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The Developmental Stage of Chicken Embryos Modulates the Impact of In Ovo Olfactory Stimulation on Food Preferences

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Abstract

Like mammals, bird embryos are capable of chemosensory learning, but the ontogeny of their feeding preferences has not been examined. We tested if the timing of stimulation in chicken embryos modulates the impact of in ovo olfactory stimulation on later food preferences. We exposed chicken embryos to an olfactory stimulus for a 4-day period in the middle or toward the end of the incubation period. The chicks were tested for their preference between foods with and without the olfactory stimulus in 3-min choice tests and on a 24-h time scale. Regardless of the type of food (familiar or novel) or the duration of the test, the control chicks not exposed to the olfactory stimulus consistently showed significant preferences for non-odorized foods. Chicks that were exposed in ovo to the olfactory stimulus did not show a preference for odorized or non-odorized foods. Only those chicks that were exposed to the olfactory stimulus toward the end of the incubation period differed from the controls and incorporated a higher proportion of odorized food into their diets on a 24-h time scale. This result indicates that olfactory stimulation at the end of embryonic development has a stronger impact on later feeding preferences. Our findings contribute to the growing pool of recent data appreciating the impact of olfactory signals on behavior regulation in avian species.

Key words: chicken embryos, feeding preferences, food neophobia, fowl, *Gallus gallus domesticus*, olfaction

Introduction

In several mammalian species, in utero experiences with chemosensory stimuli influence later food preferences (Schaal et al. 2001). Specifically, the fetus encounters food flavors that are transmitted from the mother's diet to the amniotic fluid. Experiences with such flavors lead to heightened preferences for them, shortly after birth and at weaning (e.g., Hepper 1988; Schaal et al. 2000). According to the transnatal chemosensory continuity hypothesis (Schaal and Orgeur 1992), prenatal odor acquisition adaptively guides the behavior of animals in their postnatal niches (e.g., discrimination, preferences, or orientations). This phenomenon remains largely unexplored in avian species, possibly because the sense of smell in birds has been widely underestimated and overlooked (Steiger et al. 2008). However, many recent reports have indicated that birds from several species use olfactory cues in a variety of natural contexts such as homing (Ioale et al. 1990; Jorge et al. 2009), nest building and recognition (Bonadonna et al. 2003; Gwinner and Berger 2008; Mennerat 2008), foraging (Cunningham et al. 2009), predator detection (Hagelin and Jones 2007), and social recognition (Bonadonna and Nevitt 2004; O'Dwyer and Nevitt 2009). In domestic bird species, the implication of olfactory cues in behavior regulation has received far less attention. However, in adult chickens, the neurons of the olfactory bulb respond to very minor changes in stimulus concentration (McKeegan et al. 2002). The sense of smell is implied by social discrimination in domestic chicks (Porter et al. 2005) and by sexual behavior in the Japanese quail (Balthazart and Taziaux 2009; Caro and Balthazart 2010).

In addition, there are physiological and behavioral data showing that the olfactory sense is developed in ovo and that chicken embryos are capable of chemosensory learning. In chicks, the morphogenesis of the olfactory epithelium begins at embryonic day (ED) 3 (Lalloué et al. 2003) and synaptogenesis between the sensory axon terminals and the main

olfactory bulb neurons begins between ED 8 and ED 10 (AyerLeLievre et al. 1995). By ED 13, the sensory neurons of the olfactory epithelium appear to be functional, and these neurons respond to odorant mixtures by ED 18, as in other vertebrates (Jung et al. 2005). The olfactory bulb bulges out on approximately ED 7 (Lalloué et al. 2003). By the time of hatching (ED 21), the neurons are fully mature (Gomez and Celii 2008). As air diffuses across the eggshell (Board 1982), volatile compounds can penetrate the egg and influence the behavior of the hatchlings. When tested in a T-maze after hatching (Gomez and Celii 2008) or in a choice test in a familiar environment (Sneddon et al. 1998), the chicks were found to be attracted by the odor to which they were exposed during the latter stages of incubation.

Despite the economical and ecological importance of the Galliformes species, development of their chemosensory system and its implications for feeding behavior remain to be investigated. The introduction of a novel odor commonly affects food avoidance in naïve domestic chicks (Jones 1987; Bertin et al. 2010). Chicks are able to associate specific food odors with illness (olfactory aversion conditioning), and they consequently reduce their food intake (Turro et al. 1994; Porter et al. 2002). Removal of the olfactory bulb disturbs feed intake and influences the preference for dietary lipid compounds in young chickens (Robinzon et al. 1977; Mabayo et al. 1996). Sneddon et al. (1998) and Józsa et al. (2005) exposed chicken embryos from ED 15 to ED 20 to strawberry odor via the eggshell and reported an increase in the intake of strawberry-flavored water in the 4- and 6-day-old chicks compared with the control chicks. In a similar procedure, we recently demonstrated that prehatch chemosensory experiences modify the later responses of chicks toward solid foods. In short-term choice tests, at 4 and 5 days of age, chickens that were exposed from ED 12 to ED 20 to a low concentration of an essential oil blend of orange and vanillin spent a higher proportion of time eating a familiar or unfamiliar food bearing this olfactory stimulus compared with the nonexposed control chickens. Conversely, chickens that were previously exposed to a higher concentration of olfactory stimulus avoided all foods bearing the olfactory stimulus (Bertin et al. 2010). In all the studies cited above, the embryos were exposed to odors during 2 distinct developmental stages: while they swallowed amniotic fluid (about halfway through incubation until ED 17–18 Romanoff 1960) and when they breathed with their lungs in the egg air cell (from ED 17 to ED 18). Because of the maturation of the chemosensory system throughout incubation, it is possible that the time window during which odors are applied differentially impacts subsequent behaviors, an area that has not yet been examined.

In this study, we tested whether olfactory stimulation differentially affects later feeding behavior in chicks depending on the stage of embryonic development at the time of exposure. To this end, we exposed chicken embryos to an olfactory stimulus for a 4-day period either at the middle (from ED 13 to ED 16) or toward the end (from ED 17 to ED 20) of the incubation period. Because the sensory neurons appear to be functional by ED 13, we expected that the treatment would have an effect on the food preferences of the chicks that were exposed to the olfactory stimulus at the middle of the incubation period. However, given that there is an increase in the sensitivity of the olfactory mucosa to chemicals by ED 17–18 (Lalloué et al. 2003), we expected a stronger impact of olfactory stimulation in chicks that were exposed to the olfactory stimulus at the end of the incubation period. Our expectation was that chicks familiarized with the olfactory stimulus in ovo would be more willing to accept foods bearing the olfactory stimulus compared with controls. Birds can exhibit neophobic responses when a single sensorial property of their food is changed (Jones 1987; Lecuelle et al. 2010). In addition, the amplitude of neophobic responses is enhanced when multiple sensorial properties of food are changed simultaneously. For example, the visual and tactile properties of food interact and potentiate the reaction of animals toward novel odors (e.g., Siddall and Marples 2008). Therefore, we tested the effect of the treatment when chicks were exposed to a change in the olfactory properties of their familiar food (single sensorial modality) and to a change in the multiple sensorial properties of the food (unfamiliar food).

Materials and methods

Embryos and treatment

Fertilized domestic fowl eggs (Label chicken T451NA) were obtained from a commercial hatchery. Two hundred eggs were incubated in 4 incubators (model Maya; FIEM Incubatrici) maintained at 37.6 ± 0.4 °C and 50% relative humidity while being turned automatically until day 12. At day 12 of incubation, the clear eggs were discarded and the remaining eggs (n = 174) were incubated in 3 incubators maintained in the same room (room 1). The ambient temperature in the room was maintained at 24 ± 2 °C. On ED 19, the rotation was stopped and the humidity was maintained at 80% in all incubators until the day of hatching (ED 21).

The olfactory complex was supplied by Phodé Laboratories. The exact composition of the olfactory stimulus cannot be revealed because it is protected by a confidential clause. It is composed of 0.37% of 4 pure compounds and 2 essential oils. The compounds were chosen on the basis of the findings from previous studies showing that they are detected by chickens. In addition, pilot tests performed by Phodé Laboratories showed that chicks consume the same quantity of food irrespective of the presence of the olfactory stimulus (Noirot, unpublished data). We used a liquid form of the olfactory stimulus in the incubators to ensure stable long-lasting diffusion and to avoid dispersion of the olfactory stimulus by the fan system of the incubators. The odorless liquid solvent was composed of glycerol tricaprylate and tricaprate.

We studied 3 groups: 2 groups of odorant-treated embryos and 1 group of nonexposed control embryos. The first group

(58 embryos randomly selected from the 3 incubators) was exposed to the olfactory stimulus in the middle of the incubation period from ED 13 to ED 16 (ED 13–16 group). At ED 13, the embryos were transferred to another room (room 2) in a similar incubator maintained at 37.6 ± 0.4 °C and 50% relative humidity. We followed the same protocol as Bertin et al. (2010). Two milliliters of the olfactory stimulus were deposited in 2 vials that were attached to the right and left sides of the incubator, and the solution was replenished daily. At ED 16, the eggs were removed from the incubator. The olfactory stimulus on the eggshells was not detected by the human nose (from 3 naive persons). However, as a precautionary measure as well as to ensure that the embryos were not exposed to the olfactory stimulus beyond ED 16, all the eggshells were rubbed softly with an absorbent paper that was soaked in pure water. The 58 eggs were then placed back in room 1 and dispatched in the 3 incubators. A second group of 58 embryos was exposed to the olfactory stimulus at the end of incubation from ED 17 to ED 20 (ED 17–20 group) by the same method. Similar to the first group, the embryos were transferred to room 2 and exposed to the olfactory stimulus for 4 days. At ED 20, the eggs were removed from the incubator, rubbed softly with wet absorbent paper, and placed back in room 1 (dispatched in the 3 incubators). Eggshells from the control nonexposed group (dispatched in the 3 incubators) were also rubbed with wet absorbent paper to ensure similar treatment.

Chicks and housing conditions

As soon as the chicks hatched, they were placed in pairs in plastic tubs measuring 50 × 40 × 40 cm (length × width × height) with a wire top and a floor covered by wood shavings. Only chicks that hatched on the 21st day of incubation were used for the experiment. On hatching, each chick (30 per group) was weighed and numbered with a leg band. Each chick was also weighed at 9 days of age. Each experimental group consisted of 15 pairs of chicks (15 pairs for the control group, 15 pairs for the ED 13–16 group, and 15 pairs for the ED 17–20 group). The chicks were randomly allocated into 2 rooms that had not been contaminated with the olfactory stimulus. Both rooms were maintained at 32 ± 2 °C on a 10-h:14-h light:dark cycle, and water was provided ad libitum. The chicks were fed with the conventional starter mash (Experimental Unit PEAT, INRA Centre de Tours). The food was dispensed in 50 cm–long feeding troughs. The troughs were covered by a metallic roof with 12 circular holes (diameter, 5 cm), which allowed the chicks sufficient access to food while avoiding food spillage.

Behavioral procedures

In birds, a novel odor can induce a short-lived (several minutes long) aversion to contact with a food—termed food neophobia. A prolonged reluctance to add a novel food to the diet is termed food conservatism and is particularly well described in birds (Marples and Kelly 1999). Thus, we tested the reaction of chicks toward foods in both short (3 min) and long (24 h) food choice tests. Because chicks are extremely distressed when isolated, we examined 2 chicks from the same exposure conditions together in all choice tests.

Three-minute choice test with familiar food

This test was carried out in chicks at 4 days of age. The aim was to test whether prenatal olfactory experiences could modify the reaction of the chicks to the variations in the olfactory properties of their familiar food and to assess their preference between non-odorized and odorized familiar foods. To odorize the food, we used a protocol previously described by Bertin et al. (2010). To odorize the food without modifying the visual or tactile characteristics of the starter mash, we used a translucent powder form of the olfactory stimulus. The substrate was composed of an odorless mixture of wheat flour and calcium carbonate. Immediately before testing, 0.25 g of the olfactory stimulus was mixed with 1 kg of the starter mash.

The test box, identical to the home box, was located in a different room. The floor was covered with wood shavings, and two 50 cm–long feeding troughs were located at opposite sides. These troughs were identical to the familiar trough. One trough contained 200 g of the familiar starter mash food and the other contained 200 g of the odorized familiar starter mash food. The location of the troughs was balanced across test trials. Each pair of chicks was placed in the test box after 1 h of food deprivation. They were transported in a 15 × 15 × 15 cm container, deposited in the center of the test box, and observed for 3 min. An observer (blind to the treatment) hidden behind a curtain with small observation windows recorded the behavior of 1 focal bird of each pair. Focal birds were chosen randomly beforehand and identified by a colored mark on the head. The experimenter recorded the latency to eat each food (the bird was considered to eat when the movements of the mandible, neck, and throat due to swallowing were observed), the time spent eating each food, and the number of feeding sequences initiated at each trough (the uninterrupted sequence during which the bird was eating). The quantity of food eaten could not be recorded with precision over such a short period because of the very small weight of the food particles. One pair of control birds was excluded from the analysis because neither bird in the pair approached a trough.

Three-minute choice test with unfamiliar food

The aim was to test whether prenatal olfactory exposure could modify the reaction of birds to an unfamiliar food and whether the presence of an olfactory stimulus enhanced the acceptance of that unfamiliar food in chicks that were prenatally familiarized with the odor.

This test was carried out in chicks at 8 days of age. The procedure of the test was similar to the 3-min choice test with familiar food. In this test, 1 trough contained a mixture of mashed seeds of wheat and corn (an unfamiliar food for

all groups) and the other trough contained the same mixture of mashed seeds plus the olfactory stimulus (powder form, as used previously). The experimenter recorded the behavior of the same focal birds as in the familiar food test and the same variables were recorded. Pilot studies showed that at 1 week of age, chicks showed sufficient motivation for the novel food to obtain data during a 3-min test.

Twenty four–hour choice test with familiar food

The aim was to examine whether prenatal olfactory exposure could modify feed preferences and feed intake over a longer time span (24 h).

This test was carried out at 11–12 days of age. In their home cage, each pair of chicks was provided a choice between their familiar starter mash and the starter mash with the olfactory stimulus (same method as the 3-min choice test). Two familiar troughs were placed on 2 sides of the box for 24 h. The location of the troughs was balanced across pairs. The weight of each trough was recorded before and after 24 h of the test to determine the consumption of each food by each pair.

Twenty four–hour choice test with unfamiliar food

This test was carried out in chicks at 15–16 days of age. The procedure was similar to the previous test, except that mashed seeds of wheat and corn and mashed seeds with the olfactory stimulus were used.

Statistical analyses

Cochran and Shapiro–Wilk tests were used to test for the normality and the equality of variances in our data. Hatching success was analyzed using chi-square tests. The data on the mass of chicks were analyzed by one-way repeated measures analysis of variance (ANOVA; treatment × age) and subsequent post hoc Tukey's honestly significant difference (HSD) tests. In the familiar and unfamiliar 3-min food choice tests, data were not normally distributed even after transformations; therefore, Wilcoxon matched-pairs signed-rank tests were applied on raw durations for intragroup comparisons. For intergroup comparisons, due to the nonindependence of the duration scores (time spent eating each type of food) during the tests, the duration scores were converted into the proportion of total duration scores (time spent eating the odorized food/total time spent eating). The latencies to ingest each type of food were also converted to latency scores by the following formula: latency to eat odorized food – latency to eat non-odorized food. Thus, negative scores indicated that the birds touched the odorized food faster than the non-odorized food. Intergroup comparisons were performed using nonparametric Kruskal–Wallis and post hoc Mann–Whitney U tests. The quantity of food eaten followed a normal distribution in the 24-h choice tests. Paired t-tests were used for intragroup comparisons on the raw quantity of food eaten. ANOVA was used on the proportion of odorized food eaten (quantity of odorized food eaten/total quantity of food eaten) for intergroup comparisons. When ANOVA revealed significant effects, post hoc protected least significant difference Fisher tests were performed to compare the groups. Because the usefulness of using corrections for multiple comparisons in cases of low sample size is highly debated and results in a loss of power (Garcia 2004; Nakagawa 2004; Garamszegi 2006), we present the original P values. Conversely, the effect size correlational coefficients (r) were calculated for all post hoc comparisons. According to Cohen (1988), an effect size of 0.3 is viewed as moderate and 0.1 is considered small. Data are presented as box plots with median and interquartile ranges of distribution, except for the proportions of odorized food eaten in the 24-h food choice tests, which are presented as mean ± standard error. All analyses were performed using the Statview software (SAS) with significance accepted at $P < 0.05$.

Ethical note

Animal care and all experimental procedures were conducted in accordance with the French and European regulations concerning animal experimentation, including authorization no. 37–129 from the French Ministry of Agriculture. The subject chicks remained at the research centre in stable groups after experiments were completed and were sold when they were 6–7 weeks old.

Results

Hatching success and growth rate

Treatment conditions during incubation did not affect hatching success. The nonexposed control, ED 13–16, and ED 17–20 groups had hatch rates of 74.7%, 83.7%, and 76.2%, respectively (chi square = 0.6, $P = 0.73$). We found that the treatment affected the chick growth significantly (ANOVA, $F_{2,87} = 8.73$, $P < 0.01$). There was an effect of age (ANOVA, $F_{1,87} = 3955$, $P < 0.01$) and a significant interaction between age and treatment (ANOVA, $F_{2,87} = 16.44$, $P < 0.01$). The mass of chicks at hatching did not differ significantly (control chicks: 48.63 ± 0.72 g; ED 13–16 chicks: 47.53 ± 0.78 g; ED 17–20 chicks: 48.63 ± 0.77 g; HSD Tukey's tests, $P > 0.05$). An effect of the treatment on mass was observed on day 9 (control chicks: 114.64 ± 2.31 g; ED 13–16 chicks: 130.15 ± 2 g; ED 17–20 chicks: 123.14 ± 2.04 g). The body weight of ED 13–16 chicks was higher than those of both ED 17–20 (post hoc HSD Tukey's test, $P < 0.01$) and the control chicks (post hoc HSD Tukey's tests, $P < 0.01$), whereas that of ED 17–20 chicks was higher than that of the control chicks (post hoc HSD Tukey's tests, $P = 0.02$).

Three-minute choice test with familiar food

Intergroup comparisons did not reveal significant differences in the total duration of time spent eating during the testing

(controls: 69.78 ± 8.71 s; ED 13–16 chicks: 59.66 ± 9.81 s; ED 17–20 chicks: 75.06 ± 8.93 s; Kruskal–Wallis, $H = 1.73$, $P = 0.42$). Significant differences were not observed in the proportion of time spent eating odorized food (controls: 0.28 ± 0.08; ED 13–16 chicks: 0.29 ± 0.09; ED 17–20 chicks: 0.47 ± 0.12; Kruskal–Wallis, $H = 1.13$, $P = 0.56$) or in the latency scores to ingest odorized food (controls: 28.93 ± 29.90; ED 13–16 chicks: 17.27 ± 27.38; ED 17–20 chicks: –35.60 ± 27.11 s; Kruskal–Wallis, $H = 2.48$, $P = 0.28$).

Control chicks did not differ significantly in the latency to eat odorized or non-odorized familiar foods (86.93 ± 20.15 vs. 58.00 ± 12.90 s, respectively; Wilcoxon, $z = –0.97$, $P = 0.33$). However, they tended to express fewer feeding bouts on the odorized familiar food than on the familiar non-odorized food (3.21 ± 0.8 vs. 6.28 ± 0.89, respectively; Wilcoxon, $z = –1.89$, $P = 0.058$). They spent significantly less time eating odorized food compared with non-odorized food (Figure 1a; Wilcoxon, $z = –2.1$, $P = 0.03$).

The latency to eat odorized or non-odorized familiar foods did not differ significantly within both ED 13–16 and ED 17–20 groups (ED 13–16 chicks: 79.26 ± 18.53 vs. 64.00 ± 14.16 s, respectively, Wilcoxon, $z = –0.71$, $P = 0.47$; ED 17–20 chicks: 68.13 ± 16.76 vs. 103.73 ± 17.58 s, respectively, Wilcoxon, $z = –1.47$, $P = 0.14$). Within treated groups, we did not observe significant differences in the number of feeding bouts on the odorized familiar food or the familiar non-odorized food (ED 13–16 chicks: 3.06 ± 1.01 vs. 5.73 ± 1.02, respectively, Wilcoxon, $z = –1.47$, $P = 0.14$; ED 17–20 chicks: 3.06 ± 0.85 vs. 3.53 ± 0.95, respectively, Wilcoxon, $z = –0.14$, $P = 0.88$) or in the time spent eating each type of food (Figure 1a; $P > 0.05$ for all comparisons).

Three-minute choice test with unfamiliar food

Intergroup comparisons did not reveal significant differences in the total duration of time spent eating during testing (controls: 42.14 ± 12.59 s; ED 13–16 chicks: 31.86 ± 8.39 s; ED 17–20

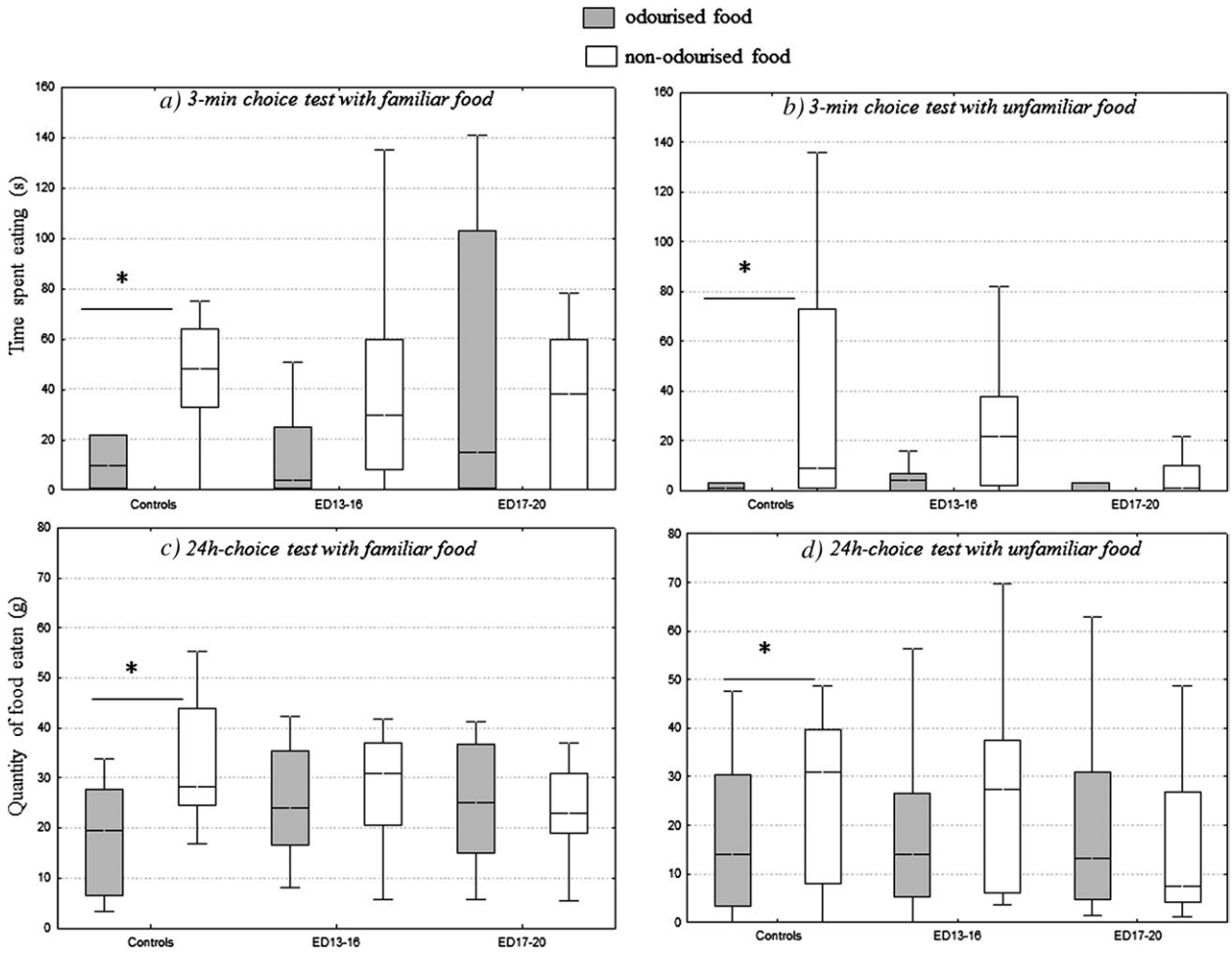

Figure 1 Median and interquartile distribution ranges of the time spent eating odorized and non-odorized food during (**a**) the 3-min choice test with familiar food or (**b**) the 3-min choice test with unfamiliar food. Median and interquartile distribution ranges of the quantity of food eaten during (**c**) the 24-h choice test with familiar food or (**d**) the 24-h choice test with unfamiliar food. Controls ($n = 15$), ED 13–16 chicks ($n = 15$), and ED 17–20 chicks ($n = 15$). *Wilcoxon, $P < 0.05$ (intragroup comparisons).

chicks: 21.2 ± 9.4 s; Kruskal–Wallis, $H = 0.72$, $P = 0.69$). The proportion of time spent eating the odorized food did not differ (controls: 0.26 ± 0.09; ED 13–16 chicks: 0.37 ± 0.11; ED 17–20 chicks: 0.33 ± 0.12; Kruskal–Wallis, $H = 2.65$, $P = 0.26$) nor did the proportional latencies to ingest food (controls: 51.21 ± 25.93; ED 13–16 chicks: 34.27 ± 27.27; ED 17–20 chicks: 30.00 ± 22.59; Kruskal–Wallis, $H = 0.68$, $P = 0.71$).

Control birds tended to have a higher latency to eat odorized unfamiliar food than to eat unfamiliar non-odorized food (110.93 ± 19.73 vs. 59.71 ± 57.90 s, respectively; Wilcoxon, $z = -1.85$, $P = 0.06$). They expressed significantly fewer feeding bouts on odorized unfamiliar food than on unfamiliar non-odorized food (1.36 ± 0.49 vs. 4.36 ± 0.79, respectively; Wilcoxon, $z = -2.67$, $P < 0.01$) and spent significantly less time eating the odorized unfamiliar food than the non-odorized unfamiliar food (Figure 1b; Wilcoxon, $z = -2.13$, $P = 0.03$).

The latency to eat unfamiliar odorized or non-odorized unfamiliar food did not differ significantly within the experimental groups (ED 13–16 chicks: 100.66 ± 17.87 vs. 66.40 ± 17.25 s, respectively, Wilcoxon, $z = -1.16$, $P = 0.24$; ED 17–20 chicks: 137.00 ± 19.14 vs. 107.00 ± 16.81 s, respectively, Wilcoxon, $z = -1.24$, $P = 0.21$). Within treated groups, we did not observe significant differences in the number of feeding bouts on odorized familiar food or familiar non-odorized food (ED 13–16 chicks: 2.2 ± 0.62 vs. 3.67 ± 0.93, respectively, Wilcoxon, $z = -1.01$, $P = 0.31$; ED 17–20 chicks: 1.4 ± 0.82 vs. 1.80 ± 0.61, respectively, Wilcoxon, $z = -1.12$, $P = 0.26$) or in the time spent eating each type of food (Figure 1b; $P > 0.05$ for all comparisons).

Twenty four–hour choice test with familiar food

The total quantity of food eaten over a 24-h time span did not differ between groups (controls: 50.41 ± 2.99 g; ED 13–16 chicks: 51.95 ± 1.56 g; ED 17–20 chicks: 48.35 ± 2.39 g; ANOVA, $F_{2,42} = 0.57$, $P = 0.57$). The proportion of familiar odorized food eaten did not differ significantly between the groups (Figure 2a: ANOVA, $F_{2,42} = 2.1$, $P = 0.13$). Although the ANOVA results were not significant, the post hoc Fisher tests revealed a significantly higher proportion of odorized food eaten by ED 17–20 chicks than by the control chicks ($P = 0.05$, $r = 0.35$).

Intragroup comparisons revealed that control chicks ate significantly less odorized familiar food than non-odorized familiar food (Figure 1c: $t = 2.8$, $P = 0.01$). The quantity of each food eaten did not differ significantly within both ED 13–16 and ED 17–20 chicks (Figure 1c: $P > 0.05$ for all comparisons).

Twenty four–hour choice test with unfamiliar food

The total quantity of food eaten over a 24-h time span did not differ between groups (controls: 44.59 ± 7.27 g; ED 13–16 chicks: 46.09 ± 5.83 g; ED 17–20 chicks: 36.15 ± 7.76 g; ANOVA, $F_{2,42} = 0.58$, $P = 0.56$). The proportion of unfamiliar odorized food eaten differed significantly between the groups (Figure 2b; ANOVA, $F_{2,42} = 3.6$, $P = 0.03$). ED 17–20 chicks incorporated a significantly higher proportion of odorized food into their diet than control chicks (Fisher's test: $P = 0.01$, $r = 0.61$), whereas no difference was found between ED 13–16 and the control chicks (Fisher's test: $P = 0.31$, $r = 0.18$).

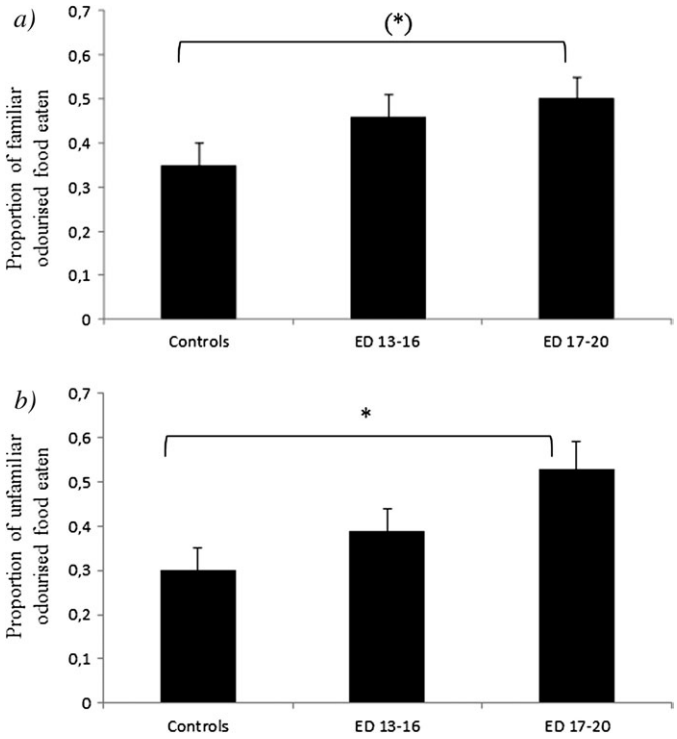

Figure 2 Mean ± standard error proportion of odorized food eaten on a 24-h time scale in pairs of controls ($n = 15$), ED 13–16 ($n = 15$), and ED 17–20 ($n = 15$) chicks. (**a**) Familiar food choice test and (**b**) unfamiliar food choice test. *Fisher's test, $P < 0.05$; (*) ANOVA: $P = 0.13$, Fisher's test, $P = 0.05$.

Intragroup comparisons revealed that control birds ate significantly less odorized unfamiliar food than non-odorized unfamiliar food (Figure 1d: $t = 2.8$, $P = 0.01$). The quantity of each food eaten did not differ significantly within both ED 13–16 and ED 17–20 chicks (Figure 1d: $P > 0.05$ for all comparisons).

Discussion

Our data show 2 main findings. First, unlike the control non-exposed chicks, chicks that were exposed in ovo to the olfactory stimulus did not show a preference between foods with and without the olfactory stimulus in any test. Second, in the 24-h choice tests, chicks that were exposed to the olfactory stimulus at the end of the incubation period were found to integrate a higher proportion of odorized food into their diet than control chicks, which indicates a stronger effect of olfactory stimulation at the end of embryonic life on later feeding behavior. Our results suggest that chemosensory stimulation affects the later reactions and the orientation

of the chicks toward food items as early as midembryonic life.

When given the choice, ED 13–16 chicks did not show any significant preference for odorized or non-odorized food. This absence of preference was observed with both familiar and novel foods, in 3-min and 24-h choice tests. This pattern was observed from 4 days of age until 15–16 days of age, which indicates stability in food preferences over time. The feeding behavior of chicks that were exposed to the olfactory stimulus at the middle of the incubation period did not show any preference as was observed in control birds, which consistently showed a preference for non-odorized foods. The introduction of a novel odor commonly affects the avoidance of foods in naïve domestic chicks (Jones 1987; Bertin et al. 2010). The fact that ED 13–16 chicks did not show any significant avoidance of odorized food suggests that they are not naïve and that olfactory continuity between the prehatch environment and food items reduced the propensity of chicks to avoid those novel foods. At ED 13–14, the back of the embryo is close to or touches the inner membrane of the shell and the vascular chorioallantoic membrane is established as close as possible to the source of oxygen (Romanoff 1960). The pores of the eggshells allow gaseous and water exchange (Board 1982), and it is very likely that the odors in the surrounding environment penetrate the egg and reach the embryo. At this stage, the embryo is surrounded by the amniotic fluid, which is swallowed by approximately ED 11–12 (Vrbitch 1924; Taylor and Saenz 1949). Because 90% of the amniotic fluid is water (Romanoff AL and Romanoff AJ 1949), it is likely that the volatile compounds surrounding avian eggs diffuse into the water and reach the chemosensory receptors through the movement of fluids from the mouth to the nasal cavity (Sneddon et al. 1998). Our behavioral data support the previous physiological description of a functional sense of smell as early as midincubation. Indeed, the neurons of the olfactory epithelium appear to be functional and respond to experimental exposure to different olfactory stimuli by approximately ED 13 (Lalloué et al. 2003). It is, however, relevant to point out that the absence of preference in ED 13–16 chicks has to be interpreted cautiously because our statistical analysis did not show significant intergroup differences. At 9 days of age, the weight of ED 13–16 chicks was higher than the weight of both ED 17–20 and control chicks. Although chemosensory stimulation during this specific time window may have influenced the daily feed intake of chicks, this was not recorded, and it remains to be investigated.

Similar to ED 13–16 chicks, chicks that were exposed at the end of the incubation (ED 17–20) showed no preference for odorized or non-odorized foods in all choice tests, regardless of the food type. In addition, ED 17–20 chicks incorporated a significantly higher proportion of odorized foods into their diet compared with nonexposed control chicks on a 24-h time scale. This result supports our expectation and suggests that the olfactory stimulation that was received at the end of the incubation period had a more pronounced impact on later feeding preferences. It is, however, important to note that our data has to be interpreted cautiously because significant intergroup differences were not observed in all choice tests. In 3-min choice tests, the distribution of our data and the use of nonparametric statistics may have masked potential differences between ED 17–20 and control chicks.

It is interesting to note that the difference between ED 17–20 and control chicks in the proportion of odorized food eaten was more pronounced in the 24-h choice test with unfamiliar food than in the 24-h choice test with familiar food. It could be thought that avoidance of the odorized food was enhanced in control birds. As mentioned by Siddall and Marples (2008), the visual and tactile properties of the novel food may have interacted and potentiated the reaction of control animals toward the olfactory stimulus. On the other hand, despite novel tactile and visual properties of the food, the olfactory stimulus was probably perceived as familiar for ED 17–20 chicks, which resulted in enhanced ingestion of the odorized novel food compared with controls. This finding suggests that in ovo exposure to an olfactory stimulus specifically during the late phase of embryos' development might reduce neophobic reaction toward food with multiple unfamiliar sensory modalities.

In this group, chicks were exposed from ED 17 to the olfactory stimulus. This is the time when the embryo begins to emerge from a fluid environment to an aerial environment and begins to breathe through its lungs. At this stage, the beak has pierced or is about to pierce the inner shell membrane in most embryos (Romanoff 1960). The embryos of domestic fowl respond to exposure to different kinds of odors shortly after penetration of the membranes by the beak (Tolhurst and Vince 1976). Interestingly, this period corresponds to the highest density of functional mature neurons in the olfactory mucosa (Leibovici et al. 1996). In addition, between ED 17 and ED 20, the respiratory movements become more regular and frequent (increase from 5 to more than 80 per minute), like their amplitude (Kuo and Shen 1937). A more mature chemosensory system, in combination with the instatement of true respiratory movements in the air chamber of the egg, possibly accounts for the more pronounced effect of our olfactory stimulus in ED 17–20 chicks than in ED 13–16 chicks. However, it should also be noted that the time between in ovo stimulation and the choice tests was shorter in ED 17–20 chicks than in ED 13–16 chicks.

Our results confirm previous findings showing that chicken embryos are sensitive to chemosensory stimulation coming from their external environment (Sneddon et al. 1998; Burne and Rogers 1999; Józsa et al. 2005; Gomez and Celii 2008; Bertin et al. 2010). Thus, they may also be sensitive to chemosensory stimulation already present in the egg. The amniotic fluid is rich in amino acids and related compounds (ten Busch et al. 1997), which could stimulate the chemosensory receptors of the embryos. The influence of the mother's diet on

the perceptual development of the avian embryo has been ignored because avian embryos develop outside the mother's body. However, Mahapatra et al. (2008) reported that free-range duck eggs from a coastal area near the seashore have a fishy scent compared with duck eggs from a plain area. In addition, several groups have reported that the inclusion of omega-3 fatty acids in the avian diet results in an increase in the fatty acids in the yolk and in a modification of the organoleptic properties of the egg. For example, eggs from hens that have been fed diets containing fish, fish oil, flax seeds, or menhaden oil were all found to have a "fishy smell" or a fish product-related flavor (Leeson et al. 1998; Gonzalez-Esquerra and Leeson 2000; Lawlor et al. 2010). Thus, it is likely that bird embryos experience a wide range of chemosensorial stimulations very early in development.

Similar to ED 13–16 chicks, ED 17–20 chicks were heavier than controls at 9 days of age. It is important to note that despite differences in growth rate between groups, the total time spent eating and the total quantity of food eaten during testing never differed between groups. Thus, the differences in feeding motivation do not explain the observed differences. The higher growth rate of treated chicks was unexpected and remains unexplained. Chicks that are exposed in ovo to the olfactory stimulus may more rapidly orient their attention toward food items following hatching; however, the daily feed intake was not recorded. It is also interesting to note that control birds showed a consistent preference for non-odorized foods despite several exposures to the same olfactory stimulus. Chicks require at least 10 min to associate specific olfactory cues with food items (Turro et al. 1994). Thus, habituation to odorized foods could have been expected at 15–16 days of age in control birds. The consistent preference for non-odorized foods may be explained by the persistent neophobic responses or an uncontrolled unpleasant taste of the olfactory stimulus.

Our data suggest that olfactory stimuli surrounding avian eggs affect the development of chemosensory systems before the embryos actually start breathing. Our findings contribute to the growing pool of recent data appreciating the impact of olfactory signals on behavioral regulation in avian species. Our data indicate that the chemosensory experience in ovo orients later feeding behavior and preferences in birds. This suggests that common principles of the sensory system development across vertebrate taxa. From an evolutionary point of view, vertebrate embryos may preprogram their sensorial systems very early during the course of their development, which would allow them to cope better with their later ecological conditions. Normally, precocial chicks benefit from the experience of their mothers to select food items, and preferences are transmitted from mothers to chicks (Wauters et al. 2002). In the absence of parental care, domestic chicks must learn to discriminate palatable from non-palatable items. Because olfaction is implied in food identification (Gentle 1985), establishing olfactory continuity between the prenatal and postnatal environments may help young birds identify food items and reduce food neophobia. Although the mechanism remains to be determined, the ontogeny of chemosensory systems and the experience-dependent feeding behavior in domestic farm birds should be taken into further consideration. These birds have long been considered anosmic and very little is known about the olfactory/gustatory regulation of their feeding behavior. Our results suggest that stimulation later in incubation might be more effective to influence posthatch feeding behaviors. Investigating olfactory/gustatory stimulation in precocial birds could yield new insights into the mechanism by which the prenatal sensory ecology influences perceptual and behavioral development of individuals in vertebrates.

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