Correction

AGRICULTURAL SCIENCES. For the article “L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon,” by Hidenobu Yambe, Shoji Kitamura, Michiya Kamio, Miho Yamada, Shigeki Matsunaga, Nobuhiro Fusetani, and Fumio Yamazaki, which appeared in issue 42, October 17, 2006, of Proc Natl Acad Sci USA (103:15370–15374; first published October 9, 2006; 10.1073/pnas.0604340103), the authors note that on page 15372, left column, first full paragraph, line 4, the sentence “It appeared that this amount of released L-kynurenine was sufficient to induce male behavioral responses when compared with that of bile acid pheromone (0.53 × 10^{-6} mole/female per hour) found in the sea lamprey (Petromyzon marinus) (8)” should instead read: “It appeared that this amount of released L-kynurenine was sufficient to induce male behavioral responses when compared with that of bile acid pheromone (0.53 × 10^{-6} mole/male per hour) found in the sea lamprey (Petromyzon marinus) (8).” Also, on page 15373, right column, in Behavioral Assay, first sentence, “The middle part of the Y-maze trough (13) was used as two experimental flumes (108 liters/min × 16 cm; water depth, 6 cm) under illumination at 50 lux” should instead read: “The middle part of the Y-maze trough (13) was used as two experimental flumes (108 × 16 cm; water depth, 6 cm) under illumination at 50 lux.” These errors do not affect the conclusions of the article.
L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon

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Many animals employ sex pheromones to find mating partners during their reproductive seasons. However, most sex pheromones of vertebrates remain to be identified. Over the past 20 years, steroids and prostaglandins have been identified as sex pheromones in several fishes. These pheromones are broadly termed “hormonal pheromones” because they or their precursors act as hormones in these fishes. Hitherto, no other type of sex pheromone has been unambiguously identified in teleost fish. Here we report the identification of a “nonhormonal pheromone” in teleost fish. The urine of the reproductively mature female masu salmon (Oncorhynchus masou) contains a male-attracting pheromone. Bioassay-guided fractionation yielded an active compound that was identical to L-kynurenine in spectral and chromatographic properties. L-Kynurenine is a major metabolite of L-tryptophan in vertebrates. This pheromone elicits a male-specific behavior at even picomolar concentrations; its electrophysiological threshold is 10^-14 M. L-Kynurenine is a reasonable substance for female masu salmon to advertise their readiness for mating.

Fig. 1. Pheromonal activity of OFU and the first purification. MMU, mature male urine; IFU, immature female urine. (A) Y-maze tests of spermiating males to the urine of adult salmon. BW, blank water. Each graph represents mean ± SEM (n = 16). *, P = 0.0100, compared by using the Wilcoxon signed rank test. (B) Activities of OFU and eluates from the polystyrene column in the new assay. CS, control solution (50% ethanol); WS, wash solution; 50, 50% ethanol eluate; 80, 80% ethanol eluate; 100, 100% ethanol eluate. Scores are represented as mean ± SEM (n = 14). *, P = 0.0011; **, P = 0.0003, compared by using the Mann–Whitney U test.

Results and Discussion

Y-maze preference tests showed that a male-attracting pheromone is present in ovulated female urine (OFU) (Fig. 1A). The pheromone is not present in their coelomic fluid as in the case of other salmonid fishes (13–15). To identify the pheromone, we devised a simple and reliable assay system (Fig. 2) based on the male behavior observed in the Y-maze tests (13). In this bioassay, precocious spermiating males clearly responded to OFU (left side of Fig. 1B); this finding coincided with their behavioral responses in the Y-maze trough. In the new bioassay as well as in previous Y-maze tests (13), immature males did not respond to OFU (data not shown).

The collected OFU was fractionated on a polystyrene resin column by using stepwise elution with aqueous ethanol. In the 50% ethanol eluate, ~85% of the pheromonal activity was recovered (right side of Fig. 1B). In thin-layer chromatography, the active fraction exhibited an intense blue fluorescent spot under a 365-nm UV lamp and gave a positive reaction with ninhydrin reagent. The fraction also showed characteristic UV

Author contributions: H.Y. and F.Y. designed research; H.Y., M.K., and M.Y. performed research; H.Y., S.K., S.M., and N.F. contributed new reagents/analytic tools; H.Y. and M.K. analyzed data; and H.Y., S.K., N.F., and F.Y. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: OFU, ovulated female urine; EOG, electroolfactogram.

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activity (Fig. 3) reverse-phase HPLC to produce a single peak with pheromonal the same pheromone. The pheromone was finally purified by high-resolution mass spectrometry ([M/H]11001). Marfey’s analysis (16) indicated that the pheromone was L-kynurenine. The UV absorption spectrum (data not shown). Information on the PNAS web site); this finding was also supported by the UV absorption spectrum (data not shown).

The isolation of a sex pheromone from the urine of ovulated female masu salmon. This figure will help in understanding the protocol of the bioassay and the behavioral response shown in Movie 1.

absorptions at 260 and 370 nm. However, these results were not observed in the corresponding fractions obtained from the urine of immature females and mature males (data not shown). The active fraction was further subjected to gel filtration on a Sephadex LH-20 column to separate active fractions (35–42 ml) that were associated with most of the pheromonal response (Fig. 3A). The first peak (13–17 ml) was later demonstrated to contain the same pheromone. The pheromone was finally purified by reverse-phase HPLC to produce a single peak with pheromonal activity (Fig. 3B).

The molecular formula of the pheromone was C10H12N2O3, as established by high-resolution mass spectrometry ([M+H]+, calculated, 209.0926; observed, 209.0929; error 1.4 ppm). Liquid chromatography–mass spectrometry detected no ion corresponding to any hormonal compounds in the active peak, as shown in Fig. 4A (right inset). One-dimensional and two-dimensional NMR spectra, including 1H-1H correlation spectroscopy, 1H-detected multiple quantum coherence, and heteronuclear multiple bond correlation, indicated that the compound was 2-aminooxy-4-(2-aminophenyl)-4-oxobutanoic acid (kynurenine) (Fig. 6 and Table 1, which are published as supporting information on the PNAS web site); this finding was also supported by the UV absorption spectrum (data not shown). Marfey’s analysis (16) indicated that the pheromone was L-kynurenine (Fig. 4B). The natural pheromone and authentic L-kynurenine (Sigma, St. Louis, MO) were coeluted in HPLC and showed the same mass spectrum (Fig. 4A, right inset). HPLC showed that L-kynurenine was specific to OFU (Fig. 4C); and its concentration was 10–6 M. The L-kynurenine concentrations in the OFU of the rainbow trout (Oncorhynchus mykiss) and the brown trout (Salmo trutta) were only one-hundredth of that in the masu salmon (data not shown). Although 10–6 M L-kynurenine detected in the urine of rainbow trout or brown trout is sufficient for electroolfactogram (EOG) recordings, fish would not encounter such a concentration in natural streams, thus indicating the species specificity of L-kynurenine, as suggested by our previous report (17).

The activity of authentic L-kynurenine or its sulfate was significantly higher than that of authentic D-kynurenine or D-tryptophan, a precursor of L-kynurenine (Fig. 5A). Based on the dose-response relationship of authentic L-kynurenine sulfate with regard to eliciting the behavioral activity (Fig. 5B), the threshold of the behavioral response was estimated to be <10–11 M (P = 0.0045) because the dilution rate of authentic L-kynurenine in the experimental flume was 105, as analyzed by HPLC. The EOG revealed that the olfaction of spermiating male masu salmon detected L-kynurenine with high sensitivity (Fig. 5C). EOG recordings demonstrated that only spermiating males detected L-kynurenine at concentrations as low as those that the fish might encounter in natural streams (Fig. 5D). The response threshold for L-kynurenine was 10–14 M in spermiating males (P = 0.0043), 10–11 M in immature males (P = 0.0368), and 10–9 M in sexually regressed males (P = 0.0032) and ovulated females (P = 0.0051). On the other hand, 10–5 M D-kynurenine elicited a negligible response in spermiating males (Fig. 5D); this finding is consistent with their low behavioral response to it (Fig. 5A). Both immature males and ovulated females exhibited no behavioral response to 10–9 M L-kynurenine (Fig. 5E). Therefore, the spermiating males were electrophysiologically more sensitive to L-kynurenine than inactive males and ovulated females. These differences in the olfactory sensitivity between male-maturity indices or between sexes may induce specific behavioral responses in spermiating males. They also appear to recognize the different chiral forms of kynurenine in both the EOG and the behavior.

Unidentified compound(s) present in OFU could produce a synergistic effect with L-kynurenine, because other animal species are known to use pheromone blends (18, 19). Liquid chromatography–mass spectrometry analysis of the single peak
L-Kynurenine and related compounds have been identified as metabolites of tryptophan. L-Tryptophan is a glucogenic amino acid. In the masu salmon, L-tryptophan accelerates the final maturation in females (30). Therefore, the secretion of L-kynurenine from the female masu salmon is possibly under hormonal control at the final stage of maturation, during which the levels of plasma sex hormones are elevated (31). Anadromous females of the *Oncorhynchus* species often enter into the fasting state after the homing migration for spawning, and they spawn just once in their lifetime and then die. L-Tryptophan is one of the glucogenic amino acids. In the masu salmon, L-tryptophan may also be used for gluconeogenesis until spawning time. L-Kynurenine is a previously unrecognized amino acid sex-attracting pheromone in vertebrates. Commonly occurring amino acids, such as glycine, alanine, and serine, act as feeding stimulants and not as sex attractants in a wide range of fish species (32). It is known that two olfactory receptor families (V1R and V2R) are present in the olfactory sense organ of mammals (33). Fish V2Rs are likely to recognize amino acids as feeding stimulants (34), whereas male-specific peptides stimulate mouse V2R-expressing neurons (35). Taken together, our results and the above findings suggest that fish V2Rs may also detect specific amino acids as sex pheromones. The hydrophilic property of the amino acid pheromone may be advantageous for

obtained in HPLC detected the presence of a trace amount of N-formylkynurenine (237 M+H+; Fig. 4A, left inset), a kynurenine precursor, in tryptophan metabolism. However, various proportions of the binary mixtures of synthetic N-formylkynurenine and L-kynurenine were as active as 10−4 M L-kynurenine alone, indicating that N-formylkynurenine was not involved in the pheromonal activity. The OFU also contained 1.4 × 10−4 M tyrosine, 3.9 × 10−4 M cystathionine, and 2.5 × 10−9 M prostaglandin F2α (13), but even these compounds were not synergistic with L-kynurenine. The natural concentration of L-kynurenine (10−4 M) accounted for ∼80% of the pheromonal activity in the original urine (Fig. 5A and B). Some test fish even achieved the maximum score in the behavioral response to 10−4 M L-kynurenine (Movie 1, which is published as supporting information on the PNAS web site), suggesting that it is the sole male-attracting pheromone in OFU. In preliminary Y-maze tests, sperminating mature males were also attracted to only the L-kynurenine (data not shown).

Based on the urine flow rate (∼4 ml/hour) of ovulated females (20) and the concentration of L-kynurenine in the urine, it was estimated that L-kynurenine was released at a rate of 0.40 × 10−6 mole/female per hour. It appeared that this amount of released L-kynurenine was sufficient to induce male behavioral responses when compared with that of bile acid pheromone (0.53 × 10−6 mole/female per hour) found in the sea lamprey (*Petromyzon marinus*) (8). Thus, the estimated behavioral threshold of L-kynurenine was presumed to be sufficient to attract the male masu salmon in natural streams, because the odor plume in the water flow would migrate with the streak before diffusing widely (21) and mature males appear to actively move in search of females during the reproductive period (22).

L-Kynurenine is an intermediate in L-tryptophan metabolism that produces a wide variety of physiologically active substances. L-Kynurenine and related compounds have been identified as various pigments in animals (23–25). Endogenous kynurenines were suggested to be modulators of glutamatergic neurotransmission in mammalian brains (26, 27). Although it is well known that various metabolites of tryptophan are secreted in mammalian urine (28), their functions have not been reported. In humans, tryptophan metabolism is controlled under the hormonal dynamics of the menstrual cycle (29). In teleosts, dietary L-tryptophan accelerates the final maturation in females (30). Therefore, the secretion of L-kynurenine from the female masu salmon is possibly under hormonal control at the final stage of maturation, during which the levels of plasma sex hormones are elevated (31). Anadromous females of the *Oncorhynchus* species often enter into the fasting state after the homing migration for spawning, and they spawn just once in their lifetime and then die. L-Tryptophan is one of the glucogenic amino acids. In the masu salmon, L-tryptophan may also be used for gluconeogenesis until spawning time.

![Fig. 4. LC-MS and stereochemical analyses of the pheromone and its distribution in masu salmon urine.](https://www.pnas.org/cgi/doi/10.1073/pnas.0604340103) Yambe et al.

(A) Liquid chromatography–mass spectrometry analysis of the major peak obtained by HPLC. The mass spectra of the pheromone and authentic L-kynurenine are shown in the insets on the right. The inset on the left indicates the precursor, N-formylkynurenine. (B) Marfey’s analysis (16) of the natural pheromone (upper part of B) and authentic D- and L-kynurenine. Inset shows the structure of the pheromone. (C) HPLC analysis of original urine samples. MMU, mature male urine; IFU, immature female urine.
the masu salmon, because reproductively mature females need to advertise their mating readiness and location more widely to both anadromous and resident river resident males in mountain streams, which is in contrast to many other species that possess relatively hydrophobic pheromones, namely, steroids and prostaglandins (2–7).

In summary, we provide evidence that teleost fish use a sex-attracting pheromone other than hormonal compounds; l-kyunurenine is a previously unrecognized amino acid sex-attracting pheromone. This study revealed not only a previously unrecognized function of amino acids in vertebrates but also the diversity of fish pheromones. Furthermore, our finding may provide a practical application as a powerful tool for aquacultures, for example, as a pheromone-trap to select sexually active males from parental fish in salmon hatcheries.

Materials and Methods

**Y-Maze Test.** A Y-maze trough constructed from an acrylic board (290 × 35 cm; water depth, 10 cm) was used as described (13).

Two spermiating males were acclimatized for 15 min in the downstream section under a gate, and 500 μl of urine and control solutions were simultaneously introduced into each channel during a 2-min period. The observations were started when the gate was opened 12 s after introducing the solutions. The frequency of the entry of the two males into each channel was observed for 6 min. After one trial, the sides receiving the urine and control solutions were switched. New test fish were used for each trial.

**Behavioral Assay.** The middle part of the Y-maze trough (13) was used as two experimental flumes (108 liters/min × 16 cm; water depth, 6 cm) under illumination at 50 lux. A ruler was placed on the floor plate of each flume to estimate the swimming distance of the test fish. Well water was supplied to the head of the flume (20 liters/min, 9.5°C). Before each experiment, two spermiating males were acclimatized for 15 min in each flume. We used a test fish that settled down and stayed in the downstream area. The test fish was first exposed to 100 μl of well water as a negative control, followed by 100 μl of the test solution that was equivalent to 100 μl of OFU or only the solvent (ethanol solution/distilled water), and finally to 100 μl of OFU as a positive control. These solutions were applied by using a micropipette that was not visible to the test fish. The test solution reached the downstream end of the flume in ~20 s, as estimated by using a dye. The test fish was exposed to the odor for ~10 s. After the introduction of the odor, the behavioral responses of the fish were observed for 60 s by using a remote camera system. The responses were scored according to the degree of the fish activity (1 point, rapidly beating pectoral fins; 2 points, same as 1 point plus turning in place or advancing to a distance less than half the total length of the test fish; 3 points, same as 1 point plus advancing to a distance more than half its total length). All bioassays were conducted as blind tests. The dilution rate of the test samples in the flume was estimated to be ~10^5. After exposure to the three solutions, the test fish was replaced with a new one. In the assays of the fractions, the results were omitted if the test fish did not respond to the positive control or responded to the negative control.

**Isolation of the Pheromone.** Urine (20 ml) from eight ovulated females was filter-sterilized (porosity, 0.2 μm; Millipore, Bilslerica, MA) and passed through a polystyrene resin column (1 g of TSK-G3000S; Tohsio Chemical, Tokyo, Japan; prewashed with 20 ml of ethanol followed by 60 ml of distilled water) at a rate of ~30 ml/min. The column was washed with 20 ml distilled water and eluted in a stepwise manner with 20 ml each of 50% ethanol, 80% ethanol, and absolute ethanol. The active 50% ethanol eluate was applied onto a Sephadex LH-20 column (10 × 500 mm; Amersham, Piscataway, NJ) and then eluted with 70% ethanol at a flow rate of 0.5 ml/min. We tested 18 fractions of 3 ml each. Active fractions were purified by reverse-phase HPLC on a SCL-AR-II column (10 × 250 mm; Naclacil Teseque, Tokyo, Japan) with 28% methanol at a flow rate of 1.0 ml/min. The yield of the final product was 1.1 mg per 100 ml of urine. Before the assays, the solvent of the HPLC fractions was replaced with 70% ethanol.

**Thin-Layer Chromatography.** Aliquots (10 μl) of each fraction were dissolved in 70% ethanol and spotted onto a silica gel plate (catalog no. 1.05715; Merck, Nottingham, U.K.). The plate was developed with chloroform/methanol/water (70/30/5, vol/vol/vol) and observed under a UV lamp. The plate was then sprayed with 5% ninhydrin in propanol and heated at 100°C for 3 min.

**Spectroscopic Analysis of the Pheromone.** Fraction no. 3 from the HPLC was analyzed by liquid chromatography–mass spectrometry with electrospray ionization [JEOL (Tokyo, Japan)] JMS-
T100LC AccuTOF mass spectrometer equipped with an Agilent 1100 Series liquid chromatography] using a 5C18-AR-II column (4.5 × 250 mm) with 28% methanol at a flow rate of 0.2 ml/min. The planar structure of the major peak was determined by NMR. The solvent peaks were recorded on a JEOL A600 NMR spectrometer at 300 K. The 1H and 13C NMR, and all two-dimensional NMR spectra were recorded on a JEOL A600 NMR spectrometer at 300 K. The 1H and 13C NMR chemical shifts were referenced to the following solvent peaks: δH 3.30 and δC 49.0 for CD3OD.

**Determination of Stereochemistry.** Natural kynurenine and authentic DL-kynurenine were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide and then analyzed by HPLC (16); this analysis was performed on a 5C18-AR-II column (4.5 × 250 mm) by gradient elution with 10–50% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min.

**Determination of L-Kynurenine in Salmon Urine.** Urine sample (100 μl) was centrifuged at 7,000 × g for 1 min, and 10 μl of the supernatant was analyzed as described for isolation of the pheromone by using a 5C18-AR-II column (4.5 × 250 mm).

**EOG Recordings.** EOG recordings were performed according to established methods (7). In brief, the olfactory lamellae of immobilized fish were exposed by removing the tissues covering the right naris. The recording electrode was placed just above the olfactory epithelium. The position of the electrode was adjusted to obtain the maximal response to the standard, 10−5 M L-serine. The nasal cavity was perfused with L-kynurenine solutions ranging from 10−15 to 10−5 M in a stepwise manner at a rate of 10 ml/min for 5 s with 3-min intervals. To check the reliability of the preparation, the standard was examined at the beginning and end of each concentration series of L-kynurenine. The amplitude of each response was expressed as a percentage of the most recent response to the standard. To evaluate the response thresholds, we compared the relative responses to the expected zero value by using the t test.

We thank the staff of the National Research Institute of Fisheries Science, Nikko Station, for arranging the test fish and setting up the experimental equipment; members of the Hokkaido Fish Hatchery for providing adult salmon; and Drs. M. Satou, K. Sato, M. Amano, and T. Yada for their comments on the manuscript. The Movie 1 file was prepared by Mr. T. Usami. H.Y. was supported by the Hokkaido Foundation for the Promotion of Scientific and Industrial Technology, Akiyama Memorial Life Science Foundation, Northern Advancement Center for Science and Technology, Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists, and the 21st Century Centers of Excellence Program at the University of the Ryukyus.