A releaser pheromone that attracts methyltestosterone-treated immature fish in the urine of ovulated female rainbow trout

HIDENOBU YAMBE* AND FUMIO YAMAZAKI

SUMMARY: Behavioral experiments concerning a releaser pheromone in the urine of female rainbow trout were performed using immature fish administered orally with 17α-methyltestosterone (MT) during the non-spawning season. The urine was collected by catheter. The frequency of entries of test fish was recorded in each channel scented by test and control solutions in a Y-maze trough. The behavior of both MT-treated and control fish demonstrated that they could not discriminate the differences between distilled and environmental water as control solutions. There was also no difference between MT-treated and control fish when distilled and environmental water were introduced. The MT-treated immature fish were attracted to the channel scented by ovulated female urine. Neither coelomic fluid nor the immature female urine had any effect on the behavioral responses of MT-treated fish, while immature control fish had no preference for the urine of ovulated females. These results suggest in rainbow trout that ovulated female urine contains a releaser pheromone to attract mature males, and that androgens are involved in the sensory mechanisms detecting the releaser pheromone in fish.

KEY WORDS: androgen, behavior, coelomic fluid, pheromone, rainbow trout, sex attractant, urine.

INTRODUCTION

In a number of fish species, sex pheromones have important roles in the chemical communication between mature males and females during the spawning season. Two types of sex pheromones exist. One is a primer pheromone which relates to inducing physiological changes in the recipient and the other is a releaser pheromone which elicits sexual behaviors of the recipient.1 Over the past few decades a considerable number of studies have been made in sex pheromones of fishes (see reviews2,3). Especially, the studies on sex pheromones in goldfish Carassius auratus are informative,4,5 giving concise explanations: (i) 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) from preovulated females having a priming effect to stimulate gonadotropin secretion in males and increase the milt volume, and (ii) F-type prostaglandins (PGF) from ovulated females having a releasing effect that induces male reproductive behavior.

In relation to the sources of sex pheromones, the urine has been reported in a number of animals including fish species.6–12 In salmonids, it is known that mature males prefer the water conditioned by ovulated females.13,14 However, many researchers have quoted the result of Honda15 and Emanuel and Dodson16 who reported that a sex pheromone which attracts males and induces courtship behaviors to a female is present in the coelomic fluid of ovulated female rainbow trout Oncorhynchus mykiss. Recently, Scott et al.17 and Vermeirssen et al.18 in rainbow trout, and Waring et al.19 in Atlantic salmon Salmo salar reported that mature female urine contains a priming pheromone. Further, in the case of masu salmon O. masou, Yambe et al.20 showed that a releaser pheromone which attracts males during the reproductive season is present in the mature female urine but not in the coelomic fluid. In the past, mixture of the coelomic fluid and ovulated female urine was used as an urogen-
ital fluid in experiments, because either solution is easily mixed with the other when collecting the two solutions.\textsuperscript{21,22} Therefore, this discrepancy in the results concerning the coelomic fluid remains a matter to be elucidated in salmonids.

On the other hand, it is recognized that sex steroids influence and change behavior.\textsuperscript{23} In goldfish, Yamazaki and Watanabe\textsuperscript{24} showed that males treated with 17\(\alpha\)-methyltestosterone (MT) have the ability to recognize normal mature females or males treated with estradiol-17\(\beta\) and discriminate them from non-treated control fish. The male ability to detect a sex pheromone from females is suggested to be under the control of androgens. It is also indicated that experiments on sexual behaviors in fish are possible to conduct regardless of the spawning season and genetic sex.\textsuperscript{24,25}

In this study, behavioral experiments concerning a releaser pheromone that attracts males in rainbow trout were performed using a Y-maze trough. We aimed first to demonstrate whether immature fish administered orally with MT are attracted by the ovulated female urine or the coelomic fluid, and second to indicate that small immature MT-treated fish are useful for behavioral experiments because precocious male rainbow trout are unobtainable and the adult mature males are too large to use as test fish in a limited experimental space.

\section*{MATERIALS AND METHODS}

\textbf{Experimental fish and test solutions}

Three ovulated females (400–450 mm in fork length), one immature female (390 mm in fork length) and immature males and females (180–230 mm in fork length, \(n=60\)) of rainbow trout were used in this study. They were obtained from a private trout farm near Hakodate in February and April 1999 and July 2000. All fish were maintained in outdoor tanks (1000 L) supplied with well water at 11–18°C under the natural photoperiod and fed commercial pellets prior to the experiments. Immature and ovulated females were catheterized to collect the urine according to the previous study.\textsuperscript{20} Coelomic fluid of ovulated females was collected carefully by squeezing the abdomen to avoid contamination with the urine after urine collection. The collected urine was frozen at \(-80^\circ\text{C}\) prior to use in experiments. Test solutions (urine or coelomic fluid) were made by diluting 500\(\mu\)L sample with 300 mL distilled water. Three hundred milliliters of environmental water (from well water) was used as a control solution.

\textbf{Steroid treatment}

Immature fish of 1\(\frac{1}{2}\) years were used as test fish in behavioral experiments, because they were small (about 100 g in bodyweight) and easier to handle than normal-sized adult males. The test fish were divided into two groups. One group was fed commercial pellets supplemented with MT (Sigma Chemical Co., St Louis, MO, USA) at the rate of 100 \(\mu\)g MT/kg fish per day (MT-treated fish, \(n=20\)). The concentration of MT dissolved into ethanol in the diet (10 \(\mu\)g/g diet) was followed from Yamazaki and Watanabe.\textsuperscript{24} The other group was fed the pellets that had been supplemented with equivalent ethanol and dried (control fish, \(n=20\)). Each group was held in outdoor tanks (500 L) supplied with well water under the natural photoperiod. They were fed to satiation daily for 4 weeks (total amount of MT was 2.8 mg/kg fish). The normal reproductive season of rainbow trout is from December to March in northern Japan. During the non-reproductive season (May 1999) MT-treated fish were tested for behavioral responses to immature female urine (IFU) versus environmental water (EW), ovulated female urine (OFU) versus EW and coelomic fluid (CF) versus EW, and control fish were tested to OFU versus EW, further MT-treated fish (\(n=20\)) were also tested to OFU versus IFU in September 2000.

\textbf{Behavioral assay}

The behavioral experiments were conducted in a Y-maze trough that has two channels (290 x 35 x 30 cm, 10 cm in water depth, 50 L/min in flow of each channel) under the same conditions as the previous study.\textsuperscript{20} Tests using a dye solution showed that test solutions reached the downstream section of the trough in less than 90 s. Two test fish were acclimatized for 15 min in the downstream section of the trough. At the end of the acclimation, test and control solutions were simultaneously introduced into each channel head of the trough by pouring from separation funnels for a 120 s period. The channels scented by test and control solutions are referred to as the experimental and control channels, respectively. After 12 s of pouring of the test and control solutions into channels, the experiment was initiated by opening the gate of the trough to free the test fish. The test fish swam freely back and forth in the trough after the opening of the gate.

The frequency of entries of two fish into each channel, which include movements that appeared to be forced by agonistic behaviors, was counted for 6 min, because the frequency of these agonistic
behaviors was too low to have an effect on the statistics. First selection (the number of fish that entered either channel first) and no choice (the number of fish which selected the downstream section from the beginning to the end in each trial) were recorded for each individual fish. The positions of the test fish at the end of each trial were also recorded as the final selection for each individual fish. The location of the test solution was reversed in each trial to avoid location bias, so that both channels were alternately scented by the test solutions. Test fish and test solution were changed after two trials. In 1999, one set of experiments regarding the responses of MT-treated fish to distilled water (DW) versus EW, OFU versus EW, IFU versus EW and CF versus EW, and control fish to DW versus EW and OFU versus EW, was conducted with every two trials being repeated 10 times. The trough was washed with EW after each day of the testing. All test fish were immature, but to be certain, six each from MT-treated and control fish used in 1999 and all 20 of MT-treated fish used in September 2000 were checked for the developmental condition of the gonad by histological observation after the experiments.

For statistical analysis, ‘the frequency of entries’ to each channel by two test fish was pooled for the respective replications. The channel selection behavior of the test fish was compared by Wilcoxon-signed rank test between the channels scented by test and control solutions. Differences between entries of MT-treated and control fish were compared using the Mann–Whitney U-test (i.e. DW to MT-treated fish versus DW to control fish; EW to MT-treated fish versus EW to control fish). First and final selections of each test fish were compared by χ² test. The significant level was determined at \( P = 0.05 \). Significant values are expressed as arithmetic mean ± SEM.

**RESULTS**

In both MT-treated and control fish, no significant differences were found between frequencies of entries to channels scented each by DW and EW (Fig. 1). Therefore, they did not discriminate DW from EW (i.e. introduced DW and EW into channels did not make any difference in odor between the channels). There were also no statistical differences in the behavioral responses to DW or EW between MT-treated and control fish (Mann–Whitney U-test; \( P > 0.05 \); Fig. 1). This shows that oral administration with MT had no effect on the fish behavior when DW and EW were introduced.

However, MT-treated fish discriminated the difference between OFU and EW or IFU. The mean frequency of entries of MT-treated fish was 5.2 ± 1.1 to OFU and 1.8 ± 0.6 to EW (Fig. 2). These values indicate that MT-treated immature fish had significantly more entries to the channel scented by OFU.
Pheromone in the urine of ovulated female trout

0.19 ± 0.01] and non-treated controls were three females (GSI = 0.26 ± 0.08) and three males (GSI = 0.24 ± 0.08). A total of nine females in MT-treated and control fish were all immature having oocytes at the perinucleolus stage in their ovaries and three

than into the channel scented by EW ($z = -2.241, P = 0.0146$). The mean frequency of entries of MT-treated fish to OFU and IFU was 8.8 ± 1.4 and 4.2 ± 0.9, respectively (Fig. 2). The MT-treated fish also made significantly more entries into the channel scented by OFU than into the channel scented by IFU ($z = -2.703, P = 0.0069$). On the other hand, responses of MT-treated fish to CF or IFU showed no statistical differences between the entries into experimental and control channels. Consequently, neither coelomic fluid nor immature female urine was preferred by MT-treated fish. No statistical difference was also shown between the entries of immature control fish to OFU and EW (Fig. 3). Therefore, immature control fish had no preference for the urine of ovulated females.

The first and the final selections of two test fish into the channels are listed in Tables 1 and 2. In all test categories, many test fish were swimming into channels during the first half of the observation period (~3 min). More than half of the MT-treated fish first selected the channel scented by OFU and they also tended to select the same channel at the end of the experiment. In both the first and the final selections of MT-treated fish, significant differences were found between OFU and EW (Table 2). In all experiments, however, many fish selected the downstream section in the trough at the end of the observation.

Twelve experimental fish checked had thread-like undeveloped gonads. In the fish used in 1999, six MT-treated immature fish were casually all females [mean gonad somatic index (GSI) ± SEM =

### Table 1

<table>
<thead>
<tr>
<th>Odorants</th>
<th>First selection</th>
<th>Final selection (position)</th>
<th>No. of trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-treated fish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DW</td>
<td>16</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>EW</td>
<td>10</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>No choice</td>
<td>14</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Control fish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DW</td>
<td>11</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>EW</td>
<td>16</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>No choice</td>
<td>13</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

DW, distilled water; EW, environmental water; MT, 17α-methyltestosterone.

### Table 2

<table>
<thead>
<tr>
<th>Odorants</th>
<th>First selection</th>
<th>Final selection (position)</th>
<th>No. of trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-treated fish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovulated female urine</td>
<td>24*</td>
<td>10*</td>
<td>20</td>
</tr>
<tr>
<td>EW</td>
<td>10</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>No choice</td>
<td>6</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Immature female urine</td>
<td>14</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>EW</td>
<td>13</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>No choice</td>
<td>13</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Ovulated female urine</td>
<td>20</td>
<td>12*</td>
<td>20</td>
</tr>
<tr>
<td>Immature female urine</td>
<td>15</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>No choice</td>
<td>2</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Coelomic fluid</td>
<td>14</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>EW</td>
<td>14</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>No choice</td>
<td>12</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Control fish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovulated female urine</td>
<td>7</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>EW</td>
<td>8</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>No choice</td>
<td>25</td>
<td>37</td>
<td></td>
</tr>
</tbody>
</table>

* Significant differences between test and control solutions.

* P < 0.05, $\chi^2 > 5.76$. 

**Fig. 3** Frequency of entries of control fish (non-treatment) to the water scented by the ovulated female urine (OFU) in a Y-maze preference trough. Record was made for 6 min in each experiment. Vertical bars show SEM. Number of trials in each experiment was 20. EW, environmental water.
males in control fish had immature testes with spermatogonia, scattered spermatocytes and a few spermatids. From checking the sex of all MT-treated immature fish used in September 2000, 14 were females (GSI = 0.23 ± 0.07) having immature oocytes at the perinucleolus stage in their ovaries and six were males (GSI = 0.20 ± 0.05) with vacuolated immature testes showing scarcely observed spermatogonia and some spermatids.

**DISCUSSION**

The present study strongly suggests from using MT-treated immature fish that ovulated female urine contains a releaser pheromone which attracts mature males in rainbow trout. This result supported the previous studies that showed the presence of a releaser pheromone for attracting mature males in the urine of ovulated female masu salmon. In the case of masu salmon, the rates of urine production in mature females were higher than those in immature females (Yambe H & Yamazaki F, unpubl. data, 1998). Recently, Scott and Liley, Scott *et al.* and Vermeirssen *et al.* reported that mature female urine of rainbow trout contains a priming pheromone which enhances plasma sex steroids and gonadotrophin II or milt amounts in males. It has also been shown that adding coelomic fluid to holding water has no effect on the enhancement of plasma levels of testosterone and 17,20β-P in rainbow trout males. Consequently, it is assumed in salmonids that the urine of ovulated females has specific functions for their reproduction and contains not only primer pheromones but also releaser pheromones.

Emanuel and Dodson showed in rainbow trout, that coelomic fluid obtained from spawning females stimulates the upstream movement of ripe males. Moreover, it has been reported that a sex pheromone, which attracts mature male rainbow trout and induces courtship behaviors, is present in the coelomic fluid (egg washings and homogenized ovaries) of conspecific females. In contrast, Newcombe and Hartman reported that washed water from eggs stripped from ripe females (nearly equal to the coelomic fluid) was not effective as a sex attractant in rainbow trout. The present study supports the latter result and indicates that the coelomic fluid of ovulated rainbow trout contained no sex attractant. These contradictory results may be due to contamination of coelomic fluid with the urine and also differences in experimental methods used in the studies. In salmonids, however, coelomic fluid contains many steroids and species-specific proteins which have been proposed as a putative pheromone. From previous reports we hypothesize that mature salmonid males will approach a female by a chemical signal of a releaser pheromone from mature female urine and male spawning behaviors may be facilitated by her coelomic fluid at the spawning. In any case, the behavioral role of the coelomic fluid for males may still remain a matter for debate and further experiment will be needed.

Effectivity of androgens to the behavior demonstrated in this study is supported by some other reports. For example, Yamazaki and Watanabe showed in goldfish that MT-treated immature or hypophysectomized males had enhanced olfaction and discriminated males treated with estradiol-17β. They also reported that olfactory epithelia of the MT-treated fish became thick. Stacey and Kobayashi reported in detail the induction of male sexual behaviors in female goldfish by treatment with testosterone and 11-ketotestosterone. From these studies, it seems that MT undergone by an unknown metabolic pathway may operate directly on the central nervous system or may develop the pheromone receptor in olfactory epithelia to induce male sexual behavior.

The present data indicated that MT-treated immature rainbow trout, ranging from 180 to 230 mm fork length and hardly identified the sex from the outer appearance, were attracted specifically to the ovulated female urine. Test rainbow trout, which were sampled randomly from MT-treated and control fish, included relatively many immature females (9/12) having oocytes at the perinucleous stage in their ovaries in 1999. Moreover, out of 20 MT-treated fish used as test fish in 2000, 14 fish were immature females with oocytes at the perinucleous stage in their ovaries. Therefore, the rate of females was unexpectedly high in all the test fish. Further, it was reported that the sexual bipotentiality (i.e. masculinization in female) of gonad and brain was induced by the combination of incomplete ovariectomy and 11-ketotestosterone implantation in female goldfish. Furthermore, sexual behavior in goldfish is controlled by sex steroids or PGF regardless of the genetic sex. From these results, it might be also true in salmonids that even females with immature ovaries, treated with MT would be attracted to the ovulated female urine.

In the case of masu salmon, we administered about 40 μg MT/kg fish per day for 25 days (total amount, 1 mg/kg fish) to immature parr and obtained significant results in behavioral response to sex attractant. In the preliminary experiment using rainbow trout, we obtained significant results when giving 100 μg MT/kg fish per day for 28 days (total amount, 2.8 mg/kg fish), but not for
21 days (total amount, 2.1 mg/kg fish). This difference in effective amount of MT between the two species may be attributed to species specificity or the rate of females in the test fish. Nevertheless, the present method on the treatment with MT seems to be appropriate, because frequencies of behavioral responses of MT-treated immature male masu salmon parr to the urine were similar to those of mature male or MT-treated immature male masu salmon parr to the urine of ovulated female masu salmon.20–30 At any rate, further investigations concerning the term of treatment or administration with other androgens may be needed for bioassay to identify a sex attractant in the releaser pheromones. In this study, the method using MT treatment induced higher preference of the test fish to the ovulated female urine and facilitated the behavioral response resulting in reproduction even in the non-spawning season. These steroid-treated immature fish are useful for bioassay to study sex pheromones in any season.

ACKNOWLEDGMENTS

The authors thank Drs K. Arai and A. Goto, Hokkaido University for their encouragements throughout this study, and Mr. T. Sohtome, Fukushima Prefectural Fisheries Research Station, Ocean and Fisheries Research Division for his help in making the histological preparations of the gonads.

REFERENCES

24. Yamazaki F, Watanabe K. The role of steroid hormones in sex recognition during spawning behaviour of the goldfish,