Female Sex Pheromone of the Ayu, *Plecoglossus altivelis*,
Involved in Courtship Behaviour

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This report deals with the courtship behaviour and the presence of a female sex pheromone of the Ayu, *Plecoglossus altivelis*. Increased ventilatory frequencies and fin display reactions in the male Ayu were elicited by the water sample inclusive of post-ovulatory females. It was noticed that the males were able to discriminate post-ovulatory females from pre-ovulatory females without actually sighting the female.

Some highlighted aspects derived from these experiments are listed as follows;
1. Post-ovulatory females released a female sex pheromone, which males detect by olfaction.
2. The female sex pheromone, produced in the ovaries of post-ovulatory females, is secreted through the urogenital papilla.
3. The female sex pheromone was found to be a water-ether soluble basic substance, having an Rf value of 0.43 on the thin layer chromatogram (silica gel) with a cyclohexane-ethyl alcohol (4:1) solvent.
4. The female sex pheromone from post-ovulatory females played the role of first trigger, inducing a series of courtship behaviour of the Ayu.

In Japan, the Ayu, *Plecoglossus altivelis*, is one of the most important fish in commercial and game fishing. A progressive culturing technology of it has been made in recent years and therefore, the exploration of its spawning behaviour has become a serious subject for cultivating resources and river farming. It is due to the above reason that there have been many published papers concerning its spawning behaviour.

The male Ayu, having been reared in an aquarium placed in a laboratory in October 1978, had its courtship behaviour observed when a post-ovulatory female was put into the aquarium. This fact suggests that the chemical communicant might be used as an olfactory cue in the courtship behaviour of the Ayu. There has been no previous report published, describing the biological phenomenon as mentioned above.

In many insects, however, sexual recognition and attraction have been shown to involve chemical communicants called sex pheromones, and these results are widely applied in the control of insect pests. In fishes, Tsukahara reported a possibility of the presence of a sex pheromone in the courtship of the loach *Misgurnus anguillicaudatus*. Tavolga demonstrated the olfactory cue in the reproductive behaviour of the gobiid fish, *Bathygobius soporator*. Todd reported on the sex pheromones of two blennies, *Hypoblephius jenkinsi* and *H. gilberti*, and finally, Partridge and his collaborators reported on the presence of a sex pheromone of goldfish, *Carassius auratus*. So far, however, there has not been much data available on the chemical communicant involved in a series of courtship behaviour of the Ayu.

This report explores the presence of a sex pheromone as closely related to the courtship behaviour of the Ayu.

**Materials and Methods**

**Fish Used**
The Ayu, obtained from a fish culturist in Chiba prefecture (during the period May to September, 1978), were reared in 15 m³ concrete pools on appropriate diet for Ayu. After rearing, the mature fish ranging from 130 to 184 mm in body length were used for tests. Ovulation occurred naturally in the pools during October to December.

Anosmic males were readied by plugging the nostrils with an adhesive agent (Semedain 3000 gold). MS 222 was used to anaesthetize fish dur-
Preparation of Stimulus Sources

Water samples: Water samples were readied by placing either a pre-ovulatory female (Bw. 1) or a post-ovulatory female (Bw. 2) in a polyethylene bucket (containing 8 l of water) for 2 hours.

Fragments of blotting paper: Fragments of blotting paper (ca. 50 mm²) were applied either to the skin of the trunk or to the urogenital papilla of the post-ovulatory female.

Test solutions: Three test solutions were prepared using the post-ovulatory female by the procedures as shown in Fig. 1. Three ml of each solution was used in the trials.

Extraction and isolation: Ether extract procedures were used for separating the secretion fractions from the post-ovulatory female as shown in Fig. 2. Test samples were prepared by removing ether from 5 ml of each fraction and filling up to 30 ml with distilled water for the biological tests. Three ml of each solution was used in the trials. For isolating the ether extracted fraction-3, thin layer chromatography (TLC) was used by the ordinary method, in which a cyclohexane-ethyl alcohol mixture (4:1) was used as a solvent. The spots were detected by the following two methods; one was burning the plate on an electric heater until the spots on TLC were carbonized to a brownish color, and another was spraying a bromothymol blue-ethyl alcohol solution. Small pieces of the silica gel plate, inclusive of spots, were torn off from the glass plate and added to ca. 5 ml of distilled water.

Biological Tests

1. Tests for eliciting fin display

An aclilite aquarium (60 x 30 x 35 cm) equipped with a filter, containing 40 l of water with the floor

Ovulated eggs (15 g, wet weight) —washed with water

Washings
—extd. with ethyl ether

Ether soln.
—dried with Na₂SO₄ and ether was evaporated

Aqueous soln.
—added dil. HCl and extd. with ethyl ether

Fr. 1

Ether soln.
—dried and ether was evaporated

Aqueous soln.
—added NaOH and extd. with ethyl ether

Fr. 2

Ether soln.
—dried and ether was evaporated

Aqueous soln.
—pH was adjusted to pH = 7.0 with HCl

Fr. 3

Fig. 1. Preparation procedures of test solutions from the post-ovulatory female Ayu.

Fig. 2. Ether extract procedures of substances from washings of ovulated eggs.

extd: extracted, soln: solution, Fr.: fraction
covered with sand, was used. Either a pair of males or a sole male and a pre-ovulatory female were simultaneously placed in the aquarium. After 30 minutes, 3 l of the water sample either Bw. 1 or Bw. 2 was poured into the aquarium through a siphon and observations were made for 30 minutes.

2. Y-maze tests
A wooden Y-maze, consisting of 2 stimulus chambers (as shown in Fig. 3) with its floor covered with sand (5 mm or less in diameter, 1 cm in thickness), was used. Five l of water were added every minute into each stimulus chamber, which held either a pre-ovulatory or post-ovulatory female as a stimulus source.

![Fig. 3. Plane figure of the Y-maze device. A & A': Stimulus chambers (left open 1 cm under the non-transparent partition). B & B': Gates. C: Buffle. D: Start area (covered with a wooden plate). Arrows: Stream direction. Water depth: 10 cm.](image)

3. Ventilatory frequency tests
The aquarium used was the same as described under “Tests for eliciting fin display”. Two males were simultaneously placed in the aquarium. Thirty minutes after the placement of fish and 5 minutes after providing each stimulus, the ventilatory frequency of each fish was obtained by measuring the time required for 20 cycles of movement of the jaws.

4. Spawning tests
The aclilite aquaria (150 x 30 x 35 cm and 200 x 60 x 45 cm), both equipped with a filter and the floor covered with sand (5 mm or less in diameter, 5 cm in thickness), were used. Either a pre-ovulatory or post-ovulatory female was put into each aquarium holding normal or anosmic males (2~7 individuals) during 13:00-17:00. These were kept over night until 9:00 the morning. Having observed whether the fish spawned or not, the final conclusion was drawn the next morning.

Results and Discussion

Observation of the Courtship Behaviour
The courtship behaviour of the Ayu was observed on 26 October and 2 November in aquaria placed in the laboratory. The same aquaria, as mentioned in the spawning tests, were used for the observation. The courtship was elicited by putting a post-ovulatory female into the aquarium holding males (5 males, on 26 Oct. and 3 males, on 2 Nov.). The courtship behaviour observed in the aquaria is summarized as follows.

Before a post-ovulatory female was put into the aquarium, the males retained their normal body color phase and swam slowly in loose aggregation. Within a few minutes after placement of the post-ovulatory female, the males exploded into courtship, beginning to chase the female and extend all of their fins with arching and quivering bodies under or in front of the female. Courtship color phase (blackish brown body with an orange band at the lowest part) began to appear. One of males then swam in parallel with the female, inserting his pectoral fin into the posterior part of the female’s, and attempted to hold her down on the bottom.

On the other hand, the female swam all about the aquarium just after placement. In due course of time, the female began to touch her genital area on the bottom occasionally. At a particular moment, one of males dashed in and swam in parallel with the female. Next, they rushed into the sand at the bottom and released the gametes as shown in Fig. 4. Spawning commenced at 16:30, three hours after the placement of the female on

![Fig. 4. Photograph showing spawning behaviour of the Ayu. The Ayu in the photograph are as follows; Lowest, Female; Others, Males.](image)
26 October, and at 14:50, thirty minutes after placement on 2 November. Each female spawned 16 and 27 times until 19:00, in each observation. As described above, the placement of a post-ovulatory female changed the behaviour and coloration of males in a few minutes, before the female recovered from the effects of being handled with a net. These facts indicated that a chemical communicant might be released from post-ovulatory females.

**Biological Tests**

1. Tests for eliciting fin display

To examine if a chemical communicant was released from post-ovulatory females, the water samples (Bw. 1 and 2) were tested to see whether they allowed males to elicit fin display or not. The response of males to the water samples and the result of "Fisher's exact probability test" are shown in Table 1. The water obtained from the post-ovulatory female (Bw. 2) elicited the fin display reactions from 6 males within 30 minutes after providing stimuli to males. The water from the pre-ovulatory female (Bw. 1), on the other hand, did not produce fin displays. After anosmic treatment, the fin display reactions were not shown by the males, though they did respond positively before the treatment.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>No. of males</th>
<th>Fisher's exact probability test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bw. 1</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Bw. 2</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

It became clear that the water sample from the post-ovulatory female contained a chemical substance known as sex pheromone, that elicited fin displays from males, who noticed it with olfaction. These facts corresponded to the two reports, one describing on the olfactory cue of the gobid fish and the other describing the chemical cue of goldfish in the courtship behaviour of each species.

2. Y-maze tests

The preference tests were carried out by using the wooden Y-maze device. A male had 15 minutes to choose between 2 gates of the maze and if failing to do this they were discarded. The position of the female in each stimulus chamber was switched after a trial with 10 males. Four trials were carried out following the first.

<table>
<thead>
<tr>
<th>Female as a stimulus source</th>
<th>No. of males responded</th>
<th>χ²-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-ovulatory</td>
<td>29</td>
<td>sign</td>
</tr>
<tr>
<td>Pre-ovulatory</td>
<td>4</td>
<td>(χ²=18.94, p=0.001)</td>
</tr>
</tbody>
</table>

The response of males in the Y-maze are shown in Table 2. The males distinguished between pre-ovulatory and post-ovulatory females without actually sighting the female. It became evident that the sex pheromone, released from post-ovulatory females, attracted males.

3. Ventilatory frequency tests

(1) Response to stimulus sources

Owing to limitation of the spawning season of the Ayu, further experiments had to be carried out efficiently. Ventilatory frequency of male therefore, examined to see whether it represents a sexual excitement of the male or not. Eight mates and the water samples (Bw. 1 and 2, using 100 ml/trial) were used in the tests. As shown in Table 3, the ventilatory frequencies of males were increased by the water sample from the post-ovulatory female.

<table>
<thead>
<tr>
<th>Olfaction of male</th>
<th>Stimulus</th>
<th>Ventilatory frequency (cycle/min)</th>
<th>U-test (α=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>range</td>
<td>mean</td>
</tr>
<tr>
<td>Normal</td>
<td>Control</td>
<td>95.2–111.1</td>
<td>104.5</td>
</tr>
<tr>
<td></td>
<td>Bw. 1</td>
<td>101.7–109.1</td>
<td>105.1</td>
</tr>
<tr>
<td></td>
<td>Bw. 2</td>
<td>118.8–139.5</td>
<td>127.2</td>
</tr>
<tr>
<td>Anosmic</td>
<td>Control</td>
<td>100.0–107.1</td>
<td>104.8</td>
</tr>
<tr>
<td></td>
<td>Bw. 2</td>
<td>100.8–109.1</td>
<td>104.4</td>
</tr>
</tbody>
</table>

Table 1. Elicited fin display of males responding to the water samples obtained from the pre-ovulatory female (Bw. 1) and from the post-ovulatory female (Bw. 2). +: Positive response. −: Negative response.

Table 2. Response of males in the Y-maze.

Table 3. Ventilatory response of normal and anosmic males to the water samples obtained from the pre-ovulatory female (Bw. 1) and the post-ovulatory female (Bw. 2). Water temperature: 14.3–15.4°C.
The water from the pre-ovulatory female (Bw. 1), on the other hand, did not increase the ventilatory frequencies of males. After anosmic treatment, Bw. 2 also did not increase the ventilatory frequencies of males. These facts indicate that the female sex pheromone not only elicited the courtship behaviour from males but also increased the ventilatory frequencies of males.

In order to determine the region secreting the female sex pheromone, 10 males were tested with fragments of blotting paper applied to two regions of the post-ovulatory female. As shown in Table 4, the ventilatory frequencies of males were increased by the fragments when applied to the urogenital papilla of the post-ovulatory female, whereas those applied to the skin of the trunk did not increase the frequency. It was thus determined that the female sex pheromone was secreted through the urogenital papilla of the post-ovulatory female. This conclusion was consistent with TAVOLGA reporting on the gobiid fish.

Table 4. Ventilatory response of males to the fragments of blotting paper when applied to the skin of the trunk (SK) and when applied to the urogenital papilla (UP) of the post-ovulatory female. Water temperature: 15.2~15.5°C.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Ventilatory frequency (cycle/min)</th>
<th>U-test (α=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>range</td>
<td>mean</td>
</tr>
<tr>
<td>Control</td>
<td>101.7~109.1</td>
<td>106.9</td>
</tr>
<tr>
<td>SK</td>
<td>103.4~112.1</td>
<td>108.7</td>
</tr>
<tr>
<td>UP</td>
<td>120.0~139.5</td>
<td>128.5</td>
</tr>
</tbody>
</table>

To detect the origin of the female sex pheromone, three test solutions (Ts. 1~3, shown in Fig. 1) were used. Eight males were tested with Ts. 1 and 2, and another 8 males were tested with Ts. 3. The ventilatory response of males to the test solutions are shown in Table 5. The ventilatory frequencies of males were increased with Ts. 2 and 3. Ts. 1, however, did not increase the frequency. It could reasonably be assumed that the female sex pheromone did not originate in the ovulated eggs but in the ovaries of post-ovulatory females.

Four males were tested with four ether extracted fractions (Fr. 1~4, shown in Fig. 2). As shown in Table 6, the ventilatory frequencies of males were increased by only Fr. 3.

Table 6. Ventilatory response of males to the ether extracted fractions. Fr. 1~4: Fractions shown in Fig. 2. Water temperature: 15.0°C

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Ventilatory frequency (cycle/min)</th>
<th>U-test (α=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>range</td>
<td>mean</td>
</tr>
<tr>
<td>Control</td>
<td>100.8~109.1</td>
<td>105.6</td>
</tr>
<tr>
<td>Fr. 1</td>
<td>100.0~105.3</td>
<td>102.6</td>
</tr>
<tr>
<td>Control</td>
<td>105.3~109.1</td>
<td>107.9</td>
</tr>
<tr>
<td>Fr. 2</td>
<td>101.7~110.1</td>
<td>106.3</td>
</tr>
<tr>
<td>Control</td>
<td>100.8~106.2</td>
<td>104.4</td>
</tr>
<tr>
<td>Fr. 3</td>
<td>127.7~133.3</td>
<td>131.2</td>
</tr>
<tr>
<td>Control</td>
<td>101.7~107.1</td>
<td>104.4</td>
</tr>
<tr>
<td>Fr. 4</td>
<td>100.0~108.1</td>
<td>104.3</td>
</tr>
</tbody>
</table>

On a thin layer chromatogram, two spots (Rf value: 0.43, 0.20) were detected with burning and spraying a bromothymol blue-ethyl alcohol solution. The ventilatory frequency of the male was increased up to 134.8 (before stimulus, 108.1) cycles per minute by the spot having Rf value of 0.43. The other spot did not affect the ventilatory frequency.

From the above reason, the female sex pheromone of the Ayu was shown to be a water-ether soluble basic substance.
(2) Minimum effective concentration

One ml of Fr. 3 contained 0.025 mg of chemical substances. The relationship between concentration of Fr. 3 (crude female sex pheromone) and mean ventilatory frequency of 2 males is shown in Fig. 5. Although Fr. 3 contained another non-effective substance, 10^{-11} mg/l concentration of Fr. 3 was shown to be the minimum effective concentration.

4. Spawning tests

Three groups of Ayu were tested to determine whether spawning take place or not. Each group consisted of combinations, such as between a post-ovulatory female and males having normal olfaction (6 lots were tested); a post-ovulatory female and males having anosmic olfaction (3 lots) and a pre-ovulatory female and males having normal olfaction (4 lots).

Table 7. Effect of males intact or impaired on spawning.

<table>
<thead>
<tr>
<th>Olfaction of male</th>
<th>Sexual condition of female</th>
<th>Spawning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-ovulatory</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Post-ovulatory</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Pre-ovulatory</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

As shown in Table 7, spawning was completed by the post-ovulatory females with the males having normal olfaction by the next morning. With anosmic males, on the other hand, the post-ovulatory females never completed their spawning, nor did the pre-ovulatory females spawn.

These facts indicate that the female sex pheromone played the role of first trigger, inducing a series of courtship behaviour of the Ayu.

Conclusion

The above-mentioned facts demonstrate that post-ovulatory female Ayu release a sex pheromone, eliciting courtship behaviour from males. As described in the observation item, the release of gametes is made at a particular moment. Taking a look at the female touching her genital area to the bottom, a male takes immediate action. This behaviour (touching the bottom) may be a visual cue from females to males as NISHIDA described in the spawning habits of the dwarf Ayu-fish in Lake Biwa. Therefore, as shown in Fig. 6, the outline of the courtship behaviour of the Ayu up to its spawning period is considered.

![Fig. 6. Proposed ethogram of courtship behaviour up to spawning of the Ayu.](image-url)

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References