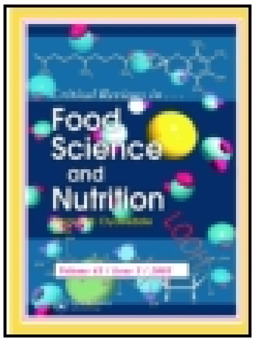

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A minireview of effects of maternal diet during pregnancy on postnatal vegetable consumption: implications for future research (a new hypothesis) and recommendations

Paloma Rohlfs Domínguez

A minireview of effects of maternal diet during pregnancy on postnatal vegetable consumption: implications for future research (a new hypothesis) and recommendations

Paloma Rohlfs Domínguez[1,2]

[1]Department of Psychology and Anthropology, University of Extremadura, Spain

[2]Department of Social Psychology and Methodology of Behavior, University of Basque Country-Euskalherriko Univertsitatea, Spain

CONTACT: Paloma Rohlfs Domínguez. University of Extremadura, School of Nursing and Ocuppational Therapy and Faculty of Teaching Training. Campus Universitario, s/n 10071, Cáceres (Spain).**E-mail:** prohlfsdomnguez@yahoo.es

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Abstract

The flavor of the maternal diet is transferred to the amniotic fluid and in this way the amniotic fluid becomes a transmitter of flavor-related information, developing babies can perceive in uterus. We aimed to review the available evidence regarding the impact of prenatal exposure to flavor on postnatal vegetable-related eating behavior. Studies of our focus have been the ones that have ad hoc examined the association of prenatal experience with vegetable-related vegetables with vegetable consumption in children. We have identified that some studies have found this association, against others. Particularly, the first group of studies found an increased vegetable intake at weaning and late childhood associated with prenatal flavor exposure. The second set, instead, did not find this association of variables at early childhood. We propose here that the vegetable type used in these last studies might explain this disparity of results, and suggest the following hypothesis to be tested in the future: prenatal exposure to non-bitter vegetables leads to an increased vegetable consumption at early childhood in comparison to prenatal exposure to bitter vegetables, postnatal exposure to vegetables and no exposure. The experimental design of this hypothesis as well as subsequent recommendations for future research and maternal diet are also here suggested.

Keywords

Amniotic fluid**,** Children, Food preferences, Prenatal exposure, Vegetables.

1. Introduction

Vegetable consumption is considered by the World Health Organization (WHO) as being essential for maintaining a good health and preventing diseases, in general, and cardiovascular diseases and certain cancers, in particular (WHO, 2017). In addition, vegetable consumption during childhood promotes cognitive development (Contento et al., 1995) and, together with physical activity, counteracts childhood obesity (Aranceta-Batrina and Pérez-Rodrigo, 2016). Childhood vegetable consumption also prevents the development of diseases in adulthood, such as coronary disorders, hypertension and cancer (Van Duyn and Pivonka, 2000). Despite these benefits, rates of children's vegetable consumption do not reflect the recommendations by the WHO or national recommendations in Europe (Yngve et al., 2005) or in the Unites States (Johnson and Kennedy, 2000). Regarding the European recommendations, they vary according to the country in question. For example, Spanish recommendations for daily vegetable intake in ≤ 6 year-old, 6- to 8-year-old, 9- to 11-year-old and ≥ 12 year-old children are 150g, 200g, 220g, and 250g, respectively (Aranceta et al., 2008). However, the actual average vegetable consumption of 2- to 13-year-old Spanish children has been estimated as being a mean of 66.1 grams per day (Serra and Aranceta, 2002). Dutch recommendations for daily vegetable intake in 0- to 4-year-old, 4- to 8-year-old and 8- to 12-year-old children are 50-100g, 100-150g and 150-200g, respectively (Gubbels et al., 2014). However, 2- to 3-year-old, 4- to 6-year-old and 7- to 13-year-old Dutch children currently eat only 41, 43 and 60 grams of vegetables per day, respectively (Ocke et al., 2008; Van Rossum, et al., 2011). Several reports have also indicated low consumption rates of vegetables in young children in the United States (Johnson and Kennedy, 2000; Lorson et al., 2009). Therefore, research on determinants of children's vegetable

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consumption is warranted because this effort is expected to help to better understand the problem at hand as well as to discover strategies that may increase young children's vegetable intake, and thus to reinforce children's healthy eating behavior.

Human behavioral research has shown that the foods that are consumed by children directly match their food preferences (Nasser, 2001; Sorensen et al., 2003; Zeinstra et al., 2007; Mennella and Beauchamp, 2010). The flavor (taste/s plus odor/s) of a food is its most salient feature (Beauchamp and Mennella, 2009). The term *preference* refers to the choice of one item over another item and differs from liking, which refers to a positive affective reaction (Rozin and Vollmecke, 1986). Young children prefer to eat what they like over what they do not like, and thus only eat foods that taste and smell "good" rather than "bad" (Birch, 1979; Birch, 1999; Burguess-Champoux et al., 2006; Mennella et al., 2016).

Human behavioral research has also shown that a mixture between genetic predisposition and experience-driven learning ultimately determines children's food likings and preferences. Indeed, we know that there is a generalized innate predisposition for children to like sweet foods and reject bitter ones (Mennella et al., 2005). For example, cruciferous (green) vegetables, such as broccoli and spinach tend to be rejected by young children due to their bitter taste (Anliker et al., 1991; Aranceta and Pérez Rodrigo, 1996; Cooke and Wardle, 2005). We also know that this innate vegetable rejection may vary due to children's genetic level of sensitivity to 6-n-propylthiouracil (PROP) that accounts for the bitterness of some green vegetables (Wardle et al., 2001). This variation lies on the high polymorphism of the bitter taste gene TAS2R38, which explains individual differences in the preference for the bitter taste. Individuals who are genetically sensitive to the bitter taste commonly reject bitter-tasting edibles, such as nonnutritive glucosinolate-content vegetables, more than do individuals who are genetically bitter insensitive (Sandell and Breslin, 2006; Rohlfs Domínguez, 2011; Hoppu et al., 2016). Children and their taste likings are also affected by this genetic variation in bitter taste sensitivity (Bobowski et al., 2016). Apart from the TAS2R38, other bitter taste genes that may show polymorphisms -TAS2R16, TAS2R19 and TAS2R31- may also impact human consumption of bitter edibles (see Hayes et al., 2013, for a review). Liking the umamy and salty tastes also appears to rely on genetic basis (Beauchamp and Mennella, 2011). Together with this genetic basis, experience-driven learning, through the action of different experience-related mechanisms (see Krølner et al., 2011; Rohlfs Domínguez, 2014; Whadreva et al., 2015; Johnson, 2016, Mennella et al., 2016, for reviews), may shape infants' and children’s taste and olfactory preferences and thus food acceptance, due to the behavioral plasticity typically found in these age groups. These mechanisms mainly refer to repeated exposure to food- and drink-related flavors (tastes plus odors) during early developmental stages. Indeed, repeated exposure to any stimulus, such as a food, usually generates an increased familiarity with the flavor of this food, and consequently, an increased liking of that flavor and consumption of this food (Zajonc, 1968; Hill, 1978; Bornstein, 1989; Wadhera et al., 2015a). In some cases, this repeated exposure refers to mere exposure, that is, when exposure to a neutral flavor does not follow any consequence. In some other cases, instead, exposure refers to associative conditioning. Here, exposure to the neutral flavor is followed by a given consequence, either positive, such as nutritive (caloric) ingestion or hedonic pleasure, either negative, such as nausea or emesis, due to repeated pairings of the neutral flavor with a high caloric substance or an already preferred flavor, or an aversive substance, respectively (see Rozin and Vollmecke, 1986 and Wadhera et al., 2015a, for reviews).

In addition, mere exposure to flavors may occur in two ways: through substances naturally generated by the mother, such as the amniotic fluid (during the prenatal phase of development) or milk (during weaning), or through industrialized formulas during weaning as well as foods and drinks during later developmental stages at home and at the school.

Food preferences and acceptance -including preferences for and acceptance of vegetables- as well as the eating habits that are established during early childhood have been shown to be reflected at least until young adulthood (Nu et al., 1996; Skinner et al., 2002; Nicklaus et al., 2004; Nicklaus et al., 2005; Pearson et al., 2009: Wadhera et al., 2015b), and thus may be the basis for future eating behavior patterns. For this reason, acquiring knowledge on the effects of mere exposure during early stages of development on vegetable consumption during childhood may be useful finding and/or to improving strategies that promote healthy eating patterns throughout life.

This minireview aims to identify the effects of the exposure-related mechanisms that may determine development of preferences for vegetables in children and therefore, their vegetable acceptance, and presents implications for further research, including a new working hypothesis. This minireview is exclusively focused on studies that include vegetables as targeted stimuli as well as prenatal mere exposure as indirect research method. This is, to the best of our knowledge, the first minireview exclusively focused on the effect of prenatal exposure to vegetable-related flavors on childhood preferences for and acceptance of vegetables.

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2. On how the flavor of the maternal diet is transferred to the amniotic fluid and how the amniotic fluid becomes a transmitter of flavor-related information

A woman's egg that is penetrated by sperm leads to the emergence of the fertilized egg, and thus to pregnancy (Sachdeva et al., 2009). The fertilized egg or also called zygote then begins to divide by successive mitotic cell divisions --two, four, eight, sixteen and thirty-two, etc.- into a growing conglomerate of cells, and it travels the path between the Fallopian tube and the uterus, in which walls it is implanted (Rosenberg and Rosenberg, 2012; Bury et al., 2016; Rohlfs Domínguez, 2016). During approximately the first eight weeks of life it is known as embryo, and from here onwards, it is called as fetus (Jones and López, 2014). When the embryo is already implanted into the wall of uterus, the placenta starts to be formed. The placenta is the biological structure that enables, through the umbilical cord, the transfer of several substances, as many as there are in the mother's blood stream, that is, from O_2, amino acids, fats, vitamins, minerals and hormones to toxic substances, such as drugs or polluting substances, where appropriate, to the embryo's and fetus' blood stream (Chen and Morris, 2007; Sachdeva et al., 2009; Kippler et al., 2010; Veid et al., 2011; Schalkwijk et al., 2016; Penn, 2017). Moreover, small molecules derived from the foods and drinks that are ingested by the mother, and therefore, processed by her digestive system (Sauer, 2010; Fearon, 2014), are also absorbed and transferred to her blood stream, and hence, to that from the fetus (Smith and Lau, 2016).

The food and drinks that are consumed by humans, in general, and pregnant women, in particular, are flavored in nature thanks to the taste and smell molecules that are contained in those foods and drinks. These taste and smell molecules are responsible for the taste and smell qualities, humans perceive. While there are five commonly accepted tastes that can be perceived

-sweet, salty, bitterness, umami and sourness- (Yoshida et al., 2006; Briand and Salles, 2016, Heymann and Ebler, 2017) and an already controversial sixth one -the taste of fat- (see Dransfield, 2008; Besnard et al., 2016 for review), and different transduction mechanisms for these five basic tastes (Medler, 2008), the number of odorants has been estimated to be between half a million (Kringelbach, 2007) and more than one trillion (Bushdid et al., 2014), also with multiple transduction mechanisms for these odorants, although the exact amount of perceived olfactory stimuli remains unknown. Examples of taste molecules are those of sugars (sweet taste), such as sucrose (molecular formula (MF) = $C_{12}H_{22}O_{11}$), fructose (MF = $C_6H_{12}O_6$), glucose (MF = $C_6H_{12}O_6$) and lactose (MF = $C_{12}H_{22}O_{11}$) (Yudkin et al., 1971; Kanou et al., 2017); salt (MF = NaCl) (salty taste) (Kanou et al., 2017), quinine (MF = $C_{20}H_{24}N_2O_2$) (Raheem et al., 2004) and caffeine (MF = $C_8H_{10}N_4O_2$) (Oestreich-Janzen, 2016; Wang et al., 2016), among others (bitter taste); monosodium glutamate (MF = $C_5H_8NO_4Na$) and aspartate (MF = $C_4H_7NO_4$) Haynes (2014) (umami taste) and acids, such as citric (MF = $C_6H_8O_7$), tartaric (MF = $C_4H_6O_6$) or acetic (MF = $C_2H_4O_2$) (Dziezak, 2016) (sour taste). Examples of smell molecules are those of different odorants, such as benzyl acetate (MF = $C_9H_{10}O_2$), eucalyptol ($C_{10}H_{18}O$), eugenol (MF = $C_{10}H_{12}O_2$) limonene (MF = $C_{10}H_{16}$), menthol (MF = $C_{10}H_{20}O$) and vanillin (MF = $C_8H_8O_3$), among others, which generate the strawberry (Api et al., 2015), eucalyptus (Dörsam et al., 2015), honey (Api et al., 2016), lemon (Kim et al., 2013), menthol (Bathia et al., 2008) and vanilla fragrances (Sinha et al., 2008), respectively. These taste and smell molecules are retained during mastication and ingestion (Guichard and Salles, 2016), and they are also retained during digestion. They are, then, transferred to the pregnant woman's blood stream and, therefore, to the fetus' blood stream. They even reach the amniotic sac within which the fetus develops. This

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explains why the amniotic fluid may be flavored, but how does this occur? The key is in the fetal urine. Developing babies regularly urinate into the amniotic fluid. Indeed, Touboul et al., (2008) estimated fetal urine production rates of 7.5, 22.2, 56.1, and 125.1 mL/h at 25, 30, 35, and 40 gestational weeks, respectively. In other words, fetal urine transports maternal diet's smell and taste molecules to the amniotic fluid (Mennella and Beauchamp, 1998). Therefore, the fetus remains surrounded by an amniotic fluid that is flavored by the maternal diet during pregnancy.

We know that maternal diet during pregnancy may flavor the amniotic fluid thanks to a well-designed experiment by Mennella et al., (1995). These authors, by testing samples of amniotic fluid that were collected from women who had to undergo a routine amniocentesis, found that women who had swallowed a capsule that contained garlic extract before the amniocentesis showed a garlic-flavored amniotic fluid, in comparison to women who had swallowed a milk-containing capsule. The examination of the amniotic fluid samples consisted of a group of 13 people having to give their value judgment, in a blind manner, regarding which samples smelled more like garlic. This resulted in the choice of the amniotic fluid samples from the women who ingested the garlic capsule as being the most garlic-like smelling.

Developing babies regularly breath and take amniotic fluid into the lungs and out of the lungs (through the mouth and the nose), and swallow amniotic fluid. Swallowing amniotic fluid regulates the amount of the fluid. If a fetus is not able to swallow because a disease, such as esophageal atresia, that is, lack of continuity in the course of the esophageal tract, or a neurological damage does not let him swallow, this would lead to polyhydramnios, that is, an excess of amniotic fluid in the amniotic sac (personal communication with Dr. Israel Shapiro). The next two figures show these processes:

Because fetuses regularly perform these functions (breathing and swallowing), the tastes and smell molecules contained in the amniotic fluid may stimulate the baby’s taste and smell receptors. Indeed, the taste receptors, that is, the taste buds, emerge by the 7th to 8th week of gestation and are adult-like functional by 13 to 15 gestational weeks, that is, by third gestational trimester (Bradley, 1972; Mennella and Beauchamp, 1998).

Several studies on preterm babies indicate that they can detect some tastes, such as sweet tastes. In one of these studies (Tatzer et al., 1985), it was showed that preterm babies displayed more nonnutritive sucking in response to glucose than water, after having been exclusively fed via gastric tubes and then presented with tiny amounts of either glucose or water solutions intraorally. In another study, the authors (Maone et al., 1990) found that preterm infants, who had been exposed to taste substances in a nipple-shaped gelatin medium, demonstrated more recurrent and robust sucking responses when offered a sucrose-sweetened nipple in comparison to a latex nipple.

3. Evidences of Prenatal exposure to flavors and its effects on children’s preferences for and consumption of vegetables

Studies examining the relationship of these variables are scarce but highly informative. Gustatory and olfactory prenatal experience may positively change infants’ hedonic reaction to vegetables. For example, 5.7 ± 0.2 month-old infants' positive reaction, measured through infants' oro-facial activity, to carrot-flavored cereals increases if their mothers repeatedly consume vegetable-flavored edibles, such as carrot juice during pregnancy, as demonstrated by Mennella et al. (2001). These results are explained by the fact that in uterus-living fetuses are exposed to the flavor (carrot-like flavor) of the chemical stimuli (carrot-related tastes and odors)

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derived from the mother's diet (carrot juice). These flavors are transferred to the amniotic fluid, as we have described in the previous section, and thus perceived by fetuses (Mennella et al., 2001). After all, fetuses' taste and olfactory receptors are stimulated by those chemical stimuli, and are supposedly functional by the third semester (Mennella and Beauchamp, 1998). In a previous study, Schaal et al., (2000) reported that infants that had been prenatally exposed to the anise flavor as a result of their mother's anise consumption during pregnancy, displayed positive facial responses to the anise presentation immediately after birth and on postnatal fourth day, while infants who had not prenatally exposed to anise showed either negative or neutral facial reactions to that stimulus over that period of time. In addition to infants' hedonic reaction, infants' food acceptance patterns may also be influenced by maternal feeding during pregnancy. For example, in an interview-based study (Mennella et al., 2005), it was found that the type of foods accepted by Mexican infants at weaning, including vegetables, correlated with the type of foods their mothers ate during pregnancy. Therefore, maternal vegetable-based dietary during pregnancy may induce positive effects on learning to accept vegetable flavors at infancy. However, when trying to check these effects at early childhood, the findings obtained are complementary rather than similar. Indeed, Ashman et al., (2016) compared the effect of maternal diet --a diet based on fruits and vegetables- during pregnancy, and during their children's early childhood, on young children's acceptance of vegetables in an Australian cohort, placing maternal diet during early childhood as notoriously effective against maternal diet during pregnancy. A previous study by Nicklaus et al., (2014) did not find either an effect of vegetable variety-based mother's diet during pregnancy on children's acceptance of vegetables at 2 years of age. Nevertheless and surprisingly, the targeted positive influence of prenatal exposure may be observable at later childhood (at the age of 8-9 years). In fact, Hepper et al., (2013), showed that children who were in uterus exposed to the garlic flavor during their mothers' pregnancy ate more garlic-flavored potato than compared to non-exposed children. What these authors particularly measured was the weight of the remaining gratin potato to which fresh garlic was added, of which the ration (113 g) was provided to the children along with a beefburger and baked beans at the usual dinner.

The association of prenatal exposure to tastes, smells and flavors with postnatal development of preferences for theses flavors may prepare the developing subjects for living in a postnatal environment where the available flavors are similar to the flavors present in uterus, and thus may be seen as an adaptive mechanism (Nicklaus, 2016). In other words and focusing on vegetables, the association between prenatal exposure to vegetable flavors and postnatal development of preference for these or similar vegetables, as found by Mennella et al., (2001), may serve as an adaptive mechanism that induces developing individuals' to learn to eat the vegetables that are present in their postnatal life-surrounding environment against those that are not present, thus increasing individuals' nutritional survival during postnatal life.

On the other side, the evidences provided by the whole of these studies give rise to the interesting question of why this effect has been shown at some stages of development, such as weaning and late childhood, but it has not been shown at others, such as early childhood, in particular, at the age of 2 years. Given the fact that fresh-cut vegetables differ according to their attributes --color, flavor, texture, etc.- (Barrett et al., 2010), a possible answer to that particular question might be that the prenatal stage of development might be a sensitive period of development of preferences for some types of vegetables, such as those with a particular flavor.

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Future research should test this particular new hypothesis. Doing this would require varying the vegetable type that is consumed by the mother during pregnancy and the timing of exposure (prenatal versus postnatal) as well as testing its effect on children's vegetable intake at 2 years of age by means of a longitudinal experimental paradigm. The following argument helps us to support that particular hypothesis. A recent review by Wadhera et al., (2015a) has identified that postnatal mere exposure may increase liking for non-bitter vegetables in all ages, while associative conditioning is required for improving liking for bitter vegetables in 2-year-old and older children. Therefore, this affirmation suggests that the effectiveness of postnatal exposure in increasing children's vegetable consumption is age-dependent as well as to depend on vegetable type. However, no study has ad hoc varied the vegetable type (bitter versus non-bitter vegetable) that is consumed by the mothers during pregnancy, and thus developing babies are prenatally exposed to, and the timing of exposure (prenatal versus postnatal), and consequently checked the effect of maternal diet during pregnancy on children's vegetable intake at the age of 2 years by means of a longitudinal study. Moreover, and in a subsequent and broader investigative approximation, existence of different sensitive periods of development of preferences for different vegetable types should also be examined in the future. Finally, the sensory systems (olfactory, gustatory and somatosensory systems) directly implicated in flavor perception «are completely anatomically separate, [but] the sensory impression is unitary» (Beauchamp and Mennella, 2011, p. 1). In other words, when we eat or drink any edible, for example, a given vegetable, such as spinach, we do not separately perceive its taste, smell, texture, etc. but perceive a unique and unitary sensation. This reflection becomes more relevant, if possible, if we consider the combination of a vegetable of particular sensorial characteristics or type with other

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vegetables of different sensorial characteristics, in which case the final perceived flavor-related sensation might be different from the case in which a given vegetable is eaten alone. If this is, in fact, the case, we wonder, whether different sensitive periods of development of preferences for different perceived vegetable-related unitary sensations might also exist.

4. Sensitive periods of human gustatory and olfactory development

For all the reasons given in the last paragraph of the previous section, in order to situate the reader in the context of the investigation of sensitive periods of sensory development, we present here a brief review of some of the available evidence for the existence of sensitive periods of development of human sensory functions, with special attention to the gustatory and olfactory systems, which are the focus of this minireview, due to their implication in flavor perception.

Sensitive periods can be defined as developmental stages, during which there is a biological display of an extreme neural sensitivity to the storage of experience-driven sensory information (Rohlfs Domínguez, 2011), such as visual or auditory information. A more rigid version of this term refers to the term *critical period*, this meaning that the extreme neural sensitivity is not present in other time periods (Hensch, 2004; Takesian and Hensch, 2013). Sensory experience during sensitive periods or critical periods of development has relatively greater impact on brain and behavioral development than outside these developmental stages (Voss, 2013).

In comparison to the visual and auditory systems, from which study derives most of the knowledge that we have gained about the impact of sensory experience during sensitive periods of development on brain and behavior (Rohlfs Domínguez, 2014), sensitive periods of development of these two systems have been less studied. Lack of an experimental model to enable the examination of those sensitive periods probably explains this fact. However, there are some indications that point out a long-lasting impact of early learning experiences with odors and tastes on the acquisition of gustatory and olfactory preferences.

Within the gustatory domain, high correlations have been reported (see Trabulsi and Mennella, 2012) between the food preferences acquired during early infancy - 2 to 3 years of age -, and the status of such preferences in later childhood (Skinner et al., 2002), adolescence (Nu et al., 1996) and young adulthood (Nicklaus et al., 2005). Moreover, there is evidence showing high correlations between the vegetable exposure during early childhood -- 2--3 years of age -- and the learned vegetable choices that are made later --at the age of 4 years- (Nickalus et al., 2004). Results of these studies show the influence of vegetable exposure during early childhood on later vegetable choices. In contrast to this demonstrated positive learning-related effect, that the vegetable choices made at early childhood resemble the vegetables that the mother consumed during pregnancy is still to be examined, and therefore, warranted. The same applies to the question of whether the prenatal stage of development is a sensitive period of development of preferences for vegetables. Results from the study by Mennella et al., (2011) are another evidence indicating that early consumption of edibles help to learn to like them. Moreover, these authors ascertained for the first time the time course of a sensitive period for infants to learn to accept an edible, particularly high sour- and bitter-tasting hydrolyzed protein hydrolysate formulas (PHFs) in terms of timing and duration was found. The duration of the formula-based exposure and the type of formula, infants were offered during the exposure, was controlled, and the timing of the PHF-based exposure was varied. All of the infants initially rejected the PHF. It was found that, in contrast to infants aged $\geq$ 3.5 months, infants aged at least 1.5 months but younger than 3.5 months may learn to accept PHFs if they are exposed to this type of formula for ≥ 1 months.

In the case of olfactory preferences, the available evidence points to the perinatal time (beginning 4-37 min after birth) as a sensitive period for postnatal olfactory learning. In fact, Romantshik et al., (2007) found that perinatal exposure to a given odor correlated with higher preferences for that odor in full-term neonates, as they showed their heads more time oriented to this odor than to a novel one or an odorless stimulus. However, if the olfactory exposure was offered later, this olfactory preference response was absent. In previous studies, indications of an association between prenatal exposure to substances and postnatal preferences for the scents of those substances had already been showed. In one of these studies, Marlier et al., (1998) encountered that full-term 2- and 4-month-old and formula-feeding newborns spent more time oriented to the odor of their mother's amniotic fluid than to the fragrance of the formula, they were consuming, and to a control stimulus. Prior to this, they found that the odor of the amniotic fluid as well as the odor of the formula also elicited more positive orientation than if each of both stimuli were simultaneously offered with a control stimulus. In another study, Schaal et al., (1998) found that 3-month-old neonates turned their head more to the odor of their mother's amniotic fluid compared with the odor of a non-familiar amniotic fluid and the scent of a control stimulus. Finally, Faas et al., (2000) found significant higher rates of motor responsiveness (overall body and head and facial activity) in response to ethanol odor in 24- to 48-hour-old infants whose mothers reported to have been frequent drinkers during pregnancy, in comparison to infants whose mothers reported to have been infrequent drinkers during pregnancy, although

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no differences appeared when comparing the motor responsiveness of both infant groups in response to lemon odor, a non-ethanol stimulus.

5. On the targeted new hypothesis

This section and all its subsections are exclusively focused on aspects related to the particular hypothesis suggested in the third section of this work.

5.1. The hypothesis

According to the research question indicated on the last paragraph of the third section of this manuscript, and using a longitudinal intra- and between-subject sample-based experimental design, it should be examined, whether prenatal exposure to vegetables of particular sensory features or type induces increases on children's vegetable intake at early childhood. Our hypothesis, in particular, is: if we repeatedly expose two different groups of developing babies (exposure groups, EGs), by means of maternal ingestion during pregnancy, to two different vegetables, that is, two vegetables that are different in terms of their sensory characteristics (bitter vegetables, such as chard versus non-bitter vegetables, such as carrot), respectively, and afterwards we measure vegetable consumption at the age of 2 years, then we would find different vegetable exposure-related patterns of vegetable intake, thus reflecting different vegetable exposure-related degrees of preference developed for those vegetables. Specifically, the 2-year-old children who were exposed to the non-bitter vegetable (carrot) during their prenatal life would show higher rates of carrot consumption than the 2-year-old children who were exposed to the bitter vegetable (chard) and the control groups (CGs), that is, non-exposure groups and groups postnatally exposed to the targeted vegetables. These differences in the 2-year-old children's exposure-related patterns of preferences developed for the targeted vegetables would give us new insights into the way in which those preferences may develop during early life as well as which strategies are more useful for inducing that increasing vegetable consumption in early childhood (Fig.3).

5.2. Implications of the hypothesis

To the best of our knowledge, testing the present hypothesis would contribute to cover the following important research gaps. (1) The studies that have tried to find an association between an increased vegetable consumption in 2-year-old children and prenatal exposure to vegetables ingested by the mothers during pregnancy are scarce. (2) Those available studies did not take into account the sensory characteristics of vegetables. (3) The methodological designs of those available studies have used cross-sectional and transversal studies, which makes it more difficult to observe intra-subject behavioral changes. (4) Data supporting strategies to increase vegetable consumption in 2-year-old children, that is, in early childhood are scarce.

For all these reasons, we consider that investing research effort in testing the hypothesis described here would contribute, among others, to the following advances in knowledge: (1) A more thorough understanding of how the development of preference for vegetables is developed during early life, specially, of its mechanisms, in particular, prenatal exposure to particular vegetables, such as non-bitter vegetables. (2) To identify, and therefore, predict the existence of a sensitive period for vegetables of particular sensory attributes, that is, non-bitter vegetables. (3) Therefore, generation of new knowledge about sensitive periods of development of preferences for vegetables at behavioral level. (4) New recommendations on the health-related utility of exposing developing babies to vegetables of particular sensory attributes during prenatal stage of development. These recommendations would be specially useful for parents, in general, and mothers, in particular, and could be applied as a strategy to increase 2-year-old children's vegetable consumption.

5.3. Experimental design required

To test the hypothesis, and during a first phase, the two different vegetable-related EGs should be prenatally exposed to either carrot (EG1) or chard (EG2) via maternal vegetable consumption. For this purpose, the daily diet of the mothers of these babies should contain 400 to 660 grams of the targeted vegetable (carrot versus chard). This is the daily amount of vegetables recommended for adults to maintain a good state of health (WHO, 2003; Boeing et al., 2012; Elmadfa and Meyer, 2016). In contrast to the EGs, CG1 and CG2 should not be exposed to either vegetable. Finally, CG3 and CG4 should be postnatally and before 2 years of age exposed to carrot and chard, respectively, instead of prenatally. For this purpose, there are two ways: 1) Breast-feeding regimen, in which case the mothers should intake the same vegetable amount as the mothers of the EGs. Here, the vegetable flavor is transferred to the babies via maternal milk, as several studies have demonstrated (i.e. Mennella and Beauchamp, 1991a; Mennella and Beauchamp, 1991b; Mennella and Beauchamp, 1996; Mennella and Beauchamp, 1999). 2) Bottle-feeding regimen. In this case, juice of the relevant vegetable (carrot juice versus chard juice) can be added to the milk contained in the bottle, depending on the corresponding CG. This regimen type has been commonly used to test the development of flavor preferences as a consequence of exposure to formula-milk-based feeding (i.e. Mennella and Beauchamp, 2002). Time exposure should be controlled across conditions. During the second phase, all groups' total vegetable intake, either carrot either chard intake should be measured at 2 years of age and compared to each other (Table 1).

A similar number of participants should be randomly assigned to each condition. Specific instructions on how to present the vegetables to be eaten by children of all conditions at 2 years of age, that is, at the second study phase, should be given to the adults who are responsible for children' feeding. In particular, it would be desirable for the vegetables to be cooked with a pinch of oil and salt. This is the way in which vegetables have been provided to young children in past studies that have examine strategies that increase young children’s vegetable consumption (i.e. Rohlfs Domínguez et al., 2013). In addition, 50- to 100-grams should be offered to the children. This is the daily amount of vegetables recommended for 0- to 4-year-old children (Gubbels et al., 2014).

Final conclusions and recommendations

We are aware of the importance of good nutrition, that is, a diet rich in vegetables, during early childhood, to avoid childhood health problems, such as childhood obesity as well as health problems in adulthood, such as cancer. For this reason, we aimed to contribute, on the one side, to understand, why young children, in particular, 2-year-old children do not consume satisfactory of vegetable amounts and, on the other side, to search for possible solutions to the problem of reduced vegetable consumption during early childhood. The present work is the result of the effort invested in achieving these two objectives. In view of the evidences here reviewed, we conclude that the effect of the maternal diet during pregnancy on children’s vegetable choice and acceptance does not appear to be linear throughout postnatal life. Some studies have found this effect at weaning and late childhood and in so far eating vegetables during pregnancy is positive to increase infants’ vegetable acceptance and children’s vegetable consumption at both of those stages of development. However, observation of that effect at early childhood, in particular, at

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the age of 2 years, has not yet been found. This is probably due to the fact that the experimental studies that have intended to check the effect of the maternal diet during pregnancy on children's vegetable acceptance at 2 years of age, did not take into account the vegetable type. In other words, it is probably that the vegetables consumed by mothers during pregnancy were not adequate to induce 2-year-old children to satisfactorily intake vegetables. To solve this gap, it is here for the first time recommended to conduct future research to ascertain which vegetable types should be eaten during pregnancy, for children to continue to make vegetable choices when faced with daily meals in early childhood. Moreover, we suggest here that the consumption of non-bitter vegetables, against bitter vegetables, during pregnancy might give rise to the positive effect sought in 2-year-old children's vegetable consumption because the taste of such vegetables lies within the sensitive period that is appropriate to develop preferences for some vegetables. We, therefore, hypothesized that children who are prenatally exposed to non-bitter vegetables would show higher rates of vegetable intake at 2 years of age than children prenatally exposed to bitter vegetables and children postnatally exposed to vegetables and non-exposed children. If this hypothesis is confirmed, we would better understand why children do not eat vegetables at 2 years of age, and we might recommend eating particular vegetables, such as non-bitter vegetables during pregnancy. Confirmation of this hypothesis would additionally give rise to test in the future, if there are different sensitive periods of development of preferences as a function of vegetable type.

Conflict of interest

There is no conflict of interest to declare.

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Table 1 Longitudinal intra- and between-subject sample experimental design for testing the targeted hypothesis.

1° Phase (repeated exposure to vegetable)				**2° Phase (measurement of vegetable intake at 2 years of age)**		
Vegetable consumed group	EGs (during pregnancy)	CG1 and CG2 (no exposure during pregnancy)	CG3 and CG4 (postnatal exposure before 2 years of age)	EGs	CG1 and CG2	CG3 and CG4
Non-bitter (carrot)	+EG1	- CG1	+ CG3	+EG1	+CG1	+ CG3
Bitter (chard)	+EG2	- CG2	+ CG4	+EG2	+CG2	+ CG4

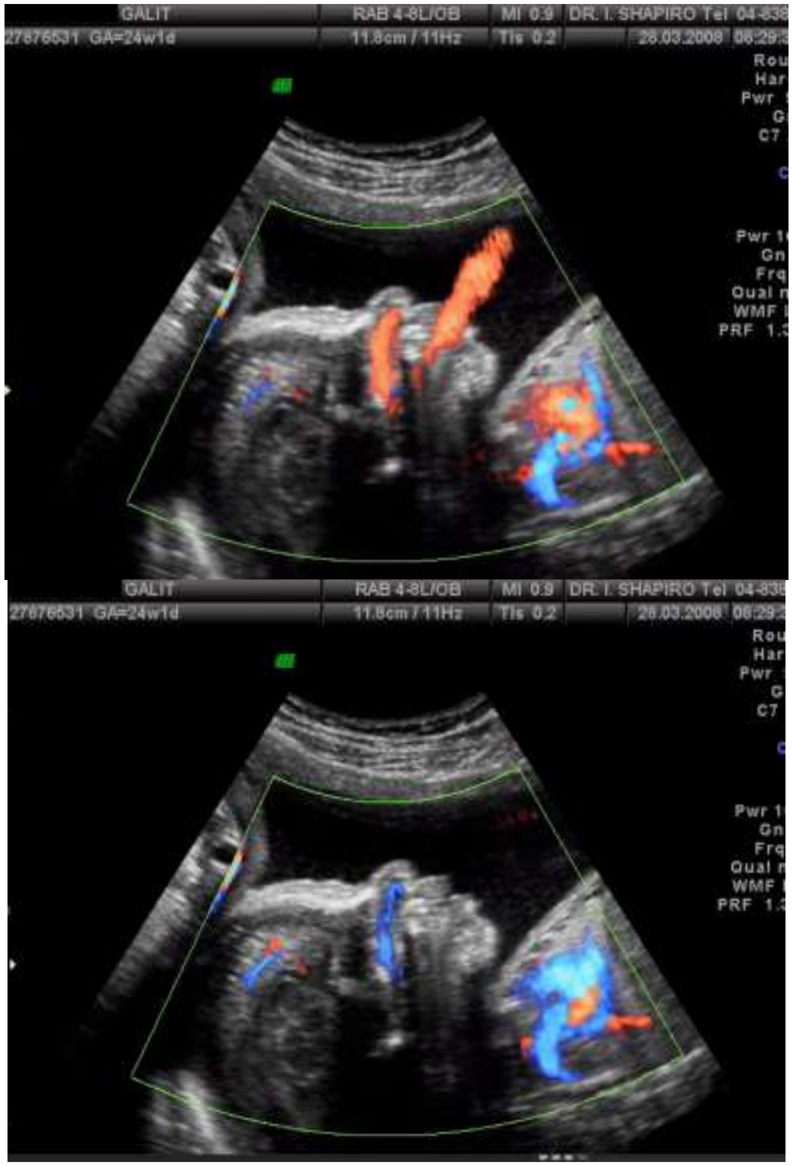

Figure 1: Breathing fluid out (in red color) and in (in blue color) from mouth and nose from a 24 gestational weeks old fetus (courtesy by Dr. Israel Shapiro, from the Department of Obstetrics and Gynecology, Bnai-Zion Medical Center, Haifa, Israel). The red and blue colors are inherent in all ultrasound apparatus that have a Doppler capability. Red is movement toward the transducer and blue is movement way from the transducer. Click here to watch the video of fetal breathing in color Doppler. (Press Ctrl + left mouse)

Figure 2: Stool in the stomach (conglomerate see on the bottom left of the image) from a 15 gestational weeks old fetus (courtesy by Dr. Israel Shapiro, from the Department of Obstetrics and Gynecology, Bnai-Zion Medical Center, Haifa, Israel). Stool particles are to see particles in the amniotic fluid. Sometimes they are also swallowed by the fetus, and then we can see the stool in the stomach (personal communication with Dr. Shapiro). Click here to watch the video of stool in fetal stomach in 2D ultrasound. (Press Ctrl + left mouse)

<u>1. What we know</u>

a) Prenatal exposure to vegetables may lead learning to like and thus to eat vegetables. This effect has been observed at weaning and late childhood, but it has not been observed at early childhood.
b) The effectiveness of postnatal exposure in increasing children's vegetable intake seems to be age-dependent as well as depend on vegetable type.

<u>2. What we do not know</u>

a) Whether the positive effect of prenatal exposure to vegetables on postnatal vegetable consumption (at 2 years of age) depends on vegetable type.
b) Whether the prenatal stage of development might be a sensitive period of development of preferences for some types of vegetables.

<u>3. Hypothesis</u>

Children who have been prenatally exposed to non-bitter vegetables would show higher rates of vegetable intake at 2 years of age than children who have been prenatally exposed to bitter vegetables and children that have been postnatally (before 2 years of age) exposed to vegetables and non-exposed children.

Fig.3. Graphic representation of the logic reasoning process followed to reach our hypothesis. This begins with the knowledge, we already have (1), from what we can then deduce what we still do not know (2), giving rise to the new working hypothesis (3).