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# Prenatal Flavor Exposure Affects Flavor Recognition and Stress-Related Behavior of Piglets

**Marije Oostindjer, J. Elizabeth Bolhuis, Henry van den Brand and Bas Kemp**

Adaptation Physiology Group, Wageningen Institute of Animal Sciences, Wageningen University, Marijkeweg 40, PO Box 338, 6700 AH Wageningen, The Netherlands

*Correspondence to be sent to: Marije Oostindjer, Adaptation Physiology Group, Wageningen Institute of Animal Sciences, Wageningen University, Marijkeweg 40, PO Box 338, 6700 AH Wageningen, The Netherlands. e-mail: marije.oostindjer@wur.nl*

## Abstract

Exposure to flavors in the amniotic fluid and mother's milk derived from the maternal diet has been shown to modulate food preferences and neophobia of young animals of several species. Aim of the experiment was to study the effects of pre- and postnatal flavor exposure on behavior of piglets during (re)exposure to this flavor. Furthermore, we investigated whether varying stress levels, caused by different test settings, affected behavior of animals during (re)exposure. Piglets were exposed to anisic flavor through the maternal diet during late gestation and/or during lactation or never. Piglets that were prenatally exposed to the flavor through the maternal diet behaved differently compared with unexposed pigs during reexposure to the flavor in several tests, suggesting recognition of the flavor. The differences between groups were more pronounced in tests with relatively high stress levels. This suggests that stress levels, caused by the design of the test, can affect the behavior shown in the presence of the flavor. We conclude that prenatal flavor exposure affects behaviors of piglets that are indicative of recognition and that these behaviors are influenced by stress levels during (re)exposure.

**Key words:** behavioral tests, chemosensory learning, neophobia, pigs, preference

## Introduction

Young animals face a major challenge around weaning. Instead of relying on their mother for the provisioning of healthy food, they need to start selecting healthy and nutritious food types by themselves. Olfactory cues that are transmitted from parents to offspring by means of skin, feathers or fur, feces, diet, or breath may be used to select appropriate food sources and foraging sites (Morrow-Tesch and McGlone 1990; Bilko et al. 1994; Galef 1996).

Learning about nutritious food types may already take place before birth by transmission of chemosensory cues from the maternal diet to the offspring. Flavors can be transmitted to amniotic fluid and may be perceived by the fetus during mouthing movements and ingestion of the fluid (Mennella et al. 1995; El-Haddad et al. 2005). In addition, flavors from the maternal diet can enter the fetal blood stream after crossing the placental barrier and may be perceived through the fetal nasal capillaries (Schaal et al. 1995). Schaal et al. (2000) showed that babies of mothers that ingested anise-flavored foods during the last 2 gestational weeks showed a higher preference for anise compared with nonexposed infants in the first 4 days after birth. Similar effects of prenatal exposure to a particular flavor on olfactory preference have been shown in rats (Smotherman 1982a; Hepper 1988) and dogs (Wells and Hepper 2006). Moreover, prenatal flavor exposure resulted in enhanced acceptance of similarly flavored food around weaning in rabbits (Bilko et al. 1994) and sheep (Simitzis et al. 2008). Effects of prenatal flavor exposure on later preference might be strengthened if combined with flavor exposure through the maternal milk during lactation (Galef and Henderson 1972; Bilko et al. 1994; Désage et al. 1996; Mennella et al. 2001; Hepper and Wells 2006), although the relative effectiveness of prenatal as opposed to postnatal exposure may be dependent on the species under study. Prenatal exposure appears to affect preference in all species tested so far (see Schaal and Orgeur 1992 and Bolhuis et al. 2009 for review), but it can be postulated that postnatal exposure without prenatal exposure may have the strongest effect in altricial species, in which the brain and the olfactory system are still relatively immature and plastic as compared with precocial species (Brunjes 1983). Precocial animals, on the other hand, generally start exploring and feeding relatively early in life, and therefore, postnatal programming of preference, that is, changing the structure and function of tissues such as the brain through early experiences (Seckl 2001), may be less beneficial for those species.

Early chemosensory learning may be of particular interest for piglets in animal husbandry, which are generally weaned abruptly and at a much younger age than would happen under (semi)natural conditions (Jensen 1988). As a consequence, piglets often display a very low food intake and high stress levels in the first days postweaning (Bruininx et al. 2002). Increasing the preference for a certain type of food may be helpful in motivating piglets to ingest solid food and thereby reduce welfare and health problems, such as diarrhea and weight loss, that are associated with the low food intake around weaning (Jarvis et al. 2008).

Apart from influencing flavor preference, (early) exposure to flavors may also affect emotionality and stress responsivity during reexposure to these flavors. For instance, flavors associated with traumas increase alpha activity in the electroencephalogram of veterans suffering from posttraumatic stress disorder (McCaffrey et al. 1993). Flavors that are of personal significance affect emotionality through increased activity in the amygdala (Herz et al. 2004). A neutral familiar flavor, such as vanilla, or a positive familiar flavor such as mother's milk reduces crying and grimacing after administration of a heel stick and overall body movement during the heel stick procedure in babies (Rattaz et al. 2005). This means that during a test for flavor preference, which often takes place in a novel environment, the behavior of previously exposed animals may be different from that of control animals due to the mere presence of the familiar flavor in the environment.

These potential stress-reducing effects of familiar flavors are often overlooked in experiments on the effect of early flavor exposure on preference. There is a large variation in the paradigms and observations used to assess flavor preference. For instance, in some paradigms, the ingestion of the flavor (Hepper and Wells 2006) or feeding-related behaviors, such as pecking in flavored substrate (Sneddon et al. 1998), are measured, whereas in others exploration and time spent in differentially flavored locations in a Y-maze are assessed (Morrow-Tesch and McGlone 1990). In the different paradigms used to assess flavor preference, stress levels induced by the test situation may vary due to, for instance, novelty of the test environment or social isolation. The stressfulness of a test situation may in turn moderate the expressed flavor preference of animals that have pre- or postnatally been exposed to this flavor. For instance, in a relatively stressful test setting, exposed animals could be less inhibited to explore or ingest both the test flavor and control flavors than unexposed animals. So, on the one hand, relatively stressful test situations may increase the contrast in behavior of exposed and unexposed animals. On the other hand, a stressful test situation may also mask flavor preferences if the mere presence of a familiar flavor in the test situation reduces neophobia and results in a lower reluctance of exposed animals to explore the unfamiliar control flavors.

It is important to know whether the intrinsic stressfulness of a test situation interferes with the expressed preference within a paradigm in order to facilitate interpretation of the response of the experimental animals. Comparing stress-related behaviors of previously exposed and unexposed animals within a test provides information on the changes in stress that are induced by the familiarity of the flavor. A comparison between different tests may provide information on different motivations of previously exposed compared with unexposed animals and can be used in the interpretation of flavor preference data.

The aim of the current study was to test whether flavors experienced by piglets pre- and/or postnatally are determinants of flavor recognition before weaning. As the pig is a relatively precocial species that starts to explore and forage early in life, we hypothesized that prenatal flavor exposure and the combination of pre- and postnatal exposure would lead to behaviors indicating recognition, but postnatal exposure alone may not. We furthermore investigated whether the stressfulness of the flavor recognition tests used would affect behavior of the animals during (re)exposure.

## Materials and methods

### Animals and housing

The experiment was approved by the Animal Care and Use Committee of Wageningen University. The experiment was set up in a 2 × 2 factorial arrangement and was carried out in 2 successive batches. A total of 18 multiparous gestating Great Yorkshire × Dutch Landrace sows were used. An overview of all experimental treatments and procedures is given in Figure 1.

Sows were either exposed to flavored (F, $n = 9$) or control (C, $n = 9$) feed during days 98–108 of gestation (see below). During the last trimester of gestation, fetuses of several mammalian species appear to be able to detect and retain chemosensory information (see Schaal and Orgeur 1992). Flavor treatment ceased on day 108, a week before farrowing, to prevent flavor exposure through mother's feces, milk, or breath before the postnatal flavor treatment started (see below). All sows were provided with feed without any additions between day 109 of gestation and day 6 of lactation. In this period, sows were gradually switched from gestation feed to feed for lactating sows. From farrowing onward, sows received only lactation feed.

From day 95 of gestation onward, sows were individually housed in 4 different stables: 2 control stables and 2 flavor stables. On day 110 of gestation, sows were moved to 4 clean stables in which the test flavor had not been present before and were placed individually in a farrowing pen of 3.54 × 2.20 m. The sow was placed in a farrowing crate (2.16 × 0.62 m) in the front center of the pen. All sows farrowed within a 4-day range (gestational day 115–118), and 1 day after the last sow farrowed, all piglets were cross-fostered to another sow.

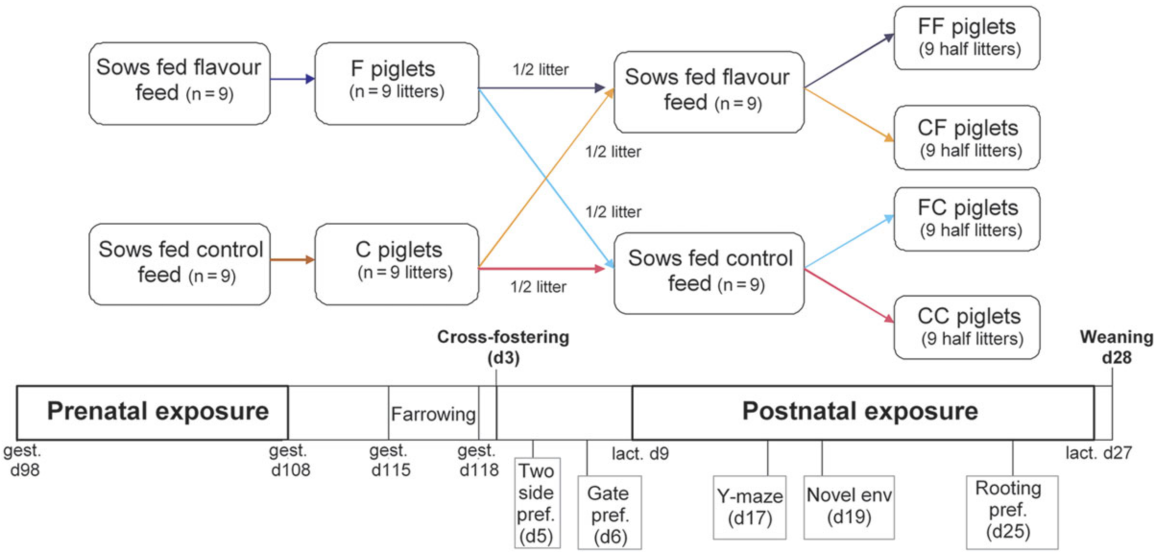

**Figure 1** The experimental design and the timing of the different treatments, procedures, and behavioral tests. Average days of age are given for each test; for exact age and variation per treatment group, we refer to Table 1.

Piglets remained with their biological mother between birth and cross-fostering.

Each sow fostered 10 piglets that she did not give birth to 5 from an F-fed sow and 5 from a C-fed sow. Both male and female piglets were used, and the distribution of piglets of different sexes between half litters was balanced for treatments. A half litter generally consisted of piglets originating from the same sow. In some cases of small litter sizes, the half litter consisted of offspring from 2 different sows; piglets were always cross-fostered together with at least one sibling. Four additional sows participated in the experiment (1 fed F and 3 fed C feed from days 98 to 108 of gestation) but were not included in this analysis. These sows only provided some piglets for the cross-fostering.

Half of the sows of each prenatal treatment were provided with F feed from days 9 to 27.0 ± 1.1 of age (mean ± standard deviation) and the other half with C feed. Hence, piglets were exposed, through their (foster) mother's diet, to flavor from days 98 to 108 of gestation (FC), from days 6 to 24 of lactation (CF), during both gestation and lactation (FF), or not at all (CC, see Figure 1), $n = 9$ half litters per treatment combination. See Table 1 for an overview of the characteristics of the animals within the different treatments.

Piglets were weaned at 28.0 ± 1.1 days of age. No feed was provided to the piglets during the lactation period.

## Flavor exposure

Anise was chosen as the experimental flavor in this experiment. Anise is known to be accepted in the feed by pigs (Langendijk et al. 2007), and previous research in dogs and humans has shown that addition of this flavor to the maternal diet during gestation or lactation results in recognition by the offspring (Schaal et al. 2000; Wells and Hepper 2006). All sows in the flavor groups received a daily dose of 350 mg *trans*-anethol (99%, Sigma-Aldrich), the molecule responsible for the anisic flavor (Karaali and Başoğlu 1995), given in 2 daily portions of 175 mg. This dose was based on a study done in humans (average anethol dose of 121.2 mg/day, Schaal et al. 2000), scaled for the average body weight of sows during late gestation. The portions of 175 mg anethol were dissolved into 20 mL soy oil and kept in 20-ml syringes in the dark. Syringes containing 20 mL of soy oil were prepared for the control sows and were kept at a considerable distance from the anethol solutions to prevent any contamination.

**Table 1** Characteristics of animals exposed to anise pre- and postnatally (FF), only prenatally (FC), only postnatally (CF), or never (CC)

| | FF | FC | CF | CC |
|---|---|---|---|---|
| Number of half litters | 9 | 9 | 9 | 9 |
| Number of piglets | 44 | 44 | 45 | 44 |
| Number of males | 24 | 22 | 21 | 20 |
| Age at cross-fostering (days) | 2.7 (0.9) | 2.6 (0.9) | 3.0 (1.1) | 3.0 (1.3) |
| Body weight at cross-fostering (kg) | 1.7 (0.3) | 1.7 (0.3) | 1.7 (0.3) | 1.8 (0.4) |
| Body weight at weaning (kg) | 8.7 (1.0) | 8.7 (1.3) | 8.6 (1.2) | 8.5 (1.2) |

Standard deviations are given within parenthesis.

The anethol solution (or plain oil) was sprayed on top of a portion of 300 g of food (standard commercial sow diets), which was between 4% and 10% of the total daily food intake depending on the gestational and lactational stage. Sows were fed this mixture in separate feeding troughs that could be placed into the trough of the home pen. Additional food was not given until the sow finished the treatment food, which resulted in an anethol uptake of 100% for sows in the experimental groups.

When the sow finished eating, the trough was removed from the pen and transported in closed plastic bags to a separate room for cleaning. A protocol was followed to prevent exposure of the control sows and piglets to the anethol. This protocol included feeding the control sows before the flavor sows; wearing gloves; keeping the exposure of anethol-contaminated objects, such as the troughs and syringes, to the air to a minimum by transporting them in plastic bags; and changing clothes between the morning and afternoon feeding period.

## Behavior during flavor exposure

The first 2 behavioral tests described below were carried out before the postnatal flavor treatment and thus only tested effects of prenatal exposure. The final 3 tests described were carried out during the postnatal exposure and thus tested both pre- and postnatal exposure effects. In most tests (see below), peppermint was used as a control scent. A small pilot study, performed before the current experiment, showed that piglets did not differ in preference for anisic and minty flavors. Peppermint was not used to flavor the food of the sows so that effects of flavor exposure on flavor preference, food intake, and growth could be examined after weaning (results will be reported elsewhere). Concentrations of the different flavor stimuli were determined before the experiment by a human panel so that strengths of the scents were matched for anise and mint.

During the experiment, 36 half litters were used, 9 of each treatment. In each half litter, one piglet was kept naive to the anisic flavor in the tests. The other 4 piglets all participated in both the 2-side preference test (day 2) and the gate preference test (day 3 or 4). The naive piglet was also handled in the 2-side preference test and exposed to the gate preference test but in the absence of the anisic or minty flavors. Subsequently, out of the 4 tested piglets, 1 was tested in the Y-maze test (day 14), 2 other piglets were tested in pairs in the rooting preference test (day 22 or 23, $n = 20$, only second batch), and the fourth piglet was tested in the novel environment test on day 16. The naive piglet was used as the control in the novel environment test. The timing of the different behavioral tests is given in Figure 1.

Each of the tests was classified as inducing low, moderate, or high stress levels before the experiment, based on whether the test was conducted alone or with siblings, in the home pen or elsewhere, and with or without habituation to the test environment and procedure. To validate this classification, the number of vocalizations given by the control animals (CC) as well as the percentage of control animals that defecated or urinated or tried to escape from the test environment were assessed to make a post hoc classification of stress levels within a test. The Y-maze test and the rooting preference tests had higher post hoc stress levels than were previously assigned, perhaps due to less effective habituation than assumed. The number of vocalizations per piglet per minute as well as the percentage of animals that defecated, urinated, or tried to escape are given in Table 2.

The order of testing the half litters and placement of the experimental flavors within a behavioral test were always balanced for treatment.

### *Two-side preference test*

The 2-side preference test was carried out when piglets were 5.0 ± 1.1 days of age and was done on a half litter basis. This test was based on the study by Hepper (1987), in which proximity to a scented object was considered a determinant of preference. The test was assumed to induce only low stress levels because piglets were subjected to the test in their home pen together with siblings. The other half litter plus the naive piglet of the focal half litter remained behind a wooden board in the home pen during the test. The second half litter of the pen was tested immediately after the first half litter was tested.

The focal piglets were presented with 2 test tube holders, each holding a cotton swab dipped in either anise seed infusion (33 g anise seed per liter) or peppermint infusion (8 g peppermint leaves per liter). Flavor infusions were used because of the close proximity of the animals to the stimuli. The test tube holders were placed against one wall of the pen with an equal distance between the test tube holders and the front and back of the pen. The test started at the moment the test tube holders were in place and lasted 5 min. The behaviors of interest during this test were sniffing the swabs, chewing on the swabs, sniffing and/or chewing on the holders, and time spent on either the anise or control side of the test area (see Figure 2C for an impression). Two people were positioned outside the pen against the wall in front of which the test tube holders were placed, one on each side of the area. Behavior of one half litter could not be analyzed due to technical problems with video recording.

### *Gate preference test*

The gate preference test was carried out when piglets were 6 or 7.0 ± 1.1 days of age. Piglets were separated from the sow and given the opportunity to return to the sow via either an anise-scented gate or a mint-scented gate. We expected piglets to have a preference for a familiar smelling gate to move from a nonsafe environment to a safe environment (the sow). The test was assumed to induce moderate stress levels due to a separation from the sow and siblings in a part of the home pen, which may not be favored by piglets at that age.

The sow was confined to the farrowing crate, and a wooden board of 0.8 × 0.6 × 0.03 m with 2 ellipse-shaped holes (gates) on each side of the center (center of the holes at 21.5 cm

**Table 2** Mean vocalizations per piglet per minute, percentage of animals that urinated and/or defecated, percentage of animals that made an attempt to escape the test setup, and the preassigned and post hoc assigned stress levels for the 5 different behavioral tests

| | Mean vocalizations/ piglet/minute | Percentage of animals defecating/ urinating | Percentage of animals showing escape behavior | Preassigned stress level | Post hoc stress level |
|---|---|---|---|---|---|
| Two-side preference test | 0 (0) | 0 | 0 | Low | Low |
| Gate preference test | 16.3 (14.6) | 11 | 0 | Moderate | Moderate |
| Y-maze test | 72.6 (21.9) | 44 | 0 | Moderate | High |
| Novel environment test (without anise) | 65.3 (26.7) | 55 | 22 | High | High |
| Rooting preference test | 27.7 (5.2) | 10 | 0 | Low | Low–moderate |

Standard deviations of the means are given within parenthesis.

above the floor, hole diameter = 32.5 cm, distance between holes = 25 cm) was placed on the outside of the farrowing crate, behind the sow. A hook was present above each gate on the side of the board facing the sow to which a cotton swab dipped in anise or peppermint infusion (see 2-side preference test for concentrations) could be attached. Placement of anise- and mint-flavored swabs (left or right) was balanced for treatments. Two wooden boards (170 × 0.6 m) were used to close off the area behind the sow and were placed diagonally, leading to a trapezium-shaped arena of 60 cm near the sow and 258 cm on the far side of the sow (see Figure 2A). Two people were positioned on the outside of the trapezium, one on each side. The gate above which the anise was placed was kept the same within a pen to avoid mixture of scents. The 2 naive piglets of the pen went through both gates, without the anise and mint flavor present, to provide scent marks from piglets on both gates. The naive piglets remained behind wooden screens in the home pen during the test. Piglets that were already tested were kept separate behind a wooden screen from the piglets that still were to be tested to avoid contamination through body contact.

The test piglet was taken individually from the compartment left of the farrowing crate and was placed in the center of the arena. It had to choose one of the gates (anise- or mint scented) to return to the sow. The test ended when the piglet's entire body went through the gate or when 5 min had passed (Figure 2B). The gates were cleaned with water after all piglets of the pen were tested. The test was videotaped with a camcorder, and the following parameters were scored afterward: choice of gate, sniffing and chewing on the edge of the gate, as well as being on the left or right side of the test arena. Interest in the gates was calculated as time spent sniffing and chewing on the gate divided by the total test time.

### Y-maze test

The Y-maze test was carried out when piglets were 17.0 ± 0.9 days of age and was done on an individual basis. The test was adapted from Morrow-Tesch and McGlone (1990) in which the time spent in the arm containing the test scent was a measure of preference. The test was assumed to induce somewhat higher stress levels than the previous tests due to temporary social isolation away from the home pen after habituation. A maze was built that consisted of 3 arms of 1.2 m × 25 cm and with walls of 1 m high. The maze was divided into 4 compartments: the start arm, the center, the flavor arm, and the control arm (Figure 2E). A wooden trapdoor was located at the end of the flavor and control arms, with a small ventilator placed in the middle of the door and with the center of the fan at 31.5 cm height. The flavor and control arms were switched between batches but not within a batch to minimize contamination risks. A petri dish filled with 4 drops of anethol (99%) and peppermint oil was placed behind the flavor and control arms, respectively, in front of the ventilator that blew the scents slowly into the flavor and control arms (0.9 $m^3$/min). Pure anethol and peppermint oil were used because the scent needed to be strong enough to carry throughout the respective arm of the Y-maze.

Piglets were habituated to the Y-maze on day 12 after cross-fostering without the flavors present, together with the other focal piglet from the same foster sow. During the actual test, the focal piglet was placed into the start arm of the maze and was left to explore the maze for 3 min. Vocalizations were scored live. The observer was positioned 5 m behind the start arm of the maze, outside of the visual range of the piglet. Time spent in each arm, locomotion, and latency to enter arms were scored afterward with video observations using focal sampling and continuous recording with the Observer 5.0 (Noldus B.V., Wageningen, The Netherlands). The maze was cleaned between trials.

### Novel environment test

The novel environment test was carried out when piglets were 19 ± 1 days of age. The test was assumed to induce, as compared with the other tests, relatively high stress levels due to isolation in a strange environment without habituation. A walled arena of 2.2 × 2.2 × 1.0 m was set up in a room unfamiliar to the piglets. All 4 walls contained

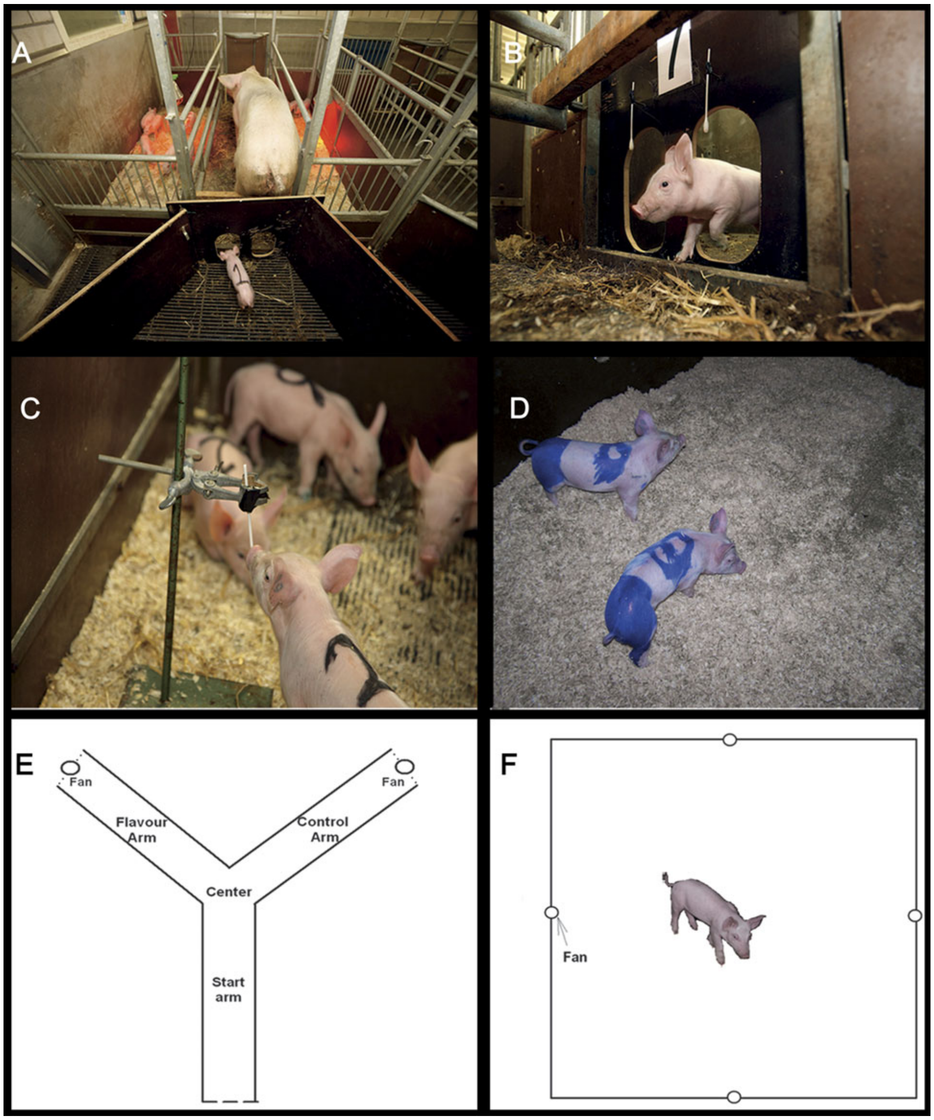

**Figure 2** Photographs of 3 of the behavioral tests and general setup of the other 3 behavioral tests. Panel **A** shows the general setup of the gate preference test, with the piglets to be tested on the left side of the farrowing crate and the piglets that have been tested on the right. Panel **B** shows the 2 flavors above the different gates. Panel **C** shows chewing behavior in the group preference test. Panel **D** gives an impression of the rooting preference test. Panel **E** shows the setup for the Y-maze test. Panel **F** shows the setup for the novel environment test.

a fan (see Y-maze test). The naive piglet of the half litter was first subjected to the novel environment for 3 min in absence of any test flavor but with the fans turned on. After the trials with the naive piglets, the arena was scented with anethol (99%) through the fans in each wall of the arena (Figure 2F). Anethol was used to ensure the scent being present in the entire test arena. The same test procedure as described for the naive piglets was followed for the test piglets. Number of vocalizations, escape attempts, defecation, and urination were scored live from a video screen positioned outside the visual range of the focal piglet. The arena was cleaned after each trial with water and dried afterward.

### *Rooting preference test*

The rooting preference test was carried out when piglets were 25.0 ± 0.4 days of age and done with sibling pairs on 2 consecutive days. The test was based on the "olfactory preference test" for chickens as described by Sneddon et al (1998) in which the amount of time spent on scented shavings was taken as a measurement of preference. This test was assumed to induce a relatively low level of stress due to the piglets being tested in pairs after habituation. A walled arena of 2.2 × 2.2 × 1.0 m was used to test the pairs of piglets. The piglets were habituated to the arena with unscented substrate on day 21 after cross-fostering for 5 min, together with the test pair of the other half litter from the same pen. During the actual test, half of the floor of the arena was covered with substrate scented with anisic flavor and the other half with peppermint-scented substrate (175 mg of 99% anethol dissolved in 20 mL oil and 20 mL peppermint oil, respectively, shaken in a bag with 18% of the substrate for the top layer). The 2 halves of the arena were divided by a low wooden beam (3.5 cm high). Piglets were introduced to the arena with the front legs on one scented half and the hind legs on the other half. One piglet faced the anise-scented half, and the other piglet faced the mint-scented half of the arena. Behavior was scored live by 2 observers, using the Observer 3.0 installed on a Psion Organizer II LZ64, by continuous focal sampling. Two observers were positioned on the outside of the arena at both ends of the wooden beam that divided the 2 halves of the arena. Time spent on either side was scored, as well as exploration of the floor (rooting, sniffing, and chewing substrate), standing alert (pricked ears and raised head), and contact behavior (nosing head or body of the other piglet). The test time was 5 min after which the substrate was completely renewed. The side with the anise-flavored substrate was constant between trials but reversed between days. See Figure 2D for an impression.

### Statistical analyses

Effects of pre- and postnatal flavor treatments were analyzed with general linear models (GLMs) in SAS (SAS 9.0, SAS Institute Inc). Data from tests in which more than one piglet from the same half litter performed the same test (2-side preference test, gate preference test, and rooting preference test) were averaged per half litter. Latencies and the proportion of time spent on a behavior were log- and arcsine square root transformed, respectively, when the residual variance was not normally distributed.

Behaviors in the 2-side preference test and gate preference test were analyzed using a model with prenatal treatment and batch as main effects. Preliminary analysis of the behavioral data from the gate preference test tended to show a choice bias for the left gate, irrespective of the flavor associated to the gate ($P = 0.08$, logistic regression), and therefore, the left and right gate were analyzed separately. The choice for the anise or mint gate was analyzed using a logistic regression model with prenatal exposure as class variable.

Behavior in the Y-maze was analyzed with a model including prenatal exposure, postnatal exposure, their interaction, and batch as class variables. A logistic regression using the same factors was used to investigate differences between treatments in which arm of the Y-maze was entered first. Behavior in the novel environment test was analyzed in a GLM with prenatal treatment, postnatal treatment, and anise presence included, as well as their interactions and batch as class variables. The data in the rooting preference test were analyzed with a model including prenatal treatment, postnatal treatment, and their interaction.

Data are presented as mean ± standard error of the mean unless stated otherwise.

## Results

### Two-side preference test

In total, 79% of piglets tested showed any exploratory behavior directed to the cotton swabs in the 2-side preference test. Out of these piglets, 84% explored the anise-flavored swab, and 82% of the piglets explored the control-flavored swab.

Time spent chewing or sniffing the anise-flavored cotton swab or the holder of the swab did not differ between half litters exposed to anise prenatally and control half litters (chewing: 7.5 ± 2.4 s, sniffing: 4.7 ± 1.0 s, holder: 21 ± 3.9 s). There was also no effect of prenatal treatment on chewing and sniffing the control-flavored swab nor in exploring the holder of the control swab (chewing: 6.9 ± 2.2 s, sniffing: 3.7 ± 0.9 s, holder: 19.9 ± 3.8 s). Finally, no differences were observed between treatment groups in the time spent on the anise or control side of the pen (anise side: 154.8 ± 10.6 s, control side: 144.4 ± 10.7 s, all $P$'s >0.21).

### Gate preference test

Three piglets did not choose any gate to return to the sow within the allotted time of 5 min in the gate preference test. The proportion of piglets choosing the anise gate with anise either above the left or right gate did not differ between piglets that were prenatally exposed to anise and controls ($P = 0.15$, see Figure 3A,B).

Treatment did not affect interest in the anise and control gate or time spent on the left or right side of the test arena when anise was placed above the left gate (all $P$'s >0.53, Figures 3C,E). When the anise was placed above the right gate, piglets exposed to anise prenatally tended to show a higher interest (i.e., sniffing and chewing) in the anise gate ($F_{1,15} = 4.1$, $P = 0.06$) and showed a lower interest in the control gate ($F_{1,15} = 5.1$, $P = 0.04$, see Figure 3D) than control piglets. Piglets that were prenatally exposed to anise spent more time on the anise side of the test arena than control piglets, who spent more time on the control side of the arena when anise was above the right gate ($F_{1,15} = 5.2$, $P = 0.04$, Figure 3F).

### Y-maze test

Treatment did not affect the time spent in the flavor arm or the control arm of the Y-maze test nor the latency to enter the flavor arm (all $P$'s >0.26). Piglets that were never exposed to anise through their mother's diet (CC) tended to enter the control arm sooner for the first time than piglets from the other 3 treatments (CC: 25.1 ± 4.8 s, FF: 57.2 ± 18.6 s, FC: 57.9 ± 14.3 s, CF: 53.6 ± 15.6 s, prenatal × postnatal interaction, $F_{1,35} = 3.14$, $P = 0.086$). Piglets exposed to anise prenatally only (FC) showed a longer latency to enter the center of the Y-maze than piglets exposed to anise both

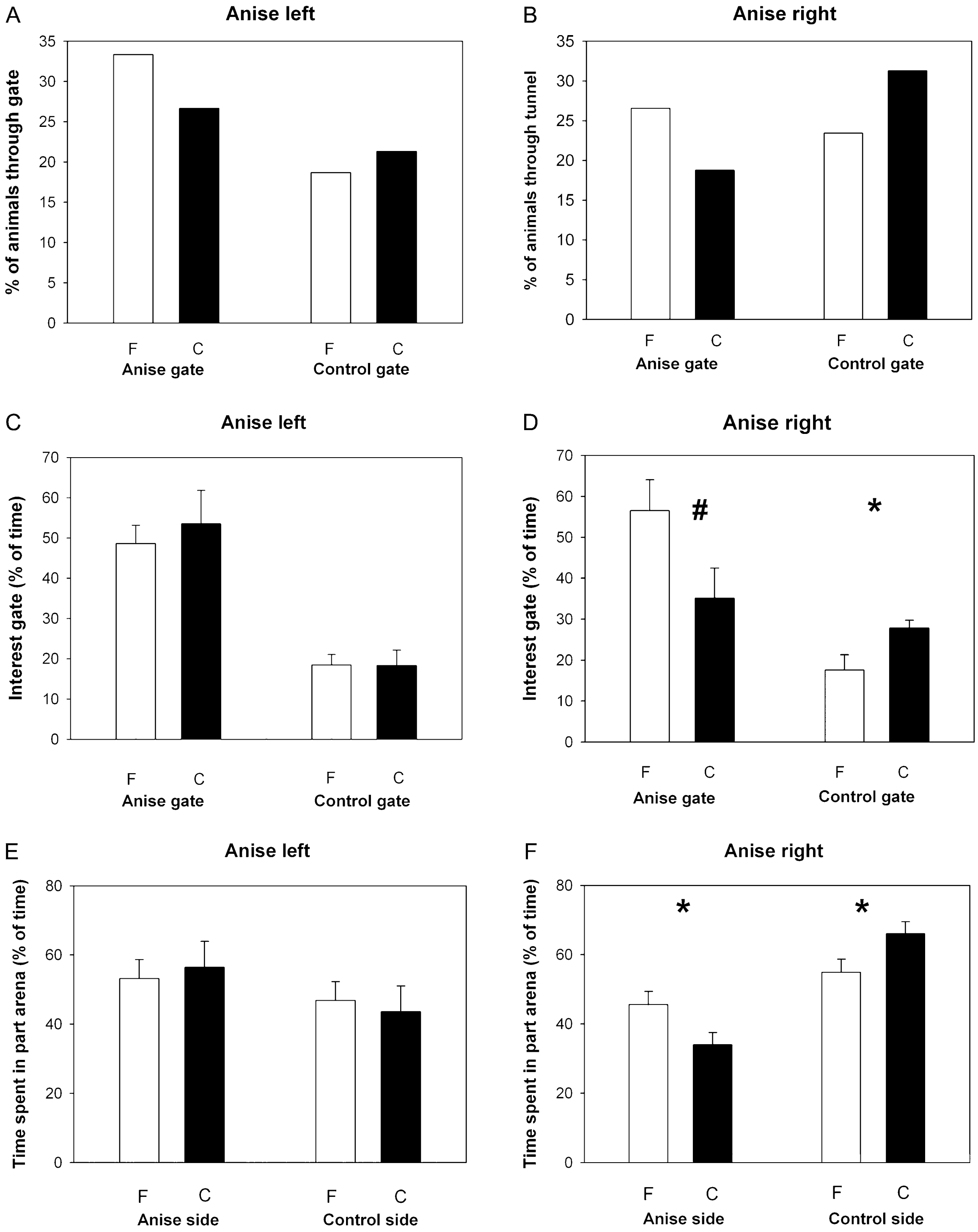

**Figure 3** Percentage of animals choosing the anise-scented gate or the control-scented gate to return back to the sow for anise located above the left and right gates, respectively **(A + B)**; interest in anise and control gates as percentage of time for anise above the left and right gates **(C + D)**; and time spent on either the anise half or control half of the test arena for anise above the left and right gates **(E + F)** in the gate preference test for animals exposed to flavor prenatally (F) or control animals (C). $^*P < 0.05$, $\#P < 0.1$.

pre- and postnatally (FC: 16.4 ± 3.1 s, FF: 6.4 ± 1.8 s, prenatal × postnatal interaction, $F_{1,35} = 4.40$, $P = 0.04$). There was no effect of treatment on time spent walking and standing ($P > 0.23$) nor on vocalizations during the test ($P > 0.15$). No effects of the treatments on the percentage of piglets entering the anise arm first were found ($P > 0.56$).

### Novel environment test

Piglets that had prenatally been exposed to anise (FF/FC groups) showed fewer escape attempts in the novel environment test when anise was present in the test area compared with their siblings that were tested without the anise present (prenatal treatment × anise present interaction, $F_{1,71}$ = 4.7, $P = 0.04$, Figure 4A). No effect of anise presence on behavior was found in the CF/CC groups. Piglets exposed to anise both pre- and postnatally (FF) had fewer vocalizations when anise was present than their siblings tested without anise in the test area ($F_{1,71}$ = 5.2, $P$ = 0.03, Figure 4B). No effects of pre- or postnatal exposure were found on defecation and urination during the test ($P > 0.23$).

### Rooting preference test

Pre- and postnatal treatment did not affect total time spent on either the anise or control side of the test arena nor total exploration (wall + floor) of either side in the rooting preference test ($P > 0.19$). Piglets that were prenatally exposed to anise (FF + FC) spent relatively less time exploring the floor on the anise side of the arena and more time exploring the floor on the mint side compared with CF and CC piglets ($F_{1,19}$ = 4.8, $P$ = 0.04, Figure 5A). Treatment did not affect the time spent on contact behavior ($P > 0.1$), but prenatal anise exposure (FF + FC) tended to increase the latency to initiate contact with the other piglet of the pair on the anise side ($F_{1,19}$ = 3.8, $P$ = 0.07, Figure 5B). Piglets exposed to anise only postnatally (CF) tended to initiate contact later on the control side than piglets from the other treatment groups ($F_{1,19}$ = 3.2, $P$ = 0.09, Figure 5B). No effects of treatment on time spent standing alert were found ($P > 0.47$).

## Discussion

### Effects of pre- and postnatal exposure on behavior during reexposure

This study demonstrates that piglets prenatally exposed to anisic flavor through the maternal diet differed in behavior from nonexposed pigs during reexposure to this flavor in various behavioral tests up to 23 days after birth, indicating recognition of the flavor. For instance, previously exposed piglets showed fewer vocalizations and escape attempts in the novel environment test and less exploration of the anise substrate as well as a longer latency to initiate contact with the other piglet on the anise half of the arena in the rooting preference test. In the gate preference, piglets showed increased exploration of the anise-scented gate after prenatal exposure, though only when the anise was present above the right gate, likely due to the piglets always being taken from the left side of the pen.

These results are in unison with those in other species, where prenatal exposure to a flavor led to recognition and a reduced aversion of this flavor later in life (Smotherman 1982b; Hepper 1988; Bilko et al. 1994; Schaal et al. 2000; Wells and Hepper 2006; Simitzis et al. 2008).

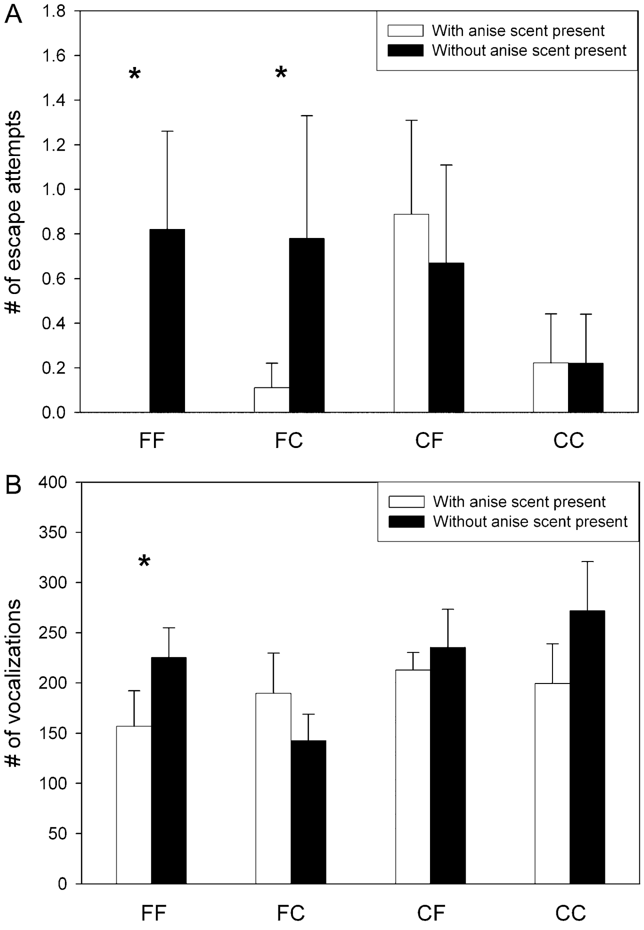

**Figure 4** Number of escape attempts **(A)** and number of vocalizations **(B)** during the novel environment test with anise scent present (empty bars) or without the anise scent present (solid bars) in the environment for animals exposed to anise prenatally and postnatally (FF), only prenatally (FC), postnatally (CF), or never (CC). *$P < 0.05$.

Providing flavor to piglets both prenatally and postnatally through the maternal diet had a larger effect on behavior in the novel environment test than prenatal flavor exposure alone. Prenatal exposure alone may already organize the olfactory system to such an extent that receptor density, sensitivity, and reactivity to a flavor in the maternal diet are increased, as hypothesized by Hepper and Wells (2006). Indeed, it has been shown that prenatal exposure to juniper results in an increased sensitivity of the olfactory epithelium during reexposure in rabbits (Semke et al. 1995). The continuity of flavor exposure after birth may be important to activate the changes in the olfactory system, as is seen in dogs, in which preference was shifted only after both prenatal and postnatal exposure (Hepper and Wells 2006). Continuity may also be important in increasing flavor preference in piglets, but organization by prenatal exposure alone already significantly affected flavor preference in pigs.

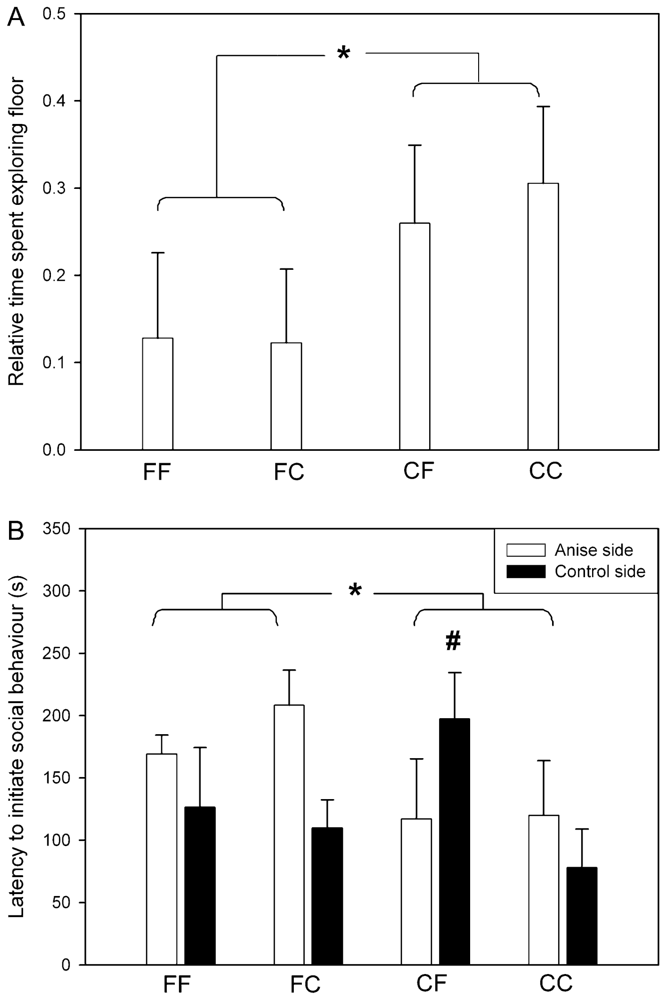

**Figure 5** Relative time spent exploring the floor **(A)** and latency to initiate contact with the other piglet **(B)** on the anise (empty bars) and control (solid bars) side in the rooting preference test arena for animals exposed to anise prenatally and postnatally (FF), only prenatally (FC), postnatally (CF), or never (CC). $*P < 0.05$, $\#P < 0.1$.

Addition of the anisic flavor to the maternal diet during lactation alone, that is, without addition during gestation did not lead to behavioral changes in exposed piglets as compared with unexposed animals in the different tests. This is in contrast to studies in humans, rats, and rabbits, in which exposure through mother's milk alone led to a shift in preference (Galef and Henderson 1972; Bilko et al. 1994; Mennella et al. 2001). It is possible that the anethol ingested by the sows did not appear in their milk or in too low concentrations only. A study by Hausner et al. (2008) showed, however, that ingested anethol can be found in human breast milk after 2 h and is still present 8 h after ingestion. It is therefore unlikely that piglets were not exposed to anethol at all through their mother's milk. Another possibility is that all piglets, including the control piglets, have been postnatally exposed through the experience with anise in the group preference and gate preference tests, which would explain the lack of contrast between the nonexposed piglets and the postnatally exposed piglets. Experience with anise in these tests may have been associated with a positive environment (the home pen) and may therefore have caused similar effects on stress and preference as postnatal exposure through the maternal diet. In line with this, infants that live with alcoholic parents show more behavior directed at toys scented with ethanol, whereas infants whose mother often uses vanilla products directed more behavior to vanilla-scented toys (Mennella and Beauchamp 1998). Mennella and Beauchamp (1991), however, suggest that the exposure to a flavor through mother's milk may have stronger effects on preference than mere olfactory exposure to the flavor because the suckling and chewing movements made during milk ingestion enhance retronasal stimulation. Furthermore, suckling is a highly rewarding experience for a young animal (Nowak et al. 1997), and the intake of the flavor in combination with milk may also result in satiety. The rewarding and satiating effects of milk intake may lead to a stronger association of the flavor to food, which is more relevant for the animal than mere exposure to flavors in the environment. The stronger association with food may induce a larger change in structures and functioning of the brain, resulting in a stronger preference for the flavor than when the flavor is only present in the environment. Also, in dogs, postnatal exposure to anise did not change the preference for this flavor (Hepper and Wells 2006). This suggests that, in some species, postnatal exposure alone may not be an important mechanism to modify the offspring's preference, whereas in others it is. The pig is a relatively precocial animal, and the brain of pigs, which has a perinatal growth spurt (Book and Bustad 1974), is probably less plastic in the period after birth than that of altricial species (Brunjes 1983) and therefore less sensitive to postnatal modification of flavor preference through milk. Furthermore, under (semi)natural conditions, piglets leave the nest to start exploring food items together with the sow starting a few days after birth (Jensen 1988). It may be adaptive to have a preference for healthy and available food types already before this period to avoid the intake of toxins, and this may be established mainly by prenatal experience. Humans, rats, and rabbits start exploring food types later than piglets, whereas dogs are intermediates with their first exploration of solid food at 2 weeks of age (Scott and Fuller 1965). Consequently, there is more time available for postnatal programming of flavor preference before the first intake of nonmilk food types in humans, rats, and rabbits, and thus, postnatal flavor exposure could have, in contrast to pigs and dogs, additional benefits for programming.

### Effects of pre- and postnatal exposure on stress during reexposure

In this experiment, we found several effects of prenatal exposure to a flavor on behaviors that are indicative of stress during reexposure to the flavor. For instance, vocalizations and escape attempts were reduced in the novel environment test, and contact behavior with the other piglet was reduced in the rooting preference test. This indicates that stress levels within the test setting were different for animals previously exposed to the flavor than for unexposed animals. This is in line with studies in pigs, as well as in chickens, in which a scent to which animals were familiarized during rearing reduced stress-related behaviors in a novel environment (Jones 1985; Jones et al. 2000). The results of the current experiment suggest that investigating stress levels or stress-related behaviors during (re)exposure in a stressful test setting can serve as a recognition test, irrespective of preference for the flavor, and may be a useful tool in research on prenatal and postnatal olfactory learning.

The reduced stress levels, caused by the familiarity of the flavor present in the test setting, may in turn have affected the behaviors measured as indicators of preference in different ways, as seen in the gate preference test and the rooting preference test. Animals that were prenatally exposed to anise directed more sniffing and chewing behavior toward the anise gate in the gate preference test. A higher percentage of time spent exploring a flavor indicates a higher preference for the anise flavor for prenatally exposed animals compared with unexposed animals (Hepper 1988; Schaal et al. 2000; Wells and Hepper 2006). In the rooting preference test, however, animals prenatally exposed to anise showed less exploration of the anise-scented substrate and more exploration of the unfamiliar mint-scented substrate than unexposed animals, suggesting lower neophobia for the unfamiliar mint flavor. This finding may also point to recognition of the anise in previously exposed piglets. Pigs are neophilic in nature and explore familiar objects or individuals less than strange objects or individuals (Wood-Gush and Vestergaard 1991; Kristensen et al. 2001). Although both the rooting preference and gate preference tests did not induce high stress levels, there are several factors linked to the setup of the tests that can explain the differences in the expression of anise recognition found in both tests. On the one hand, the design of the test itself may have affected the response of the animals. Animals were forced to solve a task in the gate preference test: make a choice and escape the test situation, thereby returning to the sow. In the rooting preference test, animals were not forced to choose as the test was designed to induce explorative behavior and the expression of their neophilic nature. This may have led to different motivations of the animals in both tests and thus a different expression of the preference resulting from prenatal exposure to the flavor.

On the other hand, the rooting preference test may have induced lower stress levels than the gate preference test, thereby inducing more neophilic behavior for prenatally exposed animals. Animals were older in the rooting preference and thus less sensitive to stress induced by separation from the sow than piglets of 6 days old (Worobec et al. 1999), as well as more eager to explore relatively novel environments. Also, piglets were tested in pairs in the rooting preference test, whereas animals in the gate preference test were tested individually. Though the number of vocalizations per piglet was comparable for both tests, the motivation of the animals to vocalize may have differed greatly. Animals that are separated from sow and littermates, as in the gate preference test, may have vocalized more to reinstate contact with their pen mates and sow on the other side of the barrier, which may be indicative of stress. Animals in the rooting preference test may have had more social contact vocalizations, which are not indicators of stress. The lower stressfulness of the rooting preference test, further lowered by the familiarity of the anise flavor, likely induced the neophilic behavior toward the mint flavor. In the gate preference test, however, the design of the test and the stress-reducing effect of the familiar flavor likely led to the observed increased expression of preference for anise by prenatally exposed animals. When designing a flavor preference test, it is thus important to consider the design of the test, the stress level it induces, the motivations of the animals within the test, as well as the effect the familiar flavor may have on the stress levels and the consequent expression of preference.

### Conclusions and implications

In conclusion, prenatal flavor exposure through the maternal diet affected behavior of piglets during reexposure tests, indicating recognition of the flavor. Adding the flavor to the maternal diet after birth had an additive effect to prenatal exposure yet did not result in familiarization without prenatal exposure. Behavioral effects in preference and recognition tests may be modulated by the design of those tests, however. The stressfulness of the test, which may be lower for experienced animals because of the mere presence of a familiar flavor in the test environment, may interfere with the expression of preference. A next step will be to see whether prenatal exposure to a flavor also reduces stress and subsequently increases the acceptance and intake of flavored food after weaning in piglets. If this proves true, the welfare and performance of piglets around weaning under the current husbandry conditions may be significantly improved.

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