CHRONIC EFFECTS OF METHYLMERCURY ON THE REPRODUCTION
OF THE TELEOST FISH, ORYZIAS LATIPES

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
THE FACULTY OF GRADUATE STUDIES
Department of Zoology

We accept this thesis as conforming to the
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THE UNIVERSITY OF BRITISH COLUMBIA
August, 1977
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ABSTRACT

This study evaluates the toxicity, accumulation, chronic effects and mode of action of methylmercury on the reproduction of the teleost fish, Oryzias latipes.

The median lethal concentration, 96h-LC50, for adult Oryzias was $88 + 9.8 \mu g \text{CH}_3\text{Hg}^+/l$ as determined in a static system. Residue analysis by gas chromatography showed that fish exposed to 43 to 1000 $\mu g \text{CH}_3\text{Hg}^+/l$ had tissue levels below 40 $\mu g \text{CH}_3\text{Hg}^+/g$ while fish exposed to more than 1000 $\mu g \text{CH}_3\text{Hg}^+/l$ accumulated methylmercury steadily and reached levels as high as 408.1 $\mu g \text{CH}_3\text{Hg}^+/g$. Death seems to occur when tissue level reaches 25 $\mu g \text{CH}_3\text{Hg}^+/g$. Studies on long-term exposure to 0.0, 4.3, 10.7 and 21.5 $\mu g/l$ of methylmercury in a flow-through system confirmed this observation.

Four-hour exposure of 8.5 and 42.9 $\mu g/l$ of methylmercury on alternate days during the fish's normal oviposition period resulted in inhibition of oviposition. This observation occurred only on days when fish were exposed to methylmercury but not on days when fish were returned to clean water. However, at a concentration of 85 $\mu g \text{CH}_3\text{Hg}^+/l$, complete inhibition was observed even on days when fish were returned to clean water. High rates of accumulation with low rates of excretion of methylmercury were suggested explanations for these observations.

Six-week exposure to 4.3, 10.7 and 21.5 $\mu g/l$ of methylmercury resulted in inhibition of spawning. This inhibition was directly related to the log of exposure concentrations. At the end of six weeks, both male and female gonads showed reduction in size; the females were more sensitive. However, hatchability of the spawned eggs was not affected by the exposure.
Juvenile fish were very sensitive to methylmercury. After two weeks of exposure, one-week old juvenile exposed to 0.0, 4.3, 10.7 and 21.5 µg CH₃Hg⁺/l had mortality rate of 2.2%, 54.3%, 64.9% and 99.4% respectively.

Synthetic LH-RH, at concentrations of 100 and 1000 ng/g body weight, was effective in stimulating ovarian development in Oryzias. This shows that the LH-RH (synthesis based on structure of porcine LH-RH) has biological activity in Oryzias.

When exposed to methylmercury, spawning activities were inhibited. LH injections were able to restore the spawning activities inhibited by the methylmercury treatment, but not LH-RH. However, histology of the pituitary gland showed stimulation of gonadotropic cells by LH-RH injection with no restoration of spawning activities. This suggests that methylmercury may be blocking the release of gonadotropin.

In vitro ovulation was affected by previous exposure to methylmercury. Addition of methylmercury directly to the incubation medium further reduced the percentage of in vitro ovulation in the previously treated fish. Using oocytes from untreated donor fish, the percent inhibition of in vitro ovulation by methylmercury was directly related to the log of doses used. A possible bioassay with in vitro ovulation was suggested. Among the various steroids used (progesterone, cortisone, estradiol and testosterone), cortisone was the only steroid effective in restoring in vitro ovulation blocked by the presence of methylmercury in the incubation medium.

Ecological implications of these findings are discussed.
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ACKNOWLEDGEMENTS

I would like to extend my sincere thanks and indebtedness to my supervisor, Dr. W.S. Hoar, for his interest, advice and encouragement throughout this study.

Special thanks are also extended to the members of my doctoral committee: Drs. P. Larkin, T. Northcote and H. Kasinsky of the University of British Columbia, Vancouver, Dr. P. Oloffs of Simon Fraser University, Burnaby and Dr. J. Thompson of Marine Sciences Branch, Environment Canada, Victoria, for their generous assistance and for reading the thesis. The use of Dr. J. Thompson's laboratory for residue analysis is deeply appreciated.

I am obligated to the visiting research fellows: Dr. T.J. Lam, Dr. S. Pandey and especially Dr. Y. Nagahama for many stimulating discussions. Helpful advice and encouragement from fellow graduate students: Dr. K. Khoo, Dr. N. Stacey, Dr. W. Marshall, Mr. J.-G. Godin, Mr. R. Neuman and Ms. M. Hurlburt, throughout the entire study are also deeply appreciated.

Financial support for this research was from the National Research Council of Canada through grants-in-aid to Dr. W.S. Hoar and a postgraduate scholarship from the National Research Council of Canada and a B.C. Summer Fellowship to myself.

Last but not least, I would like to express my deepest gratitude to my wife, Bernadette, for her constant encouragement and patience throughout this study.
GENERAL INTRODUCTION

Since the outbreak of Minamata Disease in Japan, methylmercury has been recognized as one of the most hazardous environmental pollutants (Katsuki et al., 1957, Takuomi, 1961; Takeuchi et al., 1962, Okinaka et al., 1964). The sources of many contaminating incidences have been well documented (Fimreite 1970, Aaronson, 1971; Goldwater, 1971; Nelson 1971; Saha 1972) and the cycling of mercury through the environment has been reviewed (Gavis and Ferguson 1972).

Poisoning by methylmercury has been demonstrated at all levels in phylogeny (Skerfving 1972; Clarkson 1972; Katz 1972). Since the Minamata episode, studies have shown that fish are clearly sensitive to methylmercury (see Literature Review). Accumulation of methylmercury in fish is very high, sometimes as high as 5000 times over the environment (Johnels et al., 1967), while excretion is very slow (Burrows and Krenkell 1973). This high accumulation in fish probably affects the physiological processes of the animal. However, some information does exist on the long term, chronic effects of methylmercury in fish; but information of its effects on fish reproduction is particularly sparse (Sprague 1971).

The present work investigates the toxicity, accumulation of methylmercury under long and short term exposures and the effects of low concentrations of methylmercury on oviposition, gonadal development, spawning activity, hatchability of eggs and survival of hatchlings in Oryzias latipes. Since methylmercury has been shown to be a bioaccumulative toxicant, correlation will be made between the amount of accumulated methylmercury in the fish and its effects on the reproductive processes. The latter part of this study will examine the mode of action of methylmercury on the
reproductive physiology in *Oryzias*. The possible effects of methylmercury on the endocrine system concerned with reproduction will be investigated. An attempt will be made to determine whether a blockage of hormone activity by methylmercury occurs in the hypothalamic–hypophyseal–gonadal axis. This study is not only of academic interest but might also suggest a possible remedy for fish that may be affected similarly in nature. *Oryzias* is a much hardier fish than most other fishes that are of commercial value, like the salmon. However, this fish is a convenient animal for laboratory studies on reproduction. It is hoped that the present study with *Oryzias* will suggest some possible effects that methylmercury may have on commercially important fishes.

*Oryzias* offers several advantages as an experimental animal. Its small size (30 mm adult) allows handling of large numbers in relatively limited spaces. By manipulating temperature and photoperiod, reproductively mature individuals can be obtained throughout the year. Spawning, once induced, occurs daily for 4 to 6 weeks. Since *Oryzias* is oviparous, the number of spawnings, number of spawned eggs, hatchability of the eggs and mortality of the hatchlings can be easily quantified. The spawning habits of this fish are also ideal for the study. Females ovulate daily between 0100 and 0400 hours and oviposition occurs about two hours after daybreak; and the eggs hatch in about two weeks. This not only allows studies of the effects of methylmercury on the reproductive physiology of the adults but also, if desired, on the second generation. Finally, there is much to be said for working with an animal which has been the subject of so much behavioural, physiological, genetical and endocrinological research.
Levels of mercury in natural waters are generally low except in contaminated areas (Voege 1971, Gavis and Ferguson 1972, Fitzgerald and Lyons 1972). In fish, mercury may reach levels above 25 μg/g when collected from contaminated areas while specimens from uncontaminated areas are generally below 1.0 μg/g, but frequently above 0.2 μg/g, the maximum natural background concentration (Fimreite and Reynolds 1973). It appears that most of the mercury in fish is present as methylmercury (Westby 1969). The source of methylmercury has not been well determined, but it has been observed that microorganisms, especially those from sediments, can methylate mercury (Fagerstrom and Jernelov 1971; Jensen and Jernelov 1969; Wood et al., 1968). The methylmercury may then be accumulated to a 'harmful' concentration in the higher trophic levels, e.g., fish, via the food chain (Jernelov and Lann 1971).

A maximum permissible level of mercury in fish of 0.5 μg/g was established by the U.S. Food and Drug Administration in 1970. At the same time people were cautioned that the human hazard depends largely on the quantity of contaminated fish eaten (Katz 1972).

In mammals, tissue accumulation, distribution and excretion of methylmercury have been documented (Iverson et al., 1973; 1974; Casterline Jr. and Williams 1972; Skerfving 1974; Ikeda 1973). Effects of methylmercury in humans are well known. Methylmercury accumulates in the central nervous system. Severe poisoning results in gross constriction of the visual field, cerebellar ataxia, dysarthria, sensory changes and impairment of hearing. Memory and intelligence are unaffected. These symptoms are generally irreversible but the motor disturbances may improve after
rehabilitation. Methylmercury has also the unique property of crossing the blood-brain barrier and the placental-foetal barrier (Clarkson 1972). Skerfving et al. (1970) observed chromosome breakage in leukocytes from humans with elevated blood mercury concentrations. Experimental studies with other mammals have shown that methylmercury interacts with erythrocytes (White and Rothstein 1973; Mykkanen and Ganther 1974), damages liver (Chang and Yamaguchi 1974; Desnoyers and Chang 1975a, b; Lucier et al., 1972), kidney (Hirsch 1971, Chang and Sprecher 1976; Fowler et al., 1974) and the central nervous system (Kim 1971, Herigstad et al., 1972, Albanus et al., 1972; Ikeda et al., 1973; Diamond and Sleight 1972; Barthoud et al., 1976; Berlin et al., 1975); it also affects behaviour (Spyker et al., 1972; Hughes 1975) and reproduction (Casterline Jr. and Williams 1972, Khera 1973; Skerfving 1974).

In birds, methylmercury poisoning impaired reproduction in the hen (Tejning 1967) and mallard duck (Heinz 1974), and the hatchability of hen eggs (Tejning 1967). Unlike mercuric chloride (Stoewsand et al., 1971), eggshell thinning was not observed in the methylmercury treated ring dove, American kestrel (Peakdall and Liner 1972) and mallard duck (Heinz 1974). However, methylmercury was shown to be both embro-lethal and teratogenic to early chick embryogenesis (Gilani 1975) and also to affect mallard duckling behaviour (Heinz 1975).

Very little work has been done on the effects of toxicants on fish reproduction (Sprague 1971). Crandall and Goodnight (1962) observed a delay in sexual maturity in the guppy by lead and zinc. Inhibition of spawning by copper was detected in fathead minnows (Mount 1968). Also, near complete elimination of egg production by zinc was found in fathead
minnows (Brungs 1969). Resistance of fish eggs and fry to toxicants, e.g., detergents, zinc, copper and malathion, has been studied and it seems that eggs are less sensitive to toxicants than fry (Sprague 1971).

Methylmercury uptake, distribution and excretion rate have been well studied in fish because of its potential hazard to the fish eating population (Jarvenpää et al., 1970; Olson and Fromm 1973; Miettinen et al., 1969; Olson et al., 1973; Freeman and Horne 1973; Rucher and Amend 1969; Burrows 1973). Other studies of methylmercury in fish include its toxicity (Akiyama 1970), its effects on gill metabolism (O'Connor and Fromm 1975; Kendall 1972), blood parameters and erythrocytes (O'Connor and Fromm 1975; Olson and Fromm 1973), kidney (Kendall 1972; Matsumura et al., 1975) and fertilization of eggs (McIntyre 1973). Generally, studies on effects of toxicants on fish reproduction has been sparse (Sprague 1971). For organic mercury, the work of Kihlström and his co-workers remains a unique piece of information on the sublethal effects of phenylmercury on fish reproduction (Kihlström, Lundberg and Hulth 1972; Kihlström and Hulth 1972).
SECTION I

THE TOXICITY AND ACCUMULATION OF METHYLMERCURY
INTRODUCTION

Mercury and many of its compounds have long been known to be highly toxic to both plants and animals. The main sources of mercury contamination in the environment are from industries such as mining, pulp and paper, the manufacture and use of fungicide, plastic and chlor-alkali. Incidences of mercury poisoning and sources of mercury contamination have been well documented (Fimreite 1970; Aaronson 1971; Goldwater 1971; Nelson 1971; Saha 1972). The cycling of mercury through the environment has been reviewed (Gavis and Ferguson 1972).

Though occurring in several forms, mercury in fish is mainly present as methylmercury (Westöö 1969). This is because methylmercury is readily absorbed (ability to cross cell membranes), is relatively resistant to biotransformation, has a low clearance rate and a strong affinity for protein (Clarkson 1972). The source of methylmercury has not been well determined, but it has been observed that microorganisms, especially those in sediments, can methylate mercury (Fagerstrom and Jernelöv 1971, Jensen and Jernelöv 1964; Wood et al., 1968). This methylmercury may then be accumulated via the food chain to a harmful concentration in the higher trophic levels, e.g., fish and ultimately man (Jernelöv and Lann 1971). Thus, it is worthwhile to investigate the possible effects, both lethal and sub-lethal, of methylmercury in fish.

In this section, the toxicity of methylmercury and its accumulation in the tissues of the adult freshwater teleost Oryzias latipes are investigated.
MATERIALS AND METHODS

Maintenance of Fish

Adult medaka, *Oryzias latipes* were obtained via air-shipment from Nagoya Aquarium Company, Nagoya City, Japan. Following arrival, the fish were distributed into 35-litre capacity plastic tanks equipped with side filters and aerated with compressed air. Dechlorinated Vancouver City water was used (hardness 2-10 mg CaCO\(_3\)/l, pH 6.8-7.0). Temperature of the water was maintained at 13 ± 1°C by standing the tanks in a trough of running cold water (10 ± 1°C). These fish were exposed to short photoperiods of eight hours light (0900 hr to 1700 hr) alternating with sixteen hours darkness (8L/16D). Light was provided by fluorescent lamps suspended 60 cm above the tanks and the photoperiod regulated by time clocks (Intermatic, Marr Electric Ltd., Toronto). Fish were fed daily *ad libitum* with frozen brine shrimp. All imported fish were subjected to the above conditions for at least a month before they were slowly adapted to different conditions depending on the various experiments.

Gravid Fish

After this initial treatment with low temperature and short photoperiod for a month, a group of medaka was slowly adapted to and kept at a warm temperature (23 ± 1°C) and under long photoperiod (16L/8D). After about three weeks of such treatment most of the fish become gravid and spawning occurs in the fourth week. The cold temperature and short photoperiod pretreatment allows the fish to pass out of its refractory stage while the warm temperature and long photoperiod induces gonadal development (Yoshioka 1962, 1963). Fish treated in this manner were used in acute toxicity tests and studies of oviposition.
Sexually regressed fish

For studying gonadal development, fish should be sexually regressed at the beginning of the experiment. Yoshioka (1962, 1963) observed that treatment with low temperature (4-15°C) and short photoperiod (8L/16D) was effective in inducing *Oryzias latipes* to a sexually regressed state. After this treatment, fish would have passed its refractory period and can easily be brought to spawning state with warm temperature (18-23°C) and long photoperiod (16L/8D). In the present study, treatment of low temperature (13 ± 1°C) and short photoperiod (8L/16D) for two months was used to ensure that fish were sexually regressed. Fish in the regressed state were used in all long term exposure studies.

Acute toxicity tests

Toxicity tests were static, conducted in 18-litre capacity glass aquaria with 10 fish per tank. The fish ranged from 2.6 to 3.0 cm in length and weighed 0.25 to 0.35 g; this resulted in a loading density of 6 litres test solution per gram of fish. Test water was dechlorinated Vancouver City water warmed to 23 ± 1°C. Fish were acclimated to the test conditions for three days and fed daily with frozen brine shrimp. The test period began on Day 4 when methylmercuric chloride was first added to the tanks and feeding discontinued. Observations were continued for seven days and death was defined as a permanent cessation of opercular movement. All fish were frozen immediately for residue analysis.

A stock solution of methylmercuric chloride (1.71 g CH$_3$Hg$^+$ /l) was prepared by dissolving the proper amount of chemical in distilled water. Appropriate volumes of this stock solution were added to the different aquaria to achieve the desired concentrations. To maintain proper concen-
tration in the tanks, about 90% of the solution in each tank was renewed daily.

At each concentration, the cumulative percentage mortality was plotted on a probit scale against the survival time in hours on a logarithmic scale, and the median lethal time (LT50) and its 95 percent confidence limits estimated (Litchfield, 1949). From the eye-fitted regression of log LT50 against log concentration (Litchfield 1949) and by plotting the partial mortality at 96 hr (Litchfield and Wilcoxon 1949), the concentration required to produce 50 percent mortality (LC50) and its 95% confidence limits at 96 hr were calculated.

Long-term exposure

Adult medaka weighing 0.25–0.35 g were divided into four groups with 35 fish per group. Each group was exposed in a flow-through system to one of four different concentrations of methylmercury (0, 4.3, 10.7 and 21.5 µg CH$_3$Hg$^+$ /l) at 23 ± 1°C under 16L:8D light-dark regime. The loading density in the tanks was approximately one litre of test solution per gram fish and the approximate time for 99% replacement of test solution was 3.5 hours. Fish were fed daily with frozen brine shrimp. Mortality was checked each day and fish were sampled at 0, 2, 4 and 6 weeks for methylmercury residue determination.

The flow-through system is illustrated in Fig. 1. Dechlorinated Vancouver City water was first heated to approximately 18°C by stainless steel coils carrying flowing hot water. The 18°C water was then pumped to a head tank and further heated to and maintained at 23 ± 1°C with two 1000-watt stainless steel immersion heaters. The warmed water was then delivered at a constant rate (regulated by flowmeters; Jencons, England)
Figure 1. Diagram of the experimental apparatus used for continuous exposure of fish to low concentrations of methylmercury, showing the pattern of water flow, heating system, one Mariotte bottle for the metering of toxicant and one test-tank.
to mixing buckets situated on top of the test-tanks. The methylmercury chloride solutions were metered into the mixing buckets by Mariotte bottles (Leduc 1966). The mixed solution of water and methylmercury entered the different test-tanks by gravity. The final concentrations in the test-tanks were determined by the concentrations of methylmercuric chloride solution in the Mariotte bottles. Test-tanks used were of translucent, white, polyethylene equipped with plastic outlets (2 cm I.D.) on one side of the tank positioned to maintain a water volume of about 9 litres. The outlets were fitted with fibreglass screens to prevent fish from escaping. There were four of the above set-ups for different methylmercury chloride concentrations of 0.0, 4.3, 10.7 and 21.5 μg CH₃Hg⁺/l.

Residue analysis

Extraction and purification

This is a modification of the extraction method developed by Newsome in 1971. Since weight of individual fish was small (0.2 to 0.3 g per fish), fish samples were pooled (approximately 1.5 to 2.5 g per determination) and homogenized in a Sorval homogenizer for 15 min with a solution of 1 N hydrobromic acid and 2.1 N potassium bromide (40 ml). The homogenate was filtered through glass wool under gravity on a Buchner funnel and washed with a further portion (40 ml) of hydrobromic acid-potassium bromide solution. The filtrates were pooled and extracted three times (3 x 50 ml) with benzene (nanograde, Caledon). The benzene layers were combined and a portion (100 ml) of this combined benzene layer was then extracted twice (2 x 6 ml) with freshly prepared cysteine acetate solu-
tion (2.0 g cysteine hydro-chloride monhydrate, 4.0 g sodium acetate and 12.5 g anhydrous sodium sulphate in 100 ml distilled water). An aliquot (8 ml) of the combined cysteine layer was recovered and acidified with 1 ml of 48% hydrobromic acid. The mercury was then extracted with benzene (either 5 ml or 9 ml) and the benzene extract subjected to analysis by gas chromatography.

Gas chromatography.

Gas chromatography was performed on a Hewlett-Packard Model 7620 Gas Chromatograph fitted with a $^{63}$Ni foil electron capture detector. The glass column was 182 cm X 4 mm and packed with 10% DEGS (diethylene glycol succinate) on 80-100 mesh Chromosorb W. For the determination of methylmercury, typical operating temperatures were: injection port 200°C, oven 170°C and detector 200°C. The carrier gas was argon-methane (95:5); gas flow was 60 ml/min at 40 psi. Under these conditions methylmercury had a retention time of 150 sec. Samples and standard were injected as benzene solutions in 2.0 µl aliquots.

Standards were run daily with each batch of samples and a calibration curve was plotted for each individual batch of samples. The concentration of methylmercury in the samples were calculated as ug CH$_3$Hg$^+$/g based on fish wet weight.
RESULTS

Nine spiking tests were performed during the entire period of fish tissue analysis and the calculated percent recovery varied between 83% to 89% with an average of $86.5 \pm 0.8\%$. This factor ($86.5\%$) was used to adjust the residual methylmercury ($\text{CH}_3\text{Hg}^+$) concentration in the fish samples accordingly.

**Acute toxicity tests**

In the acute toxicity tests, no mortality occurred in the control group and it was concluded that any mortality observed in the treated groups was due to the effects of the methylmercury.

Results are shown in Fig. 2 to 4. Fish exposed to lower than 80 $\mu$g $\text{CH}_3\text{Hg}^+/l$ survived for more than four days (Fig. 2). The median lethal concentration, 96 hr-LC50, of methylmercury for medaka is $88 \pm 9.8$ $\mu$g $\text{CH}_3\text{Hg}^+/l$ (Fig. 3). Levels of methylmercury accumulated in fish exposed to 43 to 1000 $\mu$g $\text{CH}_3\text{Hg}^+/l$ varied but remained below 40 $\mu$g $\text{CH}_3\text{Hg}^+/g$ (Fig. 4). When fish were exposed to concentrations higher than 1000 $\mu$g $\text{CH}_3\text{Hg}^+/l$ the amount of methylmercury accumulated in the tissue increased steadily and reached as high as 408.1 $\mu$g $\text{CH}_3\text{Hg}^+/g$ as in the case of 42900 $\mu$g $\text{CH}_3\text{Hg}^+/l$ exposure (Fig. 4).

**Long-term exposure**

Results are shown in Table 1 and Fig. 5. No mortality was observed in the control group during the entire six weeks of exposure. Fish exposed to 4.3 and 10.7 $\mu$g $\text{CH}_3\text{Hg}^+/l$ had one death each during the 4th week and the 2nd week respectively (Table 1). At the highest concentration (21.5 $\mu$g $\text{CH}_3\text{Hg}^+/l$), the fish began dying during the second week and mortality reached a peak during the fourth week of exposure when over 83%
Figure 2. Effects of different concentrations of methylmercury on the LT50 of *Oryzias latipes* at 23 ± 1°C. Values reported are mean and 95% confidence limits.
TIME (hr) TO 50% MORTALITY

EXPOSURE CONCENTRATION \( \times 10^3 \) \( \mu g \text{CH}_3\text{Hg}^+ / l \)

LC 50

96 hrs
Figure 3. Determination of the median lethal concentration (LC50) at 96 hr of methylmercury for Oryzias latipes.
PERCENT KILLED AT 96 hr.

CONCENTRATION

μg CH₃Hg⁺/l

0 20 40 60 80 100 200
Figure 4. Accumulation of methylmercury in *Oryzias latipes* exposed to different concentrations of methylmercury during the acute tests (each value represents the average of two determinations on groups of five fish).
Tissue Residue (µg CH₃Hg⁺/g)

Exposure Concentration (x10³ µg CH₃Hg⁺/l)
Figure 5. Accumulation of methylmercury in *Oryzias latipes* under long-exposure to different concentrations of methylmercury. Each point represents the mean of two methylmercury determinations on groups of five live fish.
Table 1. Mortality of *Oryzias latipes* exposed to different concentrations of methylmercury over a 6-week period (35 fish in each concentration at start of test).

<table>
<thead>
<tr>
<th>Exposure concentration (µg CH₃Hg⁺/l)</th>
<th>1st wk.</th>
<th>2nd wk.</th>
<th>3rd wk.</th>
<th>4th wk.</th>
<th>5th wk.</th>
<th>6th wk.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10.7</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21.5</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>21</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>
of the fish had died. At the end of the exposure, only three out of the initial 35 fish were alive from this group (Table 1).

Tissue methylmercury level of the control remained below 1 µg CH$_3$Hg$^+$/g (Fig. 5). Both of the 4.3 and 10.7 µg CH$_3$Hg$^+$/l exposed groups accumulated methylmercury steadily during the entire exposure and reached levels of 19.4 and 30.7 µg CH$_3$Hg$^+$/g respectively at the end of the exposure (Fig. 5). A high, probably lethal, level of methylmercury (67.1 µg CH$_3$Hg$^+$/g) was accumulated within two weeks in fish exposed to 21.5 µg CH$_3$Hg$^+$/l; during this period of two weeks, the fish started dying (Fig. 5 and Table 1). At the end of the fourth week over 83% of the fish from this group were dead (Table 1) and the tissue methylmercury level was 63.4 µg/g.
DISCUSSION

The present study has shown that methylmercury is toxic to the madaka (the 96h-LC50 was \(88 \pm 9.8 \mu g \text{ CH}_3\text{Hg}^+/l\)). This value is high compared to the 10 \(\mu g \text{ Hg}^{++}/l\) (96h-LC50) for rainbow trout (Lock 1974). Thus, the medaka is at least eight times more tolerant to methylmercury than the rainbow trout; salmonids have frequently been observed to be more sensitive to toxicants than many other fishes (Jones 1964).

At concentrations above 1000 \(\mu g \text{ CH}_3\text{Hg}^+/l\), the rate of mercury accumulation increases steadily suggesting possible damage to the cell membranes, especially in gill epithelial cells (Rucker and Amend 1969), which would allow a sharp increase in uptake of methylmercury. Although the effect of methylmercury at the molecular level has not been completely elucidated, it is apparent that this compound has a strong affinity for sulphur, particularly for the sulfhydryl group (-SH) in proteins (Saba 1972). This chemical binding of methylmercury to proteins in cell membrane may alter the distribution of ions, change electric potential and thus interfere with movement of fluids across the membrane (Passow et al., 1961). Methylmercury enters the fish mainly through the gills, although some may be absorbed through the skin (Olson et al., 1973); once inside the fish, it binds tightly with the sulfhydryl group in proteins, causing a steady increase of methylmercury accumulated. At concentrations below 1000 \(\mu g \text{ CH}_3\text{Hg}^+/l\), accumulation of methylmercury appears variable. Death seems to occur in the medaka when
the tissue level of methylmercury reaches 25 μg CH₃Hg⁺/g (Fig. 4). This value is quite high when compared with levels found in fish from uncontaminated areas or from salt water (Fimreite and Reynolds 1973; Peterson et al., 1973; Childs and Gaffke 1973). However, it is not uncommon for fish from contaminated areas like Clay Lake, Ontario, to reach levels as high as 20 μg/g of mercury (Fimreite and Reynolds 1973).

Unlike many other chemicals, methylmercury can be accumulated to a lethal level in fish exposed to a low concentration for long duration. In the acute toxicity study, medaka were observed to survive over a week in 75 μg/l methylmercury solution; in the long-term study, it was observed that fish exposed to 21.5 μg CH₃Hg⁺/l solution accumulated a lethal level of methylmercury (over 25 μg CH₃Hg⁺/g body weight) and started dying after the second week of exposure.

From the foregoing, it can be seen that the toxicity and effects of methylmercury depend not only on the exposure concentration but also on the amount of toxicant the fish accumulates during the exposure. This raises serious doubts about the reliability of acute toxicity tests for bioaccumulative chemicals like methylmercury. The use of 96h-LC50 for non-accumulative toxins would be valid but not for bioaccumulative chemicals like most pesticides (Sprague 1969). The definition of an acceptable sublethal concentration for bioaccumulative toxin cannot be some concentration below or a particular fraction of the 96h-LC50. Sublethal concentrations, where uptake of toxin equals its excretion without causing death, cannot be regarded as 'safe' concentration be-
cause sublethal effects may occur at relatively low tissue levels.
To solve this particular problem, residue determinations will have to be done concurrently with toxicity studies. "Safe" levels which could be much lower than the sublethal concentration, will have to be determined with long-term, continuous-flow experiments. We have only partially accomplished this and found that for medaka, the upper sublethal concentration of methylmercury lies between 10.7 and 21.5 μg CH₃Hg⁺/l.

For chemicals that accumulate in tissues, exposure to sublethal concentration of such chemicals will have long-lasting effects. Release of such chemicals into the environment, even in minute quantities, will ultimately bring about drastic changes in the organisms that come in contact with them. Section II examines the effects of long-term exposure to low levels of methylmercury on the reproductive physiology in the medaka.
SECTION II

CHRONIC EFFECTS OF METHYLMERCURY ON REPRODUCTION
INTRODUCTION

Very little work has been done on the effects of aquatic pollutants on fish reproduction (Sprague 1971). Crandall and Goodnight (1962) observed a delay in sexual maturity of guppy treated with lead and zinc. Mount (1968) reported inhibition of spawning of fathead minnows treated with copper, while Brungs (1969) found almost complete elimination of egg production by zinc. Fish eggs appear to be less sensitive than fry to toxicants such as detergents, zinc, copper, and malathion (Sprague 1971). A decrease in number of eggs and hatched young by phenylmercuric acetate at concentrations as low as 1.0 μg/l was observed in zebrafish (Kihlström et al., 1971). Decreased egg production was attributed to the affinity of mercurials for sulfhydryl groups thus inhibiting mitosis, enzyme reactions and protein hormones necessary for egg production and egg laying. In another study by Kihlström and Hulth (1972), increased frequency of hatching of zebrafish eggs was reported in water containing 10 to 20 ng/g phenylmercuric acetate; these workers suggested that this was due to a decrease in effects of microorganisms upon the developing eggs and also that low concentrations of mercuric compound may "stimulate" hatchability.

Kihlström's work remains a unique piece of information pertaining to the effects of organic mercury on fish reproduction. However, there are still many unanswered questions concerning the effects of mercury on fish, especially on their reproduction and the mechanism behind these effects.
This section investigates the effects of low concentrations of methylmercury on oviposition, gonadal development, spawning activity, hatchability of eggs and survival of juveniles of *Oryzias latipes*.
MATERIALS AND METHODS

On oviposition

Spawning fish were used in this experiment. To obtain actively spawning fish, *Oryzias* were maintained in dechlorinated Vancouver City water for over a month at 23 ± 1°C under long photoperiod (16L/8D) with the light period between 0800 and 2400 hours.

The experiments were performed with ten 18-litre capacity glass aquaria divided into two series. The first series consisted of five tanks, each holding a suspended cage (dimension 36 x 20 x 26 cm) made of fibreglass screening. These cages allowed rapid transfer of fish from one aquarium to another with minimum disturbance. The second series of five tanks contained various concentrations of methylmercury (0.0, 4.3, 8.58, 42.9 and 85.8 µg CH$_3$Hg$^+$/l).

Twenty-five pairs of spawning medaka were chosen from the warm temperature and long photoperiod group (see Section I). They were distributed, 5 males and 5 females per tank, into the cages of the first series of five aquaria. In the morning before the light came on, the caged fish were rapidly transferred to freshly prepared solution of methylmercury (0.0, 4.3, 8.58, 42.9 and 85.8 µg CH$_3$Hg$^+$/l) in the second series of five tanks. Spawning was checked at hourly intervals until noon at which time the fish were returned to clean water in the first series of five tanks and observation continued for another four hours. Fish were fed daily with frozen brine shrimp during the afternoon while the fish were in clean water. The fish were exposed to methylmercury on alternate days only; on other days, the cages were agitated.
mechanically to simulate the transfer. Spawning was checked each day. Spawning activity was recorded as number of females with eggs attached (number of spawning) and the total number of eggs collected in one tank.

**On gonadal development and spawning**

A flow through system as described in Section I was employed in this part of the experiment. Fish used had regressed gonads since they had been exposed to low temperature (13 ± 1°C) and short photoperiod (8L/16D) for over two months. These fish were allowed to warm to room temperature (approximately 23°C) overnight and on the next day were distributed to four test-tanks at the rate of 14 males and 20 females per tank, resulting in a loading density of approximately one litre of test solution per gram fish. The acclimation period consisted of four days with running warm water (23 ± 1°C) and long photoperiod (16L/8D, 800 to 2400 hours) but no methylmercuric chloride added. On the fifth day, methylmercuric chloride solutions were metered into the test-tanks and the desired concentrations (0.0, 4, 3, 10.7 and 21.5 μg CH₂Hg⁺/l) reached in about 4 hr; the approximate time for 99% replacement of test solution was 3.5 hr (Sprague 1969). Fish were fed daily with frozen brine shrimp. Spawning activity was recorded as number of females with eggs attached and number of eggs collected each day. Observations were continued for six weeks.

The eggs collected were allowed to hatch in disposable plastic petri dishes containing the same solutions from which they were collected. At the end of six weeks, the fish were killed, weighed, measured and the gonadosomatic indices calculated by the following formula:
The Gonadosomatic Index is calculated as follows:

\[
\text{Gonadosomatic Index} = \frac{\text{wt. of gonad}}{\text{wt. of fish}} \times 100
\]

There were two experiments performed and since both of them showed similar results, the data were pooled.

**On survival of juvenile fish**

To raise juveniles to maturity in different concentrations of methylmercury, one-week-old juvenile medaka were collected from untreated parents and distributed, approximately 50 to 80 fish per tank, into four test-tanks containing 0.0, 4.3, 10.7, and 21.5 μg CH₃Hg⁺/l. However, high mortality occurred in all treated groups within the first week and consequently the experiment was terminated in two weeks.

Duplicate experiments were performed. Both experiments showed similar results and the data were pooled.
RESULTS

Effects on oviposition

Control fish and fish experiencing 4-hr exposure of 4.3 μg CH$_3$Hg$^+$/l on alternate days consistently showed over 60% of oviposition activity (Table 2). At a concentration of 8.58 μg CH$_3$Hg$^+$/l the number of spawnsings was reduced whenever the fish were exposed to the toxic solution; however, spawning was unaffected on the following day when fish were returned to clean water. A similar but more marked effect was observed in the group exposed to 42.9 μg CH$_3$Hg$^+$/l. Exposure to the highest concentration, 85 μg CH$_3$Hg$^+$/l, not only prohibited spawning completely (except for Day 3) but also affected spawning the next day in clean water. There seems to be an increased reduction of spawning in clean water as this group experienced more exposure of the toxic solution.

Effects on gonadal development and spawning

No sign of spawning was observed during the first two weeks of exposure. Hence Figure 6 reports only spawning activity during the last four weeks of exposure. The fish fed normally but there was mortality in the 21.5 μg CH$_3$Hg$^+$/l exposed group; two fish and six fish died during the first and second week, respectively. During the third week of exposure spawning was observed in all groups while mortality was observed only at the highest concentration. During the 3rd, 4th, 5th and 6th week of exposure, treated groups showed a consistent reduction of spawning activity (Fig. 6); also there seemed to be some relationship between the degree of reduction of spawning activity and the concentrations of methylmercury. Figure 7 shows an inverse linear relationship between
Table 2. Effects of 4-hour exposure to different concentrations of methylmercury on the oviposition of Oryzias latipes. Five males and five females per tank. Positive treatment represents presence of the 4-hour exposure to the toxic solution on that day; negative treatment represents no such exposure. Numbers without brackets represent number of spawning females while the total number of eggs laid were stated within brackets.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Control</th>
<th>Concentration μg CH₃Hg⁺/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4.3</td>
<td>8.58</td>
</tr>
<tr>
<td>1</td>
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<td>5(62)</td>
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<td>2</td>
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<td>4(44)</td>
<td>3(31)</td>
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<tr>
<td>3</td>
<td>+</td>
<td>3(35)</td>
<td>2(20)</td>
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</tr>
<tr>
<td>5</td>
<td>+</td>
<td>4(42)</td>
<td>2(23)</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>3(37)</td>
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<tr>
<td>7</td>
<td>+</td>
<td>4(39)</td>
<td>4(48)</td>
</tr>
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<td>8</td>
<td>-</td>
<td>5(43)</td>
<td>3(40)</td>
</tr>
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<td>9</td>
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<tr>
<td>10</td>
<td>-</td>
<td>4(38)</td>
<td>4(50)</td>
</tr>
</tbody>
</table>
Figure 6. Weekly observations, from the 3rd to the 6th week, on spawning and egg-laying of Oryzias latipes for the different concentrations of methylmercury. Numbers in brackets represent number of fish in each group at the end of the week.
Figure 7. Effects of different concentrations of methylmercury on the total number of spawning and total number of eggs laid during six weeks of exposure.
the total number of spawnings (number of females with eggs attached) as well as the total number of eggs collected over the four weeks and the log exposure concentrations. Considering the control as 100%, during the four weeks of observation, egg-laying ability and spawning activity for fish exposed to 4.3, 10.7 and 21.5 μg CH$_3$Hg$^+$/l were respectively 58%, 49% and 10% (Fig. 8). When transformed to percent inhibition and plotted against log exposure concentrations, these values showed a direct linear relationship (Fig. 9). At the end of six weeks the gonadosomatic indices of both males and females showed reduction in relation to the increase in exposure concentration; females were more sensitive than males (Fig. 10). Spawned eggs were incubated in the media where they were collected; hatchability of these eggs bears no relationship to the concentrations of methylmercury previously used (Fig. 11). The percent hatchability for the four groups (0.0, 4.3, 10.7 and 21.5 μg CH$_3$Hg$^+$/l) did not deviate from the control and varied between 55% and 68%.

**Effects on juvenile fish**

Mortality occurred in all groups, treated and untreated. During the two weeks of exposure, 1-week old juveniles exposed to 0.0, 4.3, 10.7 and 21.5 μg CH$_3$Hg$^+$/l had mortality rates of 2.2%, 54.3%, 64.9% and 99.4% respectively (Table 3). This shows a high sensitivity of juvenile medaka to methylmercury toxicity.
Figure 8. The percent spawning activity and egg-laying ability of *Oryzias latipes* exposed to different concentrations of methylmercury. Control is 100 percent.
- EGG-LAYING
- SPAWNING

CONCENTRATION

µg CH₃Hg⁺/l
Figure 9. The percent inhibition of spawning in *Oryzias latipes* in water containing different concentrations of methylmercury.
% INHIBITION

CONCENTRATION

$\mu g \text{ CH}_3\text{Hg}^+ / l$

$Y = -11.6 + 30.5 \ln X$
Figure 10. Gonadosomatic indices of male and female *Oryzias latipes* exposed to different concentrations of methylmercury for 6 weeks. Numbers in brackets represent sample size; Asterisk, statistical significance (t-test, P <0.05) compared to control.
The image shows a bar chart comparing the GSI (Gonadosomatic Index) of male and female fish at different concentrations of methylmercury (CH$_3$Hg$^+$) in the water. The concentrations are 0.0, 4.8, 10.7, and 21.5 µg CH$_3$Hg$^+/l$. The chart indicates a higher GSI in male fish compared to female fish at the 4.8 and 10.7 concentrations, with the GSI decreasing at the 21.5 concentration. The number in parentheses above each bar indicates the sample size.
Figure 11. The percent hatchability of eggs exposed to different concentrations of methylmercury.
% HATCHABILITY

CONCENTRATION

μg CH₃Hg⁺ / l
Table 3. Mortality of one-week old juvenile *Oryzias latipes* exposed to different concentrations of methylmercury for two weeks.

<table>
<thead>
<tr>
<th>Concentration (μg CH$_3$Hg$^+$/l)</th>
<th>0.0</th>
<th>4.3</th>
<th>10.7</th>
<th>21.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual Mortality</td>
<td>3/134</td>
<td>57/105</td>
<td>100/154</td>
<td>157/158</td>
</tr>
<tr>
<td>% Mortality</td>
<td>2.24</td>
<td>54.28</td>
<td>64.93</td>
<td>99.37</td>
</tr>
</tbody>
</table>
DISCUSSION

Under normal conditions, female *Oryzias* ovulate between 0100 and 0400 hr; in the presence of a male oviposition takes place on the same day, soon after daybreak. In the present investigation, medaka were exposed to different concentrations of methylmercury during their normal period of oviposition, i.e., between 0800 and 1200 hr (light period, 0800 to 2400 hr). Control fish and fish exposed to 4.3 µg CH$_3$Hg$^+$/l were not affected but exposure to a higher concentration of 8.58 µg CH$_3$Hg$^+$/l reduced the number of spawning females; on return to clean water normal oviposition occurred the next day. This suggests that the concentration of 8.58 µg/1 methylmercury was strong enough to induce a stress that reduced the number of spawning females. Although there may be some accumulation of methylmercury this seems to have been partially excreted to a level whereby normal spawning could be achieved on the next day. This concentration (8.58 µg CH$_3$Hg$^+$/l) is approximately one-tenth of the 96h-LC50 dose (88 ± 9.8 µg CH$_3$Hg$^+$/l, Section 1). Similar results were observed at a higher concentration, 42.9 µg CH$_3$Hg$^+$/l, with further reduction in number of spawning females. However, at the highest concentration, 85.8 µg CH$_3$Hg$^+$/l, oviposition was abolished after the third day, and this effect was evident on subsequent days when the fish were in clean water. Thus, the 4-hour exposure to this high concentration resulted in an accumulation of methylmercury that blocked spawning. A similar phenomenon is probably true for all bioaccumulative toxins where accumulation is faster than the excretion of the toxin.
Kihlström et al. (1971) observed a decrease in number of zebra-fish eggs in water containing 1 ng or more phenylmercuric acetate per gram of water. In the present investigation using a similar range of concentrations of toxicant as Kihlström's, similar results were observed in Oryzias latipes exposed continuously to methylmercury for six weeks. Spawning occurred during the third week of exposure and continued until the end of the experiment. The percent inhibition of spawning for fish exposed to 4.3, 10.7 and 21.5 μg CH₃Hg⁺/l was 42, 48 and 88% respectively. Both spawning activity and egg-laying ability were related to the log of exposure concentrations. Unlike that of zebrafish (Kihlström and Hulth 1972), hatchability of Oryzias eggs from treated parents was not affected by the exposure to methylmercury. One-week old fish were especially sensitive to the toxicant. This is often true for other species of fish (Sprague 1971).

In rainbow trout, exposure to methylmercury for up to 12 weeks (10 μg Hg⁺⁺/l) did not significantly affect the in vitro metabolism of the gill or the concentration of plasma electrolytes (O'Connor and Fromm 1975). The only deleterious effects observed by these workers was a significant increase in hematocrit after 12 weeks exposure. Oryzias latipes is more "hardy" than the rainbow trout, and yet in the present investigation, the reproduction of Oryzias was adversely affected by even lower concentrations of methylmercury than that used on the rainbow trout by O'Connor and Fromm (1975); these findings suggest that reproduction is much more sensitive to environmental pollution than other physiological functions. Reproduction is indispensible to the survival of the species and any environmental contamination that adversely af-
Effects reproduction will have long lasting effects on the species and the ecological systems connected with it.

Mercury compounds inhibit mitosis by interacting with sulfhydryl groups (Hughes 1950). Since *Oryzias* depends on constant replacement of new oocytes for its daily spawning, inhibition of mitosis is a possible explanation for the decreased number of eggs spawned in contaminated water. Biochemically, methylmercury may inhibit enzyme systems for steroidogenesis by reacting with sulfhydryl groups of the enzymes, thus making them inactive. Fish reproduction depends on proper balance of hormones in the hypothalamic-hypophysial-gonadal system. It is not known where methylmercury acts. It may act at the hypothalamic-hypophysial level because methylmercury has been observed to accumulate in large quantities in the brain, but the possibility that it may also act at the gonadal level cannot be disregarded. These possibilities are examined in the following section.
SECTION III

MODE OF ACTION OF METHYLMERCURY ON REPRODUCTION
INTRODUCTION

Previous sections have demonstrated that exposure to methylmercury resulted in reduction of spawning activity in Oryzias latipes. However, it is not certain where methylmercury acts. This section attempts to elucidate the action of methylmercury on reproduction at the organ level.

In fish, the spawning period corresponds to a season most favourable for the development of the offspring. The fish synchronizes its reproductive physiology with the season by using environmental cues, like photoperiod and temperature. These environmental cues are transmitted to the hypothalamus via the eye, the pineal and possibly the skin. The hypothalamus in turn stimulates the hypophysis to produce and release hormone(s) capable of stimulating gonadal development; thus forming the hypothalamic-hypophysial-gonadal axis. There is also a feedback mechanism in this system whereby levels of the hormones are in proper balance. Fig. 12 shows such a relationship.

So far we have observed the effects of methylmercury treatment on the end product of the entire reproductive process. But it is not known at what point methylmercury acts on this hypothalamic-hypophysial-gonadal axis. To elucidate this, it was assumed that methylmercury blocked or rendered inactive some link in this system; thus, some hormone was not produced. It was further assumed that "replacement therapy" with appropriate hormones would restore reproduction in methylmercury treated fish. In the experiments that follow, different hormones from the hypothalamus,
Figure 12. Diagram showing the interrelationship between environmental cues, hypothalamus, hypophysis and gonads.
ENVIRONMENTAL CUES
- temperature
- photoperiod
- water chemistry, etc.

via eye, pineal, skin

HYPOPHYSIS
- gonadotropin(s)

GONADS
- gonadal development
- steroidogenesis

HYPOTHALAMUS
- hypothalamic hormones (releasing or inhibiting hormones)
hypophysis and gonads are "replaced" in the methylmercury affected
fish to determine whether these hormones restore reproductive activity.

Mammalian luteinizing hormone is effective in inducing matura-
tion and ovulation in a variety of fishes (Hirose 1971, Goswami and
Sundararaj 1972; Donaldson 1973; Hoar 1969), but piscine hypothalamic
hormone, possibly similar to mammalian luteinizing hormone-releasing
hormones (LH-RH) has not been well studied. Synthetic luteinizing hor-
mones-releasing hormone (LH-RH), recently synthesized based on the pro-
posed structure of porcine and ovine LH-RH, is highly effective in sti-
mulating the release of luteinizing hormone and in inducing ovulation
in mammals (Schally et al., 1973; Humphrey et al., 1973; Foxcroft et al.,
1975), in chickens (van Tienhoven and Schally 1972) and in amphibian
(Thornton 1974; Mazzi et al., 1974; Vellano et al., 1974). In teleost
fishes, administration of synthetic LH-RH causes release of secretory
granules from the gonadotropic (GtH) cells in the proximal pars distalis
and induces ovulation in the goldfish (Lam et al., 1976) and in the ayu
(Hirose and Ishida 1974). Gonadotropin release by synthetic LH-RH was
also observed in the carp (Breton and Weil 1973) but the reaction was
less than that elicited by carp hypothalamic extracts. In trout, treat-
ment with mammalian gonadotropin releasing hormone elevated plasma
gonadotropin concentration as determined by radioimmunoassay (Crim and
Cluett 1974). However, it is not known whether synthetic LH-RH would
stimulate the synthesis and release of gonadotropin(s) in sexually re-
gressed fish and subsequently gonadal maturation. This phenomenon was
observed in hens (Reeves et al., 1973).
Oryzias latipes kept under warm temperature \((23 \pm 1^\circ C)\) and short photoperiod \((8L/16D)\) for 6 to 12 weeks showed little or no gonadal development (Chan 1976, Yoshioka 1962, 1963). This system permits tests to show whether synthetic LH-RH can stimulate activity in the pituitary gonadotropic cells and consequently gonadal maturation. This part of the study first examines the effect of synthetic LH-RH on ovarian development in the Japanese medaka, Oryzias latipes.

After establishing the effectiveness of synthetic LH-RH on gonadal maturation in Oryzias, we may then determine whether hypothalamic and hypophysial hormones are capable of restoring reproductive activity in methylmercury treated fish. By injecting hypothalamic and hypophysial hormones into fish exposed to methylmercury, it is possible to determine not only which of the hormones is effective in restoring reproduction in methylmercury poisoned fish but also at what level of the hypothalamic-hypophysial-gonadal axis methylmercury inhibition occurs. Histological examination of the pituitary may also help to explain the action of methylmercury on the synthesis and release of gonadotropin(s). Needless to say, an important outcome of this experiment is of ecological significance since it may show whether hormonal treatments can restore the spawning activity inhibited by methylmercury when indeed such a contamination does occur in nature.

Hirose (1971) devised a simple in vitro system for studying ovulation in Oryzias. Using this system, the rate of ovulation at various starting hours of incubation was determined (Hirose & Hirose 1972) and also effects of various hormones on ovulation were studied (Hirose 1972a, b,
1973). Similar studies were performed on the Indian catfish by Goswami and Sundararaj (1972a, b, 1973, 1974). Studies by both groups showed that hypophysial gonadotropic hormone and the corticosteroids were effective in inducing ovulation in vitro. This in vitro system provides a fast, convenient method for studying the effects of methylmercury on ovulation and may be developed into an effective bioassay.

This part of the study investigates the effects of long term exposure of female fish to methylmercury on in vitro ovulation. The effects of various concentrations of methylmercury on in vitro ovulation of untreated fish and the effects of exogenous luteinizing hormone on methylmercury blocked ovulation in vitro of untreated fish will also be examined. The effects of various steroids on methylmercury affected ovulation will be investigated, thereby some postulations can be made on the effects of methylmercury on the ovary level.
MATERIALS AND METHODS

On ovarian maturation by synthetic LH-RH

In this part of the experiment, only female fish were used because they provide better indices for gonadal development than male fish. Adult regressed fish were obtained by pretreating fish with low temperature (13 ± 1°C) and short photoperiod (8L/16D) for three months (May 6 to Aug. 6, 1974). On Aug. 6, 1974, fish weighing about 0.3 g each were warmed to room temperature (23 ± 1°C) overnight under short photoperiod (8L/16D = 0800 to 1700 hours); they were distributed, 10 to 12 fish per tank, into five 22-liter capacity all glass aquaria with subgravel filters. The fish were maintained under the above conditions for six weeks and fed once-a-day with a slight excess of frozen brine shrimp during the morning.

Four groups received injections of synthetic LH-RH (AY-24, 031. Ayerst Laboratory) at doses of 1, 10, 100 and 1000 ng/g body weight respectively; the fifth group was saline-injected and acted as a control. Injections were intraperitoneal using a microsyringe (Hamilton) equipped with a 32 gauge hypodermic needle. The synthetic LH-RH was dissolved in 0.6% NaCl and the injection volume was 5 µl per fish. Fish were injected twice a week for 6 weeks (Aug. 8 to Sept. 23, 1974).

At the end of the experiment, all fish were sacrificed, body weights and gonad weights were recorded to the nearest 0.2 mg and the gonadosomatic index (GSI = gonad weight X 100/body weight) calculated. For histological observations, the ovaries and the pituitary glands were fixed in Bouin's solution, embedded in paraffin and sectioned at 5-7 µm.
Ovarian sections were stained with Erlich's hematoxylin-eosin; aldehyde fuchsin counter-stained with Halmi's was used for the pituitary glands. Oocytes were classified into three categories according to Chan (1976), and only percent distribution of Class III oocytes (number of oocytes with yolk formation x 100/total number of oocytes in a mid-section of the ovary) was calculated because appearance of Glass III oocytes represents mature or maturing fish.

On effects of LH-RH and LH injection in methylmercury treated fish

This experiment was performed in a flow-through system described in Section I.

Sexually regressed fish were obtained by maintaining fish at 13 ± 1°C under short photoperiod (8L/16D) for four months (May 11 to Sept. 10, 1974). The fish weighing 0.25 to 0.30 g were warmed to 23 ± 1°C overnight and divided into four groups, 15 males and 15 females in each group. Loading density of the tanks was approximately one litre of test solution per gram fish. Acclimation consisted of two days with running clean water at 23 ± 1°C and under long photoperiod (16L/8D) for all four groups. The same photoperiods and water temperature were used during the entire experimental period.

On the third day, methylmercuric chloride solution was added to three of the four groups and the desired concentration of 10.7 μg/l as methylmercury was reached in about four hours; the approximate time for 99% replacement of test solution was 3.5 hours (Sprague 1969). The fourth group, kept in clean water, acted as the control. Starting on the fourth day, the three groups exposed to methylmercury received
twice-a-week either saline (0.6% NaCl) or luteinizing hormone (10 µg/g body weight; porcine luteinizing hormone, NIH-LH-S8) or synthetic luteinizing hormone-releasing hormone (1 µg/g body weight; AY-24, 031, Ayerst Laboratory). The control group received only saline injections. Injections were performed intraperitoneally using a micro-syringe (Hamilton) equipped with a 32 gauge hypodermic needle, the hormones were dissolved in 0.6% NaCl and the injection volume was 5 µl per fish. Exposure to methylmercury and the twice-weekly injections lasted for 6 weeks. Fish were fed daily with frozen brine shrimp.

Spawning activity was recorded as number of females with eggs attached and total number of eggs collected each day. Observations were continued for 6 weeks. At the end of six weeks, the fish were killed, weighed, measured and the gonadosomatic indices (GSI) calculated. For histological observation of the pituitary glands, the tissues were fixed in Bouin's fixative, embedded in paraffin and the sections (6 µm) were stained with aldehyde fuchsin, AF, counter-stained with Halmi's solution. Analysis of variance was used in the statistic analyses.

On effects of methylmercury on in vitro ovulation

All fish were maintained at 23 ± 1°C under long photoperiods (16L/8D; 0800-2400 hour light). Spawning females with eggs attached were removed each day at noon and kept in a separate tank until required for the experiment.

All instruments and media were sterilized before use. Each fish was swabbed with 70% alcohol and killed by decapitation. The ovary was removed, and placed in a glass petri dish containing Medium 199 (Hirose
The eggs were individually separated as free eggs with dissecting pins. Only eggs with diameter greater than 0.8 mm were used. Incubation was carried out at 23 ± 1°C. For each treatment, 10-15 eggs were placed in 5 ml of medium in a watch glass covered with a petri dish. Incubation started either at 1700 hours or 2300 hours—the two best times for commencing incubation with or without LH (Hirose and Hirose 1972). Percent ovulation was recorded at 1000 hours the following morning with the help of a dissecting microscope. Ovulated eggs were characterized by their detachment from the follicles and presence of filaments around the ovulated eggs (Hirose 1971). Normal ovulation was confirmed by artificial fertilization and observation of development.

Pre-exposure of fish to methylmercury was similar to that described in Section I. At the end of the exposure period (6 weeks), all female fish were killed, the ovaries were dissected and all oocytes with diameters greater than 0.8 mm from the same treatment group were pooled. The eggs were then incubated with 0 or 10 µg/ml porcine luteinizing hormone (NIH-LH-S8) and various concentrations of methylmercury in Medium 199 at 1700 hours and ovulation checked at 1000 hours the following morning. The experiment was duplicated and since similar results were observed, the data were pooled.

Additional experiments were performed using various concentrations of methylmercury in the incubation medium with eggs from untreated fish. To study the effects of steroids, progesterone, estradiol, cortisone and testosterone, were individually dissolved in ethanol: propylene glycol (1:1) and added to the incubation media just prior to incubation, result-
ing in a final concentration of 1 \( \mu g/1 \) steroid. Procedure of incubation was similar to that previously described. All experiments were duplicated and the results were pooled.
RESULTS

Effect of Synthetic LH-RH on ovarian maturation

The effects of synthetic luteinizing hormone-releasing hormone on the gonadosomatic index, percent distribution of Class III oocytes and ovulation are shown in Fig. 13. The ovary at the initiation of this study was mostly composed of small oocytes (diameter 0.1 mm or less); there were no Class III oocytes. The mean gonadosomatic index (+ S.E.) of this initial group was low (1.08 ± 0.17). This value is characteristic of sexually regressed fish.

After six weeks of injections, the control group (saline injected) showed only a slight increase in GSI (from 1.09 to 1.42) with the appearance of some Class III oocytes in less than 4% of the fish (Figs. 13 and 14). Groups injected with synthetic LH-RH, with the exception of 1 ng/g, showed an increase in both gonadosomatic index and percent distribution of Class III oocytes (Figs. 13, 15, 16, and 17); however, only the two higher doses showed significantly different results from the saline control (P < 0.05, t-test). Furthermore, there appeared to be a linear relationship between the gonadosomatic index and log of the injection doses (Fig. 13). Ovulation was also observed in 5 out of the 12 fish injected with the highest dose, 1000 ng/g.

The pituitary gland of Oryzias has already been described and the globular basophils, located at the most ventral portion of the proximal pars distalis, identified as the gonadotrophs (Aoki and Umeura 1970; Kasuga and Takahashi 1971). The activity of these cells can be estimated by their staining affinity with aldehyde fuchsin (AF), and is related to the changes of reproductive activities; the staining affinity
Figure 13. Effects of different dosages of synthetic LH-RH on the gonadosomatic index and the percent distribution of Class III oocytes. Numbers in brackets represent number of fish in each group. Values reported are means ± standard errors. Asterisk represents statistical significance (P <0.05, t-test) compared to saline injected control, a, ovulation observed.
INJECTION DOSES
ng/g

INJECTION DOSES
ng/g

INJECTION DOSES
ng/g

INJECTION DOSES
ng/g

INJECTION DOSES
ng/g

INJECTION DOSES
ng/g

INJECTION DOSES
ng/g

INJECTION DOSES
ng/g

INJECTION DOSES
ng/g

INJECTION DOSES
ng/g

INJECTION DOSES
ng/g
Figure 14. Section (7 μm) of ovary from *Oryzias* injected with saline twice-a-week for 6 weeks at 23 ± 1°C under 8L/16D. Hematoxylin-eosin, X 100.

Figure 15. Section (7 μm) of ovary from *Oryzias* injected with 10 ng/g synthetic LH-RH twice-a-week for 6 weeks at 23 ± 1°C under 8L/16D. Hematoxylin-eosin. X 100
Figure 16. Section (7 μm) of ovary from *Oryzias* injected with 100 ng/g synthetic LH-RH twice-a-week for 6 weeks under $23 \pm 1^\circ C$ and 8L/16D. Notice the appearance of Class III oocytes with yolk vesicles as indicated by arrow. Hematoxylin-eosin. X100

Figure 17. Section (7 μm) of ovary from *Oryzias* injected with 1000 ng/g synthetic LH-RH twice-a-week for 6 weeks under $23 \pm 1^\circ C$ and 8L/16D. Notice the presence of Class III oocytes with yolk formation as indicated by arrow, and also the enlarged ovarian cavity (C) due to the ovulated oocytes. Hematoxylin-eosin. X100
is highest during the spawning season from May to August, less evident during the post-spawning period from September to October and absent during the resting period from November to February (Kasuga and Takahashi 1971). In the present study, the gonadotrophs of the saline-injected control fish were not stained with AF, suggesting inactivity of these cells (Fig. 18). Similar results were observed in the 1 ng/g injected fish and the photomicrograph was omitted here. As the injection dose increases, the gonadotropic cells show an increase in stainability and in the area stained (Figs. 18, 19, 20 and 21). These results suggest that synthetic LH-RH at doses of 10 to 1000 ng/g were effective in stimulating activity of the gonadotropic cells resulting in ovarian development.

Effects of Synthetic LH-RH and LH injections on methylmercury treated fish

Spawning was first observed in groups injected with hormones (either LH or syn. LH-RH) during the second week of the methylmercury exposure; while groups injected with saline (both methylmercury treated and clean water control) started spawning only during the fourth week of the exposure (Fig. 22). Though showing an early start in spawning, the methylmercury-exposed LH-RH injected group showed a decline in spawning activity after the fourth week, while the methylmercury-exposed LH-injected group showed increasing spawning activity throughout the treatment period. Methylmercury-exposed saline-injected fish showed spawning only in the last three weeks of exposure and these activities were lower than that of methylmercury-exposed LH-injected groups. Though spawning
Figure 18. Section (6 μm) of pituitary gland from *Oryzias* injected with saline twice-a-week for 6 weeks under 23 ± 1°C and 8L/16D. P, prolactin cell; T, thyrotroph; G, gonadotroph; N, neurohypophysis; S, somatotroph. Aldehyde Fuchsin. X410.

Figure 19. Section (6 μm) of pituitary gland from *Oryzias* injected with 10 ng/g synthetic LH-RH twice-a-week for 6 weeks under 23 ± 1°C and 8L/16D. Notice the slight staining of AF in gonadotrophs, G and increased AF staining in neurohypophysis, N. Aldehyde Fuchsin, X410.
Figure 20. Section (6 μm) of pituitary gland from Oryzias injected with 100 ng/g synthetic LH-RH twice-a-week for 6 weeks under 23 ± 1°C and 8L/16D. Note the increased AF staining in both gonadotrophs, G and neurohypophysis, N. Aldehyde Fuchsin. X410.

Figure 21. Section (6 μm) of pituitary gland from Oryzias injected with 1000 ng/g synthetic LH-RH twice-a-week for 6 weeks under 23 ± 1°C and 8L/16D. Note the further increase of AF staining in both gonadotrophs, G and neurohypophysis, N. Aldehyde Fuchsin. X410.
Figure 22. Weekly observation on spawning and egg-laying of *Oryzias* for the various hormonal treatment in the presence of methylmercury (10.7 µg/l).
occurred later than in the hormone injected groups, the clean water saline-injected control group showed substantially higher spawning activity than any of the methylmercury exposed groups. The percent of spawning activity over the entire 6-week treatment period of the methylmercury treated groups were less than the clean-water control group (Fig. 23). Injection of LH was effective in restoring part of the spawning activity inhibited by methylmercury but not LH-RH.

At the end of six weeks, the female gonadosomatic indices of the methylmercury treated groups were less than the control (Fig. 23). Male gonadosomatic indices of the different groups were variable. Since the female gonadosomatic indices provide a better indication of the effects of the different treatment, statistical analysis was performed on data collected from female fish only (Table 4). The methylmercury exposed groups were all statistically different (P <0.05) from the clean water control group. Among the methylmercury exposed groups, the LH-injected group showed significant differences from the saline injected group and the LH-RH injected group, while no significant difference was observed between the saline injected and LH-RH injected group.

The pituitary gland of *Oryzias* has been described and the globular basophils, located at the most ventral portion of the proximal pars distalis, identified as the gonadotrophs, GTH cells (Aoki and Umeura 1970). The activity of these cells can be estimated by their staining affinity with aldehyde fuchsin and is related to the reproductive activities (Kasuga and Takahashi 1971). The present study showed that saline injected clean water control fish had high activity in gonadotrophs and neurosecretion as indicated by more intense staining with AF
Figure 23. Effects of various hormonal treatments on percent spawning, male and female gonadosomatic indices at the end of 6-weeks methylmercury exposure. Numbers in brackets represent sample size and the vertical bars, standard error.
Table 4. Statistical significance of female gonadosomatic indices among the various treatments by analysis of variance

<table>
<thead>
<tr>
<th></th>
<th>Clean Water Saline inj.</th>
<th>CH$_3$Hg$^+$ Saline inj.</th>
<th>CH$_3$Hg$^+$ LH inj.</th>
<th>CH$_3$Hg$^+$ LH-RH inj.</th>
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<tbody>
<tr>
<td>Clean Water Saline inj.</td>
<td>---</td>
<td>P &lt;0.05</td>
<td>P &lt;0.05</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>CH$_3$Hg$^+$ Saline inj.</td>
<td>P &lt;0.05</td>
<td>---</td>
<td>P &lt;0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>CH$_3$Hg$^+$ LH inj.</td>
<td>P &lt;0.05</td>
<td>P &lt;0.05</td>
<td>---</td>
<td>P &lt;0.05</td>
</tr>
</tbody>
</table>
(Figs. 24, 25, 26 and 27). Very little activity was observed in the methylmercury-exposed saline-injected group showing inhibition of GTH cell activity by methylmercury. Methylmercury-exposed LH-injected fish showed moderate activity in gonadotrophs and neurosecretion while LH-RH injected fish showed high activity in both gonadotrophs and neurosecretion, suggesting that this hormone was effective in stimulating the activity of the pituitary gland (Figs. 24 to 27).

Effects of methylmercury on in vitro ovulation

Hirose (1971b) observed that when donor medaka were maintained under a long photoperiod (16L/8D, 0800 to 2400 hr) in vitro ovulation required gonadotropic hormones if incubation commenced at 1700 hours but when incubated at 2200 hours, ovulation occurred naturally. These two time schedules were used in the present study depending on the requirements of the individual tests.

Pre-exposure to methylmercury for 6 weeks. When incubated at 1700 hours, little or no ovulation was observed unless LH was present in the media (Table 5). This confirms Hirose's observations. Even without methylmercury added to the incubation media, pre-exposure of female fish to methylmercury for 6 weeks reduced the percentage of ovulation in accordance with the pre-exposure concentrations. When methylmercury was added to the incubation media, further reduction in percent ovulation was observed. Methylmercury in the incubation media seems to have an additive effect on the concentration of methylmercury to which the fish were previously exposed.
Figure 24. Section (6 μm) of pituitary gland from *Oryzias* exposed to clean water and injected with saline twice-a-week for 6 weeks under 23 ± 1°C and 16L/8D. T, thyrotroph; G, gonadotrophs; N, neurohypophysis. Note the intense AF staining in the gonadotrophs. Aldehyde fuchsin. X410.

Figure 25. Section (6 μm) of pituitary gland from *Oryzias* exposed to methylmercury and injected with saline twice-a-week for 6 weeks under 23 ± 1°C and 16L/8D. G, gonadotrophs; N, neurohypophysis. Note the loss in AF staining in the gonadotrophs. Aldehyde fuchsin. X410.
Figure 26. Section (6 μm) of pituitary gland from *Oryzias* exposed to methylmercury and injected with luteinizing hormone twice-a-week for 6 weeks under 23 ± 1°C and 16L/8D. G, gonadotrophs; N, neurohypophysis. Note the moderate AF staining in gonadotrophs. Aldehyde fuchsin. X410.

Figure 27. Section (6 μm) of pituitary gland from *Oryzias* exposed to methylmercury and injected with luteinizing hormone-releasing hormone twice-a-week for 6 weeks under 23 ± 1°C and 16L/8D. G, gonadotrophs; N, neurohypophysis. Note the intense AF staining in gonadotrophs. Aldehyde fuchsin. X410.
Table 5. Effects of different concentrations of methylmercury on in vitro ovulation in *Oryzias* previously treated with methylmercury for 6 weeks. Values reported as percent ovulation (no. of oocytes ovulated/no. of oocytes used).

<table>
<thead>
<tr>
<th>6-wk pre-exposure to CH$_3$Hg$^+$ media</th>
<th>Conc. of CH$_3$Hg$^+$ in µg/l</th>
<th>No LH Added</th>
<th>0</th>
<th>0</th>
<th>4.8</th>
<th>10.7</th>
<th>21.5</th>
<th>215</th>
<th>480</th>
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<tr>
<td>From Control</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>3.3</td>
<td>83.3</td>
<td>76.9</td>
<td>70.1</td>
<td>53.8</td>
<td>27.3</td>
<td>19.1</td>
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<td></td>
<td></td>
<td></td>
<td>(1/30)</td>
<td>(20/24)</td>
<td>(30/39)</td>
<td>(22/30)</td>
<td>(21/40)</td>
<td>(9/33)</td>
<td>(8/42)</td>
</tr>
<tr>
<td>From fish exposed to 4.8 µg/l CH$_3$Hg$^+$</td>
<td></td>
<td></td>
<td>5.5</td>
<td>73.7</td>
<td>68.4</td>
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<td></td>
<td></td>
<td>(2/36)</td>
<td>(28/38)</td>
<td>(20/36)</td>
<td>(20/36)</td>
<td>(16/32)</td>
<td>(9/32)</td>
<td>(8/30)</td>
</tr>
<tr>
<td>From fish exposed to 10.7 µg/l CH$_3$Hg$^+$</td>
<td></td>
<td></td>
<td>70.0</td>
<td>24.0</td>
<td>33.3</td>
<td>20.0</td>
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<td></td>
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<td></td>
<td>(14/20)</td>
<td>(6/25)</td>
<td>(9/27)</td>
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<tr>
<td>From fish exposed to 11.5 µg/l CH$_3$Hg$^+$</td>
<td></td>
<td></td>
<td>54.5</td>
<td>28.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(12/22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Effects of methylmercury on normal oocytes from untreated fish.

Normal oocytes from untreated fish incubated in methylmercury solutions showed a reduced percentage of ovulation (Table 6). More than 50% reduction of ovulation was observed in oocytes exposed to 48 μg/1 of methylmercury. Percent observed ovulation converted into percent inhibition showed a direct relationship with the logarithm of the doses used (Fig. 28).

Effects of different steroids on methylmercury inhibited ovulation in vitro. Under normal conditions, ovulation occurs naturally when incubated at 2200 hours. A pilot study was undertaken to determine an effective dose of methylmercury whereby ovulation would be inhibited at this hour. Table 7 showed the results of such a study. Normal oocytes from untreated fish when incubated at 2200 hours ovulated without LH while the addition of methylmercury at concentration of 192 μg/1 and 215 μg/1 was effective in reducing not only this reaction but also that induced by the addition of 10 μg/ml LH to the incubation media.

Using the above modified system for an in vitro ovulation study, four steroids (progesterone, cortisone, estradiol and testosterone) were tested for their effectiveness in restoring methylmercury inhibited in vitro ovulation. Table 8 shows the results. When incubated without methylmercury at 2200 hours, normal oocytes from untreated fish ovulated naturally and this was stimulated by the addition of cortisone. Progesterone, estradiol and testosterone had little or no effect. When methylmercury at concentrations of 215 μg/1 was present in the incubation media, ovulation was reduced in all cases with the exception of cortisone which remained slightly potent in inducing a certain degree of ovulation.
Table 6. The effects of various concentrations of methylmercury on ovulation in vitro of *Oryzias latipes* incubated with 10 µg/ml porcine LH at 1700 hours.

<table>
<thead>
<tr>
<th>Conc. of CH$_3$Hg$^+$ µg/l</th>
<th>No. of oocytes used</th>
<th>No. of ovulated eggs</th>
<th>Percent Ovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>21</td>
<td>84.0</td>
</tr>
<tr>
<td>4.8</td>
<td>38</td>
<td>28</td>
<td>73.7</td>
</tr>
<tr>
<td>10.7</td>
<td>26</td>
<td>17</td>
<td>65.4</td>
</tr>
<tr>
<td>21.5</td>
<td>33</td>
<td>20</td>
<td>60.6</td>
</tr>
<tr>
<td>48.0</td>
<td>35</td>
<td>17</td>
<td>48.6</td>
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<tr>
<td>96.0</td>
<td>45</td>
<td>18</td>
<td>40.0</td>
</tr>
<tr>
<td>192.0</td>
<td>51</td>
<td>15</td>
<td>29.4</td>
</tr>
<tr>
<td>215.0</td>
<td>40</td>
<td>7</td>
<td>17.5</td>
</tr>
</tbody>
</table>
Figure 28. Percent inhibition of *in vitro* ovulation by various concentrations of methylmercury in incubation media at 1700 hours.
% INHIBITION

CONCENTRATION

μg CH₃Hg⁺ / l

Γ = -17.2 * 16.2 lnX

r = 0.96
Table 7. The effects of methylmercury on ovulation *in vitro* of *Oryzias latipes* incubated with and without LH (10 µg/ml) commencing at 2200 hours.

<table>
<thead>
<tr>
<th>Conc. of CH$_3$Hg$^+$ µg/l</th>
<th>LH µg/ml</th>
<th>No. of oocytes used</th>
<th>No. of ovulated eggs</th>
<th>Percent Ovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>25</td>
<td>15</td>
<td>60.0</td>
</tr>
<tr>
<td>192.0</td>
<td>10</td>
<td>30</td>
<td>8</td>
<td>26.6</td>
</tr>
<tr>
<td>192.0</td>
<td>0</td>
<td>32</td>
<td>8</td>
<td>25.0</td>
</tr>
<tr>
<td>215.0</td>
<td>10</td>
<td>34</td>
<td>8</td>
<td>23.5</td>
</tr>
<tr>
<td>215.0</td>
<td>0</td>
<td>35</td>
<td>6</td>
<td>17.1</td>
</tr>
</tbody>
</table>
Table 8. The effects of steroids on ovulation in vitro of *Oryzias latipes* oocytes incubated with and without methylmercury at 2200 hours as the time of incubation.

<table>
<thead>
<tr>
<th>Conc. of CH$_3$Hg$^+$ µg/l</th>
<th>Steroid</th>
<th>No. of oocytes used</th>
<th>No. of ovulated eggs</th>
<th>Percent Ovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>28</td>
<td>15</td>
<td>53.6</td>
</tr>
<tr>
<td>215</td>
<td>0</td>
<td>34</td>
<td>6</td>
<td>17.6</td>
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<tr>
<td>0</td>
<td>Progesterone</td>
<td>35</td>
<td>22</td>
<td>62.8</td>
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<tr>
<td>215</td>
<td>Progesterone</td>
<td>30</td>
<td>6</td>
<td>20.0</td>
</tr>
<tr>
<td>0</td>
<td>Cortisone</td>
<td>38</td>
<td>32</td>
<td>84.1</td>
</tr>
<tr>
<td>215</td>
<td>Cortisone</td>
<td>41</td>
<td>18</td>
<td>43.9</td>
</tr>
<tr>
<td>0</td>
<td>Estradiol</td>
<td>30</td>
<td>16</td>
<td>53.3</td>
</tr>
<tr>
<td>215</td>
<td>Estradiol</td>
<td>29</td>
<td>5</td>
<td>16.1</td>
</tr>
<tr>
<td>0</td>
<td>Testosterone</td>
<td>30</td>
<td>17</td>
<td>56.6</td>
</tr>
<tr>
<td>215</td>
<td>Testosterone</td>
<td>32</td>
<td>6</td>
<td>18.7</td>
</tr>
</tbody>
</table>
DISCUSSION

Effect of synthetic LH-RH on ovarian maturation

Results of the present study show that synthetic LH-RH is effective in stimulating the activity of gonadotropic cells in the pituitary as indicated by the affinity to AF staining (Kasuga and Takahashi 1971), and in inducing ovarian maturation and ovulation in *Oryzias latipes* maintained at 23 ± 1°C under short photoperiods (8L/16D). This agrees with the results obtained in the ayu (*Plecoglossus altivelis*) (Hirose and Ishida 1974), goldfish (Lam et al., 1976; Kaul and Vollrath 1974), carp (Breton and Weil 1973), and trout (Crim and Cluett 1974).

Teleost pituitary gonadotropic cells appear to be under the control of a releasing hormone from the hypothalamus (Peter 1970). However, radioimmunoassay studies of Deery (1974) have shown that hypothalamic extracts of goldfish do not have any immunological cross reaction with labelled synthetic LH-RH while rat hypothalamic extracts do. Also, Breton and Weil (1973) found that carp hypothalamic extract is more potent than synthetic LH-RH in stimulating release of gonadotropin(s). These observations suggest the existence of a fish gonadotropin(s)-releasing hormone that may be chemically different from mammalian LH-RH but have overlapping biological activities.

The effective dose of synthetic LH-RH used, although similar to dosages used in other fish studies, was much higher than the effective dose in mammals. This difference could be due to the methods of administration of the hormone. Intraperitoneal injection has been shown to be less effective than perfusion of the pituitary *in situ* (Vellano et al., 1974) or
intracranial injection (Lam et al., 1976). Though not known for fish, LH-RH has a very short half-life in mammals (Redding et al., 1973); hence, in this study, twice-a-week may not be frequent enough to stimulate the pituitary at the lower doses. At a low dose of 10 ng/g, a slight increase (though not statistically significant) was observed in gonadosomatic index with no increase in percent distribution of Class III oocytes over the control (Fig. 13). It is possible that low doses only stimulate mild synthesis and release of gonadotropin(s) leading to development of Class I and II oocytes only.

Under natural spawning conditions, where male and female fish are present together, the gonadosomatic index of female fish is approximately 6% (Chan 1976). In the present study, the GSI was much lower. This may possibly be due to the absence of males resulting in retention of ovulated oocytes in the ovarian cavity; these unspawned eggs may have an inhibitory effect on the development of the rest of the ovary (Egami and Hosokawa 1973). Moreover, the effect of handling the fish during frequent injections cannot be disregarded.

In this experiment, stainability with aldehyde fuchsin was used as a criterion of activity in gonadotropic cells. Since synthetic LH-RH is effective in releasing gonadotropin(s) (Hirose and Ishida 1974; Lam et al., 1976; Kaul and Vollrath 1974; Breton and Weil 1973), the increased AF staining of gonadotropin cells observed in the present study probably represents the excess of gonadotropin synthesis over its release from the pituitary. Ovarian maturation probably depends on a high rate of synthesis of gonadotropin(s) with a low but sustained release of
hormone(s) as observed in the present study while ovulation depends on a large release (surge) of the accumulated gonadotropin(s) in the pituitary as observed in goldfish (Lam et al., 1976) and ayu (Hirose and Ishida 1974). Under favourable conditions of long photoperiod, warm temperatures and good supply of food, Oryzias spawns daily throughout the spawning season (Egami and Hosokawa 1973). Thus it seems possible that gonadotropic cell activity remains high in Oryzias throughout the entire spawning season. The large release of gonadotropin(s) by LH-RH as observed in goldfish (Lam et al., 1976) and ayu (Hirose and Ishida 1974) will probably be observed in Oryzias only during its daily ovulation between 0100 and 0400 hr.

Of interest also in the present study is the increased neurosecretion (increased AF staining of neurohypophysis) associated with increasing doses of synthetic LH-RH (Figs. 18 to 21). The cause is not known. However, it has been observed that neurohypophysial activity correlates with reproductive activity in Oryzias (Kasuga and Takahashi 1971), and that neurohypophysial secretions affect spawning behaviour in the killifish (Macey, Pickford and Peter 1974) and stimulate activity of the oviduct and ovarian smooth muscles in the guppy (Heller 1972). There are two possible explanations for the stimulation of neurosecretion by synthetic LH-RH: it is possible that the stimulation of the pituitary by mammalian LH-RH may be mediated through yet another system in the neurohypophysis, and secondly this stimulation may be the result of a feedback by the maturing ovary on the hypothalamus. These highly speculative explanations require further research.
In summary, one point seems clear; a long photoperiod is essential for ovarian development in the medaka (Yoshioka 1962; 1963; Chan 1976) and this long photoperiod triggers the secretion of a hormone, probably from the hypothalamus (Peter 1973), similar in activity to synthetic LH-RH, which in turn stimulates the activity of the gonadotropic cells in the pituitary.

Effects of synthetic LH-RH and LH injections on methylmercury treated fish.

Previous experiments demonstrated that a 6-week exposure to 10.7 μg/l of methylmercury reduced spawning by about 50% compared to the control value (Fig. 8, Section II). In the present study, a further reduction of spawning by about 20% was probably due to frequent handling during the twice-weekly injections (see Figs. 8, 10 and 23).

The reduction of spawning activity by methylmercury was partially prevented by injection of LH (Fig. 23) suggesting that the gonads remained receptive to LH stimulation while exposed to methylmercury. Both hormone injected groups started spawning earlier than the saline injected groups suggesting that hormone injections accelerated gonadal development and that the fish may not have accumulated enough methylmercury during the first two weeks of exposure to block reproduction.

Gonadal maturation depends on the synthesis and release of gonadotrophin(s), and synthetic LH-RH has been demonstrated effective in doing both. In the present study, however, pituitary cytology showed a slightly different picture. Synthetic LH-RH was effective in stimulating gonadotropic cell activity in methylmercury treated fish even though spawning activity was not restored (Fig. 24 to 27). Previously, it was suggested that the
activity shown by pituitary gonadotrophs represents the net result between synthesis and release of gonadotropin(s) in GTH cells. Thus injection of synthetic LH-RH into methylmercury poisoned fish, which resulted in stimulation of gonadotropic activity with no increase in spawning activity, suggests a possible blockage in the release of gonadotropin(s). The lowering of receptivity at the gonadal level by methylmercury has been ruled out because exogenous LH was effective in removing the inhibition of spawning activity by methylmercury (Fig. 23).

David and Ramasawmi (1971) observed an increase in granulation of LH cells in the langur pituitary following cadmium-induced testicular necrosis suggesting possible blockage in the release of the hormone. Present findings show similar results with *Oryzias*. Since both cadmium and methylmercury have a strong affinity for sulphydryl groups and since in both animals the release of gonadotropin were affected, it seems possible that the action on reproduction for these two chemicals are similar. Furthermore, gonadotropin release in these two animals may well be similar, under the influence of sulphydryl group containing compound or enzyme. Since the mechanism controlling release of hormones from the pituitary in fish is not known, this suggestion is highly speculative and requires further investigation.

However, it is clear that reproduction may be partially restored in methylmercury poisoned fish with luteinizing hormone injections. In other words, the gonads of methylmercury treated fish were still receptive to exogenous luteinizing hormone stimulation. Reproductive damages incurred in nature by methylmercury contamination may thus be remedied
partially by injecting the appropriate hormone(s).

**Effect of methylmercury on in vitro ovulation**

Ovulation in non-mammalian vertebrates has been described as a process whereby follicular layers immediately surrounding the apex of the oocytes are dissociated, forming a rupture which is smaller in diameter than the oocytes, and through which the oocytes is squeezed out (Asdell 1962). In *Oryzias latipes*, this process can occur in vitro in isolated, intact follicles (Hirose 1971). The mechanism behind this process is not well understood. Pendergrass (1976) observed an increase in microfilaments in the thecal layer prior to ovulation in vitro and suggested that these microfilaments, being contractile, are involved in cell movement during ovulation. However, in vitro ovulation in *Oryzias* seems to be under the control of several gonadotropic hormones (Hirose 1971, 1972c, Hirose and Donaldson 1972) and steroids (Hirose 1972a) and the follicular envelope is indispensable for both protection and hormonal action (Hirose 1972b).

Treatment with methylmercury affects the release of gonadotropin in the pituitary while the ovary remains receptive to stimulation of luteinizing hormone. Pre-exposure of fish to 21.5 µg/l of methylmercury for six weeks reduced in vitro ovulation by about 40% even under the stimulation of exogenous LH (Table 5). The mechanism behind this inhibition is not known. It is possible that methylmercury affected the enzyme system for steroid production. Methylmercury has a strong affinity for sulphydryl groups and the enzymes involved in steroidogenesis may possess such properties. Methylmercury added directly to the incubation media further
reduced the percent in vitro ovulation for fish previously exposed to the same toxicant (Table 5). This may be possible because the enzyme system may not be completely blocked by methylmercury during the pre-treatment; thus uncubation with methylmercury in the in vitro system allowed a more complete inhibition.

A dose response was observed between percent inhibition of in vitro ovulation and the logarithm of the concentration of methylmercury used. Fifty percent inhibition occurs at about 55 µg/l of methylmercury in the incubation medium. This method provides a fast convenient system for testing effect of a toxicant on reproduction and may well develop into a bioassay for general toxicity studies.

When incubations commenced at 2200 hr or 2400 hr a very high percent of oocytes ovulated naturally without LH (Hirose & Hirose 1972). Similar results were obtained in the present study (Table 7). However, if the incubation media contained 192 µg/l or 215 µg/l of methylmercury, this reaction was reduced suggesting that methylmercury added to the medium may be inhibiting the synthesis, release or action of the hormone(s) responsible for ovulation. LH could not be one of the hormones in question, since the addition of 10 µg/ml of LH was not effective in increasing the percent ovulation (Table 7).

Corticosteroids are effective in inducing ovulation in Oryzias in vitro (Hirose 1972a, c, 1973), in Indian catfish in vivo and in vitro (Goswami and Sundararaj 1972a, b, and 1974) and in goldfish in vivo (Khoo 1974). The present experiment showed similar results. Cortisone was effective, at least partially, in overcoming the inhibitory effect of
methylmercury (Table 8). Columbo et al. (1973) demonstrated corticosteroid synthesis in *Gillichthys* and suggested that endocrine control of ovulation acts by pituitary gonadotropin stimulation of the synthesis of corticosteroids in the ovary. Sundararaj and Goswami (1974) in a coculture study showed that the interrenal contributes the major portion of corticosteroids for ovulation. However, both of these phenomena have not been shown for other species. The present study has not clarified this point. Since the addition of corticosteroid to the media can restore some of the ovulation inhibited by methylmercury, it seems possible that the follicular cells of *Oryzias* are capable of producing some corticosteroids. Hirose (1972b) also made such a suggestion in another *in vitro* ovulation study with afofolliculated oocytes. Presence of methylmercury in the medium probably blocked corticosteroidogenesis in the follicular tissues, while addition of exogenous cortisone was effective in inducing some ovulation. When both methylmercury and cortisone were present, balance of the two resulted in a slight prevention of the inhibitory effect of methylmercury as observed in the present study. It seems possible that once corticosteroid is present, even just prior to ovulation, ovulation can ultimately occur.

Sex steroids have not been effective in inducing ovulation and maturation (Goswami and Sundararaj 1972b; Hirose 1972a). The present study confirmed this. The fact that progesterone was observed to be slightly effective in stimulating ovulation and ovarian maturation in both *Oryzias* (Hirose 1972b) and the Indian catfish (Goswami and Sundararaj 1972b) was also observed in this study. However, the addition of methylmercury completely abolished this effect (Table 8). This suggests that progesterone,
a precursor of corticosteroids, cannot be converted to corticosteroids
because of inhibition of steroidogenesis by methylmercury. From the
foregoing discussion, it seems that methylmercury may be a general inhi-
bitor of enzymes, especially enzymes with sulfydryl groups. Since a
majority of proteins possess sulfydryl groups, methylmercury remains a
very potent toxicant for all living organisms.

In this section, we have shown that methylmercury acted at two levels,
pituitary and gonad, of the hypothalamic-hypophysial-gonadal axis. How-
ever, *in vitro* ovulation studies strongly suggested that methylmercury
is a cellular inhibitor of an enzyme system. This is probably true be-
cause chemical reactions form the basis of all biological activities.
GENERAL DISCUSSION

The median lethal concentration, 96hr-LC50, of methylmercury for adult *Oryzias latipes* was found to be $88 \pm 9.8 \mu g \text{CH}_3\text{Hg}^+/l$. Compared to rainbow trout, this value is quite high suggesting that *Oryzias* is a much more resistant fish.

Tissue accumulation of methylmercury increases with exposure time and concentration of the chemical in the external medium. Death seems to occur once tissue methylmercury levels reached about 25 $\mu g/g$ as methylmercury. Such levels were reached in two weeks for fish exposed to 21.5 $\mu g/l$ of methylmercury and 6 weeks when exposed to a lower concentration of 10.7 $\mu g \text{CH}_3\text{Hg}^+/l$. However, in the present study, this level was never reached even at the end of six-weeks exposure to 4.8 $\mu g/l$ of methylmercury.

Four-hour exposure of spawning fish to methylmercury during the fish's normal oviposition time affected spawning at concentrations equivalent to one-tenth of the medial lethal concentration. However, this effect was not carried over to the time when the fish were returned to clean water. Oviposition was completely abolished when exposure concentration was at the median lethal concentration. This effect was carried over to the time when the fish were returned to clean water. This short exposure of 4 hours to the median lethal concentration of methylmercury may have allowed enough accumulation of the chemical to effect a change in spawning activity when returned to clean water. This phenomenon may occur with bioaccumulative toxicants. If so, this type of study not only provides information on the toxicity but also indicates whether the toxicant in question is bioaccumulative. This suggestion is hypothetical and
requires further investigations. Since *Oryzias* has a fixed pattern of spawning which is easily quantified, this behavioral response may be useful for monitoring environmental changes like water pollution.

Long-term exposure to methylmercury at concentrations approximately equal to one-eighth of the median lethal concentrations resulted in about 50% inhibition of reproductive ability. At a lower concentration of about one-twentieth of the median lethal concentration, 40% inhibition on reproductive ability was observed. These results clearly showed that reproduction is extremely sensitive to environmental contamination, and since reproduction is indispensable for the survival of the species, it seems reasonable that studies of environmental contaminations should include more thorough examinations on reproduction of the species in question. Sometimes it may not be practical to study larger species like the salmon, but studies on fish like *Oryzias* may provide an insight into what might happen in the more valuable food fishes. For bioaccumulative toxicants, the use of "safe" factors does not seem to hold, since the "sublethal" concentration may vary with different types of bioaccumulative toxicant (Sprague 1971).

For methylmercury, it seems that the accumulation of toxicant by parent fish does not affect the hatchability of the eggs. This seems to suggest that the toxicant may not have been accumulated in spawned eggs. Since no residue analysis was performed on the spawned eggs, this suggestion remains speculative. The observation that juvenile fish are more sensitive to methylmercury than adult fish agrees with Sprague's reasoning (1971).
Synthetic LH-RH at doses of 100 µg/g and 1000 µg/g was effective in inducing ovarian development at warm temperatures (23 ± 1°C) and short photoperiods (8L/16D). When exposed to methylmercury (10.7 µg/l) even at warm temperatures (23 ± 1°C) and long photoperiods (16L/8D), inhibition of spawning activity was observed in *Oryzias*. This inhibition was not removed by the injection of synthetic LH-RH. Pituitary cytology revealed high activity in gonadotrophs as stimulated by the injection of synthetic LH-RH. This suggests a possible blockage in the release of gonadotropin(s). Partial restoration of spawning activity in methylmercury-treated fish by injection of LH suggests that the gonads were still receptive to LH. This study demonstrated effects of methylmercury on both the hypophysis and gonads but failed to show the effects of methylmercury at the hypothalamic level.

Ovulation *in vitro* was used to elucidate further the mode of action of methylmercury at the gonadal level. A log dose response was observed in the percent inhibition of ovulation *in vitro*. Fifty percent inhibition occurred at concentrations of 55 µg/l of methylmercury in the incubation medium. Once a block developed with methylmercury, luteinizing hormone was not effective in removing this block. Cortisone was the only steroid tested that was effective in restoring methylmercury-blocked *in vitro* ovulation. This observation confirms reports that corticosteroids are involved in ovulation, and that in *Oryzias* corticosteroids may be produced by the follicular layer of the oocytes. Methylmercury may have blocked the synthesis of corticosteroids in the follicular layer of the oocytes and thus blocked ovulation while the addition of exogenous corticosteroid to the medium restored some ovulation in the methylmercury treated oocytes.
The present study has shown that methylmercury, at concentrations as low as 4.8 to 21.5 μg/l as CH$_3$Hg$^+$, has detrimental effects on the reproduction in *Oryzias*. These physiological effects are of ecological importance, not only because survival will be reduced but also because other biological systems closely related to it may be impaired. *Oryzias* is more resistant than some other fish and, if methylmercury has such detrimental effects on *Oryzias*, one can expect substantially greater effects of methylmercury on other more "delicate" fishes such as salmon.

In the present study, methylmercury seems to act at two levels, the pituitary and the gonad. Though inhibition may have occurred at these two levels, "replacement therapy" seems to be effective in overcoming some of these effects. Reproductive damages occurring in nature by methylmercury contamination might thus be partially remedied by injecting the appropriate hormones. The same may be true for other physiological functions.
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