EXPOSURE TO THE MATERNAL ODOR ENHANCES INTAKE OF A TASTE THAT MIMICKS THE SENSORY ATTRIBUTES OF ETHANOL

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Abstract:

Early exposure to ethanol increases subsequent acceptance of this drug. Little attention, however, has been devoted to the interaction of the taste of the drug with other, familiar or non-familiar, odors contingent with ethanol access, particularly early in ontogeny. ~~The~~ This ~~current~~ study assessed the influence of exposure to maternal odor on intake and grasp responses to an artificial nipple providing a solution (a sucrose-quinine mix) that emulates the taste of alcohol, in 4-day old rat pups. The results showed that the mother's odor enhanced intake from and seeking responses to an artificial nipple that provided the solution that mimicked the taste of alcohol (Experiment 1). This pattern of results was not evoked by the odor of an unrelated dam (Experiment 2), nor was observed when the nipple delivered water. The main new finding of the present study is that 4-day old ~~animals~~ rats tested in the presence of the mother (and hence exposed to its odor cues) exhibited enhanced seeking and intake of a solution that mimics the chemosensory properties of ethanol. ~~This suggests that, in 4 day old ratsduring the neonatal period, the exposure to familiar odors may facilitate acceptance of flavors with aversive components (i.e., with a bitter taste), and therefore may act as a permissive factor of ethanol intake.~~

Keywords: odor - taste - ontogeny – rats

The roots of alcohol use problems (i.e., alcohol abuse and dependence) can be traced to very early life stages (Molina, Spear, Spear, Mennella & Lewis, 2007). Human fetuses or babies can be involuntarily exposed to alcohol through different cultural practices; e.g., through breast milk or amniotic fluid, after maternal intoxication with the drug. Epidemiological and pre-clinical research indicates that these early experiences significantly modulate subsequent, voluntary, alcohol drinking (March, Abate, Spear & Molina, 2009).

Not so long ago (Mennella & Beauchamp, 1993a), it was considered that drinking small quantities of alcohol before nursing increased milk yield, facilitated milk let-down, or induced relaxation of the mother-baby dyad. Abundant evidence, nevertheless, indicates that drinking before nursing disrupts lactation performance and alters infant behavior and development (Mennella & Gerrish, 1998; Zeanah, Boris & Larrieu., 1997). Besides, ethanol alters the flavor of breast milk, a change that results in an altered suckling behavior pattern. Mennella & Beauchamp (1991a, also see Mennella, 1999) observed that babies whose mothers consumed alcohol before nursing performed, when compared to babies whose mothers had not consumed alcohol, more suckling during the first minute of meals, yet they consumed significantly less milk by the end of the observation.

These studies (Mennella & Beauchamp, 1991a,; Mennella, 1999), inferred that ethanol changed the flavor of the milk or induced sedation or activation. The influence of the maternal odor in the context of suckling, and the possibility of this odor promoting or coming into association with ethanol intake or exposure has not been, however, considered. The present study employed an animal (rat) model in which tastes

and odors interact in the context of suckling for a substance (a quinine-sucrose compound) that emulates the taste of alcohol. Preweanling and neonate rats detect and discriminate between flavors, even when taste buds are not fully developed (Ganchrow, Steiner & Canetto, 1986; Nizhnikov, Petrov, Varlinskaya & Spear, 2002). In preweanling rats, familiarization with the odor of ethanol (Bannoura, Kraebel, Spear & Spear, 1998) or with ethanol's unconditional effects (Arias & Chotro, 2005a; Diaz-Cenzano & Chotro 2010; Kiefer & Lawrence, 1988) results in (a) greater preference for ethanol's odor and (b) greater intake of either ethanol or a sucrose-quinine mix, that mimics the psychophysical properties of ethanol (Kiefer, Bice, Orr & Dopp, 1990; López & Molina, 1999).

Olfactory stimuli are critical during early stages of life of development in mammals. Altricial species learn behaviors that facilitate approach towards the caregiver (Moriceau & Sullivan 2005). The maternal odor changes depending on the diet, and rat pups are born without fully developed visual and auditory systems. Such plasticity in odor learning allows pups to find the nest and maternal nipple through smell (Pedersen, Williams & Blass, 1982). This species exhibits a sensitive period that lasts about 10 days since the time of birth, in which odor pre-exposure results in long-lasting learned preferences (Moriceau, Roth, Okotoghaide & Sullivan, 2004; Upton & Sullivan, 2010). Such olfactory learning continues repeatedly throughout the early postnatal period, which presumably allows the infant to adjust to the changes in the mother's odor (Landers & Sullivan, 2012). The relevance of this age-specific predisposition declines as subjects undergo transition into adolescence and adulthood. Previous data from our lab indicated that stimulation with a familiar, pre-exposed odor (i.e., lemon scent) either during the gestational or postnatal life, increased seeking and intake of quinine (an aversive solution, Berridge, 2000) in neonate rats tested via an

artificial nipple (Kamenetzky, Suárez, Pautassi, Mustaca & Niznikov, 2015; Kamenetzky, Suárez, Ifran, Nizhnikov & Pautassi, 2018). Furthermore, others studies from our lab showed that the presence of maternal odor also increased these ingestive behaviors toward a surrogate nipple at posnatal day (PD) 4 (Ifran, Suárez, Pautassi, Kamenetezky, 2018~~accepted~~). This suggested that familiar odors can switch the hedonic value of aversive solutions in rats, and reproduced results of greater acceptance of unpalatable flavors seen in studies with human babies. During the first 4 months of life, human infants exhibit a sensitive period in flavor programming, characterized by enhanced predisposition to accept unpalatable flavors. Protein hydrolysate formulas (which are bitter and have a rancid smell) are accepted before the 4 months, yet strongly rejected by older infants and children (Beauchamp & Mennella, 2011; Mennella & Beauchamp, 1996). The mechanisms of this flavor programming in infancy and the action of both senses (smell and taste) working jointly during early ontogeny have not been widely explored.

The present study assessed seeking and drinking of a mixed solution (quinine + sucrose) that mimics the taste of ethanol. Prior research has taken advantage of this solution to study responsivity to ethanol's sensory properties without the confounding factor of ethanol's pharmacological (e.g., motor activating or depressing) effects (López & Molina, 1999; Bachmanov et al., 2003~~)~~, and the influence of the maternal odor in the testing situation. The hypothesis of our study was that the stimulation with a pre-exposed, biologically relevant odor (i.e., mother's odor), would enhance seeking (i.e., grasping of the nipple) and drinking of a solution that mimics the taste of alcohol. Rats were given access to a nipple providing sucrose+quinine, either with or without the anesthetized mother. A second experiment replicated the procedure yet adding further

controls (i.e., animals stimulated with the odor of another, unrelated mother and animals given only water at test).

This work provides valuable information to understand how the olfactory and gustatory systems work in unison during early life and how this interaction can influence alcohol intake.

General methods

Subjects. Eighty-seven Wistar, 4-day old, rats ~~(male and female, 4 days of age at the start of the experiments)~~ were used. The animals, male and female, ~~s~~ were derived from 18 dams, mated at the vivarium of Instituto de Investigaciones Médicas Dr. Alfredo Lanari (IDIM-CONICET, Argentina) and given ad libitum access to water and lab chow (Cooperación, Buenos Aires, Argentina). Litter representation and number of subjects ~~used~~ in each experiment were as follows: Experiment 1 (24 animals derived from 6 litters, 12 animals in each group), Experiment 2 (63 animals, 12 litters). The day of parturition was considered PD 0 and, within each litter, only one male or female was assigned to a given treatment condition (Holson & Pearce, 1992). The vivarium had a 12 hr/12 hr light/dark cycle, with lights on at 7:00 am, and controlled temperature (22˚C) and humidity. Rats used in these experiments were maintained and treated in accordance with the guidelines for animal care and use established by the National Institutes of Health (1996). The experimental protocol (N 010-14) was approved by the institutional animal care committee (CICUAL).

Apparatus.

Surrogate nipple. The surrogate nipple was cast from rubber latex (AMACO rubber latex, Indianapolis, IN) and molded into a conical form to measure 12 mm long with a rounded tip measuring 1 mm in diameter and the base measuring 2.5 mm in diameter. The base of the surrogate nipple was attached to the end of an angled dental probe to

facilitate presentation by the experimenter (Petrov, Varlinskaya & Smotherman, 1997). Polyethylene tubing (Clay Adams, Sparks, MD) run throughout the length of the nipple. The tubing was attached to a syringe, which was filled with the corresponding solutions. The nipple was in continuous contact with the mouth of the animal, which was gently stimulated on the lips with the tip of the device ~~on the lips~~. The pressing of ~~the tip of~~ the nipple by the pup's mouth was associated with a negative pressure that in turn allowed voluntarily intake. The pup was clamped in a semi-supine posture into a ''vest'', fashioned out of an ultra-thin, elastic rubber. This light restraint prevented righting attempts but did not otherwise produce discomfort nor hinder the pups' movements.

Solution.

The nipple delivered a mixture of quinine and sucrose, which has been shown to emulate the taste of alcohol (Di Lorenzo et al., 1986). Following previous studies (Arias & Chotro, 2005a; Diaz-Cenzano & Chotro 2010; López & Molina, 1999), this solution was prepared by diluting sucrose 0.1 M + quinine 0.0001 M (Sigma-Aldrich, Buenos Aires, Argentina) ~~or~~ on distilled water.

Procedure.

Experiment 1. Responsiveness to a surrogate nipple providing a solution that emulates the taste of alcohol (i.e., sucrose and quinine) in the presence of the mother's odor.

We explored the effects of the mother's odor on attachment to an artificial nipple delivering a solution that mimics the taste of alcohol. Two groups were stimulated during 6 min with the artificial nipple providing this sweet and bitter solution in the presence (Odor Group) or absence (No odor Group) of the dam. The test involved delivery of the quinine-sucrose solution via the artificial nipple in the presence (Odor

Group, the anesthetized dam was about 2 cm away from the nose of the pup) or absence (No odor Group) of the mother's odor.

Experiment 2. Responsiveness toward a surrogate nipple providing sucrose-quinine or water in the presence of the own mother's or another mother's odor.

In Experiment 1 the experimental, but not the control, pups~~animals,~~ were exposed to the odor ~~of the mother~~ by the presence of anprovided by an anesthetized mother.~~,~~ There was no physical contact between the pups and the dam, yet the pups ~~therefore they~~ were ~~also~~ exposed to the social stimulus ~~without physical contact~~ and to the heat provided by

~~; but they were also exposed to social contact and to the heat provided by~~ the dam. It was not clear, then, if the odor was the only factor inducing differential responsiveness to the nipple. ~~To control for these confounding factors,~~ Experiment 2 controlled these confounding factors by ~~assessed~~ assessing responsiveness to the nipple in the presence of the anesthetized mother or in the presence of another, unrelated, anesthetized dam. Moreover, to assess the specificity of the promoting effect of the dam on nipple attachment, a sub-group of pups was offered water ~~by the nipple~~, instead of the sucrose-quinine mixture, through the nipple. A 2 (female present during the test: own mother or other mother) × 2 (solution given via the nipple) factorial design was employed in Experiment 2. Each of the four groups had 15-16 animals.

In both Experiments, the test was performed ~~from~~between 9 ~~am~~AM ~~to 12~~and noon. ~~pm.~~ A ~~a~~t DP 4, the dam was anesthetized with a mix of ketamine (40 mg/kg) and xylazine (5 mg/kg). The pups were then placed into a heating chamber (kept at 35 °C, Simen, Buenos Aires, Argentina) and, 15 min later, gently stimulated in the urogenital region with cotton, to induce urination and defecation. Each animal was weighed,

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equipped with the vest and attached to a tempered mirror. The experimenter stimulated the perioral area of the pup with the tip of the surrogate nipple. The oral grasp response, which allowed suckling and hence access to the solution, involved an active movement of the pup's head toward the surrogate nipple, which resulted in the tip of the nipple entering the oral cavity and the mouth closing around it. From this response the following measures were obtained: latency to grasp, total time spent on the nipple (sum of the duration of all grasps), frequency of grasps (attachments initiated) and mean duration of an individual grasp response (total time of grasps / number of grasps). The consumption of the solution was measured via the percentage of body weight gain: [(post testing weight - pre testing weight)/pre testing weight * 100].

All experiments were videotaped and subsequently analyzed by two observers (observer's reliability > 85%) who were blind to the experimental conditions.

Data analysis.

In Experiment 1 each variable was analyzed via t-T Tests tests for independent samples. The grouping factor was the odor condition during the test (mother's odor or no odor exposure). Between-group analyses of variance (ANOVAs) were employed in Experiment 2. Odor condition (own mother's odor or other mother's odor) and Solution (sucrose-quinine or water) were the independent factors. The significant main effects or significant interactions were further analyzed by Tukey's HSD *post-hoc* comparisons, and planned comparisons were used when justified by *a priori* hypotheses. Data were collapsed across sex since, across variables, this factor exerted no significant main effect nor interacted with the remaining variables. The alpha level was set at ≤ 0.05.

Results.

Experiment 1.

Fig. 1 shows latency to grasp the nipple, number of grasps, total time of grasps, mean of grasp duration, and percentage of body weight gain during the test. It seems that the presence of the mother significantly facilitated approach and contact with the nipple. The statistical analysis supported these observations. Subjects evaluated in the presence of the mother showed, when compared to animals in the no odor group, significantly greater total time attached to the nipple, $t_{(22)} = 4.81$, $p < .00008$; number of grasps, $t(22)_{22} = 4.14$, $p < .0004$; mean duration of grasps, $t(12.58)22 = 3.02$, $p < .01$ and a significantly lower latency to grasp the nipple, t_{22} (14.99) $= -5.71$, $p < .00004$. Percentage of body weight gained was greater in the odor vs. the no odor group, yet this difference did not reach statistical significance ($p > .05$).

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Experiment 2.

Figure 2 illustrates the behaviors measured in Experiment 2. The ANOVA for percentage of body weight gain revealed a significant interaction between odor condition and solution, $F_{(1, 59)}=7.60$, $p < .01$. 07. The *post-hoc* tests revealed significantly greater intake of sucrose-quinine than water in those pups stimulated with the odor of the mother. On the other hand, the *post-hoc* tests indicated that pups stimulated with the odor of an unrelated dam consumed as much sucrose-quinine as water. Also important, intake of sucrose-quinine was significantly greater in the presence of the dam than in the presence of the non-related dam.

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The ANOVAs for total time spent grasping the nipple and mean grasp duration indicated the lack of significant main effects or significant interactions. Fig. 2 suggests, however, an exacerbated response in the group given sucrose-quinine than in the other groups. Guided by these impressions, and by our a priori hypotheses, we conducted planned comparisons and observed significantly greater time spent on the nipple ($F_{(1, 31)}=4.06$, $p < .053$) and mean grasp duration, ($F_{(1, 31)}=4.14$, $p < .05$)1 for in the group

that ~~receiving~~ received the ~~solution of~~ sucrose~~+ and~~ quinine solution in the presence of the own mother, ~~compared to~~than in the group assessed in the presence of another mother. Number of grasps and latency to grasp ~~was~~ were not affected by the factors under ~~study~~ analysis ($p > .05$).

Discussion.

Studies have shown that ~~odors (e.g. cineole)~~ initially neutral odors (e.g. cineole) came to elicit alcohol intake in rat pups following pairings with ethanol ~~(~~(Abate, Spear & Molina, 2001; March, Abate & Molina, 2011). The main new information added by the present study is that 4-day rats tested in the presence of the mother (and hence exposed to maternal odor cues previously experienced in-utero) exhibited enhanced seeking and intake of a solution that mimics the chemosensory, but not the post-absorptive, properties of ethanol. This suggests that, during the neonatal period, the mere presence of familiar odors may facilitate acceptance of flavors with aversive components (i.e., bitter taste), and therefore may act as permissive factor for ethanol seeking and intake.

In Experiment 1, the experimental group had access to a heat source and social contact, via the anesthetized dam. Experiment 2 included control animals stimulated with the odor provided by an anesthetized, yet unrelated dam, and thus dispelled the alternative explanations brought by these issues. The animals assessed in the presence of their mother exhibited greater percentage of body weight gain and performed significantly more responses toward the artificial nipple than those assessed in the presence of an unrelated dam. This difference was not observed when the artificial nipple dispensed water, suggesting that the promoting effect of pre-exposed odors upon fluid seeking and intake is expressed only when the solutions have an aversive component. These results replicate studies in newborn rats (Kamenetzky et. al., 2015)

that reported greater intake and grasp responses to an artificial nipple (scented with a pre-exposed lemon odor) that dispensed a moderate concentration of quinine, but not to a nipple providing sucrose. The present study generalizes this phenomenon to a biologically relevant odor (i.e., mother's odor), and to a solution that emulates the taste of a drug of abuse (ethanol) that can be readily transferred to breast milk after maternal intoxication.

The taste of milk can be altered by the diet ingested by the mother. It has yet to be assessed how the tastes experimented in the maternal milk could interact with previously learned odors that are present in the suckling context. Studies with human babies, however, showed that when mothers consume a capsule of garlic, babies display more responses towards the breast (Mennella & Beauchamp, 1991b; Mennella & Beauchamp, 1993b). In addition~~,~~, babies who were exposed to milk contaminated with alcohol consumed more of the breast milk compared to babies exposed to uncontaminated milk (Mennella, 1999; Mennella and Beauchamp, 1991a). In light of these ~~results~~results, it is interesting to hypothesize that odors present in the context of breastfeeding could modulate ~~the~~ responses to the maternal breast when two conditions are met: 1. A familiar odor is present and 2. A substance with a bitter taste is offered.

Several pre-clinical (Molina, Spear, Spear, Mennella & Lewis, 2007; Fabio et al., 2013) and epidemiological (Windle and Windle, 2012~~1~~~~2~~; Dawson et al, 2008~~0~~~~8~~; Jenkins et al., 2011~~1~~) studies indicate that early exposure to ethanol increases subsequent acceptance of this drug. These studies have analyzed several factors that can modulate the effect of an early-onset ethanol use upon subsequent ethanol abuse and dependence (e.g., dose of first ethanol exposure). Yet, little attention has been devoted so far to the interaction of the taste of the drug with other, familiar or non-familiar, odors contingent with ethanol access. The results of the present study show that a

biologically relevant pre-exposed odor, the scent of the own mother, enhanced the acceptance of the sucrose-quinine mixture. The sweet component of ethanol has been traditionally thought to induce or facilitate alcohol consumption, yet the data from this research and those obtained by Kamenetzky et. al. (2015) suggest that the bitter component could also play an important role, particularly early in life in the context of breastfeeding. This is a preliminary hypothesis that requires more evidence to be confirmed.

It has been shown that experimental contamination of the amniotic fluid with alcohol increases later, postnatal, consumption and palatability of that substance (Arias & Chotro, 2005a, Arias & Chotro, 2005b). It could be proposed that, in those experiments, the animals went through the two stages of our protocol. In phase 1, the animals were naturally pre-exposed to the mother's odor during the last stage of gestation. In phase 2, in turn, the animals were exposed to ethanol through an amniotic fluid that also provided exposure to the mother's odor. When tested postnatally, these pups exhibit, akin to those in the present report, greater acceptance of alcohol or alcohol-related cues. A large amount of evidence shows that both human (Mennella, Jascow & Beauchamp, 2001; and rat (Abate, Pueta, Spear & Molina, 2007; Chotro & Arias, 2006) fetuses are capable of perceiving odors present in the amniotic fluid during the last stage of gestation. The present results suggest that the intake of the amniotic fluid contaminated with alcohol is enhanced in these subjects, due to the interaction of the bitter taste and maternal odor.

In summary, the results indicated that the presence of the maternal odor significantly enhanced attachment to, and intake from, an artificial nipple dispensing a solution that emulated the taste of alcohol. It is noteworthy that the sucrose-quinine mix

employed was novel, which indicates that these results cannot be the consequence of previously learned associations involving such taste.

~~These results contribute to the development of an animal model to assess the hedonic ontogeny of taste. Furthermore, t~~This study contributes to the scarcely explored area of the interaction between olfaction and taste during early ontogeny. ~~The results further confirm that the first days of life constitute a sensitive period for olfactory learning and that, in turn, this learning is a key factor in taste programming.~~ The results~~is~~ are consistent with ~~agrees with a previous research~~previous work ~~,~~from our laboratory in which ~~it was found that~~ 3~~-~~ ~~and~~or 12~~--~~day old rats exhibited an increase in the consumption of a bitter solution when tested in the presence of their own mother, but not when tested in the presence of another, unrelated, ~~mother~~dam (Ifrán, Suárez, Pautassi & Kamenetzky, 2018). ~~Likewise, unpublished data showed that this phenomenon could be framed within a sensitive period, given that 9 PD rats exhibited an increase in quinine consumption when a familiar odor was present; this was not observed in 15 DP. Therefore~~rats. Therefore, ~~i~~It seems that these early experiences are key for the development of eating habits or the consumption of psychoactive substances. The animal model outlined in the present study should be useful for assessing strategies aimed at solving problems related to the early rejection or acceptance of unpalatable substances, including those subjected to abuse. ~~Pre-clinical studies, such as the one submitted, will enhance understanding of mechanisms related to this important phenomenon.~~

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Funding

This work was supported by the PIP-CONICET-2012 [grant number 11220110100228], BID-PICT 2014 [grant number 3212] and the Universidad Abierta Interamericana, awarded to Giselle Kamenetzky.

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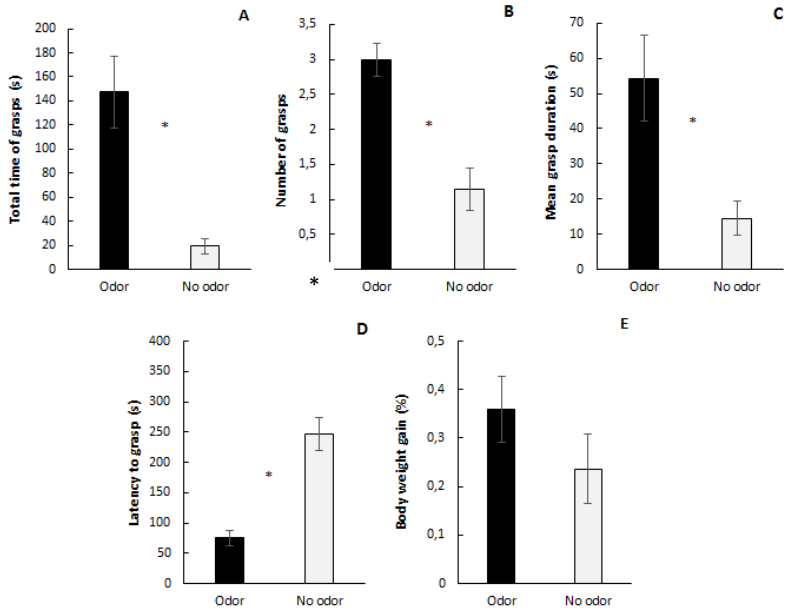
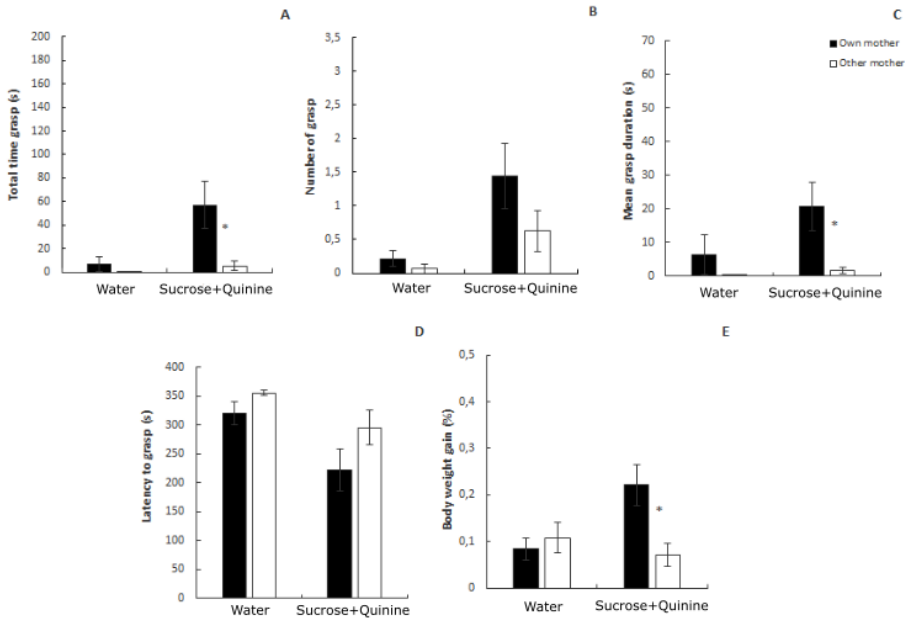

A
Total time of grasps (s)
200
180
160
140
120
100
80
60
40
20
0
*
Odor
No odor
B
Number of grasps
3,5
3
2,5
2
1,5
1
0,5
0
*
*
Odor
No odor
C
Mean grasp duration (s)
70
60
50
40
30
20
10
0
*
Odor
No odor
D
Latency to grasp (s)
400
350
300
250
200
150
100
50
0
*
Odor
No odor
E
Body weight gain (%)
0,5
0,4
0,3
0,2
0,1
0
Odor
No odor


A
B
C
Total time grasp (s)
Number of grasp
Mean grasp duration (s)
Own mother
Other mother
Water
Sucrose+Quinine
D
E
Latency to grasp (s)
Body weight gain (%)

Captions.

Figure 1. Mean (±SE) of (A) total time grasp, (B) mean grasp duration, (C) number of grasp, (D) latency to grasp and (E) percentage of body weight gained, during the 6-min presentation of the artificial nipple containing quinine and sucrose solution. *Indicates *p* values < .05.

Figure 2. Mean (±SE) of (A) total time grasp, (B) mean grasp duration, (C) number of grasp, (D) latency to grasp and (E) percentage of body weight gained, during the 6-min presentation of the artificial nipple containing water (left bars) or quinine and sucrose solution (right bars). *Indicates *p* values < .05.