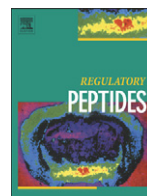




Contents lists available at ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep

Appetite suppression through smelling of dark chocolate correlates with changes in ghrelin in young women

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ARTICLE INFO

Article history:

Received 16 August 2009

Received in revised form 6 December 2009

Accepted 18 January 2010

Available online xxxx

Keywords:

Appetite

Cephalic phase response

Ghrelin

Satiation

ABSTRACT

Cephalic effects on appetite are mediated by vagal tone and altered gastrointestinal hormones. The objective of this study is to explore the relationship between appetite and levels of gastrointestinal hormones after smelling chocolate and after melt-and-swallow 30 g chocolate (1.059 oz, 85% cocoa, 12.5 g of sugar per 100 g product). Twelve female residents (BMI between 18 and 25 kg/m²) all participated in two 60-minute study sessions. In the first session, all 12 women ate chocolate; for the second session, they were randomized either to smell chocolate ($n=6$) or to serve as a control (no eating or smelling; $n=6$). At the start of the sessions, levels of insulin, glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK), but not glucose, correlated with appetite scored on a visual analogue scale (VAS). In contrast, ghrelin levels correlated inversely with scored appetite. Chocolate eating and smelling both induced a similar appetite suppression with a disappearance of correlations between VAS scores and insulin, GLP-1 and CCK levels. However, while the correlation between VAS score and ghrelin disappeared completely after chocolate eating, it reversed after chocolate smelling, that is, olfactory stimulation with dark chocolate (85%) resulted in a satiation response that correlated inversely with ghrelin levels.

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1. Introduction

Considerable advances have been made in understanding the pathways regulating energy homeostasis and how gastrointestinal hormones are involved in the crosstalk with brain areas for the regulation of appetite [1,2]. Energy homeostasis dictates energy intake by sharing common circuits with the nucleus of the solitary tract (NTS) of the brainstem (hindbrain) and the arcuate nucleus (ARC). ARC neurons project to other hypothalamic areas such as the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA). Both PVN and LHA convey neuronal signals to the brainstem where they become integrated into the vagal output heading toward adipose tissues and the digestive system [3–5]. The connection between brain and gastrointestinal tract was classically pioneered by Pavlov who discovered the functional significance of the vagal nerve in the regulation of gastric and pancreatic secretion [6]. The term ‘cephalic phase response’ refers to anticipatory physiological regulation related to feeding. This regulation actually pertains to

digestive and metabolic responses to food cues generated by the central nervous system in order to be prepared to ingest, digest, absorb and metabolize food [6–8]. This efferent part of the crosstalk between brain and gut in the regulation of food intake probably enables the processing of food-related signals from the brain to the gut by the vagal nerve [9–11]. The physiological role of gastrointestinal hormones as mediators of appetite regulation in this cephalic crosstalk between brain and gut remains controversial. However, high hunger scores have been demonstrated to be accompanied by high ghrelin and low obestatin cephalic responses in eating disorders, i.e. anorexia nervosa [12,13]. Enhancement of cephalic ghrelin responsiveness has been demonstrated also in women with bulimia nervosa [14].

In the current study, we used dark chocolate eating/smelling to explore some aspects of the cephalic response of feeding. Dark chocolate contains cocoa solids (also called cocoa liquor), cocoa butter and various amounts of sugar. The ‘cocoa percentage’ in bars of chocolate refers to the total amount of cocoa solids and cocoa butter contributed by ground-up cocoa beans. Sugar accounts for the remainder of the content, along with minute amounts (typically less than 2 wt.%) of emulsifiers, vanilla flavoring, salt and sometimes milk fat. Pure cocoa, which contains no sugar at all, is hardly edible. The U.S. Food and Drug Administration (FDA) states that dark chocolate,

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whether labeled bittersweet, semisweet or dark, must contain at least 35% cocoa.

The aim of the study was to relate some cephalic appetite scores with a number of gastrointestinal hormone responses in healthy females. Our research question was whether the strong odor of cocoa (85%) could provoke cephalic phase feeding responses, while looking at the combination of appetite perception and gastrointestinal hormonal regulation. We studied strongly odorous chocolate with a very low sugar content in order to avoid carbohydrate cravings, since particularly women report extreme liking of or craving for foods that are both sweet and high in fat, such as milk chocolate [15]. Moreover, we were specifically interested in the physiology of cephalic response regulation rather than in the pathophysiology of food cravings.

2. Materials and methods

2.1. Subjects

Twelve healthy female subjects participated in the study. Exclusion criteria were eating disorders or frequent chocolate cravings, drug abuse, existing bile stones, previous gallbladder surgery or pancreatitis, pregnancy or a body mass index (BMI) above 25 kg/m² or below 18 kg/m². Informed consent was given by all participants. The study was approved by the review board of the medical ethics committee of the Reinier de Graaf Group of Hospitals, The Netherlands.

2.2. Experimental design

All subjects were tested individually at a random time point in their menstrual cycle; 4 subjects were using contraceptives and 1 individual had a levonorgestrel-containing IUD. They were all residents of the Department of Internal Medicine, and underwent two tests sessions on two distant working days. Attempts were made to mimic regular working habits as much as possible. As subjects reported to have breakfast irregularly or skip it altogether, to lunch daily around 12:30 h at work and to have an evening meal around 19:30 h, all were instructed on a test day to lunch ad libitum between 12:00 h and 12:30 h, then fast, except for drinking water, until 1 h before the experimental session, then fast until the start of the session. The time gap between lunch and the experimental sessions was logged. Experimental sessions started exactly at 18:00 h. One of the investigators (clinical biochemist) was responsible for the timing of each VAS scoring, blood sampling, and securing the exact timing of each session.

At the beginning of the first session at 18:00 h, each subject was asked to complete a visual analogue scale (VAS) designed to assess appetite, as previously described [16]. The VAS consisted of 4 domains: VAS1: How satiated are you? VAS2: How is your appetite? VAS3: How full are you? VAS4: How hungry are you? Each domain had a scale of 100 mm, ranging from 'hardly' to 'very much'. VAS domains were quickly completed at baseline before (at $t = -5$ min) and after insertion of an indwelling catheter in the cubital vein (at $t = 0$ min). Immediately thereafter, all subjects were requested to eat a standard 30 g portion of dark chocolate containing a total energetic value of 157 kcal (700 kJ)¹ and to complete a VAS at 5, 10, 20, 30, 40, 50 and 60 min, each time swiftly followed by blood sampling and measurement of gallbladder volume.

For the second session, taking place at least 4 weeks later, 6 subjects were randomized to smell chocolate for exactly 5 min, while the remaining 6 subjects served as controls (there were no chocolate-related cues during the control sessions).

¹ Lindt Excellence chocolate; 85% cocoa, 12.5 g of sugar per 100 g. http://www.globalchocolates.com/excellence_nutrition.htm.

2.3. Laboratory protocols

Glucose, insulin and triglyceride concentrations in plasma were measured within 2 h after sampling, while the other (aliquoted) serum and plasma samples were stored at -20°C and thawed once for final analysis.

To preserve glucagon-like peptide-1 (GLP-1), stoppers of the 4 ml serum Vacutainer tubes (with clot activator; CAT: REF369032, Becton Dickinson) were quickly and carefully punctured by a 23G Becton Dickinson Microlance 3 needle on the luer hub of a 250 μl syringe (Kloehn, CA, USA) and 40 μl ice-cold protease inhibitor DPP-IV (Nuclilab, NL) was injected. This procedure was performed within 1 h prior to venous sampling.

To preserve ghrelin, stoppers of the 4 ml K2-EDTA Vacutainer tubes (K2E 7.2 mg PLUS; REF368861, Becton Dickinson) were fast and carefully punctured by a 23G Becton Dickinson Microlance 3 needle on the luer hub of a 250 μl syringe (Kloehn, CA, USA) and 200 μl ice-cold protease inhibitor Aprotinin (MP Biomedicals, NL) was injected. In both protocols, vacuum was not disturbed as validated by using water.

Immediately after collection of each blood sample, the meniscus of blood in the tube – spiked with protease inhibitor (see above) – was measured and the measured concentrations of GLP-1 and ghrelin in each tube corrected both for actual meniscus and added volume of protease inhibitor.

Glucose: Serum glucose levels were determined by quantitative enzymatic measurements and photometry on NADH with the use of Hexokinase and Glucose-6-Phosphate Dehydrogenase on a cArchitect Systems (Abbott Diagnostics, IL, and USA).

Insulin: Serum insulin levels were determined by quantitative measurements with the use of a solid-phase, two-site chemiluminescent immunometric assay on an Immulite 2500 (Siemens Medical Solutions Diagnostics, LA, USA).

GLP-1: Mammalian GLP-1 was assessed with an (active) ELISA (Millipore, MA, USA). The in-house validated GLP-1 test showed imprecisions (CV%) of <5 at concentrations >2 pmol/l (inter-assay and intra-assay).

Ghrelin: Human ghrelin was assessed with a RIA (Millipore, MA, USA). The verified total ghrelin assay showed good sensitivity over the range of 100–10,000 pg/ml and imprecisions (CV%) of 14.7–17.8 (inter-assay) and 3.3–10.0 (intra-assay); accuracy was 90–96%.

CCK: CCK concentrations were measured using a highly specific radioimmunoassay [17].

2.4. Determination of gallbladder contractility

Gallbladder size was visualized, always after blood sampling, by serial ultrasonographic imaging (Antares Sonoline, Siemens) at baseline and at 10, 20, 30, 40, 50 and 60 min after challenge. Images along the x-, y- and z-axes were recorded to determine the gallbladder volume with a mathematical ellipsoid formula ($VE = 2/3\pi \cdot x \cdot y \cdot z$), measuring diameters being x maximal longitudinal, y antero-posterior and z transverse.

2.5. Statistical analysis

Data were analyzed using Statgraphics Centurion XV software (Version 15.2.00 for MS Windows; Statpoint, Inc., VA, USA) and using Analyse-It (Version 1.71 for MS Excel; Analyse-It Software, Leeds, UK). A Shapiro–Wilks test was used to check whether nominal parameters were normally distributed, prior to analysis. The means, standard deviations, coefficients of variation and medians (25th–75th percentile) of all data and of changes versus results at baseline ($t = 0$ min) were calculated by subset analysis. To compare VAS scores, levels of serum parameters, and gallbladder volumes between chocolate eating, chocolate smelling and control, paired non-parametric

Wilcoxon signed rank tests were performed for separate time lags. Friedman tests were used to compare changes within groups, non-parametrically. Correlations between VAS scores and serum parameters, gallbladder volumes and time were calculated by Pearson linear product moment and Spearman rank correlation tests in grouped chocolate-eating subjects, chocolate-smelling subjects and controls. P -values <0.05 were considered statistically significant. The U-shaped VAS-versus-time curves were fitted to the best P -value using Statgraphics' modalities. Areas-under-the-curve (AUCs) for any relevant parameter-versus-time curve were calculated using the trapezoidal rule method.

3. Results

The 12 subjects had a mean age of 26.6 years (range: 25–30 years). Repeated measures for non-parametric outcomes (Friedman tests) comparing changes during each test session within one of the groups, revealed significantly higher VAS 1 (more satiated) and VAS 3 (more full) and lower VAS 2 (less appetite) and VAS 4 (less hungry) scores after chocolate eating ($P < 0.01$ for all scores), no VAS changes after chocolate smelling, and opposite VAS changes in controls (they became less satiated, less full, with more appetite, and more hungry during the session; $P < 0.02$ for all scores). Wilcoxon signed rank tests for each time lag disclosed appetite suppression after chocolate ingestion (higher VAS 1 meaning more satiated and higher VAS 3 meaning more full (for all time lags $P < 0.05$) and lower VAS 2 at 10, 20, 30 and 60 min meaning less appetite ($P < 0.05$) and lower VAS 4 at 30 and 40 min meaning less hungry ($P < 0.05$)). There were no significant changes of VAS scores per time lag, neither in the smelling group nor in controls (Wilcoxon signed rank tests). However, appetite

suppression differed significantly between groups, demonstrating more satiation and fullness and less appetite and hunger after chocolate eating and smelling as compared with controls (Wilcoxon signed rank tests, $P < 0.0001$). The data are graphically shown in Fig. 1.

Levels of CCK, ghrelin and GLP-1, as well as gallbladder volumes, did not change in controls or after smelling. Notably, glucose levels increased significantly after smelling ($P < 0.05$) but not in controls, while insulin levels decreased both after smelling and in controls ($P < 0.07$ and $P < 0.05$; Friedman tests, respectively). The picture was different after chocolate eating; like in controls or after smelling there were no significant changes in CCK, ghrelin and GLP-1 levels, but both glucose and insulin levels increased significantly ($P < 0.0001$ for glucose and $P < 0.05$ for insulin). Gallbladder volumes decreased after chocolate eating in contrast with controls or after smelling ($P < 0.0001$). The data are graphically shown in Figs. 2 and 3.

Integrated gallbladder volume over 60 min were analyzed for each subject comparing the total effects of chocolate eating with that of either smelling or no challenge at all (controls). In 6 subjects we found a lower integrated gallbladder volume (subjects 1, 3, 5, 8, 9 and 12: high responders), while in 6 other subjects we found a (slightly) higher integrated gallbladder volume (subjects 2, 4, 6, 7, 10 and 11: non/low responders), see Fig. 4. There were no relationships between CCK levels and gallbladder volumes, neither for all chocolate eaters, nor for the separate groups of high or non/low gallbladder volume responders.

The correlations between the four VAS domains and all parameters and gallbladder volumes are listed in Tables 1a, 1b, and 1c. The controls revealed positive correlations between VAS domains 1 and 3 (fullness and satiation) and insulin, GLP-1, and CCK concentrations, while these were negative for ghrelin (Table 1a). In contrast, negative correlations were found between domains 2 and 4 (appetite and

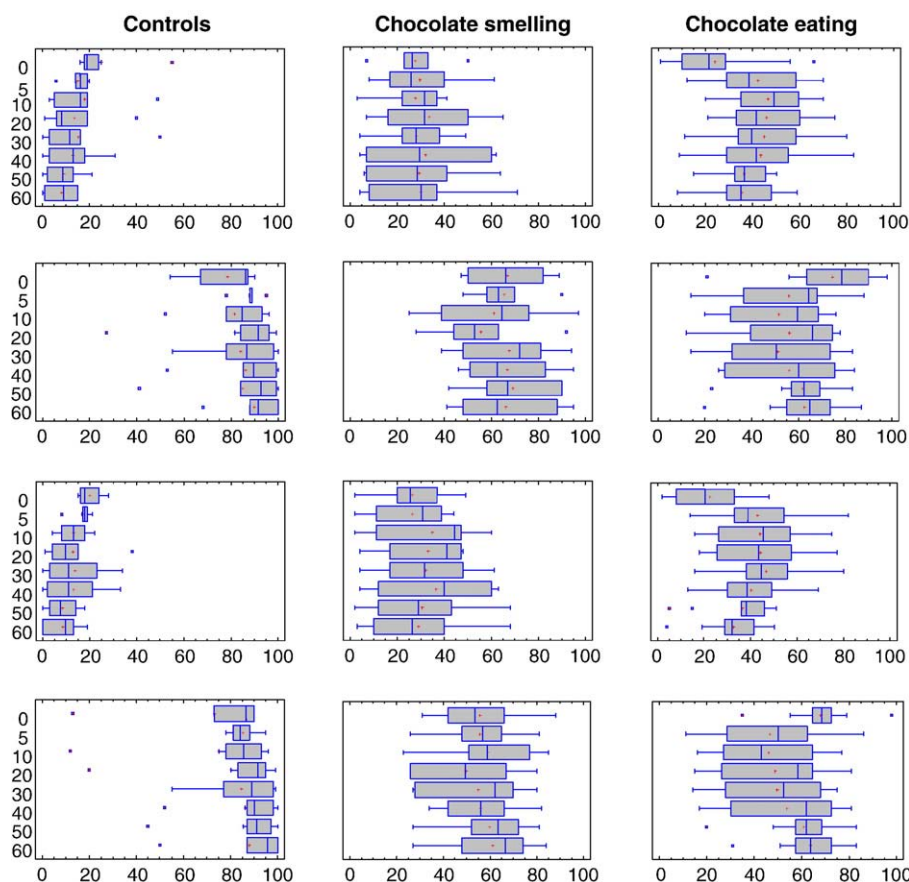


Fig. 1. Visual analogue scale scores (VAS1-4; 100 mm) in controls (left), chocolate smelling (middle) and chocolate-eating probands (right) with time presented in Box-and-Whisker plots. The time (min) per VAS 1 to 4 score is represented at the vertical axis in the upper, second, third and bottom row of each horizontal cartoon panel. Symbols: vertical line in box: median; mean: *; outlier: □.

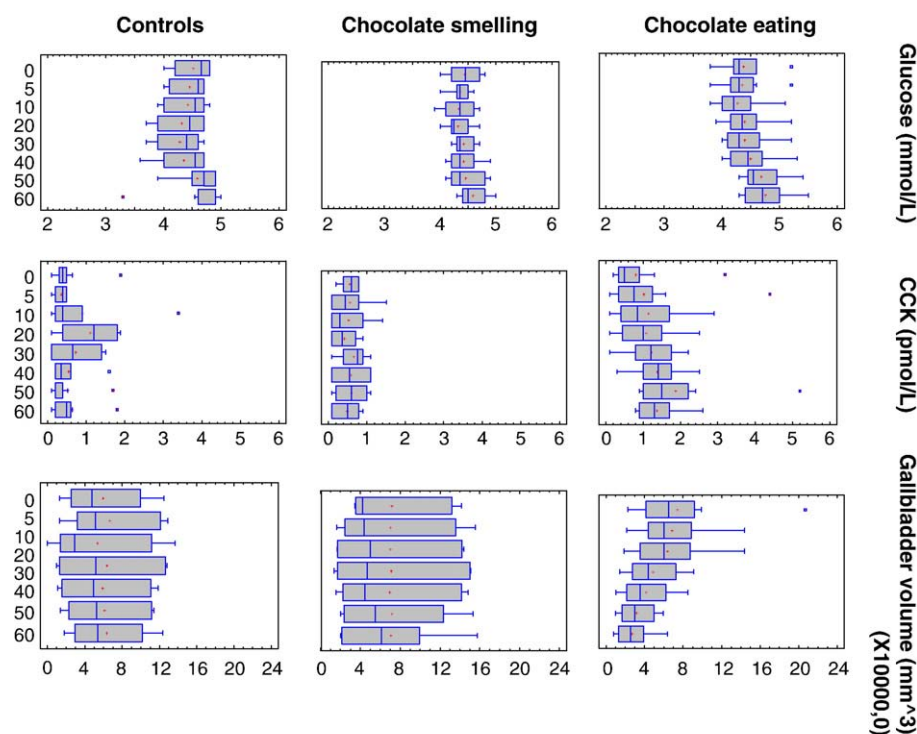


Fig. 2. Laboratory parameters (glucose, CCK and gallbladder volume) in controls (left), smelling (middle) and eating probands (right) with time presented in Box-and-Whisker plots. The time (min) is represented at the vertical axis of each horizontal panel. Symbols: vertical line in box: median; mean: *; outlier: □.

hunger) and insulin, GLP-1 and CCK concentrations, while these were positive for ghrelin (Table 1a). A different picture emerged after chocolate smelling or eating. We found a significant inversion of all correlations between VAS domains and ghrelin (Table 1b), while all other correlations disappeared both after smelling and after eating (Tables 1b and 1c).

4. Discussion

This study examined whether the ingestion or the smell of dark chocolate alters the levels of several gastrointestinal hormones with special focus on appetite. It confirmed that subjects who have fasted are less satiated (VAS 1) and full (VAS 3) and have more appetite (VAS 2)

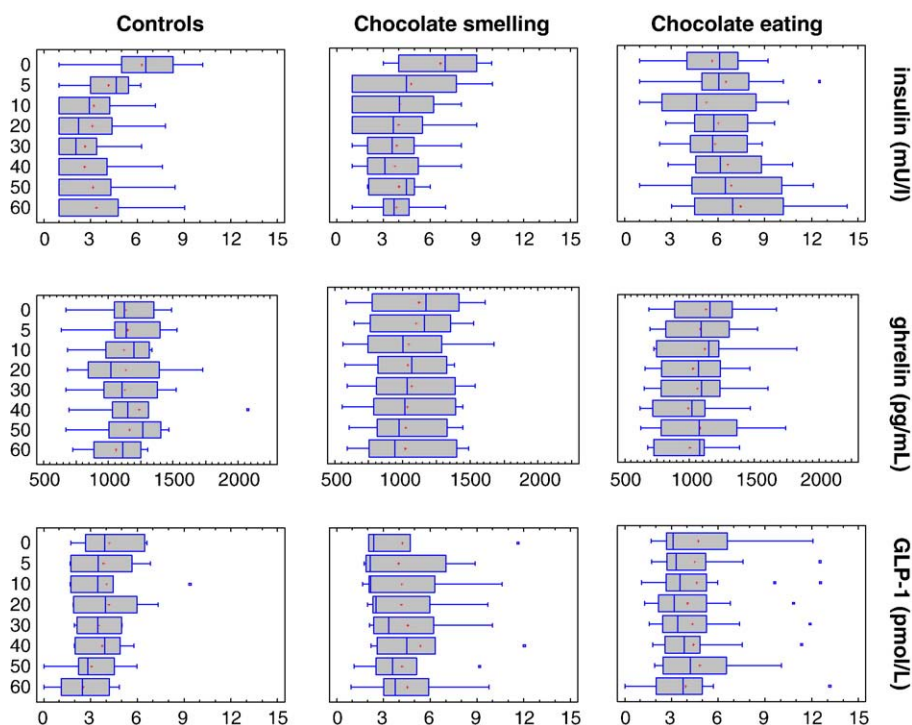


Fig. 3. Laboratory parameters (insulin, ghrelin and GLP-1) in controls (left), smelling (middle) and eating (right) probands with time presented in Box-and-Whisker plots. The time (min) is represented at the vertical axis of each horizontal cartoon panel. Symbols: vertical line in box: median; mean: *; outlier: □.

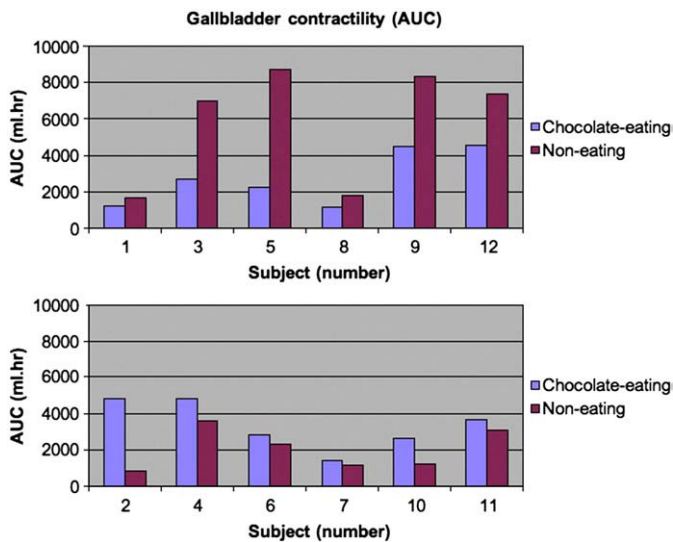


Fig. 4. Integrated gallbladder volumes over 1 h for each subject comparing the total effect of chocolate eating with that of no eating over time. Subjects were grouped, according to changes in gall bladder contractilities post-challenge, into high responders (upper panel) and low/non-responders (lower panel).

and hunger (VAS 4) than when fed. Subjects were obviously more satiated and full as well as less hungry after the ingestion of 30 g of dark melted chocolate, but satiation increased also after chocolate smelling. This olfactory effect was not accompanied by changes of glucose, insulin, CCK and GLP-1, but with an inversion of the relationship between appetite and ghrelin.

The effects of chocolate smelling and eating on appetite were similar according to the VAS scores. These findings are different from data showing that thought, sight, smell and taste of food resulted in less powerful cephalic responses than sham feeding [18]. Food-related cephalic ghrelin responses vary from decreasing [19], unaffected as currently demonstrated, up to increasing ghrelin levels [20]. Whether palatability-affected appetite can alter ghrelin levels is still unknown. On the other hand, co-existing eating disorders clearly affect cephalic ghrelin responses. Patients with anorexia nervosa reported to be hungry with concomitantly high ghrelin levels, while at the same time they suffered from underweight. Moreover, in patients with bulimia nervosa, ghrelin levels induced by sham eating correlate positively with the weekly frequency of bingeing [12–14].

Since our primary objective was to study physiology, not pathophysiology, healthy subjects were asked to smell/eat dark chocolate containing only small amounts of added sugar (12.5 g of sugar per 100 g). It is of note that high-sugar high-fat foods may provoke food cravings [15], which were intentionally avoided in the current study. Ingesting a 30 g portion of dark chocolate induced satiation after 5 min that lasted for the duration of the test. Gallbladder contractions

Table 1a
Correlation controls.

	Glucose	Insulin	GLP-1	Ghrelin	CCK	Gallbladder
VAS 1	0.16	0.74	0.55	-0.45	0.67	-0.38
	0.35	<0.0001	<0.001	<0.01	<0.0001	<0.05
VAS 2	-0.14	-0.77	-0.61	0.44	-0.68	0.41
	0.41	<0.0001	<0.001	<0.01	<0.0001	<0.05
VAS 3	0.08	0.67	0.45	-0.50	0.48	-0.30
	0.63	<0.0001	<0.01	<0.01	<0.01	0.07
VAS 4	-0.21	-0.77	-0.71	0.36	-0.80	0.45
	0.21	<0.0001	<0.0001	<0.05	<0.0001	<0.01

Pearson product moment correlations between each pair of variables. Rho (r), ranging between -1 and $+1$, measures the strength of the linear relationship between the variables. p is the P -value of the estimated correlation. Data with significant correlations of all 4 VAS domains are in boldface type.

Table 1b
Correlations chocolate smelling.

	Glucose	Insulin	GLP-1	Ghrelin	CCK	Gallbladder
VAS 1	0.44	-0.08	0.07	0.69	-0.35	-0.06
	<0.01	0.59	0.65	<0.0001	<0.05	0.72
VAS 2	-0.15	0.51	0.30	-0.50	0.30	-0.42
	0.33	<0.001	0.06	<0.001	0.06	<0.01
VAS 3	0.36	-0.11	0.16	0.64	-0.37	-0.03
	<0.05	0.48	0.33	<0.0001	<0.05	0.84
VAS 4	0.15	0.30	0.01	-0.34	0.16	-0.48
	0.34	0.05	0.98	<0.05	0.33	<0.01

Pearson product moment correlations between each pair of variables. Rho (r), ranging between -1 and $+1$, measures the strength of the linear relationship between the variables. p is the P -value of the estimated correlation. Data with significant correlations of all 4 VAS domains are in boldface type.

were observed after 30 to 40 min similarly as previously reported [21]. Contractions were only noted in half of the subjects, which might be related with portion size. However, a larger portion size would have been problematic, since even 30 g chocolate was difficult to melt-and-swallow for most subjects.

We found that appetite scores decreased similarly after 30 g of dark chocolate either smelling or eating, while smelling altered appetite apparently mediated by ghrelin. All ghrelin data in this study were assayed with a polyclonal RIA that measures total plasma ghrelin, which consists of an estimated mixture of desacylated and acylated ghrelin peptides (ratio roughly 9:1) [22]. Biologically active ghrelin requires post-translational n -octanoylation of the Ser 3-hydroxyl group (Ser³) [23,24]. Non-octanoylated ghrelin is presumed to be inactive [23]. The peptide becomes acylated with n -decanoic acid and was found in octanoylated and decanoylated forms in human stomach (at a ratio of about 3:1). Classified into four groups by the type of acylation observed at Ser³, the ghrelin peptides were found to be non-acylated, octanoylated (C8:0), decanoylated (C10:0) and decenoylated (C10:1). The most abundant form of human active ghrelin is (C8:0) [25]. Important but also confusing, is a recent publication on non-acylated ghrelin forms, suggesting that these peptides are actively involved in appetite regulation and homeostasis [26].

The reason for us to measure total ghrelin is the robustness of available assays, while a number of precautions are required when active forms of ghrelin are to be assayed. Moreover, measuring acylated ghrelin (C8:0) remains cumbersome because of its instability in plasma [27]. By assaying total ghrelin, we were able to show a reverse and disappearance of correlations for each VAS domain but no longitudinal changes after cue exposure. Nonetheless, all VAS domains and ghrelin were found to correlate before and after smelling with consequent inversions from plus to minus and vice versa, while all correlations disappeared after eating. These findings argue against statistical bias of outcome data.

The notice of a feeding preparation response (cephalic phase response), which occurs prior to the actual ingestion of food, was

Table 1c
Correlations chocolate eating.

	Glucose	Insulin	GLP-1	Ghrelin	CCK	Gallbladder
VAS 1	0.08	-0.29	0.18	0.04	0.12	0.05
	0.47	<0.05	0.12	0.72	0.31	0.66
VAS 2	-0.01	-0.15	-0.03	0.06	-0.21	-0.21
	0.89	0.19	0.80	0.63	0.07	0.07
VAS 3	-0.01	-0.27	0.18	0.24	0.01	-0.15
	0.95	<0.05	0.12	<0.05	0.97	0.19
VAS 4	0.01	-0.19	-0.15	0.14	-0.37	-0.15
	0.99	0.10	0.21	0.23	<0.01	0.21

Pearson product moment correlations between each pair of variables. Rho (r), ranging between -1 and $+1$, measures the strength of the linear relationship between the variables. p is the P -value of the estimated correlation.

conceptualized by Pavlov at the beginning of the 20th century. In the 1970s it was postulated that the cephalic phase response is also involved in appetite regulation [28,29]. It has been shown that sham feeding in humans, that is, stimulation of receptors of the oral-pharyngeal cavity, induces a reflexory rise of insulin and pancreatic polypeptide secretion [30]. Moreover, palatable foods generally result in more robust cephalic phase responses than do less preferred foods, while blocking of cephalic phase responses resulted in the ingestion of smaller meal sizes in animals as well as in humans [29]. A cephalic phase response has been initially reported for insulin, which occurs on chewing and food tasting [28–30] and depends entirely on the degree of its palatability [31–33]. For example, the tasting of sweeteners in humans does not evoke an insulin response, in contrast to robust insulin responses after tasting but not ingesting palatable sweet food, e.g. apple pie [34].

The present study design differs from most protocols for sham feeding in humans. Subjects are usually instructed to smell and chew food, avoiding swallowing, and to spit it out to avoid post-ingestion effects. In our study, sham feeding was different, as melting chocolate is creamy and sticky and covers large parts of the oral cavity and tongue, so that spitting it out is cumbersome and swallowing is practically inevitable. However, the composition of chocolate is highly variable and more studies on different chocolate flavors and compositions may help to understand causal relationships between palatability, appetite modification and gastrointestinal hormones.

In conclusion, this study shows that appetite was suppressed after smelling or ingesting dark chocolate. The satiation effects after smelling seems to find its biological basis in the involvement of ghrelin, possibly mediating vagal tone. Satiating food odors are therefore challenging to study further and may be of medical interest by influencing appetite in clinical conditions.

Acknowledgements

The authors are grateful to all participants of the study, to Mr. Chris van der Bent and to all laboratory technicians of Leiden University, Rigshospitalet Copenhagen and Reinier de Graaf Groep of Hospitals (RdGG) Delft, who were dedicatedly involved in the study and to Mr. Roelof Broekhuysen of RdGG, for his expertise and dedication in ultrasound assessments.

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