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Repeated Exposures to Gustatory Stimuli Produce Habituation or Positive Contrast Effects in Perinatal Rats

ABSTRACT: *Adult rats exhibit a decrease in consummatory responses following repeated presentations of a taste (habituation) and an increase in consummatory responses if they experience an upward shift in the magnitude or intensity of a gustatory stimulus (e.g., sucrose or saccharin). These responses do not represent a direct sensorimotor reaction to a gustatory cue, but rather reflect a change in responding based on the memory of a previous taste. Here, we sought to determine if fetal rats could (like adults) adjust their orofacial motor responses based on a memory of recent gustatory experience. Embryonic Day 18 (E18) or Day 19 (E19) rat fetuses received oral lavage with either 0.15 or 0.30% saccharin (SAC). Subsequently, observations of orofacial movements (mouthing and licking) following oral lavage with 0.30% SAC were made 50 min later, 24 hr later, or on postnatal Day 3 (P3). Thus, some animals were in a "shifted" condition in which they first experienced a relatively low concentration of SAC and then a higher one while control rats ("nonshifted") received 0.30% SAC during both taste exposures. Fetuses exhibited evidence of both habituation (with repeated presentation of the 0.30% SAC) and positive contrast effects (PCEs) (following an upward shift in SAC concentration) when retested 50 min after their first exposure to SAC on E19. However, these animals did not exhibit PCEs 24 hr later or 5 days later (on P3). Contrast effects were not observed when the initial SAC exposure was on E18, and habituation responses were variable depending on the time interval between the taste presentations to these animals. Rats with a 5- to 6-day latency between the two taste presentations showed neither PCEs nor habituation. Our data indicate that PCEs and habituation effects emerge at different ages, and their demonstration is dependent upon the latency between the taste presentations.*
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Keywords: *rat fetus; habituation; positive contrast effects; early experience; fetal behavior; neonatal behavior; development; learning and memory*

INTRODUCTION

An early view that newborn infants are born into a "blooming, buzzing confusion" (James, 1890) no longer reflects the data now at hand. Infants and fetuses are now recognized as having a diverse behavioral repertoire and intellectual abilities to perceive and interact with their environment (Smotherman & Robinson, 1988b). Because the gustatory and olfactory systems are somewhat functional late in gestation (Teicher & Blass, 1977), our laboratory has been studying conditioned taste aversion (CTA) formation, taste recognition memory formation,

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and other gustatory memory phenomena in fetal and neonatal rats (Mickley, Lovelace, Farrell, & Chang, 1995; Mickley, Remmers-Roeber, Crouse, & Peluso, 2000a, 2000b; Mickley, Remmers-Roeber, Crouse, Walker, & Dengler, 2000; Mickley, Remmers-Roeber, Dengler, Kenmuir, & Crouse, 2001; Mickley et al., 1998). The studies reported here extend this work to a different behavioral paradigm designed to assess behavioral indicators of gustatory positive contrast and long-term habituation in fetal and neonatal rats.

Gustatory positive contrast is represented by a change in consummatory behaviors (drinking or ingestive behaviors such as mouthing or licking) as a result of an upward shift in the hedonic value of a taste stimulus. Typically, a weak taste (e.g., low concentration of sucrose or saccharin) is first presented, and after a time interval, a second stronger and more ''preferred'' concentration is administered—therefore eliciting an increase in response to the favorable stimulus (Flaherty, 1982, 1999; Weinstein, 1978). This enhancement in consummatory behaviors is not seen in animals that had experienced the high concentration taste during both presentations. Therefore, the increased responding by the ''shifted'' animal is presumably based on the memory of the previous taste experience (Grigson, Kaplan, Roitman, Norgren, & Grill, 1997).

Positive contrast effects (PCEs) are pervasive in flavor preference learning, but they produce results that are sometimes paradoxical when compared to traditional reinforcement learning (Capaldi, Sheffer, & Pulley, 1989). However, gustatory contrast can serve as an effective measure of nonassociative memory over time (Grigson et al., 1997). The interval between the two taste stimuli can be brief enough to effectively test short-term memory, but contrast detection also can persist after a long duration. Research in adult animals indicates that contrast learning can occur with intervals between taste presentations ranging from 20 min to 17 days (Flaherty, 1982).

Although gustatory contrast paradigms have been used extensively with adult animals (Flaherty, 1982; Grigson et al., 1997), limited work has been done with younger rats (Fagen & Shoemaker, 1979; Stanton, Lobaugh, & Amsel, 1984; see Flaherty, 1982 for review). Studies with infant/preweanling rats have shown that some forms of contrast learning (i.e., negative contrast) emerge at about postnatal Day 17 (Stanton et al., 1984). However, we are not aware of any gustatory positive contrast studies involving fetal rats.

The contrast paradigm necessarily involves the sequential presentation of stimuli to the same animal; therefore, an analysis of the resulting behaviors also may reveal information about habituation or sensitization. Smotherman and Robinson (1988c, 1992) previously explored some of these phenomena and documented the ability of the rat fetus to habituate to tastes presented repeatedly over seconds or a few minutes. However, using procedures consistent with the assessment of contrast phenomena, the current study employed just two taste presentations with much longer taste-exposure intervals ranging from 50 min to 5 to 6 days.

The current study sought to determine if habituation and PCEs could be observed in fetal rats first exposed to a novel taste on E18 or E19. Previous research from this lab has shown that fetuses at these ages exhibit differences in their ability to learn and remember taste stimuli (Mickley et al., 2000a, 2000b; Mickley et al., 2000). This research has shown that E19 fetuses are capable of recognizing a previously presented taste whereas E18 fetuses are unable to demonstrate this taste-recognition memory. Therefore, we examined if this age-related discrepancy also would be observable within a contrast-learning paradigm.

METHODS

Subjects

The subjects were fetal and neonatal Sprague-Dawley rats (male and female) obtained from timed-pregnant dams supplied by Zivic Laboratories (Zelienople, PA). The date of conception (i.e., the first day that a vaginal plug was detected) was designated as embryonic Day 0 (E0). Pregnant animals (from which our subjects were derived) were individually housed in plastic ''shoe box'' cages (44.45 cm long $\times$ 21.59 cm wide $\times$ 20.32 cm high). Following birth, litters were not culled, and except for the period of behavioral testing, the neonatal rats were housed with the dam. Home-cage temperature was maintained at 23 to 26°C under a 12:12 hr light:dark cycle (lights on at 0600 hr). Rodent chow (Purina 5001) and water were available ad libitum.

Tastant Preparation

Saccharin was mixed in deionized water to create solutions of 0.15 and 0.30%. Previous studies have shown that PCEs may be obtained using nonnutritive sweeteners (e.g., saccharin) (Flaherty & Rowan, 1986; Weinstein, 1978).

Overview of Experimental Design

During Taste Exposure 1 (TE1), rats received oral lavage with either 0.15 or 0.30% saccharin (SAC) on either E18 or E19. Subsequently, behavioral observations following oral lavage with 0.30% SAC (Taste Exposure 2: TE2) were made 50 min later, 24 hr later, or on postnatal Day 3 (P3). Slightly different procedures were required depending on the time of behavioral testing. See Table 1 for the experimental design, number of subjects per group, and group nomenclature used in this study.

Preparation of the Pregnant Rat

Our procedures required conscious fetuses; therefore, we used spinal-block procedures to provide appropriate analgesia for the

Table 1. Treatment Groups, Numbers of Subjects per Group, and Nomenclature Used to Identify the Groups

Experimental Condition	Fetal Age at First Taste Exposure (TE1)	Subject Age at Second Taste Exposure (TE2)	Number of Subjects (Number of Litters)	Group Nomenclature[a]
Shifted[b]	E18	E18[c]	10(3)	E18-E18-S
Shifted	E18	E19	11(3)	E18-E19-S
Shifted	E18	P3	22(4)	E18-P3-S
Shifted	E19	E19[c]	7(2)	E19-E19-S
Shifted	E19	E20	10(2)	E19-E20-S
Shifted	E19	P3	19(3)	E19-P3-S
Not shifted[d]	E18	E18[c]	12(3)	E18-E18-NS
Not Shifted	E18	E19	10(3)	E18-E19-NS
Non shifted	E18	P3	29(5)	E18-P3-NS
Non shifted	E19	E19[c]	9(3)	E19-E19-NS
Non shifted	E19	E20	9(2)	E19-E20-NS
Non shifted	E19	P3	13(2)	E19-P3-NS

[a]Groups are identified by two numbers representing subject age at TE1 and TE2 (E18, E19, E20 = Embryonic Days 18, 19, 20, respectively; P3 = postnatal Day 3) and then the shifted (S) or non shifted (NS) condition.
[b]Shifted rats received oral lavage with 10 µl 0.15% saccharin (SAC) during TE1 and then 10 µl 0.30% SAC during TE2.
[c]When TE1 and TE2 occurred on the same day, there was a 50-min latency between the two taste exposures.
[d]Non shifted rats received oral lavage with 10 µl 0.30% SAC as their first taste and then 10 µl 0.30% SAC as their second taste.

dam during fetal taste exposures. Pregnant rat dams carrying E18 or E19 fetuses were briefly anesthetized with Isoflurane before they underwent either a reversible or irreversible (chemomyelotomy) spinal-block procedure.

The reversible spinal-block procedure was employed on E18 or E19 when our two taste exposures were to be administered on different days (i.e., TE1-TE2 latencies of 24 hr or 5 to 6 days; see Table 1). Here, a 30-gauge needle was used to inject Lidocaine HCl 2% and Epinephrine 1:100,000 (in a volume of 0.1 ml) between the first and second lumbar vertebrae. This procedure is effective in producing (a) complete abdominal and hindlimb paralysis, (b) consistently long periods of spinal anesthesia (~45 min), and (c) complete recovery after the anesthesia (Smotherman, Robinson & Miller, 1986). There is no indication that litters are adversely affected by this procedure (Smotherman, Richards, & Robinson, 1984; Smotherman & Robinson, 1988a, 1988b).

A chemomyelotomy was performed when a longer period of analgesia was required (i.e., when behavioral testing was to be conducted). Procedures were similar to those used to produce the reversible spinal block, but in this case, following Isoflurane anesthesia, 0.1 ml of 100% ethyl alcohol was injected between the first and second lumbar vertebrae.

Following the analgesic treatment, the pregnant dam was restrained in a plastic holding apparatus and her vision restricted. Uterine horns were exposed through a midline laparotomy, and the dam's hindlegs and lower abdomen were immersed in a warm bath (37.5 ± 1°C) containing isotonic saline (Locke's solution) (Galigher & Kozloff, 1971). Both horns of the uterus were exteriorized through the abdominal incision and were allowed to float freely in the bath. To reduce the residual effects of the Isoflurane anesthesia (Smotherman et al., 1986), we allowed 15 min to elapse following placement of the dam in the bath before we began fetal injections or behavioral testing (discussed next).

Fetal Injections and Behavioral Testing

50-Min Latency. In this part of the experiment, two exposures to saccharin (TE1 and TE2) occurred on the same day (either E18 or E19), separated by approximately 50 min. Following chemomyelotomy of the dam (procedure discussed earlier), each fetus was carefully removed from the uterus (to ensure that the umbilical cord remained securely attached) and floated in the temperature-controlled Locke's solution. Each fetus, in turn, was individually placed on a submerged platform where baseline motor activity was videotaped for 1 min. The rat then received oral lavage with 10 µl of either 0.15% (the shifted group) or 0.30% (the nonshifted group) of SAC from a blunt, 30-gauge injection needle. The SAC was delivered via an automated syringe pump. Videotaping continued for an additional minute following the injection. At the end of this first observation, the fetuses were tagged with a number (on a loose-fitting, soft-plastic ring placed around the umbilical cord) to keep track of the SAC concentration they received. All animals in the right or left uterine horn were randomly assigned to either the shifted or the nonshifted condition. All fetuses in the opposite horn received the alternate treatment. Thus, both treatment conditions were represented in each litter. Approximately 50 min from the time that the first pup received its first taste stimulus, a second round of baselines and behavioral responses to oral injections (in this case, 10 µl of 0.30% SAC) were videotaped.

24-Hr Latency. In this paradigm, TE1 occurred on either E18 or E19, and then TE2 and behavioral testing occurred approximately 24 hr later. TE1 followed a reversible spinal block of the pregnant dam (see procedure discussed earlier); all fetuses in a particular uterine horn received oral lavage with either 10 µl of 0.15% or (in the opposite horn) 0.30% SAC. Thus, both treatment conditions were represented in each litter. Over the course of the study, we alternated the assignment of the 0.15 or

0.30% SAC treatments to the fetuses in the right or left horns. Fetal injections were administered, in utero, using a 30-gauge needle and an automated syringe pump. The mouth of the fetus can be visualized through the uterine membranes and oral injections accurately placed (Revta, Remmers-Roeber, & Mickley, 1999). After the injections, the uterus was replaced, the abdominal wall and the skin of the pregnant rat were sutured, and the wounds were infused with a local analgesic (Bupivicaine; 0.25%) to produce postsurgical analgesia.

The behavioral responses of the fetuses were tested on E19 or E20. The pregnant dams were provided analgesia using an irreversible spinal block (0.1 ml of 100% ethanol) via the general method described previously. Both horns of the uterus were exteriorized through the abdominal incision (created before the TE1 fetal-injection procedure), and the horns were allowed to float freely in the Locke's solution bath. At least 15 min were allowed to elapse before onset of behavioral observations to allow the pregnant female and fetuses to fully recover from the Isoflurane anesthesia used during the spinal block procedure. While still attached to the dam via the umbilical cord, fetuses were individually removed from the uterus and floated in the Locke's solution bath. A blunt, 30-gauge stainless-steel injection tube was placed in each rat's mouth, and 10 μl of 0.30% SAC was injected into the oral cavity. Behavior was videotaped for 1 min immediately before (baseline) and 1 min after oral SAC injection.

Neonatal Behavioral Testing. In this paradigm, TE1 occurred on either E18 or E19. TE2 and behavioral testing occurred on P3. Following a reversible spinal block of the pregnant dam on E18 or E19 (procedure discussed earlier), all fetuses in a particular litter received oral lavage with either 10 μl of 0.15 or 0.30% SAC (Note that this procedure required special statistical analyses to partition out the litter effect; see data analysis section for details). Fetal injections were administered, in utero, using a 30-gauge needle and an automated syringe pump. After the injections, the uterus was repositioned within the abdomen, the abdominal wall and the skin of the pregnant rat were sutured, and the wounds were infused with a local anesthetic (Bupivicaine; 0.25%) to produce postsurgical analgesia.

These animals were later born via a normal vaginal delivery on E21, and on P3 were separated from the dam 20 min before the behavioral test. While awaiting testing, pups were placed adjacent to and on top of littermates (to aid in maintaining body temperature) within a small gauze-covered, plastic container. The container sat on a warm (38.5 ± 0.5°C) heating pad and was maintained within a temperature-controlled incubator (ambient temperature $= 28 \pm 1$°C; mean relative humidity $\pm$ *SEM* $= 42.74 \pm 2.42$%) until immediately before testing of the litter began. For the behavioral observations, neonates were placed in a warm, humid chamber (ambient temperature $= 28 \pm 1$°C; mean relative humidity $\pm$ *SEM* $= 42.74 \pm 2.42$%) on a glass plate warmed (via constantly circulating water) to 36 ± 1°C. They were allowed 5 min to acclimate to this environment before behavioral observations were begun. Pups received oral lavage with 10 μl of 0.30% SAC through a blunt/smooth, 18-gauge, stainless-steel infusion needle. Subjects were then placed (ventral side down) on the glass plate. Using a mirror, behavior was videotaped from below the animal for 1 min before (baseline) and after oral injection.

Dependent Variables and Data Analysis

Rat behaviors were recorded on videotape and later reviewed by a practiced observer and scored with the help of The Observer computer program developed by Noldus Information Technology (Observer, 2002). Using a modification of the methods described by Smotherman et al. (1984), we counted mouth movements and licks. In our experiments, the reliability of behavioral scoring is high. In several assessments of our methods throughout the period of data analysis, the interrater correlations ranged from $r = 0.92$ to $r = 0.98$.

Mouthing and tongue protrusions are considered ''ingestive'' responses since they allow contact with the taste stimulus and are usually accompanied by consumption of the tastant (Schwartz & Grill, 1985). Gapes and head shakes are labeled as ''aversive'' responses since they facilitate removal of infused fluids. These characterizations of ingestive and aversive behavioral responses have been supported by studies indicating that a highly palatable taste (e.g., sucrose) elicits ingestive mouthing and licking responses, but once the taste has been associated with an illness-inducing stimulus, the behavioral reaction to sucrose becomes aversive (Breslin, Spector, & Grill, 1992). Likewise, the natural aversive responses to quinine can be reduced through conditioning (i.e., if quinine signals a preferred taste like sucrose; Breslin, Davidson, & Grill, 1990). The orofacial responses of fetal and neonatal rats seem to represent ingestive or aversive reactions similar to those of adult rats (Reilly, Robertson, MacLennan, & Smotherman, 1997; Smotherman & Robinson, 1985).

In our study, some animals were tested as fetuses while others were tested using the neonatal procedure. Comparisons of different-aged rats may be challenged by the fact that the quantity of spontaneous movements generally increases as rats progress through the perinatal period. In an attempt to control for differences in the level of overall activity demonstrated by fetal versus neonatal rats, our treatment of the data included an analysis of covariance (ANCOVA). The covariate employed was a combination of each animal's mouthing and licking movements during the 1-min baseline period immediately before oral lavage with SAC on the test day. This factor was a component of a three-way ANCOVA (Kirk, 1982; SPSS (2002)-compensating for unequal *n*) of mouthing and licking responses during TE2, which took the form: Taste Exposure Interval [50-min or 24-hr] $\times$ Age During First SAC Taste [E18 or E19] $\times$ Contrast Treatment [Shifted (0.15 then 0.30% SAC) or Nonshifted (0.30 then 0.30% SAC)]. If the effect of the covariate was not statistically significant ($p > 0.05$), then subsequent ANOVAs were run without this factor.

As described earlier, the animals assigned to the 50-min and 24-hr taste-exposure intervals were randomly assigned a treatment (shifted or nonshifted), and both treatments were represented in each litter. However, all rats in a particular litter were necessarily the same age and assigned to the same taste-exposure interval. Therefore, we included litter as an independent, random, and nested factor (within-subject age and taste-

exposure interval). This approach uses the litter MS_{error} term for the denominator of the F ratios, controls for litter effects, and offers a direct statistical test of the significance of such effects (Denenberg, 1976; Holson & Pearce, 1992). All rats/litter in the 5- to 6-day TE interval group (tested on P3) were either in the shifted or nonshifted group. Therefore, these data were analyzed within an ANCOVA: Age During First SAC Taste [E18 or E19] × Contrast Treatment [Shifted (0.15 then 0.30% SAC) or Nonshifted (0.30 then 0.30% SAC)], with both factors nested within litter.

Orofacial responses from pre-TE1 (baseline), TE1, pre-TE2 (baseline), and TE2 were available for rats assigned to the 50-min TE1-TE2 interval group. Therefore, we were able to perform additional analyses on the mouthing and licking responses of these animals before and following the first taste exposure. These ANOVAs took the form: Age During First SAC Taste [E18 or E19] × Contrast Treatment [Shifted (0.15% then 0.30% SAC) or Nonshifted (0.30 then 0.30% SAC)], with the "age" factor nested within litter.

We used t tests to make several a priori and a posteriori paired comparisons between treatment groups. When the broad ANOVA/ANCOVA analyses revealed statistically significant differences and multiple t tests were part of an a posteriori comparison, we employed the Bonferroni correction for multiple comparisons to reduce the risk of a Type I error (Kirk, 1982).

RESULTS

TE2 Orofacial Responding

A positive contrast effect was defined as a significant increase in the behavioral response of subjects experiencing an increasing shift in SAC concentration (i.e., 0.15 to 0.30%) over a given time period (either 50 min, 24 hr, or 5–6 days) relative to the behavioral response of subjects that have experienced two exposures to the same SAC concentration (0.30%). Figure 1 suggests that a positive contrast effect was evident in rats tested 50 min later after first exposure to SAC on E19 (E19-E19-S). However, these animals did not exhibit contrast effects 24 hr later (E19-E20-S) or 5 days later (on P3; E19-P3-S). Likewise, contrast effects were not observed 50 min, 24 hr, or 6 days (on P3) after a first SAC exposure on E18.

The ANCOVA of the combined mouthing and licking responses following the second SAC exposure revealed a significant three-way interaction (Taste Exposure Interval × Age During First SAC Taste × Contrast Treatment), $F(1, 6) = 19.17$, $p = 0.005$, a significant litter effect, $F(7, 63) = 5.91$, $p < 0.001$, as well as significant variation associated with the covariate (i.e., baseline mouthing and licking), $F(1, 49) = 7.43$, $p = 0.009$.

Post hoc analyses indicated that rats in the shifted condition that were first exposed to SAC on E19 significantly changed their mouthing and licking responses as compared to their nonshifted controls following TE2. When a 50-min interval existed between TE1 and TE2, E19 fetuses *increased* their mouthing and licking to SAC. However, if the TE1-TE2 interval was lengthened to 24 hr, shifted fetuses significantly *decreased* mouthing and licking responses (as compared to nonshifted controls).

Rats first exposed to SAC on E18 did not show these effects and exhibited no reliable changes in mouthing and licking in response to a shift in SAC concentration. Although there was a trend for E18-E19-S rats to exhibit more mouthing and licking than E18-E19-NS animals, this effect did not achieve statistical significance.

Likewise, P3 neonates with TE1 on either E18 or E19 failed to exhibit reliable adjustments in mouthing and licking following a shift in SAC concentration on the test day. The baseline mouthing and licking responses recorded immediately prior to TE2 were not significantly different between the shifted/nonshifted animals of the same age.

Habituation effects were more variable and depended on the age of the animal and the TE1-TE2 interval. E19-E19-NS rats exhibited significantly fewer mouthing and licking responses during TE2 than they did during their initial exposure to 0.30% SAC (TE1) (see Figures 1 and 2). Moreover, E19 rats that received two exposures to 0.30% SAC within a 50-min interval showed significantly fewer mouthing and licking responses following TE2 than did E18 rats receiving this same treatment. These data suggest that habituation may be more readily observed in E19 rat pups than in E18 fetuses (but see different conclusions when the TE1-TE2 interval is lengthened, next).

Changes in Orofacial Responding to SAC as the TE1-TE2 Interval Changes from 50 Min to 24 Hr

Further post hoc analyses of the significant three-way interaction (Taste Exposure Interval × Age During First SAC Taste × Contrast Treatment) revealed reliable changes in mouthing and licking responses that depended on both the age of the rat at the time of the test and the TE1-TE2 interval. As Figure 1 illustrates, nonshifted fetuses first tasting SAC on E18 significantly decreased their orofacial responding if the TE1 to TE2 interval was lengthened to 24 hr (comparison of E18-E18-NS with E18-E19-NS). The opposite was true of the fetuses first tasting SAC on E19 (comparison of E19-E19-NS with E19-E20-NS). These rats significantly increased their mouthing and licking responses to SAC when TE2 was 24 hr after TE1 (as compared to the animals in the 50-min interval group). This same effect was revealed in a direct comparison of the nonshifted animals of different ages following the 1-day latency (comparing E18-E19-NS with E19-E20-NS). These data suggest that fetuses, separated

by just 1 day in age, respond very differently to a repetitive exposure to 0.30% SAC when there is a 24-hr interval between TE1 and TE2.

TE1 Orofacial Responding

Since TE1 occurred in utero for animals in the 24-hr and 5- to 6-day interval groups, the rats were not visible to allow analysis of orofacial responding. However, the rats experiencing a 50-min TE1-TE2 interval (E18-E18 and E19-E19 groups) were provided their first exposure to SAC ex utero. Therefore, we were able to videotape these subjects for 1 min before the first taste exposure as well as immediately following the oral lavage with SAC. Two-way ANOVAs comparing baseline mouthing and licking indicated no significant differences between the animals that would eventually be randomly assigned to the shifted or nonshifted groups (see Figure 2). Likewise, there was not a significant difference in the TE1 baseline orofacial movements of E18 and E19 fetuses.

Similar results were recorded for mouthing and licking responses following the TE1 SAC lavage. Both E18 and E19 fetuses exhibited similar levels of orofacial responding following this first taste of either 0.15 or 0.30% SAC (see Figure 2).

DISCUSSION

Fetuses exhibited evidence of both habituation (with repeated presentation of the 0.30% SAC) and positive contrast (following an upward shift in SAC concentration) when retested 50 min after their first exposure to SAC on E19. However, these animals did not exhibit PCEs 24 hr later or 5 days later (on P3). PCEs were not observed when the initial SAC exposure was on E18, and habituation responses were variable depending on the time interval between the taste presentations to these animals. Rats with a 5- to 6-day latency between the two taste presentations showed neither PCEs nor habituation. To the best of our knowledge, these data represent the first demonstration of positive contrast effects in fetal rats and help define developmental and temporal parameters that are predictive of simple learning phenomena in perinatal animals.

Definitive SAC concentration preference functions are not available for fetal rats. However, we report here that there is little difference between the initial orofacial responses of E18 or E19 fetuses to 0.15 and 0.30% SAC (see Figure 2). This result was unexpected since, within a fairly broad range, adult rats typically prefer drinking higher concentrations of saccharin or sucrose over lower concentrations (Mickley et al., 2002; Weinstein, 1978). Orofacial movements have been used successfully to gauge palatability in both adult and perinatal rats (Schwartz & Grill, 1985). Mouthing increases monotonically with increasing sucrose concentration over a broad range (5.1–10.2% sucrose; Schwartz & Grill, 1985); note that 2% sucrose's hedonic value approximates that of 0.15% SAC; Flaherty, 1982), and neonatal behavior parallels that seen in the adult.

We were surprised, therefore, when the initial mouthing and licking responses to 0.15 and 0.30% SAC were similar in our E18 and E19 fetuses. It may be the case that increasing concentrations of flavored solutions also are perceived as increasingly novel, and taste preferences interact with taste novelty to make the predictability of a new taste preference a complicated matter (Domjan & Gillan, 1976). If the normal preference for a higher concentration of a tastant is antagonized by neophobia the first time the taste is experienced, our initial assessment of SAC palatability may have produced results different from those that might be observed following multiple presentations.

It also may be the case that the mouthing and licking responses following 0.15 and 0.30% SAC may have both reached a maximal level (i.e., ceiling effect). The number of mouthing and licking responses of our E18/E19 fetuses was approximately 10 to 11 per min. Our previous work with fetal animals at this stage of development has never revealed a rate of responding higher than this (Mickley et al., 2000; Mickley et al., 2001). Likewise, spontaneous

FIGURE 1 Mean mouthing and licking responses (±*SEM*) of rats after a taste of 0.30% SAC (Taste Exposure 2: TE2) which followed oral lavage with either 0.15% SAC or 0.30% SAC administered on E18 or E19 (Taste Exposure 1: TE1). Rats experiencing a shift in the SAC concentrations presented (0.15–0.30%) are identified with an "S" while those receiving two exposures to 0.30% SAC are identified as nonshifted ("NS"). Results are presented for three different TE1-TE2 intervals: 50 min (rats experiencing both TE1 and TE2 on the same day: E18-E18 or E19-E19 groups); 24 hr (E18-E19 or E19-E20 groups), or 5 to 6 days (i.e., TE2 on P3: E18-P3 or E19-P3 groups). See Table 1 for all group assignments and naming conventions. *Significantly different from nonshifted control animals of the same age and TE1-TE2 interval. +Significantly different from nonshifted animals in the 50-min versus 24-hr TE1-TE2 interval. #Significantly different from the E19 nonshifted group within either the 50-min or 24-hr TE1-TE2 interval. Paired group comparisons followed ANCOVAs and employed *t* tests using the Bonferroni correction, when applicable, to reduce the chance of Type I errors (see Results section for details). $\alpha = 0.05$ throughout.

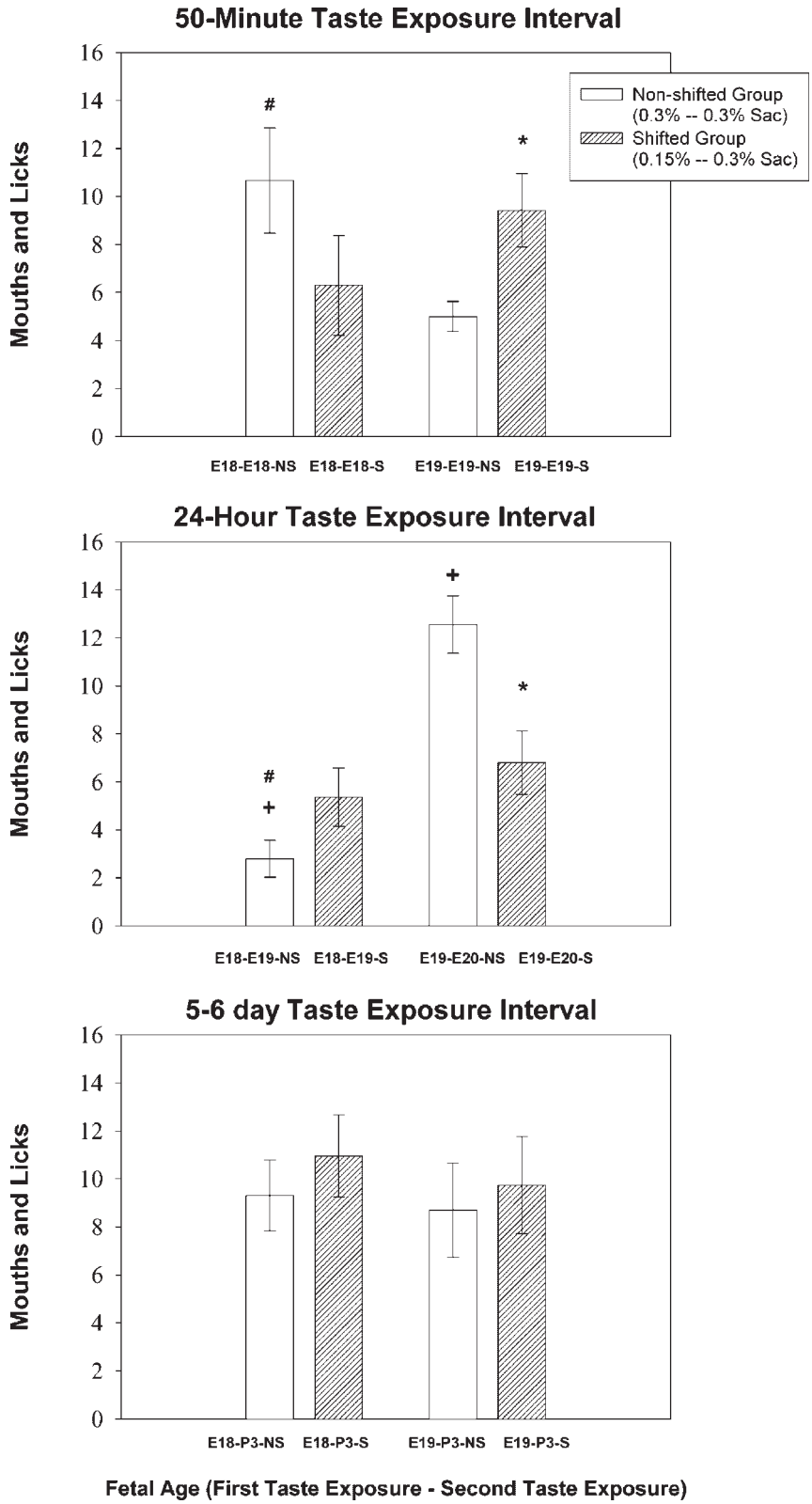
50-Minute Taste Exposure Interval
Mouths and Licks
Non-shifted Group (0.3% -- 0.3% Sac)
Shifted Group (0.15% -- 0.3% Sac)
#
*
E18-E18-NS
E18-E18-S
E19-E19-NS
E19-E19-S
24-Hour Taste Exposure Interval
Mouths and Licks
+
#
*
E18-E19-NS
E18-E19-S
E19-E20-NS
E19-E20-S
5-6 day Taste Exposure Interval
Mouths and Licks
E18-P3-NS
E18-P3-S
E19-P3-NS
E19-P3-S
Fetal Age (First Taste Exposure - Second Taste Exposure)

FIGURE 1

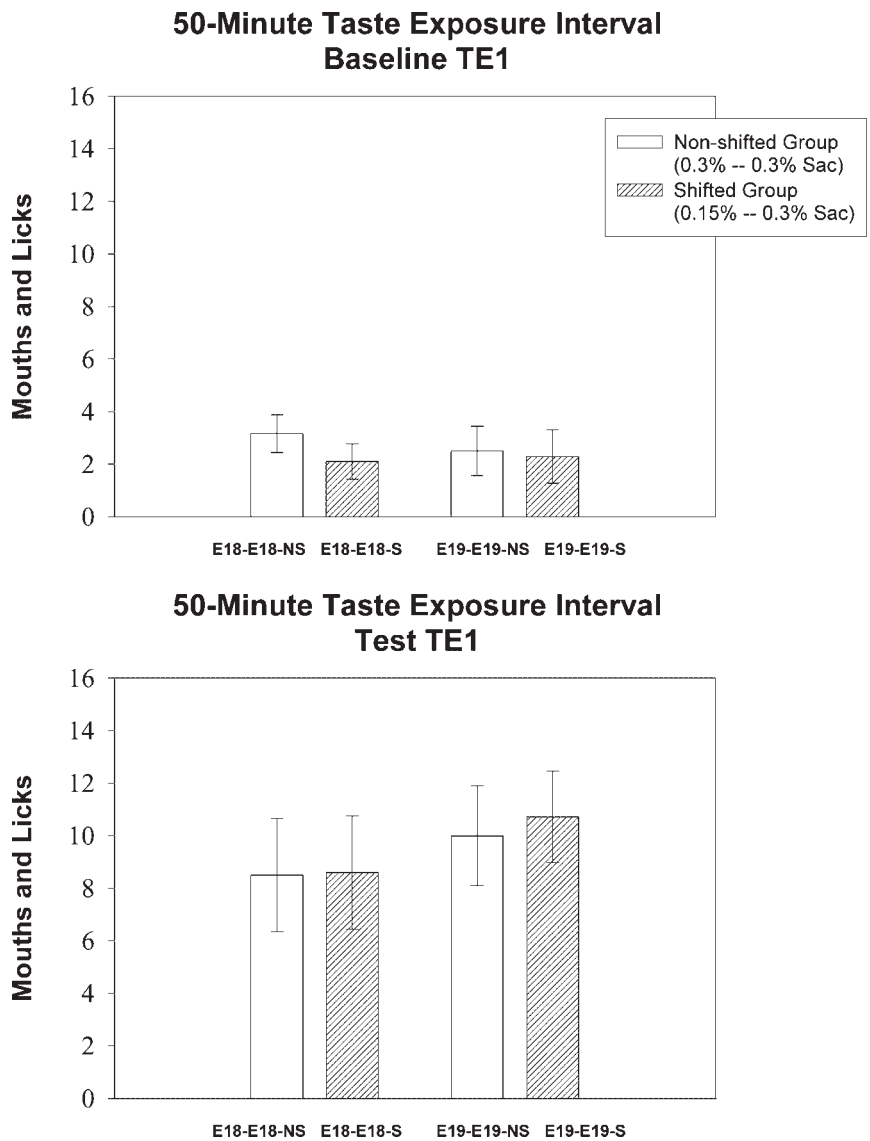

FIGURE 2 Mean mouthing and licking responses (±*SEM*) of E18 or E19 rat fetuses immediately before (baseline; see top panel) or after (bottom panel) the first infusion with either 0.15 or 0.30% SAC during TE1. These animals were in the 50-min taste-exposure-interval condition (E18-E18; E19-E19). Rats that will eventually experience a shift in the SAC concentrations presented (0.15–0.30%) are identified with an "S" while those that will receive two exposures to 0.30% SAC are identified as nonshifted ("NS"). In the bottom panel, "NS" animals had just received 0.30% SAC while the "S" animals had just tasted 0.15% SAC. (see Table 1 for all group assignments and naming conventions). As expected, pretaste-exposure baselines were similar independent of the concentration of SAC that was to follow. Orofacial responses to 0.15 and 0.30% SAC also were similar following this initial exposure to the sweet taste.

mouthing rates significantly lower than those reported here ($\leq$1/min; Smotherman et al., 1984; Smotherman et al., 1986) have been reported. Mouthing rates increase to approximately 2 to 3 per min following SAC or mint infusions (Smotherman & Robinson, 1985) (Note that according to our observations, licking movements are a small percentage, $<$5%, of the total mouthing + licking statistic.) While not conclusive, these data suggest that mouthing and licking rates of 10 per min may represent an upper limit of responding for fetuses of this age (Note that dramatic increases in these limits occur over the next few days and may be observed in P3 neonates; see discussion later.) If this is the case, our failure to observe different levels of orofacial movements following 0.15 or

0.30% SAC may not be representative of palatability or taste preferences. Instead, these data may be attributed to a ceiling effect which may have obscured our ability to detect a difference between fetal perceptions of the two SAC concentrations.

There is ample evidence to suggest that the greater the difference between large and small rewards, the greater the contrast effect (for review, see Flaherty, 1982). If 0.15 and 0.30% SAC were, in fact, perceived as similar in their hedonic value, this may have contributed to the limited demonstration of PCEs reported here.

Despite our use of a paradigm designed to reveal PCEs, portions of our data also may be interpreted in the context of stimulus habituation. The animals in our nonshifted groups received two presentations of 0.30% SAC over a 50-min, 24 hr, or 5- to 6-day interval. E19-E19-NS rats exhibited fewer mouthing and licking responses during TE2 than they did during their initial exposure to 0.30% SAC (TE1). In fact, it should be noted that this habituation effect may have contributed to the appearance of a PCE when the orofacial responses of rats in Groups E19-E19-NS and E19-E19-S are contrasted. A further comparison of the E18-E18-NS and the E19-E19-NS groups suggested that mouthing and licking is reduced in the E19 fetuses as compared to the E18 fetuses (see Figure 1). This is not a general maturational effect since baseline spontaneous orofacial movements of the E18 and E19 rats are similar (see Figure 2). This result is consistent with the interpretation that E19 fetuses are more likely to show habituation effects than are E18 fetuses. However, over the longer taste-exposure intervals, it is difficult to discern consistent habituation effects. E18-E19-NS fetuses exhibited significantly less mouthing and licking than the E18-E18-NS rats (suggesting habituation), but the same type of comparison between the E19-E20-NS and E19-E19-NS rats actually revealed a significant increase in orofacial movements following two 0.30% SAC exposures separated by 24 hr (suggesting sensitization). This 24-hr taste-exposure interval has not been attempted before in fetal rats, and there may be some unique characteristics associated with it (e.g., see data from other taste learning paradigms: Paschall, Clancy, & Batsell, 1998).

It should be noted that fetal taste-habituation effects have been reported previously (Smotherman & Robinson, 1988c, 1992), but under very different methodologies than those used here. Fetal (E20, E21) motor movements increase immediately following oral infusion of a lemon taste and then return to baseline after 30 s (Smotherman & Robinson, 1992). A significant decrease in fetal activity takes place if lemon infusions occur every 15 s, over 10 min (Smotherman & Robinson, 1988c). Thus, habituation paradigms have classically involved frequent stimulus presentations over a short period of time. This is quite different from our procedure aimed at revealing contrast effects that employed only two taste exposures separated by a minimum of 50 min and as much as 5 to 6 days.

Why did our P3 rats not exhibit a PCE? Fagen & Shoemaker (1979), working with juvenile and adult rats, suggested that the probability of detecting PCEs increases with age. However, we did not record contrast effects in the oldest animals used in this study. Clearly, this may be due to the relatively long interval between TE1 and TE2 in our P3 rats. Positive contrast effects diminish as retention intervals are lengthened (for review, see Flaherty, 1982). However, other explanations also might be proposed. Sometimes the failure to observe a PCE has been discussed in the context of ceiling effects (Brazier & Dachowski, 1991). Is the fact that we did not observe enhanced mouthing and licking responses in up-shifted P3 neonates due to their inability to emit these orofacial responses at a level significantly higher than nonshifted controls? The mean number of mouthing and licking movements in our shifted P3 rat pups was on the order of 10 to 12 per min. This is clearly below the motor capacity for an animal of this age (e.g., Mickley et al., 2000b). Thus, it is unlikely that ceiling effects obscured the observation of PCEs in our animals. A more plausible explanation is that the P3 neonates failed to recall the SAC concentration tasted 5 to 6 days earlier.

In addition to the extended TE1-TE2 interval employed in our P3 tests, other factors also may have reduced the likelihood of detecting PCEs or habituation effects in these animals. It was impossible to treat these animals in a manner identical to the other groups in which we detected a PCE since the final behavioral test was scheduled after birth. Therefore, the context of TE2 was dissimilar to the context of TE1. Moreover, the neonatal test chamber was very different from the uterinelike environment of the temperature-controlled Locke's solution bath. There is clear evidence that conditioned fear (Bouton & King, 1983) and appetitive conditioning (Boakes, Westbrook, Elliott, & Swinbourne, 1997; Bouton & Sunsay, 2001) can be influenced by the contextual stimuli available during training and testing. Less work has addressed the role of context in nonassociative learning paradigms such as the one used here. If nonassociative learning is indeed context dependent, then the dissimilar fetal and neonatal environments may have reduced the chance of detecting evidence of a PCE in our subjects at age P3.

Other environmental factors may have contributed to the different behavioral responses seen in the animals experiencing a 50-min versus a 24-hr TE1-TE2 latency. Animals in the 24-hr TE1-TE2 groups tasted SAC in utero while the 50-min latency fetuses received oral lavage ex utero while floating in the Locke's solution bath. PCEs are influenced by the duration of the first exposure to a tastant (Pinel & Rovner, 1977). Perhaps the in utero exposure

allowed the fetuses to taste the SAC for a longer period of time before it was cleared away.

Beyond contextual stimuli, the P3 rats may have experienced variable amounts of maternal care and nutrition during the first few days after birth. These uncontrolled random factors were not experienced by the fetuses in our study and may have had the effect of enhancing the variability in the E18-P3 and E19-P3 groups. Enhanced variability would reduce our ability to detect PCEs or other types of learning in these animals.

The fetuses in this study were tested surrounded by fluid whereas the neonates were not. Thus, there is the potential for different orofacial responses elicited by the two testing methods. Could the difference in methodology limit our ability to detect, in P3 neonates, the behavioral indicators of learning that we saw in E19 fetuses? We attempted to address this concern in our earlier studies of perinatal animals. Previous work from our lab compared the orofacial responses of E21 fetuses tested before parturition (using the same fetal methods as described here) to E21 neonates tested in the same paradigm as our P3 neonates in the current study (see Mickley et al., 2000). When assessed for demonstration of a taste recognition memory (TRM), no difference was found between the E21s tested as fetuses and the E21s tested as neonates. This provides some evidence that the methods of P3 testing used here do not necessarily obscure behavioral demonstrations of learning phenomena.

Maturation of the fetus occurs in the context of a changing uterine environment, and therefore, development during the perinatal period is not always uniformly linear or progressive. Data suggesting waxing and waning of the ability to learn and retain new information (i.e., the existence of ''periods of learning readiness'') are well-known (Campbell & Campbell, 1962; Spear, 1984). For example, although virtually all rat fetuses exhibit a facial wiping response on E21, the incidence of facial wiping is reduced by 50% in newborn rat pups tested only a few hours after birth. Within 24 hr, the wiping response disappears almost completely and remains absent until the end of the second postnatal week when it reappears (Smotherman & Robinson, 1989).

Such information fosters a view of the developing organism as occupying a succession of ontogenetic niches (West & King, 1987). Periods of adaptation to a particular niche are interrupted by transitions to subsequent niches. Development within an ontogenetic niche may be represented by increasing behavioral diversity and organization while periods of transition between niches may result in a temporary slowing or regression in measures of development (Smotherman & Robinson, 1990). Perhaps the pressures of the E19 ontogenetic niche are different from those experienced by an E20 fetus. The findings that rats in the nonshifted condition exhibit opposite mouthing and licking responses depending on the TE1-TE2 interval (50 min or 24 hr) and the age of the animal at TE2 also is consistent with the concept that fetuses may adjust their behavioral responses in accordance with environmental pressures imposed at a particular age of development. The fact that we observed such different orofacial responses over just 24 hr (E18-E19; E19-E20) may illustrate how rapidly the uterine environment may be changing during this period of perinatal development.

Previous work from this laboratory has indicated that perinatal rats can distinguish between novel and familiar tastes (Mickley et al., 2000). Using procedures similar to those employed here, E17, E18, or E19 fetuses received an oral injection of 10 μl, 0.30% SAC while in utero. These animals were then reexposed to the same concentration of SAC either 2 days later or on P3, and observations of orofacial motor responses were recorded. Rats that first experienced SAC on E19 later (on E21 or P3) exhibited an SAC-induced stimulation of mouthing and licking, as compared to animals experiencing novel SAC (i.e., no previous taste exposure in utero). These data suggest that a TRM is maintained for up to 5 days (i.e., E19-P3). The data also indicate that E19 rat fetuses can acquire this TRM and retain it for at least 2 to 5 days while E17 and E18 fetuses cannot.

There are clear similarities and differences between the TRM paradigm/phenomenon and the PCE described in this article. Like the TRM, positive contrast effects can be observed in E19 fetuses, but not in animals a day younger. However, the TRM effect was recorded in P3 neonates while animals of this age did not exhibit a PCE. These data verify the rapid transition of gustatory-based memory capacity over a 24-hr period (E18-E19). Moreover, they distinguish between the rat's ability to retain specific types of gustatory memories. TRM is relatively long-lived as compared to memories associated with positive contrast, which fade between 1 and 5 days after TE1.

Solution novelty may be a factor in the demonstration of contrast effects (Meinrath & Flaherty, 1988) and in the production of orofacial movements more generally (Mickley et al., 2000). In fact, PCEs may be obscured by orofacial movements reflecting neophobia. Mickley et al. (2000) previously reported a reduction in mouthing and licking responses to a novel taste as compared to those observed following exposure to a familiar taste. Since the 0.30% SAC concentration was new to the shifted animals, we would expect an inhibition of orofacial movements. Thus, neophobic effects may be reducing the likelihood of observing PCEs in the current studies. This makes the demonstration of a PCE more impressive (e.g., in rats with a 50-min TE1-TE2 interval), but also makes failure to find a PCE more difficult to assert with confidence (e.g., in animals having 24-hr or 5- to 6-day retention intervals).

Our finding indicating rapid development of PCE from E18 to E19 evokes parallels with other data indicating that perinatal rats at these ages respond very differently to N-methyl-D-aspartate (NMDA) glutamate receptor-blocking drugs. Administration of the NMDA receptor-blocking drug ketamine before CS to US pairings potentiated CTA formation and conditioned motor responses in E18 fetuses (Mickley et al., 1995; Mickley et al., 2001). However, when injected with equivalent doses of ketamine at the time of conditioning, E19 and older rats later failed to exhibit a CTA or conditioned motor responses (Mickley et al., 1998; Mickley et al., 2001). Thus, there may be critical periods in the developmental process when the NMDA receptor blockade produces very different effects on learning and memory. These drug effects may build upon and/or highlight the normal development of memory capacity during the late embryonic period.

Investigators have proposed a variety of neural, pharmacological, and psychological mechanisms to explain contrast effects (Flaherty, 1982, 1990; Flaherty, Lombardi, Wrightson, & Deptula, 1980; Flaherty, Turovsky, & Krauss, 1994; Grigson et al., 1997; Leszczul & Flaherty, 2000; Meinrath & Flaherty, 1988). For example, contrast effects have been attributed to generalization decrements, neophobic reactions, adaptation level averaging, and frustration (Flaherty, 1982). Our data are not aimed at revealing the underlying bases for this phenomenon in perinatal rats. In fact, the data presented here may be interpreted in several ways. It may be the case that E18 rat fetuses cannot sense SAC with sufficient acuity to allow them to notice a shift in the concentration minutes, hours, or days later. Alternatively, the memory capacities of E18 fetuses may not have matured sufficiently to allow the animal to recall the previous SAC taste upon experiencing TE2. There also may be attentional and motivational factors that could influence the expression of contrast effects (Meinrath & Flaherty, 1988). These potential mediators of PCE in perinatal rats are worthy of investigation and are currently being explored in our laboratory.

In summary, our data indicate that fetal rats can remember and compare two tastes they experience late in gestation and adjust their behavioral responses accordingly. The ability to retain a taste memory and make a comparison emerges late in gestation, on E19, and depends not only on the age of the animal but also on the interval between the taste presentations.

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