PHEROMONAL STIMULATION AND METABOLITE INHIBITION OF OVULATION IN THE ZEBRAFISH, *BRACHYDANIO RERIO*[1]

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ABSTRACT

Female zebrafish, *Brachydanio rerio*, would not ovulate in the absence of males in waters previously inhabited by the fish. Chemical presence of males and fresh, dechlorinated tap water each induced ovulation in about half of the trials. Application of the two factors in combination gave 100% ovulation. These results suggest that in the zebrafish a pheromone released by the males stimulates ovulation and that metabolites produced by the fish repress ovulation. It is postulated that metabolites restrict spawning of the fish to the rainy season and that the pheromone functions in synchronizing reproductive readiness between sexes or in conserving courtship energy expenditure.

In a study concerning egg size, incubation period, and growth in the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan), we encountered the problem of having to strip eggs from the females to synchronize the fertilization of the eggs artificially. We tried the method described by Hart and Messina (1972) without successes. We have regularly been able to induce natural spawning by introducing ripe individuals of both sexes from their holding tanks at 27°C together into a bowl of fresh, dechlorinated tap water at 21°C. Some of the changes associated with this introduction, such as the chemical presence of the males (pheromone), the physical presence of the males (visual, auditory, tactile, and lateral line), fresh tap water (absence of accumulated metabolites), and temperature shock (from 27° to 21°C), may be capable of inducing ovulation.

The roles of these factors in controlling reproduction in fishes have been well documented. Some of the examples are:

Pheromones–Aronson 1945; Tavolga 1956; Amouriq 1965; Gandolfi 1969; Rossi 1969; and Chien 1973.

Visual–Aronson 1945, 1965; Tavolga 1956; Rossi 1969; and Chien 1973.

Auditory–Tavolga 1956; Brawn 1961; Gray and Winn 1961; and Myrberg and Spires 1972.

Tactile–Egami and Nambu 1961.

Metabolites–Swingle 1956; and Greene 1966.

Temperature–Harrington 1959; Aronson 1965; and de Vlaming 1972a, b.

Much of the information in the literature, however, does not clearly distinguish between gonad development, ovulation, and spawning. The present study was undertaken to single out such ovulation-inducing factors.

MATERIALS AND METHODS

Female zebrafish were kept at 27.0 ± 1.0°C in 40- or 60-liter aerated aquaria subdivided into three or four compartments by perforated plastic dividers, one female per comparment to enable identification. Male zebrafish were isolated in aerated 20-liter aquaria at room temperature (21.0 ± 1.0°C). No visual contact was permitted between sexes. All individuals were subjected to 12 h of light per day and were generously fed "Tetramin"[3] in the morning and frozen brine shrimp in the evening. To ensure fertility, each fish was initially permitted to spawn naturally. This was done by introducing the fish into fresh, dechlorinated tap water at room temperature in a 20-cm finger bowl with another individual of the opposite sex. A total of 27 fertile females and 19 fertile males were used.

Eight experiments were designed to test the relative contribution of each factor individually, and in combination, on ovulation in the zebrafish (Table 1). In experiments 1 to 7, a female was transferred from the holding compartment into

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TABLE 1.—Design and results of experiments to test factors suspected of influencing ovulation in the zebrafish. Symbol "+" indicates presence of the factor and symbol "0" indicates absence of the factor. The chi-square values are for comparing results of any experiment with that of the control (experiment 8), and the 0.01 level critical limit is 6.63.

Item	Experiment								
	1	2	3	4	5	6a	6b	7	8
Factors:									
Pheromone	+	+	+	+	0	0	0	0	0
Temperature shock	0	+	+	+	+	+	+	0	0
Fresh tap water	+	+	+	0	+	+	+	+	0
Visual image of male	0	0	?	0	+	0	0	0	0
Auditory and/or lateral line stimulation	0	0	+	0	0	0	0	0	0
Number of females used	18	14	19	14	9	19	18	16	14
Results:									
Trials eggs obtained	20	15	21	8	5	17	10	7	0
Trials no eggs obtained	0	0	1	8	5	18	13	10	17
Chi-square	33.1	28.1	31.4	8.7	7.4	10.2	7.7	6.5	—
% positive responses	100	100	95	50	50	49	44	41	0

the experimental chamber for 12 to 18 h in nearly all the trials but for only 4 h in several instances in experiment 2, and then stripped by applying gentle pressure onto the abdomen. The release of ripe ova indicated that the fish had completed ovulation. Failure to give eggs, or the release of immature or ruptured eggs, was considered a negative response. Ripe ova are nearly translucent, round, and about 0.8 mm in diameter before taking on any water, and are not attached to each other. Immature ova are often opaque, may be undersized and irregularly shaped, and are often in clumps. In experiment 8, the female was stripped immediately upon removal from the holding compartment.

After successful stripping, the female was immediately permitted to spawn naturally with a male in order to assure release of all ovulated ova. After approximately 4 to 8 h, the male was removed, and the female was returned to her compartment the following day. Between experiments each female was allowed to rest for 8 to 12 days, and males 4 to 6 days.

After an unsuccessful attempt of stripping eggs, the female was presented with either male pheromone and/or fresh tap water, whatever was lacking originally in the experimental chamber, and then stripped again 4 to 18 h later. If the second stripping failed again, then the female was permitted to spawn naturally with a male. In cases where natural spawning failed, data pertaining to that female were rejected. This procedure assured that an unsuccessful attempt at stripping a female of eggs was due to the subjected treatment and not due to an unripe condition of the female.

Trials of different experiments were alternated randomly without any definite chronological sequence.

The experimental chambers in experiments 1, 2, 5, 6, and 7 were 5-liter all-glass aquaria. In experiment 3, 20-liter aquaria were used and were partitioned into two halves, one the experimental chamber and the other the male chamber. The partitionings were done with 1-mm thick opaque plastic divider perforated with holes 1.5 mm in diameter and 3 mm apart. In experiment 4, a 60-liter aquarium was used and was partitioned with nonperforated black plastic dividers into a 30-liter metabolite chamber, a 15-liter male chamber, and a 15-liter experimental chamber. Water was circulated from the metabolite chamber into the male chamber, and then to the experimental chamber and back to the metabolite chamber by means of pumping and siphoning.

The presence of the male pheromone in experiments 1 and 2 was established by air lifting into the experimental chambers water from a 5-liter aquarium into which a male was introduced simultaneously with the introduction of the female into the experimental chamber, the water then was siphoned back into the male aquarium. In experiments 3 and 4, male pheromone was provided by placing, during the experimental period, a male into the male chamber which was chemically continuous with the experimental chamber because of the perforations or water circulation.

The presence or absence of temperature shock was established by maintaining the experimental chamber respectively at room temperature (21.0± 1.0°C) or at the temperature of the holding compartments (27.0 ± 1.0°C).

The absence of metabolites in experiments 1, 2, 3, 5, 6, and 7 was attained by filling the experimental chamber and the male chamber with fresh, dechlorinated tap water. In experiment 4, metabolites were presented by conditioning the system for at least 2 wk with 75 mature zebrafish of both sexes in the metabolite chamber which is

chemically continuous with the experimental chamber because of the water circulation.

Visual stimuli from a male were provided in experiment 5 by allowing the test female to be in visual contact with a mature male in a separate, chemically discontinuous all-glass aquarium. In experiment 3, the perforations of the divider partitioning the male chamber and the experimental chamber provided questionable visual stimuli from the male. In all other experiments (1, 2, 4, 6, and 7), visual stimuli from males were screened by visually isolating the experimental chambers with cardboard or black plastic sheet.

Possible auditory and lateral line stimuli from male were allowed in experiment 3 through the perforated condition of the partition separating the experimental chamber from the male chamber.

In experiment 6a, the experimental chamber was a simple 5-liter all-glass aquarium, whereas in experiment 6b a current similar to that in experiments 1, 2, 4, and 7 was provided by air lifting and back-siphoning of water between the experimental chamber and a vacated 5-liter aquarium.

Prior to each experiment, the glass aquaria and hoses were scrubbed, soaked, and thoroughly rinsed with tap water. Experiments testing the pheromonal responses utilized a different set of hoses from those used in experiments lacking the male pheromones. Different nets were used for netting males and females as a precaution against contacting a test female with the slime of a male.

RESULTS

The number of positive responses (trials eggs obtained) and negative responses (trials no eggs obtained) are given for each of the eight experiments in Table 1. The results of each experiment were compared with those of experiment 8 and the chi-square value was calculated. The percent of trials resulting in a positive response is also given for each experiment.

It is apparent from the results that presence of pheromone and absence of metabolites are the two most influential factors stimulating ovulation in the zebrafish. Experiments 1, 2, and 3 in which pheromone was provided and metabolites were absent invariably gave nearly 100% ovulation. Experiments 4, 5, 6, and 7 in which either pheromone was provided or metabolites were absent gave 40-50% ovulation. However, in experiment 8 in which pheromone was absent and metabolites were present, no ovulations were observed. The roles of metabolites and male pheromone are further indicated by the fact that females which initially responded negatively in experiments lacking the male pheromone and/or fresh tap water (experiments 4, 5, 6, and 7) gave eggs in the second stripping in all cases upon being presented with the missing factor(s).

None of the other factors tested, including temperature shock and auditory and/or lateral line stimulations seem important in controlling ovulation, as in no cases did their presence or absence significantly alter the results. The influences of water movement between the experimental chamber and the male chamber were insignificant, as there is no difference between results of experiments 6a and 6b.

Successful stripping was recorded at all times over the morning, afternoon, and early evening. After the stripping, without exception, a pair would commence natural spawning immediately upon introduction regardless of the time of day.

DISCUSSION

In the absence of the male pheromone and the presence of the metabolites, females consistently failed to ovulate (experiment 8). Under similar condition, Eaton and Farley (1974) also failed to strip eggs from isolated females. Histological studies of zebrafish ovaries by Hisaoka and Firlit (1962) indicated that oocytes are not released from the ovarian stroma into the central lumen and oviducts (ovulation) until stimulated by males during the breeding process. Eaton and Farley (1974) were able to obtain ripe ova from isolated females in the morning only after a brief (7 h) introduction of a male into the female's tank on the previous day. They suggested that the vigorous chasing behavior exhibited by the male toward the female might have provided the stimulus, although no supporting data were provided.

The results of the present study clearly establish that a male pheromone stimulates ovulation of the female. Little is known about the nature of the male pheromone. It appears not to be species specific since circulation of water between aquarium containing male *Brachydanio albolineatus* and aquarium containing female *B. rerio* elicited ovulation in the female in four out of four trials. Intrageneric interspecific effec-

tiveness of sex pheromone has also been demonstrated by Rossi (1969) in *Colisa lalia* and *C. labiosa*. In this case a female pheromone can induce nest building in heterospecific males.

As to the chemical nature of piscine sex pheromones, Amouriq (1965) identified an estrogen as the pheromone inducing hyperactivity in male *Lebistes reticulata*, and Tavolga (1956) identified the internal fluid of the ovary as the source of the chemical stimulus eliciting courtship behavior of male in *Bathygobius soporator*. In the present study, of six trials consisting of placing test females into fresh tap water previously occupied for 24 h by a male, only four positive responses were recorded. Since the metabolites produced by a male in 24 h are far below the threshold level for inhibiting ovulation, as we have experienced and as Greene (1966) has reported, the two failures out of six trials in the above experiment suggest either that the male pheromone is short lived or that less male pheromone was released in the chemical absence of the female.

The selective advantage of an ovulating pheromone in the zebrafish is not clear. Although Hart and Messina (1972) claimed to be able to obtain sperm from male zebrafish at all times under laboratory conditions, we often encountered unsuccessful milking of males. If both sexes are not sexually ready at all times, it would be advantageous to synchronize sexual readiness between sexes. If release of the ovulating pheromone corresponds with male readiness, synchronization would be guaranteed. However, males appeared to release the pheromone quite regularly, even while in the presence of metabolites as suggested by the 50% ovulation in experiment 4. Yet, it is possible that while the metabolites may repress testicular development or spermiation and the release of the pheromone by the males, the test male introduced at the same time as the test female into the two small chambers of experiment 4 was often already sexually ready at that time and would thus release the pheromone regardless of the presence of metabolites.

Such a pheromone, if functioning in synchronization, would be advantageous for a species with a long spawning interperiod. Although female zebrafish can spawn every 1 or 2 days under laboratory conditions (Eaton and Farley 1974), the spawning interperiod in the native habitat is not known.

In some fishes, it is possible that the active chasing of the female by the male prior to spawning takes part in stimulating ovulation. An ovulating pheromone would conserve such chasing energy and therefore, be selectively advantageous.

The inhibitory effect of metabolic wastes on fish reproduction has been reported by Greene (1966) who found that an increase in metabolite concentration resulted in a decrease in the number of successful natural spawnings in the zebrafish. Lin (1935) observed that grass carp, *Ctenopharyngodon idellus*, would spawn only after a rise in the river water due to rain. Similar observations confirming the coincidence of heavy rain and spawning have been made by von Ihering and Wright (1935) and Lake (1967). Lake suggested that the stimulatory effect of rain on fish spawning was through addition of soil elements through runoffs. However, according to Swingle (1956), draining a pond crowded with goldfish or largemouth bass and refilling it subsequently with new water could induce spawning in the pond fish. One of us (Chen) had observed on numerous occasions that goldfish spawned during or after rain in outdoor concrete tanks. In these cases, spawning occurred without input of soil elements. Swingle (1956) suggested that the effect of rain was to dilute a spawning repressive factor. It is obvious from the present experiment that this repressive factor is metabolites.

Tang (1963) noted that the testes of silver carp, *Hypophthalmichthys molitrix*, would develop only after the volume of the reservoir had been increased by rain, thus suggesting that maturation of testes may be retarded by waste products from fish and that new water, or dilution of these wastes, is necessary for sexual development. It is possible that removal of the metabolites can also induce the release of the pheromone by the male zebrafish, indirectly stimulating ovulation in the females. In the present study, however, removal of the metabolites apparently had a direct effect on the females, as mere exposure of the females to fresh tap water resulted in ovulation in nearly half of the trials (experiment 7). Tang (1957) reported that female common carp inhibited from spawning by metabolic wastes would release eggs in the absence of males upon introduction of new water.

The chemical nature of the inhibiting metabolites is not known. Greene (1966) believed that they were ammonia. From the observation made by Swingle (1965) that crowding of bluegill inhibited spawning in the largemouth bass in the

same pond, the inhibiting metabolites cannot be species specific.

As discussed earlier, many of the freshwater fishes in the tropics spawn only in the rainy season. During this period, there is an addition of flooded lowland suitable for the deposition of eggs, an increase in the dissolved oxygen favorable for embryological development, and an increase of organic and inorganic nutrients which promote growth of food plankton. Rain would also dilute any metabolic wastes accumulated during the dry season. In this context, metabolites may serve as a controlling factor, repressing ovulation until the rainy season when environmental conditions are more favorable for both embryo development and larval growth.

The results of the present study clearly indicate the stimulatory effect of the male pheromone and the inhibitory effect of metabolites on ovulation in the zebrafish. As gonadotropin is known to be effective in inducing ovulation in fishes, either directly, or via stimulating the synthesis of corticosteroids and/or progesterone (Donaldson 1973; de Vlaming 1974), the action of the ovulating pheromone and the metabolites is probably to activate or to deactivate the hypothalamus-pituitary-gonad axis. A pheromonal facilitation of gonadotropin-induced ovulation has been reported in mouse (Zarrow et al. 1973). Further studies are needed to clarify the route of action of the pheromone and the metabolites.

Aronson (1965) cited numerous examples in fishes in which gonadal development and subsequent spawning were stimulated by either an increase or a decrease in temperature. An increase in temperature has been reported to affect the gonadal response to treatment with gonadotropin in *Lepomis cyanellus* by Kaya (1973) and in *Gillichthys mirabilis* by de Vlaming (1972c). In the present study, a sudden decrease in temperature alone does not seem important in stimulating ovulation in the zebrafish.

In the zebrafish, visual or auditory and lateral line stimuli between sexes do not seem important in enhancing ovulation, although some of these factors may be pertinent in eliciting the proper behavior during the actual spawning act.

The onset of light alone is not sufficient to stimulate ovulation, as demonstrated by the complete failure to strip eggs during the morning hours from females tested directly from their holding compartments (experiment 8). Furthermore, these females were stimulated to ovulate later that day, after exposure to the male pheromone and fresh tap water. Ovulation and natural spawning were induced regardless of time of day. One of us (Chen) has observed natural spawning of zebrafish to commence at midnight in darkness and continue for hours. These observations conflict with all previous accounts that the onset of light is important to trigger ovulation and spawning in the zebrafish (Legault 1958; Hisaoka and Firlit 1960; Eaton and Farley 1974).

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