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Effects of Petroleum Hydrocarbons on Length of Incubation and Hatching Success in the Japanese Medaka[1]

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The effects of crude oil and its water-soluble fraction (WSF) on fish eggs range from direct mortality (RICE et al. 1975; MIRONOV 1969), to the retardation or acceleration of growth of embryos (KUHNHOLD 1972), to the development of morphogenic abnormalities in newly hatched larvae (STRUHSAKER et al. 1974). The specific effect depends, in part, on the concentration of crude oil and the age of the embryos at time of exposure. While testing different crude oils on various life stages of Japanese medaka, Oryzias latipes, LEUNG (1977) noted a significant decrease in length of the egg incubation period and an increase in the opercular movement of unhatched embryos. These changes and their probable relation are discussed in this paper.

The Japanese medaka is a small, oviparous freshwater killifish indigenous to areas of Japan, Taiwan, and South East Asia (BRIGGS and EGAMI 1959, KIRCHIN and WEST 1975). The fish has two characteristics that make it a useful species for determining the effects of various toxicants on reproduction: The adult female spawns daily for 3-4 months in the laboratory and thereby provides a continuous supply of test specimens for various experiments; and the chorion is transparent so that embryos can be observed during developmental stages without removal of the egg shell. The effects observed can than be tested on fish species that have a much shorter annual reproductive period, an opaque chorion, or both.

MATERIALS AND METHODS

Eggs used in our experiments were collected from the golden strain of Japanese medaka obtained from Carolina Biological Supply

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Company and raised in aquaria at 25°C. After collection, eggs were incubated in petri dishes with embryo-rearing medium (KIRCHEN and WEST 1975) before use in experiments. The medium is composed of equal parts of 10% NaCl, 0.30% KCl, 0.40% $CaCl_2.2H_2O$, 1.63% $MgSO_4.7H_2O$, and 0.01% methylene blue mixed with 95 parts of glass-distilled water. The medium was replaced daily. Length of the incubation period at 25°C is about 12 days. Plastic petri dishes measuring 1.5 cm high and 9 cm in diameter capacity, (about 80 ml) were used as the assay containers.

Crude oil used in the experiments was furnished by the Chevron Oil Company from the Birch Creek Field, Sublette County, Wyoming. Chemical and physical properties of the oil were as follows: specific gravity at 16°C, 0.809; A.P.I. gravity, 43.4°; sulphur, 0.02%; nitrogen, 0.006%; Saybolt Universal Viscosity 35.0 at 21°C, and 33.0 at 38°C; pour point, 16°C; carbon residue of residuum, 1.0% by weight; color, NPA 6; total distillate, to 150°C, 26.5% by weight, to 275°C, 67.7%, and to 300°C 93.1%; total gasoline and naphtha, 39.7% by weight. Composition of water used for mixing with oil was: specific conductance, 77 µmho/cm; dissolved solids, 529 mg/liter; phenolphthalein alkalinity, 0; total alkalinity, 283 mg/liter (as $CaCo_3$); ph, 7.0; NO_3, 0.1 mg/liter; Cl, 17 mg/liter; HCO_3, 345 mg/liter; CO_3, 0; hardness 410 mg/liter (as $CaCO_3$). Oil was added by pipette to water in a beaker and then mixed mechanically for 5 min with a high-speed (1725 rpm) beater fitted with a Teflon propeller. Mixing was violent, but within a few minutes after it was completed the oil separated from the water to form a surface slick. Water below the slick, containing only the WSF, was then removed with the aid of a separatory funnel and used in the experiments. Concentrations given in this paper are expressed in terms of the amount of oil added, not by analysis of WSF in the resulting solution.

To determine the relation between calculated and actual concentrations of WSF in fresh solutions, we added 5 ml of 50% sulphuric acid and 5 g of sodium chloride and then extracted 1-liter samples three times in carbon tetrachloride (GRUENFELD 1973). A Beckman infrared spectrophotometer Model IR 4250 was used for determinations. Known concentrations of crude oil in carbon tetrachloride provided a standard for quantification.

In the first set of experiments, 25 8-day-old eggs were placed in each of two petri dishes containing 50 ml of WSF solution at each of four concentrations--65, 87, 155, and 155 ml/l-- and were exposed for 96 h without change of the solution. Embryos in two additional petri dishes, without test solution, served as controls. No aeration was supplied because dissolved oxygen remained at 8-9 mg/liter. Dead eggs and hatched embryos were counted daily and removed. After 96 h of exposure, the eggs were transferred to clean petri dishes and maintained in incubation medium. The medium was replaced daily until hatching. Hatching time and survival of eggs exposed to different concentrations of crude oil were determined.

In the second set of experiments, eggs of different ages were exposed to 155 ml/liter of WSF. Eighteen to 25 eggs for each day of age from 0 to 9 days old were placed in separate petri dishes with test solution. Four petri dishes of eggs were used for each day of age: one control, and one each for 24-, 48-, and 96-h test periods. The solution was not renewed during the test period. After exposure,

the remaining eggs were transferred to fresh medium, renewed daily, and hatching time and success were determined. Differences were tested by chi square.

In the third set of experiments, 8-day-old eggs were placed individually in a petri dish containing 20 ml of embryo-rearing medium. Opercular movements (yawns, coughs, distinct flickers) were then counted for three 1-min periods under a optical microscope to establish a base rate of movement for each embryo. After the base rate was obtained, the test egg was transferred to a petri dish containing 20 ml test solution containing 155 ml/liter WSF. After a 10-min exposure, opercular movements were counted for three additional 1-min periods as exposure continued. Ten eggs were tested; 10 others, the controls, were tested similarly except that no oil was added to the rearing medium.

The effects on opercular movement of certain aromatic components of crude oil (benzene, toluene, and xylene) were tested by procedures similar to those for oil except that, after obtaining the base rate for each embryo, we added the chemical directly to the surface of the rearing medium to produce a calculated ratio of 100 µl/liter. Opercular movement of the test embryo was then counted for 1 min at about 5-min intervals for up to 40 min. Three eggs were tested for each control and each experimental group.

RESULTS

Concentrations of WSF in freshly mixed solutions maintained a nearly linear relation with quantities of crude oil used over the range of test concentrations. The ratios of concentrations (ml/liter) of crude oil to WSF at the beginning of experiments were: 65 vs. 0.090, 87 vs. 0.120, 115 vs. 0.168, and 155 vs. 0.206. The amount of WSF in solution during and at the end of individual test was not determined, but response of the embryos indicated that it was considerably less than at initial exposure.

Hatching success of 8-day-old embryos (Figure 1) was not affected by exposure to WSF of Sublette, Wyoming crude oil for 96 h (at days 8-12). The only mortality of test embryos was one specimen each in concentrations of 65 and 155 ml/liter. Control eggs began hatching on the 12th day of incubation, whereas exposed eggs began 1-3 days earlier. By the time the first control egg hatched, 48% of eggs exposed to the 155 ml/liter solution had hatched, 26% in the 115 ml/liter, and 13% in the 87 and 65 ml/liter solutions. Hatching was also completed 2 to 4 days earlier in all experimental groups than in control groups. In terms of 50% hatch, there was little difference, however, among concentrations. The controls and eggs exposed to all concentrations, except 155 ml/liter, required 15 days to 50% hatch; the group exposed to 155 ml/liter required only 13 days. Average length at hatching was about 4.0 mm for early hatching medaka as compared with about 4.5 mm for control larvae. The yolk mass appeared to be larger in the early hatching embryos (however, no measurements were made).

When embryos of different ages (0-9 days old) were immersed in WSF of crude oil at a concentration of 155 ml/liter for 24, 48, and 96-h, time to 50% hatch was usually shorter than for controls (Table 1). The notable exception was eggs exposed during the first day after being laid (day 0). Fifty percent of both control eggs and day 0 eggs

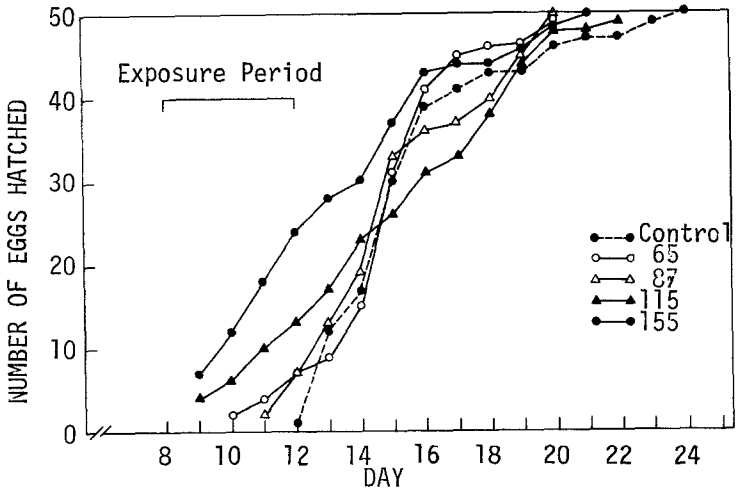

Figure 1. Hatching time of 50 medaka eggs exposed after 8 days incubation to fresh water-soluble fractions of Sublette, Wyoming, crude oil for 96 h at 25°C.

TABLE 1

Difference in days to 50% hatch between groups of control and test eggs exposed to 155 ml/liter WSF of Sublette crude. Each figure represents 18-25 eggs.

Age of Egg (days)	Length of Exposure (h)		
	24	48	96
0	0	1	2
1	-6[a]	-4	-3
2	-2	-2	-2
3	-3	-7	-5
4	-5	-6	-5
5	-1	-2	0
6	-2	-3	-3
7	-2	0	-1
8	-2	-2	-3
9	-3	-4	0

[a] 50% of control eggs hatched by 18 days; 50% of eggs exposed for 24 h after first day of incubation hatched by 12 days (12-18 = -6 days).

exposed for 24 h hatched 12 days after fertilization. Eggs exposed for an additional 24 h required 13 days for 50% hatch, and eggs exposed for an additional 48 h (96-h total) required 14 days. Statistical comparison based on total chi square for the 10 age groups revealed significantly shorter incubation time ($P < 0.05$) than for controls for eggs exposed for 24 and 48 h, but not for those exposed 96 h.

Embryos were examined more closely during exposure in an effort to determine a reason for the shortened incubation period. Visual

observation of the embryos suggested that respiratory activity was increasing. A significant increase in opercular movement was observed when 8-day-old eggs were exposed to WSF at a concentration of 155 ml/liter (Table 2). Movements increased from 0.13 per min before exposure to 4.33 per min after 10 min of exposure. Mean number of movements per minute was significantly different ($P < 0.01$, $t = 8.4$, d.f. = 58). Opercules of test embryos moved much more frequently than those of control embryos ($P < 0.01$, $t = 7.8$, d.f. = 58). Opercular movement was deep and irregular. The embryos also showed signs of stress during the exposure period, such as shallow, but increased, rate of mouth movement and periodic rapid fluttering of pectoral fins.

Exposure to 100 µl/liter of benzene, toluene, and xylene caused an increase in rate of opercular movement of 8-day-old embryos (Table 3), but the increase was significant only for benzene ($P < 0.05$, $t = 2.7$, d.f. = 8). Magnitude of the increased movement varied tremendously over the exposure period (Figure 2). Rates of opercular movement in embryos immersed in toluene and xylene were sometimes 3 to 4 times greater than in the controls, but differences were not statistically significant.

TABLE 2

Mean number of opercular movements per minute of 8-day-old medaka embryos after exposure for 10 min to 155 ml/liter WSF of Wyoming crude oil. Standard deviation is in parentheses.

Exposure Concentration	Mean number of opercular movements			
	Base line[a]		Test[b]	
Control	0.00	(0.00)	0.20	(0.55)
155 ml/liter	0.13	(0.25)	4.33	(3.84)

[a] Average of three 1-min counts before exposure to WSF.

[b] Average of three 1-min counts beginning 10 min after exposure started (i.e., 10, 11, 12, min of exposure period).

TABLE 3

Mean number of opercular movements per minute of 8-day-old medaka embryos exposed to 100 µl/liter of three crude oil aromatic components. Standard deviation is in parentheses.

Toxicant	Mean number of opercular movements			
	Base line[a]		Test[b]	
Control	0.00	(0.00)	0.01	(0.68)
Benzene	0.55	(0.00)	8.46	(2.63)
Toluene	0.00	(0.00)	2.28	(2.26)
Xylene	1.22	(0.88)	3.70	(2.69)

[a] Average of three 1-min counts before exposure to toxicant.

[b] Average of eight 1-min counts taken at ca 5-min intervals for 40 min of exposure.

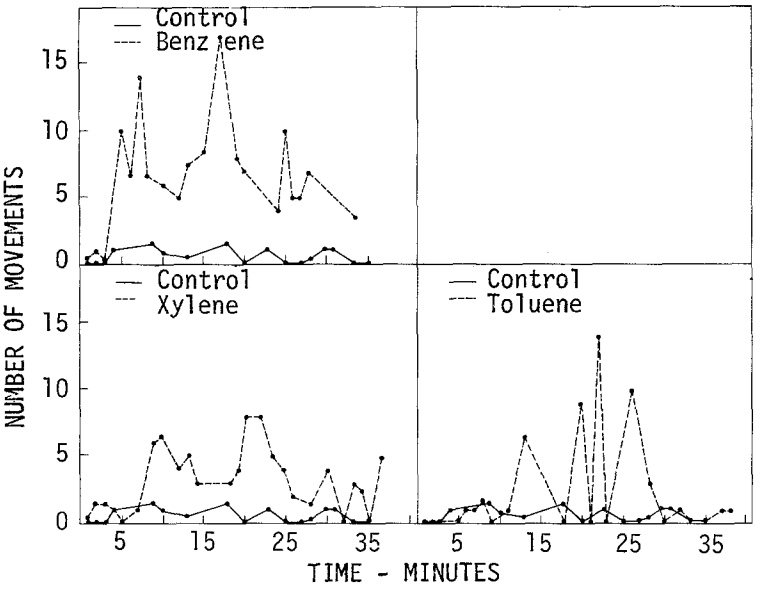

Figure 2. Opercular movement of medaka embryos in 8-day-old eggs after exposure to three aromatic components of crude oil.

DISCUSSION

Exposure to medaka eggs (other than newly fertilized ones) to water-soluble components of Sublette crude oil resulted in a reduction in incubation time of as much as 33% (12 vs. 18 days). KÜHNHOLD (1972) found that exposure of cod eggs (Gadus morhua) to Venezuelan and Iranian crube oil extracts caused delayed hatching. Most of the larvae that hatched had deformed bodies or abnormal flexures of the tail. STRUHSAKER et al. (1974) reported that hatching was delayed in herring eggs (Clupea pallasi) exposed to 45 ppm benzene for 48 to 96 h and that the larvae were deformed in various ways. In contrast, ANDERSON et al. (1977) reported that petroleum hydrocarbons stimulated hatching of eggs of Fundulus simulus at lesser concentrations. No detailed mechanism was discussed. The contrasting results reported in these studies could be explained on the basis of different concentrations of the water-soluble components (STRUHSAKER et al. 1974). In additional studies with benzene, not reported here, we found that we could stimulate hatching with low concentrations of benzene, and depress heart beat and opercular movement and produce a narcotizing effect at greater concentrations, which presumably would delay hatching.

Premature hatching as demonstrated with medaka eggs probably is seriously detrimental to survival of wild larvae because they are less developed than are individuals that incubate full term. Early hatching frequently produces smaller larvae with larger-than-normal yolk sacs, as we believe we observed among medaka larvae. Larvae would thus spend a longer time with a fragile yolk sac and, because of their small size, would be subjected to predation for a longer time until reached the size of larvae incubated under normal conditions. Once hatched, larvae also are more susceptible to environmental

pollutants than are eggs (LINDEN 1974, SCHIMMEL et al. 1974, STRUHSAKER et al. 1974). LEUNG (1977), using the method of mixing crude oil and water described in this paper, found that concentrations of WSF that were tolerated for 96 h by eggs were lethal to newly hatched medaka larvae.

An explanation for the premature hatching of eggs of medaka, and perhaps other species, and the mechanism involved is now available. RICE et al. (1977) reported that exposure of fry of pink salmon (Oncorhynchus gorbuscha) to WSF of Cook Inlet and Purdhoe Bay crude oils at sublethal concentrations resulted in an elevated metabolic rate. They proposed that the increased rate of metabolism, reflected by greater oxygen consumption, results from efforts of the body to metabolize and excrete the absorbed hydrocarbons. BROCKSEN and BAILEY (1973) postulated a similar reason for increased respiration rate of oil-exposed fish. The shorter incubation period of medaka exposed early in the developmental period could therefore result from stimulation of metabolism of the young embryo.

The mechanism of effect from exposure to WSF on hatching time of older medaka embryos is better documented. Premature hatching of advanced eggs results from stimulation of the hatching mechanism by oil components. The hatching glands of medaka are first found (ISHIDA 1944a) at the stage of melanin formation in the eye (3-4 day embryo at 25°C). The glands are found within the mouth and pharyngeal cavities and on the inner surface of the operculum of the embryo. By the time eggs are 5 to 6 days old (25°C), hatching glands contain enzymes capable of dissolving the chorion. Under normal conditions, forceful opercular movement starts shortly before hatching and initiates a current of water flowing from mouth to the gill openings. The pressure of this current ruptures the hatching glands. The released enzymes perforate and soften the chorion. The embryo then straightens its body, ruptures the chorion, and springs out of the membrane. The hatching process is thus completed. ISHIDA (1944a, 1944b) precipitated opercular movement and premature hatching by exposing medaka eggs to M/8 KCl and M/4 NaCl. Other workers have found an increase in rate of opercular movement or "coughing" of fish from exposure to WSF of crude oil (ANDERSON et al. 1974, BROCKSEN and BAILEY 1973, STRUHSAKER et al. 1974, THOMAS and RICE 1975, RICE et al. 1977). Thus, premature hatching of advanced medaka embryos to WSF of crude oil probably results from rupture of the hatching glands by opercular movement brought on by stimulation of respiration or irritation from the soluble organocarbons. This mechanism of effect needs to be examined in commercially important fish species exposed to oil-contaminated water because small premature fish larvae are relatively vulnerable to the external environment and are more susceptible to pollutants than are embryos still within the egg.

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