mercuric chloride	avoidance	avoidance tube	no detection	Jones, 1947
Pygosteus pungitius	copper sulfate	avoidance	avoidance tube	avoided high concn., at low concn. fish swim into toxicant and become stupified, interferes with fish's ability to detect other substances	Jones, 1947
Gasterosteus aculeatus	lead nitrate	avoidance	avoidance tube, reverse flow after time interval	avoidance followed by persistant swimming into toxicant and eventually prefer the lead solution	Jones, 1948
Phoxinus phoxinus	lead nitrate	avoidance	avoidance tube	avoidance at all concns.	Jones, 1948
Gasteroseus aculeatus	pH	avoidance	avoidance tube	avoided water more acid than pH 5.6 and more alkaline than pH 11.4; indifferent or positive reaction to pH 5.8 to 11.2	Jones, 1948
Gasteroseus aculeatus	ammonia	avoidance	avoidance tube	attraction to low concn., avoidance of high concn.	Jones, 1948

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Cyprinodon variegatus	2,4-D	avoidance	Y-maze apparatus	avoided concn. near the 24 hr. LC50; fish did not sense an increase in concn.	Hansen, 1969
Cyprinodon variegatus	malathion	avoidance	Y-maze apparatus	did not avoid	Hansen, 1969
Cyprinodon variegatus	Sevin	avoidance	Y-maze apparatus	did not avoid	Hansen, 1969
Gasterosteus aculeatus	low oxygen, 13^{o} C	avoidance	avoidance tube	swim into low oxygen water with no hesitation; remaining in it causes distress and random movement; when random movement takes fish to well-oxygenated water, the fish stops swimming and quickly recovers	Jones, 1947
Gasterosteus aculeatus	low oxygen, 13^{o} C	avoidance	avoidance tube	results similar to above study but reaction time slower	Jones, 1947
Gasterosteus aculeatus	low oxygen, 13^{o} C	avoidance	avoidance tube	fish will usually not swim into low oxygenated water, turns away or swims backwards, may make repeated attempts each time retreating	Jones, 1947
Cymatogaster aggregata	chlorinated effluent	avoidance	avoidance tank, steep gradient	avoided 96 hr LC50 concn. but was attracted to concns. shown to produce sublethal damage	Dinnel, Stober, and DiJulio, 1979

Species	Toxicant	Behavior measured	Methods	Effects	Authors
Coregonus clupeaformis	mercury	avoidance-preference; food seeking behavior	avoidance preference trough, fish treated 1 to 2 weeks with mercury then tested for food preference	mercury interfered with fish detection of food; no change in activity	Kamchen and Hara, 1980
Ictalurus nebulosus *Ictalurus natalis*	cysteine hydrochloride	searching patterns in relation to time and space	large open field tank	fish need no current to locate source	Bardach, Todd, and Crickmer, 1967
Ictalurus natalis	alkyl benzene sulfonate	food seeking and general activity	exposed fish offered food, observation	exposed fish did not detect food; increased activity	Bardach, Fujiya, and Holl, 1965
Cymatogaster aggregata	chlorine	feeding behavior	observation in tanks	fish developed "turned in" eyes, unsuccessful in attempts to pick up food pellet	Thatcher, 1979
Cyprinodon variegatus	kepone	feeding behavior	observation	cessation of feeding	Hansen, *et al.*, 1977
Salvelinus fontinalis	DDT	cold-block temperature of propeller tail reflex	exposed fish tested at different temperatures	DDT altered cold block temperature	Anderson and Peterson, 1969
Gambusia affinis	DDT	salinity preference	fish exposed to DDT for 24 hours, then tested in a salinity gradient	fish at higher DDT concns. selected higher salinities than controls	Hansen, 1972
Gambusia affinis	malathion	salinity preference	fish exposed to malathion for 24 hours and then tested in a salinity gradient	no difference between control and exposed fish	Hansen, 1972

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Salmo salar	fenitrothion	feeding desire	observation	feeding discontinued, digestion slowed or prevented, reguritation, mucous	Symons, 1973
Jordanella floridae	ABS	feeding behavior	observation	approach food with same intensity as controls but spit food out rather than eat it; length of time to finally eat food was related to concn.	Foster, Scheier, and Cairns, 1966
Salmo salar	DDT	feeding behavior	field study, stomach analysis	feeding habits changed after spraying so that salmon ate surviving biota	Elson and Kerswill, 1966
Leiostomus xanthurus	toxaphene	feeding behavior	tank study using natural seawater	at sublethal concn. did not interfere with feeding	Lowe, 1964
Carassius auratus	parathion	locomotor orientation to a food odor	used flowing water with food odor and water flow alone at two different rates in a multiple choice situation, before and after short term treatment with a subacute concn.	total number of entries decreased, angles of orientation changed, average duration of pathways not significantly different	Rand, 1977

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Branchydanio rerio	zinc	pheromonal perception in females	T-maze: control water from one arm; pheromone-containing water from the other	controls selected selected pheromone-containing water; treated fish did not respond to pheromone-containing water	Bloom, Perlmutter, and Seeley, 1978
Lepomis macrochirus	zinc	spawning	observation	fish with ripe eggs only spawned once in 77 days in zinc solution; eggs from other fish died within 3 days at this same concn.	Sparks, Cairns, and Heath, 1972
Pimphales promelas	copper	spawning	observation	prevented spawning	Mount and Stephan, 1969
Pimphales promelas	zinc	spawning	observation; counted number of spawnings	frequently demonstrated typical spawning behavior but eggs were rarely laid; less spawnings at all concns.	Brungs, 1969
Oncorhynchus kisutch	bleached kraft pulpmill effluent	territorial behavior and social hierarchy	measured growth and biochemical changes	may be inhibited by the dark color or the toxicity of the effluent	McLeay and Brown, 1974
Salmo salar	fenitrothion	ability to hold territory	fish exposed during dark periods, then replaced in the stream; observation	treated fish showed a large decline in numbers defending territories after return to stream than did controls; exposed fish tock longer to regain their territories	Symons, 1973

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Salmo salar	copper-zinc	premature downstream returns;	counting fences, tagging	increased downstream returns	Saunders and Sprague, 1967
Moxostoma macrolepidotum Catostomus commersoni Alosa pseudoharengus	chlorinated sewage effluent	migration	stream sampling using nets	fish did not migrate through effluent discharge	Tsai, 1970
Cyprinus carpio Salmo gairdneri Oncorhynchus kisutch Oncorhynchus tshawytscha Salmo trutta Salvelinus fontinalis Salvelinus namaycush	temperature	migration	mark and recapture study using heat sensitive tags	Brown trout showed strongest attraction to discharge waters; spawning is not interrupted by plume, but some fish may spend considerable time in plume interrupting migration schedules	Romberg, *et al.*, 1974
Cyprinus carpio	DDT and phenol	schooling	laboratory observation	schooling present with just phenol but disappears when DDT is added	Besch, *et al.*, 1977
Cyprinus carpio	gasoline	schooling	laboratory observation	schooling present in all tests	Besch, *et al.*, 1977

Species	Toxicant	Behavior measured	Methods	Effects	Authors
Micropterus salmoides *Pimphales promelas*	cadmium	predator-prey interactions	circular observation tanks, total number of prey eaten, prey exposed	prey exposed to toxicant showed increased vulnerability	Sullivan, *et al.*, 1978
Micropterus salmoides *Gambusia affinis*	mercury	predator-prey interactions	square tanks, total number of exposed prey eaten compared to total number of prey	greater numbers of exposed prey than unexposed prey eaten	Kania and O'Hara, 1974
Oncorhynchus kisutch Oncorgynchus *nerka*	temperature	predator-prey avoidance	mean survival time of prey after brief exposure to elevated temperature	elevated temperature decreased survival time	Sylvester, 1972
Gambusia affinis *Micropterus salmoides*	irradiation	consumption of prey	deepwater chamber with small shallow refuge for prey	more prey consumed after 20 days in exposed group than in controls	Goodyear, 1972
Salvelinus fontinalis *Oncorhynchus tshawytscha*	ammonia chloride	consumption of prey	artificial streams, both predator and prey exposed to toxicant, various prey densities used	prey more sensitive to toxicant, consumption rates increased as prey density increased and as toxicant increased	Hedtke and Norris, 1980
Lagodon rhomboides	mirex	predator/prey interaction	grass shrimp were exposed to mirex, then pinfish were introduced as the predator	number of deaths due to predation were higher in treated tanks than controls	Tagatz , 1976

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Salvelinus fontinalis	fenitrothion	swimming velocity	tube within a tube, electrified grid	critical velocity lowered after exposure as a function of concn. and length of rest period prior to exercize	Peterson, 1974
Oncorhynchus nerka	sodium pentachlorophenate	swimming performance	tunnel respirometer increasing velocity steps, terminating at fatigue	swimming performance unaffected at the concn. used	Webb and Brett, 1973
Carrassius auratus	parathion	total number of entries, average duration of entries and orientation angles	circular tank divided into compartments, photocell counts	overall decline in activity, larger angles for some fish	Rand, 1977
Salmo gairdneri Salvelinus fontinalis Oncorhynchus kitsutch	malathion	swimming ability	water tunnel	brook trout and rainbow trout showed a reduced ability to perform	Post and Leasure, 1974
Pimphales promelas	coal-conversion gasifier condensate	swimming ability and unidirection-avoidance	observation	avoided unidirectional flow, dose dependent behavior: rapid swimming, loss of equilibrium, resting on side, erratic swimming	Shultz, Davis, and Dumont, 1978
Salmo gairdneri	copper, pH and hardness	swimming speed	recirculating water tunnel	pH and hardness can modify critical swimming speed	Waiwood and Beamish, 1978
Carrassius auratus	copper	amount of time spent in toxicant, average size of turns	photocell responses triggered by presence of the fish	all increased in the polluted area	Kleerekoper, et al., 1972

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Salmo gairdneri	zinc	swimming ability	fish held in paddlewheel apparatus for two days at 85% maximum velocity	did not affect the toxicity	Herbert and Shurben, 1963
Salmo gairdneri	ammonia	swimming ability	fish held in paddlewheel apparatus for two days at 85% maximum velocity	did not affect the toxicity	Herbert and Shurben, 1963
Phoxinus phoxinus	phenol	swimming ability	observation	loss of equilibrium, wild movements, followed by feeble attempts at swimming	Jones, 1951
Phoxinus phoxinus	para-cresol	swimming ability	observation during avoidance study	even momentary contacts dur-avoidance produced some stress on the fish	Jones, 1951
Phoxinus phoxinus	ortho-cresol	swimming ability	observation during avoidance study	entry into toxicant caused wild rushes up and down, loss of equilibrium, followed by recovery, resulting in eventual loss of coordination	Jones, 1951
Salmo salar	organo-phosphorus insecticides	swimming behavior	static test, observation	loss of balance, rapid and gulping respiratory movements, whole body spasms	Wildish, et al., 1971
Pimphales notatus	endrin	swimming ability	chronic exposure study, observation	jerky movements, increased sensitivity to external stimuli, fish swim around and around, frequently backwards, convulsions, loss of equilibrium	Mount, 1962

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Oncorhynchus nerka	temperature	swimming ability	respirometer tube, flow increased in steps	when acclimated to 15C fish exhibited only a 4% reduction in swimming speed at 10°C and 20°C	Brett, 1964
Oncorhynchus kisutch	Bleached kraft pulp-mill effluent	swimming performance	respirometer tube velocity increased in steps	effluent concn. reduced swimming speed, but after 18 hr. exposure no further reduction appeared	Howard, 1975
Salmo salar	fenitrothion	swimming ability	observation	some fish swam stiffly, fins extended, convulsive flexing developed	Symons, 1973
Cyprinodon variegatus	malathion	swimming ability	eggs hatched in toxicant, observation	hyperactive movements, uncontrolled and uncoordinated, often upside down	Weiss and Weiss, 1976
Pimphales promelas	pulpwood fiber	swimming endurance, and maximum swimming speed	paddle-wheel swimming chamber	swimming endurance reduced, no effect on swimming speed	MacLeod and Smith, 1966
Micropterus salmoides	silver	swimming ability	observation	some body tremors, erratic swimming, open mouth, extended fins, expanded branchiostegals	Coleman and Cearley, 1974
Carassius auratus	phenol	swimming performance	photocell counts	immediate onset of decreased performance followed by normal behavior	Besch *et al.*, 1977
Carassius auratus	DDT	swimming performance	photocell counts	lag time before decreased performance	Besch *et al.*, 1977
Saccobranchus fossilis	Chlordane Ekalaux Ekatin Sumithion	swimming ability	observation	erratic swimming, convulsions, swimming to surface, gulping air	Verma, Bansal, Dalela, 1978

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Salmo gairdneri	diquat and simazine	frequency of positive rheotaxis, negative rheotaxis, and no-response; Swimming speed	water current simulated with background, optomotor tank, observation	elevated frequencies of no-response and decreased swimming speed	Dodson and Mayfield, 1979
Notropis cornutus	temperature and light	frequency of positive rheotaxis	water current simulated with background, optomotor tank, observation	temperature more important than photoperiod in producing greatest frequency of upstream movements	Dodson and Young, 1977
Leuciscus rutilus	methyl-mercuric hydroxide	reduced ability of the fish to maintain upright position	water enters tube and rotates around longitudinal axis	sublethal poisoning decreases ability of fish to compensate for the torque	Lindahl and Schwanbom, 1971
Gaddus morrhua	polluted water high in mercury	rheotaxis	rotary-flow apparatus to test fitness of fish	decreased time to rotate fish	Lindahl, Olofsson, Schwanbon, 1977
Carassius auratus	temperature	loss of equilibrium (LE)	held for time period at specific current velocity	50% LE is related to acclimation level and is highest at highest acclimation; fish losing equilibrium settle to bottom of tanks	Schneider, *et al.*, 1974
Salmo gairdneri	temperature	loss of equilibrium (LE)	held for time period at specific current velocity	exercized fish lose equilibrium more readity than rested fish, smaller fish most vulnerable	Schneider, *et al.*, 1977
Salvelinus fontinalis	cyanide	swimming ability	used rotating circular chamber with inner core, velocity increased in steps	reduced ability to maintain swimming; exposed fish were reluctant to swim when first placed into tanks	Neil, 1957

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Cyprinodon variegatus	kepone	swimming ability	observation	uncoordinated swimming	Hansen, *et al.*, 1977
Micropterus salmoides	total residual chlorine	swimming ability	observation during sublethal exposure	changes in behavior occurred in the following sequence: increases in rates of swimming, opercular activity, and coughing; reduced swimming activity near the surface; rapid swimming with thrashing; lethargic swimming, frequent collisions with the tank walls and other fish; bobbing with dorsal surface of head exposed at water surface; resting on tank bottom with some erratic swimming; turning over	Larson and Schlesinger, 1977
Pimphales notatus	endrin	swimming ability	paddle-wheel, rounded corners on tank	little effect on swimming ability against current	Mount, 1962
Lepomis macrochirus	TNT manufacturer's waste	total activity	fed fish vs unfed fish exposed to toxicant; activity detected by photocells	fed fish had increased sensitivity to toxicant	Prather, 1975
Carassius auratus	temperature and toxaphene	total movement	66 hour exposure to toxaphene, then temperature progressively increased	toxaphene increased the heat sensitivity of the fish	Engineering-Science, Inc., 1964

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Lepomis macrochirus	zinc	abnormal movement patterns	light beam interruption, photocells	abnormal movement detected	Cairns, Sparks, and Waller, 1973
Lepomis macrochirus	zinc	abnormal movement patterns	light beam interruption, photocells	abnormal movement prior to death	Waller and Cairns, 1972
Micropterus salmoides	mercury	abnormal movement patterns	photoelectric sensors	increased activity with toxicant	Morgan, 1979
Micropterus salmoides *Lepomis macrochirus*	cadmium	abnormal movement patterns	observation	exhibited erratic, uncoordinated swimming, muscle spasms, and convulsions; loss of equilibrium, periods of quiescence and paralysis	Cearley and Coleman, 1974
Gambusia affinis	chlorpromazine	surfacing and sinking	fish placed in horizontal galss tubes, observation	surfacing increases with increasing concn.	Avivi and Chari-Britron, 1970
Carassius auratus	zinc	surfacing activity	photocell counts	surfacing occurred at the 96 hour LC50	Cairns, Sparks, and Waller, 1970
Lepomis macrochirus	methyl parathion, Akton, Dyrene, phosalone	excitability to outside disturbances	observation	the magnitude of excitability in decreasing order as listed under "Toxicant"	McCann and Jasper, 1972
Lepomis macrochirus	Dylox, Neguvon, demeton	excitability to outside disturbances	observation	no different than controls	McCann and Jasper, 1972
Salmo salar	DDT	abnormal behavior	observation, field study with cages suspended in water	beaching resulting from extreme convulsive activity, swimming to surface	Kerswill and Edwards, 1967

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Salvelinus fontinalis	Chlorine	activity	activity measured by probe	activity increased at high concn. initially, fish swam to surface, coughing. Later movements spasmotic, loss of equilibrium	Dandy, 1972
Salmo trutta	sulfite pulp and paper mill effluent	activity	live boxes placed in river below mill discharge, observation	tendency to swim near surface, sluggish movements, would lie on their sides for several hours before death	Grande, 1964
Carrassius auratus	toxaphene	total movement	Conditioned avoidance response apparatus (CARA)	increased general movement after 96 hour exposure	Warner, Peterson and Borgman, 1966
Alburnus alburnus	chlorinated parafins	activity	observation in tanks	sluggish movements, absence of shoaling behavior, and abnormal vertical postures	Bengtsson, et al., 1979
Lepomis macrochirus	heptachlor	activity	observation in pools	general nervousness, hyper-sensitivity to light or move-ment followed by rapid, erratic swimming frequently at surface, later became listless, display-ing progressive loss of equili-brium followed by death	Andrews, Van Vallin, and Stebbings, 1966
Salmo salar	fenitrothion	upstream and downstream movement	observation	no different than controls	Symons, 1973
Carassius auratus	zinc	abnormal movement patterns	light beam photocell counts	increased movement toward surface of tank, altered light-dark movement patterns in some fish	Shirer, Cairns, and Waller, 1968

Species	Toxicant	Behavior measured	Methods	Effects	Authors
Salvelinus fontinalis *Salmo gairdneri* *Salmo clarki* *Oncorhynchus kisutch*	carbaryl; malathion	abnormal behavior	static test in jars, observation	fish became irritable and moved sluggishly when the jar was tapped; muscular spasms and convulsions, loss of equilibrium	Post and Schroeder, 1971
Carassius auratus	temperature	spontaneous activity and total movement	fish acclimated to to 12°C and 25°C then subjected to progressively elevated temperatures, CARA	Marked difference in activity	Engineering-Science, Inc., 1964

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Carrassius auratus	toxaphene	learned avoidance	used conditioned avoidance response apparatus, light followed by shock	exposed fish learned response significantly faster than controls	Warner, Peterson, and Borgman, 1966
Carassius auratus	toxaphene	ability to retain a learned response	light only used one week after light-shock training	all fish responded to light only, memory was not impaired	Warner, Peterson, and Borgman, 1966
Carassius auratus	toxaphene	ability to unlearn a response	light-shock reversed on previously taught fish	exposed fish learned reverse significantly faster	Warner, Peterson, and Borgman, 1966
Carrassius auratus	toxaphene	habituation to light only stimulus	exposed to light stimulus only	all fish showed increased response then reached a plateau	Warner, Peterson, and Borgman, 1966
Salvelinus fontinalis	DDT	conditioned learning	Light-Shock, response propellerlike movement of the tail	more than half of the exposed fish could not be conditioned at all	Anderson and Prins, 1970
Salvelinus fontinalis	DDT	conditioned learning	light-shock, response avoidance	fish could be trained after exposure; fish rose to the surface in response to the light but prior to the shock	Jackson, Anderson, and Gardner, 1970
Salmo salar	Sumithion Abate DDT methoxychlor	learning ability and learning improvement on second conditioning	shuttlebox conditioning apparatus	learning capabilities altered if dose is sufficient, but all alterations are not permanent	Hatfield and Johansen, 1972
Carassius auratus	lead	ability of fish to recall prior training	fish taught to escape in response to light, then exposed to lead	lead interfered with performance of memory	Weir, *et al.*, 1970 as cited in LaPorte and Talbott, 1977

Species	Toxicant	Behavior measured	Methods	Effects	Authors
Salvelinus fontinalis	DDT	visual conditioned avoidance, light-shock	light followed by shock	naive controls took about thirty trials to become conditioned, not one of the naive DDT-treated fish became conditioned	Anderson and Peterson, 1969
Salvelinus fontinalis	DDT	visual conditioned avoidance, light-shock	light followed by shock, trained fish exposed to DDT, then retrained	controls retrained in less trials than exposed fish, but exposed fish did retrain	Anderson and Peterson, 1969
Carassius auratus	toxaphene	learning	fish exposed for 264 hours to low level toxaphene; tested in CARA system; light-shock	increased aversion to light	Engineering-Science, Inc., 1964
Carassius auratus	Sulfite pulp waste liquor	learning	fish exposed for 30 days to low levels	no effect	Engineering-Science Inc., 1964
Carassius auratus Cichlasoma nigrofasciatum Phoxinus phoxinus	temper-ature	ability to retain a learned discrimin-ation when temperature is reduced	trained fish sub-jected to lower temperature, then retested; food-rewarded	low temperature during retention period caused an increase in retention in C. carassius and C. nigrofasciatum but not in P. phoxinus; memory retention related to metabolism	Stascheit, 1979
Salmo salar	DDT	shuttlebox learning	light-shock; treated fish tested after 24 hour exposure	enhanced learning	Hatfield, 1970

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Salmo salar	methoxychlor	shuttlebox learning	light-shock; treated fish tested after 24 hour exposure	no effect on learning	Hatfield, 1970
Salmo salar	Sumithion	shuttlebox learning	light-shock; treated fish tested after 24 hour exposure	inhibited learning	Hatfield, 1970
Salmo salar	Abate	shuttlebox learning	light-shock; treated fish tested after 24 hour exposure	retarded learning	Hatfield, 1970
Salmo salar	DDT	retrained shuttlebox learning	fish trained, treated, then retrained	ability to retrain less than controls	Hatfield, 1970
Salmo salar	Sumithion and Abate	retrained shuttlebox learning	fish exposed to toxicant, allowed to recover for 7 days, then trained	after recovery, fish learned as rapidly as controls	Hatfield, 1970

Species	Toxicant	Behavior Measured	Methods	Effects	Authors
Salmo salar	coal dust, wood pulp, zinc sulfate	coughing frequency, amplitude of cough	closed respirometer, electromyograms, pressure manometers	cough frequency related to particle size, wood pulp coal dust zinc sulfate	Hughes, 1975
Lepomis macrochirus	treated complex effluents	cough response	electrode chamber, impulses recorded on strip chart	increased coughing as effluent concn. increased	Carlson and Drummond, 1978
Jordanella floridae	alkyl benzene sulfonate	duration and sequences of behavior	viewing ports, event strip recorder	increased gasping, reduced chasing	Foster, Cairns, and Kaestler, 1969
Oncorhynchus kisutch	DDT	cough frequency	tube inserted into buccal cavity to measure pressure	cough frequency related to DDT concn. and exposure time	Schaumberg, Howard, and Walden, 1967
Oncorhynchus kisutch	Kraft pulp-mill effluent	cough frequency	tube inserted into buccal cavity to measure pressure	cough frequency related to effluent concn. and exposure time	Schaumberg, Howard, and Walden, 1967
Lepomis macrochirus	sodium hypo-chlorite	coughing	electrodes in tank, recorded coughing on strip chart and simultaneously filmed fish and chart	increased coughing at high concn.	Gruber, *et al*. 1978
Pimphales promelas	pulp-wood fiber	gill cleaning reflexes	observation	swimming close to surface, increased gasping, cleaning reflexes related to concn. of fiber	MacLeod and Smith, 1966
Lepomis macrochirus	zinc	coughing	pressure changes in buccal and opercular cavity, cannulas	mean cough frequency increased, coughing peaked 2-3 hours after introducing toxicant	Sparks, *et al*., 1972

APPENDIX B.

Table 1. Water Chemistry Analysis. (All values in mg/l unless otherwise indicated.)

PARAMETER	CONCENTRATION
Hardness as CaCO3	47
Alkalinity	33
Iron as Fe	Ø.1Ø
Chloride as Cl	3.8
pH (pH units)	7.Ø
Turbidity (turbidity units)	Ø.1Ø
Conductivity (umhos/cm)	124
Sulfate	1Ø
Ammonia nitrogen	Ø.Ø2
Kjeldahl nitrogen	Ø.Ø6
Nitrates	Ø.25
Nitrites	Ø.ØØ1
Total phosphorus	Ø.ØØ2
Copper	Ø.Ø5Ø
Nickel	Ø.ØØ6
Lead	Ø.ØØ1
Zinc	Ø.ØØ9

Table 2. Mean Temperature, pH, and Dissolved Oxygen during the 1st run (±standard deviation).

Nominal EtOH conc. (g/liter)	Temperature (°C)	pH (pH units)	DO (mg/liter)
0.0	28.1±Ø.7	7.2±Ø.2	7.5
0.0	27.9±Ø.8	7.1±Ø.1	7.4
0.0	27.9±1.1	7.1±Ø.9	- **
0.0	27.5±Ø.8	7.5±Ø.3	7.3±Ø.7
0.5	28.1±Ø.7	7.2±Ø.2	7.4
0.5	28.Ø±Ø.7	7.2±Ø.1	7.4
0.5	27.9±1.Ø	7.1±Ø.9	- **
0.5	27.7±Ø.8	7.5±Ø.3	7.4±Ø.7
1.0	28.Ø±Ø.7	7.1±Ø.3	7.3
1.0	27.9±Ø.7	7.2±Ø.1	7.2
1.0	28.1±Ø.8	6.9±Ø.7	- **
1.0	27.6±Ø.6	7.4±Ø.3	7.1±1.Ø
1.5	28.2±Ø.7	7.1±Ø.2	7.6
1.5	28.1±Ø.7	7.2±Ø.1	7.4
1.5	28.2±Ø.7	7.1±Ø.8	- **
1.5	27.6±Ø.8	7.4±Ø.3	7.4±Ø.7
2.0	28.2±Ø.7	7.1±Ø.3	7.6
2.0	28.1±Ø.8	7.1±Ø.2	7.1
2.0	28.1±1.4	7.Ø±Ø.7	- **
2.0	27.7±Ø.8	7.4±Ø.3	7.5±Ø.8
3.0	28.3±Ø.6	7.1±Ø.2	7.7
3.0	28.Ø±Ø.7	7.1±Ø.2	7.5
3.0	28.1±Ø.6	7.1±Ø.2	- **
3.0	27.8±Ø.7	7.4±Ø.3	7.4±Ø.6

**No data collected

Table 3. Test tank water chemistry results during the 2nd run (±standard deviation).

Nominal EtOH conc. (g/liter)	Temperature (°C)	pH (pH units)	DO (mg/liter)
0.0	27.7±Ø.7	7.4±Ø.1	7.3±Ø.1
0.0	27.7±Ø.7	7.3±Ø.Ø	7.3±Ø.4
0.0	27.2±Ø.7	7.5±Ø.1	7.5±Ø.3
0.0	27.2±Ø.7	7.5±Ø.Ø	7.7±Ø.4
0.5	27.7±Ø.7	7.5±Ø.1	7.4±Ø.3
0.5	27.6±Ø.7	7.5±Ø.Ø	7.5±Ø.4
0.5	27.1±Ø.7	7.6±Ø.1	7.4±Ø.3
0.5	27.3±Ø.6	7.5±Ø.Ø	7.7±Ø.4
1.0	27.7±Ø.7	7.3±Ø.Ø	7.3±Ø.3
1.0	27.7±Ø.7	7.3±Ø.1	7.3±Ø.3
1.0	27.4±Ø.6	7.3±Ø.1	7.2±Ø.3
1.0	27.3±Ø.8	7.5±Ø.Ø	7.7±Ø.4
1.5	27.8±Ø.7	7.3±Ø.Ø	7.3±Ø.3
1.5	27.8±Ø.7	7.3±Ø.1	7.4±Ø.4
1.5	27.3±Ø.3	7.4±Ø.Ø	7.4±Ø.3
1.5	27.2±Ø.7	7.4±Ø.Ø	7.7±Ø.4
2.0	27.8±Ø.7	7.3±Ø.Ø	7.4±Ø.3
2.0	27.9±Ø.7	7.3±Ø.Ø	7.4±Ø.4
2.0	27.3±Ø.6	7.5±Ø.1	7.5±Ø.4
2.0	27.3±Ø.7	7.4±Ø.1	7.6±Ø.6
3.0	27.8±Ø.7	7.3±Ø.Ø	7.3±Ø.3
3.0	27.7±Ø.7	7.4±Ø.1	7.5±Ø.4
3.0	27.3±Ø.7	7.4±Ø.2	7.3±Ø.4
3.0	27.4±Ø.7	7.4±Ø.Ø	7.6±Ø.4

Table 4. Mean ethanol concentration (±standard deviation).

	1st RUN	2nd RUN
Control	Ø.Ø±(Ø.Ø)	Ø.Ø ±(Ø.Ø)
Tanks 1	Ø.4±(Ø.Ø)	Ø.5±(Ø.1)
Tanks 2	1.Ø±(Ø.Ø)	1.Ø±(Ø.1)
Tanks 3	1.4±(Ø.Ø)	1.5±(Ø.1)
Tanks 4	2.Ø±(Ø.1)	2.1±(Ø.1)
Tanks 5	3.Ø±(Ø.5)	2.9±(Ø.1)

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