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## The role of urine in sex discrimination in the goldfish *Carassius auratus*

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### Abstract

Pheromones are important chemical cues for sex discrimination and spawning behavior in goldfish. Urine of mature males and females, and that of gonadectomized goldfish treated with estradiol 17 $\beta$  (E<sub>2</sub>) was tested whether or not it would induce prespawning behavior of pecking or nudging using blinded males. Chasing behavior of blinded males was observed when they were paired with ovariectomized females treated with E<sub>2</sub>. It was concluded that urine of mature females contains a chemical cue or pheromone which stimulates male prespawning behavior that includes the pecking, nudging, or chasing of females. This pheromone is female specific and is released by the kidney under the control of estradiol 17 $\beta$ . Estrogen in females, and androgen in males, have important roles in sex discrimination in the spawning behavior of goldfish.

It is well established that newly ovulated goldfish release pheromones which stimulate the spawning behavior of males. Stacey et al. (1988) and Sorensen et al. (1989) have substantiated the pheromones from goldfish in a series of their works. They proposed a dual hypothesis. First, that 17 $\alpha$ -20 $\beta$  dihydroxy-4-pregnen-3-one (17, 20 $\beta$ p) has a priming action to stimulate GTH secretion from males. This results in increasing milt volume. Secondly 15 K prostaglandin F<sub>2 $\alpha$</sub>  has a releasing action that stimulates male spawning behavior. This hypothesis indicates that sex discrimination of males toward females is done through two female secreted pheromones. The first primer pheromone is supposedly secreted from ovaries and it is not clear from what organ the second pheromone is released. Yamazaki and Watanabe (1979) reported that hypophysectomized male goldfish treated with estradiol 17 $\beta$  were chased by hypophysectomized male goldfish that had been treated with methyltestosterone. They suggested that the origin of the pheromone which attract males is not from the ovary but from the kidney through urine excretion.

In this paper, the role of urine in sex discrimination is analyzed using eyectomized male goldfish.

### Materials and Methods

The materials used in the present study were two year old goldfish *Carassius auratus*. All males used in the experiments were eyectomized. The eyectomy operation was carried out without anesthesia. Epithelial membrane connected with the tunica fibrosa bulbi was cut with scissors circularly around the eye ball.

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Scissors were then inserted into the eye cavity along the sclera of the anterior part of the eye. Then the optic nerve was cut, and the eye ball was removed from the cavity. Both eyes were removed continuously. The operation required only a few minutes. The eye cavity closed and healed within three weeks.

A glass aquarium (30 cm × 45 cm × 30 cm) was used to observe fish behavior. The six eyectomized males were put into the aquarium. They were fed occasionally with a diet containing 10 ppm methyltestosterone. A float with a fine pipette was set on the surface of the water in the aquarium.

Twenty  $\mu$ l urine, collected from experimental fish, was poured into the pipette. Then, 3 ml of rearing water was gradually added into the pipette to force the urine to flow into the aquarium. Urine was collected from the following 4 fish groups containing 2-3 individuals each. 1: mature males, 2: mature gravid females, 3: ovariectomized females fed commercial diets containing 10 ppm estradiol 17 $\beta$  for about 2 weeks before urine collection. 4: castrated males fed diets containing 10 ppm estradiol 17 $\beta$  also for about 2 weeks before urine collection.

During urine collection, the region of the urino-genital pore was washed with clean gauze. Then, a pointed capillary was inserted a few mm into the urinary duct. Urine flowing into the capillary was collected 2-3 times a day.

Gonadectomies were conducted using an operation board to fix the fish anesthetized with MS 222. The left side of the belly was cut with scissors from the anterior part of the peritoneal cavity to the posterior just before the urino-genital pore. Then the gonadal lobe of the left side was carefully removed. The operated fish were transferred without stitches into a clean aquarium. The same operation was done on the right side of the belly to remove a right side gonadal lobe after the left side operation had completely healed. If the operation of both sides was done at one time, most fish did not survive the operation. However, since the operations were done separately, no fish died and were then used as gonadectomized fish.

Observation of behavior of six blinded male goldfish that had been treated orally with 10 ppm methyltestosterone, was done for 15 minutes after the 20  $\mu$ l urine was dropped into the aquarium. The male fish were stimulated and swam around actively. Then they displayed pecking, or nudging around the belly of the other males with the tip of the mouth. The number of the pecks, or nudges against others were counted during a 15 minute observation period. This activity is considered a pre-spawning response for these six males.

The water of the aquarium was changed with running dechlorinated tap water passed through a calcium sulphite column. The water temperature ranged between 18°-23°C. If the behavioral change was induced by urine, the next experiment was not conducted until several hours later. This was done after confirming all males had become quiet and no pecking or nudging behavior was observed.

Three ovariectomized fish were treated orally with 10 ppm estradiol 17 $\beta$  ( $E_2$ ) for three weeks. At the start of feeding  $E_2$ , each ovariectomized fish was paired with a spermated and eyectomized male having clear secondary sexual characters of pearl organs on the operculum. The total duration of chasing behavior of blinded males was measured during a 15 minute observation period.

**Results**

*1. Urine of mature males and females*

The control responses of blinded male fish to the experimental aquarium rearing water are shown in Fig. 1. Observations were repeated 4 times as blank tests. In these four observations, 3-5 instances of pecking behavior were observed. This behavior would be random phenomena, without any significant meaning.

The responses to the urine of intact mature males are shown in Fig. 2. Between two to five peckings were observed during the observation periods. This response was not significantly different from the control responses. The responses to urine of

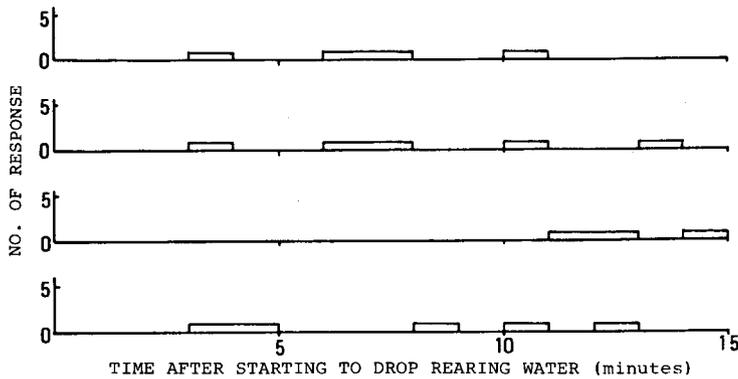


Fig. 1. Frequency of pre-spawning response of blind mature males to rearing water (Blank test).

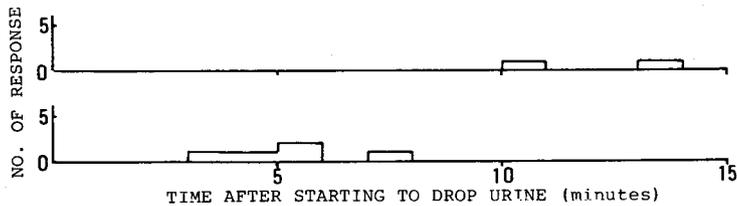


Fig. 2. Frequency of pre-spawning response of blind males to diluted urine of mature males.

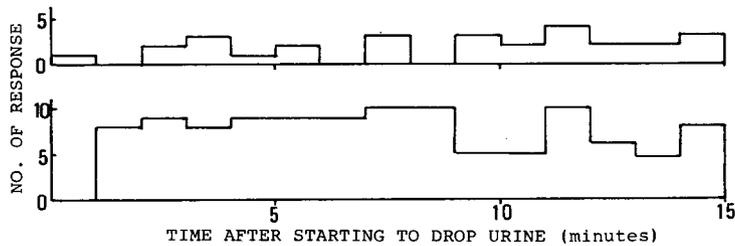


Fig. 3. Frequency of pre-spawning response of blind males to diluted urine of mature females.

mature gravid females are shown in Fig. 3. Conspicuous responses were observed for the urine of gravid females. The total numbers of prespawning behavior of pecking or nudging were 28 and 111 times, respectively. The maximum responses of pecking or nudging behavior were recorded as 10 times per minute. Blinded males were stimulated by gravid female urine to chase each other. The chased males moved quickly from the fish.

2. *Urine of castrated and ovariectomized fish treated with estradiol 17 $\beta$*

The responses to the urine of two castrated males treated with estradiol 17 $\beta$  (E<sub>2</sub>) are shown in Fig. 4. The total responses of blinded males to the urine of castrated males treated with E<sub>2</sub> was 20 and 7, respectively.

Their response was greater than that of the controls (Figs. 1 and 2). The response to the urine of three ovariectomized females treated with E<sub>2</sub> are shown in Fig. 5. Responses were induced 2-6 minutes after introducing the urine. The total number of responses to the urine were 28, 39, and 19, respectively. These responses clearly indicate that blinded males can identify the urine of fish treated with E<sub>2</sub>. As a result, they start to peck and chase the other fish.

3. *Chasing behavior*

Males chasing females are characteristic prespawning behavior in goldfish. This behavior continues several hours intermittently before spawning or oviposition. Observation of this chasing behavior of blinded mature males toward ovariectomized females before and after oral treatment of E<sub>2</sub> was made on three fish. The total

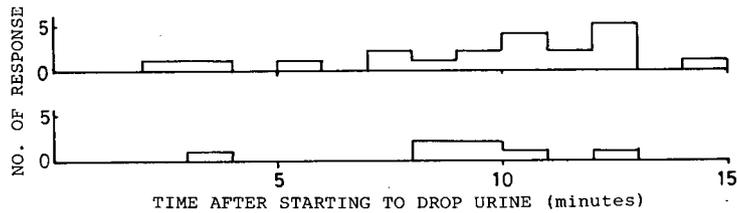


Fig. 4. Frequency of prespawning response of blind males to diluted urine of castrated males treated with estradiol 17 $\beta$ .

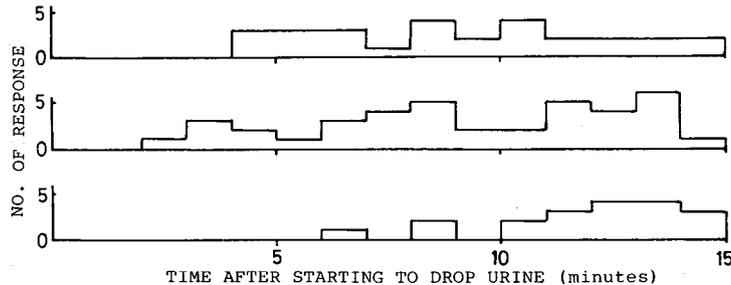


Fig. 5. Frequency of prespawning response of blind males to diluted urine of ovariectomized females treated with estradiol 17 $\beta$ .

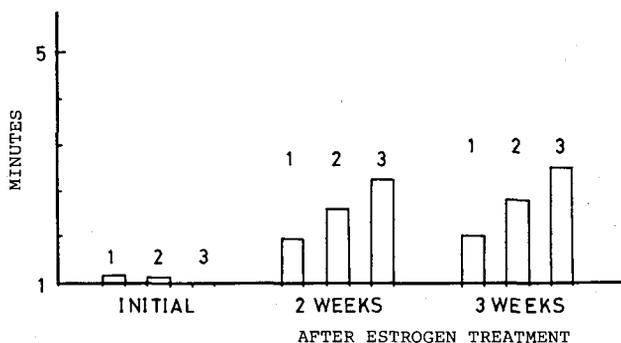


Fig. 6. Total duration of chasing behavior of blinded mature males toward ovariectomized and estradiol  $17\beta$  treated females during a 15 minute observation period. Fish are numbered in the figure.

chasing behavior duration during the observation period is shown in Fig. 6.

Initially, fish No. 1 and No. 2 were chased 10-12 seconds in total but fish No. 3 was completely ignored. At 2 weeks after the  $E_2$  treatment, the duration of chasing behavior of blinded males increased greatly. The behavior was especially prominent toward No. 3 fish after  $E_2$  treatment. This fish was not chased by paired mature male at the starting of the  $E_2$  treatment. However, two weeks after the treatment, this fish was chased 2.3 minutes in total during the observation period. Almost the same results were obtained 3 weeks after  $E_2$  treatment (Fig. 6)

### Discussion

When fish spawn, pheromones may participate in functions that contribute to sex discrimination, courtship, or mating behavior (Liley, 1982). The site of pheromone production in females appears to be ovaries in gobiid fish (Tavoluga, 1956), pondsmelt (Okada et al., 1978), ayu (Honda, 1979), guppy (Meyer and Liley, 1982), rainbow trout (Honda, 1980) and in other fish (Stacy et al., 1986). However, Yamazaki and Watanabe (1979) reported that hypophysectomized male fish which had been treated with estradiol  $17\beta$  ( $E_2$ ) were chased by methyltestosterone treated hypophysectomized male fish. This suggested that the pheromone secreted from female fish is of kidney origin and urine is involved. Using blinded males, the present study provides strong evidence that urine of intact mature females contains important chemical cues by which males identify females or otherwise stimulates males into pre spawning behavior. This pheromone in urine is female specific and the urine of mature males had no effect on male behavior. The present study confirmed that the releaser pheromone is not of gonadal origin. The urine of castrated and ovariectomized  $E_2$  treated goldfish stimulated pecking or nudging behavior of mature males. Also ovariectomized  $E_2$  treated fish were chased vigorously, almost naturally, by blinded mature males. Meyer and Liley (1982) indicated that in guppies, pheromone is produced in the ovary under the control of  $E_2$  using ovariectomized and  $E_2$  treated guppies. In this case,  $E_2$  secreted from ovaries may act on the ovary itself to produce pheromone. However, in goldfish  $E_2$  might

act on the kidney to release the pheromone as previously suggested (Yamazaki and Watanabe, 1979) because in the present study, the test fish chased had no ovary. Oral treatment of this steroid at a concentration of 10  $\mu\text{g}$  per gram diet given for 1-2 weeks, appeared effective in producing the pheromone in urine. This hormone is secreted from ovaries into the circulatory system and accelerates the synthesis of yolk protein in the liver at vitellogenic stages of maturation. The highest plasma  $\text{E}_2$  level is recorded in April just before spawning in goldfish (Kobayashi et al., 1986). This peak in  $\text{E}_2$  level in maturing females may accelerate the production of the releaser pheromone from their kidneys, resulting in stimulation of chasing or nudging behavior by mature males. This behavioral pheromone might be released in urine several days later than the  $\text{E}_2$  peak in the circulatory system, but is a real preovulatory pheromone.

Sorensen et al. (1988) proposed the hypothesis that prostaglandins and/or their metabolites function as goldfish postovulatory releaser pheromone. F type prostaglandin increases in circulating and ovarian levels at the time of ovulation (Goetz, 1983). Possibly this circulatory prostaglandin is excreted from the kidneys and acts as a pheromone in the urine under the control of the  $\text{E}_2$  function. This pheromone, so called postovulatory pheromone by Sorensen et al. (1988), may be the same substance as the preovulatory releaser pheromone observed in the present study. In this case, the term pre or post is not significant, and we should call it just a releaser pheromone in goldfish. However, the mature males chased ovulated females more actively than nonovulated gravid females. This may suggest that there are at least two different releaser pheromones. In this case, one acts mainly as a preovulatory pheromone from urine and the other as a postovulatory releaser pheromone like prostaglandin as suggested by Sorensen et al. (1988). To clarify this problem, the urine excreted from gonadectomized  $\text{E}_2$  treated goldfish should be chemically analyzed to examine what kind of chemicals are involved in preovulatory chasing behavior of mature males. The chemical substances in the urine might also explain the species-specific characteristics of chasing behavior. Urine secreted at the reproductive mating periods in other species should also be examined to determine the generality of the role of urine as a chemical cue in fishes.

In the present study, methyltestosterone (mt) was occasionally administered to the experimental blinded mature males. This treatment was necessary to keep the males sensitive to odor in the experimental aquarium. The olfactory epithelium of mt treated goldfish becomes thick (Yamazaki and Watanabe, 1979). This suggests that androgen secreted from testes of maturing males makes their olfactory sense organs more sensitive to waterborne pheromones. It is concluded from the results of this and other studies (Yamazaki and Watanabe, 1979) that, in goldfish, estrogen in females and androgen in males have important roles in sex discrimination in the spawning behavior.

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**Enzyme Linked-Immunosorbent Assay (ELISA)  
of Vitellogenin in Whitespotted Charr,  
*Salvelinus leucomaenis*\***

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and Juro YAMADA\*\*

**Abstract**

A specific and sensitive enzyme-linked immunosorbent assay (ELISA) was developed for the measurement of low levels of serum vitellogenin (Vg) of the whitespotted charr (*Salvelinus leucomaenis*). Serum Vg was purified by precipitation with distilled water, followed by Sepharose 6B column chromatography. Two egg yolk proteins, E1 (lipovitellin) and E2, were also isolated by Sephadex G-200 gel filtration. The molecular weights of Vg, E1 and E2 were estimated by gel filtration to be approximately 540,000, 300,000 and 30,000, respectively. An ELISA was developed using an antiserum to E1 of whitespotted charr. The enzyme-labeled antibody was prepared by coupling the Fab' fragment of the antibody to horseradish peroxidase with an aid of maleimide groups. The assay was performed by the so-called "sandwich" method using a microtiter plate as a solid phase. The assay could be run in one day and could routinely detect Vg in a concentration as low as 10 ng/ml. The appearance of serum Vg was detected 8 to 10 hr after a single injection of estradiol-17 $\beta$  into males or immature fish of unknown sex. The amplification effect on vitellogenesis of successive injections of estradiol-17 $\beta$  was observed in the serum. The Vg level varied throughout the annual reproductive cycle from 5-10  $\mu$ g/ml to 15-25 mg/ml in whitespotted charr. The development of an ELISA for Vg made possible the quantification of serum Vg and thereby the ability determine the sex of fish in early gametogenesis at least one year prior to spawning.

**Introduction**

Vitellogenin is a sex specific protein present in sexually maturing female blood of oviparous and viviparous animals. The name "vitellogenin" was first coined for female specific protein in hemolymph of insects by Pan et al. (1969). The term has then become generally accepted name for the female specific protein in fish as well as other oviparous vertebrates. Vitellogenin (Vg) is a calcium- and iron-binding glycolipophosphoprotein which is produced by the liver in response to circulating estrogen, released into the bloodstream, and transported to the developing ovary where it serves as a precursor to the egg yolk (see reviews: Wiegand, 1982; Ng and Idler, 1983; Wallace, 1985; Mommsen and Walsh, 1988).

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