Influences of Pre- and Postnatal Early Life Environments on the Inhibitory Properties of Familiar Urine Odors in Male Mouse Aggression

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Abstract

For group-living animals, discriminating among individuals and chasing unfamiliar strangers away from the home range are important to protect their territory. Previously, we reported that the familiar individual information conveyed by urine results in less aggressive behavior by resident male mice toward intruders. A resident male is aggressive toward an intruding unfamiliar castrated C57BL/6J mouse (unfamiliar castrated male [UFC]), whereas there is less aggression by the resident male when the UFC is swabbed with urine collected from the resident's cage mate. Urine is affected by various factors, including the environment. In this study, we investigated the effect of 2 living environments, the early developmental environment and the adult diet, on individual information conveyed in urine. Aggressive behavior toward UFCs was lower when UFCs were swabbed with cage mate urine or urine from a cage mate's littermate that was not living with the resident male (UFCL). Litters were cross-fostered, and we examined whether the pre- or postnatal period was important for formation of individual urine odor. The resident male displayed attack bites toward UFCs that were his cage mate's littermates but were fostered by another C57BL/6J dam. In addition, a castrated male that was reared with a cage mate (sharing the same postnatal environment) but that was not his littermate was also attacked by the resident male, suggesting that littermates that share the same pre- and postnatal environments provide similar (or identical) information, which inhibits aggression. In adulthood, even after dietary changes, the resident male showed less aggression toward UFCs when the UFCs were swabbed with the cage mate's urine, which was collected before a dietary change, indicating that individual information was not affected by dietary conditions in adulthood. In a habituation–dishabituation test, resident mice could discriminate among all pairs of mouse urine from each group. These results suggest that olfactory cues containing individual information are shared among littermates, and both the pre- and postnatal environments are important for formation of the information that inhibits aggressive behavior. This individual information might differ from the odor that is used for discriminating in the habituation–dishabituation test.

Key words: aggression, early life environment, mice, urine

Introduction

Male mice are highly territorial and deposit urine scent marks to defend their territory (Desjardins et al. 1973; Hurst 1990). Mice can obtain much information from urine, such as species, sex, social status, and the individual identity of the owner (Brown 1979). It is thought to be important for mice to discriminate between their group members and unfamiliar strangers so they can defend their territory. Urine might be involved in this discrimination. Previously, we found that group-living male mice are less aggressive toward unfamiliar males that have been swabbed with a group member's urine. This finding indicates that information about familiarity that is conveyed by urine diminishes aggressive behavior by resident male mice (Nakamura et al. 2007). In the previous study, inbred mice were used as group members and unfamil-

iar strangers. Thus, there might be some factors that change individual information in individuals from the same genetic background. In this study, we investigated the effects of 2 living environments, the adult diet and the early developmental environment, on the information conveyed in urine.

Urine odor is influenced by various factors, including genetic, hormonal, microbiological, and dietary factors (Brown 1995; Hurst and Beynon 2004). In mice and rats, these individual urinary odors have been linked to genetic and environmental factors. For example, mice can discriminate among the urine odors of conspecific strains that are genetically identical except for major histocompatibility complex (MHC) genes (Yamazaki et al. 1979; Yamaguchi et al. 1981). However, rats can discriminate between the

urinary odors of MHC-congenic mice or genetically identical mice that had been maintained at different diets (Schellinck et al. 1992; Brown et al. 1996). Therefore, dietary changes might lead to changes in the information conveyed in urine.

The early life environment, both the prenatal (Maccari et al. 1995; Vallee et al. 1997; Zimmerberg and Blaskey 1998; Lemaire et al. 2000) and the postnatal maternal environment (Liu et al. 1997; Vallee et al. 1997; Kikusui et al. 2006; Veenema et al. 2006), can have a great effect not only on behavioral development but also on neuroendocrine development. Based on the fact that hormonal changes influence urine odor, sharing the maternal environment early in life might influence the individual information conveyed in urine. Additionally, other early maternal environment, such as intestinal microflora that is thought to be affected by maternal milk (Martin et al. 2003; Gronlund et al. 2007), might influence the formation of urine odor.

To examine our hypothesis, we first determined the effect of dietary changes on the information in urine that inhibits aggressive behavior toward unfamiliar castrated male (UFC). Second, we determined whether resident males could discriminate among littermates. To reveal the critical period for the development of individual information (i.e., the prenatal or postnatal environment), we cross-fostered offspring. Additionally, to confirm that mice could discriminate among urine odors, habituation–dishabituation tests were performed.

Materials and methods

Animals

Male ($n = 28$) and female ICR mice ($n = 27$), C57BL/6J male mice ($n = 54$), and pregnant female C57BL/6J mice ($n = 6$) were obtained from Clea Japan (Yokohama, Japan). In experiment 3, pups from pregnant female C57BL/6J mice were weaned at 3 weeks of age, and only male pups were used. Male A/J mice ($n = 27$) were obtained from Japan SLC. (Shizuoka, Japan). The mice were kept at a constant temperature (23°C) and humidity (40%), on a 12-h light–dark cycle. Food (rodent pellets, MM3, Funabashi Nojyo) and water were given ad libitum. The animals were handled in accordance with the "Policies Governing the Use of Live Vertebrate Animals" set by The University of Tokyo, based on "The Public Health Service Policy on Humane Care and Use of Laboratory Animals" (revised in 1985) and The National Institutes of Health's "Guide for the Care and Use of Laboratory Animals." All experiments were conducted during the light period in the colony room.

Rearing conditions

Following our previous study (Nakamura et al. 2007), to simulate natural rearing conditions, each male was reared with a female mouse and allowed to produce pups. A castrated male was added to each pair to simulate a juvenile

or a subordinate group member. In social units, dominant house mice show aggressive behaviors toward subordinates or females; however, this aggression is less than is exhibited toward an unfamiliar intruder (Mugford and Nowell 1970; Harvey et al. 1989). The androgen levels and aggression pheromones of prepubertal juvenile subordinate males are known to be lower than those of intact males (Poole and Morgan 1975; Svare and Gandelman 1975). In our study, castrated male mice can be considered comparable to subordinate or prepubertal males in terms of androgen status. At 4.5 weeks of age, each resident male was paired with a 5.5-week–old castrated C57BL/6J mouse that had been castrated at 4.5 weeks of age; each pair was placed in a polycarbonate cage (17.5 × 24.5 × 12.5 cm) with an acrylic dome (Mouse Igloo, Bio-Serv, Co. Ltd, Frenchtown, NJ). The cages were lined with soft chip bedding made from wood and corncobs. When a castrated cage mate was introduced into the resident cage, the mice soon settled after sniffing each other and engaging in occasional minor agonistic encounters without any serious aggressive behaviors. One week later, a 6-week–old female ICR mouse was introduced into the cage, and these mice were raised together. Twenty-seven C57BL/6J mice that were used as intruders also had been castrated at 4.5 weeks of ages and reared in groups of 6–7 in cages (17.5 × 24.5 × 12.5 cm) until use.

Aggression acclimation phase

When a resident reached 9 weeks of age, it was subjected to a series of resident–intruder tests. Some studies have indicated that aggressive behavior toward intruder changes as a result of experience and aggression becomes stable after about 4 repeated tests (Parmigiani and Brain 1983; Winslow and Miczek 1984); therefore, resident males were trained via 4 aggression exercises to minimize the effect of experience. The partner female, the castrated male cage mate, any pups, and acrylic dome were removed from the home cage immediately before each training bout and then an intruder male (A/J mouse, 7–9 weeks old) was introduced into the cage to confront the resident for 10 min. Aggression training was repeated 4 times at 3-day intervals for each resident male. After the aggression acclimation phase, each animal was used in the following experiments. Through these aggression exercises, resident aggressiveness becomes stable and some environment effects (such as the presence of pups during the trials) would be diminished.

Urine collection

Urine was collected by gently picking up the animals by the scruff of the neck and massaging the bladder region to induce urination. Collected urine was stored at –20°C.

Experiment 1: The effect of diet on individual information in urine

A series of aggression tests was conducted when the resident males ($n = 7$) reached 11 weeks of age. All intruders,

including cage mates, were 12 weeks old at that time. The partner female, cage mate castrated male, any pups, and acrylic dome were removed from the cage 10 min before each test and then an intruder was placed in the resident cage for 10 min. As intruders, unfamiliar castrated C57BL/6J mice (UFC, $n = 7$) that had been swabbed with either UFC urine (urine from castrated male mice that were the cage mates of other resident males) or the cage mate's urine. Urine was applied to the back and anogenital area of the intruder immediately before introducing it into the resident male's cage. To swab urine, a cotton fluff (3×3 mm) was soaked in 60 µL of urine and held with tweezers. After the aggression test toward this intruder was performed, food was changed in resident's cage from pellets for rodents to pellets for piglets (M-16, Clea Japan). One week after the food change, the aggression test was performed again using UFCs that had been swabbed with UFC urine or cage mate urine that was collected before the food change.

Based on our previous study (Nakamura et al. 2007), all experimental sessions were recorded on videotape, and the behavior parameters were later analyzed using a Microsoft Excel based Visual Basic Editor. To measure the resident's aggressiveness, we used the number of attack bites and the latency to the first attack bite. The durations of other aggressive (i.e., sideways threats and tail rattles) and nonaggressive (i.e., anogenital contact, locomotion, rearing, digging, self-grooming, and sniffing) behaviors were also measured. For attack bites, we observed the 5 min following the first bite; the other behavior's parameters were measured for whole test session (10 min).

Experiment 2: The effect of early life environment on individual information in urine

A series of aggression tests were conducted when the resident males ($n = 11$) reached 11 weeks of age. All intruders, including cage mates, were 12 weeks old at that time. Aggression tests were performed in which the cage mate and female were removed from the cage and 10 min later a UFC ($n = 11$), a UFC that was born to and raised by the same mother as the cage mate (UFCL), or the cage mate was placed in the resident male's cage (Figure 1). UFCs were swabbed with UFC, the cage mate, or UFCL urine and introduced into the resident male's cage. The urine application, recording, and analysis procedures were the same as in experiment 1.

Experiment 3: The effect of prenatal or postnatal environment on individual information in urine

Nine male ICR mice were used as the residents. A series of aggression tests were conducted when the resident males reached 11 weeks of age. All intruders, including cage mates, were 12 weeks old at that time. C57BL/6J litters were divided into 2 groups that were reared by their parents or were reciprocally cross-fostered within 16 h after birth to parents of other C57BL/6J mice. Pups were gently removed from

their cage; the tails of half of the litter were cut and they were introduced into the foster mother's cage. The remaining pups were returned to their biological mother's cage. All litters were left undisturbed until weaning. Individuals were weaned at 3 weeks of age. The castration procedure, rearing conditions, and aggression test procedures were the same as in experiment 1. As intruders, we used a UFC (control), the cage mate, a UFC that was the cage-mate's littermate but fostered to a different mother (littermate, not fostermate; LNF), or a UFC that was fostered to the cage-mate's dam (fostermate, not littermate; FNL; see Figure 1). UFCs that had been swabbed with FNL, LNF, or UFC urine were also used as intruders. The urine application, recording, and analysis procedures were the same as in experiment 1.

Experiment 4: Habituation–dishabituation test

Ten male ICR mice (9 weeks old) were used for the habituation–dishabituation test. When animals become habituated to stimulus odors by repeated presentation, investigation time of the odor decreases. An increase in investigation time following a new stimulus indicates that animals can discriminate the 2 stimuli (Brown 1988; Johnston et al. 1993). The tests were conducted for 4 consecutive days. Subjects were placed in an acrylic test box ($17.5 \times 24.5 \times 12.5$ cm). After a 3-min acclimation period, the odor stimulus was presented to the subject by hanging a cotton swab soaked with 40 µl of urine from the wire cage top. In the course of each daily test session, each subject received three 1-min presentations of water (at 30-s intervals), followed by three 1-min presentations of the first urine sample and three 1-min presentations of the second urine sample.

We tested 4 combinations of 2 urine samples.

a. Urine samples from mice born to the same mother and reared by the same mother.
b. Urine samples from mice born to the same mother and reared by different mothers.
c. Urine samples from mice born to different mothers and reared by the same mother.
d. Urine samples from mice born to different mothers and reared by different mothers.

Statistical analyses

Statistical analyses were conducted in StatView + Graphics 5.0J (Abacus Concepts, Berkeley, CA, no longer available). Values of $P < 0.05$ were considered significant. Each parameter, except latency, was analyzed using a repeated measures 1-way analysis of variance followed by a Tukey test. The latency to the first attack bite was analyzed using a Friedman test followed by a Tukey test.

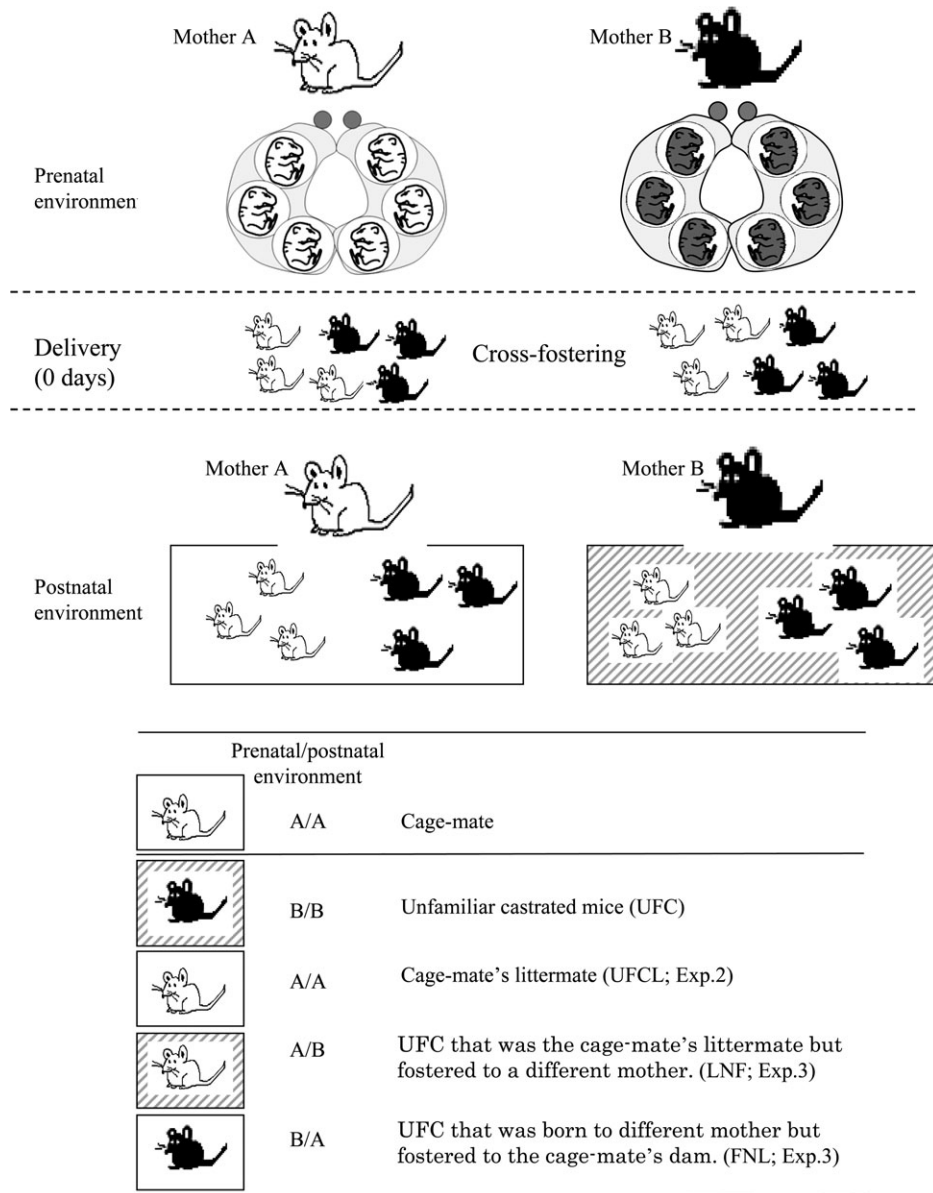The duration of investigation for each odor stimulus in the habituation–dishabituation test was analyzed. The

Figure 1 Relationships between cage mates and intruders. UFC: Unfamiliar castrated C57BL/6J mouse that was born to and raised by a different mother. UFCL: unfamiliar castrated male that was born to and raised by the same mother as the cage mate. LNF: unfamiliar castrated male that was the cage mate's littermate but fostered to a different mother. FNL: unfamiliar castrated male that was born to another mother but fostered to the cage mate's dam.

investigation times between the third water presentation and the initial presentation of the first urine and between the third presentation of the first urine and the first presentation of the second urine were analyzed using Wilcoxon rank tests.

Results

Experiment 1

In experiment 1, to observe resident aggression toward UFC with cage mate urine in which the odor profile was changed by diet, the effect of dietary condition on the individual in-

formation in urine was investigated. Following application of urine from the cage mate, the number of attack bites toward the UFC was significantly lower than those toward UFCs with UFC urine. Even 1 week after the food change, the resident male was less aggressive toward UFCs when the UFCs were swabbed with cage mate urine (Figure 2; $F[2, 12] = 6.04$, $P < 0.05$ both of before and after food change; UFC with UFC urine vs. UFC with cage mate urine, $P < 0.05$). The latency to the first attack bite also differed among intruder types, with a shorter latency toward UFC with UFC urine than toward UFC with cage mate urine both before and after the food change (Figure 2; $\chi^2 = 12.33$, $P < 0.01$
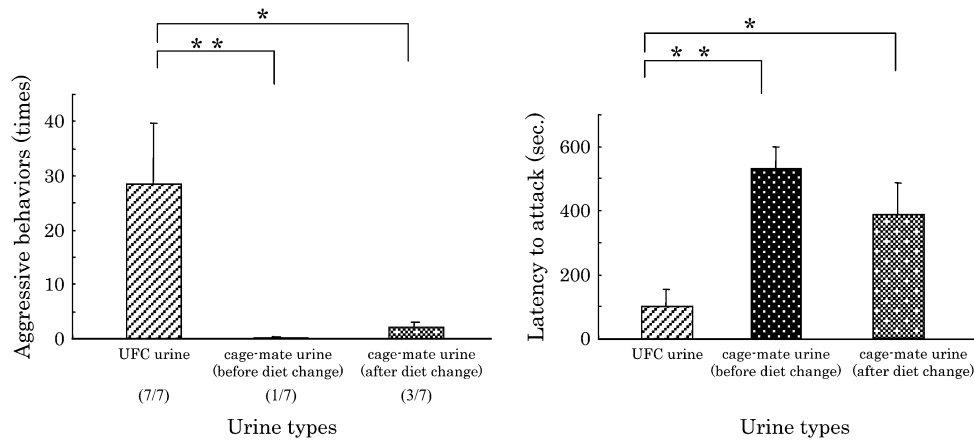
Figure 2 Experiment 1. The effect of a resident diet change on the number of attack bites (left) and latency to the first attack bite (right) against UFCs that were swabbed with cage mate urine. When UFCs were swabbed with cage mate urine before and after the food change, the number of attack bites toward them was lower and the latency to the first attack bite was longer (*$P < 0.05$ and **$P < 0.01$, repeated 1-way analysis of variance for frequency and Friedman test for latency followed by Tukey test; mean + standard error of the mean; $n = 7$). The number of resident mice that showed attack bites is shown in parentheses.

before food change; UFC with UFC urine vs. UFC with cage mate urine, $P < 0.01$, after food change; UFC with UFC urine vs. UFC with cage mate urine, $P < 0.05$). No significant difference was seen in any other behavior among groups (Table 1). In summary, the individual information in urine that inhibits resident aggression was not influenced by diet.

Experiment 2

In experiment 2, the effect of early life environments on the individual information in urine was examined using the cage mate's littermate. Resident males displayed many attack bites toward UFCs, but fewer toward their cage mates and UFCLs (Figure 3: $F[2, 20] = 12.422$, $P < 0.001$; UFC vs. cage-mate, $P < 0.01$; UFC vs. UFCL, $P < 0.01$). The latency to the first attack bite also differed among intruder types, with a shorter latency toward UFC mice than toward cage mates (Figure 3; $\chi^2 = 13.15$, $P < 0.01$; UFC vs. cage-mate, $P < 0.01$). Sniffing was also lower for the cage mate than for the other 2 groups (Table 2; $F [2, 20] = 7.309$, $P < 0.05$; UFC vs. cage mate, $P < 0.01$; cage mate vs. UFCL, $P < 0.05$). Following application of a small amount of urine from the cage mate or UFCL, the number of attack bites toward UFCs was significantly lower than those toward UFCs with UFC urine (Figure 4; $F [2, 20] = 8.456$, $P < 0.001$; UFC with UFC urine vs. UFC with cage mate urine, $P < 0.01$; UFC with cage mate urine vs. UFC with UFCL urine, $P < 0.05$). The latency to the first attack bite also differed among intruder types, with a shorter latency toward UFC mice than toward cage mates and UFCL mice (Figure 4; $\chi^2 = 14.00$, $P < 0.001$; UFC vs. cage mate, $P < 0.01$; UFC vs. UFCL, $P < 0.05$). No significant difference was seen in any other behavior among the groups (Table 3). In summary, the UFCL urine inhibited resident aggression toward UFC.

Table 1 The effect of urine on the total time of each behavioral parameter which were observed in the aggression tests in the experiment 1

Behavior	Urine types		
	UFC urine	Cage mate urine (before diet change)	Cage mate urine (after diet change)
Locomotion	269.00 ± 21.51	169.00 ± 20.33	168.50 ± 13.57
Rearing	131.00 ± 11.94	152.00 ± 22.49	129.75 ± 30.77
Digging	37.50 ± 4.84	48.25 ± 4.99	68.00 ± 18.52
Self-grooming	6.00 ± 1.78	6.75 ± 1.89	4.75 ± 1.75
Sniffing	49.50 ± 4.87	25.25 ± 7.44	23.25 ± 2.50
Sideway threats	39.25 ± 11.01	3.25 ± 3.25	5.50 ± 2.60
Tail rattles	16.00 ± 2.94	0.25 ± 0.25	2.50 ± 1.5
Anogenital contact	11.00 ± 2.38	20.25 ± 5.74	19.50 ± 3.07

Values are mean ± standard error of the mean.

Experiment 3

In experiment 3, the effect of prenatal or postnatal environments on the individual information in urine was investigated by cross-fostering. There was no difference in the number of attack bites from residents between UFC, FNL, and LNF as intruders (Figure 5). However, the latency to the first attack bite differed almost significantly among intruder types, with a shorter latency toward control mice (UFC), as compared with the LNF and FNL (Figure 5, right; $\chi^2 = 6.24$, $P < 0.05$; control vs. LNF, $P < 0.05$). There was no significant difference following the application of a small amount of urine from the UFC, FNL, and LNF (Figure 6). No significant difference was seen in any other behavior among the groups (Tables 4 and 5). By
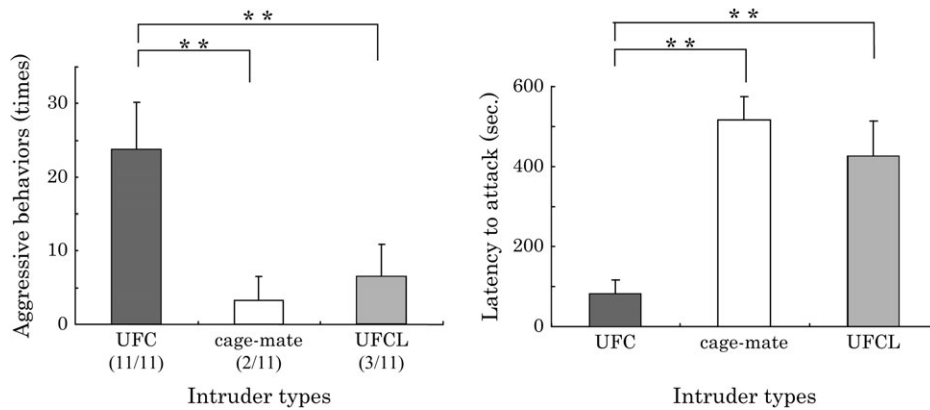
Figure 3 Experiment 2. The effect of the early life environment on the number of attack bites (left) and latency to the first attack bite (right). The number of attack bites was lower and the latency to the first attack bite was longer when the cage mate or UFCL was the intruder compared with when a UFC was the intruder (**$P < 0.01$, repeated 1-way analysis of variance for frequency and Friedman test for latency followed by Tukey test; mean + standard error of the mean; $n = 11$). The number of resident mice that showed attack bites is shown in parentheses.

cross-fostering, similarity in individual information in urine might disappear.

Experiment 4

In experiment 4, to determine whether mice could discriminate cage mates, UFC, UFCL, LNF, and FNL odors, habituation–dishabitiation tests were performed. In all combinations of urine samples, the investigation time significantly increased after changing from the third water stimulus to the initial presentation of the first urine and from the third presentation of the first urine to the first presentation of the second urine (Figure 7, $P < 0.05$). Mice could discriminate all pairs of urine odors.

Discussion

In this study, we examined the effects of diet and the early life environment on the formation of individual information in male mouse urine. Even after a food change, cage mate urine inhibited resident aggression toward UFC. Moreover, resident males showed less aggression toward UFCs when the UFCs were swabbed with UFCL urine. When a UFCL was cross-fostered and fostered by a different mother, its urine did not lower the resident male's aggression toward the UFC. These results suggest that there is individual information in urine that is not influenced by dietary changes in adulthood and that both the pre- and postnatal maternal environments are important for the formation of the individual information conveyed in urine.

In experiment 1, after a dietary change, the resident male showed less attack bites toward UFCs when the UFCs were swabbed with the cage mate's urine that had been collected before the dietary change. Although they were not statistically significant, other aggressive behaviors, such as a sideways threats or tail rattles, were also reduced. These results suggest low aggressiveness by the resident toward the in-

Table 2 The total time of each behavioral parameter in the aggression tests in the experiment 2

Behavior	Intruder types		
	UFC	Cage mate	UFCL
Locomotion	288.67 ± 14.25	288.67 ± 14.25	235.78 ± 12.22
Rearing	116.33 ± 11.15	106.22 ± 13.35	114.33 ± 7.08
Digging	53.56 ± 10.76	35.11 ± 8.32	39.33 ± 6.89
Self-grooming	7.33 ± 1.35	7.22 ± 1.15	8.89 ± 1.77
Sniffing	50.33 ± 7.50**	23.44 ± 4.13	48.67 ± 6.02*
Sideway threats	37.22 ± 13.96	10.89 ± 6.96	19.89 ± 11.74
Tail rattles	11.56 ± 6.32	3.78 ± 2.52	6.22 ± 3.27
Anogenital contact	25.33 ± 4.54	24.67 ± 3.07	30.44 ± 4.90

Values are mean ± standard error of the mean.
*$P < 0.05$, **$P < 0.01$ compared with the cage mate group for UFC and the UFCL group (repeated 1-way analysis of variance followed by Tukey's test).

truder. Urine odor can be influenced by diet (Schellinck et al. 1992; Brown et al. 1996); thus, cage mate urine might have changed after the dietary change. Nevertheless, the resident males could modulate their aggression toward UFCs that were swabbed with the cage mate's urine from before the dietary change. This result indicates that the individual information in urine is not affected by a food change during adulthood.

Regarding the early life environment, we observed the effects of pre- and postnatal environments on the individual information conveyed in urine. In experiment 2, the resident showed fewer attack bites and other aggressive behaviors (e.g., sideways threats and tail rattles) toward UFCLs and cage mates than UFCs, suggesting low aggressiveness by the resident. Moreover, the resident males showed less
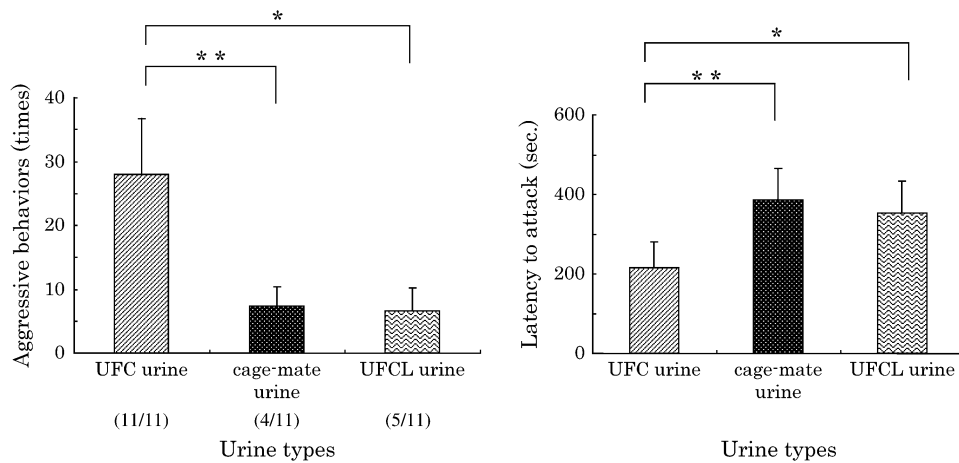
Figure 4 Experiment 2. The effect of UFCL urine on the number of attack bites (left) and latency to the first attack bite (right) against UFCs. When UFCs were swabbed with cage mate urine or UFCL urine, the number of attack bites toward them was significantly lower (*$P < 0.05$ and **$P < 0.01$, repeated 1-way analysis of variance for frequency and Friedman test for latency followed by Tukey test; mean + standard error of the mean; $n = 11$). The number of resident mice that showed attack bites is shown in parentheses.

aggressive behavior when UFCs were swabbed with UFCL urine. This finding indicates that common early life environments (both pre- and postnatal environments) might produce similar individual information in the urine, making it difficult for mice to distinguish such individual information between cage mates and UFCLs under social confrontation.

In addition, in experiment 2, resident mice spent longer periods sniffing UFCL and UFC than cage mates. Because sniffing is one means of discriminating intruders by odor, long sniffing durations might mean that residents were interested in UFCL and required more time for accurate and careful investigation to find any differences (or similarities) between UFCL and cage mates in social confrontational situations. When urine was swabbed, sniffing duration decreased and residents showed almost identical sniffing periods toward each intruder. This might suggest that individual odor information was presented strongly from swabbed urine and residents required less time to sniff intruders themselves.

Although mice did not distinguish the UFCL from the cage mate and showed less aggression toward UFCs with UFCL urine, they could discriminate between the urine of their cage mate and a UFCL in the habituation–dishabituation test. These results suggest that the individual information conveyed in urine that was used for familiarity recognition and that inhibited resident aggression is different from the odor that was used for discrimination in the habituation–dishabituation test.

In experiment 3, the cross-fostering manipulation was performed to reveal the critical period for individual urine cue development. The number of attack bites did not differ among groups. As for attack latency, although no statistically significant difference was observed, the residents tended to have longer attack latencies toward LNF and FNL compared with UFC. One explanation for this is individual

Table 3 The effect of urine on the total time of each behavioral parameter in the aggression tests in the experiment 2

Behavior	Urine types		
	UFC urine	Cage mate urine	UFCL urine
Locomotion	176.56 ± 9.88	227.43 ± 11.11	183.78 ± 13.83
Rearing	125.11 ± 10.07	141.29 ± 12.44	120.44 ± 12.81
Digging	49.89 ± 10.48	59.71 ± 13.37	52.33 ± 8.52
Self-grooming	8.56 ± 1.76	6.29 ± 0.97	8.22 ± 1.97
Sniffing	20.89 ± 9.33	26.29 ± 6.96	15.78 ± 2.75
Sideway threats	9.44 ± 7.28	6.86 ± 5.04	10.33 ± 5.04
Tail rattles	2.00 ± 1.56	2.57 ± 1.72	2.78 ± 1.89
Anogenital contact	15.44 ± 7.02	23.57 ± 6.15	9.11 ± 1.82

Values are mean ± standard error of the mean.

differences among the residents. Some of the residents showed longer attack latencies toward LNF and FNL. Comparing the similarity of individual information between UFCL and cage mates, the similarities between FLN or LNF and cage mates might be diminished by cross-fostering, and therefore, some residents showed aggressive behaviors toward LNF and FNL.

Although no significant differences were detected, the durations of sideways threats and tail rattles toward FNL were less than those toward UFC and LNF. Sharing of postnatal environments might be slightly more important for the formation of individual information. Further study is required.

Urine odor is influenced by various factors, including genetic, hormonal, microbiological, and dietary factors (Brown 1995; Hurst and Beynon 2004). In this study, we used inbred C57BL/6J mice, so the genetic background was identical, and all animals were given the same food as in
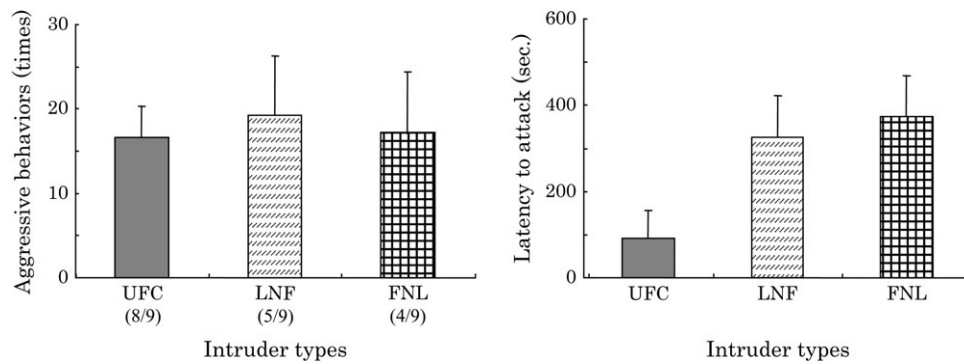
Figure 5 Experiment 3. The pre- and postnatal environments on the number of attack bites (left) and latency to the first attack bite (right) against UFCs. There was no difference in the number of attack bites or the latency to the first attack bite between intruder types (mean + standard error of the mean; $n = 9$). The number of resident mice that showed attack bites is shown in parentheses.
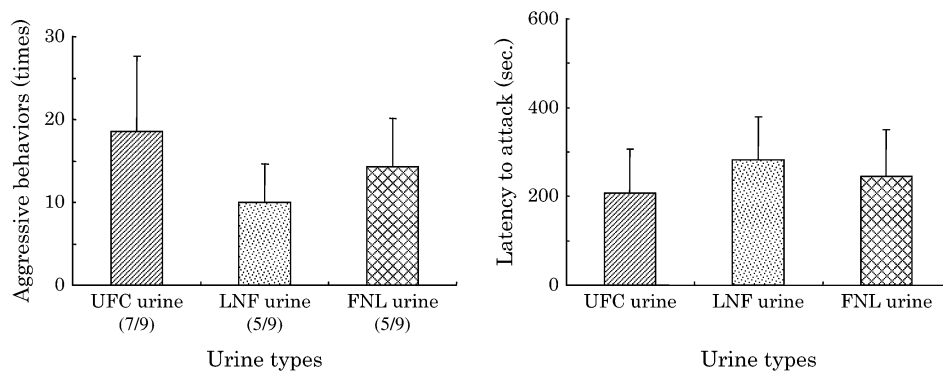

Figure 6 Experiment 3. The effects of LNF and FNL urine on the number of attack bites (left) and latency to the first attack bite (right) against UFCs. There was no difference in the number of attack bites from residents or the latency to the first attack bite between urine types (mean + standard error of the mean; $n = 9$). The number of resident mice that showed attack bites is shown in parentheses.

experiments 2 and 3. The results of experiment 1 indicate that the individual information in urine that inhibits aggression toward UFCs is not affected by dietary changes. Commensal microflora are also important in determining the unique urine odors in rats, and these microflora are established by genotype, maternal antibodies, and early diet (Brown 1995). Therefore, littermates might have similar urine odors. Hormonal effects are another important factor, suggesting that the pre- and postnatal environments affect a pup's behavior and neuroendocrine function. Thus, the environmental effect of these 2 factors (i.e., commensal microflora and neuroendocrine function) might be related to the individual information conveyed in mouse urine.

We will discuss the effect of these 2 odor-related factors during each developmental period, i.e., the pre- and postnatal environments. In the prenatal environment, regarding microflora, maternal stress affects the behavior and macrophage activity of mice (Fonseca et al. 2002). Regarding neuroendocrine factors, prenatal stressed animals show high stress responses (Bosch et al. 2007). Prenatal maternal stress also affect on pup's testosterone concentration (Kemme

et al. 2007). Rodents can discriminate androgen-dependent odors, such as aggression pheromones (Novotny et al. 1985), and discriminate between urine from dominant and subordinate individuals (Huck et al. 1981; Hurst 1990; Drickamer 1992). Moreover, major urinary protein expressions in mice urine depend on testosterone concentration and are also involved in urine odor information (Hurst et al. 2001). Although cage mates and intruders were castrated in our study, a change in testosterone concentration before castration might still have had a large effect on individual odor information in urine.

In the postnatal period, nursing is one source of infant intestinal microbiota (Gronlund et al. 2007). Because maternal corticosterone decreases maternal antibody levels in milk (Yorty et al. 2004), a pup's microflora might be influenced by maternal stress, and littermates might have the same microflora. Regarding a hormonal effect in the early life environment, the mother–pup interaction plays an important role in the offspring neuroendocrine response in adulthood. For example, hypothalamic–pituitary–adrenal and testosterone responses to stress are heightened in offspring

Table 4 The total time of each behavioral parameter in the aggression tests in the experiment 3

Behavior	Intruder types		
	UFC	LNF	FNL
Locomotion	245.00 ± 17.22	249.44 ± 11.19	241.22 ± 13.34
Rearing	89.89 ± 12.52	96.11 ± 10.39	100.44 ± 14.55
Digging	38.67 ± 12.06	41.11 ± 14.02	50.11 ± 15.60
Self-grooming	8.22 ± 1.36	11.56 ± 2.38	11.89 ± 2.26
Sniffing	45.44 ± 9.28	31.67 ± 8.23	31.89 ± 4.57
Sideway threats	50.56 ± 13.36	52.22 ± 14.14	26.11 ± 17.96
Tail rattles	13.89 ± 3.18	16.22 ± 4.23	6.78 ± 4.49
Anogenital contact	32.44 ± 6.89	31.67 ± 8.23	31.89 ± 4.57

Values are mean ± standard error of the mean.

Table 5 The effect of urine on the total time of each behavioral parameter in the aggression tests in the experiment 3

Behavior	Urine types		
	UFC urine	LNF urine	FNL urine
Locomotion	292.89 ± 25.12	251.89 ± 21.36	258.67 ± 13.75
Rearing	106.78 ± 9.59	96.00 ± 11.99	117.89 ± 18.40
Digging	84.11 ± 22.63	69.67 ± 13.39	70.78 ± 20.71
Self-grooming	11.78 ± 6.32	5.89 ± 1.58	6.00 ± 1.40
Sniffing	21.56 ± 3.24	22.44 ± 3.13	25.67 ± 7.04
Sideway threats	40.78 ± 19.22	25.66 ± 9.43	27.56 ± 12.28
Tail rattles	20.67 ± 11.84	5.89 ± 2.23	8.11 ± 3.70
Anogenital contact	17.89 ± 4.83	16.67 ± 3.71	19.89 ± 3.51

Values are mean ± standard error of the mean.

whose mothers showed low maternal care, weaned earlier, or showed maternal separation (Liu et al. 1997; Vallee et al. 1997; Kikusui et al. 2006; Veenema et al. 2006). To our knowledge, the relationship between corticosterone and odor is unknown. However, rats can discriminate urine odors from mice in stressful conditions (Mackay-Sim and Laing 1981). Corticosterone concentration in the early life environment might be important in the formation of individual information in urine.

These 2 periods; (i.e., the pre- and postnatal environments) are important for the development of individual information, and sharing these periods might lead to similarity of individual information in urine. Bowers and Alexander suggested that mice could discriminate among urine from within the same strain (Bowers and Alexander 1967). Moreover, Francis et al. found that both the pre- and postnatal
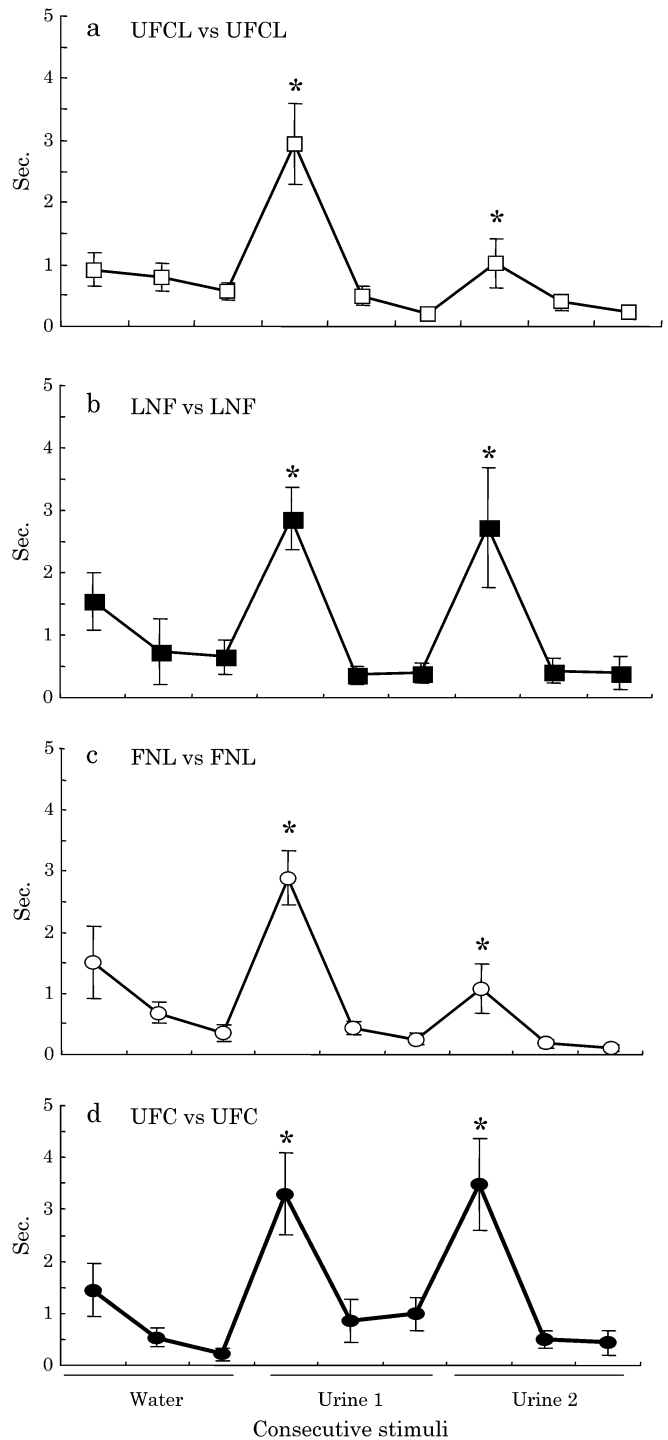

Figure 7 Investigation time for 2 types of urine in the habituation–dishabituation test (experiment 5). *$P < 0.05$; Wilcoxon signed-ranks test comparison with the third presentation of the previous stimulus (mean ± standard error of the mean). **(a)** Urine samples from mice born to and reared by the same mother. **(b)** Urine samples from mice born to the same mother and reared by different mothers. **(c)** Urine samples from mice born to the different mothers and reared by the same mother. **(d)** Urine samples from mice born to different mothers and reared by different mothers.

environments are involved in behavior development in 2 in-
bred strains (Francis et al. 2003). Our results support these
reports and suggest that even in an inbred strain, individual
variance arises, and gathering small changes in the pre- and
postnatal environments is needed to develop them. However,
because inbred C57BL/6J mice were used as intruders and
cage mates in our study, if embryo transfer is performed be-
tween different inbred strains and protocols used here
are followed, resident males might be able to discriminate
between odors from mice that shared pre- and postnatal
environments.

It is also possible that resident male mice use a "group
odor" to discriminate among other group males. Ants can
discriminate between nestmates and non-nestmates via che-
mosensory sensilla (Ozaki et al. 2005). In the wild, consum-
ing the same food would be a good indicator by which to
discriminate between group members and unfamiliar strang-
ers. In this study, cage mate urine inhibited resident aggres-
sion both before and after a dietary change. Moreover, the
resident male showed less aggression toward UFCLs even
though they were not group members. These results suggest
that resident males do not use a group odor common to all
group members, but recognize their cage mate's information
in urine and decide whether to fight. Consequently, environ-
mental changes after growth, such as a dietary change, do
not seem to be involved in conveying individual information
in mouse urine.

In summary, our results suggest that the individual infor-
mation conveyed in urine is not affected by dietary changes
but is affected by both the pre- and postnatal environments.
Even within an inbred strain, genomic information might be
modified by the pre- and postnatal environments, and indi-
vidual information in urine that inhibits aggression by resi-
dent male mice could be changed. Our results also suggest
that mice could distinguish odors involved in sociosexual be-
haviors from other unrelated odors.

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