Does the nose know?

Physiological and behavioural responses upon food odours

Paulina Morquecho Campos

Propositions

1. The nose knows, but the brain is the one taking the final food choice.
   (this thesis)

2. Our sense of smell is not well-adapted to identify single macronutrients in our complex food environment.
   (this thesis)

3. Medical Ethical Committees unethically expect foreign staff to be proficient in Dutch.

4. Universities should compensate self-funded PhD students who earn less than the national minimum wage.

5. Gender stereotypes act as a road block in the pursuit of higher positions in STEM fields.

6. Preserving native dialects or languages is, at least, as important as learning English.

7. Rewarding healthy lifestyles should be more effective than penalizing unhealthy ones.

8. It is better to sail waving a fool's flag. (My father's saying, that we should be more humble and not act all-knowing: "Más vale navegar con bandera de pendejo")

Propositions belonging to the PhD thesis entitled:
Does the nose know? Physiological and behavioural responses upon food odours

Paulina Morquecho Campos
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Thesis

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A mi mamá, papá y hermana por ser mi pilar
y mi mayor inspiración en esta aventura.
Los amo

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Chapter 1

General introduction

Background

Eating is a behavioural process that might be influenced by our physical and social environment (Bellisle, 2003). Nowadays, we are living in an obesogenic environment full of sensory food cues that constantly tempt us to overeat (Bellisle, 2003; Boesveldt & de Graaf, 2017; King, 2013). Sensory food cues such as odours may play a crucial role in eating behaviour by detecting and attracting our attention towards food. For instance, imagine yourself walking in the main street of your own town and suddenly the smell of freshly baked chocolate cake catches your attention. The exposure to that food odour could induce physiological and behavioural responses such as salivation secretion (Mattes, 2000) and an increase of appetite for food with similar characteristics, which is known as *sensory-specific appetite* (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Lakemond, van Boekel, & Luning, 2014; Zoon, de Graaf, & Boesveldt, 2016). Sensory-specific appetite may be generalized across foods within taste and energy-density categories (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Gort, et al., 2014; Ramaekers, Boesveldt, Lakemond, et al., 2014; Zoon et al., 2016). For example, our appetite for sweet and high-calorie food products (but not for savoury and low-calorie products) increases upon smelling the freshly baked chocolate cake. This may infer that food odours may signal information related to the composition of the food, such as taste or even macronutrient content (Boesveldt & de Graaf, 2017; McCrickerd & Forde, 2016; Ramaekers, Boesveldt, Gort, et al., 2014; Smeets, Erkner, & de Graaf, 2010; Zoon et al., 2016). However, the effect of specific food odours on subsequent physiological and behavioural responses, and the conditions under which they exert them, are still not fully understood. Therefore, the aim of the research described in this thesis was to investigate the role of food odours on physiological (by means of salivation) and eating behaviour responses (self-reported appetite ratings, food preferences, choice, and intake).

Eating behaviour

Eating behaviour is a learned process, which involves a cascade of responses from hunger to satiety, and is influenced by metabolic and sensory factors (Blundell et al., 2010; de Graaf & Kok, 2010). Eating behaviour includes appetite, food preferences, food choice, food intake, satiation (within a meal), and satiety (between meals).

On one hand, metabolic signals may determine how often we eat and how much, and are responsible for energy balance, drive to eat, and adequate energy ingestion (Blundell et al., 2010; de Graaf & Kok, 2010). Neural and hormonal processes (i.e. the sensation of fullness, release of gut hormones, etc.) are involved in these metabolic signals. Metabolic consequences are linked in the brain to sensory signals during eating (de Graaf & Kok, 2010).

On the other hand, sensory factors – as visual and odour cues – may determine what we eat and how much, and are responsible for variety in our diet (Blundell et al., 2010; de Graaf & Kok, 2010). Also, they are crucial drivers of (dis)likes, food preferences, and food choice, and may play a functional role in signalling nutrient content (Brunstrom, 2007; McCrickerd & Forde, 2016; Sclafani, 1997). Through repeated exposure which leads to learned conditioning (Pavlovian conditioning), food cues may act as a cue (conditioned stimulus) that informs us about the post-ingestive consequences of the foods composition (unconditioned stimulus) (Brunstrom, 2005, 2007; Sclafani, 1997; Yeomans, 2006b). For example, we can determine the satiation of a meal via expectations and previous encounters, while planning that meal (Fay et al., 2011).

The main measures to investigate eating behaviour are:

- **Appetite:** the internal driver to search, choose, and ingest food (de Graaf, Blom, Smeets, Stafleu, & Hendriks, 2004). Appetite feelings can be induced by metabolic signals and by external sensory cues (de Graaf et al., 2004; Egecioglu et al., 2011).
- **Food preferences:** the choice of one food over others (Rozin & Vollmecke, 1986) taking into account intrinsic and extrinsic factors such as liking, healthiness, value, convenience, context (Mela, 2006).
- **Food choice:** the decision to select and consume a food product (Sobal, Bisogni, Devine, & Jastran, 2006)**.**
- **Food intake:** the amount of a specific food consumed in a particular context (de Graaf et al., 2004).

One of the greatest challenges is to understand eating behaviour through these available measures. Most of the research done involves explicit measures, i.e. self-reported appetite ratings (Blundell et al., 2010). Nevertheless, the use of implicit measures could be useful to detect more unconscious and spontaneous eating

responses (De Houwer & Moors, 2010; Hofmann, Gawronski, Gschwendner, Le, & Schmitt, 2005). On one hand, explicit (and conscious) exposure to food cues may mainly influence explicit (and conscious) measures such as self-reported appetite. While implicit (and unconscious) exposure may influence implicit measures such as food preference and intake (Boesveldt & de Graaf, 2017). Decision-making and choice may be mediated by visual attention (Orquin & Mueller Loose, 2013).

The sense of smell

The human sense of smell has long been falsely underestimated; however, its relevance is now gaining higher importance (McGann, 2017). Humans are estimated to be able to discriminate between more than one trillion olfactory stimuli (Bushdid, Magnasco, Vosshall, & Keller, 2014). Human olfaction has three major functions: avoiding environmental hazards, social communication, and ingestion (Stevenson, 2010).

Olfaction is a dual sensory modality that can be perceived through two different routes: orthonasal and retronasal (Rozin, 1982). Both routes contribute in complementary manners to ingesting behaviour. Orthonasal olfaction can detect volatile objects from the external environment through the nose, while retronasal olfaction senses objects from the inside of the body: via the oral cavity through the nasopharynx during eating and drinking (Rozin, 1982; Small & Green, 2012). Focusing on orthonasal olfaction, it plays a crucial role in anticipation of food such as food detection and appetite (Boesveldt & de Graaf, 2017; Small, Gerber, Mak, & Hummel, 2005; Small & Green, 2012). Importantly, beyond the nose, the olfactory bulb is considered the first central relay of the olfactory pathway, where information is integrated in glomeruli and plays an important role in odour processing (Landis, Hummel, & Lacroix, 2005; Yeshurun & Sobel, 2010). Subsequently, the signal is transferred to several brain areas: the piriform cortex is involved in odour identification, attention, memory, and valence of the odours (Gottfried, Smith, Rugg, & Dolan, 2004; Lundström, Boesveldt, & Albrecht, 2011; Zelano et al., 2005; Zelano, Montag, Johnson, Khan, & Sobel, 2007); the amygdala is involved in emotion, motivation, and cravings; and the orbitofrontal cortex is important for cognitive odour processing, evaluation, and multisensory integration and processing (Landis et al., 2005; Lundström et al., 2011; Rolls & Baylis, 1994).

Furthermore, olfaction may influence metabolic processes, playing an important role in appetite, meal initiation, and regulation (Palouzier-Paulignan et al., 2012; Stevenson, 2010; Yeomans, 2006a). As mentioned before, exposure to food cues, such as visual and odour stimuli, can increase appetite for foods with similar properties and decrease appetite for foods with dissimilar properties; the term *sensory-specific appetite* (SSA) has been coined to describe this (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Lakemond, et al., 2014; Zoon et al., 2016).

SSA has been shown within taste categories – exposure of savoury odours enhance appetite for savoury foods while reduce appetite for sweet foods, and *vice versa* (Ramaekers, Boesveldt, Lakemond, et al., 2014; Zoon et al., 2016) – and within energy-density categories – high-calorie-dense odours increase appetite for high-calorie-dense food while decrease appetite for low-calorie-dense food, and *vice versa* (Zoon et al., 2016). This may infer that food odour cues may transmit crucial information related to the macronutrient content of the associated food and thus influence specific appetite and even food choice and actual intake (H. Berthoud, Münzberg, Richards, & Morrison, 2012; Boesveldt & de Graaf, 2017; McCrickerd & Forde, 2016; Ramaekers, Boesveldt, Lakemond, et al., 2014; Smeets et al., 2010; Zoon et al., 2016). However, there is still uncertainty regarding how and under what circumstances food odours that signal specific information (such as taste or macronutrient content) may impact various measures of eating behaviour.

The role of odour awareness in eating behaviour

The level of awareness of food odours may exert different eating responses (McCrickerd & Forde, 2016; Smeets & Dijksterhuis, 2014). *Conscious* odour exposure may influence self-reported appetite (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Gort, et al., 2014), but not food preference and intake (Zoon, He, de Wijk, de Graaf, & Boesveldt, 2014). The increase in appetite feelings upon aware (or conscious) food odour exposure could be due to a learned Pavlovian response to an appetitive stimulus. In turn this may activate cognitive control systems (such as health goals and self-regulation) which may disrupt the eating process in the appetite stage, so that it does not continue into food choice and intake (Boesveldt & de Graaf, 2017).

For example, our appetite for high-calorie and sweet foods may be triggered by smelling that freshly baked chocolate cake from the bakery; however, whether or not we enter the bakery shop and buy something may depend on activation of our cognition control system ('*Wait! You already ate a piece of cheesecake during the coffee break. Hmmm ok! I will not buy anything then*'). On the contrary, when we are not aware of the smell (and thus our appetite), this cognitive control might not be activated, and we then will buy and consume the food. Some studies suggested that *non-conscious* and low-intense odour exposure could lead towards selecting a congruent food (Chambaron, Chisin, Chabanet, Issanchou, & Brand, 2015; de Wijk & Zijlstra, 2012; Gaillet-Torrent, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2014; Gaillet, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2013). This unconscious odour exposure may trigger an undisturbed food decision, outside of cognitive control (Köster, 2009; Smeets & Dijksterhuis, 2014). Proserpio and collaborators have shown that *non-conscious* and mild intense odour exposure may also influence food intake (Proserpio, de Graaf, Laureati, Pagliarini, & Boesveldt, 2017). Sub-threshold and unattended odours could thus act as prime, thereby unconsciously influencing cognitive and behavioural responses (Gaillet-

Torrent et al., 2014; Gaillet et al., 2013). Therefore, the exposure of this prime leads towards a certain food choice and potentially intake related to the target odour (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Smeets & Dijksterhuis, 2014; Tulving & Schacter, 1990). However, the impact of odour awareness on subsequent eating responses is not fully understood.

Macronutrients and their digestive routes

The food that we choose and eat consists mainly of a mix of macronutrients. Macronutrients – carbohydrates, protein, and fats – are fundamental to preserve the structure and functions of the body, providing it with the needed energy (Hofheins, 2007). Each macronutrient has exclusive chemical and physical properties, nutritional value, and function, and are present in specific foods (Hofheins, 2007; Velíšek, 2014).

Carbohydrates are based on monosaccharides connected to each other by glycosidic linkages to form di-, oligo-, and polysaccharides. Its conformation depends on the type of linkages and number of monosaccharides bonded together, offering a large variety of chemical and physical properties (Belitz, Grosch, & Schieberle, 2009; Damodaran, Parkin, & Fennema, 2007). They are the energy fuel of the body, essential to the brain. The main sources of carbohydrates are fruits, vegetables, and grains (Hofheins, 2007). Digestion of carbohydrates starts in the oral cavity with the activity of the salivary α-amylase (Bender, 2014). The salivary α-amylase is an endoglycohydrolase secreted by the parotid gland that breaks the α1→4 glycosidic bonds of polysaccharides into maltose, dextrin, and oligosaccharides (Levine, 2010; Mattes, 2000; Robyt & French, 1970). Some studies have shown a higher release of α-amylase from the parotid gland after a sucrose solution compared to other taste solutions (starch > sodium chloride > citric acid) (Froehlich, Pangborn, & Whitaker, 1987) and after mastication of bread (high-in-starch food) compared to other products (non-food products as parafilm and low-in-starch food as celery) (Mackie & Pangborn, 1990). This suggests that salivary α-amylase may specifically facilitate the digestion of carbohydrates. Furthermore, pancreatic amylase is secreted into the small intestine to continue the digestion of the starch. Hydrolysis of the disaccharides occurs in the brush border of the intestinal mucosa by disaccharidases (Bender, 2014). Additionally, an increase of the hormone insulin has been shown upon modified sham feeding of food rich in carbohydrates compared to sweet-tasting solutions such as aspartame, saccharin, and sucrose (Teff, Devine, & Engelman, 1995), or to food rich in other macronutrients (Zhu, Hsu, & Hollis, 2014).

The building blocks of **proteins** are amino acids bonded through amide linkages. Depending on the amino acid sequence and interactions, the structure of the proteins could vary (primary to quaternary) and this determines its functional properties (Belitz et al., 2009; Brady, 2013; Turgeon & Rioux, 2011; Velíšek, 2014).

Proteins have several functions in the body: source of nitrogen, energy, and essential amino acids; production of antibodies and enzymes; components of hormones and transport proteins; and a source of amino acids for reparation and growth of muscles, bones, skin, and other tissues (Hofheins, 2007; Velíšek, 2014). Proteins are mainly available in milk, meat, eggs, cereals, legumes, and seeds (Belitz et al., 2009; Damodaran et al., 2007; Hofheins, 2007). The digestion of protein starts at the stomach level, where the gastric acid decomposes the structure of the proteins and the enzyme pepsin hydrolyses them. Then, exo- and endopeptidases, secreted by the pancreas and small-intestinal mucosa, and di- and tripeptides in the intestinal mucosa brush border, hydrolyse proteins into amino acids in the small intestine (Bender, 2014). Besides, it has been shown that some hormones also play a role in the digestion of proteins. The secretion of ghrelin, a gastrointestinal hormone, significantly increased upon modified sham feeding of a food rich in protein compared to foods rich in other macronutrients (Zhu et al., 2014). Also, the hormone pancreatic polypeptide may increase upon modified sham feeding and actual meal ingestion of protein-rich foods (Witteman et al., 1994) and fat (Crystal & Teff, 2006; Simonian, Kresge, Boden, & Parkman, 2005; Witteman et al., 1994) compared to food rich in carbohydrates or a fasting state.

Fats are compounds with fatty acids as building blocks (Belitz et al., 2009; Hofheins, 2007; Velíšek, 2014). The main characteristic of fats is their hydrophobicity (Belitz et al., 2009; Velíšek, 2014). The functions of fat are: source of essential fatty acids and vitamins; formation of cell membranes; transmission of nerve signals for muscle contraction; and protection of vital organs (Hofheins, 2007; Velíšek, 2014). Fat can be classified in saturated, monounsaturated, and polyunsaturated depending on the absence or presence of one or more C–C double bonds, respectively (Hofheins, 2007; Velíšek, 2014). Therefore, fats or lipids can be found in two different physical states at room temperature, referred to as oils in a liquid state and fats in a solid state (Damodaran et al., 2007). Some of the main sources of fats are oils, butter/margarine, fatty fish (such as herring or sardines), and nuts (Hofheins, 2007). It has been speculated that the digestion of fats starts in the oral cavity with the presence of lingual lipase, but only to a minimal degree (Mattes, 2000; Schiffman, Graham, Sattely-Miller, & Warwick, 1998; Spielman, D'Abundo, Field, & Schmale, 1993). Lipase breaks down triglycerides into free fatty acids (Kupirovič et al., 2017; Mese & Matsuo, 2007; Pedersen, Sørensen, Proctor, & Carpenter, 2018). However, fats are hydrolysed in the stomach by gastric lipase and in the small intestine by lipase, phospholipase, and esterase, which are pancreatic enzymes. Fats should be emulsified to micelles – very small droplets – to be absorbed. The emulsification occurs by the hydrolysis of triacylglycerol to mono- and diacylglycerols and free fatty acids and with the addition of bile salts, which coat fat droplets to help create a stable emulsion (Bender, 2014).

Finally, the absorption of the products of digestion – monosaccharides, amino acids, fatty acids, and glycerol – occurs in the small intestine. The undigested macronutrients are metabolized by bacteria in the large intestine (Bender, 2014).

The (macro)nutrient content on our food may be signalled by the sensory properties of the food such as smell – as previously discussed – or taste (Rozin & Vollmecke, 1986; Viskaal van Dongen, van den Berg, Vink, Kok, & de Graaf, 2012). For instance, taste is closely associated with nutrient sensing: sweet products like fruit juices or biscuits contain high levels of carbohydrates; savoury and salty foods such as meat or cheese are high in protein and sodium, respectively; and fatty sensations are related to foods like white chocolate or butter with a high fat content (Lease, Hendrie, Poelman, Delahunty, & Cox, 2016; Teo et al., 2018; van Langeveld et al., 2017; Viskaal van Dongen et al., 2012). The sensory properties of the food may act as a nutrient sensor and could generate certain expectations about the macronutrient content of the food. This may subsequently induce specific (anticipatory) physiological – such as cephalic-phase responses – and behavioural responses as appetite and food intake (Mattes, 1997; McCrickerd & Forde, 2016; Nederkoorn, Smulders, & Jansen, 2000; Power & Schulkin, 2008; Viskaal van Dongen et al., 2012).

Cephalic-phase responses

Cephalic-phase responses (CPR) are a cascade of anticipatory physiologic responses induced by sensory food cues such as sight, smell, and taste, or even with the thought of food (Mattes, 1997; Nederkoorn et al., 2000; Smeets et al., 2010). CPR were first described by Pavlov in his famous studies with dogs where he demonstrated that salivation flow and gastric secretions could be triggered by mere sensory cues (Pavlov, 1904; Power & Schulkin, 2008; Smith, 2000). CPR are mediated by the activation of the vagus nerve, and consequently autonomic responses. These responses prepare the body for digestion, absorption, and metabolism of the ingested food towards the maintenance of homeostasis (Mattes, 1997; Power & Schulkin, 2008; Smeets et al., 2010; Zafra, Molina, & Puerto, 2006). CPR can be linked to certain expectations based on learning and memory of post-ingestive or rewarding consequences through Pavlovian conditioning (Berthoud, 2007; Davidson, Sample, & Swithers, 2014; Nederkoorn et al., 2000; Woods & Ramsay, 2000). CPR involve a series of hormonal, metabolic, and enzymatic responses such as gastric activity, release of gut hormones as ghrelin, and saliva secretion (Mattes, 1997; Nederkoorn et al., 2000; Smeets et al., 2010). The latter is known as cephalic-phase salivary response (Mattes, 2000).

Cephalic-phase salivary responses

Saliva secretion is a non-invasive technique to measure CPR known also as cephalic-phase salivary response (Klajner, Herman, Polivy, & Chhabra, 1981; Mattes, 2000). Cephalic-phase salivary response is the release of saliva produced upon cognitive and/or sensory cues (Mattes, 2000).

Saliva is a non-Newtonian fluid, i.e. the viscosity of saliva decreases upon increasing force or stress. It is composed mainly of water (99%) and 1% of solids which consist of some organic (proteins such as glycoproteins, enzymes, immunoglobulins, and peptides) and inorganic (electrolytes such as sodium chloride and sodium bicarbonate) compounds (Levine, 2010; Mese & Matsuo, 2007; Pedersen et al., 2018; Schipper, Silletti, & Vingerhoeds, 2007). The main functions of saliva are: lubrication in the mouth, oesophagus, and food by the action of proteins as mucins and statherins; bolus formation and initiation of digestive processes through salivary enzymes action and mucins; digestion of starch (α-amylase) and lipids (lingual lipase); buffer action by the action of bicarbonate, phosphate, urea, some proteins, and enzymes; antimicrobial activity through proteins and peptides; and assistance of speech, mastication, and swallowing (Carpenter, 2013; Dawes et al., 2015; Humphrey & Williamson, 2001; Levine, 2010; Mattes, 2000; Mese & Matsuo, 2007; Pedersen et al., 2018).

Saliva is produced by three paired major glands – parotid, submandibular, and sublingual – and several minor glands in the mucosa of the tongue (Von Ebner glands), cheeks, lips, palate, and pharynx (Mese & Matsuo, 2007; Schipper et al., 2007). Salivation is regulated by some reflex pathways that consist of afferent activation by sensory stimuli, a connection centre (salivation centre), and an efferent part. The latter is based on parasympathetic and sympathetic autonomic nerve fibres which innervate the salivary glands (Mese & Matsuo, 2007; Pedersen, Bardow, Jensen, & Nauntofte, 2002). The secretion of water and electrolytes are released through the activation of the parasympathetic nerve, while secretion of proteins is triggered by the sympathetic nerve (Carpenter, 2013; Humphrey & Williamson, 2001; Mese & Matsuo, 2007).

Different factors such as type and duration of stimulation, dietary patterns, type and size of salivary glands, circadian rhythms, etc. may influence the salivary flow rate and composition of the saliva (Pedersen et al., 2002; Schipper et al., 2007; Wolff et al., 2017). This may also depend on the nature of the stimulus which will lead to specific gland stimulation and secretion of different compounds (Davies, Wantling, & Stokes, 2009; Engelen, de Wijk, Prinz, van der Bilt, & Bosman, 2003; Guinard, Zoumas-Morse, & Walchak, 1998; Stokes & Davies, 2007). Each gland contributes with specific components towards giving unique properties to the secreted saliva:

1) **Parotid glands** are the largest ones and produce a watery and thin saliva (Mese & Matsuo, 2007; Pedersen et al., 2018). The saliva secreted by parotid glands is characterized by being rich in α-amylase, electrolytes, and proteins, mainly proline-rich proteins (Carpenter, 2013). It contributes to 20% of the total flow under unstimulated conditions; however, under stimulated conditions it could contribute to 45% or >50% (produced by acid citric and mechanical stimulation, respectively) of the total flow (Aps & Martens, 2005; Humphrey & Williamson, 2001).
2) **Submandibular and sublingual glands** produce a thick, serous, viscous, and mucin-rich saliva (Carpenter, 2013; Mese & Matsuo, 2007; Pedersen et al., 2018; Stokes & Davies, 2007). Mucins are high molecular weight glycoproteins that have an impact on the visco-elasticity of saliva (Bansil & Turner, 2006; Davies et al., 2009; Stokes & Davies, 2007). MUC5B is the highest molecular weight mucin and the predominant one in saliva, which is responsible for its gelling properties (Bansil & Turner, 2006; Schipper et al., 2007; Thornton et al., 1999). Submandibular glands contribute to 65% of the total flow under unstimulated conditions, and 33% to 45% under stimulated conditions (produced by mechanical and acid citric stimulation, respectively) (Aps & Martens, 2005; Humphrey & Williamson, 2001). In contrast, sublingual glands only produce 7–8% of the unstimulated saliva and 1–2% of the stimulated saliva (Humphrey & Williamson, 2001; Levine, 2010; Pedersen et al., 2018).
3) **Minor glands** produce less than 10% of the total flow. These glands secrete mucin-rich saliva. Contrary to the major glands, their secretion is not related to stimulation (Carpenter, 2013; Humphrey & Williamson, 2001; Pedersen et al., 2018).

Although it is well known that taste and chewing are good sialagogues, other sensory cues may also trigger salivary responses (Mattes, 2000; Spence, 2011). Following the findings from Pavlov, food cues such as sight and smell may act as a conditioned stimuli and produce anticipatory salivation as a conditioned response (Jansen, Boon, Nauta, & van den Hout, 1992). Several researchers have shown that the odour exposure of (palatable) foods could enhance salivary flow (Ferriday & Brunstrom, 2011; Kerr, 1961; Klajner et al., 1981; Lashley, 1916a; Pangborn, 1968; Pangborn & Berggren, 1973; Pangborn, Witherly, & Jones, 1979; Rogers & Hill, 1989; Sahakian, Lean, Robbins, & James, 1981; Shannon, 1974; Wooley & Wooley, 1973). Morevoer, a series of work from Lee and Linden showed that olfactory cues stimulate saliva exclusively from submandibular and sublingual glands, and not from parotid glands (Lee & Linden, 1991, 1992a, 1992b). A recent study inferred that the mere exposure to food odours could enhance whole saliva secretion compared to the control condition (Proserpio et al., 2017). Very little is currently known about the influence of food odours (signaling specific food properties) on salivary responses in terms of amount and composition.

Aim and thesis outline

Exposure to food odours impacts our eating behaviour. However, the influence of food odours that signal specific information such as (macro)nutrient content, is not yet clear. Therefore, the overall aim of this thesis was to investigate the role of specific food odours on physiological and behavioural responses and to disentangle the role of odour awareness on eating responses. To understand this aim, two main research questions were defined (**Figure 1.1**):

1. To what extent can food odours (and other sensory food cues) trigger specific cephalic-phase salivary responses? **(Chapters 2 and 3).**
2. How does the level of awareness of food odours influence specific eating behaviour responses (appetite, preference, choice, and intake)? **(Chapters 4–6).**

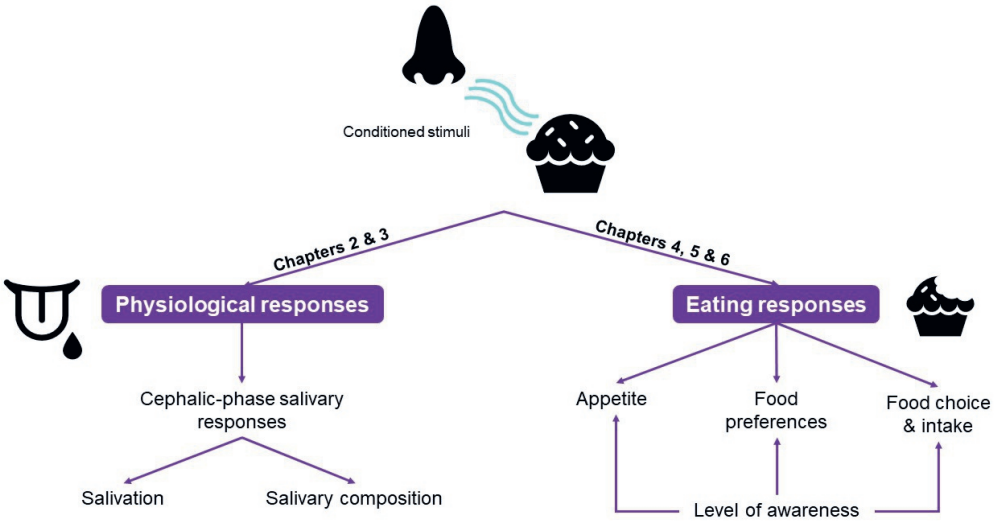

Figure 1.1 Schematic overview of this thesis.

We first examined whether and how food odours that signal specific food properties such as taste qualities and macronutrient content influence saliva secretion and its composition **(Chapter 2)**. Following up on this, we investigated how (multi)sensory cues (from solely odour exposure up to mastication, which includes visual, odour, taste, and chewing sensory information) influence saliva secretion and its composition **(Chapter 3)**. Next, behavioural studies were performed to understand the impact of specific odour exposure and odour awareness on eating responses **(Chapter 4–6)**. Firstly, we examined how conscious exposure to macronutrient-related odours influences various eating behaviour responses (appetite, food

preference, and food intake) **(Chapter 4)**. Secondly, we examined how non-conscious exposure of macronutrient-related odours impacts the same eating behaviour responses **(Chapter 5)**. Then, we determined how non-conscious exposure to odours that signal specific taste qualities affect snack selection, and whether this is modulated by visual attention **(Chapter 6)**. In the final chapter of this thesis **(Chapter 7)**, the main findings of all studies are summarized, interpreted, and discussed. Also, methodological considerations are discussed and implications of the findings and recommendations for further research are given.

The results of these studies can shed new light on how and to what extent food odours influence our eating responses. This knowledge could help us to steer people towards healthier food choices.

Chapter 2

Impact of food odours signaling specific taste qualities and macronutrient content on saliva secretion and composition

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Abstract

Olfactory food cues can induce appetite for similar food products in humans. Odours may thus signal essential information about a food's composition such as taste or even macronutrient content and may stimulate specific physiological responses in anticipation of food intake. Several studies have shown that sensory food cues could stimulate saliva secretion. However, potential differences between food odours in their effect on saliva secretion, or the effects of olfactory stimulation on changes in saliva composition remain to be elucidated. To gain more insight, we conducted two studies to determine the influence of various odours, representing different taste qualities (study 1) and macronutrients (study 2), on salivary biomarkers. In study 1, 36 participants were randomly exposed to no-odour, non-food, and odours signalling sweet, savoury, and sour tastes. In study 2, 60 participants were randomly exposed to no-odour, non-food, and odours signalling carbohydrates, protein, fat, and low-calorie food. For each condition, whole mouth saliva was collected and saliva secretion rate determined. Furthermore, we determined mouth-watering perception (subjective salivation), visco-elasticity (study 1 only), mucin concentration, α-amylase, and lingual lipase activity (study 2 only). For both studies, linear mixed model analyses showed that saliva secretion rate significantly increased by food odour exposure compared to no-odour and non-food conditions. However, no changes in salivary composition were observed. These findings indicate that food odours play a crucial role in anticipatory saliva responses and can thereby affect subsequent eating behaviour.

Introduction

Olfaction plays a crucial role in eating behaviour (Boesveldt & de Graaf, 2017; Stevenson, 2010). Food odour exposure appears to increase appetite for products similar in taste and calorie density (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Lakemond, van Boekel, & Luning, 2014; Zoon, de Graaf, & Boesveldt, 2016). For example, smelling a chocolate increases the appetite for sweet or high-calorie products rather than appetite for savoury or low-calorie products (Zoon et al., 2016). This suggests that humans may detect information related to the composition of a food, such as taste or even macronutrient content, via odours (Boesveldt & de Graaf, 2017; McCrickerd & Forde, 2016; Ramaekers et al., 2014; Smeets, Erkner, & de Graaf, 2010; Zoon et al., 2016). Based on repeated food exposures throughout life, the odours of the respective foods could become conditioned stimuli. Exposure to these conditioned stimuli (i.e. food odours, such as chocolate odour) may then trigger specific anticipatory responses of the body to facilitate subsequent ingestion and digestion of the associated food (Christensen & Navazesh, 1984; Jansen, Boon, Nauta, & van den Hout, 1992; Mattes, 2000; Spence, 2011).

Saliva secretion is part of this cascade of anticipatory physiological responses to maintain body homeostasis, which are collectively known as cephalic-phase responses (Mattes, 1997; Nederkoorn, Smulders, & Jansen, 2000; Pavlov, 1904; Smeets et al., 2010). Several classic, as well as more recent studies, have demonstrated an increase in saliva secretion upon multisensory exposure to various foods (Ferriday & Brunstrom, 2011; Kerr, 1961; Klajner, Herman, Polivy, & Chhabra, 1981; Pangborn, 1968; Pangborn, Witherly, & Jones, 1979; Rogers & Hill, 1989; Sahakian, Lean, Robbins, & James, 1981; Shannon, 1974; Wooley & Wooley, 1973). In addition, food odours *per se* have also been shown to increase saliva responses compared to no-odour control conditions (Ilangakoon & Carpenter, 2011; Lee & Linden, 1992a; Neyraud, Septier, Tournier, & Chambaron, 2015; Proserpio, de Graaf, Laureati, Pagliarini, & Boesveldt, 2017). However, it is yet not clear whether differences between food odours exist, in that specific properties of a food (odour) may alter the overall salivary response.

Besides saliva secretion itself, saliva composition may be altered during this anticipatory, cephalic phase, and serve different functions (Levine, 2010; Pedersen, Sørensen, Proctor, & Carpenter, 2018). One of most versatile components in saliva is MUC5B, a highly glycosylated salivary mucin. MUC5B is fundamental to lubrication of the mouth, bolus formation, speech, and swallowing. Due to its high hygroscopic capacity, the presence of MUC5B strongly impacts salivary visco-elasticity (Humphrey & Williamson, 2001; Levine, 2010; Mese & Matsuo, 2007; Thornton et al., 1999). The viscosity of unstimulated saliva may be 2–3 times higher compared to that of stimulated saliva (Mese & Matsuo, 2007; Rantonen & Meurman, 1998). Furthermore, salivary enzymes such as α-amylase and lingual lipase may

play an initial role in the digestion of food specific compounds such as carbohydrates and fat, respectively (Mattes, 2000). α-amylase is involved in the hydrolysis of polysaccharides (Levine, 2010; Mattes, 2000), and Mackie and Pangborn have shown that α-amylase secretion rate is higher after chewing carbohydrates compared to food low in carbohydrates and non-food controls (Mackie & Pangborn, 1990). Moreover, recent research showed a positive correlation between α–amylase and reducing sugar concentrations upon a 2-min mastication of starchy chewing gum, demonstrating the role of α -amylase in starch digestion (Kusuma Aji, Warren, & Roura, 2019). On the other hand, lingual lipase has the ability to break down triglycerides (Mese & Matsuo, 2007; Pedersen et al., 2018). Although it is present only in low concentrations in human saliva, lingual lipase activity may have a positive correlation to fat perception (Mattes, 2000; Neyraud, Palicki, Schwartz, Nicklaus, & Feron, 2011; Spielman, D'Abundo, Field, & Schmale, 1993). Salivary composition may change upon (specific) food exposure and thereby play an essential role in anticipatory physiological response to enhance specific digestion.

Little is known about how food odours may signal specific information regarding taste and/or macronutrient content and could thereby alter anticipatory saliva secretion and composition. The aim of the current research was therefore to determine to what extent odours representing different types of foods would influence saliva secretion and composition, in particular, visco-elasticity, MUC5B content, α-amylase, and lingual lipase activity, in healthy participants. Consequently, we conducted two studies: study 1 focused on odours associated with three basic taste qualities (sweet, savoury, and sour); while in study 2 we investigated odours representing foods of different macronutrient content (high in carbohydrates, fat, protein, or low-calorie).

It was hypothesized that odours have a critical and specific role in anticipatory physiological response and eating behaviour. Therefore, we tested three main hypotheses. Firstly, saliva secretion rate would increase upon exposure to food odours compared to controls. Secondly, exposure to odours would decrease saliva visco-elastic properties compared to unstimulated saliva, to aid bolus formation. Lastly, the activity of specific salivary enzymes would increase depending on specific odour exposure to facilitate specific digestion: an increase in α-amylase activity after exposure to sweet (study 1) or carbohydrate-related odours (study 2), and increased activity of lingual lipase upon exposure to fat-related odours (study 2).

Materials and methods

Participants

Study 1

Forty-four international men and women aged between 18 and 55 years were recruited around the Wageningen area. International participants were recruited taking into account that the basic tastes are easier to identify and similar across the globe. Participants were included when having a self-reported normal weight (BMI 18.5–25 kg/m^2) and were English speakers. They were excluded when they were habitual smokers, vegetarian, were pregnant or had the intention to become pregnant during the study, or were breastfeeding. The study was performed by 44 participants. However, after finishing the study, data from eight participants were excluded from the analyses: one due to extreme hyposalivation (unstimulated saliva secretion ≤ 0.01mL/min (Pedersen et al., 2018)), one due to an orofacial cleft, and six participants due to low performance on the Sniffin' Sticks 16-item odour-identification test (score <12 (Hummel, Kobal, Gudziol, & Mackay-Sim, 2007)). The characteristics of the remaining 36 participants are shown in **Table 2.1**.

Study 2

A total of 60 healthy Dutch participants (**Table 2.1**) were recruited. Only Dutch participants were included to ensure a homogenous cultural and dietary background. Furthermore, considering the challenge to properly classify an odour into a specific macronutrient category and the low presence of lingual lipase in human saliva, the number of participants recruited was higher compared to study 1. Participants interested in the study were invited to a screening session to determine their eligibility. Inclusion and exclusion criteria were similar to study 1. For this study we also evaluated their subjective dry mouth sensation (excluded when score ≥ 33 on the Xerostomia Inventory, 11-item scale (Thomson, 2015; Thomson, Chalmers, Spencer, & Williams, 1999).

In both studies, participants signed a written informed consent before they participated in the study. They received compensation via a voucher or an electrical toothbrush upon completion of the study. The studies were conducted in accordance with the Declaration of Helsinki (revised in 2013). The protocols were approved by the Medical Ethical Committee of Wageningen University (NL51747.081.14).

Table 2.1. Characteristics of participants in both studies.

Characteristic	Study 1	Study 2
	Value (Mean ± SD)	
Number of participants	36 (29F:7M)	60 (47F:13M)
Age (years)	23.7 ± 3.4	27.2 ± 11.4
BMI (kg/m^2)	21.4 ± 1.9	21.7 ± 1.8
Odour Identification	13.2 ± 0.9	13.2 ± 0.9
Dry mouth experience*	2.7 ± 0.9*	-
Xerostomia Inventory Score**	-	19.6 ± 4.1**

*Dry mouth experience on a 5-point scale (always = 4; often = 3; sometimes = 2; seldom = 1; never = 0). **Xerostomia Inventory classification: 11–23 = 'does not suffer' to 'suffer slightly' from xerostomia (Thomson, 1999).

Odour stimuli

Study 1

A pilot study was conducted in a separate sample of participants to select odours for each of the following four categories: sweet, savoury, sour, and non-food, and to determine similar intensities (70–80 mm on 100 mm visual analogue scale, VAS, clearly detectable but not overwhelming). At least 60% of the participants classified the following stimuli into their appropriate category: vanilla (sweet; International Flavours and Fragrances, IFF 10860896; 2% in propylene glycol, PG); beef (savoury; IFF 10878095; 0.1% in demineralized water, DW); lime (sour; IFF 70802446; 9% in PG); fresh green (non-food; AllSens–Voit Aroma Factory No. 819; 0.5% in PG); and a no-odour control (100% PG).

Study 2

The odours were selected to represent foods differing in macronutrient composition (i.e. high in protein, carbohydrate, fat, or low-calorie), and a non-food odour as control. The selection of odours was based on its proper macronutrient association and the nutritional value of the food that they represented. At least 50% of total energy of the food was derived from the specific macronutrient; low-calorie products contained less than 60 kcal/100 g (de Bruijn, de Vries, de Graaf, Boesveldt, & Jager, 2017; RIVM, 2016).

A pilot study was carried out to confirm the intended macronutrient associations (≥ 55 mm on 100 mm VAS, assessed by 'How many *[macronutrients]* do you think a product with this odour contains?'), and determine similar intensities and liking of the odours (70–80 and 60–70 mm on 100 mm VAS, respectively). Participants from this pilot study were not included in the actual study. Two odours per category were selected: honey (IFF 10915585; 0.01% in PG) and bread (Symrise 205361; 10% in PG) for carbohydrates; beef (IFF 10925205; 0.04% in DW) and chicken (IFF

10913579; 0.06% in DW) for protein; butter (IFF 10922603; 0.5% plus diacetyl from Sigma-Aldrich; 0.01% in PG) and cream (IFF 10923144; 10% in PG) for fat; cucumber (IFF 15311331; 100%) and melon (IFF 15025874; 2% in PG) for low-calorie foods; fresh green (AllSens–Voit Aroma Factory No. 819; 0.04% in PG) and wood (AllSens–Voit Aroma Factory No. 821; 0.6% in PG) as non-food odours; and a no-odour control (100% PG).

Ten and fifteen mL (study 1 and 2, respectively) of the odour stimuli were placed in brown 50 mL glass bottles. The bottles were prepared at least one day before the experiment and coded with three-digit numbers. They were kept at 4°C until used. At least 1 h prior to the test session, the bottles were taken out of the refrigerator to reach room temperature.

Procedure

Both studies consisted of two sessions, and were held in well-ventilated and semi-isolated sensory booths. Participants were asked to avoid drinking any alcohol, use of drugs, or eating any spicy food, garlic, or onions the evening before the session, not to wear fragranced products on the day of the sessions, refrain from brushing their teeth, using mouthwash, or chewing gum 3 h before the study, and not to eat or drink (except water) at least 2 h prior to the study. Moreover, participants in study 2 were also asked to avoid vigorous exercise on the morning of the test session days, refrain from sour and caffeinated beverages 4 h before the study, eat their usual lunch, and avoid stressful activities at least 30 min before the test session (Lapis, Penner, Balto, & Lim, 2017; Rohleder & Nater, 2009). Participants were scheduled at the same time for the two sessions, with at least one day between them.

Participants followed instructions via an online questionnaire using EyeQuestion® (Version 3.11.1, Logic8 BV). The timeline of both studies' procedure is shown in **Figure 2.1**.

Study 1

During the first test session, participants were instructed to rinse their mouth with deionized, distilled water, and disposed their saliva in a container. Participants were allowed a 1-min break, during which they had to breathe through their nose with their mouth closed and swallow before receiving the odour samples. They then collected their unstimulated saliva according to the spitting method by Navazesh (1993), for 3 min in an empty pre-weighed 25 mL transparent plastic tube (Sterilin Ltd, Newport, UK). Participants were told to sit down straight with a slightly tilted head, allowing the saliva to pool in the mouth and spit it out into a container every 30 s. They were instructed not to swallow, speak, or do mouth movements during collection as supervised. After collection of the unstimulated saliva, participants waited for 1 min

before they received an odour sample. Participants were then exposed to the different odour conditions in a randomized order. Each exposure lasted 3 min, while participants breathed normally and collected their saliva as previously instructed. One-min breaks were given between exposures. After saliva was collected for all the conditions, participants evaluated the odours' attributes on liking, intensity, familiarity, intention to eat a product with that smell, and mouth-watering perception (all 100 mm VAS scale anchored by 'not at all' – 'very much'). Finally, they were asked to categorize each odour (sweet, savoury, sour, or non-food related) and to identify each odour by an open question. Participants had a 15-s break between each evaluation. The session lasted 30 min.

During a second test session, olfactory capabilities of the participants were assessed using the extended Sniffin' Sticks test battery consisting of an odour threshold, discrimination and identification test (Hummel et al., 2007). Participants also reported the frequency of their dry mouth experience on a 5-point scale and performed a breath odour test by means of the Rosenberg Scale (Rosenberg & McCulloch, 1992; data is not reported here). Female participants indicated the start of their last menstruation.

Study 2

Prior to the actual test sessions, participants filled out the Dutch Eating Behaviour Questionnaire (DEBQ, (van Strien, Frijters, Bergers, & Defares, 1986)).

For the actual experiment, participants were exposed to 6 conditions (no-odour, and one odour from each of the 5 odour categories) divided over two sessions. Participants were randomly exposed to 3 conditions per session.

Upon arrival of each session, female participants indicated their first day of their last menstruation. Then, participants rated their hunger, fullness, prospective consumption, desire to eat, appetite for something sweet, and appetite for something savoury on 100 mm VAS ('not at all' – 'very much'). Next, participants were asked to rinse their mouth as previously described and to wait for one min. A 30-s trial was performed to familiarize participants with the collection technique (spitting method). Saliva was collected according to the procedure described in study 1 with the following modification: in this study, saliva was collected for 5 min with a 2-min break between odour samples. Saliva was collected in empty pre-weighed polypropylene 25 mL cups (Böttger, Germany), which were kept on ice during the 5 min collection. After collection of unstimulated saliva and a 2-min break, participants randomly received a bottle containing an odour stimulus and an empty pre-weighed cup to collect their saliva. After being exposed to 3 different conditions, participants were presented with the stimulus again and assessed the odours' attributes. Also, participants were asked to match an image to the odour, out of a series of 12 different pictures that represented the used odours (e.g. honey, bread, beef, chicken).

Participants had a 30-s break between each evaluation. Each session last around 45–50 min.

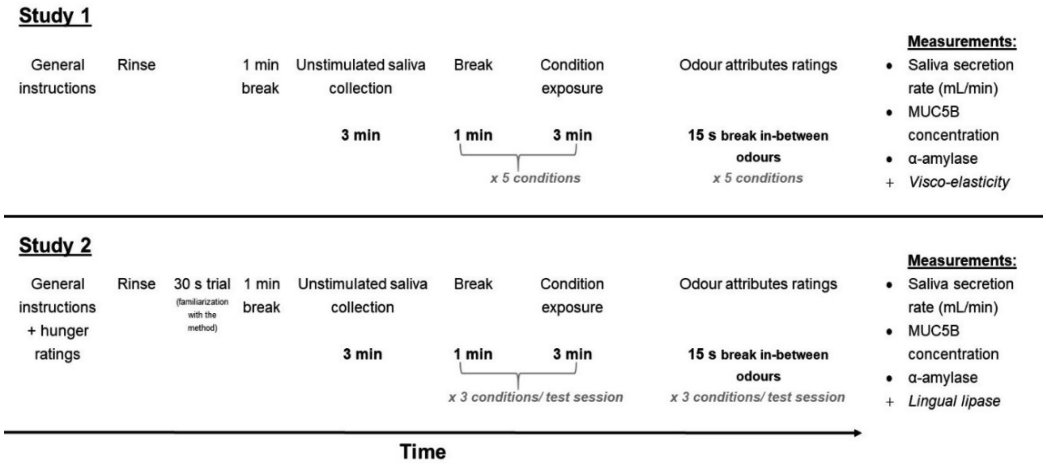

Figure 2.1. Timeline of both studies' procedure. In study 1 participants attended one test session which involved saliva collection upon odour exposure (30 min), while in study 2 they attended two test sessions (45–50 min each).

Measurements

Saliva secretion rate

Whole mouth saliva was weighed shortly after collection by an analytical gravimetric scale (Adventurer™ Pro, OHAUS Europe GmbH, Greifensee, Switzerland). The pre-weighed average of a subset of containers was subtracted to the saliva weight. It was assumed that 1 g of saliva represents 1 mL (Chicharro, Lucía, Pérez, Vaquero, & Ureña, 1998). Saliva secretion was divided by the 3 or 5 minutes (study 1 or 2, respectively) of the saliva collection to obtain mL/min as final units. After weighing, samples were stored on ice, until viscosity and elasticity measurements were done, within the next 4 h (study 1 only). After these measurements, samples were homogenized by vortexing for 20 s. Samples were clarified by centrifugation (10 min, 4°C, 10,000 *g*) to remove epithelial cellular debris, bacteria, and food residues. The resulting clarified saliva was diluted 1:1 v:v with 150 mM NaCl, to avoid protein aggregation and precipitation, aliquoted, and stored at −20°C until further analysis (Prodan et al., 2015).

Visco-elastic properties

The viscosity and elasticity (study 1 only) were measured in 500 µl of the saliva samples using the Vilastic-3 Viscoelasticity Analyzer (Vilastic Scientific Inc, Austin, TX, USA) according to the manufacturer's instructions. Four measurements were

done for each sample and the mean was calculated. Viscosity and elasticity were expressed as Pa/s.

An enzyme-linked immuno sorbent assay (ELISA) was performed to determine the amount of MUC5B (both studies) in each of the saliva samples following the procedure described previously (Veerman et al., 1997). In brief, samples were diluted 1:200 or 1:100 (study 1 or 2, respectively) with coating buffer (100 mM $NaHCO_3$; pH 9.6) after which 200 µl was pipetted into the wells of a Greiner Bio-One™ 96 Well ELISA Microlon F-shape Microplate (Greiner Bio-One B.V, Alphen aan den Rijn, The Netherlands). Subsequently, samples were diluted in coating buffer in two-fold serial dilutions. The last row of the microplate was used as blank. An overnight incubation at 4°C allowed the adherence of the mucin proteins to the wells. Then, the coating solution was dispensed after which the microplates were rinsed 3 times with phosphate buffered saline supplemented with 0.1% v:v Tween-20 (PBS-T) and blocked with 1% gelatine dissolved in PBS-T (PBS-T-G) and incubating at 37°C for 1 h, with gentle shaking. Next, PBS-T-G supplemented was added with a MUC5B specific antibody 1:40 F2 (Veerman et al., 2003) and the microplates were incubated at 37°C for 1 h. Subsequently, the microplates were rinsed 3 times with PBS-T to remove unbound F2 antibody. A 1:1000 dilution of conjugant rabbit α-Mouse – HRP (GeneTex, Inc., CA,USA) in PBS-T-G was added to each well and the microplates were incubated at 37°C for 1 h. Then, the microplates were rinsed with PBS-T (3 times) and distilled water (1 time). The substrate solution was made by a 4 mg tablet of O-phenylenediamine dihydrochloride (OPD) (Sigmafast™OPD, Sigma-Aldrich, St Louis, MO, USA) dissolved in 20 mL OPD buffer. A total of 100 µl of this solution was added to each well. Ten minutes after the reaction started, it was stopped by adding 50 µl of 2 N sulfuric acid (H_2SO_4) to each well. The absorption was measured using a Multiskan™ FC Microplate Photometer using SkanIt™ Software 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) at 492 nm. The concentration of MUC5B is expressed as AU/mL (AU = arbitrary unit), taking into account the dilution factor. All measurements were performed in duplicate.

Salivary enzyme activities

α-amylase activity was measured by colorimetric enzymatic activity assays. Saliva samples were diluted 1:50 or 1:10 (study 1 or 2, respectively) with sterilized water. In each well, 10 µl of diluted saliva was added plus 90 µl of amylase substrate, 2-chloro-4-nitrophenyl-α-D-maltotrioside (Sigma-Aldrich, St Louis, MO, USA). α-amylase cleaves the substrate into 2-chloro-4-nitrophenol, a yellow compound. Human saliva α-amylase standard of 0.5 or 2.5 U/mL (study 1 or 2, respectively; BIODESIGN international, Saco, Marine) was used for the calculation of α-amylase activity of the saliva samples. The kinetics of the α-amylase activity was measured by Multiskan™ FC Microplate Photometer using SkanIt™ Software 3.1 at 405 nm directly after the amylase substrate was added. The α-amylase activity is expressed

as units per millilitre (U/mL), taking into account the dilution factor. All measurements were performed in duplicate.
Lingual lipase activity (study 2 only) was measured following a fluorescence-based enzymatic activity method defined by Neyraud et al. (2017). A substrate solution was prepared by 20 mM of 4-methylumbelliferyl-7-oleate (Sigma-Aldrich, The Netherlands) in ethanol, and was diluted to 1 mM with a buffer containing 50 mM Tris–HCl, pH 7.5, 4 mM $CaCl_2$, 2 mM EDTA, 0.2% (w/v) sodium taurodeoxycholate (NaTDC), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM DTT and 0.02% (w/v) sodium azide. On each microplate, a control of the linearity and proportionality of the reaction was performed using commercial lipase (*Aspergillus niger* Lipase, Sigma-Aldrich, The Netherlands). The hydrolysis reaction was performed by adding 37.5 µL of saliva, 150 µL of the 1 mM substrate solution, and 1.5 µL ethanol. The inhibition reaction was done by adding 1.5 µL of a 56 mM ethanolic solution of tetrahydrolipstatin (THL) instead of ethanol. The absorption intensity was followed by the kinetics of the reaction for 30 min at 37°C (excitation filter: 355 nm; emission filter: 460 nm) using a microplate fluorometer and controlled by FLUOstar Galaxy software (Version 4.11-0). A standard curve of umbelliferone was performed as reference. Lingual lipase activity was calculated with the difference in the slopes of each saliva sample with ethanol and THL. The activity was expressed, in reference to the standard curve, as units per millilitre (U/mL). International Enzyme Unit Activity (U) is defined as the amount of enzyme that catalyses the conversion of 1 nM of substrate per minute. All enzyme measurements were performed in duplicate.

Statistical analyses (both studies)

Parameters are shown as mean and standard error, unless otherwise specified. Results were considered statistically significant when $p < 0.05$. Necessary assumptions (homoscedasticity and normal distribution of error terms, and correct specifications of the fixed and random parts of the model) were checked for each model. All statistical analyses were carried out with RStudio (RStudio Team, 2016), and graphs were made using GraphPad Prism 5.0 (GraphPad Prism Software).

In study 1, data were analysed per odour. In study 2 we were interested in the effect of condition and not of single odours, therefore data were pooled over the two odours of the same condition (e.g. data resulting from exposure to ‘beef’ and ‘chicken’ odour were collapsed into a ‘protein’ condition). For both studies, odours were also collapsed across all food odours to compare saliva secretion rate between no-odour, non-food, and food conditions.

For both studies, saliva secretion rate of unstimulated salivation was subtracted from the saliva secretion rate of each exposure condition (food odours, non-food odours, no-odour condition) and within the same test session. This adjustment was done per participant, resulting in ‘change in saliva secretion rate’. For the visco-elastic

properties, unstimulated data was considered as the exposure condition, and for enzyme activities it was added as covariate in the model. We considered only the data above the limit of detection (LOD = 0.005 U/mL; 64%) for the analysis of lingual lipase.

Linear mixed models using *lme4* package (Bates, Mächler, Bolker, & Walker, 2015) were performed to analyse odour ratings (liking, intensity, familiarity, intention to eat, and mouth-watering perception), change in saliva secretion rate, visco-elastic properties, and enzyme activities as dependent variables. Odour condition was included as a fixed factor. Participants were included as random factors in study1. For study 2, session nested within participants, and evaluation order (order in which each exposure condition was provided) were included as random factors. Gender, age, BMI, menstrual cycle, dry mouth sensation, and odour attributes ratings (both studies), and restrained eaters (results from the DEBQ) and appetite feelings (in study 2) were evaluated as covariates. The modelling followed a backward approach, and the best fitting models were selected by comparing AIC and log-likelihood of the models. Post-hoc tests with Bonferroni correction, by means of *lsmeans* package (Lenth, 2016), were performed when the fixed factor was significant.

Pearson correlation analysis was done to determine the correlation between mouth-watering perception (subjective salivation) and change in saliva secretion rate (objective salivation) for all data points. Moreover, a second Pearson correlation analysis correcting for random effects (participants in study 1; session nested within participants in study 2) was performed.

Results

Odour induced stimulation of saliva (change in saliva secretion rate)

Study 1

Mixed model analyses showed that the change in saliva secretion rate significantly increased upon food odour exposure ($F(2,175) = 13.6$, $p < 0.0001$; **Figure 2.2A**) and upon taste-related odour exposure ($F(4,173) = 7.1$, $p < 0.0001$; **Figure 2.2B**) compared to no-odour and non-food conditions. However, there was no difference in change in saliva secretion rate between the taste-related odours (sweet, savoury, and sour) (**Figure 2.2B**).

Study 2

Mixed model analyses, including liking as a covariate, showed that change in saliva secretion rate increased significantly in response to food odour exposure ($F(2,349) = 15.2$, $p < 0.0001$; **Figure 2.3A**), and to macronutrient-related odours ($F(5,346) = 8.0$, $p < 0.0001$; **Figure 2.3B**), compared to no-odour and non-food exposure. Although exposure to carbohydrate-related odours resulted in the least change in saliva secretion rate (mean ± SE = 0.036 ± 0.01), there were no differences between the macronutrient-related odour categories.

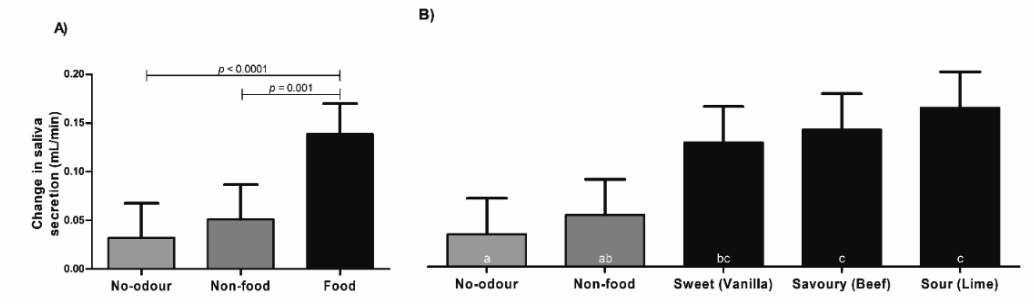

Figure 2.2. Change in saliva secretion rate (mL/min), saliva secretion rate corrected for unstimulated saliva (0.51 ± 0.04) upon odour exposure, collapsing food odours into a category (A) and per taste-related category (B). Values are expressed as mean and standard error. Similar letters indicate no significant differences ($p > 0.05$).

Figure 2.3. Change in saliva secretion rate (mL/min), saliva secretion rate corrected for unstimulated saliva (0.59 ± 0.02) upon odour exposure; collapsing food odours into a category (A) and per macronutrient category (B). Values are expressed as mean and standard error. Similar letters indicate no significant differences ($p > 0.05$).

Odour ratings for both studies can be found in the supplementary material (**Table A2.1**).

Visco-elastic properties

Viscosity and elasticity (study 1)

Exposure condition did not significantly affect viscosity ($F(5,206) = 1.0$, $p = 0.44$) nor elasticity ($F(5,206) = 0.9$, $p = 0.50$; **Table 2.2**).

Mucin 5B (MUC5B) concentration

In study 1, exposure condition significantly affected the concentration of MUC5B ($F(5,207) = 6.9$, $p < 0.0001$, **Table 2.2**). Except for the savoury condition, MUC5B concentration was significantly lower in stimulated saliva compared to unstimulated saliva. However, in study 2 exposure condition did not affect the concentration of MUC5B ($F(6,467) = 1.5$, $p = 0.23$; **Table 2.2**).

Salivary enzyme activity

α-amylase

In both studies, exposure condition did not significantly affect α-amylase activity (study 1: $F(4,128) = 2.1$, $p = 0.09$); study 2: $F(5,343) = 0.9$, $p = 0.49$; **Table 2.2**).

Lingual lipase (study 2)

Exposure condition did not significantly affect lingual lipase activity ($F(5,216) = 1.17$, $p = 0.33$, **Table 2.2**).

Correlation between mouth-watering perception (subjective salivation) and change in saliva secretion rate (objective salivation)

Subjective salivation and objective salivation were significantly positively correlated in both studies (study 1: $r(178) = 0.29$, $p < 0.0001$; study 2: $r(354) = 0.30$, $p < 0.0001$), also when correcting for random effects (study 1: $r(178) = 0.50$, $p < 0.0001$; study 2: $r(354) = 0.35$, $p < 0.0001$).

Table 2.2. Visco-elastic properties and salivary enzyme activity per study. Values are expressed as mean and standard error. Similar letters indicate no significant differences ($p > 0.05$).

Condition	Visco-elastic properties			Salivary enzyme activity	
	Viscosity (Pa/s)	Elasticity (Pa/s)	MUC5B[1] (AU/mL)	α-amylase[2] (U/mL)	Lingual lipase[3] (U/mL)
Study 1					
Unstimulated	0.06 ± 0.004*	0.04 ± 0.006*	2753.56 ± 308.99[a*]	8.29 ± 1.42	-
No-odour	0.05 ± 0.004	0.03 ± 0.004	1887.65 ± 301.63[b]	6.54 ± 0.99	-
Non-food	0.05 ± 0.004	0.03 ± 0.004	1537.72 ± 301.62[b]	7.72 ± 1.86	-
Sweet (Vanilla)	0.08 ± 0.017	0.11 ± 0.074	1716.60 ± 301.62[b]	6.42 ± 0.81	-
Savoury (Beef)	0.08 ± 0.030	0.09 ± 0.063	1988.73 ± 301.62[ab]	6.16 ± 0.77	-
Sour (Lime)	0.05 ± 0.004	0.03 ± 0.003	1815.98 ± 301.66[b]	7.27 ± 0.93	-
Study 2					
Unstimulated	-	-	1054.91 ± 36.51*	94.12 ± 6.11	0.024 ± 0.002
No-odour	-	-	922.65 ± 52.46	86.45 ± 8.41	0.028 ± 0.003
Non-food	-	-	893.83 ± 48.96	85.20 ± 8.18	0.024 ± 0.003
Carbohydrates	-	-	943.34 ± 54.36	78.16 ± 7.44	0.023 ± 0.002
Protein	-	-	878.64 ± 49.91	73.71 ± 7.49	0.024 ± 0.002
Fat	-	-	893.22 ± 50.37	83.80 ± 6.91	0.024 ± 0.003
Low-calorie	-	-	918.82 ± 52.85	82.43 ± 6.83	0.023 ± 0.002

Mixed models adding [1]age (study 2 only); [2]mouth-watering perception (study 1 only), menstrual cycle, and α-amylase activity of unstimulated saliva (both studies); [3]lingual lipase activity of unstimulated saliva, as covariate(s). *Unstimulated data were considered as the exposure condition in the visco-elastic properties models.

Discussion

This study aimed to determine to what extent food odours representing specific taste qualities and macronutrient content would influence salivary secretion rates and salivary composition as an essential and specific anticipatory response towards digestion of food. This study confirms that total saliva secretion rate increases upon exposure to food odours. However contrary to our hypotheses, exposure to odours signalling different food properties (i.e. taste and macronutrient content) did not specifically affect saliva secretion or composition.

Our findings are in line with previous literature that showed an increase in saliva secretion rate upon food odour exposure (Chambaron, Chisin, Chabanet, Issanchou, & Brand, 2015; Ilangakoon & Carpenter, 2011; Lee & Linden, 1992a; Proserpio et al., 2017). Contrary to our hypothesis, saliva secretion was not significantly different between the specific food categories (taste- and macronutrient-related odours). Firstly, sour odour (lime) was not significantly different compared to sweet and savoury odours. A higher saliva secretion upon sour odour exposure could be expected because sour food (or citric acid solution) is the most potent sialagogue (Froehlich, Pangborn, & Whitaker, 1987; Hodson & Linden, 2006) and triggers more saliva to dilute acidity and thus to prevent dental erosion and protect the digestive system (Matsuo, 1999; Pedersen, Bardow, Jensen, & Nauntofte, 2002; Watanabe & Dawes, 1988). Secondly, macronutrient-related odours secreted a similar amount of saliva as the low-calorie related odours. Macronutrient-related odour exposure may secrete a higher salivation because these food products are higher in calories and tend to be more palatable compared to low-calorie products. Klajner showed that palatable food plays an important role in saliva secretion (Klajner et al., 1981). However, all food odours in the current study were well liked, and pleasantness of the odours was taken into account in our model.

We aimed to provide an overview of whole mouth saliva secretion and composition, rather than from specific glands. Salivary responses upon odour exposure could be linked to parasympathetic nerves that innervate the salivary glands – responsible for secretion of water – and therefore modify the amount of saliva secretion rate (Carpenter, 2013; Humphrey & Williamson, 2001; Mese & Matsuo, 2007; Pedersen et al., 2018). Parasympathetic nerves consist of facial (innervating the submandibular and sublingual glands) and glossopharygneal nerves (in the parotid glands) (Mese & Matsuo, 2007; Pedersen et al., 2018). According to previous studies, olfactory stimuli induce saliva secretion from submandibular and sublingual glands, but not from parotid glands (Ilangakoon & Carpenter, 2011; Lee & Linden, 1991, 1992a, 1992b). This could mean that odours could act as an afferent sensory input and mainly activate the facial nerves of the parasympathetic branch, secreting saliva exclusively from submandibular and sublingual glands. These results further

support the idea that food odours *per se* are potent anticipatory food cues and trigger cephalic-phase responses, in this case salivation.

Surprisingly, there were no changes in saliva composition between the different food odour conditions. A possible explanation for this might be that sole odour exposure may not activate the sympathetic nerves – responsible for protein secretion – and thus does not affect saliva composition (Carpenter, 2013; Humphrey & Williamson, 2001; Mese & Matsuo, 2007; Pedersen et al., 2018). While we expected a decrease in visco-elastic properties upon odour exposure compared to unstimulated saliva, viscosity and elasticity (study 1) did not vary between exposure conditions. Moreover, unstimulated saliva tended to be richer in mucins compared to stimulated saliva. However, this was only significantly different in study 1, but not in study 2. Perhaps sole odour exposure is not sufficient to affect viscosity, and stronger stimulation such as multisensory or mechanical stimulation would be required to detect alterations in protein secretion. A two to three-fold decrease in viscosity upon mechanical stimulation compared to unstimulated saliva has been reported (Rantonen & Meurman, 1998). Furthermore, we expected that activity of salivary enzymes would increase depending on specific odour exposure to facilitate specific digestion of those macronutrients. Contrary to our hypothesis, α-amylase and lingual lipase activity was not modified by specific odour exposure (carbohydrates and fat, respectively). Though the presence of lingual lipase in saliva and its further role in digestion remains uncertain (Mattes, 2000; Neyraud et al., 2011), studies involving basic tastes or modified sham feeding of carbohydrate-related products did show an increase in salivary α-amylase compared to other food products (Froehlich et al., 1987; Mackie & Pangborn, 1990). Additionally, macronutrient-specific cephalic-phase responses of gastrointestinal hormones (such as ghrelin, insulin, and pancreatic polypeptide) have been shown after modified sham feeding of meals rich in carbohydrates, protein, or lipids (Crystal & Teff, 2006; Monteleone, Bencivenga, Longobardi, Serritella, & Maj, 2003; Witteman et al., 1994; Zhu, Hsu, & Hollis, 2014). Modified sham feeding, or actual eating of foods comprises greater sensory stimulation and more vivid experiences than the mere odour exposure in our current studies, and thus could have a more pronounced impact on specific cephalic-phase responses. These responses are learned responses, based on previous experiences with (multi)sensory food cues and their post-ingestive and/or reward consequences (Mattes, 1997; Nederkoorn et al., 2000; Smeets et al., 2010).

The salivary proteins play a role in oral health such as antibacterial action and remineralization (Carpenter, 2013; Humphrey & Williamson, 2001). We aimed to investigate the specific saliva composition involved in digestion, therefore we did not perform proteomic analyses of the collected saliva. The whole saliva proteome could vary depending on the gland stimulation. For instance, as we mentioned odours may stimulate submandibular and sublingual glands, we could expect a cystatin-rich saliva (Carpenter, 2013). Furthermore, the proteins could also be modified

depending on the nature of the stimulus. Neyraud and colleagues showed that the whole saliva proteome varies between tastants, where citric acid produced the greater differences in protein patterns compared to glucose (Neyraud, Sayd, Morzel, & Dransfield, 2006).

An underrepresentation of males (around 20% in both studies) limits generalizability to the overall population. Salivary gland size is larger in males which may influence saliva secretion and composition (Heintze, Birkhed, & Björn, 1983; Inoue et al., 2006; Prodan et al., 2015). However, adding gender to our statistical models did not improve the fit.

Moreover, the positive correlation between subjective and objective salivation we found here was in healthy participants. While it is known from patient studies that subjective dry mouth sensation (scoring low on xerostomia assessment) does not always relate to objective hyposalivation or salivary gland hypofunctioning (Ekström, Khosravani, Castagnola, & Messana, 2017; Pedersen et al., 2018; Thomson, 2015).

Conclusion

Exposure to food odours induces saliva secretion rate in anticipation of actual eating behaviour but did not affect the specific composition of the saliva. This does not support the hypothesis that changes in salivary composition upon olfactory cues are taste- or macronutrient specific. A combination of different sensory modalities (multisensory cues) could be necessary to produce more evident and specific anticipatory physiological responses to facilitate digestion and maintain body homeostasis. Together this will shed new light on the role of sensory food cues in anticipatory eating responses and may impact actual eating behaviour.

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Appendix 2.1

Table A2.1. Intensity, liking, familiarity, intention to eat a product with that odour, and mouth-watering ratings (on 100 mm VAS) and percent of the correct categorization of the used odours. Values are expressed as mean and standard error. Similar letters indicate no significant differences ($p > 0.05$) between conditions.

Condition	Intensity	Liking	Familiarity	Intention to eat a product of that odour	Mouth-watering	Correct categorization
			Study 1			
No-odour	26.11 ± 3.14^{a}	45.56 ± 2.65^{a}	29.34 ± 3.22^{a}	30.18 ± 3.50^{a}	28.61 ± 2.62^{a}	58.33%
Non-food	68.83 ± 3.21^{b}	49.60 ± 3.86^{ab}	50.33 ± 4.60^{b}	19.61 ± 2.74^{a}	33.78 ± 3.40^{a}	88.89%
Sweet (Vanilla)	76.48 ± 2.47^{bc}	77.45 ± 3.36^{d}	79.26 ± 2.97^{c}	75.34 ± 3.11^{b}	69.61 ± 2.61^{b}	97.2%
Savoury (Beef)	80.14 ± 1.79^{c}	62.71 ± 4.19^{bc}	77.54 ± 3.23^{c}	64.91 ± 4.19^{b}	65.96 ± 3.65^{b}	94.44%
Sour (Lime)	75.25 ± 1.88^{bc}	73.23 ± 2.74^{cd}	78.45 ± 2.04^{c}	62.08 ± 3.99^{b}	63.66 ± 3.19^{b}	58.33%
			Study 2			
No-odour	11.39 ± 2.10^{a}	44.69 ± 2.49^{a}	40.53 ± 3.68^{a}	31.39 ± 3.16^{a}	15.11 ± 2.22^{a}	78.33%
Non-food	70.03 ± 1.94^{bc}	58.76 ± 3.31^{bc}	50.21 ± 2.61^{ab}	23.31 ± 2.81^{a}	24.82 ± 2.69^{a}	76.27%
Carbohydrates	66.46 ± 2.56^{b}	48.98 ± 3.56^{ab}	58.88 ± 3.27^{bc}	44.72 ± 3.76^{b}	43.42 ± 3.58^{b}	57.63%
Protein	74.84 ± 1.94^{bc}	51.64 ± 3.74^{ab}	68.95 ± 2.74^{cd}	55.75 ± 3.60^{bc}	50.84 ± 3.48^{bc}	89.83%
Fat	76.51 ± 1.89^{c}	65.29 ± 2.65^{c}	65.99 ± 2.62^{cd}	61.31 ± 2.76^{c}	57.24 ± 2.99^{c}	42.37%
Low-calorie	71.08 ± 2.36^{bc}	64.39 ± 3.06^{c}	74.82 ± 2.44^{d}	62.30 ± 2.92^{c}	56.67 ± 3.09^{c}	96.61%

Appendix 2.2 – α-amylase

To be in line with the outcomes in Chapter 3, we also calculated the α-amylase secretion rate (U/min) considering the salivation rate (mL/min). In both studies, exposure condition did not significantly affect α-amylase secretion rate (study 1: $F(4,140) = 1.66$, $p = 0.16$); study 2: $F(5,345) = 0.8$, $p = 0.55$; **Table A2.2**).

Table A2.2. α-amylase concentration (U/mL) and secretion rate (U/min) per study. Values are expressed as mean and standard error.

Condition	α-amylase concentration (U/mL)[1]	α-amylase secretion rate (U/min)[2]
	Study 1	
Unstimulated	8.29 ± 1.42	4.37 ± 0.77
No-odour	6.54 ± 0.99	3.37 ± 0.50
Non-food	7.72 ± 1.86	5.39 ± 2.16
Sweet (Vanilla)	6.42 ± 0.81	4.06 ± 0.60
Savoury (Beef)	6.16 ± 0.77	3.98 ± 0.55
Sour (Lime)	7.27 ± 0.93	4.66 ± 0.70
	Study 2	
Unstimulated	94.12 ± 6.11	56.82 ± 4.49
No-odour	86.45 ± 8.41	49.54 ± 4.82
Non-food	85.20 ± 8.18	52.63 ± 5.78
Carbohydrates	78.16 ± 7.44	48.38 ± 4.60
Protein	73.71 ± 7.49	48.99 ± 5.16
Fat	83.80 ± 6.91	50.98 ± 4.18
Low-calorie	82.43 ± 6.83	55.06 ± 5.23

Mixed models adding [1]mouth-watering perception (study 1 only), menstrual cycle, and α-amylase concentration of unstimulated saliva (both studies); [2]mouth-watering perception (study 1 only) and amylase concentration of unstimulated saliva (both studies).

Chapter 3

A stepwise approach investigating salivary responses upon multisensory food cues

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Abstract

Exposure to sensory food cues such as smell, vision, taste, and/or texture may trigger anticipatory physiological responses such as salivation, anticipating on adequate metabolism of the signalled food. However, the individual contribution of each sensory modality as well as the impact of particular food products on salivation and salivary composition remains unclear. Therefore, by systematically varying sensory modalities and nutrient content of food stimuli, we investigated their effect on saliva secretion, α-amylase activity and other salivary characteristics (pH level, buffering capacity, MUC5B concentration, and total protein content).

Over 3 sessions, 46 normal-weight healthy participants were exposed to 12 conditions, consisting of 4 levels of sensory stimulation (odour, odour + vision, odour + vision + taste, and odour + vision + taste + mastication) and 3 types of stimuli (bread, high-in-starch; cucumber, low-in-starch; and parafilm as a non-food control) during which saliva was collected.

Linear mixed models showed a significant increase in salivation with increasing levels of sensory stimulation. α-amylase secretion rate increased upon the highest level of stimulation, which involved mastication, compared to odour and odour + vision level of stimulation. Other salivary characteristics varied with the level of sensory stimulation, which might be related to the total volume of salivation. The type of stimuli did not influence the saliva composition (α-amylase concentration nor other salivary components).

Our findings indicate that cumulative sensory information, rather than specific (food) product, play a vital role in anticipatory salivary responses.

Introduction

We are continuously exposed to sensory food cues that trigger physiological responses thereby affecting our appetite and, as a consequence, food intake (Boesveldt & de Graaf, 2017; McCrickerd & Forde, 2016). (Multi)sensory food cues, such as sight, smell, or taste of a food, may induce a rapid release of saliva in the oral cavity, this response is known as cephalic-phase salivary response (Mattes, 1997; Smeets, Erkner, & de Graaf, 2010; Spence, 2011; Zafra, Molina, & Puerto, 2006).

Salivation depends on a complexity of factors such as food related cues, general health, sex, etc. (Levine, 2010; Pedersen, Sørensen, Proctor, & Carpenter, 2018). On top of that glandular differences contribute to saliva properties. For example, saliva secreted by parotid glands is characterized by being serous and rich in α-amylase, while submandibular and sublingual glands produce visco-elastic, mucin-rich saliva (Carpenter, 2013; Dawes et al., 2015; Mese & Matsuo, 2007; Pedersen, Bardow, Jensen, & Nauntofte, 2002; Stokes & Davies, 2007). Each component of the saliva is attuned to serve a particular function. Salivary α-amylase is involved in the digestion of starch (hydrolysis of polysaccharides into maltose and dextrin) (Levine, 2010; Mattes, 2000). Levels of electrolytes, mainly bicarbonate, increase on mastication and stimulation of the parotid glands, provide a buffering action against acidic foods in order to maintain a neutral pH. Mucins, mainly MUC5B, impact viscosity of the saliva and are responsible for lubrication of the food bolus during mastication and swallowing, and during speaking. Lastly, proteins lubricate and protect teeth surfaces by forming a thin layer (pellicle) in the oral cavity (Carpenter, 2013; Dawes et al., 2015; Humphrey & Williamson, 2001; Levine, 2010; Pedersen et al., 2018). The secretion of mucins and protein is likely constant and its concentration may decrease upon a high saliva secretion (Levine, 2010).

Although some research suggests that salivation might not be conditioned to sensory cues (Lashley, 1916b) but mainly produced by muscle movements (Carpenter, 2013; Ilangakoon & Carpenter, 2011), others have shown that salivary responses could be conditioned (Hayashi, 1968; Holland & Matthews, 1970; Kershaw & Running, 2018; White, 1978). Moreover, research has demonstrated that salivation increases upon (multi)sensory exposure to various foods as an anticipatory response (Ferriday & Brunstrom, 2011; Keesman, Aarts, Vermeent, Häfner, & Papies, 2016; Klajner, Herman, Polivy, & Chhabra, 1981; Lee & Linden, 1992a; Pangborn, 1968; Pangborn, Witherly, & Jones, 1979; Proserpio, de Graaf, Laureati, Pagliarini, & Boesveldt, 2017; Rogers & Hill, 1989; Sahakian, Lean, Robbins, & James, 1981; Shannon, 1974; Wooley & Wooley, 1973). However, little is known about the influence of sensory cues on salivary composition, in particular, related to food digestion. Our previous research showed that exposure to unisensory (olfactory) cues, representing foods varying in taste quality and macronutrient content, enhanced saliva secretion

(Morquecho-Campos et al., 2019). Yet these cues did not result in alterations in salivary visco-elasticity and α-amylase and lipase activities. A recent study has shown similar α-amylase concentration after bread odour exposure and after mastication of bread (Carreira et al., 2020). Others have shown increased levels of α-amylase secretion rate or starch hydrolysis products by modified sham feeding of high-starch food products (Kusuma Aji, Warren, & Roura, 2019; Mackie & Pangborn, 1990; Woolnough, Bird, Monro, & Brennan, 2010). Modified sham feeding encompasses all sensory modalities including smell, sight, taste, and mastication of a stimulus but the bolus is spat out before swallowing it (Robertson, Jackson, Williams, Fielding, & Frayn, 2001). Nevertheless, to the best of our knowledge the contribution of individual sensory modalities associated with specific food products to cephalic-phase salivary responses has not yet been investigated.

Previous reports suggest that (multi)sensory food cues may signal the nutrient composition of the foods leading to cephalic-phase salivary responses to facilitate ingestion and further digestion (Boesveldt & de Graaf, 2017; Smeets et al., 2010). Therefore, the aim of the current study was to systematically determine the influence of different levels of sensory stimulation (2 anticipatory levels (odour and odour + vision) and 2 consummatory levels (odour + vision + taste and odour + vision + taste + mastication)), and specific food products (bread and cucumber, which vary in their starch content) on cephalic-phase salivary response. It was hypothesized firstly, that saliva secretion rate would increase with adding levels of sensory stimulation. Secondly, we expected increased α-amylase secretion rate when exposed to bread (food high-in-starch) compared to cucumber (low-in-starch) or non-food control stimuli with higher levels of sensory stimulation. Thirdly, we expected that salivary characteristics such as pH and buffer capacity would increase, while mucin and total protein concentration would decrease with the increase of saliva secretion rate, thus upon the level of sensory stimulation.

Materials and methods

Participants

Healthy Dutch female participants between 18 and 35 years old were recruited in Wageningen and surrounding area. We recruited only female participants due to physiological differences in the salivary gland size between the sexes (Inoue et al., 2006; Prodan et al., 2015). After registration, potential participants were invited to a screening session to determine their eligibility. Participants were included when having a body mass index (BMI) of 18.5–25 kg/m^2 (overweight and obese people tend to have greater salivation upon food cues (Ferriday & Brunstrom, 2011)), classified as normosmic (scoring ≥12 on the Sniffin' Sticks 16 items odour identification test (Oleszkiewicz, Schriever, Croy, Hähner, & Hummel, 2019)), liked the investigated food products (>40 mm on a 100 mm visual analogue scale; VAS, anchored by 'Not at all' to 'Very much'; reported in **Table A3.1**), liked and were familiar with bread and cucumber odour (>40 mm on a 100 mm VAS), and when they correctly identified these odours in a multiple forced-choice task. Smokers were excluded, as well as participants who had any allergy, intolerance or oversensitivity to the foods used in this study, used medication other than paracetamol and hormonal contraceptives, were pregnant or had the intention to become pregnant during the experiment or were currently breastfeeding, or suffered from dry mouth (score >33 out of 55, where 24 to 39 represents 'suffers moderate' to 'suffers a lot from xerostomia', we adjusted the cut-off to >33, occasional dry mouth, for our healthy participants) assessed through the Xerostomia Inventory (Thomson, 2015; Thomson, Chalmers, Spencer, & Williams, 1999). After screening 64 volunteers, a total of 46 female participants were included to the study (**Table 3.1**).

This study was conducted in accordance with the Declaration of Helsinki (revised in 2013) and approved by the Medical Ethical Committee of Wageningen University (NL51747.081.14). Participants signed a written informed consent at the beginning of the screening session. They were compensated via a monetary voucher at the end of the study.

Table 3.1. Characteristics of the 46 participants in the current study.

Characteristic	Mean ± SD
Age (years)	21.9 ± 2.0
BMI (kg/m^2)	20.9 ± 1.6
Odour Identification Score	13.7 ± 1.2
Xerostomia Inventory Score*	20.9 ± 3.8

*Xerostomia Inventory classification: 11–23 = 'does not suffer' to 'suffer slightly' from xerostomia (Thomson et al., 1999).

Design

This study consisted of a 3 stimuli type x 4 levels of sensory stimulation cross-over design. Over 3 test sessions, participants were exposed to 12 conditions differing in type of stimuli, including bread (food high-in-starch), cucumber (low-in-starch), and parafilm (as control), as well as levels of sensory stimulation encompassing odour, odour + vision, odour + vision + taste, and modified sham feeding (odour + vision + taste + mastication). During each test session, the participants were randomly exposed to four conditions, one of each level of sensory stimulation (see **Figure 3.1**).

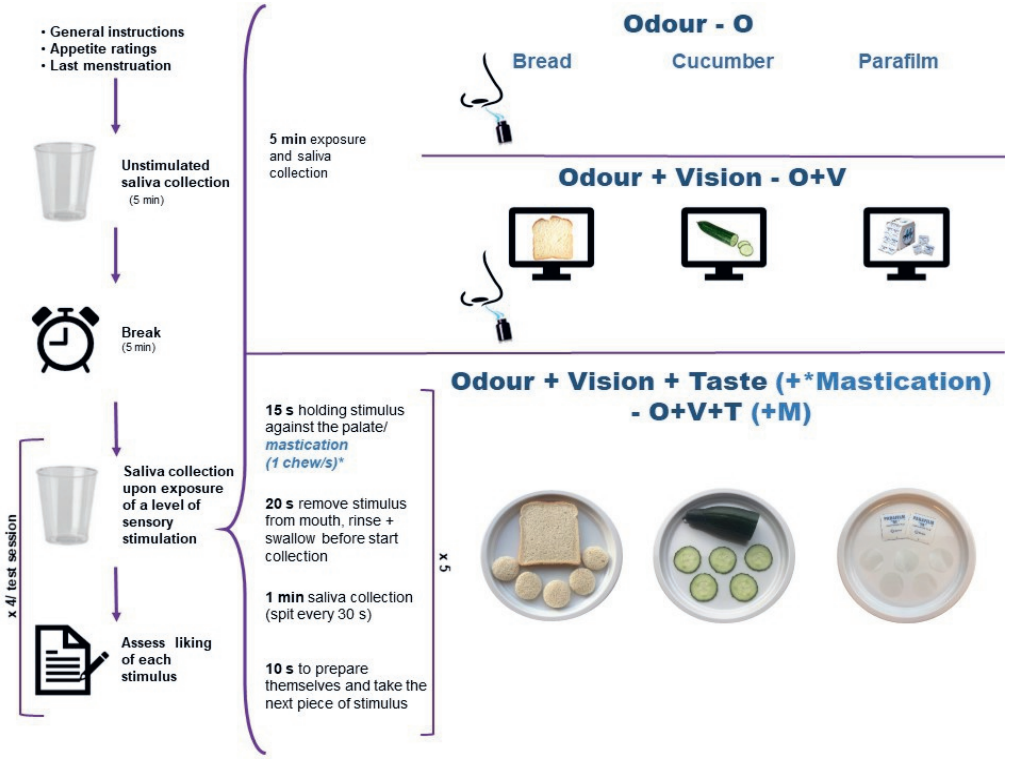

Figure 3.1. Schematic overview of each test session. Bread and cucumber pictures in the O+V condition were taken from food-pics_extended database (Blechert, Lender, Polk, Busch, & Ohla, 2019).

Stimuli and levels of sensory stimulation

Three types of stimuli were investigated: two food stimuli: bread (high-in-starch, 74% polysaccharides (RIVM, 2019)) and cucumber (low-in-starch, 0% polysaccharides (RIVM, 2019)), and parafilm (Parafilm ¨M¨, Bemis Company, Inc., North America) was used as control. Moreover, the food products were selected as they are common in a Dutch diet, and therefore familiar to the participants.

For all three stimuli, participants were exposed to four different levels of sensory stimulation. **Odour (O) –** 15 mL of the food odours were placed in amber opaque glass bottles: bread flavour (205361- Symrise, Holzmiden, Germany, 8% in PG) and cucumber flavour (15311331-International Flavours & Fragrances, IFF, New York, USA, 100%). For the control, 0.3 g (5 x 5 cm sheet) of parafilm was placed in amber opaque glass bottles. By orthonasal olfaction, participants smelled the odour stimulus and their whole mouth saliva was collected simultaneously for 5 min (spitting every 30 s and kept on ice, as described in more detail in section 2.3). **Odour + Vision (O+V) –** The odour stimuli described above were combined with a computer screen showing matching pictures from the 'Food-pics_extended' database (Blechert et al., 2019)): white bread (#439) and cucumber with slices (#267). A picture of the parafilm was created by the researchers (see **Figure 3.1**). Whole mouth saliva was collected during 5 min of simultaneous exposure to an odour stimulus and a matching picture on a computer screen. **Odour + Vision + Taste (O+V+T) –** We standardized weight and diameter of real products to minimize differences in oral experience of sample size. All stimuli were cut in a 4.5 cm diameter circle, representing a normal bite-size. Bread (white casino bread, Jumbo, Veghel, The Netherlands) was toasted 2 min before presenting it using a toaster (Tefal Principio, Groupe SEB, Rumilly, Haute-Savoie, France) set to level one. One piece of bread without crust was 3.2 g ± 0.2. A slide of cucumber was 3.5 g ± 0.2. A double layer of a round (4.5 cm diameter) piece of parafilm was 0.3 g. Participants held the stimulus against the palate with their tongue during the first 15 s. In the next 20 s they expectorated the stimulus in a pre-weighted cardboard cup, rinsed their mouth with tap water and swallowed once more before saliva collection. Whole mouth saliva was collected during the subsequent 1 min (spitting every 30 s). This procedure was repeated five times to obtain a total of 5 min saliva collection, and based on previous literature (Froehlich, Pangborn, & Whitaker, 1987; Hoebler et al., 1998, 2000; Mackie & Pangborn, 1990). As shown in **Figure 3.1**, stimuli were presented on a plate containing 5 round cut pieces of the stimulus plus a 'full version' of the stimulus (a slice of bread, half of a cucumber, two 5 x 5 cm sheets of parafilm) to maintain odour and visual stimulation during the 5 min saliva collection. **Odour + Vision + Taste + Mastication (O+V+T+M) –** We used the same real products described above. Participants were asked to chew the stimulus during the first 15 s (1 chew/s), instead of being held against their palate as described for O+V+T. The following steps were the same as described for O+V+T.

Exposure to multisensory cues and saliva collection

The test sessions followed a similar procedure as described previously (Morquecho-Campos et al., 2019) and were held at the sensory booths at Wageningen University & Research during the morning (9.30 to 11.30 h). Participants were scheduled at the same time during the three test sessions with at least one day in between. They were asked to refrain from drinking alcohol 12 h before the study; to avoid wearing

fragranced products on the day of the test session; to avoid vigorous exercise on the morning of the test session; refrain from acidic and caffeinated beverages 4 h before the study; avoid eating and drinking anything except water in the 2 h prior to the test session; refrain from daily dental hygiene measures including use of mouthwash or chewing gum 2 h before the study; avoid stressful activities at least 30 min before the test session. Each test session lasted around 60 min and instructions were given through EyeQuestion® (Version 3.11.1, Logic8 BV, Elst, The Netherlands).

On arrival to each test session, participants rated their appetite by assessing their hunger, fullness, prospective consumption, and desire to eat on 100 mm VAS anchored by 'Not at all'– 'Very much'. They then indicated the first day of their last menstruation cycle, which was considered as a potential covariate because it may influence certain salivary outcomes. Next, they were asked to rinse their mouth with deionized, distilled water, empty their mouth in a plastic container and to wait for one minute. On the first test session, they underwent a 30 s trial to get familiar with the collection technique, 'passive drooling' method (Navazesh, 1993). Instructions on the screen indicated them to sit down with a slightly tilted head, allowing the saliva to gather in the mouth and to expectorate saliva into a container once in every 30 s. The average weight of those containers was determined in advance. They were instructed to avoid swallowing or moving their mouth or tongue and not to speak during the collection. After a 1-min break, participants were asked to collect their unstimulated saliva for 5 min, spitting every 30 s, into an empty polypropylene 25 mL container (Böttger, Germany), After collecting unstimulated saliva, and between conditions, participants had a 5-min break. They were then randomly exposed to a condition, for which they received a stimulus and an empty container to collect their saliva as specified above, depending on the level of sensory stimulation. The containers were kept on ice during the saliva collection. After each condition, participants assessed liking of the stimulus on a 100 mm VAS anchored by 'Not at all' – 'Very much' (see supplementary material **Table A3.1**). An overview of the procedure is presented in **Figure 3.1**. A total of 690 samples were collected. All the saliva samples were immediately weighed and kept on ice until the determination of pH and buffer capacity. After these measurements, samples were clarified by centrifugation (10 min, 4°C, 10,000 *g*) to remove cellular debris and food residues. The clarified saliva was diluted 1:1 v:v with 150 mM NaCl, to avoid aggregation and precipitation of proteins, aliquoted, and stored at −20°C until α-amylase and mucin analysis (Prodan et al., 2015). Depending on the total amount of saliva collected, we kept at least 1 aliquot of ≤1.5 mL per sample.

Measurements

Saliva secretion rate

Whole mouth saliva was weighed shortly after collection by an analytical gravimetric scale (Adventurer™ Pro, OHAUS Europe GmbH, Greifensee, Switzerland),

assuming that 1g is equal to 1 mL (Chicharro, Lucía, Pérez, Vaquero, & Ureña, 1998). The average weight of the containers was subtracted from the final container weight with the collected saliva. Saliva secretion was divided by the 5-min of collection time and therefore we used mL/min as final units.

Salivary α-amylase concentration and secretion rate

Salivary α-amylase was measured by analysis of colorimetric-based enzymatic activity. Saliva samples were diluted 1:50 with HPLC-grade water. Ten µl of diluted saliva and 90 µl of amylase substrate, 2-chloro-4-nitrophenyl-α-D-maltotrioside (Sigma-Aldrich, St Louis, MO, USA), were added in each well of a 96-well ELISA-microplate (655101, Greiner Bio-One B.V, Alphen aan den Rijn, The Netherlands). α-amylase cleaves the substrate into 2-chloro-4-nitrophenol, a yellow compound. The absorbance of this compound was measured by Multiskan™ FC Microplate Photometer using SkanIt™ Software 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) at 405 nm directly after the addition of amylase substrate for 15 min. A reference of a known concentration of 3U α-amylase standard was included in each plate. All measurements were performed in duplicate. Salivary α-amylase concentration was expressed as units per millilitre (U/mL). Additionally, we calculated the α-amylase secretion rate (U/min) considering the salivation rate (mL/min).

Other salivary characteristics

pH and buffering capacity

Before pH was measured, the samples were homogenized for 20 s by vigorous vortexing. Subsequently, the buffering capacity of the saliva was assessed by adding 1 mL of 0.01 M HCl (pH = 2) to 1 mL of saliva and briefly vortexed (Brand, Ligtenberg, Bots, & Nieuw Amerongen, 2004). The buffering capacity could be assessed only when the collected saliva was ≥2 mL (554 out of the 690 samples). The pH of this solution was measured 30 s after mixing. A digital pH meter was used to assessed pH and buffer capacity (VOS-10001, VOS instrumenten B.V., Gelderland, The Netherlands).

Mucin 5B (MUC5B) concentration

An enzyme-linked immuno sorbent assay (ELISA) was performed to determine the amount of MUC5B in each of the saliva samples following the procedure described by Veerman and collaborators (Veerman et al., 1997). Samples were diluted 1:100 with coating buffer (0.1 M Na_2CO_3; pH = 9.6) and 200 µl was pipetted into the wells of a 96-well ELISA Microlon F-shape Microplate (Greiner Bio-One B.V, Alphen aan den Rijn, The Netherlands). Two-fold serial dilutions of each saliva sample were prepared in the previous coating buffer, in separate wells. The last row of the microplate was used as blank and only coating buffer was added. The microplates were incubated overnight at 4°C to allow the adherence of the mucins to microplate surface. The next day the microplates were rinsed 3 times with phosphate buffered

saline supplemented with 0.1% tween-20 (PBS-T) and blocked with 1% gelatine dissolved in PBS-T (PBS-T-G) and incubating at 37°C for 1 h in a mini shaking incubator, with gentle shaking. Next, the microplates were washed with PBS-T to remove the unbound gelatine. Then 100 µl of the mouse-antibody F2 diluted 1:40 in PBS-T-G was added in each well (Veerman et al., 2003). After 1 h of incubation at 37°C, the microplates were rinsed 3 times with PBS-T to remove unbound F2 antibodies. Next, 100 µl of conjugant rabbit α-Mouse – HRP (GeneTex, Inc., CA, USA) diluted 1:2000 in PBS-T-G was added to each well and the microplates were incubated for 1 h at 37°C. Then the microplates were rinsed 5 times with PBS-T and once with demineralized water. The substrate solution used was a TMB buffer mixture (3.75 mL 3,3',5,5'-tetramethylbenzidene (TMB) in dimethylsulfoxide (DMSO) dissolved in 150 mL TMB buffer supplemented with 30 µL H_2O_2). A total of 100 µl of this solution was added to each well. The reaction was stopped after all the wells turned into a light blue colour (<5 min) by adding 50 µl of 0.1 M H_2SO_4 to each well. The absorption was measured using a Multiskan™ FC Microplate Photometer using SkanIt™ Software 3.1 at 492 nm (Thermo Fisher Scientific, Waltham, MA, USA). A reference saliva sample (from a pool of unstimulated saliva from 10 healthy volunteers) was added to each microplate. The concentration of MUC5B is a relative difference taking the reference sample into account and is reported as absorbance units (AU). All measurements were done in duplicate.

Total protein concentration and secretion rate

Total protein concentration was measured by means of a Protein Assay Kit (Pierce BCA, Thermo Fisher Scientific, Waltham, MA, USA), according to the recommendations of the manufacturer. Samples were diluted 1:1 with HPLC-grade water and 20 µl was pipetted into the wells of a 96-well polystyrene microplate (Greiner Bio-One B.V, Alphen aan den Rijn, The Netherlands). A standard curve of bovine serum albumin was performed in the first two rows of each microplate. The reaction was made by adding 180 µl of BCA reagents (50:1, BCA reagent A:B) into each well and incubating at 37°C for 30 min in a mini shaking incubator. The absorbance was measured by Microplate Photometer using SkanIt™ Software 3.1 at 562 nm (Multiskan™ FC, Thermo Fisher Scientific). All measurements were performed in duplicate. The total protein content was expressed as mg/mL. Due to the limited amount of saliva, this experiment was performed in 70% of the total samples (483 samples out of the 690 samples). Total protein concentration was expressed in mg/mL and we calculated the total protein secretion rate (mg/min) taking the salivation rate (mL/min) into account.

Food recovery

Food recovery in O+V+T and O+V+T+M sensory levels was measured to confirm the compliance of the participants to refrain from swallowing the stimuli. Food recovery was measured taking into account the final weight of the cardboard cups

where participants spat out the stimuli (held or masticated stimuli with saliva), the weight of the 5 stimuli which were weighed while preparing the plate, and the average weight of cardboard cups. Food recovery of the stimuli after O+V+T level was 115.8% ± 2.1 for bread, 98.1% ± 0.7 for cucumber and 119.9% ± 1.8 for parafilm. The recovery after O+V+T+M level was 132.6% ± 2.5 for bread, 109.7% ± 2.7 for cucumber and 127.7% ± 2.2 for parafilm. Most of the food recoveries exceeded the 100% due to saliva expectorated with the stimuli.

Statistical analyses

Parameters are shown as mean and standard error, unless otherwise specified. Results were considered statistically significant when $p < 0.05$. All statistical analyses were carried out with R (version 0.99.902, RStudio Inc, Boston, MA, USA (RStudio Team, 2016)) and graphs were made using Prism GraphPad 5.0 (GraphPad Prism Software). The R codes for all final models can be found in the article online.

After data collection, 8 outliers were removed from the whole data set due to low or high percentage of food recovery (6 data points from cucumber in O+V+T+M were ≤85% of food recovery, and 2 data points from parafilm in O+V+T+M were ≥200% of food recovery; (Wijlens, Erkner, Mars, & de Graaf, 2015)).

Within the same test session, unstimulated saliva secretion rate was subtracted from the saliva secretion rate upon each condition obtaining 'change in saliva secretion rate' as final outcome.

Linear mixed models using *lme4* package (Bates, Mächler, Bolker, & Walker, 2015) were performed to analyse liking of the stimuli, change in saliva secretion rate, salivary α-amylase concentration and secretion rate, pH, buffer capacity, MUC5B concentration, and total protein concentration and secretion rate. The modelling followed a backward approach, and the most parsimonious models were selected by comparing AIC and log-likelihood of the models. For each individual model, homoscedasticity and normal distribution of error terms, and correct specifications of the fixed and random parts of the model were checked. Square root transformation was performed when the model violated these assumptions, this was done for the α-amylase concentration and secretion rate models. In a first stage of the analyses, the level of sensory stimulation, was included as a fixed factor. In a second stage of the analyses, data was divided into the level of sensory stimulation. Individual models were performed for each level of sensory stimulation, with 'type of stimuli' as a fixed factor. Participants and evaluation order nested in test sessions were considered as random factors, indicated by (1|*random effect*) in the results. However, after checking the random part of each model, most of the final models only included participants as random factors, except when indicated. potential

covariates were systematically removed following this order: 1) participants characteristics (age, BMI, dry mouth sensation score, and phase of menstrual cycle which was categorized depending on the day of cycle in follicular, ovulation, or luteal phase); 2) appetite ratings; 3) liking of the stimuli; 4) for α-amylase concentration and secretion rate, pH, and buffer capacity, their respective unstimulated data was also added as covariate; saliva secretion rate was added as covariate in the MUC5B concentration models. The unstimulated data of MUC5B concentration and total protein concentration and secretion rate were considered as a level of sensory stimulation condition. Post-hoc tests with Bonferroni correction using the *lsmeans* package (Lenth, 2016) were performed when the fixed factor was significant. Pearson correlation analyses were done to determine the correlation between pH/buffer capacity and change in saliva secretion rate, on saliva measurements corrected for participants. First, we corrected change in saliva secretion rate by using a mixed model with change in saliva secretion rate as a dependent variable and participants as random effects (Correction_Saliva = lmer(ChangeinSaliva ~ (1|Participant)). The residuals of that model (Correction_Saliva) were saved and used to correlate with pH/buffer capacity.

Results

Change in saliva secretion rate

Levels of sensory stimulation had a significant impact on change in saliva secretion rates ($F(3,495) = 156.7$, $p < 0.0001$, change in saliva secretion rate ~ level of sensory stimulation + liking of the stimuli + (1| participants); **Figure 3.2A**). O and O+V stimulation resulted in the lowest saliva secretion rate and did not differ significantly from each other; while O+V+T and O+V+T+M stimulation produced significantly higher saliva secretion rates.

Analysing the data within each level of sensory stimulation, change in saliva secretion rate differed significantly between the different stimuli (O: $F(2,90) = 4.2$, $p = 0.019$; O+V: $F(2,90) = 3.6$, $p = 0.031$; O+V+T: $F(2,90) = 8.9$, $p < 0.001$; O+V+T+M: $F(2,83) = 36.7$, $p < 0.0001$, change in saliva secretion rate ~ type of stimuli + (1| participants); **Figure 3.2B**). In general, salivation was highest upon exposure to bread, then cucumber, and then parafilm.

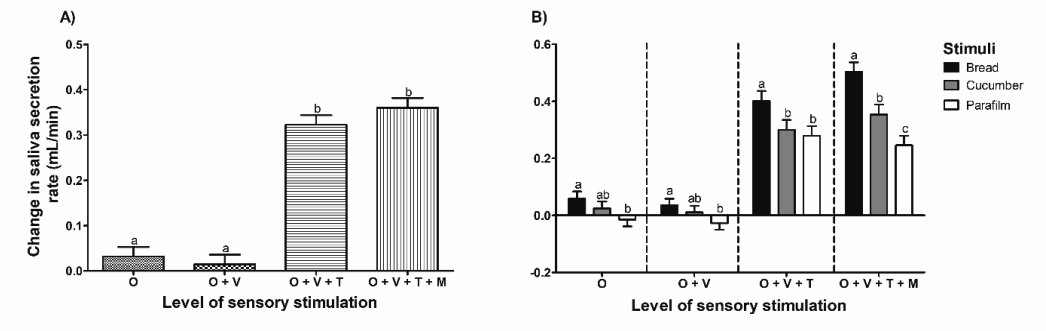

Figure 3.2. Change in saliva secretion rate (mL/min; corrected for unstimulated saliva (mean 0.60 ± 0.02 mL/min)) upon different levels of sensory stimulation (A) and upon different stimuli within each level of sensory stimulation (B). Values are expressed as mean and standard error. Similar letters indicate no significant differences ($p > 0.05$) across level of sensory stimulation (A) and across stimuli type within each level of sensory stimulation (B). O = odour; O+V = odour + vision; O+V+T = odour + vision + taste; O+V+T+M = odour + vision + taste + mastication.

Salivary α-amylase concentration and secretion rate

The square root of α-amylase concentration (U/mL) of the secreted saliva significantly decreased upon the level of sensory stimulation ($F(3,494) = 18.44$, $p < 0.0001$, sqrt (α-amylase concentration) ~ level of sensory stimulation + sqrt (α-amylase concentration) + (1| participants); for back-transformed data see **Figure 3.3A)**. However, square root of α-amylase secretion rate (U/min) significantly

increased upon the level of sensory stimulation (F(3,493) = 3.75, p = 0.011, sqrt (α-amylase secretion rate) ~ level of sensory stimulation + sqrt (α-amylase secretion rate) + liking of the stimuli + (1| participants); for back-transformed data see **Figure 3.3B**). O+V+T+M stimulation produced significantly higher α-amylase secretion rate (U/min) compared to O (p = 0.046) and O+V stimulation (p = 0.011).

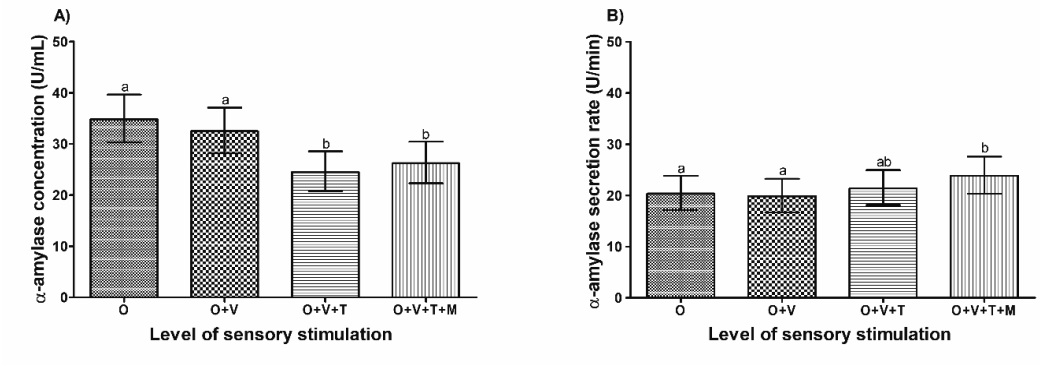

Figure 3.3. Back-transformed α-amylase concentration (U/mL; A) and secretion rate (U/min; B) of secreted saliva upon different levels of sensory stimulation. The data were analysed in a square root scale; however, the data has been back-transformed by means of square, values are thus expressed as median (mean of square root data^2) and 95% CI ((mean ± 1.96*SE)^2). Similar letters indicate no significant differences (p > 0.05). O = odour; O+V = odour + vision; O+V+T = odour + vision + taste; O+V+T+M = odour + vision + taste + mastication.

When the data was analysed within each level of sensory stimulation, only α-amylase secretion rate (U/min) after exposure to O+V+T was significantly different between the stimuli (F(2,97) = 5.16, p = 0.007, sqrt (α-amylase secretion rate) ~ type of stimuli + sqrt (α-amylase secretion rate) + liking of the stimuli + (1| participants); for back-transformed data see **Table 3.2**), where the exposure of parafilm and bread significantly increased the α-amylase secretion rate (U/min) compared to cucumber (p = 0.016 and p = 0.027, respectively).

Other salivary characteristics

pH level and buffering capacity

Similar to saliva secretion rate, level of sensory stimulation had an impact on salivary pH (F(3,447) = 38.63, p < 0.0001, pH ~ level of sensory stimulation + pH from the unstimulated saliva + (1| participants)) and salivary buffering capacity (F(3,349) = 14.52, p < 0.0001, buffer capacity ~ level of sensory stimulation + buffer capacity from the unstimulated saliva + liking of the stimuli + (1| participants)). The pH and buffering capacity after exposure to O+V+T+M were significantly higher compared to the other levels of stimulation (pH: O = 7.01 ± 0.03; O+V = 7.00 ± 0.03; O+V+T =

7.14 ± 0.03; O+V+T+M = 7.26 ± 0.03, $p < 0.0001$; buffering capacity: O = 3.98 ± 0.10; O+V = 3.88 ± 0.10; O+V+T = 3.94 ± 0.10; O+V+T+M = 4.47 ± 0.10, $p < 0.0001$). Moreover, saliva secretion rate was positively correlated with pH ($r(498) = 0.26$, $p < 0.0001$) and buffer capacity ($r(443) = 0.20$, $p < 0.0001$; cor.test(lmer(change in saliva secretion rate ~ (1| participants)), pH/buffer capacity)).

Within each level of sensory stimulation, with the exception of O+V, pH level of saliva differed after exposure to the different stimuli (**Table 3.2**; O: $F(2,83) = 3.32$, $p = 0.041$; O+V: $F(2,80) = 0.18$, $p = 0.84$; O+V+T: $F(2,82) = 25.71$, $p < 0.0001$; O+V+T+M: $F(2,78) = 9.98$, $p = 0.0001$; pH ~ type of stimuli + pH from the unstimulated saliva + (1| participants) for all the models, also desire to eat and satiety were respectively included in O and O+V model). Buffering capacity of secreted saliva was significantly different between the different stimuli after exposure to O+V+T ($F(2,65) = 10.12$, $p = 0.0002$) and O+V+T+M ($F(2,60) = 21.42$, $p < 0.0001$; **Table 3.2**, all models: buffer capacity ~ type of stimuli + buffer capacity from the unstimulated saliva + (1| participants)). In both levels of stimulation, the exposure of bread increased the buffer capacity of the saliva compared to cucumber and parafilm.

Mucin 5B (MUC5B) concentration

The concentration of MUC5B in the secreted saliva decreased significantly upon increasing levels of sensory stimulation ($F(4,77) = 12.58$, $p < 0.0001$, mucin concentration ~ level of sensory stimulation + satiety + prospective consumption + (1| participants) + (1| test session/evaluation order)). MUC5B concentration was significantly lower after O+V+T and O+V+T+M exposure compared to O and O+V exposure and unstimulated saliva. Moreover, MUC5B concentration in O and O+V-stimulated saliva did not differ from unstimulated saliva (**Figure 3.4A**). MUC5B concentration significantly differed among the stimuli in the O+V sensory level ($F(2,86) = 3.12$, $p = 0.049$, mucin concentration ~ type of stimuli + (1| participants)), showing that the exposure of parafilm secreted a saliva with higher MUC5B concentration compared to the exposure of cucumber (**Table 3.2**, all models: mucin concentration ~ type of stimuli + (1| participants)).

Total protein concentration and secretion rate

Total protein concentration of secreted saliva significantly decreased upon increasing levels of sensory stimulation ($F(4,423) = 46.92$, $p < 0.0001$, total protein concentration ~ level of sensory stimulation + (1| participants)). Unstimulated and O and O+V-stimulated saliva had a higher protein concentration compared to O+V+T and O+V+T+M (**Figure 3.4B**).

Protein secretion rate of secreted saliva is modified upon the levels of sensory stimulation ($F(4,421) = 3.68$, $p = 0.006$, protein secretion rate ~ level of sensory stimulation + (1| participants), **Figure 3.4C**). Post-hoc testing revealed that the total

protein secretion rate upon O+V+T sensory level was significantly lower compared to the total protein secretion rate upon unstimulated saliva and upon O+V+T+M (both p = 0.02). However, total protein secretion rate upon unstimulated, O, O+V, and O+V+T+M did not differ significantly.

Within each level of sensory stimulation, total protein concentration was not affected by the different stimuli (**Table 3.2**, total protein concentration ~ type of stimuli + (1| participants) for all models). For protein secretion rate, there was a significant difference among the stimuli in the O+V+T+M sensory level ($F(2,69) = 9.80$, p = 0.0002, no covariates contributed to the fit of this mixed model), showing that the saliva secreted upon bread contained more protein compared to parafilm (**Table 3.2**, all the models: protein secretion rate ~ type of stimuli + (1| participants)).

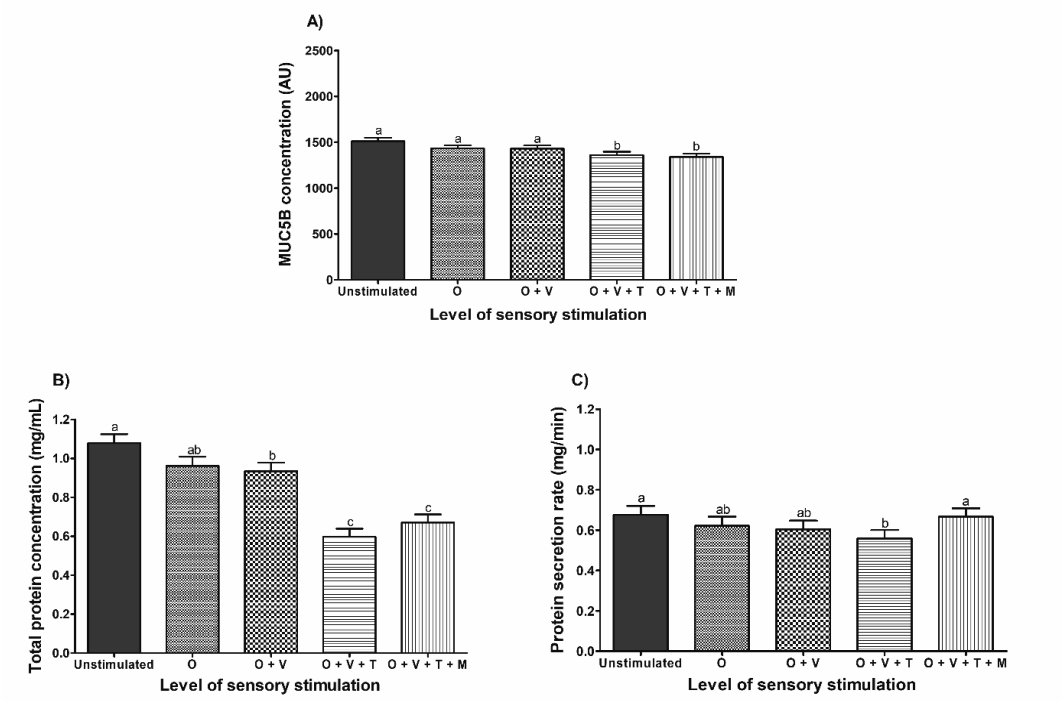

Figure 3.4. MUC5B concentration (AU; A), total protein concentration (mg/mL; B) and protein secretion rate (mg/min; C) of secreted saliva upon different levels of sensory stimulation. Values are expressed as mean and standard error. Similar letters indicate no significant differences ($p > 0.05$). O = odour; O+V = odour + vision; O+V+T = odour + vision + taste; O+V+T+M = odour + vision + taste + mastication.

Table 3.2. pH, buffer capacity, back-transformed α-amylase concentration (U/mL) and secretion rate (U/min), MUC5B concentration (AU) and total protein concentration (mg/mL), and secretion rate (mg/min) of the secreted saliva upon all exposure conditions. Values are expressed in median (mean of square root data^2) and 95% CI ((mean ± 1.96*SE)^2) for α-amylase data and in mean and standard error for the other outcomes. Similar letters indicate no significant differences ($p > 0.05$) within level of sensory stimulation.

Stimuli	Level of sensory stimulation			
	O	O+V	O+V+T	O+V+T+M
α-amylase concentration (U/mL)				
Unstimulated saliva	35.28 (29.70, 41.34)			
Bread	37.58 (31.88, 43.75)	36.24 (30.54, 42.43)	23.52 (19.37, 28.08)	23.23 (19.12, 27.74)
Cucumber	39.56 (33.71, 45.89)	39.56 (33.80, 45.78)	21.72 (17.78, 26.04)	24.21 (19.61, 29.29)
Parafilm	33.18 (27.90, 38.92)	34.81 (28.97, 41.18)	25.70 (21.37, 30.44)	33.06 (26.50, 40.34)
α-amylase secretion rate (U/min)				
Unstimulated saliva	20.16 (16.79, 23.83)			
Bread	22.56 (19.01, 26.42)	21.34 (17.50, 25.57)	21.81 (17.90, 26.10)^a	24.98 (20.64, 29.73)
Cucumber	22.37 (18.85, 26.19)	23.26 (19.39, 27.48)	17.06 (13.41, 21.15)^b	22.80 (18.28, 27.82)
Parafilm	18.40 (15.26, 21.85)	19.72 (15.89, 23.97)	26.11 (20.84, 31.98)^a	29.48 (23.24, 38.48)
pH				
Unstimulated saliva	7.06 ± 0.03			
Bread	7.00 ± 0.03^{ab}	7.01 ± 0.04	7.26 ± 0.04^a	7.36 ± 0.03^a
Cucumber	7.08 ± 0.03^a	6.99 ± 0.04	6.97 ± 0.04^b	7.17 ± 0.03^b
Parafilm	6.97 ± 0.03^b	6.98 ± 0.04	7.16 ± 0.04^a	7.25 ± 0.03^b
Buffering capacity				
Unstimulated saliva	3.93 ± 0.07			
Bread	3.99 ± 0.11	4.01 ± 0.11	4.43 ± 0.15^a	5.09 ± 0.16^a
Cucumber	4.01 ± 0.11	3.70 ± 0.11	3.85 ± 0.14^b	4.32 ± 0.16^b
Parafilm	3.88 ± 0.12	3.83 ± 0.11	3.66 ± 0.14^b	3.98 ± 0.16^b

	MUC5B concentration (AU)			
Unstimulated saliva	1513.59 ± 23.62			
Bread	1439 ± 34.80	1435 ± 35.40[ab]	1337 ± 39.50	1336 ± 40.40
Cucumber	1433 ± 34.80	1386 ± 36.00[a]	1330 ± 39.40	1354 ± 41.80
Parafilm	1420 ± 34.50	1476 ± 35.70[b]	1365 ± 39.30	1339 ± 41.20
	Total protein concentration (mg/mL)			
Unstimulated saliva	1.08 ± 0.05			
Bread	0.93 ± 0.07	0.95 ± 0.07	0.54 ± 0.04	0.70 ± 0.04
Cucumber	1.00 ± 0.07	0.89 ± 0.08	0.64 ± 0.04	0.68 ± 0.05
Parafilm	0.87 ± 0.07	0.96 ± 0.08	0.55 ± 0.04	0.63 ± 0.05
	Total protein secretion rate (mg/min)			
Unstimulated saliva	0.68 ± 0.05			
Bread	0.69 ± 0.06	0.61 ± 0.06	0.58 ± 0.05	0.79 ± 0.05[a]
Cucumber	0.67 ± 0.06	0.62 ± 0.06	0.61 ± 0.05	0.67 ± 0.06[ab]
Parafilm	0.58 ± 0.06	0.57 ± 0.06	0.51 ± 0.05	0.54 ± 0.06[b]

Discussion

The aim of our study was to investigate different levels of sensory stimulation and specific food products (differing in starch content) in relation to saliva secretion, α-amylase (concentration and secretion rate) and other salivary characteristics including pH and buffering capacity, MUC5B concentration and total protein (concentration and secretion rate). Our results confirm that saliva secretion increases upon the level of sensory stimulation. Moreover, the highest level of sensory stimulation (odour + vision + taste + mastication) induces the greatest α-amylase secretion rate compared to anticipatory sensory cues (odour and odour + vision). Interestingly, our results suggest that the level of sensory stimulation may play a more critical role for α-amylase concentration and secretion rate rather than the specific food product. Salivary characteristics such as pH and buffer capacity increased while other characteristics such as MUC5B and total protein concentration decreased upon the level of sensory stimulation and the secretion of saliva. Moreover, protein secretion rate seems to remain similar across the levels of sensory stimulation, except of O+V+T which secreted significantly lower protein compared to unstimulated saliva and O+V+T+M.

As expected, saliva secretion rate increased upon the levels of sensory stimulation. Remarkably, we observed a clear distinction difference between anticipatory sensory cues (odour and odour + vision) versus consummatory sensory cues (odour + vision + taste and odour + vision + taste + mastication). These results are in line with previous research that showed a higher saliva secretion upon taste or mastication compared to smell and sight (Ilangakoon & Carpenter, 2011; Pangborn et al., 1979). Saliva secretion rate did not increase from the odour to the odour + vision condition, even though extra sensory information was added. Previous studies have shown that salivation upon exposure to food pictures is similar to unstimulated saliva (Birnbaum, Steiner, Karmeli, & Ilsar, 1974; Hayashi, 1968; Ilangakoon & Carpenter, 2011; Richardson & Feldman, 1986). However, when using a real food product as visual stimulation, salivation is significantly higher compared to unstimulated saliva or to odour exposure (Christensen & Navazesh, 1984; Ilangakoon & Carpenter, 2011; Klajner et al., 1981; Rogers & Hill, 1989; Sahakian et al., 1981; Wooley & Wooley, 1973). We postulate that a digital picture may not add additional anticipatory information on top of the odour cue, whereas the use of a real food product could be related to more realistic expectations of consumption. Moreover, the combination of odour + vision + taste may provide sufficient information to secrete a higher amount of saliva and adding more sensory information (by mastication) would not increase it any further. It is noteworthy to mention that the similar results between the two consummatory levels (odour + vision + taste and odour + vision + taste + mastication) might be related to the procedure. We collected the saliva after ‘the activation of the saliva’ upon the stimuli rather than during the tasting and chewing of the stimuli. There is no golden standard procedure for the collection of saliva

during/after taste or mastication exposure, which is a complex procedure. The experiment was designed based on our research questions, and the selected procedure was based on previous literature (Froehlich et al., 1987; Hoebler et al., 1998, 2000; Mackie & Pangborn, 1990). Moreover, previous research performed on taste solutions or mastication (food or non-food stimuli) has shown an increase in salivation (e.g. Engelen et al., 2003; Ilangakoon & Carpenter, 2011; Pangborn & Lundgren, 1977). Although we strictly controlled the (multi)sensory exposure in each level of sensory stimulation (e.g. participants were simultaneously exposed to the smell and sight of the stimuli with a plate containing the stimuli in front of them, while having a stimulus in the oral cavity), it is possible that results from the consummatory levels are driven solely by taste and/or mastication, rather than the added combination of all levels of sensory stimulation.

Bread (food high-in-starch) enhanced salivation compared to the non-food control (parafilm), regardless of the level of sensory stimulation. However, only for both consummatory levels of stimulation (odour + vision + taste and odour + vision + taste + mastication) did saliva secretion increase upon exposure to bread compared to cucumber (low-in-starch). These results may be explained by the fact that these food products differed in their water content. Bread is a dry food product which contains 7.5 and 37.3 g of water/100 g of product for toasted and untoasted bread, respectively; while cucumber contains 96.7 g of water/100 g of product (RIVM, 2019). It has been suggested that dry food products, such as bread, toast, etc., may require longer mastication cycles, increasing saliva production for a proper lubrication, bolus formation, and further swallowing (Engelen, Fontijn-Tekamp, & van der Bilt, 2005; Gavião, Engelen, & van der Bilt, 2004; Guinard, Zoumas-Morse, & Walchak, 1998; Mackie & Pangborn, 1990; Pangborn & Lundgren, 1977). Moreover, others have suggested that liking of a food product plays a role in salivation (Christensen & Navazesh, 1984; Klajner et al., 1981). However, both food products were selected to be moderately liked (>60 mm on a 100 mm VAS), and individual liking ratings were considered in data analyses, thus discounting this as a potential confounding factor.

Salivary α-amylase concentration (U/mL) decreased upon the level of sensory stimulation while α-amylase secretion rate (U/min) increased after odour + vision + taste + mastication sensory stimulation compared to the anticipatory levels (odour and odour + vision). Carreira and colleagues showed a similar α-amylase concentration after bread odour exposure and after mastication of bread compared to unstimulated saliva (Carreira et al., 2020). The α-amylase secretion rate (U/min) results are in line with previous research which also compared modified sham feeding to smell and sight stimulation and showed a higher secretion of gastric acid with consummatory stimulation compared to the anticipatory ones (Feldman & Richardson, 1986). Perhaps surprisingly, this study has not been able to demonstrate a specific effect of starch content on α-amylase concentration nor on

secretion rate. Mackie and Pangborn showed a higher α-amylase secretion rate (U/min) upon chewing food high-in-starch (bread) for 15 sec compared to food low-in-starch (celery) and parafilm (non-food control). However, the α-amylase concentration (U/mL) was similar across the different conditions (Mackie & Pangborn, 1990). Additionally, in contrast to the present findings, they collected unilateral parotid saliva, while we collected whole mouth saliva. α-amylase is one of the most abundant proteins in saliva and is particularly secreted by the parotid gland (Carpenter, 2013). α-amylase can be directly collected from the parotid gland, where the highest percentage of α-amylase is produced, by means of Lashley cups (Lashley, 1916a; Mackie & Pangborn, 1990; Navazesh, 1993). However, we focused on whole mouth saliva secretion to test our hypotheses that involved salivation from the different salivary glands. Moreover, the 'passive drooling' method (collection method for the whole mouth saliva) is less complicated to collect and less invasive for participants compared to the use of the Lashley cup (Navazesh, 1993).

Further research could analyse the starch breakdown of the food products to give more insights about the amount of starch that could already have been hydrolysed by the α-amylase. Some researchers suggest that around half of the total starch content in food (e.g. bread and wheat) is hydrolysed into oligosaccharides upon a short modified sham feeding exposure (Hoebler et al., 1998, 2000; Woolnough et al., 2010).

Regarding the salivary characteristics, we found a positive weak correlation between saliva secretion and pH/ buffering capacity. Our results are in line with previous studies that showed a linear relation between salivation and the release of bicarbonate ions, modifying the pH and buffering capacity of secreted saliva (Bardow, Madsen, & Nauntofte, 2000; Bardow, Moe, Nyvad, & Nauntofte, 2000; Thaysen, Thorn, & Schwartz, 1954). Upon activation of the parotid gland through the consummatory levels of stimulation, the levels of bicarbonate increase leading to a slightly increased pH and stronger increased buffer capacity. The bicarbonate ions are converted upon the release of the watery portion of the saliva through the ducts (Edgar, Dawes, & O'Mullane, 2012). These bicarbonate ions produce a more basic environment, increasing the pH which supports prevention of enamel demineralization (Pedersen et al., 2018).

Moreover, MUC5B concentration significantly decreased upon exposure to the more consummatory levels of stimulation compared to the anticipatory sensory cues and unstimulated saliva. As reported in literature, unstimulated saliva is more visco-elastic, suggesting a saliva richer in mucins, compared to stimulated saliva (Mese & Matsuo, 2007; Rantonen & Meurman, 1998). Also, saliva upon chewing exposure has been found to be significantly less elastic compared to saliva upon citric acid exposure and unstimulated saliva (Stokes & Davies, 2007). MUC5B may be continuously secreted and less prone to stimulation (Dawes et al., 2015; Stokes &

Davies, 2007). Our results suggest that the composition of saliva stimulated by anticipatory sensory cues (odour and odour + vision) is similar in mucin concentration to unstimulated saliva.

Furthermore, the total protein concentration decreased significantly upon exposure to the consummatory levels of stimulation (odour + vision + taste and odour + vision + taste + mastication) compared to the anticipatory sensory cues and unstimulated saliva. Carreira and colleagues showed that the protein concentration of the unstimulated saliva was significantly higher compared to the saliva upon bread odour exposure but similar after the mastication of bread or rice (Carreira et al., 2020). Except for the protein secretion rate in the saliva upon odour + vision + taste, our results suggest that the protein secretion rate remains stable over the different levels of sensory stimulation. It could be argued that the decreased protein secretion rate in the saliva upon odour + vision + taste was due to the saliva secretion rate induced for that level of sensory stimulation.

The proteins predominantly present in saliva are α-amylase, proline-rich proteins, and mucins. Stimulation of saliva by means of sensory cues can immediately enhance the secretion of water but not of other components, resulting in a watery and serous saliva with low percentage of proteins and other components (Levine, 2010). Some researchers have suggested that the decrease of total protein concentration upon some sensory cues is related to a dilution effect (Carreira et al., 2020; Neyraud et al., 2009). Therefore, a comparison between the total protein concentration and the protein secretion rate results may suggest that the decrease in the total protein concentration for the consummatory levels is in fact a dilution effect. A moderate negative correlation between total protein concentration and salivary secretion rate upon stimulation has been reported (Levine, 2010; Neyraud, Heinzerling, Bult, Mesmin, & Dransfield, 2009; Prodan et al., 2015). A recent systematic review suggests that mastication has little or a negative effect on salivary proteins concentration (α-amylase, mucin, and total protein), which could be mainly affected by salivation flow (Al-Manei, Almotairy, Bostanci, Kumar, & Grigoriadis, 2019).

Conclusion

Exposure to multisensory consummatory cues induces larger changes in saliva secretion rate and its composition than anticipatory sensory cues. However, changes in composition may be influenced by the combination of several sensory modalities (mainly gustatory and mechanical, via mastication) rather than by specific (food) products. This study has provided deeper insight into the role of (multi)sensory food cues in anticipatory eating responses.

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Author Contributions

P. Morquecho-Campos, F. J. Bikker, C. de Graaf, M. L. Laine, and S. Boesveldt jointly conceptualized and developed the study design. P. Morquecho-Campos collected and analysed the data. K. Nazmi and P. Morquecho-Campos determined the salivary components. P. Morquecho-Campos drafted the manuscript under the supervision of S. Boesveldt, F. J. Bikker, K. Nazmi, and M. L. Laine.

Appendix 3.1

Table A3.1. Liking of the stimuli (on 100 mm VAS). Values are expressed as mean and standard error. Similar letters indicate no significant differences ($p > 0.05$) within level of sensory stimulation.

Stimuli	Level of sensory stimulation			
	O	O+V	O+V+T	O+V+T+M
Bread	59.7 ± 3.1[a]	60.6 ± 3.0[a]	64.4 ± 2.3[a]	73.45 ± 2.04[a]
Cucumber	45.3 ± 3.1[b]	53.1 ± 3.0[a]	71.9 ± 2.3[b]	77.29 ± 1.87[a]
Parafilm	40.0 ± 3.1[b]	31.4 ± 3.0[b]	27.6 ± 2.3[c]	28.74 ± 2.50[b]

Chapter 4

Smelling our appetite? The influence of food odours on congruent appetite, food preferences and intake

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Abstract

We are surrounded by sensory food cues, such as odours, that may trigger (un)conscious decisions and even lead to (over)eating, it is therefore crucial to better understand the effect of food odours on behavioural responses. Food odour exposure has been shown to enhance appetite for food products with similar properties: sensory-specific appetite. This suggests that based on previous encounters with foods, we have learned to detect the nutritional content of foods, through our sense of smell. We investigated the influence of aware exposure of macronutrient-related odours on various measures of eating behaviour, in a cross-over intervention study. Thirty-two normal-weight healthy and unrestrained Dutch females took part in five test sessions. On each test session, they were exposed to one of five conditions (active smelling of clearly noticeable odours representing food high in carbohydrates, protein, and fat, low in calories, and a no-odour condition for 3 min) and assessed on specific appetite, food preferences, and intake. Odour exposure increased congruent appetite after protein-related odour exposure. Similarly, protein-related odour exposure influenced the liking for protein foods and the preference ranking for savoury products. However, food intake was not affected by smelling congruent food odours. Together this indicates that exposure to (aware) food odours may mostly influence appetite, but does not impact subsequent food intake. Moreover, appetite seems to be triggered by taste qualities rather than macronutrient information of the food, as signalled by olfactory cues. Future studies should investigate the role of awareness in more detail, to fully understand how odours might be used to steer people towards healthier food choices.

Introduction

Living in an obesogenic environment, we are surrounded by food (odour) cues that may trigger (un)conscious decisions and induce us to (over)eat (Bellisle, 2003; Boesveldt & de Graaf, 2017; King, 2013). Exposure to food cues may induce anticipatory physiological responses and may influence on our appetite and food intake (Proserpio, de Graaf, Laureati, Pagliarini, & Boesveldt, 2017; Wooley & Wooley, 1973). Olfaction plays an important role in eating behaviour by detecting food, attracting our attention, and triggering our appetite (Boesveldt & de Graaf, 2017; Stevenson, 2010). However, the effect of food odours on subsequent behavioural responses is still not fully understood.

Olfaction may play a role in appetite and meal initiation (Yeomans, 2006a; Zafra, Molina, & Puerto, 2006). Some studies have suggested that food cues exposure increases appetite for foods with similar properties and decreases for foods with dissimilar properties, known as *sensory-specific appetite* (SSA) (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Gort, et al., 2014; Ramaekers, Boesveldt, Lakemond, van Boekel, & Luning, 2014; Zoon, de Graaf, & Boesveldt, 2016). SSA may be generalized across foods within certain categories as taste and energy-density (Ferriday & Brunstrom, 2008, 2011; Ramaekers, Boesveldt, Gort, et al., 2014; Ramaekers, Boesveldt, Lakemond, et al., 2014; Zoon et al., 2016). A brief exposure to visual and odour cues of pizza showed an increase in desire to eat and prospective intake of pizza and savoury food, and decrease for sweet food (Ferriday & Brunstrom, 2008, 2011). Similarly, Ramaekers et al. showed that suprathreshold active smelling (Ramaekers, Boesveldt, Gort, et al., 2014) and ambient exposure (Ramaekers, Boesveldt, Lakemond, et al., 2014) of sweet odours may enhance appetite for sweet foods and reduce appetite for savoury foods, and *vice versa*. Moreover, Zoon et al. replicated these findings, and showed that actively smelling high-calorie odours could increase appetite for high-calorie food and decrease appetite for low-calorie food, and *vice versa* (Zoon et al., 2016). Taken together, it seems that based on previous experiences, we have learned that food odour cues may convey information related to the taste quality or caloric content of the associated food. This information may even signal the composition of the food in terms of macronutrient content and thereby induce congruent appetite to facilitate specific physiological responses and potentially steer towards congruent actual food intake (Berthoud, Münzberg, Richards, & Morrison, 2012; Boesveldt & de Graaf, 2017; McCrickerd & Forde, 2016; Ramaekers, Boesveldt, Lakemond, et al., 2014; Smeets, Erkner, & de Graaf, 2010; Zoon et al., 2016). Research to date has not yet investigated if food odours that signal macronutrient content may impact congruent appetite and food intake.

Moreover, contradictory findings have been shown between the influence of odours on appetite versus actual food choice and intake depending on the level of

awareness of the odour exposure. Studies with conscious and detectable ambient odours have shown an influence on self-reported appetite (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Gort, et al., 2014), but not on food preference and intake (Zoon, He, Wijk, Graaf, & Boesveldt, 2014). Conversely, other studies suggested that non-conscious exposure to ambient odours, also known as priming, could lead towards selecting congruent foods (Chambaron, Chisin, Chabanet, Issanchou, & Brand, 2015; de Wijk & Zijlstra, 2012; Gaillet-Torrent, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2014; Gaillet, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2013). Overall, findings suggest that (un)conscious odour stimulation may play a crucial role in the (type of) response it exerts (McCrickerd & Forde, 2016; Smeets & Dijksterhuis, 2014).

Very little is currently known about the impact of food odours that signal specific nutrient information on various measures of congruent eating behaviour within the same participants. Therefore, in the present study we aimed to investigate how actively smelling odours that signal macronutrients would impact specific appetite, food preferences, and intake in unrestrained normal-weight females. We hypothesized that appetite would be higher for food products upon the exposure of congruent food odours compared to incongruent food odour (e.g. exposure to carbohydrate-related odours will increase specific appetite for carbohydrate-rich foods, such as bread or pasta, compared to incongruent products such as meat (protein), cream (fat), melon (low-calorie), etc.). However, this specific appetite may be overruled by cognitive factors (such as knowledge of post-ingestive effects of the foods, previous meals, specific health goals, or eating habits), and not necessarily lead to similar food preferences and intake.

Materials and Methods

Participants

Statistical power was calculated based on previous research (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Proserpio et al., 2017; Ramaekers, Boesveldt, Gort, et al., 2014; Zoon et al., 2016) and led to 32 participants. Normal-weight Dutch females between 18 and 35 years old were recruited from Wageningen and surroundings. Inclusion criteria consisted of: a normal sense of smell (scoring ≥12 on the 16 items Sniffin' Sticks odour identification test (Oleszkiewicz, Schriever, Croy, Hähner, & Hummel, 2019)); self-reported normal sense of taste; being an unrestrained eater (score <3.40, out of a maximum of 5), on the Dutch Eating Behaviour Questionnaire, DEBQ (van Strien, Frijters, Bergers, & Defares, 1986); correct identification of the used odours by means of a multiple forced-choice task; odour-label association ('How well do you think this smell corresponds to *[specific label]*?') and liking for the food odours and products used in the study (>40 on 100 mm VAS). Participants were excluded when they were: smokers; had any dietary restriction towards specific foods (self-imposed or otherwise; e.g. vegetarian, vegan); used medication other than paracetamol and hormonal contraceptives; were pregnant or had the intention to become pregnant during the experiment or were currently breastfeeding; or reported weight loss or weight gain of more than 5 kg or following a special diet in the two months prior to the study.

Potential participants provided written informed consent at the start of the screening session. After analysing the data from the screening session, a total of 32 unrestrained females (DEBQ: restrained score of 2.6, SD = 0.7, range 1.1–3.3) with an average age of 21.9 year (SD = 2.2), average BMI of 21.6 (SD = 1.6) kg/m^2, and normal sense of smell (odour identification score of 13.3, SD = 1.2, range 12–16) were included in the study. They received monetary compensation for their contribution. The study was conducted in accordance with the Declaration of Helsinki (revised in 2013) and approved by the Medical Ethical Committee of Wageningen University (NL66580.081.18).

Procedure

In a cross-over study design encompassing five test sessions, participants were exposed to three macronutrient-related odours (carbohydrates, proteins, and fat), a low-calorie odour, and a no-odour control condition. On each test session, participants were exposed to either one of the two odours of a certain category (see section 'Odour stimuli' for further details). The same odour was presented twice to the participants during the test session (**Figure 4.1**). Each participant was randomly assigned to a unique sequence of odours. Test sessions took place around lunch

time (11.30–14.00). Participants attended all test sessions at the same time of the day, with at least two days in-between sessions. Participants were asked not to eat or drink anything, apart from water, at least 3 h before testing. An alternative goal ('To investigate the role of different odours on alertness') was formulated to keep participants naïve for the actual aim of the study, to avoid influences of cognitive factors and participants' expectations on study outcomes.
Upon arrival, participants were asked to rate their general and specific appetite in isolated sensory booths. Then, they received a bottle containing an odour stimulus and were instructed to smell the odour and rate it on several attributes. Next, they were instructed to hold the bottle under their nose and breathe normally for 3 min. After odour exposure, they rated their specific appetite and performed a computer-based task on food preferences. Subsequently, they received the same odour stimulus again to smell for 3 min with similar instructions. Thereafter, participants were escorted to a dining room where they were could select lunch from a salad bar to covertly measure *ad libitum* food intake. After participants finished their lunch, they were escorted back to the sensory booths to assess their general appetite and perform a bogus task to measure alertness (Psycho Vigilance Test, PVT (Basner & Dinges, 2011)). Participants followed instructions via an online questionnaire using EyeQuestion® (Version 3.11.1, Logic8 BV). On the last test session, participants were asked to complete a final questionnaire on the aim of the study. Then, they were debriefed. The procedure for each test session is shown in **Figure 4.1**.

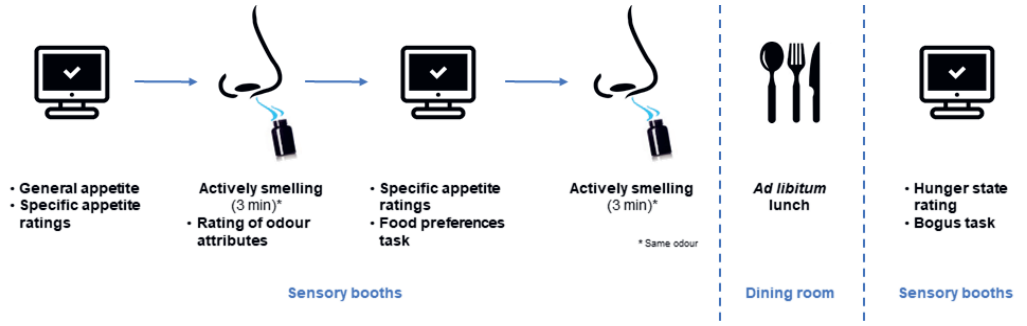

Figure 4.1. Procedure for each test session. The bogus task (alertness task) was performed as part of the alternative goal for keeping participants naïve from the actual aim of the study.

Odour stimuli

Odour stimuli encompassed a selection of eight odours that represented foods differing in macronutrient composition (i.e. high in carbohydrates, protein, fat, or low-calorie). The selection of odours was based on the nutritional value of their food counterpart: at least 50% of total energy of the food should be derived from the specific macronutrient category, and low-calorie products should contain no more

than 60 kcal/100 g (de Bruijn, de Vries, de Graaf, Boesveldt, & Jager, 2017; RIVM, 2016). Based on this and our previous study (Morquecho-Campos et al., 2019), two odours of each category were selected: corn (Symrise 653316; 0.8% in demineralized water, DW) and bread (Symrise 205361; 9% in propylene glycol, PG) for carbohydrates; duck (Symrise 619322; 0.4% in PG) and chicken (IFF 10913579; 0.06% in DW) for protein; butter (IFF 10922603; 0.5% mixed with diacetyl from Sigma-Aldrich; 0.01% in PG) and cream (IFF 10923144; 10% in PG) for fat; cucumber (IFF 15311331; 100%) and melon (IFF 15025874; 2% in PG) for low-calorie foods; and an odourless control (100% PG). Participants were counterbalanced exposed only to one odour per category. Odour stimuli (15 mL) were placed in (randomly-coded) brown 50 mL glass bottles. Odour stimuli were prepared at least one day before the experiment and stored in a refrigerator (4°C) until the morning of the test session. Odour stimuli were taken out of the refrigerator to reach room temperature on the morning of each test session.

Measurements

Odour attribute ratings

Each odour was assessed on the attributes of liking, intensity, familiarity, intention to eat a product with that odour, and mouth-watering sensation on a 100 mm visual analogue scale (VAS) anchored by 'Not at all' to 'Very much'. Then, participants were asked to identify the odour among a list of food products including 'no-odour' by a multiple forced-choice task ('Which of the following labels best fits the odour?'). Afterwards, odour-label association was assessed by 'How well do you think this smell corresponds to *[specific label]?'* on a 100 mm VAS. Ratings for the odours can be found in the supplementary materials (**Table A4.1**).

General and sensory-specific appetite

General appetite was determined by assessing hunger, fullness, prospective consumption, desire to eat, and thirst on 100 mm VAS. Specific appetite was assessed by rating 'How much would you like to eat *[specific product]* at this moment?' on a 100 mm VAS anchored by 'Not at all' to 'Very much'. Specific products consisted of 12 food items (3 per macronutrient category), that either did or did not match the odour stimuli (congruent/incongruent): pasta, bread, and corn for carbohydrates; chicken, tuna, and meat for protein; bacon, nuts, and cheese for fats; cucumber, tomato, and melon for low-calorie food products.

Food preferences

Food preferences were measured by means of the Macronutrient and Taste Preference Ranking Task (MTPRT) (de Bruijn et al., 2017), ran on E-prime (E-Prime 2.0, Psychology Software Tools, Pittsburg, PA). This validated computer-based task consists of 3 parts: practising, liking, and ranking. The practising part is meant to familiarize the participants with the ranking part. The liking and ranking part used 32

food pictures which consisted of 4 macronutrient categories (food products rich in carbohydrates, protein, fat, and low-calorie); each category consisted of 4 sweet and 4 savoury food products; except for the protein category for which the 8 products were savoury food products. The 32 food pictures are different from the ones used in the practising part. After the practising part, the participants rated their liking of the 32 food products ('How much do you like *[specific food product]?'* on a 100 mm VAS, anchored by 'Do not like at all' to 'Like extremely'; a picture of the food product was displayed below the liking question on the screen). Liking ratings were aggregated per macronutrient and taste category for analyses. The ranking part consisted of 2 sections: pictures of foods representing macronutrient categories (high-carbohydrates, high-protein, high-fat, and low-calorie; 16 different combinations), and taste categories (sweet and savoury taste; 24 different combinations). In each combination, the screen displayed 4 different pictures (1 per category in the macronutrient section and 2 per category in the taste section). Participants were asked to rank the food products depending on their desire to eat at that moment. The order and screen position of the food pictures were randomly displayed and balanced across trials. Macronutrient and taste preference scores were calculated as described in de Bruijn et al. (2017). Macronutrient preference scores can range between 1 and 4 (the total score divided by the 16 times a food product from each category was displayed), with higher scores indicating a higher preference. Taste (savoury or sweet) preference scores can range from 1.5 to 3.5 (the total score divided by the 24 times a food product from both categories were displayed). Due to the nature of the ranking part, sweet and savoury scores are opposite to each other, therefore we only report savoury taste preference scores.

Food intake

The *ad libitum* lunch consisted of a salad bar that contained 8 toppings (2 options per macronutrient: carbohydrates, protein, and fat, and 2 low-calorie products), lettuce as a base, and (optionally) 28 g of salad dressing. The selection criteria for the toppings were the same as for the odour stimuli: at least 50% of the total energy of a food product should be derived from the corresponding macronutrient category, while toppings representing the low-calorie category contained no more than 60 kcal/100 g. **Table 4.1** shows the food products per category, calories, and percentages of each macronutrient. All food products were regular products that are commercially available to consumers in a supermarket. Participants were instructed to build their own salad (with lettuce as a base) by choosing as many toppings and amount as they wanted. Participants were not allowed to go back to the buffet area or refill their plate once they started eating. Only one participant was allowed in the buffet area at any one time. They were instructed to sit in the dining area and to eat until they felt comfortably satiated and were not obliged to finish their plate. The setting of the dining area was organized in order to refrain participants from facing the buffet area. The buffet was continuously refilled, to ensure a consistent

presentation volume. Food intake was covertly measured by weighing the trays which contained each topping and lettuce before and after each participant 'built' their salad and by weighing the remaining amount on the plate of the participants after eating. Additionally, they received a glass of water (150 mL) that they were instructed to finish during the lunch.

Table 4.1. Food products offered at the *ad libitum* lunch.

Category	Food	Energy (kcal/100 g)	Carbohydrates (%)	Protein (%)	Fat (%)
Carbohydrates	Croutons	455	**57.1**	11.4	29.7
	White pasta	142	**78.0**	14.4	5.7
Protein	Ham strips	115	1.0	**69.6**	27.4
	Chicken strips	110	12.7	**72.7**	16.4
Fat	Mixed nuts	674	2.6	12.6	**82.5**
	48+ Gouda cheese	375	0.0	25.6	**74.4**
Low-calorie	Cucumber	12	63.3	20.0	15.0
	Cherry tomatoes	30	53.3	12.0	24.0
Base	Lettuce	15	29.3	34.7	18.0
	Dressing	40	95	0.1	2.3

Numbers in bold highlight the largest percentage of the total energy of the food product, which are related to their corresponding food category.

Statistical analyses

Data are shown as mean and standard error, unless otherwise specified. Results with a *p* value lower than 0.05 were considered statistically significant. All statistical analyses were carried out in RStudio (RStudio Team, 2016), and graphs were made using GraphPad Prism 5.0 (GraphPad Prism Software).

All analyses consisted of linear mixed models, carried out using the *lme4* statistical package in R (Bates, Mächler, Bolker, & Walker, 2015). The best fitting models were selected on the basis of parsimony. Necessary assumptions for mixed models were checked for each model. Post-hoc tests with a Bonferroni correction were performed given significant main or interaction effects, using the *lsmeans* statistical package (Lenth, 2016). Data were pooled over the 2 odours of the same category as described in the section 'Odour stimuli' (e.g. data resulting from exposure to 'bread' and 'corn' odour were collapsed into a 'carbohydrates' category) resulting in a variable labelled as 'odour category'.

Odour attribute ratings were analysed as a dependent variable, with odour category as fixed effects and participants as random effects and shown in **Table A4.1** of the supplementary materials.

Moreover, a variable ‘awareness of the true study aim’ was computed based on the results from the debriefing. This variable was added to all the models to test if awareness of the true study impacted our different outcomes. This variable and other potential covariates that were not significantly different were removed from the final models.

Sensory-specific appetite

Appetite data were labelled as congruent/incongruent, depending on the food products used relative to the odour condition. For example, specific appetite of carbohydrate-rich products (such as pasta, bread or corn) were considered congruent after exposure to a carbohydrate-related odour (bread or corn odour) but labelled as incongruent after exposure to other odour categories. Change in sensory-specific-appetite (SSA; difference in the specific appetite ratings before and after odour exposure) was analysed as the dependent variable. Odour category, (in)congruency of food product, and their interaction were included as fixed factors, participants were included as random factors, and general appetite ratings (hunger, fullness, prospective consumption, desire to eat, and thirst were added individually), individual odour attribute ratings, specific appetite before odour exposure, and liking of food products (as assessed during the screening session on a 100 mm VAS) were included as potential covariates. Due to this congruency variable, as there are no food products congruent with the no-odour condition, this condition was removed from the dataset when running the models. Results from the no-odour condition, descriptive statistics, and statistical analyses, were reported using food product category as the fixed factor. Covariates mentioned above were also used in this model.

Food preferences

For the liking and ranking results of the macronutrient part, congruency was similarly determined by the match between odour exposure category and each macronutrient preference score analysed. For example, when analysing preference score for carbohydrates, only exposure to carbohydrate-related odours was considered congruent, all other odour exposure conditions were incongruent. Each macronutrient preference score was analysed as the dependent variable in separate linear mixed models. Congruency and participants were included as fixed and random factors, respectively. For these models, the no-odour condition was removed from the dataset. Data from exposure to the no-odour condition are included in descriptive statistics.

For the liking and ranking results of the (savoury) taste part, odour category and participants were included as fixed and random factors, respectively.

General appetite ratings, individual odour attributes ratings, and liking of the food products, aggregated across food product category, were entered as potential covariates in both macronutrient and savoury taste preferences scores models.

Food intake

In a first model, total food intake, in g and kcal, was analysed as the dependent variable in separate linear mixed models. In a second model, odour category, congruency (depending on the food products selected and eaten relative to the odour condition), and their interaction were included as fixed factors. In a third model, odour category, food product category, and their interaction were included as fixed factors. For all the models, participants were included as random factors, and general appetite ratings, individual odour attributes ratings, and liking of food products (as assessed during the screening session on a 100 mm VAS) were included as potential covariates.

Results

Sensory-specific appetite

The main effect of congruency $F(1,1494) = 18.74$, $p < 0.0001$) was significant, suggesting sensory-specific appetite. Additionally, the effect of odour category was significant $F(3,1508) = 9.52$, $p < 0.0001$, and there was a significant interaction between odour category and congruency, ($F(3,1494) = 9.96$, $p < 0.0001$, **Figure 4.2,** mixed model included odour familiarity, liking of the food product and specific appetite before odour exposure as covariates). Post-hoc tests revealed that the change in specific appetite for (congruent) protein-rich food products after smelling protein-related odours was significantly higher compared to incongruent food products. However, for the other odour categories (carbohydrates, fat, and low-calorie) there was no significant difference in change in appetite between congruent and incongruent food products after odour exposure.

Exposure to no-odour did not affect the change in specific appetite for any of the food product categories ($F(3,347) = 0.67$, $p = 0.57$; mixed model including liking of food products and specific appetite before odour exposure as covariates); change in specific appetite by food product category (mean ± SE) = 0.31 ± 1.21 for carbohydrates, −0.60 ± 1.21 for proteins, −0.90 ± 1.20 for fat, and 0.80 ± 1.21 for low-calorie products.

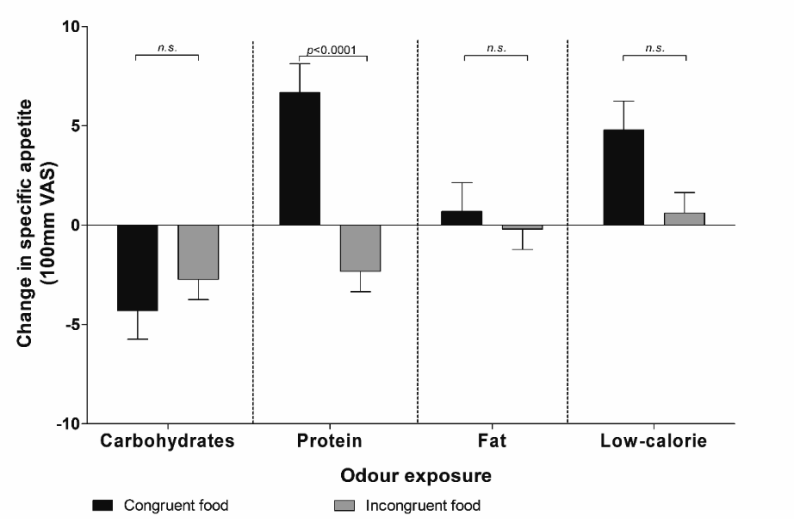

Figure 4.2. Change in specific appetite upon odour exposure for congruent (black colour) and incongruent food products (light grey colour) for the different odour categories. Values are expressed as mean and standard error.

Food preferences

Macronutrient preferences

Exposure to protein-related odours significantly increased liking for congruent compared to incongruent food products ($F(1,94) = 4.51$, $p < 0.05$; **Table A4.2**). However, exposure to other odours did not influence liking for their congruent food products ($F(1,94) = 1.92$, $p = 0.17$ for carbohydrates; $F(1,94) = 0.29$, $p = 0.59$ for fats; $F(1,92) = 0.26$, $p = 0.62$ for low-calorie; all statistical models did not include any covariate, with the exception of the low-calorie model, where odour intensity and odour-label association were included as covariates; **Table A4.2**).

Exposure to congruent odours did not affect the subsequent preference ranking for corresponding macronutrients ($F(1,94) = 0.05$, $p = 0.83$ for carbohydrates; $F(1,94) = 0.94$, $p = 0.34$ for proteins; $F(1,94) = 1.22$, $p = 0.27$ for fats; $F(1,94) = 1.15$, $p = 0.29$ for low-calorie; no covariates were included in these models; **Table A4.2**).

Savoury taste preferences

Odour exposure did not influence the liking for savoury-tasting food products ($F(4,122) = 0.91$, $p = 0.46$; savoury liking ratings upon protein-odour exposure 65.6 ± 1.63; carbohydrates 65.2 ± 1.64; fat 64.8 ± 0.05; low-calorie 64.5 ± 1.63; no-odour 64.2 ± 1.63).

However, odour exposure did influence the ranking savoury-tasting foods ($F(4,122) = 3.66$, $p < 0.01$; mixed model with odour familiarity and liking of the savoury food pictures used in the task as covariates): savoury products were significantly higher ranked upon exposure to protein-related odours compared to fat-related odours and the no-odour condition (ranking score after protein-odour exposure 2.35 ± 0.05; low-calorie 2.29 ± 0.05; carbohydrates 2.28 ± 0.05; fat 2.23 ± 0.05; no-odour 2.21 ± 0.05).

Food intake

Firstly, total food intake (in g and kcal) did not significantly differ between conditions ($F(4,124) = 0.16$, $p = 0.99$, for g; $F(4,124) = 0.13$, $p = 0.97$, for kcal; **Table A4.3**; any covariate contributed to the fit of the model). Secondly, total food intake of congruent and incongruent food products (in g and kcal) was not significantly different after odour exposure ($F(1,990) = 0.34$, $p = 0.56$ for g; $F(1,990) = 0.39$, $p = 0.53$ for kcal; both mixed models including liking of food products as covariate). Lastly, there was no interaction between odour category and food product category ($F(9,976) = 0.11$, $p = 0.99$, for g; $F(9,976) = 0.21$, $p = 0.99$, for kcal; **Figure 4.3**; both mixed models including liking of food products as covariate). The consumption of each food product category was similar after each odour exposure. As shown in **Figure 4.3A**, the amount in grams of low-calorie products was significantly higher compared to carbohydrates, protein, and fat ($F(3,988) = 74.55$, $p < 0.0001$). On the other hand,

the caloric intake of fat products was significantly higher compared to the other food product categories (F(3,988) = 125.56, $p < 0.0001$; **Figure 4.3B**).

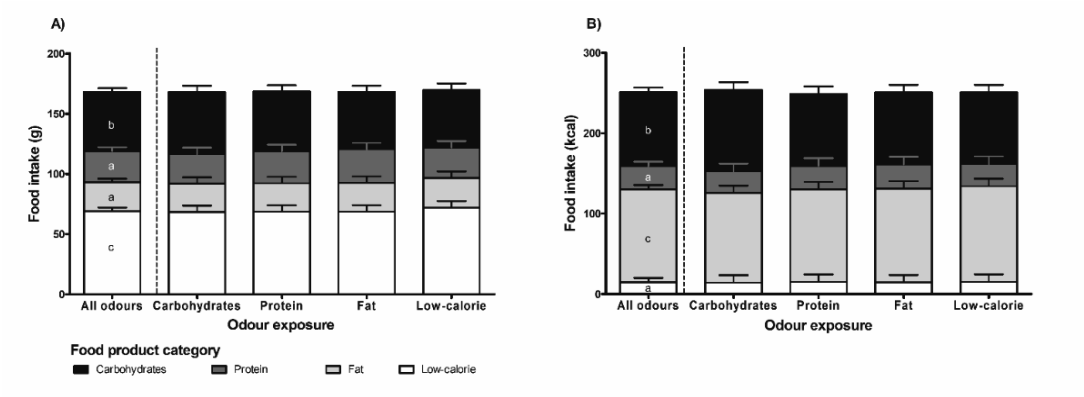

Figure 4.3. Intake of food product categories in g (A) and kcal (B) across all odours and upon the exposure of each odour. Values are expressed as mean and standard error. Similar letters indicate no significant differences ($p > 0.05$) and are in ascending order (a = lowest value and c = highest value).

Debriefing

In an open-question, participants were asked what they believed the true study aim was. Half of the participants mentioned the alternative goal (influence of odours on alertness) as the study aim, while the other half indicated something related to the influence of odours on eating behaviour.

Discussion

Our aim was to determine the influence of aware exposure to macronutrient-related odours on various measures of eating behaviour. Our results show that only protein-related odours influenced congruent appetite and liking. Odour exposure did not affect actual food intake.

The increase of congruent appetite upon odour exposure could be a direct sign of the body to activate specific metabolic routes for the smelled (macro)nutrient that is about to be ingested (Mattes, 1997; Nederkoorn, Smulders, & Jansen, 2000). Previous studies demonstrated sensory-specific appetite (SSA) after exposure to taste (sweet/savoury) and calorie related (high/low-calorie) food odours (Ramaekers, Boesveldt, Gort, et al., 2014; Ramaekers, Boesveldt, Lakemond, et al., 2014; Zoon et al., 2016). Based on these results, we hypothesized that humans are able to detect the nutritional content of foods, such as macronutrients, via their sense of smell (Boesveldt & de Graaf, 2017; Ramaekers, Boesveldt, Lakemond, et al., 2014; Zoon et al., 2016). However, our current results show that SSA was mainly driven by protein-related odours. Due to the nature of protein, this food category only represented savoury tasting products. Moreover, since most foods we encounter in daily life are a complex mix of (macro)nutrients, this may weaken the link between sensory signals (taste and odour), food and its nutrients (Martin & Issanchou, 2019; van Langeveld et al., 2017) and thereby minimize the specific appetizing effect of macronutrient-related odours. Taking this knowledge together, we postulate that olfactory SSA is perhaps mainly driven by taste quality (sweet/savoury) of the food that the odour represents, rather than its macronutrient content. Protein-related odours may have increased congruent appetite largely for its savoury taste rather than its macronutrient (protein) content. This taste association was also shown in the food preference results: exposure to protein-related odours increased the liking for protein food products compared to other food products and, similarly, the preference ranking for savoury products. Surprisingly, protein-related odours did not influence the liking for savoury food products nor preference ranking for food products rich in proteins. We could speculate that the sense of smell may be effective in protein-sensing in foods, as humans may be more prone to recognize and seek sources of this particular nutrient – compared to other macronutrients – that is crucial for body maintenance (Carreiro et al., 2016).

According to our expectations and in line with previous studies, odour exposure did not influence actual food intake. Active sniffing and explicit odour exposure has been shown to affect specific appetite (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Gort, et al., 2014; Zoon et al., 2016); while passive and implicit odour exposure affected food choice (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Proserpio et al., 2017) and intake (Proserpio et al., 2017). Moreover, a recent study showed an influence of implicit odour exposure on attentional processing of visual food cues, but this effect vanished upon explicit

odour exposure (Mas, Brindisi, Chabanet, Nicklaus, & Chambaron, 2019). The appetite ratings used in the current study (VAS scores) are explicit measurement that could be affected by conscious interaction with the environment, such as awareness of exposure to a specific odour that may result in active cravings and deliberations whether or not to choose and eat that product. Conversely, the selection and intake of food from the buffet could be considered implicit measurements that might be more influenced by non-conscious cues and are governed by emotional, impulsive, automatic decision-making, and reward responses (Cohen, 2008; Kahneman, 2011; Marteau, Hollands, & Fletcher, 2012; Mas et al., 2019; Rolls, 2011).

Even though food intake was measured within our lab facilities, we used an *ad libitum* salad buffet setting to mimic a more realistic eating environment. We offered two toppings per macronutrient category, however it is worth noting that participants seemed to follow certain strategies, even though all food products were evaluated as moderate–highly liked (60–80 on a 100 mm VAS): 1) some participants took pasta (carbohydrate) as the base of their salad instead of lettuce; 2) only one topping of the protein category was selected (chicken being the preferred one); 3) while for fat and low-calorie toppings, both options were chosen in similar amounts. This could indicate that participants built their salad according to certain habits based on their previous experiences and likings, expectations on the satiation value of each topping, and dietary patterns (Birch, 1999; Köster, 2009), leading to an overall static pattern, regardless of the odour they were exposed to. Some researchers have suggested that decision-making processes depend on the type of food, such as a full meal versus desserts or snacks (de Wijk et al., 2018; Wang, Cakmak, & Peng, 2018), where the latter two could be considered as discretionary calories and linked to reward and impulsive behaviour while decision on a full meal is likely more habit based. Other scenarios, such as performing the study in a food choice environment such as a true buffet with several assortments of meals or snacks to choose from could be more sensitive to detect impulsive eating behaviour, driven by external cues rather than habits. Nevertheless, Mors and collaborators did not find an influence of olfactory priming on food choice even though the buffet was performed in a real restaurant (Mors, Polet, Vingerhoeds, Perez-Cueto, & de Wijk, 2018). Further research should consider the use of other settings in which impulse buying is more common, such as supermarkets or convenience stores, as well as apply more implicit outcome measurements, e.g. eye-tracking, to assess food choices. These could provide more detailed and relevant information regarding real-life food appetite and selection upon odour exposure (Marteau et al., 2012; Wang et al., 2018). The potential influence of odour priming on healthier snacks or impulse buying rather than meal habits could have a greater impact in the prevention of weight gain and further healthier lifestyle.

A strong point of the current study is our cross-over design which takes into account individual characteristics that may affect differences in odour perception or eating behaviour. Furthermore, in our previous research (Morquecho-Campos et al., 2019) we optimized the selection of odours and foods for the Dutch population since the identification of macronutrients is likely related to familiarity with certain foods and thus depends on cultural culinary experiences, and these may interfere with the mental representations associated to the odours. Despite these efforts, it cannot be ruled out that the odours did not strongly signal the intended macronutrient content and therefore weakened the expected effect on eating behaviour.

A potential limitation is that about half of the participants inferred that our real aim was related to the influence of the odour exposure on appetite and/or food choice and intake. However, adding this variable to our statistical models did not modify the outcomes. Moreover, since our population only included unrestrained, normal-weight participants, the current findings cannot be generalized to other populations. Some studies have shown that individual differences in dietary restraint and body weight are decisive in the reactivity towards food. Restrained eaters (or overweight) tend to have a greater appetite and intake response upon being explicitly exposed to food cues compared to unrestrained eaters (or normal-weight participants) (Coelho, Jansen, Roefs, & Nederkoorn, 2009; Fedoroff, Polivy, & Herman, 2003; Ferriday & Brunstrom, 2011; Tetley, Brunstrom, & Griffiths, 2009).

Conclusion

Aware exposure to protein-related food odours increases congruent appetite, demonstrating sensory-specific appetite. Odour exposure may trigger specific appetite based on the taste qualities of its associated food rather than its macronutrient content. Moreover, aware exposure to macronutrient-related food odours did not affect actual food intake. Further work should focus on the impact of unaware odours on eating behaviour. Moreover, the use of implicit and more naturalistic measurements should be considered to further investigate the influence of olfactory cues on food intake.

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Author Contributions

Paulina Morquecho-Campos: Conceptualization, Methodology, Resources, Investigation, Formal analysis, Writing - original draft. Kees de Graaf: Methodology, Supervision. Sanne Boesveldt: Conceptualization, Methodology, Writing - review & editing, Supervision.

Appendix 4.1

Table A4.1. Intensity, liking, familiarity, intention to eat a product of that odour, mouth-watering ratings, odour-label associations (on a 100 mm VAS) and correct identification rates (%) of used odours, collapsed by category. Values are expressed as mean and standard error. Similar letters indicate no significant differences ($p > 0.05$).

Odour category	Intensity	Liking	Familiarity	Intention to eat a product of that odour	Mouth-watering	Correct category	Odour-label association
No-odour	29.5 ± 3.9^{a}	53.0 ± 2.8^{ab}	36.3 ± 3.1^{a}	41.4 ± 3.8^{a}	26.9 ± 3.5^{a}	40.6%	55.8 ± 6.2^{a}
Carbohydrates	74.4 ± 2.7^{b}	51.3 ± 5.0^{a}	61.0 ± 4.3^{bc}	48.3 ± 4.8^{ab}	38.7 ± 4.8^{ab}	65.6%	65.0 ± 5.3^{ab}
Protein	67.6 ± 3.6^{b}	65.5 ± 4.3^{bc}	67.8 ± 3.5^{bc}	61.7 ± 4.2^{bc}	51.6 ± 4.6^{b}	93.8%	65.6 ± 4.3^{ab}
Fat	68.1 ± 3.5^{b}	64.7 ± 3.6^{bc}	55.2 ± 3.7^{b}	48.6 ± 4.1^{ab}	42.0 ± 4.3^{b}	90.6%	58.2 ± 4.7^{a}
Low-calorie	69.1 ± 2.4^{b}	69.8 ± 3.2^{c}	73.5 ± 3.3^{c}	64.8 ± 3.7^{c}	49.8 ± 4.3^{b}	93.8%	81.0 ± 2.8^{b}

Table A4.2. Ratings of liking and preference ranking scores of each macronutrient (score range: 1–4) according to congruency with the odour exposed to, and upon no-odour exposure. Values are expressed as mean and standard error. * $p < 0.05$

Macronutrient	Congruent condition	Incongruent condition	No-odour condition
A) Liking			
Carbohydrates	70.4 ± 1.7	69.3 ± 1.6	69.1 ± 1.7
Protein*	66.8 ± 2.6	64.9 ± 2.5	64.2 ± 2.6
Fat	72.8 ± 2.0	73.3 ± 1.9	73.1 ± 1.6
Low-calorie	65.1 ± 1.8	64.6 ± 1.7	64.9 ± 1.9
B) Preference ranking score			
Carbohydrates	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
Protein	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1
Fat	2.7 ± 0.1	2.8 ± 0.1	2.7 ± 0.1
Low-calorie	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.1

Table A4.3. Total intake in g and kcal (mean ± SE) upon each odour category.

Odour category	Total intake (g)	Total intake (kcal)
No-odour	392.9 ± 21.6	449.5 ± 34.2
Carbohydrates	393.6 ± 25.7	459.6 ± 42.3
Protein	398.6 ± 21.7	467.1 ± 32.1
Fat	399.4 ± 22.5	465.4 ± 37.0
Low-calorie	398.8 ± 20.8	464.1 ± 35.9

Chapter 5

Olfactory priming for eating behaviour – The influence of non-conscious exposure to food odours on specific appetite, food preferences and intake

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Abstract

Sensory food cues in our surroundings, such as odours, trigger decisions that may lead to (over)eating. These cues occur mainly outside of people's awareness. Therefore, it is crucial to better understand the effect of (non-conscious exposure of) food odours on behavioural responses. Moreover, sensory-specific appetite suggests that food odour exposure may enhance appetite for products with similar properties in taste and calorie content, inferring that we can detect nutrient content of the food through our sense of smell. Our previous research showed that conscious exposure to macronutrient-related odours influenced specific appetite but not food preferences or intake. However, eating behaviour responses may differ depending on the level of awareness of the odour cue. Therefore, in our current study, we aimed to determine the influence of non-conscious exposure to macronutrient-related odours on specific appetite, food preferences, and food intake. Thirty-four healthy, normal-weight and unrestrained Dutch females underwent four sessions where they were non-consciously exposed to odours representing foods high in carbohydrates, protein, and fat, and low-calorie foods. Eating behaviour was assessed through a specific appetite questionnaire, a computer task on macronutrient and taste food preferences, and actual food intake by means of a salad bar which included toppings representing the different macronutrients. Results show that non-conscious exposure to macronutrient-signalling odours does not influence congruent appetite, food preferences nor food intake of a main meal. Follow-up research should focus on different odour exposure (intensity and exposure time) and outcome measures to have a better understanding of olfactory priming on eating behaviour.

Introduction

We are constantly exposed to sensory cues without being consciously aware of them: food advertisements by the road, nutritional advice on the radio, food aromas in the supermarket, etc. These sensory cues can steer our eating behaviour towards (un)healthy decisions (Köster, 2009; Stroebele & de Castro, 2004). However, the exact nature of the influence of these food cues on eating behaviour has yet remained unclear and awareness may differentially affect eating responses.

Non-conscious exposure to food cues can activate a mental representation triggering cognitive and behaviour responses, known as priming (Bargh, 2006; Tulving & Schacter, 1990). In particular, olfactory priming may influence mood, memory, consumer and eating behaviour (De Luca & Botelho, 2019; Smeets & Dijksterhuis, 2014, for reviews). Various eating behaviour outcomes such as appetite, food choice, and/or intake have been investigated in relation to olfactory priming. Literature thus far suggests that *non-conscious* exposure to odours does not influence specific appetite (Proserpio, de Graaf, Laureati, Pagliarini, & Boesveldt, 2017). However, it may impact food choices (Chambaron, Chisin, Chabanet, Issanchou, & Brand, 2015; de Wijk & Zijlstra, 2012; Gaillet-Torrent, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2014; Gaillet, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2013). E.g. in a series of studies, Gaillet et al. have shown that starters and desserts containing fruit and vegetables were selected more frequently from a menu when participants were non-consciously exposed to melon and/or pear odour (Gaillet-Torrent et al., 2014; Gaillet et al., 2013).

Furthermore, others have shown that non-conscious exposure may also influence congruent food intake, e.g. chocolate rice upon high-calorie-related odour exposure (Proserpio et al., 2017, 2019). Moreover, results from a reaction time task demonstrated that *only non-conscious* odour exposure led to attentional biases towards foods, as compared to conscious odour exposure (Mas, Brindisi, Chabanet, Nicklaus, & Chambaron, 2019).

Conversely, *conscious* odour exposure may influence self-reported appetite (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Gort, et al., 2014), but not food preference and intake (Zoon, de Wijk, de Graaf, & Boesveldt, 2014).

Awareness of the odour may thus play a crucial role in the type of response it exerts (McCrickerd & Forde, 2016; Smeets & Dijksterhuis, 2014). On one hand, barely detectable and unattended odours may act as prime and trigger congruent food choice and intake (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Proserpio et al., 2017, 2019), as these decision-making processes may occur at a non-conscious level (Bargh & Ferguson, 2000; Köster, 2009). On the other hand, conscious odours may mainly induce sensory-specific appetite (Ferriday &

Brunstrom, 2011; Ramaekers, Boesveldt, Gort, et al., 2014; Zoon et al., 2014). Awareness of the odour cue and/or the congruent appetite response may trigger cognitive control (such as self-regulation and inhibition mechanisms) which could interrupt the meal process at this stage and prevents the translation from appetite into further food choice and intake (Boesveldt & de Graaf, 2017).

Sensory-specific appetite (SSA) infers that food odour cues may convey information related to the macronutrient content, based on the taste and calorie content, of the associated food and thereby may induce congruent appetite and possibly even food choice and intake (Ferriday & Brunstrom, 2011; Ramaekers, Boesveldt, Gort, et al., 2014; Ramaekers, Boesveldt, Lakemond, van Boekel, & Luning, 2014; Zoon, de Graaf, & Boesveldt, 2016). Our own previous research showed that conscious exposure to macronutrient-related odours increased congruent appetite mainly after protein-related odour exposure, but did not impact food preferences or actual food intake (Morquecho-Campos, de Graaf, & Boesveldt, 2020). Therefore, in the current study we aimed to investigate how non-conscious macronutrient-related odours exposure would impact specific appetite, food preferences, and intake. We hypothesized that non-conscious odour exposure would enhance congruent food preferences and intake. Specifically, we hypothesized that macronutrient preferences and food intake would increase in a congruent manner after exposure to macronutrient-related odours (e.g. exposure to protein-related odours will increase preferences for foods high in protein such as ham, chicken, compared to incongruent food products such as bread/croutons for carbohydrates, nuts for fat, or cucumber for low-calorie). However, non-conscious odour exposure would not influence self-reported appetite ratings for congruent food products.

Materials and Methods

Participants

Normal-weight Dutch females between 18 and 35 years old were recruited from Wageningen and the surroundings. Initially, participants were invited to an information and screening session, during which they provided written informed consent and filled out a questionnaire to determine their eligibility. Participants with a normal sense of smell (scoring ≥ 12 on the 16 items Sniffin' Sticks odour identification test (Oleszkiewicz, Schriever, Croy, Hähner, & Hummel, 2019)), self-reported normal sense of taste, and unrestrained eaters on the Dutch Eating Behaviour Questionnaire, DEBQ (van Strien, Frijters, Bergers, & Defares, 1986) were included. Participants were excluded when they: were a smoker; disliked the food products used in the study (<40 on 100 mm visual analogue scale, VAS); had any dietary restriction towards specific foods (self-imposed or otherwise; e.g. vegetarian, vegan); used medication other than paracetamol and hormonal contraceptives; were pregnant or had the intention to become pregnant during the experiment or were currently breastfeeding; reported weight loss or weight gain of more than 5 kg or following a special diet in the two months prior to the study; or participated in our previous study (Morquecho-Campos et al., 2020). Moreover, taste ability and colour blindness were assessed during the screening session by means of Taste Strips and Ishihara's colour test, respectively (Ishihara, 1951; Landis et al., 2009; Mueller et al., 2003). These measures were taken as bogus tasks to distract potential participants from the true aim of the study that involved odour perception but were not considered as inclusion/exclusion criteria. The alternative goal communicated to participants was that we aimed to investigate the role of hunger, food cue exposure, and satiety on abilities of logic reasoning by means of psychometric tasks.

The sample size calculation was based on previous research (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Proserpio et al., 2017; Ramaekers, Boesveldt, Gort, et al., 2014; Zoon et al., 2016), resulting in a total of 34 participants included in the study (**Table 5.1**). Participants were compensated at the end of the study for their contribution. The study was conducted in accordance with the Declaration of Helsinki (revised in 2013) and approved by the Medical Ethical Committee of Wageningen University (NL 69840.081.19). This trial was pre-registered at the Netherlands Trial Register as NL7742 (https://www.trialregister.nl/trial/7742).

Table 5.1. Characteristics of the 34 participants included in the study.

Characteristic	Mean ± SD (range)
Age (years)	21.3 ± 1.8 (18–26)
BMI (kg/m^2)	21.5 ± 1.7 (18.8–24.7)
Odour Identification Score	14.1 ± 1.0 (12–16)
Restrained Score (DEBQ)	2.4 ± 0.7 (1.0–3.3)

Procedure

This study had a cross-over intervention design, where participants were (non-consciously) exposed to three macronutrients-related odours (i.e. carbohydrates, protein, and fat) and a low-calorie-related odour as a control condition. Participants visited the test location four times, once for each odour condition, with at least two days in between. Test sessions took place around lunch time (11.30–14.00) and participants attended their sessions at the same time. Participants were asked not to eat or drink (except water) at least three hours before the test sessions, to be in a mild hunger state.

Figure 5.1 provides an overview of the procedure of each test session. On arrival at each test session, participants rated their general appetite and specific appetite (non-odourised room). Then, they were escorted to a new room (odourised room) where they performed a psychometric task (#1) for 3 min. This room was scented with a non-consciously detectable concentration of the different odours (perceived intensity <35 mm on a 100 mm VAS, detailed information can be found in section 'Odour stimuli'). After the first odour exposure, participants went back to the non-odourised room where they rated their general and specific appetite again and performed a computer-based food preference task. Subsequently, they went back to the same odourised room and performed again a psychometric task (#2) for 3 min. Then, participants were escorted to a dining room where they rated their general appetite and were provided with a salad bar lunch by which their *ad libitum* food intake was covertly measured. After the participants finished their lunch (~20 min), they performed a final psychometric task (#3). After each psychometric task, participants rated their stress level after the psychometric task and the perceived level of difficulty of the psychometric task.

At the end of the last session, participants were debriefed. They were asked about their impression of the aim of the study and whether they had perceived an odour in the odourised rooms (where they had performed the first two psychometric tasks) in any of the test sessions. After completing the debriefing questionnaire, they were informed about the true aim, and were presented with the four odours to rate on various attributes.

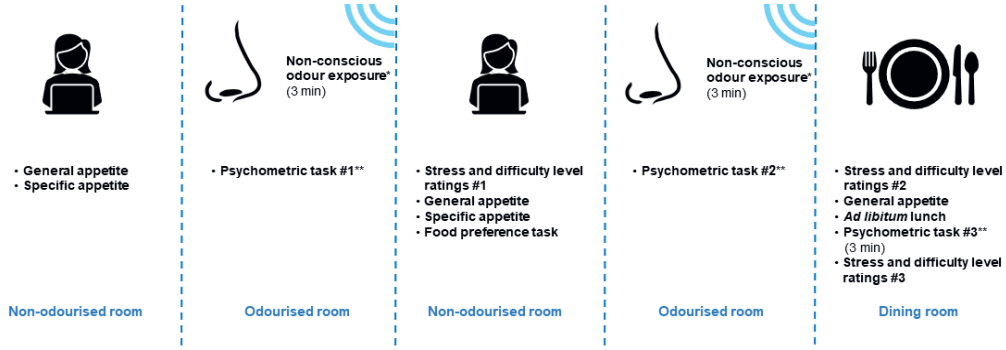

Figure 5.1. Procedure for each test session. *Participants were exposed twice to the same odour within the same session. **Psychometric tasks were bogus tasks as part of the alternative goal to keep participants naïve from the real aim of the study.

Odour stimuli

Based on our previous study (Morquecho-Campos et al., 2020), pilot studies, and familiarity for Dutch participants, we selected one odour per category and determined appropriate intensities: bread (Symrise 205361; 8% in propylene glycol, PG) for carbohydrates; duck (Symrise 619322; 0.05% in PG) for protein; butter (IFF 10922603; 60% in PG) for fat; cucumber (IFF 15311331; 100%) for the low-calorie category. The odours were distributed in air-conditioned rooms by means of vaporizers (Iscent, Zeewolde, The Netherlands). Pilot studies were carried out to achieve a non-consciously detectable concentration, which we defined as a perceived intensity lower than 35 mm on a VAS. These pilot studies consisted of several short sessions where participants (n = 10 per session, which did not participate in the actual study) were exposed to an odour dispersed in the odourised room. Participants were asked about their awareness of any odour present in the room, and if so, to assess the intensity of the ambient odour (on a 100 mm VAS anchored from 'Not at all' to 'Very much'). Only one participant was allowed in the room at the same time. The final dispersion frequency and perceived intensity of the odours can be found in the supplementary materials (**Table A5.1**).

Odours were prepared every week and stored in the fridge until the morning of the test session. On the morning of the test session, the odour stimuli were taken out of the fridge and stored at room temperature. After the test sessions of each day were completed, the room was ventilated, the surfaces were cleaned, and the odour was fully purged overnight.

Measurements

General and sensory-specific appetite

General appetite was determined by assessing hunger, fullness, prospective consumption, desire to eat, and thirst on 100 mm VAS anchored by 'Not at all' to 'Very much'. The 'General appetite score' variable was computed as the average of hunger, desire to eat, prospective consumption, and the inverse fullness score (100 – fullness).

Sensory-specific appetite was assessed by rating 'How much would you like to eat *'[specific product]'* at this moment?' on a 100 mm VAS anchored by 'Not at all' to 'Very much'. Specific products consisted of 12 food items, 3 per macronutrient category, which either did or did not match the odour stimuli (congruent/incongruent): pasta, bread, and corn for carbohydrates; chicken, ham, and beef for protein; bacon, nuts, and cheese for fats; cucumber, tomato, and melon for low-calorie food products.

Food preferences

Food preferences were measured by means of the Macronutrient and Taste Preference Ranking Task (MTPRT), a validated computer-based task (de Bruijn, de Vries, de Graaf, Boesveldt, & Jager, 2017). This task consists of rating the liking of 32 food products (represented by pictures with labels) and ranking the food products (represented only by pictures) according to participants' desire to eat at that moment. These foods are divided into four macronutrient categories – carbohydrates, protein, fat, and low-calorie – with each category consisting of 4 sweet and 4 savoury foods, except the protein category (only savoury). Liking ratings were aggregated per macronutrient and taste category for analyses. Macronutrient preference ranking score can range between 1 and 4, while taste preference scores can range between 1.5 and 3.5: higher scores representing a higher preference. As sweet and savoury preference ranking scores obtained in this task are (by definition) opposite to each other, we reported only the savoury taste results.

Food intake

The *ad libitum* lunch consisted of a salad bar, with 2 options per macronutrient (i.e. carbohydrates, protein, and fat) and 2 low-calorie products, see **Table 5.2.** The toppings for the macronutrient categories contained at least 50% of the total energy derived from the specific macronutrient category and toppings in the low-calorie category contained no more than 60 kcal/100 g (de Bruijn et al., 2017; RIVM, 2019). All food products are commercially available and familiar in the Dutch diet. Participants received a bowl with a fixed amount of salad (80 g) and were instructed to choose as many toppings and the quantity as they wanted. They also received 28 g of a natural salad dressing to add to their salad (if necessary), and a glass of water (150 mL) that they were instructed to finish during the lunch. Only one

participant was allowed in the buffet area at a time and they could only visit the buffet area once. Participants were instructed to sit in the dining area, which did not face the buffet area, and to eat until they felt comfortably satiated. They were not obliged to finish their plate. The buffet was continuously refilled, to ensure a constant volume on each topping tray. Food intake was covertly measured by weighing the trays which contained each topping before and after each participant 'built' their salad and by weighing the remaining amount on the plate of the participants after eating.

Table 5.2. Food products offered at the *ad libitum* lunch.

Category	Food	Energy (kcal/100 g)	Carbohydrates (%)	Protein (%)	Fat (%)
Carbohydrates	Croutons	455	**57.1**	11.4	29.7
	Corn	71	**59.2**	15.2	17.7
Protein	Chicken strips	105	15.2	**64.8**	21.4
	Ham strips	115	1.0	**69.6**	27.4
Fat	Mixed nuts	625	4.8	11.5	**81.4**
	48+ Gouda Cheese	360	0.0	25.6	**75.0**
Low-calorie	Cucumber	13	40.0	21.5	27.7
	Cherry tomatoes	30	53.3	12.0	24.0
Base	Lettuce	13	46.2	30.8	6.9
Dressing	Natural salad dressing	40	9.5	0.1	0.1

Numbers in bold emphasize the highest contribution to the total energy content of the food products, which represents their respective food product category.

Odour attribute ratings – debriefing session

At the end of the last test session, participants assessed the odour on liking, intensity, familiarity, intention to eat a product with that odour, and mouth-watering sensation on a 100 mm VAS anchored by 'Not at all' to 'Very much'. They were also asked to identify the odour among a list of (food) products, including 'odourless', by a multiple forced-choice task ('Which of the following label(s) best fits the odour?') and to assess the odour-label association ('How well do you think this smell corresponds to *'[specific label]'?'*) on a 100 mm VAS.

Psychometric task – as bogus task

The psychometric tasks consisted of four different type of tasks: numerical, inductive, verbal, and logical reasoning with figures. In each test session, participants randomly performed a different type of psychometric task. The psychometric tasks were retrieved from 123test.com and '501 challenging logic and reasoning problems' (123test.com, n.d.; LearningExpress (Organization), 2005). These tasks were moderate to high in difficulty to be in line with our alternative goal. However, in order to control any potential effects of different stress levels on our outcomes, the level of these tasks was similar across types of psychometric tasks and sessions. Performing these psychometric task could modify the level of stress of the participant

which may affect food choice or intake (Groesz et al., 2012; Yau & Potenza, 2013). Therefore, the stress levels after the performance of each task and the difficulty of each psychometric task were measured and added as covariates to our models.

Statistical analyses

All statistical analyses were carried out in RStudio (RStudio Team, 2016), and graphs were made using GraphPad Prism 5.0 (GraphPad Prism Software). Results with a *p* value lower than 0.05 were considered statistically significant.

All analyses consisted of linear mixed models, carried out using the *lme4* statistical package in R (Bates, Mächler, Bolker, & Walker, 2015). The best fitting models were selected on the basis of parsimony following a backward approach. Homoscedasticity and normal distribution of error terms, and correct specifications of the fixed and random parts of the model were checked for each model. Post-hoc tests with a Bonferroni correction, by means of the *lsmeans* statistical package (Lenth, 2016), were performed when the main effects or interaction were significant.

Odour attribute ratings were analysed as a dependent variable, with odour as fixed effects and participants and evaluation order as random effects. The liking of food products was analysed as a dependent variable, with food product as fixed effects and participants as random effects. This data is shown in **Table A5.2** and **A5.3** of the supplementary materials.

Data was labelled as congruent/incongruent, depending on the food products used relative to the odour category. For example, specific appetite or food intake of protein-rich products (such as ham, chicken, or meat) were considered congruent after exposure to a protein-related odour (meat), but labelled as incongruent after exposure to other odour categories.

For all the models, participants and test sessions nested in allocation groups (12 allocation groups based on participants' availability: 9 groups consisted of 3 participants and 3 groups of 1, 2, and 4 participants, respectively) were evaluated as random factors.

Change in sensory-specific appetite

Change in sensory-specific appetite (SSA; calculated as difference in the specific appetite ratings before versus after odour exposure) was analysed as a dependent variable. Odour category, (in)congruency of food product, and their interaction were included as fixed factors. Participants were included as random factor. Liking of food products (assessed during the screening session on a 100 mm VAS), specific appetite before odour exposure, individual odour attribute ratings (assessed during the debriefing on a 100 mm VAS), stress levels and difficulty of the psychometric task #1, general appetite score and thirst rating before and after the first odour

exposure, and personal characteristics (age, BMI, DEBQ, and Sniffin' sticks) were included as covariates.

Food preferences

Congruency was determined by the match between odour exposure category and each macronutrient score analysed. Each macronutrient liking and ranking score was analysed as a dependent variable in separate linear mixed models. Congruency was included as a fixed factor in the liking and ranking results of the macronutrient models. Odour category was included as a fixed factor in the liking and ranking results of the (savoury) taste models. Participants were included as a random factor for all the models. Individual odour attribute ratings, stress levels and difficulty of the psychometric task #1, general appetite score and thirst rating after the first odour exposure, and personal characteristics were entered as covariates in both macronutrient and savoury taste preferences scores models.

Food intake

Food intake was analysed in three ways. Firstly, to determine the influence of odour exposure on overall food intake, total food intake (in g and kcal) was analysed as a dependent variable, with odour category as a fixed factor. Secondly, to determine the influence of odour exposure on (in)congruent food intake, food intake (in g and kcal) was analysed as a dependent variable, and congruency (depending on the food products selected and eaten relative to the odour condition) as a fixed factor. Thirdly, to determine the influence of odour exposure on specific food intake of the different product categories, food intake (in g and kcal) was analysed as a dependent variable, with odour category, food product category, and their interaction included as fixed factors. In the models of the first part, participants were included as random factors. Allocation groups were additionally included as a random factor in the models of the second and third part.

Liking of food products available in the *ad libitum* lunch (assessed during the screening session on a 100 mm VAS), individual odour attribute ratings, stress levels and difficulty of the psychometric task #2, general appetite score and thirst rating after the second odour exposure, and personal characteristics were entered as covariates in all the models.

Moreover, Pearson correlation analyses were performed to determine the correlation between food preferences score and food intake (in g and kcal). The correlation analyses considered participants as random factors, using the residuals of a mixed model with food intake (in g and kcal) as a dependent variable and participants as random effects. The residuals of those models were correlated with food preferences scores.

Results

Change in specific appetite (SSA)

There was a significant interaction between odour category and congruency ($F(3,1588) = 2.76$, $p = 0.041$; the mixed model included liking of the food product, specific appetite before odour exposure, and general appetite score after the first odour exposure as covariates; **Figure 5.2**). However, Bonferroni post-hoc tests did not reveal any significant difference between the respective conditions (all $p > 0.05$).

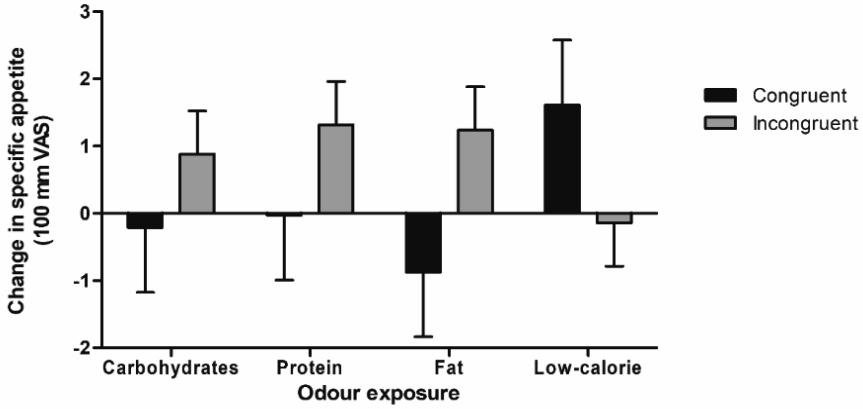

Figure 5.2. Change in specific appetite upon odour exposure for congruent (black colour) and incongruent food products (light grey colour). Values are expressed as mean and standard error.

Food preferences

Odour exposure did not influence liking for congruent food products (**Table 5.3A**). Similarly, odour exposure did not affect preference ranking for congruent food products (**Table 5.3B**).

Table 5.3. Ratings of liking (on a 100 mm VAS) and preference score (score range: 1–4) of each macronutrient after exposure to (in)congruent odours. Values are expressed as mean and standard error.

Macronutrient	Congruent condition	Incongruent condition	Statistical information
A) Liking*			
Carbohydrates	67.8 ± 2.00	66.9 ± 1.93	F(1,100) = 1.59, p = 0.21
Protein	60.6 ± 2.55	61.4 ± 2.47	F(1,100) = 0.63, p = 0.43
Fat	71.3 ± 1.83	71.3 ± 1.75	F(1,100) = 0.001, p = 0.98
Low-calorie	67.7 ± 2.29	67.1 ± 2.23	F(1,101) = 0.56, p = 0.46
B) Preference score**			
Carbohydrates	2.53 ± 0.07	2.50 ± 0.07	F(1,101) = 0.57, p = 0.45
Protein	2.27 ± 0.11	2.35 ± 0.10	F(1,98) = 3.61, p = 0.06
Fat	2.73 ± 0.07	2.77 ± 0.07	F(1,101) = 1.57, p = 0.21
Low-calorie	2.42 ± 0.11	2.39 ± 0.11	F(1,98) = 0.41, p = 0.53

*Covariates included in the liking models: general appetite score after the first odour exposure was included as a covariate in the carbohydrates, protein, and fat models; the low-calorie model did not include any covariate.

**Covariates included in the preference score models: no covariates were included in the models for carbohydrates and fat; intention to eat a product with that odour, mouth-watering sensation upon that odour, and general appetite score after the first odour exposure were included in the protein and low-calorie models.

Liking for savoury-tasting food products was not influenced by odour exposure (**Table 5.4**). The ranking of savoury-tasting foods was influenced by odour exposure (**Table 5.4**). However, Bonferroni post-hoc tests did not reveal any significant difference between the respective conditions.

Table 5.4. Ratings of liking (on a 100 mm VAS) and preference score (score range: 1.5–3.5) of savoury taste after odour exposure. Values are expressed as mean and standard error.

Odour category	Liking for savoury-tasting foods	Preference score for savoury-tasting foods
Carbohydrates	63.5 ± 1.77	2.24 ± 0.06
Protein	63.4 ± 1.78	2.21 ± 0.05
Fat	63.3 ± 1.78	2.30 ± 0.06
Low-calorie	64.0 ± 1.78	2.30 ± 0.06
Statistical information	F(3,98) = 0.30, p = 0.83*	F(3,99) = 3.38, p = 0.02**

*General appetite score after the first odour exposure was included as a covariate.

**No covariates were included in this model. Bonferroni post-hoc tests did not reveal any significant difference between these conditions.

Food intake

Firstly, total food intake (in g and kcal) did not differ significantly after exposure to the different macronutrient-related odours (**Table 5.5**).

Table 5.5. Total food intake in g and kcal after exposure to the different macronutrient-related odours. Values are expressed as mean and standard error.

Odour category	Total food intake (g)	Total food intake (kcal)
Carbohydrates	423 ± 22.7	540 ± 41.2
Protein	425 ± 22.6	494 ± 40.5
Fat	442 ± 22.9	529 ± 41.2
Low-calorie	425 ± 22.6	499 ± 40.5
Statistical information	F(3,92) = 0.51, p = 0.68*	F(3,95) = 0.78, p = 0.53**

*General appetite score after the second odour exposure was included as a covariate.
**No covariate contributed to the fit of this model.

Secondly, food intake did not differ significantly between congruent versus incongruent food products after odour exposure (g: F (1,1052) = 0.04, p = 0.85; kcal: F (1,1052) = 0.08, p = 0.77; liking of food products was included as a covariate in both mixed models).

Thirdly, there was no interaction between odour category and food product category (g: F(9,1038) = 0.58, p = 0.81, **Figure 5.3A**; kcal: F(9,1038) = 0.63, p = 0.77, **Figure 5.3B**; liking of food products was included as a covariate in both mixed models). Overall, the consumption (in grams) of low-calorie food products was significantly higher compared to the intake of carbohydrates, protein and fat (F(3,1049) = 196.56, p < 0.0001; **Figure 5.3A**), and the caloric intake of fat products was significantly higher compared to that of the other food product categories (F(3,1049) = 171.76, p < 0.0001; **Figure 5.3B**).

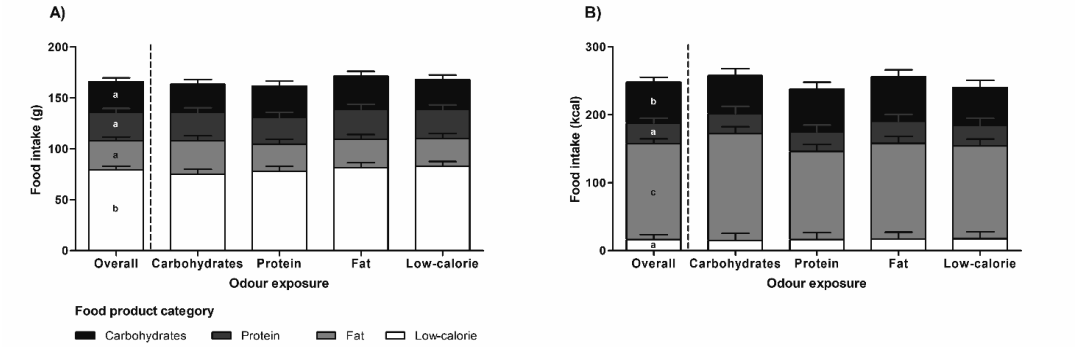

Figure 5.3. Intake of food product categories in g (A) and kcal (B) overall and per type of odour exposure. Similar letters indicate no significant differences within food product categories (p > 0.05) and are in ascending order (a = lowest intake and c = highest intake). Values are expressed as mean and standard error.

Food preference scores were positively correlated with food intake (g: $r(542) = 0.16$, $p = 0.0003$; kcal: $r(542) = 0.27$, $p < 0.0001$).

Debriefing – odour awareness

All the participants believed that the aim of the study was related to the psychometric tasks, stress levels, and/or hunger state. None of the participants reported a link between the measurements performed and the ambient odours. The data from the debriefing questionnaire was classified into four score categories as suggested by Mors, Polet, Vingerhoeds, Perez-Cueto, & de Wijk (2018). Participants did not perceive the odour in 89.0% of the test sessions, while in 2.2% of them they perceived an odour and correctly identified it. In a further 8.1% of test sessions, participants detected an odour but could not name it, and in the remaining 0.7% of the test sessions participants perceived an odour but were unable to correctly identify it.

Discussion

The current study aimed to determine the influence of non-conscious exposure to macronutrient-related odours on measures of eating behaviour. Based on available literature, we hypothesized that non-conscious exposure would not impact explicit responses such as self-reported appetite but would influence food preferences and intake. Our results show that non-conscious odour exposure did not impact appetite (albeit contrary to our hypotheses) and did not modify food preference nor food intake in a salad bar setting.

Appetite feelings can be induced by external sensory cues and may lead to an increase in searching and wanting to consume specific foods (de Graaf, Blom, Smeets, Stafleu, & Hendriks, 2004; Egecioglu et al., 2011). Therefore, when we are consciously exposed to food cues such as odours, our appetite for congruent foods may increase, demonstrating olfactory sensory-specific appetite (Ferriday & Brunstrom, 2011; Morquecho-Campos et al., 2020; Ramaekers, Boesveldt, Gort, et al., 2014; Ramaekers, Boesveldt, Lakemond, et al., 2014; Zoon et al., 2016). However, in line with the current study, results from Proserpio et al. also showed no influence of non-conscious odour exposure on specific appetite (Proserpio et al., 2017). Appetite feelings are measured by explicit, subjective ratings that involve a conscious realization of cravings and external cues (de Graaf et al., 2004; Proserpio et al., 2017). Therefore, sensory-specific appetite may only occur when the odour is being consciously perceived. However, this conscious perception may also activate cognitive processes which can disturb the decision-making beyond the appetizing stage such as choosing foods and consuming a meal (Boesveldt & de Graaf, 2017).

Contrary to our expectations, non-conscious odour exposure did not affect food preferences. Food preference is the choice of one food over other ones (Rozin & Vollmecke, 1986). Previous studies have demonstrated the influence of non-conscious odour exposure on congruent food choices (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013). Contrary to our food preference computer-based task, those studies measured food choice by means of a menu or a buffet-style where the participants selected only one option from each course category (starters, main courses, desserts). Such a forced choice procedure could be more naturalistic compared to our (computer-based) ranking task, and provides information of momentary motivation for the chosen food product over the other one(s) (Finlayson, King, & Blundell, 2008). In line with this, and similar to our current study, Mors et al. offered an assortment of different meals where the participants were able to freely select their food, and failed to show an effect of olfactory priming on subsequent food choices (Mors et al., 2018). Free selection (and consumption) of food products might lead to choices based on habits instead of being influenced by olfactory priming. Moreover, the food products displayed in the ranking task vary in a consumption context (e.g. ranking between salty sticks, chocolate bar, cod fillet,

and strawberries), which could influence the current preference selection of the products. Food choice behaviour is typically closely related to the context or appropriateness of consumption of the selected food and the odour (Chambaron et al., 2015; de Wijk et al., 2018; McCrickerd & Forde, 2016). As explained above, different methodologies (i.e. outcome measures) used to investigate similar eating responses may have led to inconsistent results. This might be a key factor in our current lack of understanding how (and to what extent) odour priming impacts eating behaviour. Moreover, previous experiments tended to focus on one outcome only (e.g. Gaillet-Torrent, 2014; Chambaron et al., 2015), which from a methodological viewpoint is more simplistic, but lacks understanding of the complete picture of eating behaviour.

Furthermore, non-conscious odour exposure did not influence food intake by means of a salad bar, overall, nor per congruency according to the odour exposure and congruent macronutrient category or food product category. Food intake is the amount of consumed food in a particular context (de Graaf et al., 2004). Previous research has shown a congruent food intake upon non-conscious odour exposure (Proserpio et al., 2017, 2019). In those studies, food intake was measured as the *ad libitum* intake of a single food.

In line with our previous study (Morquecho-Campos et al., 2020), our current results show that participants selected similar amounts of the available topping regardless of the exposed odour, leading to build their salad in a similar pattern on each test session. This might be related to habits, previous experiences, and expectations on satiation (Birch, 1999; Brunstrom, 2007; Köster, 2009). Interestingly, previous food choice studies suggest an influence of the olfactory priming mainly on starters and desserts (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013), but not on the main course (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Mors et al., 2018). Starters and desserts could be considered as rewarding foods and could be more prone to be driven by external cues, while main courses or meals may reflect more habitual choices in line with overall dietary patterns (Bellisle, 2003; Blundell et al., 2010; de Wijk et al., 2018; Wang, Cakmak, & Peng, 2018). Taken together, this suggests that olfactory priming might not be able to modify regular dietary patterns or main meal selection, but instead may impact rewarding and impulsive eating behaviour. Future work should consider the use of other settings such as forced choice response of rewarding food products in a convenience store or in a menu rather than *ad libitum* buffet style, to provide useful insights into participant´s odour-directed eating behaviour. In addition, visual attention may play a beneficial role in understanding decision-making processes (Carrasco, 2011; Krajbich, Armel, & Rangel, 2010; Orquin & Mueller Loose, 2013). Eye movements are linked to perceptual and cognitive processing to reflect visual attention. Visual attention, through the use of an eye-tracker, could be useful to detect non-conscious and spontaneous behaviour that could help to better

understand eating behaviour responses (Hummel, Zerweck, Ehret, Salazar Winter, & Stroebele-Benschop, 2017; Seo, Roidl, Müller, & Negoias, 2010; Wang et al., 2018).

It is noteworthy that the olfactory priming studies (Chambaron et al., 2015; de Wijk & Zijlstra, 2012; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Mas et al., 2019; Mors et al., 2018; Proserpio et al., 2017, 2019) have used different durations and intensities of the odour prime, which may explain some contradictory behavioural responses. Across studies, the range of exposure duration has been very broad (~3–30 min). We decided to expose the participants for the same duration (3 min) as our previous study, which has been shown to be sufficient to enhance congruent appetite when the odour is being actively sniffed (Ferriday & Brunstrom, 2011; Morquecho-Campos et al., 2020; Zoon et al., 2016). However, longer exposure (e.g. 10–30 min, (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Proserpio et al., 2017, 2019)) may be required for inattentive and low odour exposure to create a mental representation of the cued odour and influence eating behaviour. Moreover, for priming to occur, the intensity of the odour cue might be crucial: not too high to become consciously detectable, but at the same time not too low to not be perceived at all. Proserpio, et al. mentioned that the used odours were in detectable but mild concentration with a perceived intensity <50 mm on VAS (Proserpio et al., 2017, 2019). However, other studies merely mentioned that the intensity of the odour was very low (non-consciously or non-attentively noticeable) without further specification of the perceived intensity (Chambaron et al., 2015; de Wijk & Zijlstra, 2012; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Mas et al., 2019; Mors et al., 2018). Our current study was performed with low concentrations of the odours (20–30 mm VAS perceived intensity), and the vast majority of the participants did not perceive an odour during the different test sessions, which could indicate that the intensity was too low for the odour to be even physically detected and act as a prime. Taking all of the above together, it shows how differences in methodology used to understand olfactory priming on eating behaviour may have led to inconsistent outcomes. Moreover, one of the biggest (methodological) challenges in olfactory priming is to assure that the exposed odour is outside of participants' awareness. In our current study, the intensity of the odours was determined through pilot studies where the participants were aware of the presence of the odour, which likely heightened the perceived intensity, compared to participants in the actual study. The debriefing results confirmed that those participants were not aware of the presence of odours, and that the use of an alternative aim was useful to deviate the attention away from the odour as none of the participants reported a link between the measurements performed and odours. From this we infer that our odour exposure was non-conscious and that a 'priming type 7' was used (prime was not perceived, the link was not aware, but the participants were aware of the performed measurement) (Dijksterhuis, 2016).

One of the strongest points of this study is the within-subjects design, which considers the individual differences in eating behaviour, habits, and odour perception. Moreover, food intake was covertly measured in a salad bar style to offer a more realistic setting. Given our interest in macronutrient-related effects of odours exposure, we deemed a salad bar with different toppings was the best approach to measure intake of the various macronutrients. Our findings may be somewhat limited to generalize beyond the unrestrained, normal-weight, female study population. For example, obese women increased their sensory-specific appetite and *ad libitum* intake upon unaware odour exposure (Proserpio et al., 2019).

Conclusion

Exposure to macronutrient-related food odours outside of participants' awareness did not influence specific appetite, food preferences, or intake of a salad bar. Olfactory priming may not influence habitual main meals such as a lunch. Further work should focus in more detail on differences in methodology (odour exposure and outcomes) to better understand which factors play a role in how olfactory priming influences eating behaviour.

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Author Contributions

Paulina Morquecho-Campos: Conceptualization, Methodology, Resources, Investigation, Formal analysis, and Writing – original draft. Kees de Graaf: Methodology and Supervision. Sanne Boesveldt: Conceptualization, Methodology, Writing – review & editing, and Supervision.

Appendix 5.1

Table A5.1. Dispersion frequency and perceived intensity (on a 100 mm VAS) across ambient odour categories.

Odour category	Dispersion frequency	Perceived intensity (mean ± SE)
Bread (Carbohydrates)	6 times in 20 min and 1 time every 15 min	31.3 ± 2.8
Duck (Protein)	3 times in 10 min and 1 time every 15 min	31.3 ± 2.3
Butter (Fat)	3 times in 10 min and 1 time every 15 min	33.5 ± 2.9
Cucumber (Low-calorie)	20 times in 30 min and 1 time every 15 min	20.0 ± 2.6

Table A5.2. Odour ratings: intensity, liking, familiarity, intention to eat a product of that odour, mouth-watering ratings, odour-label association (on a 100 mm VAS), and correct identification rates (%) of used odours. Values are expressed as mean and standard error. Similar letters indicate no significant differences across odour categories ($p > 0.05$).

Odour category	Intensity	Liking	Familiarity	Intention to eat a product of that odour	Mouth-watering	Correct category	Odour-label association
Bread (Carbohydrates)	61.4 ± 3.6^{ab}	48.8 ± 4.2^{ab}	51.1 ± 4.0^{ab}	46.5 ± 4.3^{ab}	33.4 ± 4.1^{ab}	67.6%	52.2 ± 5.1^{a}
Duck (Protein)	57.5 ± 3.6^{a}	56.2 ± 4.2^{b}	61.4 ± 4.0^{ab}	52.5 ± 4.3^{ab}	39.7 ± 4.1^{b}	64.7%	45.8 ± 5.1^{a}
Butter (Fat)	75.1 ± 3.6^{c}	39.4 ± 4.2^{a}	49.2 ± 4.0^{a}	40.2 ± 4.3^{a}	25.3 ± 4.1^{a}	70.6%	57.7 ± 5.1^{a}
Cucumber (Low-calorie)	68.5 ± 3.6^{bc}	54.5 ± 4.2^{b}	64.2 ± 4.0^{b}	55.1 ± 4.3^{b}	40.8 ± 4.1^{b}	76.5%	58.8 ± 5.1^{a}

Table A5.3. Liking of food products (on 100 mm VAS) used in specific appetite questionnaire and *ad libitum* lunch.

Macronutrient category	Food product	Mean ± SE
Carbohydrates	Bread	73.0 ± 2.9^{ab}
	Corn	70.0 ± 2.9^{a}
	Crouton	70.3 ± 2.9^{ab}
	Pasta	76.3 ± 2.9^{ab}
Protein	Chicken	78.0 ± 2.9^{ab}
	Ham	68.5 ± 2.9^{a}
	Beef	70.7 ± 2.9^{ab}
Fat	Bacon	69.8 ± 2.9^{a}
	Cheese	82.7 ± 2.9^{b}
	Nuts	73.1 ± 2.9^{ab}
Low-calorie	Cucumber	76.3 ± 2.9^{ab}
	Melon	76.8 ± 2.9^{ab}
	Tomato	75.6 ± 2.9^{ab}

Chapter 6

Does odour priming influence snack choice? – An eye-tracking study to understand food choice process

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Abstract

Awareness of food sensory cues in our surroundings may influence our eating behaviour in different ways. For example, exposure to non-consciously perceived odours may influence food choice but not appetite. Moreover, this type of exposure may mainly influence the food choice of starters or desserts but not of main courses. This infers that odour priming may influence impulsive or rewarding food choice but may not overrule our habits concerning the choice of a main meal. It is crucial to understand the role of odour priming on eating behaviour and how people can be steered towards healthier options. Implicit measures, such as visual attention, may be central to understand the food choice process. Therefore, we aimed to determine how non-conscious exposure to odours affect congruent snack choice (i.e. with similar taste characteristics) and whether this is modulated by visual attention. A total of 53 healthy young adults took part in two test sessions in which they were non-consciously exposed to sweet and savoury odours. In each test session, visual attention was investigated by means of a wearable eye-tracker and subsequent snack choice was (covertly) measured. Our results showed that congruent snacks were fixated on first. However, sweet snacks were fixated on more frequently, and for a longer period of time, and were chosen most often, irrespective of the type of odour exposure. Our findings indicate that odour priming might steer the initial orientation towards congruent foods, but other factors (e.g. cognitive) may overrule its effect on the final choice.

Introduction

One of the main public health concerns is how to steer people towards healthier eating habits (Vecchio & Cavallo, 2019). Appetite, and even meal initiation, may be triggered by sensory cues such as food odours (Smeets & Dijksterhuis, 2014; Yeomans, 2006a; Zafra, Molina, & Puerto, 2006). However, conscious and non-conscious odour exposure may differentially impact eating responses (Boesveldt & de Graaf, 2017; McCrickerd & Forde, 2016; Smeets & Dijksterhuis, 2014).

Non-conscious exposure to ambient odours may act as a prime and lead to choosing congruent foods (Chambaron, Chisin, Chabanet, Issanchou, & Brand, 2015; de Wijk & Zijlstra, 2012; Gaillet-Torrent, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2014; Gaillet, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2013). For example, a non-consciously (or non-attentively) perceived fruity odour led to a greater selection of fruity desserts compared to the control (Gaillet-Torrent et al., 2014). Interestingly, previous research seems to suggest that odour priming may primarily influence food choice of starters (Gaillet et al., 2013) or desserts (Chambaron et al., 2015; Gaillet-Torrent et al., 2014) but does not influence the main meal (Morquecho-Campos, de Graaf, & Boesveldt, 2021; Mors, Polet, Vingerhoeds, Perez-Cueto, & de Wijk, 2018). Taking these findings together, we speculated that odour priming could mainly influence the choice of (impulsive) foods , such as snacks, but does not impact the choice of a main meal, which is related to habits and dietary patterns.

Beyond explicit measures, such as self-reported appetite ratings, the use of implicit measures, i.e. reaction time or eye-movements, could be useful to detect more unconscious and spontaneous behaviour that could lead to a better understanding of actual eating responses and the food choice process (Ayres, Conner, Prestwich, & Smith, 2012; De Houwer & Moors, 2010; Finlayson, King, & Blundell, 2008; Wang, Cakmak, & Peng, 2018). In the last decades, eye-tracking, which allows exploration of human behaviour in naturalistic environments, has been used as a reliable and well-validated tool to investigate eye movements (Casado-Aranda, Sánchez-Fernández, & Ibáñez-Zapata, 2020; Rayner, 1998). Eye movements are linked to perceptual and cognitive processing to reflect visual attention (Duchowski, 2007; Hayhoe & Ballard, 2005; Orquin & Mueller Loose, 2013; Rayner, 1998). Visual attention plays a beneficial role in understanding unconscious responses such as decision-making and choice (Carrasco, 2011; Krajbich, Armel, & Rangel, 2010; Orquin & Mueller Loose, 2013). Some eye-tracking studies have been performed to understand food choice through visual attention, for example (Hummel, Zerweck, Ehret, Salazar Winter, & Stroebele-Benschop, 2017; Peng-Li, Byrne, Chan, & Wang, 2020; van der Laan, Papies, Hooge, & Smeets, 2016; Wang et al., 2018). Moreover, Seo et al. investigated the role of odours in selective visual attention. Through a screen-based eye-tracking experiment, they showed that participants observed

images that were congruent with the exposed odour longer and more frequently compared to a non-odour condition (Seo, Roidl, Müller, & Negoias, 2010). Thus, eye-tracker technology could give a better understanding of visual attention and may predict specific food choice upon odour priming.

The 'gaze bias theory' in visual attention suggests that items that are chosen, are gazed at for longer (Pieters & Warlop, 1999; Schotter, Berry, McKenzie, & Rayner, 2010). However, it is still unclear if this theory applies to food items as well. For instance, Wang et al. showed that food choice, by means of an *ad libitum* buffet, was not predicted by total fixation time (Wang et al., 2018). Armel and collaborators found that manipulating the time of fixation may influence choice, i.e., products were chosen upon a longer fixation compared to shorter fixation (Armel, Beaumel, & Rangel, 2008). Other researchers have suggested that the direction of the first gaze, but not the total fixation time, predicts consumption (Werthmann et al., 2011), while others did not find a relation between first fixation and choice (van der Laan, Hooge, De Ridder, Viergever, & Smeets, 2015).

Eye-tracking technology could give clearer insights regarding the processes involved in food exploration and choice upon odour priming. Therefore, the aim of the current study is to determine the impact of odour priming on snack choice, and how this is mediated by visual attention processes. We hypothesized that the visual attention metrics (first fixation location and duration, fixation count, and total fixation duration) and food choice would be congruent with the exposed odour: e.g. visual attention and food choice would be in favour of sweet snacks (i.e. chocolate bar, wine gums, etc.) compared to savoury snacks (chips, meat snacks, etc.) after non-conscious odour exposure of sweet odours (i.e. chocolate or bubble-gum) and *vice versa*. Moreover, our secondary objective was to explore whether the effect of odour priming on food choices could be predicted by visual attention and snack attributes.

Materials and methods

Participants

People from different nationalities between 18 and 35 years old were recruited within the Wageningen area via social media. Potential participants provided their informed consent and filled out the online screening questionnaire – via EyeQuestion® (Version 3.11.1, Logic8 BV) – to determine their eligibility. The screening questionnaire consisted of questions regarding general lifestyle and medical information, and a colour blindness test (Ishihara, 1951). Also, eating behavior, by means of the Dutch Eating Behaviour Questionnaire (DEBQ (van Strien, Frijters, Bergers, & Defares, 1986)), impulsiveness, by the Barratt Impusiveness Scale (BIS-11 (Patton, Stanford, & Barratt, 1995)), and behavioural approach system, via the Behavioural Activation System scale (the BAS (Carver & White, 1994)), scores were assessed. Inclusion criteria consisted of: body mass index (BMI) between 18.5 and 30 kg/m^2; fluency in English; full colour vision as determined by the Ishihara's colour test (Ishihara, 1951); and self-reported normal vision or use of contact lenses. Exclusion criteria consisted of: eye limitations or a history of medical eye procedures, such as any eye surgery (e.g. corneal surgery), or dependence on any type of (bifocal) glasses; habitual smoking; any dietary restriction, allergy, intolerance, or oversensitivity to food used in this study; use of medication other than occasional use of pain medication (such as paracetamol and NSAIDs) or monophasic birth control; pregnancy; breastfeeding; participation in other medical studies; or participation in our previous studies (Morquecho-Campos, de Graaf, & Boesveldt, 2020).

Sixty participants were included in the study. However, 57 participants completed the two test sessions. Of those, four participants were excluded from the data analysis: two participants did not fulfil the inclusion criteria and two guessed the goal of the experiment. Therefore, 53 participants (39 females and 14 males; 24.4 ± SD 3.3 years old; BMI of 22.6 ± SD 2.1 kg/m^2) were included in the analysis. Further characteristics of the participants can be found in the supplementary materials (**Table A6.1**). Participants were compensated for their contribution at the end of the study. The study was conducted in accordance with the Declaration of Helsinki (revised in 2013) and approved by the Medical Ethical Committee of Wageningen University (NL51747.081.14).

Stimuli

Odour stimuli

Odours were selected to represent taste qualities: sweet and savoury. The odours were distributed in air-conditioned rooms by means of vaporizers (Iscent, Zeewolde, The Netherlands). Pilot studies were conducted in order to determine the proper

odours to be used and their concentrations. We aimed to achieve a perceived intensity of 35–50 mm on a visual analogue scale (VAS), which should be non-consciously (or inattentively) perceived by participants who were kept naïve to the odour exposure. The pilot studies consisted of several short sessions where participants – who did not participate in the actual study – were exposed to an odour dispersed in one of the rooms. At least five participants took part in each condition. Participants were asked about their awareness of any odour present in the room, and if so, to assess the odour's intensity on a 100 mm VAS ('Not at all'–'Very'), and to identify the odour and its taste category (sweet, savoury, or neutral). Based on the outcomes, we selected two odours per category and determined suitable intensities: bubble-gum (International Flavour and Fragrances, IFF SC753057; 3% in propylene glycol, PG) and chocolate (IFF 10810180; 60% in PG) for the sweet category; fries (Symrise 769462; 100%) and duck (meat) (Symrise 619322; 2% in PG) for the savoury category. The final dispersion frequency and perceived intensity of the odours can be found in the supplementary materials (**Table A6.2**).

Odour stimuli were prepared in advance and stored in the fridge until the morning of the test session. In the morning of the test session, the odour stimuli were stored at room temperature until the test session.

Snack products and food area

A pilot study was conducted to determine the familiarity, liking, and sweet or salty/savoury association of different potential snacks. Additionally, the percentage of respondents that have previously consumed the snack was assessed. These snacks were selected to match the available odour options (bubble-gum and chocolate for the sweet category and fries and duck (meat) for the savoury category). Sixty-three people living in the Netherlands, aged between 18 and 39 years old, completed the online survey (Qualtrics®, USA). Based on the results (see results in supplementary materials **Table A6.3**), we chose 12 food products (6 per taste category; 3 per sub-category) that are commonly recognized by people living in the Netherlands. The snack versions of these products were selected and used for the food area. The products and their description can be found in **Table 6.1**, where it is shown that the snack products were similar in energy density.

Table 6.1. Snack products in the food area.

Taste Category	Sub-category	Snack	Energy per package (kcal)
Sweet	**Bubble-gum**	Haribo Goldbears	257.3
		Haribo Peaches	266.3
		Skittles	178.7
	Chocolate	KitKat	213.7
		M&M's chocolate	216
		Twix	246.5
Savoury	**Fries**	Doritos nacho cheese	219.6
		Lay's Naturel	248.0
		Lay's Paprika	215.2
	Meat	BiFi	94.2
		Kettle Chips honey barbecue*	210.4
		Lay's Bolognese	213.2

*Kettle Chips Honey Barbecue was not previously piloted. It was included as an alternative to Doritos Bits Honey BBQ which was not available.

The snack products were placed in a doorless mini fridge (88 litre; 82.5 (h) × 43 (w) × 48 (d) cm). Product placement has been shown repeatedly to influence visual attention (Chandon, Hutchinson, Bradlow, & Young, 2009; Sütterlin, Brunner, & Opwis, 2008), accordingly, product placement was randomized over blocks and test sessions. The 12 snacks were distributed over 12 spots in the mini fridge (3 items per row in the 4 available rows). Three main criteria were followed to select suitable randomization schemes to prevent bias in attention and choice due to product-placement effects: 1) two snacks which belong to the same brand and category should not be next in line to each other to increase the perceived diversity; 2) the first (upper left) and the last (lower right) product had to be incongruent in taste, as people have a tendency to remember the first and the last product in a series best (Chandon et al., 2009; Dayan & Bar-Hillel, 2011) – for example, if the top left product was sweet in taste, the bottom right had to be savoury; 3) the same incongruency criteria should be applied for the two products in the centre of the food area (mid products in row two and three), as people have the tendency to pay attention to and choose products more often that are in the center of a shelf (Atalay, Bodur, & Rasolofoarison, 2012; Chandon et al., 2009). By placing taste-incongruent snack products in these specific areas, and by randomizing product placement in general, biases in attention and choice due to product-placement effects are diminished (Mantonakis, Rodero, Lesschaeve, & Hastie, 2009). Furthermore, we attempted to equalize the surface area of the snacks by piling-up smaller-sized snack items to form a stack. These size-adjustments were done because it has been demonstrated that surface size influences stimulus driven (bottom-up) attention and therefore plays a role in decision making (Marchiori, Corneille, & Klein, 2012). A visual representation of the food can be found in **Figure 6.1** – Room 2.

The mini fridge (food area) was placed on a table adjustable in height. The height of the table was matched to the participants' body height in order to assure a horizontal angle of view to the central area of the mini fridge.

Procedure

The study followed a randomized and balanced within-subjects block design. Participants visited the test location two times, once for each test condition (sweet and savoury odour). Participants were only exposed to one odour per taste category in a counterbalanced order. Individual test sessions took place in the afternoon after lunch time (14.00–17.00 h; four time slots per day) and were held with at least two days in between as a washout period and at approximately the same time of day. The participants were asked to eat their habitual lunch no later than 2 hours and no sooner than 45 min before test sessions, and to drink only water 1 hour before the test session to standardize hunger states. Participants were required to refrain from the use of any heavy make-up around their eyes (e.g. mascara, eyeliner, and eyeshadow) which could affect eye movement measures (Orquin & Holmqvist, 2019). Moreover, participants were asked to reschedule their test session if they presented any symptom related to Covid-19. An alternative goal ('To investigate the role of hunger and satiety on the performance of memory tasks using eye-tracking technology') was established to distract participants from the actual goal.

Upon arrival, participants were escorted to Room 1. This room was equipped with a vaporizer to disperse the ambient odour (Iscent, Zeewolde, The Netherlands) where participants were exposed to a non-consciously detectable odour for 10 min. In this room, we first explained the procedure and participants were fitted with a pair of eye-trackers (Tobii eye-trackers wireless glass II, Sweden). The eye-trackers were calibrated using a one-point calibration followed by a five-point validation (Duchowski, 2007). Participants were instructed to keep the eye-tracker until the end of the test session to avoid recalibration. Then, they assessed their general appetite and performed a memory task (#1) which was in line with the alternative goal. After finishing the memory task, they assessed the difficulty of the memory task and their stress level (#1). Subsequently, participants were escorted to Room 2. This room was equipped with a food area where participants could select and consume a snack. Participants stood in front of the food area – ~ 50 cm from it – while exploring the assortment of different snacks. They were instructed to select only one snack and to consume as much as they could during the break (5 min). After the break, participants were escorted back to Room 1 where they assessed their general appetite after the snack consumption, performed the memory game again (#2), and assessed the difficulty of the memory task and their stress level (#2).

At the end of the second session, a debriefing questionnaire on the aim of the study and perception of any odour during the test session was completed. Participants

were also asked to assess the odours and the snacks in their attributes and to evaluate their sense of smell through Sniffin' Sticks odour identification test (Oleszkiewicz, Schriever, Croy, Hähner, & Hummel, 2019). After participants completed the questionnaire, they were debriefed. EyeQuestion® was used to instruct participants and collect the data.

The test procedure is shown in **Figure 6.1.** Only one odour per day was used to avoid odour contamination. After the end of the day, the room was ventilated and the odour was fully removed overnight.

Figure 6.1. Procedure for each test session. *Memory tasks were bogus tasks as part of the alternative goal to distract participants' attention from the actual goal of the study.

Measurements

General appetite

General appetite was determined by assessing hunger, fullness, prospective consumption, desire to eat, and thirst on 100 mm VAS; anchored by 'Not at all'–'Very'. A 'General appetite score' variable was computed with the average of hunger, desire to eat, prospective consumption, and the inverse fullness score (100 − fullness). We used the 'General appetite score' computed with the information assessed at the beginning of the test session as a potential covariate.

Memory task – as bogus task

The memory task was performed online via https://www.improvememory.org/brain-games/memory-games/. Two types of classic memory matching games were used as bogus tasks ('Easter Memory' and 'Memory III'). These tasks were moderate in difficulty to be in line with our alternative goal. The memory tasks were randomly assigned to each test session over the blocks.

Stress and difficulty level rating

Stress and difficulty levels were assessed by rating 'How stressed do you feel at this moment?' and 'How difficult was the task you just performed?' on 100 mm VAS anchored from 'not at all' to 'very stressed/difficult'.

Visual attention – eye-tracking metrics

Area of interests (spatial region where the eye movement measures are quantified; AOI) were labelled in 'Sweet' and 'Savoury' depending on the taste qualities of the snack products in the food area. Four eye-tracking metrics were investigated. *Location of the first fixation* which is the initial orientation. *First fixation duration (ms)* is defined as the sum of all fixations on an AOI the first time it was viewed before leaving it. *Fixation count (#)* is the number of fixations recorded within an AOI. *Total duration of fixations* is defined as the sum of duration of all fixations within an AOI (Orquin & Holmqvist, 2019; Tobii, 2016).

Food choice

Congruency of food choice was determined by the snack product chosen relative to the odour category. For example, if participants were exposed to a sweet odour and selected a sweet snack from the food area, this was labelled as congruent (1), otherwise incongruent (0).

Debriefing session

Goal and odour perception

Participants completed a debriefing questionnaire where they were asked about the goal of the study and whether they had perceived an odour in Room 1 in any of the two test sessions.

Rating of odours' attributes

Participants received an odourised disposable sheet of paper with the odour to be evaluated. They assessed the two odours they were exposed to during the test sessions on their liking, intensity, familiarity, intention to eat a product of that odour, mouth-watering sensation, and sweet and salty/savoury associations on a 100 mm VAS scale anchored by 'Not at all' – 'Very much'. Identification of the odour was assessed by a multiple forced-choice task, ['Which of the following labels best fits the smelled odour?']. Afterwards, the odour-label correspondence was assessed ['How well do you think this smell corresponds to *'specific smell'*?'] on a 100 mm VAS scale. After evaluating the first odour, participants waited 30 sec before receiving the next odour. The odours' attributes were used as covariates in the statistical analyses. Odour ratings can be found in the supplementary materials (**Table A6.4**).

Rating of snacks' attributes

Participants assessed the snack products available in the food area on their liking, familiarity, and sweet and salty/savoury associations on a 100 mm VAS scale. This

was done using pictures of the snack products in the food area. The attributes were used as covariates in the statistical analyses and the ratings can be found in the supplementary materials (**Table A6.5**).

Odour identification test – Sniffin' Sticks

Odour identification scores were assessed using the identification part of the Sniffin' Sticks test which contains 16 common odours (Burghart Messtechnik GmbH, Wedel, Germany). Participants were presented (by the experimenter) with an disposable sheet of paper where a 2 cm stripe was drawn on with the Sniffin' Stick, as advised by the current (at the time of writing) Covid-19 recommendations of the German Society for Otolaryngology (MediSense, 2020). Participants selected the label which best describes the smelled odour (multiple forced-choice task). Between each odour, participants took a break of 20 seconds. A correct sum score ≥ 12 (out of 16) is considered as normosmic (Oleszkiewicz, Schriever, Croy, Hähner, & Hummel, 2019). The mean (and standard deviation) of the odour identification scores was 13.5 ± 1.3. Participants' scores were added to the statistical analyses as a covariate.

Statistical analyses

We were interested in the congruency effect, which is dependent on the taste category of the snack products, relative to the type of odours exposed (e.g. sweet AOI was defined as a congruent AOI after exposure to a sweet odour but as an incongruent AOI after savoury odour exposure, and *vice versa*). Therefore, data were collapsed over the two odours and the six snack products of the same category (i.e. 'Sweet' and 'Savoury').

Results with a p value lower than 0.05 were considered statistically significant. All statistical analyses were carried out in RStudio (RStudio Team, 2016), and graphs were made using GraphPad Prism 5.0 (GraphPad Prism Software). Most of the analyses consisted of (generalized) linear mixed models, carried out using the lme4 statistical package in R (Bates, Mächler, Bolker, & Walker, 2015), unless indicated otherwise. For these models, participants and test session nested in (13) groups based on participants' availability (3 groups consisted of 3 participants, 6 of 4 participants and 4 of 5 participants) were evaluated as potential random factors. After checking the random part of each model, all final models included only participants as random effects. The best fitting models were selected on the basis of parsimony following a backward approach. Necessay assumptions were checked for each model.

Odour and snack attribute ratings were analysed as the dependent variable, with odour or taste category as fixed effects and participants and evaluation order as potential random effects. This data is shown in **Table A6.4** and **A6.5** of the supplementary materials.

Visual attention – eye-tracking metrics

Eye-tracking data retrieved from the Tobii Pro Eye tracking glasses 2 were initially processed by means of Tobii Pro Lab (version 1.145.28180). The eye-tracker collected pictures of the eyes with a sampling rate of 50 Hz, to assess participants' gaze points based on the position of their eyes. The time of interest for our analyses was established as the time between two events, labelled as 'start of the snack search' and 'end of the snack search'. The 'start of the snack search' was defined as the time point where the participant opened the door to Room 2, right before they were exposed the food area; the 'end of the snack search' was established as the moment the participant took the chosen snack. The eye-tracker recordings were analysed by two different researchers in close cooperation. An I-VT-attention-filter and real-world mapping tool (automatic gaze coding) were performed on the raw data during this time of interest. Prior to each test session, a snapshot of the food area was taken and used for the real-world mapping. Areas of Interest (spatial regions where the eye movement measures are quantified; AOI) were defined and labelled in each snapshot. AOIs were labelled per taste category ('Sweet' and 'Savoury'). All the videos had a gaze quality above 70%, this cut-off point is in line with other eye-tracking studies (Peng-Li et al., 2020); therefore, all of them were included for further analysis. Four main parameters – *first fixation location and duration, fixation count,* and *total duration of fixations* – were investigated.

Two stages of analysis were performed for the first fixation location. In the first stage, a binomial test was performed to assess whether congruent and incongruent snacks have the same probability of being first fixated on. In the second stage, a generalized linear mixed model on the logit scale (glmer function) was performed to investigate whether the type of odour exposure (i.e. sweet and savoury) influences the congruency of the first fixation location. Congruency of the first fixation location and odour category were added as the dependent variable and fixed effects, respectively. Potential covariates were initially added and systematically removed following this order: personal characteristics (gender, age, BMI, DEBQ, BAS, BIS-11, and Sniffin' sticks); general appetite score and thirst rating before the memory task #1; stress levels and difficulty of the memory task #1; perception of ambient odour during the test session; odour attribute ratings; and snack attribute ratings.

Proportions of first fixation duration, fixation count, and total duration of fixations on congruent AOI were computed. Congruent AOI depended on the AOI relative to the odour exposure. Proportion of a parameter (e.g. total duration of fixations) on congruent AOI was calculated by dividing the total duration of fixations on congruent AOI by the sum of total duration of fixations on congruent and incongruent AOI. Furthermore, the data analysis of each parameter followed two stages. In a first stage, we assessed whether the intercept was significantly different from chance level to determine whether odour exposure influences visual attention towards congruent AOI. For this, a new variable 'Proportion of a parameter on congruent AOI above chance level' was computed by subtracting 0.5 from the proportion of a

parameter on congruent AOI. Linear mixed models (lmer function) were performed with 'Proportion of a parameter on congruent AOI above chance level' as the dependent variable. An intercept significantly different from 0 meant that the odour exposure significantly influences the (in)congruency of visual attention metrics. In a second stage, we assessed the influence of the type of odour category on the congruent AOI. A linear mixed model was performed with the proportion of each parameter on the congruent AOI as the dependent variable and type of odour category as a fixed effect. Systematic removal of the potential covariates was followed as indicated above.

Moreover, exploratory analyses were perfomed to determine the role of taste category AOI, similar to the two stages described above. Therefore, the proportion of a parameter (e.g. total duration of fixations) on the sweet AOI was calculated by dividing the total duration of fixations on the sweet AOI by the sum of total duration of fixations on sweet and savoury AOI. As mentioned above, for the first stage, 'Proportion of a parameter on sweet AOI above chance level' was computed and added as the dependent variable. For the second stage, we assessed the influence of the odour category on visual attention towards the sweet AOI, and thus the proportion of each parameter on the sweet AOI was included as the dependent variable.

Food choice

Firstly, a binomial test was performed to test whether congruent *vs* incongruent food choice (i.e. choice of a savoury snack product after the exposure of a savoury odour was labelled as congruent, otherwise incongruent) have the same probability. Secondly, a generalized linear mixed model on the logit scale was performed to investigate whether the type of odour exposure influences the congruent food choice. Congruency of the chosen category and odour category were added as the dependent variable and fixed effects, respectively. Systematic removal of potential covariates was carried out as indicated above.

Similar to the eye-tracker metrics, exploratory analyses were perfomed to determine the role of the AOI taste category on the two stages described above. Firstly, a binomial test was performed to test whether sweet *vs* savoury snacks have the same probability of being chosen. Secondly, a generalized linear mixed model was performed to investigate whether the type of odour exposure influences the food choice of sweet snacks. Sweet food choice (sweet snack was chosen/sweet snack was not chosen) and odour category were added as the dependent variable and fixed effects, respectively.

Food choice prediction

A binomial logistic regression was performed to test whether visual attention metrics and snack attributes mediate food choices. Snack choice (chosen/non-chosen) was entered as the dependent variable. Odour category, taste category of the chosen

snack, total duration of fixations (in seconds), fixation count, first fixation duration (in seconds), taste category of the first fixation, congruency of the first fixation with the exposed odour, liking, and familiarity of the snack were entered as prediction factors.

Debriefing – odour perception

Data from odour perception on each test session was classified into four score categories as indicated by Mors et al. (2018). These scores were added to the statistical models as covariates.

Results

Visual attention – eye-tracking metrics

First fixation location

The binomial test revealed that participants were more likely to first fixate on congruent snacks (= AOI) after odour exposure compared to incongruent snacks (probability = 0.63, 95% CI = [0.53, 0.7], p = 0.01), e.g. sweet products were more often fixated on first after sweet odour exposure.

The logistic mixed model showed that the probability of congruent first location did not differ between sweet *vs* savoury odour exposure (sweet odours: 0.63, 95% CI = [0.50,0.76]; savoury odours: 0.63, 95% CI = [0.50,0.77]; χ^2 (1) = 0.03; p = 0.86; model included DEBQ External eating score and BIS 11 non-planning score).

Duration of the first fixation

In the first stage, the intercept of congruent snacks was significantly lower than 0 (−0.06 ± 0.02; t(52) = −3.0, p = 0.005), meaning that the proportion of duration of first fixation on the snack products was longer for incongruent snacks compared to congruent snacks.

In the second stage, the proportion of duration of the first fixation on congruent snacks tended to be similar across odour categories (**Table 6.2A**). Exploratory analyses showed that the intercept of sweet snacks was not significantly different from 0 (0.03 ± 0.02; t(102) = 1.75, p = 0.08) and that the duration of the first fixation on sweet snacks was higher after savoury odour exposure compared to sweet odour exposure (**Table 6.2B**).

Fixation count

Firstly, the intercept of congruent snacks was not significantly different from 0 for fixation counts (0.02 ± 0.02; t(102) = −1.38, p = 0.17), meaning that after odour exposure, the number of fixations on congruent snacks was similar to the number of fixations on incongruent snacks. Secondly, participants fixated more frequently on congruent snacks after exposure to sweet odours compared to savoury odours (**Table 6.2A**). Exploratory analyses showed that the intercept of sweet snacks was significantly higher than 0 (0.06 ± 0.02; t(52) = 3.67, p = 0.001) and that participants fixated more frequently on sweet snacks after sweet odour exposure compared to savoury odour exposure (**Table 6.2B**).

Fixation duration

In the first stage, the intercept on congruent snacks was not significantly different from 0 (0.02 ± 0.02; t(102) = 0.73, p = 0.47). In the second stage, a linear mixed model with proportion of total fixation duration on congruent snacks showed that

participants fixated for a longer time on congruent snacks after the exposure of sweet odours compared to savoury odours (**Table 6.2A**).

Exploratory analyses showed that the intercept on sweet snacks was significantly higher than 0 (0.09 ± 0.02; $t(52) = 3.9$, $p = 0.0003$) and the proportion of total fixation duration on sweet snacks revealed no significant difference among odour categories (**Table 6.2B**). All these models indicate that participants fixated on sweet snack products for a longer time, regardless of the type of odour exposure.

Table 6.2. Proportion of duration of first fixation, fixation count, and fixation duration on congruent snacks (A) and on sweet snacks (exploratory analyses – B). Values are expressed as mean and standard error.

Outcome measure	Odour category		Statistical information
	Sweet	Savoury	
A) Congruent snacks			
Proportion of duration of first fixation[1]	0.47 ± 0.03	0.41 ± 0.03	F(1,51) = 3.7, $p = 0.06$
Proportion of fixation count[2]	0.57 ± 0.02	0.47 ± 0.02	**F(1,65) = 10.20, $p = 0.002$**
Proportion of fixation duration[1]	0.60 ± 0.03	0.43 ± 0.03	**F(1,51) = 19.93, $p < 0.0001$**
B) Exploratory analyses – Sweet snacks			
Proportion of duration of first fixation[3]	0.48 ± 0.03	0.59 ± 0.03	**F(1,54) = 7.67, $p = 0.01$**
Proportion of fixation count[4]	0.59 ± 0.02	0.54 ± 0.02	**F(1,50) = 4.10, $p = 0.048$**
Proportion of fixation duration[1]	0.60 ± 0.03	0.57 ± 0.03	F(1,51) = 0.87, $p = 0.35$

Covariates included in the mixed models: [1]No covariates; [2]Familiarity and liking of the congruent snack products and familiarity of the odour; [3]Odour-label association; [4]Stress after the first memory task.

Food choice

Firstly, a binomial test revealed that congruent and incongruent snacks were equally frequently chosen (probability = 0.53, 95% CI = [0.43, 0.63], $p = 0.62$). Secondly, the logistic mixed model showed that participants were more likely to choose congruent snacks after a sweet odour compared to a savoury odour (χ^2 (1) = 15.71; $p < 0.0001$; no covariate was included in the final model; **Figure 6.2A**). Exploratory analyses showed that participants were more likely to choose sweet snacks compared to savoury snacks (probability = 0.63, 95% CI = [0.53, 0.72], $p = 0.01$) and that sweet snacks were chosen with similar frequency regardless of the odour exposure (χ^2 (1) = 0.35; $p = 0.55$; mixed model included familiarity of the chosen snack products; **Figure 6.2B**).

Figure 6.2. Proportion of congruent (A) and sweet (B) food choice after sweet and savoury odour exposure.

Food choice prediction

The factors involved in the food choice prediction were 'Total fixation duration' (sec), 'Fixation count', 'Liking', and 'Familiarity of the snack'. The predicted logit snack choice follows an equation of −7.13 + (0.96*Total fixation duration in seconds) – (0.22*Fixation count) + (0.03*Liking of the snack) + (0.05*Familiarity of the snack) (see **Table 6.3**). This equation indicates that the odds of a snack being chosen (vs not being chosen) increases 2.6 times (160%) for every second that a snack is fixated upon, given that all the other variables remain constant. The odds of the snack being chosen (vs not being chosen) decreases 20% for every unit change in fixation count. The odds of the snack being chosen (vs not being chosen) increases 3 and 5% for every unit change in liking and familiarity of the snack, respectively.

Table 6.3. Binomial logistic regression for food choice prediction.

Predictor factors	β	Seβ	Wald X^2 (1)	*p*-value	e^{β} = odd ratio (mean (95% CI))
Intercept	−7.13	1.43			
Total fixation duration	0.96	0.20	23.24	<0.0001	2.60 (1.76, 3.84)
Fixation count	−0.22	0.07	11.46	0.001	0.80 (0.71, 0.91)
Liking of the snack	0.03	0.01	4.73	0.03	1.03 (1.003, 1.06)
Familiarity of the snack	0.05	0.01	13.99	0.0002	1.05 (1.02, 1.08)

Debriefing – odour perception

Two participants reported a link between odours and snack choice and they were thus removed from the analysis and results as mentioned above. The vast majority of participants believed that the aim of the study was related to the memory task and hunger state. A small percentage (13.2%; 7 out of the 53 participants included for analysis) inferred that the aim was related to food choice; however, they did not make the connection between odour exposure and true study aim. Moreover, participants did not perceive the odour in 87.7% of the test sessions, in 2.8% of the test sessions the odour was perceived but was incorrectly identified. In the remaining 9.4% of the test sessions participants perceived the odour and correctly identified it.

Discussion

The aim of the current study was to determine the impact of odour priming on congruent snack choice and how this is mediated by visual attention processes. Our results show that, overall, sweet snacks were chosen more often compared to savoury snacks, regardless of the type of odour exposure. Moreover, sweet snack products were more frequently and longer fixated on. On the other hand, the first fixation location was congruent to the odour exposure (i.e. participants first fixated on sweet snack products after being exposed to sweet odours, and *vice versa*); however, participants spent more time gazing at incongruent snack products during the first fixation. In addition, our results show that food choice is mediated by total fixation time, fixation count, and liking and familiarity of the snack products, but not by odour exposure, first fixation location, nor duration.

According to Russo and Leclerc, (food) choice processes consist of different stages: initial orientation (and screening), evaluation, and verification (Russo & Leclerc, 1994). This segmentation of the choice process may result in distinct eye-movements during the different stages (Glaholt & Reingold, 2011; Russo & Leclerc, 1994). Therefore, we investigated how visual attention evolves over these different stages. The first fixation in a food-choice paradigm is related to the orientation stage. This stage may occur implicitly, without conscious control, and is considered to be a more intuitive and spontaneous stage (Betsch, Hoffmann, Hoffrage, & Plessner, 2003; Betsch, Kaufmann, Lindow, Plessner, & Hoffmann, 2006; Russo & Leclerc, 1994). The first fixation location in this study was dominated towards snacks that were congruent with the odour exposure. We infer that this congruency effect is the result of implicit odour priming. During the orientation and screening stage an overview of the available products is acquired (Russo & Leclerc, 1994). Some of the products gazed upon during this first stage are never fixated on again, showing eliminations before moving to the following stages (Russo & Leclerc, 1994). Our results showed that incongruent snacks were fixated on longer than congruent snacks during this first fixation, suggesting an exploration of the available products

and certain (initial) eliminations. Next, cognitive processes are involved in the evaluation and verification stages which may be reflected in an increase in the number of fixations leading up to a final decision (Betsch, Plessner, & Schallies, 2004; Glaholt & Reingold, 2011; Klichowicz, Scholz, Strehlau, & Krems, 2016; Russo & Leclerc, 1994). In our data, sweet snack products were fixated on more frequently and for longer compared to savoury ones, regardless of the type of odour exposure. Although the first fixation location was congruent to the odour exposure, other snacks may have gained importance during the choice process to fulfil the task instruction. In our study, the task instruction *'choose only one snack that you want to eat now'* can be translated as a *'desire to eat in this moment'* goal. This goal could activate top-down cognitive processes that depend on the individual and their attentional and motivational states, such as previous food consumption, expectations, previous experience with the snack(s), momentary cravings, etc. (Corbetta & Shulman, 2002; Pieters & Wedel, 2004; Rayner, Miller, & Rotello, 2008). Taking this together, we can infer from our data that the first fixation location was influenced by bottom-up sensory and/or environmental factors (i.e. odour exposure) where congruent snacks were first fixated on in response to the odour priming; however, the final snack choice was influenced more by top-down controlling processes and led towards the choice of mainly sweet snacks, irrespective of the exposed odour.

The remaining question is: Why did odour priming mainly influence the first fixation location and not the complete choice process? We have hypothesized the following explanations. Firstly, participants visited the facilities in a post-lunch state, between 14–17h. According to de Graaf et al, the appetite for something sweet and savoury is relatively similar at this time point (de Graaf, Jas, van der Kooy, & Leenen, 1993). Although we did not collect any information about the type of lunch that the participants had consumed before the test session, previous research has shown an increased appetite for savoury products right before lunch, followed by a decrease in the two hours after, suggestive of sensory-specific satiety (de Graaf et al., 1993; Rolls, Rolls, Rowe, & Sweeney, 1981). Moreover, the combination of a stronger modulating effect of savoury meals on subsequent food choice compared to sweet (Griffioen-Roose, Finlayson, Mars, Blundell, & de Graaf, 2010), together with a more constant appetite for sweet across the day (de Graaf et al., 1993) might explain the predominantly sweet choices in the current study. Secondly, our odour prime could have been strong enough to steer the participants' initial attention towards a specific product, but not beyond that stage towards snack choice. More realistic settings involving *multisensory* priming may be vital to successfully steer the final decision towards choosing and consuming a congruent food product. Although, people can identify with the situation of buying an unplanned freshly baked muffin while walking in the supermarket or main street and smelling the chocolate odour, several studies have failed to replicate this effect in a laboratory setting (Morquecho-Campos et al., 2021; Mors et al., 2018). By using the wearable eye-tracker, we intended to gain

more insights to understand human food choice behaviour in real time. However, participants clearly came to the laboratory to participate in a study; therefore, the laboratory setting ambiance was still present. A real-life supermarket study that involved congruent odour, light, and sound exposure showed no effects on visit frequency or sales, suggesting that any sensory priming effect may be small (de Wijk, Maaskant, Kremer, Holthuysen, & Stijnen, 2018). The use of immersive rooms or virtual reality applications where participants can more vividly experience a real-life food setting (e.g. supermarket, restaurant, or main street), and where researchers can control the priming cues, could offer more insight into the impact of odour priming on food choice.

It is worth discussing the discrepancy between results from previous studies to understand the influence of odour priming on food choice or intake. Studies where odour priming did *not* impact food choice involved neutral (Mors et al., 2018) or macronutrient-related odours (Morquecho-Campos et al., 2021). However, studies where odour priming *did* impact food choice and intake involved sweet-fruity (Gaillet-Torrent et al., 2014; Gaillet et al., 2013), sweet-fatty (Chambaron et al., 2015), or high-calorie related odours (Proserpio, de Graaf, Laureati, Pagliarini, & Boesveldt, 2017). This may imply that 'indulgent' odours may influence congruent food choice and intake, while other odours do not. The current study involved odours that signalled sweet and savoury taste qualities. If we had merely looked at the impact of sweet odour exposure, our results would point towards a congruent influence of odour exposure on fixation count, fixation duration, and food choice. However, without a no-odour control condition, we cannot infer that sweet odour exposure increased visual attention towards sweet foods. Nevertheless, there are studies that found significant differences between sweet odours and no-odour conditions, indicating that sweet odour priming might elicit eating responses (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013). We therefore suggest that the type of odour used may signal different information and activate (or not) specific wanting process. Sweet may convey more hedonic properties suggesting a stronger reward activation (Ma, Ratnasabapathy, & Gardiner, 2017; Ventura, Santander, Torres, & Contreras, 2014, for reviews), while savoury food may be associated with protein intake and body homeostasis (Griffioen-Roose et al., 2010; O'Doherty, Rolls, Francis, Bowtell, & McGlone, 2001; Simpson & Raubenheimer, 2005). Therefore, the indulgent and reward information transmitted by the sweets might be stronger than a general odour priming effect. However, there is one study that is not in line with this hypothesis: a congruent effect of food choice was found after exposure to a fruity (citrus) odour but not after exposure to a sweet (vanilla) odour (de Wijk & Zijlstra, 2012). Finally, one limitation that should be mentioned which could have increased the choice of and attention to sweet snacks, was the lack of variety in the savoury snacks. Most of the savoury snacks consisted of chips (5 out 6 snacks); while the sweet snacks were evenly distributed over two categories (3 chocolates and 3 wine gums). The snack options of the sub-category meat were limited,

therefore we used chips with meat flavours. Another potential limitation of this study to capture impulsive behaviour was the unrestricted time for selecting the snack. The participants were allowed to freely explore the area, without any further indication. Automatic and more impulsive responses are triggered during short and limited time intervals, in an effortless manner, and may enhance priming effects (Hermans, De Houwer, & Eelen, 2001; Manippa, van der Laan, Brancucci, & Smeets, 2019).

As our secondary objective, we aimed to predict food choice based on visual attention and snack attributes. Our results showed that participants spent longer looking at options they chose compared to options they did not choose, suggesting longer examinations and verification of the ultimate choice. This is in line with previous studies that showed that more time is spent fixating on chosen options compared to not chosen options (Pieters & Warlop, 1999; Schotter et al., 2010); however, research on food choice have some contrary results (Armel et al., 2008; Manippa et al., 2019; van der Laan et al., 2015; Wang et al., 2018; Werthmann et al., 2011). Our prediction model also suggests that total fixation time positively predicts the food choice, while number of fixations decreases the chance of products to be selected. An increase in the number of fixations might be related to a higher working memory load and is considered to reflect decision difficulty (Krajbich, Lu, Camerer, & Rangel, 2012; Orquin & Mueller Loose, 2013). Finally, first fixation location and first fixation duration did not significantly contribute to our prediction model which is in line with van der Laan and collaborators who showed that first fixation did not influence choice (van der Laan et al., 2015). However, Werthmann et al. showed that the direction of the first gaze, and not the total fixation time, may predict consumption (Werthmann et al., 2011). Considerably more work will need to be done, in particular in the food domain, to determine the relationship between visual attention and food choice.

Conclusion

Odour priming did congruently influence the first fixation location, which is related to non-conscious control. However, sweet snacks were fixated on more frequently and for a longer period of time, and were chosen most often, regardless of the type of odour. The type of odour may signal different reward information (sweet vs savoury taste quality) and may thus influence (or not) eating behaviour. Future research using multisensory cues, more realistic settings, and implicit measures is needed to further understand the role of odours on eating behaviour.

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Author Contributions

Paulina Morquecho-Campos: conceptualization, methodology, resources, investigation, formal analysis, and writing – original draft. Ina M. Hellmich and Elske Zwart: methodology, investigation, data curation, formal analysis, writing – review & editing. Kees de Graaf: methodology and supervision. Sanne Boesveldt: conceptualization, methodology, writing – review & editing, and supervision.

Appendix 6.1

Table A6.1. Characteristics of the 53 participants in the study.

Characteristic	Mean ± SD
DEBQ*:	
Emotional eating score	2.6 ± 0.7
External eating score	3.1 ± 0.6
Restrained eating score	2.5 ± 0.6
BIS-11**:	
Attentional	2.2 ± 0.4
Motor	2.0 ± 0.4
Non-planning	2.1 ± 0.4
BAS***:	
Drive	2.3 ± 0.6
Fun seeking	2.1 ± 0.6
Reward responsiveness	1.7 ± 0.4

*DEBQ scale: 1–5, where higher scores indicate higher frequencies of enganging in behaviours that are associated with overeating (van Strien et al., 1986).
**BIS-11 scale: 1–4, where higher scores indicate higher frequencies of engaging in impulsive behaviours (Patton et al., 1995).
***BAS scale: 1–4, where higher scores indicate a higher motivation to approach goal-oriented outcomes, such as rewards (Carver & White, 1994).

Table A6.2. Pilot study to determine the dispersion frequency and perceived intensity (on a 100 mm VAS) across ambient odour categories.

Odour category	Odour	Dispersion frequency	Perceived intensity (mean ± SE)
Sweet	Bubble-gum	3 times in 5 min	49.0 ± 11.3
	Chocolate		48.8 ± 9.2
Savoury	Fries	6 times in 10 min	46.6 ± 10.8
	Duck (meat)		35.6 ± 13.5

Table A6.3. Pilot study to assess familiarity, percentage of previous consumption of the snack products, liking, and sweet and savoury associations (on a 100 mm VAS). Values are expressed as mean and standard error.

Taste category	Sub-category	Name of the snack	Familiarity	Percentage of previous consumption	Liking[1]	Sweet association	Savoury association
Sweet	**Fruity-Bubble-gum**	**Haribo Goldbears**	88.3 ± 3.0	96.2%	58.9 ± 3.8	85.1 ± 2.5	7.8 ± 2.1
		Haribo Peaches	79.3 ± 4.4	86.5%	63.6 ± 3.9	87.2 ± 2.4	11.1 ± 2.2
		Skittles	83.6 ± 3.4	98.1%	58.3 ± 3.8	84.3 ± 2.4	10.7 ± 2.2
	Chocolate	**Kitkat**	90.1 ± 2.8	98.0%	71.3 ± 3.3	81.4 ± 1.9	16.1 ± 2.8
		M&M's chocolate	88.5 ± 2.8	98.0%	65.7 ± 3.3	79.4 ± 2.8	9.9 ± 2.1
		Twix	85.6 ± 3.7	95.9%	71.9 ± 3.6	81.3 ± 2.6	18.6 ± 2.8
Savoury	**Fries**	**Doritos Nacho Cheese**	86.3 ± 2.9	94.3%	72.5 ± 3.4	19.3 ± 3.1	75.0 ± 3.1
		Lay's Naturel	92.7 ± 2.3	94.2%	75.7 ± 3.2	13.7 ± 2.6	82.3 ± 2.6
		Lay's Paprika	88.7 ± 3.1	94.2%	69.0 ± 3.0	23.8 ± 3.1	77.1 ± 3.2
	Meat	**Bifi**	65.2 ± 4.6	68.5%	51.1 ± 5.3	16.7 ± 2.8	77.8 ± 3.2
		Doritos BitsHoney BBQ[2]	61.7 ± 5.5	60.0%	69.1 ± 4.1	28.9 ± 3.2	69.3 ± 3.3
		Lay's Bolognese	69.9 ± 5.0	72.0%	58.8 ± 4.1	20.5 ± 3.0	76.0 ± 2.8

[1] The liking question was not shown if the respondents had never consumed the product before.

[2] This product was not included as a snack option in the study due to unavailability. It was replaced by Kettle Chips Honey Barbecue.

Table A6.4. Odour ratings: intensity, liking, familiarity, intention to eat a product of that odour, strength of the mouth-watering sensation that the odour produces, sweet, savoury, and odour-label association (on a 100 mm VAS) and correct identification rates (%) of used odours. Values are expressed as mean and standard error. Statistical results significantly differed between sweet and savoury odour categories.

Attributes	Odour category	
	Sweet	Savoury
Intensity	73.0 ± 2.5	62.4 ± 2.5
Liking	60.7 ± 3.0	40.6 ± 3.0
Familiarity	72.3 ± 3.4	61.4 ± 3.4
Intention to eat a product of that odour	47.0 ± 3.3	39.4 ± 3.3
Mouth-watering	41.8 ± 3.2	35.9 ± 3.2
Sweet association	81.5 ± 2.1	12.2 ± 2.2
Savoury association	24.7 ± 3.8	72.5 ± 3.8
Correct category	98.1%	75.5%
Odour-label association	75.0 ± 3.2	65.3 ± 3.3

Table A6.5. Snack ratings: liking, familiarity, sweet and savoury association (on a 100 mm VAS). Values are expressed as mean and standard error.

Snack category	Liking*	Familiarity	Sweet association	Savoury association
Sweet	65.1 ± 2.0	80.6 ± 1.9	82.3 ± 1.5	18.9 ± 2.4
Savoury	63.6 ± 2.0	67.9 ± 1.9	17.2 ± 1.5	77.0 ± 2.4

*Non-significant differences between sweet and savoury odour categories.

Chapter 7

General discussion

The main aim of this thesis was to investigate the role of specific food odours on physiological and behavioural responses, and to disentangle the role of odour awareness on eating responses. Physiological responses were investigated by salivary responses – salivation and saliva composition. Behavioural responses were investigated by means of eating behaviour responses – appetite, preference, choice, and intake. Firstly this final chapter summarizes the main results, followed by a discussion and interpretation of these findings. Then, several methodological considerations are addressed. Finally, implications of the results, further research, and conclusions are discussed.

Main findings – Summary of the main findings

The main findings of the research described in this thesis are summarized in **Figure 7.1** and **Table 7.1**. In **Chapter 1** (Introduction) of this thesis, two research questions were introduced.

3. To what extent can food odours (and other sensory food cues) trigger specific cephalic-phase salivary responses? **(Chapters 2 and 3).**

   The findings described in this thesis demonstrate that exposure to olfactory food cues increased saliva secretion rate compared to non-food and no-odour conditions. Saliva secretion rate was similar among food odours that signal taste qualities (i.e. sweet, savoury, and sour) and specific macronutrient content (i.e. carbohydrates, protein, fat, and low-calorie). Moreover, salivary composition remained stable upon the different odour conditions **(Chapter 2)**. Follow-up research in **Chapter 3** investigated how (multi)sensory cues (odour exposure, + vision, + taste, + mastication) and stimuli varying in nutrient content (starch) influence saliva secretion and its composition. This research demonstrated that saliva secretion rate increased with increasing levels of sensory stimulation. The high-in-starch condition (bread) secreted the highest amount of salivation compared to the control condition (parafilm). α-amylase secretion rate increased upon exposure to the highest level of sensory stimulation (odour + vision + taste + mastication) compared to odour and + vision stimulation. Other salivary characteristics differed with the level of sensory stimulation. pH and buffer capacity significantly increased upon increasing the levels of stimulation, while the concentration of mucin 5B (MUC5B) decreased upon increasing the levels of sensory stimulation. Protein secretion rate varied across the different levels of sensory stimulation. The modification of salivary characteristics may be related to the total volume of salivation. Furthermore, the nutrient content of the stimuli (i.e. high and low-in-starch and control condition) did not influence any salivary characteristics mentioned above. Level of sensory information, rather than specific nutritional

content – in this case starch –, seems to play a crucial role in salivary responses **(Chapter 3).**

4. How does the level of awareness of food odours influence specific eating behaviour responses (appetite, preference, choice, and intake)? **(Chapters 4–6).**

   The influence of conscious and non-conscious exposure of macronutrient-related odours (i.e. odours representing food high in carbohydrates, protein, fat, and low in calories) on appetite, food preference, and food intake was investigated in **Chapters 4 and 5**. Conscious exposure increased appetite for congruent food products, suggesting sensory-specific appetite. This effect was only shown after exposure to odours signalling protein content. Similarly, this type of odour enhanced the liking for protein foods and the preference ranking for savoury products. Food intake of the various macronutrients – by means of *ad libitum* salad buffet – remained stable across the different types of odour exposure **(Chapter 4)**. In **Chapter 5** we showed that non-conscious exposure of macronutrient-related odours did not influence congruent appetite, food preferences, nor food intake of a main meal. Finally, the influence of non-conscious exposure to odours signalling specific taste qualities (sweet and savoury) on snack selection and visual attention was researched **(Chapter 6)**. The results showed that non-conscious odour exposure influenced the first fixation (initial orientation) on congruent locations. However, sweet snacks were fixated on more frequently and for longer, and were mainly chosen compared to savoury snacks, regardless of the odour exposure.

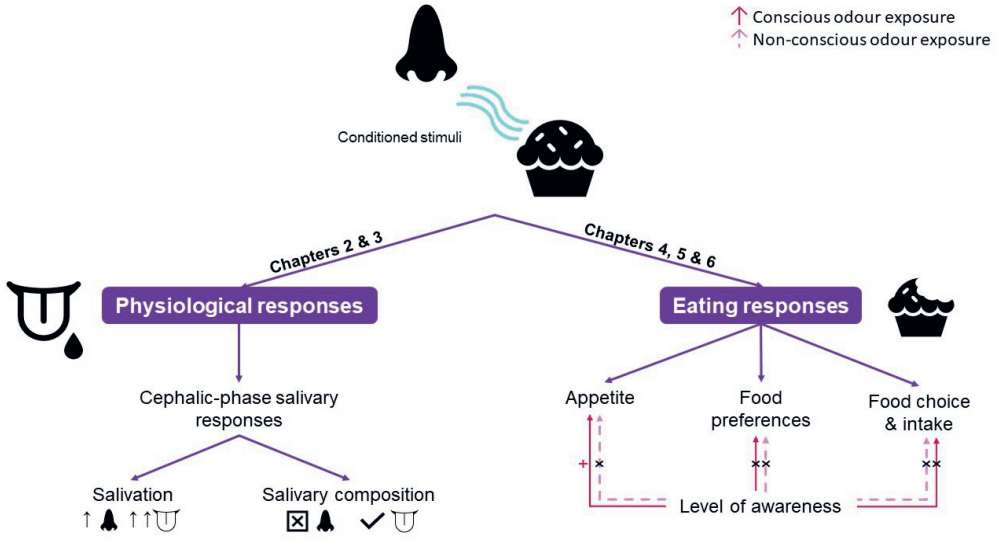

Figure 7.1 Schematic overview of the main findings of this thesis.

Table 7.1 Overview of the main findings of this thesis.

Chapter	Sensory modality	Type of stimuli	Main findings
To what extent can food odours (and other sensory food cues) trigger specific cephalic-phase salivary responses?			
2	Odours	Taste quality (study 1) and macronutrient-related odours (study 2) as food odours and control conditions (non-food and no-odour)	• Food odours enhanced salivation compared to control conditions. Saliva secretion rate was similar among food odours. • Salivary components (α-amylase, lingual lipase, and MUC5B) were similarly secreted among the different conditions.
3	Odour, + vision, + taste, + mastication	Food high- and low-in-starch, and non-food	• Salivation increased with increasing levels of sensory stimulation. Salivation was higher upon exposure to high-in-starch food compared to control. • α-amylase secretion rate increased upon the highest level of sensory stimulation compared to the lowest ones. • The type of stimuli did not influence the saliva composition.
How does the level of awareness* of food odours influence specific eating behaviour responses (appetite, preference, choice, and intake)?			
4	Odours *Conscious	Macronutrient and low-in-calories-related odours as food odours and control	• Odour exposure increased congruent appetite, mainly after protein-related odour exposure. • Liking for protein foods and preference ranking for savoury products was influenced by congruent protein-related odour exposure. • Food intake was not affected by smelling congruent food odours, following the same pattern (amount of each macronutrient), regardless of the type of odour exposure.
5	Odours *Non-conscious	Macronutrient-related odours and low-in-calories-related as control	• Specific-sensory appetite was not affected after congruent macronutrient-related odour exposure. • Liking nor preference were influenced after macronutrient-related odour exposure. • Food intake was not modified after macronutrient-related odour exposure, following the same pattern, regardless of the type of odour exposure.
6	Odours *Non-conscious	Taste-related odours	• Sweet snacks were fixated on more frequently, for longer, and mainly selected, regardless the odour exposure. • Congruent snacks were first fixated on relative to the odour exposure. However, incongruent snack products were fixated on for longer.

Discussion and interpretation of the results

The general idea underlying this thesis was the theory that we are able to detect information related to the composition of a food such as taste or macronutrient content via the sense of smell (Boesveldt & de Graaf, 2017; Ramaekers, Boesveldt, Gort, et al., 2014; Zoon, de Graaf, & Boesveldt, 2016), and the body can thus prepare for subsequent specific ingestion and digestion of the smelled food (Mattes, 2000). Our physiological results showed that saliva secretion and composition are similar across the food odours (and other sensory cues) independent of the information they signal (i.e. taste qualities or macronutrient content) but different between the level of sensory stimulation (**Chapters 2 and 3**). It suggests that anticipatory eating responses depend on the amount of accumulated information from different sensory modalities that is being perceived rather than the type of stimuli (nutrient content). It implies that specific anticipatory salivary responses might not be strictly regulated by the nutritional content of the food. Moreover, our behavioural results showed that odours that signal taste qualities (savoury – high in protein – and sweet) influence anticipatory eating responses (i.e. appetite or initial orientation; **Chapters 4 and 6**). However, odours that signal other macronutrients, such as carbohydrates and fat, did not impact eating responses (**Chapters 4 and 5**). Zoon and colleagues showed a stronger appetizing effect after odours that signal taste qualities compared to odours that signal calorie content (Zoon et al., 2016). Moreover, it seems that indulgent odours such as sweet and sweet-fatty odours do have an impact in eating responses (Chambaron, Chisin, Chabanet, Issanchou, & Brand, 2015; Gaillet-Torrent, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2014; Gaillet, Sulmont-Rossé, Issanchou, Chabanet, & Chambaron, 2013; Proserpio, de Graaf, Laureati, Pagliarini, & Boesveldt, 2017) while exposure to neutral odours did not impact them (Mors, Polet, Vingerhoeds, Perez-Cueto, & de Wijk, 2018). The type of information signal by the odour might be the key to the subsequent impact on eating behaviour and this is an important issue for future research. In general, we infer that the signalling and identification of macronutrients – via our sense of smell – might not be so accurate due to the complex mix of macronutrients in foods we are exposed to daily in our current obesogenic environment (Martin & Issanchou, 2019; van Langeveld et al., 2017; Viskaal van Dongen, van den Berg, Vink, Kok, & de Graaf, 2012). For example, our dinner may consist of a diverse mix of macronutrients such as pasta (carbohydrates) with meatballs (fat and protein) covered in tomato sauce (carbohydrates and fat), but it is clearly a savoury meal. Given that the vast majority of our food can be described as sweet or salty/savoury (Mattes, 1985), overall this may suggest that taste qualities (sweet and savoury) of foods are easier to identify – via our sense of smell – and might communicate clearer information compared to macronutrient content.

Our two main research questions are discussed in depth.

1. To what extent can food odours (and other sensory food cues) trigger specific cephalic-phase salivary responses? (**Chapters 2 and 3**).

   The two main points stemming from our results are discussed here.

   Firstly, sensory cues influence salivation. Our results clearly demonstrated a role of food cues – from olfactory to mastication – in saliva secretion rate. It was shown that sole exposure to food odours increased the saliva secretion rate compared to non-food and no-odour conditions (**Chapter 2**). Moreover, we classified the sensory food cues into *anticipatory sensory cues*, which involve the exposure to odours and visual cues, and *consummatory sensory cues* – taste and mastication. A greater saliva secretion rate was produced upon the consummatory sensory cues compared to anticipatory sensory cues (**Chapter 3**). This may suggest that *consummatory sensory cues*, taste and mastication, are needed on top of anticipatory cues in order for the body to produce a significant response towards the coming food, by activating the release of saliva from the parotid gland (Aps & Martens, 2005; Carpenter, 2013; Ilangakoon & Carpenter, 2011; Mackie & Pangborn, 1990).

   Secondly, mastication, but not the nutrient content, of the food cue impacts the secretion of α-amylase. Our results showed that mastication is required to produce a change in the secretion rate of α-amylase (**Chapters 2 and 3**). α-amylase is mainly produced in the parotid glands and the activation of these glands is related to the mastication process (Carpenter, 2013). Other studies have also shown the importance of the mastication process (vs anticipatory sensory cues or liquid food stimulation) on other cephalic-phase responses such as the secretion of gastric acid, insulin, and pancreatic polypeptide, (Feldman & Richardson, 1986; Teff, 2010; Teff, Devine, & Engelman, 1995) and on the reduction of appetite (Cassady, Hollis, Fulford, Considine, & Mattes, 2009; Zhu, Hsu, & Hollis, 2013). Contrary to our hypothesis, neither α-amylase concentration nor secretion varied with specific nutrient content (**Chapters 2 and 3**). Consequently, the hypothesis that α-amylase would be specifically triggered by nutrient content (e.g. carbohydrates), as signalled by odour exposure (and other sensory cues), was not supported by this thesis (**Chapters 2 and 3**). It might be crucial to collect the saliva directly from the parotid gland, to have a better understanding of this finding. Researchers that collected saliva directly from the parotid gland have shown an increased concentration or secretion rate of α-amylase after the oral stimulation with solutions or mastication of food high in carbohydrates compared to other foods and the control (Froehlich, Pangborn, & Whitaker, 1987; Mackie & Pangborn, 1990). On the other hand, researchers that collected whole mouth saliva have shown a similar α-amylase concentration or activity after the smell or mastication of foods high in starch compared to

unstimulated saliva (Carreira et al., 2020; Hoebler et al., 1998; Joubert et al., 2017).

In general, both unstimulated and stimulated conditions (from solely odours to the combination of different sensory modalities including mastication) produced variations in saliva secretion and salivary components (such as pH, buffer capacity, mucins, and proteins) (**Chapters 2 and 3**). These results are related to distinct stimulation of the salivary glands. Unstimulated saliva consists mainly of saliva from submandibular glands, while mechanical and gustatory (citric acid) stimulation produces saliva from the parotid glands (Aps & Martens, 2005; Edgar, Dawes, & O'Mullane, 2012). Thus, sensory modalities may act as a different afferent input and activate different salivary glands. On one hand, olfactory and visual food cues, similar to unstimulated saliva, may mainly activate the submandibular and sublingual glands, resulting in a viscous (less watery) saliva but high in mucins and other proteins such as cystatin (Carpenter, 2013; Ilangakoon & Carpenter, 2011; Lee & Linden, 1991, 1992a; Pedersen et al., 2018). On the other hand, gustatory and mechanical stimulation may also activate the parotid glands, resulting in a larger volume of saliva that is watery and rich in bicarbonate ions (more basic pH) and α-amylase, and low in other proteins such as mucins (Carpenter, 2013; Edgar et al., 2012; Ilangakoon & Carpenter, 2011; Mackie & Pangborn, 1990; Matsuo, 1999; Pedersen, Sørensen, Proctor, & Carpenter, 2018; Stokes & Davies, 2007). Therefore, the type of sensory input may thus result in a unique saliva composition.

In conclusion, food odours are important sensory cues to induce salivation but do not impact saliva composition. A more vivid combination of sensory cues (in particular mastication) is required to influence saliva composition. Although we failed to prove the relation between α-amylase and carbohydrate content of the foods, further research should focus on a more precise collection of the parotid saliva upon a broader spectrum of food varying in carbohydrate content.

2. How does the level of awareness of food odours influence specific eating behaviour responses (appetite, preference, choice, and intake)? (**Chapters 4–6**).

   Olfaction may play a crucial role in anticipatory eating responses such as appetite and meal initiation (Palouzier-Paulignan et al., 2012; Stevenson, 2010; Yeomans, 2006a). However, it has been suggested that the level of awareness of food odours may exert different eating responses (Boesveldt & de Graaf, 2017). Therefore, in this thesis we tested two different levels of awareness: conscious and non-conscious odour exposure. Two main take home messages are highlighted:

   Firstly, the eating response is related to the level of awareness of the sensory cue. Results from this thesis show that conscious odour exposure influences self-

reported appetite, which is an explicit measure (**Chapter 4**), while non-conscious odour exposure did influence the initial orientation phase of visual attention, it did not influence (implicit) eating responses such as food preferences, choice, and intake (**Chapters 5 and 6**). We speculate that initial orientation and appetite are anticipatory eating responses that may reflect automatic reactions towards the odour cue and occur prior to or in the early stage of the activation of cognitive factors (Moors & De Houwer, 2006). While food preferences, choice, and intake may be mainly governed by cognitive factors – dietary patterns, expectations, previous food consumptions, etc. – which may deviate the attention away from the odour cue *(*de Graaf, Blom, Smeets, Stafleu, & Hendriks*, 2004; Sobal,* Bisogni, Devine, & Jastran*,* 2006*)*.

Secondly, cognitive factors might be stronger than the influence of olfactory cues on eating responses. *C*ognitive factors may modify the steps in the eating (decision) process between appetite and actual food intake (Berthoud, 2007; de Graaf et al., 2004). We propose two suggestions to understand how odours could overrule the cognitive factors and promote a continuity of the eating process until food intake. 1) Additional exposure to other sensory modalities could strengthen the olfactory cue. For example, eye tracking experiments that involved more than one cue (e.g. odour + visual or music + visual) showed an increase in congruent visual attention and food choice (Peng-Li, Byrne, Chan, & Wang*,* 2020; Seo*,* Roidl, Müller, & Negoias*,* 2010*)*. The eating behaviour responses in our experiments were assessed in a non-odourised room (**Chapters 4–6**) which could have diminished the effects of the odour. Additionally, it has been suggested that exposure to multisensory priming improves the eating (and drinking) experience and might nudge people towards certain food behaviour (Oliver & Hollis, 2021; Velasco, Jones, King, & Spence, 2013; Velasco, Obrist, Petit, & Spence, 2018). A multisensory priming might represent a more realistic food environment and strength the prime cue towards the goal food. 2) Olfactory cues could mainly impact impulsive reaction towards food. Unplanned, effortless, and/or automatic selection of food is influenced by the sensory cues and might not activate the cognitive factors (Hofmann, Friese, & Wiers, 2008). Further studies that involve the food choice at the checkout counter of supermarkets – where food products are purchased on impulse (Miller, Bodor, & Rose, 2012; Thornton, Cameron, McNaughton, Worsley, & Crawford, 2012) –, or with restricted time during the food selection – where automatic responses are triggered and may facilitate priming effects (Hermans, De Houwer, & Eelen, 2001; Manippa, van der Laan, Brancucci, & Smeets, 2019) – may provide more information about impulsive responses.

Furthermore, (non-conscious) odours might mainly influence the selection of indulgent foods (i.e. snacks or desserts). Our first test food was an *ad libitum* salad lunch (**Chapters 4 and 5**). Our results suggested that the participants built

their salad in a similar pattern, which reflects dietary patterns, and were not influenced by the odour cues. In line with these results, other researchers did not find an influence of non-conscious odour exposure on the choice of main course (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Mors et al., 2018). However, they did find an influence on choice of starters and desserts (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013), which are considered as indulgent/rewarding foods and may be more prone to be driven by external cues (Berthoud, 2007; de Wijk et al., 2018; Wang, Cakmak, & Peng, 2018). Therefore, our final test food was snack products (**Chapter 6**). However, we also failed to find an effect of the odour cue here. Further research is required to understand the influence of olfactory cues on indulgent (mainly energy-dense) foods. For example, the impulsive choice whilst at a convenience store could be crucial to understanding the impulsive reaction towards indulgent foods.

Taken together, even though under our experimental conditions, odour exposure did not overrule cognitive factors or interfere in habitual decisions, we do hypothesize a role of odours in anticipatory eating responses (initial orientation and appetite), in particular on the impulsive selection of indulgent foods.

Methodological considerations

Odours as stimuli

Since we were interested in understanding the influence of odours that signal specific information such as taste qualities (study 1 of **Chapter 2** and **Chapter 6**) and macronutrient content (study 2 of **Chapter 2** and **Chapters 3–5**) on physiological and eating responses, the selection of odours that signal these qualities and proper piloting were crucial to study our research questions.

The first selection of the odours that signal a particular macronutrient content was based on the nutritional value of the food that they represented. At least 50% of total energy of the food should derive from the specific macronutrient, and the low-calorie products should contain less than 60 kcal/100 g (de Bruijn, de Vries, de Graaf, Boesveldt, & Jager, 2017; RIVM, 2019). Additionally, with the exception of protein-related odours, which by nature signal a savoury taste (e.g. meat, chicken, and seafood are by nature savoury, and high in protein), these odours should not signal a specific taste category (sweet or savoury). We thus focused on odours that signal neutral taste qualities. Some research has shown that neutral foods only represent around 28% of the most common Dutch foods, and the rest are a mix of different nutrients and taste qualities such as sweet(-fatty and -sour) and savoury-fatty foods (Teo et al., 2018; van Langeveld et al., 2018). These criteria, together with feasibility of the design (e.g. number of test sessions per participant), limited our options to only two odours per category. This selection may have been too specific and not

actually representative of the complexity of the foods and odours we encounter daily. Increasing the number of samples per category and considering the complex mix of macronutrients (and their interactions) in the (ultra-processed) food currently available may be useful to understand how our sense of smell is adapting to navigate in the modern environment.

Moreover, the pilot studies focused on having similar intensities and liking of the odours, and proper taste or macronutrient categorization. Although we carefully piloted the odours used, some odours were not equally well evaluated in the actual study. For example, the honey odour used for **Chapter 2** (study 2) was confused for some participants with 'wood', or other non-food odours, reducing its liking and intention to eat, and we thus replaced it with corn odour in **Chapter 4**. In addition, odours' association and liking may depend on cultural background and autobiographical memory which lead to learned associations (Boesveldt & de Graaf, 2017; Chrea et al., 2004; Smeets & Dijksterhuis, 2014). Therefore, odour piloting and evaluation of the odours by participants in the actual study is vital to understand how the odour's attributes are assessed and to ascertain that the (learned) association to the odour is the expected one. Further research could benefit from an initial exploration of odour-nutrient content associations (e.g. through lexical decision tasks, see (Gaillet et al., 2013; Holland, Hendriks, & Aarts, 2005) for examples) with the actual participants to ensure the learned association of the food with a specific macronutrient.

Besides food odours, non-food (such as fresh green or wood odour) and a no-odour control (propylene glycol) were used as control conditions. These conditions were crucial to understand the differences between them and food odours and to establish a baseline response. The responses upon control conditions were significantly different compared to the food odour conditions, showing that their selection was adequate (**Chapters 2 and 3**). Moreover, there was no significant difference between the control conditions, non-food, and no-odour condition (**Chapter 2**). We did not include a control in **Chapters 5 and 6** because we were mainly interested in a 'congruent' effect (e.g. savoury or meat food products would be consumed after exposure to savoury or protein-related odours). However, including a no-odour condition as a baseline could have been beneficial to assess whether the eating responses changed at all upon food odour exposure.

Level of awareness and perceived intensity

The level of awareness and perceived odour intensity varied depending on the aim of the experiment. **Chapters 2–4** involved *conscious* (and *active*) odour exposure. This exposure was performed by having participants sniff from bottles. These bottles allowed us to provide the odour in an isolated manner, without the addition of any other sensory stimulation. We aimed to achieve moderate intensities (70–80 mm VAS), which has been used by other researchers while investigating the role of active

exposure of odours in anticipatory eating responses (Ramaekers et al., 2016; Ramaekers, Boesveldt, Gort, et al., 2014; Zoon et al., 2016). Additionally, **Chapters 5 and 6** involved *non-conscious* (and *inattentive*) odour exposure, which was achieved via vaporizers. These vaporizers allowed us to disperse the odour in the environment (i.e. odourised room) without capturing the attention of the participants and to establish the frequency of odour dispersion in a selected time frame to reach the proper intensity. In **Chapter 5**, we decided to expose participants to a very low odour intensity level (20–35 mm VAS), while in **Chapter 6** we opted for a slightly higher intensity (35–50 mm VAS), as we inferred that the very low intensity could be one of the reasons why we failed to find effects on eating responses in **Chapter 5**. It is noteworthy to mention that neither intensity level was perceived by the participants. In **Chapter 6**, the influence of odours on the first fixation location suggests that the intensity was adequate to steer, at least, the initial orientation towards congruent food products. However, we should have replicated the study in **Chapter 5** to confirm that the higher intensity was needed to really act as a prime and influence the eating responses. In addition, our experiments raise questions regarding the intensity of the exposed odours which may be increasing the conflicting results on the olfactory research. A further study could assess the eating responses after different levels of perceived intensity (odour awareness) to have a deeper understanding on the adequate intensity to act as an olfactory priming.

Exposure time of the odours

Different odour exposure times were used depending on the aim of the experiment. The time of odour (or other food cues) exposure in the physiological studies (**Chapters 2 and 3**) was mainly established based on the saliva secretion, where it is recommendable to be collected for 3–5 min (Navazesh, 1993; Navazesh & Kumar, 2008). Although shorter collection time (≤2 min) has shown an increase in salivation after certain food odours vs the control, for example (Lee & Linden, 1992a; Pangborn, Witherly, & Jones, 1979; Proserpio et al., 2017; Sahakian, Lean, Robbins, & James, 1981), this time frame might not reflect the true effect in secretion of salivary composition. For instance, α-amylase activity and total protein concentration increase after sham feeding until reaching a peak at 60 and 30 min, respectively (Messenger, Clifford, & Morgan, 2003). The time of odour exposure in the eating behaviour studies (**Chapters 4–6**) varied between 3 and 10 min. We established a conscious odour exposure of 3 min in **Chapter 4**. It has been shown that this exposure time is suitable to enhance appetite when being actively sniffed (Ferriday & Brunstrom, 2011; Zoon et al., 2016), and this enhanced appetite remained stable even up to 20 min of odour exposure (Ramaekers, Boesveldt, Lakemond, van Boekel, & Luning, 2014). **Chapter 5** followed the same exposure time to be comparable with **Chapter 4**. However, we decided to extend the odour exposure in **Chapter 6** to 10 min to be in line with previous studies with non-conscious odour exposure. The time frame used in previous odour priming studies is between 10 and 30 min (Chambaron et al., 2015; Gaillet-Torrent et al., 2014; Gaillet et al., 2013; Mors

et al., 2018; Proserpio et al., 2017, 2019). A longer exposure time (10 vs 3 min) might be necessary when odours are being non-consciously exposed in order to create a mental representation and act as a prime on eating responses. It is important to mention that a series of sensory marketing studies have proposed a cross-modal sensory compensation effect between olfaction and gustatory sensory modalities (Biswas & Szocs, 2019). This effect suggests that prolonged exposure to indulgent ambient odours (>2 min) – which are related to a high reward value – compared to non-indulgent odours might induce satiation for these types of products and thus steer away from the actual food choice. However, a very brief exposure (<30 sec) to an indulgent odour produced contrary behaviour: increased desire for indulgent foods, compared to exposure to non-indulgent odours (Biswas & Szocs, 2019). A better agreement between time of exposure (short vs long) and the perceived intensity of odours to act as olfactory priming is needed to understand its role on (in)congruent eating responses and how it can be applied as a public health measure.

Study population

We aimed to include a homogenous group of people in each of our studies to focus on the effects of odours and not be confounded by other factors. Several factors might influence the reactivity to odours and foods such as gender, body weight, restrained eating, previous experiences with food, etc. (Coelho, Jansen, Roefs, & Nederkoorn, 2009; Fedoroff, Polivy, & Herman, 1997, 2003; Ferriday & Brunstrom, 2011; Hallama, Boswella, Devito, & Kober, 2016; Klajner, Herman, Polivy, & Chhabra, 1981; Larsen, Hermans, & Engels, 2012; Tetley, Brunstrom, & Griffiths, 2009). All our studies included non-obese healthy young participants. Our study population was mainly determined by the selected odours, as participants needed to be familiar with the odours and associate them to the correct category. The studies that involved odours that signal taste qualities (study 1 in **Chapter 2** and **Chapter 6**) included people from various nationalities. The selected odours (e.g. vanilla and chocolate for the sweet category and meat for the savoury one) represent food products that are commonly known and eaten in different countries. The studies that involved odours that signal specific macronutrient content recruited only Dutch people. The categorization of food in a particular macronutrient content may be related to previous encounters with the foods and thus depends on the culinary experiences and familiarity with the foods (Boesveldt & de Graaf, 2017; Chrea et al., 2004). For example, although bread is a worldwide consumer food product, each country (and region) uses different ingredients – such as the type of flour, yeast, salt, and other optional additives – and baking processes that can modify the flavour and odour experience.

Moreover, self-imposed dietary restrictions such as vegetarianism or veganism may influence the familiarity with meat products and the reaction towards food (De

Houwer & De Bruycker, 2007; Povey, Wellens, & Conner, 2001); therefore, participants that followed these dietary restrictions were excluded due to the use of meat odours and food products. Also, a homogenous population is crucial in studies that involve research on salivary α-amylase due to a potential positive relation between diets high in starch and the expression of α-amylase, which is related to the α-amylase gene (AMY1) (Méjean et al., 2015; Perry et al., 2007; Squires, 1953). Finally, a young group of participants (18–35 years old) was recruited to ensure a normal olfactory function. Young people tend to perform better on olfactory abilities compared to young children and older adults and the olfactory function declines over the years (Oleszkiewicz, Schriever, Croy, Hähner, & Hummel, 2019; Sorokowska et al., 2015). Although the inclusion criteria mentioned above allowed us to have a homogeneous study population, it also limited the generalizability of our results to other groups such as restrained, obese, or elderly people. For example, restrained (or obese) people tend to be more reactive to food cues (i.e. increased salivation, appetite, and food intake) compared to unrestrained (or normal-weight) people (Buckland, Finlayson, & Hetherington, 2013; Coelho et al., 2009; Fedoroff et al., 2003; Klajner et al., 1981; Rogers & Hill, 1989).

Experimental design

All the studies followed a cross-over design. This type of design takes account of individual characteristics as each participant acts as their own control condition and is thus best for investigating complicated concepts such as eating behaviour, which can be influenced by many (individual) factors. Moreover, the order of the conditions was always counterbalanced to prevent order effects and reduce any influence of familiarization over conditions.

Our experiments were conducted on different times of the day, depending on the typical consumption time of the food products associated with the selected odours. The time of the day may determine the appropriateness on (anticipatory) eating responses (Kramer, Rock, & Engell, 1992; Stroebele & de Castro, 2004). For example, experiments conducted in **Chapters 4 and 5** were conducted during lunch time (11.30–14.00) because the odours used are related to main meal consumption and the food cues to understand eating responses (specific appetite questionnaire, food preference task, and salad buffet for food intake) were adequate for consumption during the lunch. Furthermore, taking into account the influence of the circadian rhythm on food intake (de Castro, 2004; de Graaf, Jas, van der Kooy, & Leenen, 1993), all the test sessions for a given participant were scheduled at the same time of the day. Similarly, for all studies, participants were instructed to arrive with a similar hunger state (neither hungry nor satiated) across the test sessions to ensure comparable reaction towards the odours and food products. Finally, the behavioural studies (**Chapters 4–6**) included an alternative aim to keep participants naïve to the real aim and to deviate their attention from the presence of the odours,

to avoid any influence of cognitive factors on the outcomes. Keeping participants naïve is common in eating behaviour research for this reason, for example (Chambaron et al., 2015; Ferriday & Brunstrom, 2011; Gaillet-Torrent et al., 2014).

All the mentioned factors were carefully established and recorded to check the participants' compliance and were used as potential covariates in our statistical models. In further research, the last eaten meal could additionally be documented (amount, taste quality, macronutrient content, etc.) to be used as a potential covariate. Alternatively, a standardized meal could be provided prior to the test sessions to guarantee the hunger state and reactivity for further eating responses.

Research approach and measurements

Saliva collection

In the physiological studies performed in this thesis, we used the spitting (or passive drooling) method to collect whole mouth saliva. While other methods to collect whole mouth saliva – draining, suction, swab, etc. – are available, the spitting method is recommended for unstimulated and stimulated whole mouth saliva collection because it is a simple, non-invasive, and a high reproducible and reliable method (Navazesh & Christensen, 1982; Navazesh & Kumar, 2008). Moreover, there are other methods to collect saliva from individual glands, e.g. by using a Lashley cup and cannulation of the Wharton's duct to collect saliva from the parotid and submandibular glands, respectively. These methods could be beneficial to investigate specific salivary component analyses such as α-amylase. However, these methods are complex and invasive (Bellagambi et al., 2020; Navazesh, 1993), and we thus used the (more straightforward) spitting method as our main goal was to get an overview of whole mouth saliva secreted upon different stimuli. Nevertheless, the collection of parotid saliva could have offered us more precise results to detect the secretion of α-amylase upon high-in-starch stimuli compared to other stimuli.

Eating behaviour responses

In our three eating behaviour studies (**Chapters 4–6**) we used a combination of explicit and implicit measures to give a complete overview of how odours may influence the eating behaviour responses. We chose self-reported appetite as an explicit measure and visual attention, food preference, choice, and intake as implicit measures.

The subjective appetite VAS ratings assess the conscious realization and motivation to eat something, but may not represent actual behaviour (Blundell et al., 2010; de Graaf et al., 2004). It can be mainly related to the responsiveness to sensory or environmental cues and could even contrast with the homeostatic state (Blundell et al., 2010; de Graaf et al., 2004). These subjective ratings have been used to assess

general and specific appetite in previous research, for example (Proserpio et al., 2017; Ramaekers, Boesveldt, Gort, et al., 2014; Zoon et al., 2016). Specific appetite ratings depend on the chosen food products, and we thus carefully selected foods to match each odour category (pasta, bread, and corn matched the carbohydrates-related odours, bread and corn) and to be pleasant and familiar. For instance, several participants did not like or were unfamiliar with 'tuna' (**Chapter 4**) and was thus replaced with 'ham' (**Chapter 5**).

The food preference task – Macronutrient and Taste Preference Ranking Task (MTPRT) – is a validated computer-based task (de Bruijn et al., 2017), which includes standardized food pictures of sweet and savoury food products from the three macronutrient categories and low-calorie category. Although there are other validated questionnaires, this task was ideal to measure liking of each food product and preference by ranking four available food products differing in macronutrient content after congruent (macronutrient-related) odour exposure. However, the products displayed in the task differed in their consumption context (e.g. fries, cream pie, roast beef, and apple), which could influence the current preference of the products. Other potential food preference tasks that involve macronutrient classification are the Macronutrient Preference Checklist and Leeds Food Preference Questionnaire (Brisbois-Clarkson, McIsaac, Goonewardene, & Wismer, 2009; Finlayson, King, & Blundell, 2007; Hill, Leathwood, & Blundell, 1987). However, in the Macronutrient Preference Checklist the participants are instructed to check off all the food items they want to eat at that moment from a list of 32-food items from the different macronutrient categories (Brisbois-Clarkson et al., 2009; Hill et al., 1987) but do not consider the comparison among different food products as the MTPRT, and only two macronutrient categories (protein and fat) are available in the Leeds Food Preference Questionnaire with a similar set-up to the MTPRT (Finlayson et al., 2007; Griffioen-Roose, Mars, Finlayson, Blundell, & de Graaf, 2011).

Food intake, overall and for each respective macronutrient, was measured by means of an *ad libitum* lunch which consisted of a salad bar. As we were interested in determining the specific food intake of each macronutrient, a salad – which can be freely built and consisted of different macronutrient content – was a versatile and appropriate choice. However, potential limitations should be highlighted: 1) consumption of a salad during lunch can be related to habits and dietary patterns and thus might not be prone to influences by sensory cues (Birch, 1999; Köster, 2009); 2) a buffet style meal is mainly used to investigate food choice, while single item meals are more appropriate to assess food intake (Blundell et al., 2010). The forced choice of a single course from a limited assortment of food or menu could be more naturalistic and useful to capture the role of odours in the food environment.

Finally, food choice (**Chapter 6**), was measured by the forced choice of a snack from a food area, taking into account the limitations observed in the assessment of food intake. The food area consisted of 6 snacks per sweet and savoury category and 3 snacks per sub-category. However, a better balance of the available products could have been provided. Due to the lack of available meat snacks, the vast majority of the savoury snacks were chips. This limited the assortment of savoury snacks and might have steered the selection towards the sweet snacks.

Although these measures – food preferences, intake, and choice – are considered as implicit measures and were performed within covert manipulations, the laboratory setting could make participants more aware of their decisions performed in every task and thus reduce the implicit goal of the measures. Therefore, visual attention – an implicit measure used in **Chapter 6** – is better suited to detect more unconscious and spontaneous behaviour that is vital to investigate the food choice process (Carrasco, 2011; Duchowski, 2007; Orquin & Mueller Loose, 2013; Wang et al., 2018).

In general, all the measures were well-thought out and selected according to our hypothesis and the odours used and were appropriate for the respective study designs and research questions. Nevertheless, they assessed only a single (and momentary) eating occasion. Cognitive information of previous or upcoming eating occasions and meal planning might influence the current single eating occasion (Bilman, van Kleef, & van Trijp, 2017; de Graaf, de Jong, & Lambers, 1999; Gibbons, Finlayson, Dalton, Caudwell, & Blundell, 2014). A further analysis or potential standardization of the food consumption around the event of interest might help us to clarify the food decision made during the experiment.

Implication of results and further research

The **physiological** results of the thesis highlight the importance of being exposed to (multi)sensory cues before starting to eat and the crucial role of mastication for the beginning of digestion. This emphasizes the importance of oro-sensory exposure to invoke certain cephalic-phase responses such as salivary and insulin. For example, some researchers have shown that longer oro-sensory exposure leads to a higher response in insulin and subjective satiation, and a decrease food intake (Lasschuijt, Mars, De Graaf, & Smeets, 2020; Zhu et al., 2013). Further research in stimuli varying in nutrient content is an essential next step in confirming (or not) the role of salivary α-amylase with high-in-carbohydrate foods. The stimuli used in our studies were limited; therefore, we suggest a larger variation in starch content to fully understand this aspect.

Our results of **behavioural responses** have contributed to understanding the role of odour exposure and its impact mainly on anticipatory eating behaviour. However,

we cannot claim any direct steering effect of odours on (congruent) food choice and intake based on the scientific knowledge available and we thus are not yet ready to draw to immediate applications. However, if scientific results are able to demonstrate a steering effect, a potential application could be the use of exposure to olfactory cues to increase the appetizing effect of food products high in protein. This could motivate the consumption of foods high in protein in malnourished elderly people. Furthermore, we could increase the appealing and appetizing effect of low-calorie foods in children and young people upon the exposure of low-calorie-related odours. Placing vaporizers to disperse these type of odours in school canteens or dining rooms in the home could be beneficial to enhance the consumption of fruits and vegetables in this segment of the population. As a side note, this population might have different reactivity towards food cues compared to the young adult population tested in this thesis.

Moreover, the extensive methodological considerations raise remarkable questions regarding under which experimental circumstances olfactory priming might steer people's behaviour. Additionally, two main future considerations are addressed here. Firstly, the level of awareness should be investigated in a systematic manner to have clearer insights about the perceived intensity and exposure time. This might be crucial to understand the contradictory findings and to tackle public health concerns. Secondly, the use of implicit measures in a more naturalistic environment – for example a wearable eye-tracker in a convenience store – are needed to investigate the role of odours and their impact on eating responses up to the food intake stage. The laboratory settings might immediately active the conscious controls and thus disturb the truly effortless responses that the odours might influence.

Additionally, more work in (multi)sensory exposure is needed to understand the role of odours and their interaction with other sensory cues on physiological responses and eating responses. It would be interesting to conduct a stepwise experiment, such as the one performed in **Chapter 3**, to understand their impact on eating responses. The (multi)sensory exposure to food cues could be: smell (condition 1); smell and visual (videos – without sound – with people walking or eating and food being exhibited; condition 2); and smell, visual, and auditory (sound of food being prepared and eaten; condition 3). Multisensory exposure (condition 3) could have a stronger impact on eating responses compared to unisensory exposure (condition 1). The use of immersive technology, which can promote a similar behaviour to that in an actual store (van Herpen, van den Broek, van Trijp, & Yu, 2016), could be beneficial to control the exposure of these (multi)sensory cues. The simulation of a main street of a town, restaurants, or supermarkets via virtual reality or augmented reality in a controlled setting may help us to understand the role and contribution of each sensory cue on the decision-making process of eating a food product in a real food environment.

Finally, although taste quality seems more important and a clearer signal than macronutrient content when being perceived through odours, it would be worthwhile to further explore the hypothesis concerning the identification of macronutrient content via the sense of smell, which we were not able to prove in the current thesis. A descriptive library of different odours which represent common food products that vary in nutrient content performed by a sensory panel could shed more light on our understanding of the associations of (the mix of) nutrient content signalled via odours.

Conclusions

Olfactory cues may play a crucial role in anticipatory responses such as saliva secretion rate, initial orientation (first location fixation), and appetite. The appetizing effect may be disturbed by cognitive factors, overruling the eating process in this phase, and is not converted into food choice and intake. Moreover, physiological and behavioural responses are not specifically influenced by the macronutrient content of the food cues. The complex mix of macronutrients that we are exposed to daily in the current obesogenic environment might blur the signal of specific content information and further influence our physiological and behavioural responses. Therefore, taste qualities might give clearer information compared to macronutrient content. Moreover, multisensory cues are needed to induce a change in salivary composition, and it might be fundamental to strengthen the olfactory cues and to overrule the cognitive factors in order to impact food choice and intake.

In general, odours play a role in (anticipatory) eating behaviour. However, their effect under laboratory circumstances might be too small and therefore difficult to grasp. Nevertheless, more research is needed to better understand the potential impact of odours to steer people's healthier food choices.

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Summary

Summary

Imagine yourself walking into your preferred supermarket or in the main street of your home town. Suddenly, a smell of freshly baked chocolate cake interrupts your thoughts, your mouth starts watering, and you start craving some sweet, high-calorie food. Through repeated exposure, which leads to learned conditioning, food odours may act as a conditioned stimulus that transmits specific information about the food and produces physiological and behavioural responses in anticipation of consumption. Exposure to food cues increases the appetite for food products with similar taste and energy-density characteristics: sensory-specific appetite. This infers that food odour cues may transmit vital information associated to the macronutrient content of the food and consequently induce specific responses such as salivation, appetite, and even food intake. However, there is still uncertainty how, and under what circumstances, food odours may specifically impact physiological and behavioural responses. This thesis was geared towards a better understanding of the role of food odours and their impact on those responses, which is crucial in improving eating patterns towards healthier options.

Two main questions were addressed:

1. To what extent can food odours (and other sensory food cues) trigger specific cephalic-phase salivary responses? **(Chapters 2 and 3).**

2. How does the level of awareness of food odours influence specific eating behaviour responses (appetite, preference, choice, and intake)? **(Chapters 4–6).**

In **Chapter 2** we investigated the role of odours on saliva secretion and composition in two studies: study 1 involved odours that signal taste qualities (sweet, savoury, and sour) and study 2 involved odours that signal macronutrient content (high in carbohydrate, protein, fat, and low-calorie). Our results showed that food odour exposure significantly increased saliva secretion rate compared to non-food and non-odour conditions. Saliva secretion rate was similar across odours that signal different taste qualities (study 1) and specific macronutrient content (study 2). However, salivary composition remained stable across odour and control conditions. This demonstrated that food odours play a role in saliva secretion as part of physiological response in anticipation of food intake. Nevertheless, the use of more sensory modalities could be necessary to impact these responses more specifically, in particular salivary composition. Therefore, a follow-up study, was conducted to investigate the role of (multi)sensory cues and the type of stimuli on saliva secretion and its composition (α-amylase concentration and secretion rate, pH level, buffering capacity, MUC5B concentration, and total protein content) (**Chapter 3**). We systematically varied levels of sensory stimulation (odour exposure, + vision, + taste, + mastication) and macronutrient content of the stimuli (bread, high-in-starch;

cucumber, low-in-starch; and parafilm as a control). Our results showed that saliva secretion rate increased with increasing levels of sensory stimulation. In general, bread (high-in-starch condition) secreted the highest amount of salivation compared to parafilm (control condition). α-Amylase secretion rate increased upon exposure to the highest level of stimulation (odour + vision + taste + mastication) compared to the lowest levels (odour and odour + vision stimulation). Other salivary characteristics differed with the level of sensory stimulation, which might be related to the total volume of salivation. Importantly, the nutritional content of the stimuli did not influence any salivary characteristics. Cumulative sensory information, mainly taste and mastication, may play a crucial role in anticipatory salivary responses.

Chapters 4 and 5 were conducted to understand the role of odours that signal specific macronutrient content on eating behaviour responses and to disentangle the influence of the level of awareness. In **Chapter 4**, participants were *consciously* exposed, while in **Chapter 5** they were *non-consciously* exposed to odours in a cross-over design. In each test session, participants were (non-)consciously exposed to one odour and assessed their congruent appetite, food preferences, and intake (by means of an *ad libitum* lunch consisting of various macronutrients). *Conscious* exposure increased appetite for congruent food products. However, this effect was mainly driven by the protein-related odours. Also, these odours steered the liking towards foods high in protein and a preference for savoury products. Nevertheless, food intake was similar across the different odour exposures. On the other hand, *non-conscious* exposure did not impact specific appetite, and, although contrary to our hypothesis, also did not influence food preference nor food intake. Our results suggest that the exposure to conscious odours mainly influences sensory-specific appetite. We hypothesized that intake of a main course – salad during lunch in this case – might not be prone to modification by non-conscious odour exposure as it is part of our habitual dietary patterns. However, non-conscious odour exposure could influence food choice of rewarding foods that are more susceptible to being steered by external cues. Therefore, we conducted a final study where we investigated the role of *non-conscious* exposure of sweet and savoury odours on snack choice, as well as visual attention by means of eye-tracking (**Chapter 6**). Our results showed that sweet snacks were mainly chosen regardless of the type of odour exposure. However, congruent snacks were fixated upon first, suggesting that non-conscious exposure might influence the initial orientation. Furthermore, similar to the snack choice, sweet snacks were fixated upon more frequently and for longer compared to savoury snacks, regardless of the type of odour exposure.

In general, the type of odours, time and intensity of the exposed odour, and different measures to investigate the same outcome are crucial to understand the role of (non-) conscious odour exposure and its influence on eating responses.

In summary, food odours play a key role in anticipatory responses such as saliva secretion rate, initial orientation, and appetite. However, their effect under laboratory circumstances might be too small and therefore difficult to grasp. Physiological and behavioural responses are not specifically influenced by the macronutrient content of the food cues. The nutrient information that is signalled by the food cues might be disrupted by the complex mix of macronutrients available in our current food environment. Further research should focus on a larger sample of odours consisting of different mixtures of macronutrients and on the role of multisensory cues to fully understand the role of odours on physiological and behavioural responses. More research is needed to better understand the potential impact of odours to steer people’s healthier food choices.

Resumen

Imagina que estás entrando en tu supermercado preferido o caminando en la calle principal de tu ciudad natal; de repente, un olor a pastel de chocolate recién horneado interrumpe tus pensamientos, comienzas a salivar y tu deseo por comer algo dulce y alto en calorías incrementa. ¿Te ha pasado esto? ¿Te has puesto a pensar porque quieres comer ese pastel que aún no has visto? Esto se debe a que después de repetidas exposiciones a lo largo de tu vida, se genera un condicionamiento aprendido (clásico). Los olores de los alimentos pueden actuar como estímulos condicionados que transmiten información específica sobre los alimentos hacia el cerebro, generando respuestas fisiológicas y de comportamiento en anticipación al consumo. La exposición a los alimentos genera señales que aumentan el apetito por productos alimenticios con características similares de sabor y densidad energética, a esto se le conoce como apetito sensorial específico (sensory-specific appetite). Lo anterior, sugiere que las señales de olor de los alimentos pueden transmitir información vital asociada al contenido de macronutrimentos de los mismos y, en consecuencia, provocar respuestas específicas como la salivación, el apetito e incluso la ingesta de alimentos. Sin embargo, todavía existe incertidumbre sobre cómo y bajo qué circunstancias, los olores de los alimentos pueden afectar específicamente las respuestas fisiológicas y de comportamiento.

Esta tesis fue orientada para obtener una mejor comprensión del papel de los olores de los alimentos y su impacto en las respuestas fisiológicas y del comportamiento de la alimentación. Esto es crucial para la posible mejora de los patrones de alimentación hacia una opción más saludable.

Se abordaron dos cuestiones principales que se enlistan a continuación:

1. ¿En qué medida pueden los olores de los alimentos (además de otras señales sensoriales) desencadenar respuestas salivales específicas de la fase cefálica? (**Capítulo 2 y 3**).

2. ¿Cómo influye el nivel de consciencia de los olores de los alimentos en las respuestas específicas de la conducta alimentaria (apetito, preferencia, elección e ingesta)? (**Capítulo 4 - 6**).

En el **Capítulo 2**, investigamos el rol de los olores en la secreción y la composición de la saliva en dos estudios: el estudio 1 involucró olores que señalan cualidades gustativas (dulce, salado y ácido) y el estudio 2 involucró olores que señalan el contenido de macronutrimentos (alto en carbohidratos, proteínas, grasas y bajo en calorías). Nuestros resultados mostraron que la exposición al olor de los alimentos aumentó significativamente la tasa de secreción de saliva en comparación con las condiciones no alimentarias y sin olor. La tasa de secreción de saliva fue similar

entre todos los olores que indican diferentes cualidades gustativas (estudio 1) y contenido específico de macronutrimentos (estudio 2). Sin embargo, la composición de la saliva se mantuvo estable en las condiciones de control y olor. Esto demostró que los olores de los alimentos juegan un papel en la secreción de saliva como parte de la respuesta fisiológica en anticipación a la ingesta de alimentos. No obstante lo anterior, el uso de más modalidades sensoriales podría ser necesario para tener un impacto más específico en estas respuestas, en particular en la composición de la saliva. Por lo tanto, se realizó un estudio de seguimiento para investigar el papel de las señales (multi)sensoriales y los tipos de estímulos en la secreción de saliva y su composición (concentración y tasa de secreción de α-amilasa, nivel de pH, capacidad buffer, concentración de MUC5B y contenido total de proteínas) (**Capítulo 3**). Los niveles de estimulación sensorial (exposición al olor, + visión, + gusto, + masticación) y el contenido de macronutrimentos de los estímulos (pan, alto contenido de almidón; pepino, bajo en almidón; y parafilm como control) fueron variados sistemáticamente. Nuestros resultados mostraron que la tasa de secreción de saliva aumentó cuando los niveles de estimulación sensorial también aumentaron. En general, el pan (condición con alto contenido de almidón) secretó la mayor cantidad de salivación en comparación con el parafilm (condición control). La tasa de secreción de α-amilasa aumentó con la exposición al mayor nivel de estimulación (olor + visión + gusto + masticación) en comparación con los niveles más bajos (olor y olor + visión). Otras características salivales difirieron con el nivel de estimulación sensorial, lo que podría estar relacionado con el volumen total de salivación. Es importante destacar que el contenido nutricional de los estímulos no influyó en ninguna característica de la saliva. La acumulación de información sensorial, principalmente el gusto y la masticación, puede desempeñar un papel crucial en las respuestas salivales anticipatorias.

Los **capítulos 4 y 5** se realizaron para comprender el papel de los olores que señalan el contenido específico de macronutrimentos en las respuestas de la conducta alimentaria y para aclarar la influencia del nivel de consciencia. En el **Capítulo 4**, los participantes fueron *conscientemente* expuestos a olores, mientras que en el **Capítulo 5** fueron *no conscientemente* expuestos a olores en un diseño cruzado. En cada sesión, los participantes estuvieron conscientemente (o no) expuestos a un olor y se evaluó si su apetito, preferencias alimentarias e ingesta de alimentos (mediante una ensalada *ad libitum* durante la comida el cual consistía en varios macronutrimentos) fueron congruentes. La *exposición consciente* aumentó el apetito por alimentos similares. Sin embargo, este efecto se debió principalmente a los olores relacionados con las proteínas. Además, estos olores dirigieron el gusto hacia los alimentos ricos en proteínas y su preferencia por los productos salados. Sin embargo, la ingesta de alimentos fue similar en la exposición a los diferentes olores. Por otro lado, la *exposición no consciente* no afectó el apetito específico y, aunque contrariamente a nuestra hipótesis, tampoco influyó en la preferencia ni en la ingesta de alimentos. Nuestros resultados sugieren que la exposición *consciente*

a olores influye principalmente en el apetito sensorial específico (sensory-specific appetite). Planteamos la hipótesis de que la ingesta de un plato principal, en este caso la ensalada durante la comida, podría no ser propensa a modificarse por la exposición *no consciente* a los olores, ya que es parte de nuestros patrones alimenticios habituales. Sin embargo, la exposición *no consciente* a los olores podría influir en la elección de alimentos gratificantes que son más susceptibles a ser influenciados por señales externas. Por lo tanto, realizamos un estudio final en el que se investigó el rol de la exposición no consciente de olores dulces y salados en la elección de botanas, así como la atención visual mediante el seguimiento ocular conocido como eye-tracking (**Capítulo 6**). Nuestros resultados mostraron que las botanas dulces fueron la elección principal independientemente del tipo de olor expuesto. Sin embargo, los participantes fijaron primero las botanas dulces después ser expuestos a olores dulces (y viceversa), lo que sugiere que la exposición no consciente podría influir en la orientación inicial. Además, de manera similar a la elección de botanas, las botanas dulces fueron observadas con mayor frecuencia y durante más tiempo en comparación con las botanas saladas, independientemente del tipo de olor expuesto.

En general, los tipos de olores, el tiempo y la intensidad del olor expuesto y las diferentes medidas para investigar el mismo resultado son cruciales para comprender el papel de la exposición al olor consciente (y no consciente) y su influencia en las respuestas a los alimentos.

En resumen, los olores de los alimentos juegan un papel clave en las respuestas anticipatorias, como la tasa de secreción de saliva, la orientación inicial y el apetito. Sin embargo, su efecto en circunstancias de laboratorio puede ser demasiado pequeño y, por lo tanto, difícil de demostrar. Las respuestas fisiológicas y de comportamiento no están específicamente influenciadas por el contenido de los macronutrimentos en los alimentos. La información de nutrimentos generada por las señales sensoriales de los alimentos podría verse alterada por la compleja mezcla de macronutrimentos disponibles en nuestro actual entorno alimentario. Las siguientes investigaciones, deben centrarse en tener una muestra más grande de olores que considere diferentes mezclas de macronutrimentos y en el papel de las señales multisensoriales con el fin de comprender completamente el papel de los olores en las respuestas fisiológicas y de comportamiento. Es necesario que se lleve a cabo mayor investigación para comprender mejor el potencial impacto de los olores para que las personas puedan ser orientadas a la elección de alimentos más saludables.

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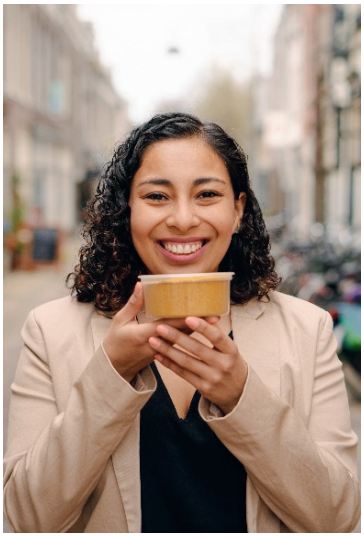
About the author

Curriculum Vitae



Paulina Morquecho Campos was born on June 28th, 1988 in Mexico City, Mexico. In 2006, she started her bachelor studies at Universidad La Salle in Mexico City, where she obtained a bachelor degree in Food Chemistry. She performed her dissertation project "Develop of cereal bar added with Agave's inulin or lactose or lactitol for Liver Cirrhosis' patients" at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. During this project, she developed a deep passion for sensory science and nutrition. During the last stage of her dissertation project in 2011, she started working as a Consumer Insights & Market Research Specialist at Takasago in Mexico State. In 2014, she was awarded a full scholarship from the National Council on Science and Technology of Mexico (CONACYT) to pursue her Master degree in Wageningen University and Research (WUR). Later that year, she moved to Wageningen, the Netherlands and started her MSc Nutrition and Health with a Nutritional Physiology and Health Status specialisation. She was selected to be part of the VLAG Research Master Track Programme- Food Structure, digestion and health- to write a PhD proposal. Along with this, she performed her MSc thesis in the Sensory Science and Eating Behaviour group where she investigated the influence of L-arabinose on glycaemic control and satiety. Paulina did an internship at Gösta Ekmans Laboratory, Department of Psychology of Stockholm University in Stockholm, Sweden where she investigated the role of odour mixture training on odour identification. In 2016, Paulina was awarded a full scholarship from CONACYT to pursue her PhD in the Sensory Science and Eating Behaviour group at WUR which she started in 2017. Her research focused on understanding the role of food odours on physiological and eating behavioural responses. During her PhD project, Paulina attended various courses and she presented her work at several international conferences. She was involved in teaching the Principles of Sensory Science course at WUR and supervising BSc and MSc students. She was part of the organizing committee of the PhD tour to East Canada in 2019. She is also a founder member and part of MEXA association which aims to guide Mexican students and promote the Mexican culture in Wageningen. Currently, Paulina is working as a Senior Market Analyst at Innova Market Insights in Arnhem, the Netherlands.

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List of publications

Publications in peer-reviewed journals

Morquecho-Campos, P., Larsson, M., Boesveldt, S., Olofsson, J. K. (2019). Achieving olfactory expertise: training for transfer in odor identification. *Chemical Senses*, 44(3), 197-203. doi:10.1093/chemse/bjz007

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Morquecho-Campos, P., Hellmich, I.M., Zwart, E., de Graaf, K., Boesveldt, S. (2021). Does odour priming influence snack choice? – An eye-tracking study to understand food choice processes. *Submitted*, 2021.

Overview of completed training activities

Discipline specific courses and activities

Name	Organizer and location	Year
NutriScience. Global nutrition: from nutrients to whole diets	VLAG; Wageningen, NL	2017
Summer School on Human Olfaction	Smell & Taste Clinic of the Department of Otorhinolaryngology of the University of Dresden Medical; Dresden, GE	2017
WIOS 1st Symposium	WIOS and International School for Advanced Studies (SISSA); Trieste, IT	2017
Sensory Perception & Food Preference: Affective drivers of food choice	VLAG; Wageningen, NL	2018
AChemS annual meeting XL	Association for Chemoreception Sciences; Bonita Springs, US	2018
AChemS annual meeting XLI	Association for Chemoreception Sciences; Bonita Springs, US	2019
PennState Symposium	PennState; State College, US	2019
WIOS 2nd Symposium	WIOS and International School for Advanced Studies (SISSA); Wageningen, NL	2019
13th Pangborn Sensory Science Symposium*	Elsevier; Edinburgh, UK	2019
Olfactometer training	Burgart; Ede, NL	2020
9th European Conference on Sensory and Consumer Research - EuroSense	Elsevier; online, NL	2020

General courses

Name	Organizer and location	Year
VLAG PhD week	VLAG; Baarlo, NL	2017
Presentations Skills course	VLAG; Wageningen, NL	2017
WGS PhD Workshop Carousel	WGS; Wageningen, NL	2018
Applied Statistics course (in R)	VLAG; Wageningen, NL	2018
Scientific writing course	Wageningen into Languages; Wageningen, NL	2018
Teaching and supervising Thesis students	Educational Staff Development; Wageningen, NL	2018
Pitch training	WUR; Wageningen, NL	2019
Career Perspectives	VLAG; Wageningen, NL	2020
Last stretch of the PhD program	WGS; Wageningen, NL	2019
Writing propositions	WGS; Wageningen, NL	2020

Optional courses

Name	Organizer and location	Year
Preparation of research proposal	VLAG; Wageningen, NL	2017
Advanced Sensory Methods and Sensometrics (MSc course)	WUR; Wageningen, NL	2017
Worldwide Wageningen Alumni Reunion (Stand in Foodlab)	Worldwide Wageningen Alumni Reunion; Wageningen, NL	2018
Applied Statistics	VLAG; Wageningen, NL	2018
Nutritional Neurosciences (BSc course)	WUR; Wageningen, NL	2019
Organization of the PhD tour to East Canada	WUR; Wageningen, NL	2018- 2019
PhD tour to East Canada	WUR; CA	2019
Meetings Sensory Science & Eating Behaviour group	WUR; Wageningen, NL	2017-2020

Colophon

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