Title page

Influence of subliminal intragastric fatty acid infusion on subjective and physiological responses to positive emotion induction in healthy women: A randomized trial

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Data described in the manuscript and analytic code will be made available upon request pending.

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Abbreviations

BGA	brain-gut axis
CCK	cholecystokinin
ECG	electrocardiograph
GLP1	glucagon-like peptide-1
HF	high frequency components
HRV	heart rate variability
LF	low frequency components
LF/HF	low frequency to high frequency ratio
PMSF	phenylmethansulfonylfluoride
PYY	peptide YY
RMSSD	root mean square of successive differences
SAM	self-assessment manikin
VAS	visual analogue scale

Abstract

Background: Subliminal intragastric fatty acid infusion attenuates subjective and brain responses to negative emotion induction. However, the underlying gut-brain signaling mechanisms remain unclear, and it is unknown whether such effect equally applies to positive emotion.

Objective: We aimed to investigate the interaction between fatty acid-induced gut-brain signaling and subjective responses to positive emotion, and the potential mediational role of gastrointestinal (GI) hormones.

Design: Twelve fasted healthy women underwent intragastric infusion of 2.5g lauric acid or saline, after which either positive or neutral emotion was induced for 30min, in 4 separate visits. Appetite-related sensations, subjective emotional state, and GI hormones were measured at baseline and every 10min after infusion. Heart rate variability was measured at baseline and at t=20–30 min to quantify vagal tone (root mean square of successive differences, RMSSD), and sympathovagal balance (low frequency to high frequency ratio, LF/HF).

Results: Fatty acid infusion did not influence appetite-related sensations (as expected), nor emotional state ratings (contrary to expectations). As anticipated, fatty acid stimulated release of CCK at t=20-40min ($p<0.001$), and GLP1 at t=30-40min ($p<0.001$), but not PYY. Interestingly, positive emotion induction suppressed plasma octanoylated ghrelin at t=20-40min ($p=0.020$). Further, both positive emotion and fatty acid attenuated RMSSD ($p=0.012$ & 0.0073, respectively). Positive emotion attenuated LF/HF after fatty acid ($p=0.0006$), but raised LF/HF after saline ($p=0.004$).

Conclusions: Subliminal fatty acid did not influence subjective responses to positive emotion induction. However, positive emotion induction suppressed octanoylated

ghrelin release. Moreover, both positive emotion and subliminal fatty acid decreased cardiac vagal tone. Further, the fatty acid reversed the effect of positive emotion on sympathovagal balance.

Keywords

gut-brain axis, gut hormones, positive emotion, ghrelin, heart rate variability

Introduction

The brain-gut axis (BGA) is part of an integrated interoceptive system which continuously conveys homeostatic information about the physiological state of the body to the brain (1). At the brain level, such information is integrated with information from affective brain circuits, after which appropriate bodily and behavioral responses are generated (1). We previously demonstrated that a purely interoceptive, subliminal 'appetitive' nutritional stimulus interacts with an exteroceptively generated negative emotional state, both at the subjective and the neural level (2). More specifically, intragastric fatty acid infusion attenuated subjective and neural responses to sad emotion induction. However, the neurohumoral gut-brain and brain-gut signaling mechanisms underlying this effect remain unclear. Furthermore, it remains unknown whether fatty acid infusion would also have an influence on responses to positive emotion (e.g. happiness) induction.

Several peptide hormones are produced in the GI tract in response to chemical stimuli, particularly nutrients, and are crucially involved in appetite regulation (1). Among these peptides, the anorexigenic hormones cholecystokinin (CCK), glucagon-like peptide-1 (GLP1), peptide YY (PYY) and the orexigenic hormone ghrelin are key candidate mediators of the attenuating effect of intragastric fatty acid infusion on responses to negative emotion induction. For example, brain responses induced by intragastric fatty acid infusion are abolished by CCK-1 receptor antagonists (3), as well as by intravenous ghrelin infusion (4). Both CCK and ghrelin respond to fatty acid infusion and reach peaks (or nadirs) within 20-30 min (5). GLP1 and particularly PYY, on the other hand, are characterized by a delayed secretion pattern in response to fatty acid intake (6, 7).

In addition, the vagus nerve innervates the stomach and conveys bidirectional gut-brain signals, whether or not in interaction with the abovementioned gut peptides (8). Researchers have used heart rate variability (HRV) to investigate the role of the efferent vagus nerve in brain-gut communication (9-11). For example, a standard meal (500kCal) reportedly caused vagal withdraw in healthy volunteers, and therefore rendered the sympathovagal balance to a sympathetic dominance (12). Moreover, HRV is also sensitive to emotions (13). For example, both sad and happy emotion induction decreased HRV (13).

Our primary aim was therefore to test the hypothesis that subliminal interoceptive stimulation induced by fatty acid would interact with positive emotion induction at the subjective level. More specifically, fatty acid infusion would enhance the effect of positive emotion induction without triggering any changes in appetite-related sensations. Moreover, we measured plasma hormone levels including CCK, ghrelin, GLP1 and PYY as secondary outcomes to explore whether hormonal responses to fatty acid would interact with the positive emotion induction in a similar pattern as the subjective responses. Further, we explored whether vagal efferent responses to positive emotion would interact with the fatty acid-induced subliminal interoceptive signals (i.e. hormone responses), using HRV measurements.

Subjects and Methods

Eligibility criteria for participants

Normal weight, healthy, non-pregnant, non-breastfeeding women (18–65 years) were included, to avoid sex as a potential confounder. Exclusion criteria included alcohol consumption >7 units/week, smoking, substance abuse, regular intake of medications with an exception of oral contraception pills, chronic medical illness, chronic pain, and any psychiatric disorder. The sample size was chosen based on our previous findings on the effects of fatty acid infusion on subjective and neural responses to negative emotion induction (2).

Ethics

This study was approved by the Medical Ethics Committee of the University Hospitals Leuven, Belgium (ML10475, 08-Apr-2014), registered at ClinicalTrials.gov (NCT02982616) and performed in the University Hospitals Leuven, Belgium, in accordance with the Declaration of Helsinki, including written informed consent.

Study design

In this randomized, placebo-controlled, single-blind, cross-over study, participants came on 4 separate visits assigned to receive either fatty acid or saline (placebo) infusion (nutrient conditions) and either positive or neutral emotion induction (emotion conditions), at least one week apart (2x2 within-subject factorial design, partially counterbalanced using Latin Square).

On each visit, participants came to the lab in the morning, after a 12-hour overnight fast. After a 10 min rest period upon arrival, HRV was measured for 6 min in resting state (sitting). After the HRV measurement, appetite-related sensations and

emotional state were rated, and blood samples were taken at fixed time points throughout the procedure (*Figure 1*). A nasogastric feeding tube was then inserted, with the catheter tip in the fundus of the stomach. After a 10 min adaptation period, either positive or neutral emotion induction was performed for 33 min. Three minutes after the emotion induction started, either 2.5g lauric acid (0.05 mol/L) or 250 mL 0.9% saline placebo was infused through the nasogastric tube over 2 min, while the participants were requested to focus on the emotion induction stimuli. Ratings of appetite-related sensations and emotional state as well as blood samples were collected before the start of the emotion induction and every 10 min after the intragastric infusion. The second HRV measurement was performed during the last 10 min of the emotion induction. Immediately after the end of the emotion induction, participants were extubated, followed by a 10 min break. Finally, hedonic food intake was measured using an *ad libitum* chocolate milkshake drinking task. ***Figure 1*** presents an overview of the study procedure for each visit.

Emotion induction

To induce positive (happiness) or neutral emotional states, we combined two validated methods of emotion induction. Eleven excerpts of classical music of 1-minute duration each (14) were randomly played through headphones for 33 minutes in total, beginning 3 minutes before the start of the intragastric infusion to allow participants to get into the desired emotional state. At the same time, 10 validated facial expressions depicting either positive or neutral emotion (15) were projected onscreen for 5 seconds each and repeated in random order, only to be interrupted briefly for obtaining ratings, as described in the study design and experimental procedure paragraphs. In order to quantify the effect of the emotion induction procedure and the intragastric infusion on emotional state, the Self-Assessment

Manikin (SAM) (16) was used to measure emotional valence, arousal, and dominance on a 9-point scale, with the anchors: 1-very negative (sad) to 9-very positive (happy) (valence); 1-very calm to 9-very excited (arousal); and 1-not having the situation under control to 9-having the situation completely under control (dominance).

Blood sample processing and laboratory analysis

Ghrelin blood samples were collected on ice in EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 500 kIU/mL aprotinin (Roche Applied Science, Penzberg, Germany) and phenylmethylsulfonylfluoride (PMSF; 0.57mM; Sigma-Aldrich, Steinheim, Germany). Tubes were centrifuged at 4° C at 3000 rpm for 10 min and plasma samples were aliquoted. Plasma samples for ghrelin measurements were immediately acidified (10%) with 1N HCL and extracted on a Sep-Pak C18 cartridge (Waters Corporation, Milford, Massachusetts, USA) and dried in a speedvac (17). The active form of ghrelin, *octanoylated ghrelin* was measured by radioimmunoassay with ^{125}I [Tyr^{24}] human ghrelin [1-23] as tracer and a rabbit antibody against human ghrelin [1-8] (final dilution 1/100000), which does not cross-react with desoctanoylated ghrelin, as described previously in more detail (17).

CCK-8 blood samples were collected in EDTA tubes filled and diluted in 10 fold in RAPID buffer pH 3.6 [0.1 M ammonium acetate, 0.5M NaCl and enzyme inhibitors: diprotinin A, E-64-D, antipain, leupeptin, chymostatin (all 1 µg/ml; Peptide International, Louisville, KY)] as previously described (18). Samples were centrifuged and the supernatant was purified on Sep-Pak C18 cartridges and dried in a speedvac. CCK-8 was measured with a commercially available RIA kit (Euro-Diagnostica AB, Malmö, Sweden).

GLP-1 samples were collected in EDTA tubes containing 500 kIU/ml aprotinin and dipeptidylpeptidase IV inhibitor (DPP4, 10µl/ml blood; Merck Millipore, Billerica, USA). GLP-1 was measured with an immunoassay kit (ver. 2, Meso Scale Discovery, Rockville, USA) which measures the active forms of GLP-1, GLP-1 (7-36) amide and GLP-1 (7-37).

PYY samples were measured with an enzyme immunoassay (Phoenix Pharmaceuticals Inc. Burlingame, CA, USA) which measures PYY_{3-36}.

Plasma was separated by centrifugation at 4℃ for 10 min at 3000 xg and stored at -80℃ until analysis.

Appetite-related sensations assessment

Validated computer-based visual analog scales (VAS) were used to rate the subjective sensations of hunger, prospective food consumption, fullness, satiety and nausea (19, 20). Subjects were instructed to indicate their subjective sensations at the present time point by clicking the left and right arrows on the keyboard (in 1-point steps). The duration of each VAS was fixed to 7 seconds. In addition, the mark was always reset to the middle of the vertical line at the beginning of each VAS question.

Milkshake drinking task

At 40 min post infusion, participants were instructed to drink chocolate milkshake [4 scoops of IJsboerke vanilla ice cream, 355 mL of 2% milk, and 2 tablespoons of Imperial chocolate syrup, 270 kcal, 13.5 g fat, and 28 g sugar per 150 mL; recipe adapted from Burger *et al*. (21) with Belgian brands.] from a 200mL glass, at their own pace until they felt comfortably satiated. The glass was immediately refilled when it was empty. The amount of milkshake before and after the task was weighed

on a scale to calculate the amount of milkshake drunk by the volunteer as a measure of hedonic eating behavior. participants were asked “How much do you like the milkshake”, with anchors “not at all” and “extremely”. All subjects liked the milkshake (VAS liking of milkshake: 83 ± 3).

Heart rate variability (HRV)

HRV is a commonly used and well established method to measure efferent vagal activity (22). Heart rate data were collected with the standard Electrocardiograph (ECG) electrodes attached to the anterior chest wall (MediFit Instruments Ltd, London, UK). The signal was sampled at 1 kHz and transduced, amplified and filtered through a Coulbourn S75-04 Isolated Bioamplifier. A low pass filter at 10 Hz and a high pass filter at 1 kHz were applied on sampling data. The raw heart rate sampling data was processed in Artiifact (23). The root mean square of successive differences (RMSSD) in inter-beat intervals were calculated in the time domain as indicators of the vagal tone. The ratio between low-frequency (0.04 – 0.15 Hz, LF) component and high-frequency (0.15 – 0.50 Hz, HF) component was calculated (LF/HF) in the frequency domain as an indicator of sympathovagal balance (22, 24).

Statistics

Analyses were performed in SAS 9.4 (SAS institute, Cary, NC, USA). Data are reported as mean ± SE. Significance was set at $p \leq 0.05$.

Changes from baseline in appetite-related sensations, emotional state ratings, and plasma hormone levels were calculated by subtracting the baseline value from the values at each post-infusion timepoint, resulting in delta values, which were then boxcox-transformed if needed to fulfill the assumption of normally distributed residuals. Linear mixed models, and generalized linear mixed models when a normal

residual distribution could not be achieved after transformation, were performed on the aforementioned preprocessed data, with main effects of time, emotion and nutrient, their second order interaction effects, and with baseline measurements and visit number as covariates. Significant interaction effects with time (nutrient-by-time, or emotion-by-time) were followed by *post hoc* contrasts at each time point, with stepdown Bonferroni (Holm) correction for multiple testing.

Delta values were calculated on HRV data (RMSSD and LF/HF) in the same way, boxcox-transformation was applied if needed, and the resulting data were then analyzed using linear mixed models with main effects of emotion and nutrient, and their two-way interaction effect, with baseline measurements and visit number as covariates.

Hedonic eating (amount of milkshake drunk) was compared in a linear mixed model with main effects of emotion and nutrient and their two-way interaction effect, and with visit number as covariate.

The emotion-by-nutrient interaction effect in all the aforementioned models, which constitutes the principal effect of interest together with the main effect of emotion, was followed up by *post hoc* contrasts using two-tailed paired t-tests testing the effect of both factors at each level of the other factor, with stepdown Bonferroni (Holm) correction for multiple testing.

Results

Study participants

Fourteen eligible female volunteers with a mean age of 23 ± 2 years and mean BMI of 21.1 ± 1.2 kg/m^2 were recruited (August 2015 – March 2016). Two volunteers did not receive the allocated intervention because they could not tolerate the nasogastric tube. Twelve volunteers (n = 12) completed all the allocated interventions and were included in the analysis. One volunteer was excluded for HRV analysis due to frequent premature beats. However, the volunteer was still included in other analyses because the volunteer's heart rate was still within the normal range, and the volunteer did not report any adverse sensations during the measurements. Minimal nausea scores (zero-inflated with very limited variability between conditions, time points and participants, not permitting formal statistical analysis) were reported. No adverse events occurred.

Appetite-related sensations

Hunger and prospective food consumption *(**Figure 2A & 2B**)*

Hunger and prospective food consumption ratings did not differ between fatty acid and placebo, nor between positive and neutral emotion. There were no emotion-by-nutrient interaction effects. The results of the mixed model analysis are summarized in *Table 1*.

Satiety and Fullness *(**Figure 2C & 2D**)*

Satiety and fullness ratings did not differ between fatty acid and placebo, nor between positive and neutral emotions. There were no emotion-by-nutrient

interaction effects. A significant time-by-nutrient interaction effect was found on fullness ratings ($F_{4,135}$=3.49, p=0.018). However, the *post hoc* contrasts did not show any significant differences between fatty acid and placebo at any time point (all p_{Holm}>0.10). The results of the mixed model analysis are summarized in ***Table 1***.

*Emotional state ratings (**Figure 3**)*

Emotional valence, arousal and dominance responses were significantly higher in positive emotion compared to neutral emotion (main effect of emotion $F_{1,30}$=16.49, 7.70 & 6.22, p<0.001, p=0.006 & 0.018, respectively), thereby confirming efficacy of the emotion induction procedure. Furthermore, there was no significant main effect of nutrient on any of the emotional ratings (all p>0.05). Moreover, there was no emotion-by-nutrient interaction effect (contrary to our hypothesis) on valence, arousal, nor dominance ratings. The results of the mixed model analysis are summarized in ***Table 2***.

Hormone responses

Plasma octanoylated ghrelin *(**Figure 4A**)*

Positive emotion significantly suppressed plasma octanoylated ghrelin compared to neutral emotion (main effect of emotion $F_{1,40}$=5.91, p=0.020). Furthermore, the time-by-emotion interaction was significant ($F_{3,134}$=3.51, p=0.017). *Post hoc* contrasts indicated significantly stronger decreases in octanoylated ghrelin level in positive emotion compared to neutral emotion at t=20, 30 & 40min (t_{134}=-2.76, -2.40, & -2.73, p_{Holm}=0.020, 0.020, & 0.020, respectively). However, plasma octanoylated ghrelin levels did not differ between fatty acid and placebo, nor was there an emotion-by-nutrient or time-by-nutrient interaction effect. The results of the mixed model analysis are summarized in ***Table 3***.

Plasma CCK *(**Figure 4B**)*

Plasma CCK was analyzed in a generalized linear mixed model, because a normal residual distribution could not be achieved after box-cox transformation. Plasma CCK levels increased after fatty acid compared to placebo ($F_{1,133}$=69.30, p<0.001). However, there was no main effect of emotion, nor was there an emotion-by-nutrient interaction. There was also no time-by-emotion or time-by-nutrient interactions. The results of the generalized linear mixed model analysis are summarized in ***Table 3.***

*Plasma GLP1 (**Figure 4C**)*

Plasma GLP1 levels increased significantly after fatty acid compared to placebo ($F_{1,40}$=8.90, p=0.0048). Further, there was no main effect of emotion induction, nor was there an emotion-by-nutrient interaction effect. There was also a significant time-by-nutrient interaction effect ($F_{3,135}$=4.04, p=0.0087). *Post hoc* contrasts revealed that the plasma GLP1 levels increased after fatty acid compared to placebo at t= 30 & 40min (t_{135}=3.16 & 3.84, p_{Holm}=0.0057 & 0.0007, respectively). The results of the mixed model analysis are summarized in ***Table 3***.

*Plasma PYY (**Figure 4D**)*

Plasma PYY levels did not differ after fatty acid compared to placebo. There was also no main effect of emotion induction, nor was there a nutrient-by-emotion interaction. Furthermore, there was a significant time-by-emotion interaction ($F_{3,132}$=2.82, p=0.041). However, *post hoc* contrasts did not show significant differences between emotion conditions at any time point (all p_{Holm}>0.10). The results of the mixed model analysis are summarized in ***Table 3***.

Heart rate variability (HRV)

*Time domain (**Figure 5A**)*

RMSSD was lower in positive emotion compared to neutral emotion ($F_{1,10}$=9.33, p=0.012). Moreover, RMSSD significantly decreased after fatty acid compared to placebo ($F_{1,10}$=11.27, p=0.0073). However, there was no emotion-by-nutrient interaction ($F_{1,10}$=2.00, p=0.19).

*Frequency domain (**Figure 5B**)*

We did not find a main effect of nutrient nor emotion ($F_{1,10}$=0.31 & 0.22, p=0.59 & 0.65, respectively) on the LF/HF ratio, but the nutrient-by-emotion interaction effect was significant ($F_{1,10}$=59.99, p=0.046). Specifically, *post hoc* contrasts indicated that the LF/HF ratio was significantly lower in positive emotion compared to neutral emotion after placebo (t_{10}=-6.18, p_{Holm}=0.004), whereas the difference between emotion conditions was reversed after fatty acid (t_{10}=5.66, p_{Holm}=0.0006).

Hedonic food intake

The amount of milkshake drunk was not significantly different between emotion or nutrient conditions (main effect of nutrient, $F_{1,11}$=0.18 p=0.68, main effect of emotion, $F_{1,11}$=0.37 p=0.56), nor was there an interaction effect between emotion and nutrient ($F_{1,10}$=0.01, p=0.91).

Discussion

In the current study, fatty acid did not influence emotion ratings, nor was there any interaction between fatty acid infusion and emotion induction. Although this is at variance with the previous study indicating that fatty acid attenuated the effect of negative emotion induction and, hence, our hypothesis, it is noteworthy that positive emotion is not merely the opposite end of the spectrum as negative emotion. According to Ekman as well as more recent emotion theorists (15, 25), positive emotions and negative emotions are emotions in different ‘themes’, rather than simply two ends of one dimension. Indeed, the valence hypothesis suggests that the right hemisphere is dominant in negative emotion, whereas the left hemisphere is dominant in positive emotion (26). Recent studies suggest that the salience network, which plays a key role in emotion generation, consists of brain regions that respond to emotions regardless of valence, as well as brain regions that response differentially depending on valence (26-28). Further investigation on the valence-general and valence-dependent brain regions will be necessary to understand the mechanism underlying the difference between the previous and our findings.

Moreover, we found that positive emotion induction significantly suppressed plasma octanoylated ghrelin, which is the active form of ghrelin, at t=20-40min. Based on the current literature, the relationship between ghrelin and emotions is ambiguous (1). The evidence of ghrelin’s role on emotions in human is especially limited (29). A recent study in healthy human indicated that overnight fasting state (with high plasma ghrelin levels) prior to fear extinction prevented the return of fear (30). In animal models, stress/negative emotion enhanced the release of ghrelin (31), thereby increasing food intake. On the other hand, increased plasma ghrelin levels had anxiolytic- and antidepressant-like effects in a rodent model of depression (32).

Ghrelin may induce anxiolytic effects by interfering with amygdala function (33). Interestingly, the amygdala is a crucial brain region that enables humans to rapidly detect and recognize positive or negative emotionally salient stimuli, especially face expressions (34). Further investigation using brain imaging techniques will be necessary to address the role of amygdala in the interaction between (positive and/or negative) emotions and ghrelin in humans. Finally, the intragastric fatty acid did not suppress octanoylated ghrelin levels in the current study. As food intake suppresses plasma active ghrelin levels in a calorie-dependent manner (35), the caloric content provided in the current study (22.5kCal) may not have been enough to suppress plasma octanoylated ghrelin levels.

Furthermore, we found that RMSSD was significantly lower in positive emotion compared to neutral emotion. RMSSD is considered as a reliable parameter reflecting efferent vagal tone (10), and is less sensitive to other factors such as breathing or baroreflex sensitivity. Therefore, the decrease of RMSSD indicated vagal withdrawal. Our finding is in line with previous findings showing vagal withdrawal and decreases of HRV parameters during happiness (13). Moreover, subliminal fatty acid attenuated RMSSD, but there was no nutrient-by-emotion interaction. Our findings indicate that the effect of subliminal intragastric fatty acid on the gastrointestinal vagal afferent nerves might trigger a vagal efferent response in another branch (cardiac branches). Further investigations on animal models will be necessary to address the exact mechanism of the nutrient induced vagal efferent responses.

In the frequency domain of HRV, we found an interaction effect between nutrient and emotion on the LF/HF ratio. An increased LF/HF indicates that sympathetic activity dominates the effects, and *vice versa (12, 36)*. Lu *et al*. (12) found that healthy

volunteers' LF/HF ratio increased 30 min after a 500 kcal meal, whereas in another study (36) the LF/HF ratio had an insignificant increase after a 250 kcal meal. Another recent study replicated the results from Lu *et al.* and furthermore, they found a weak but significant *negative* correlation between active ghrelin levels and the LF/HF ratio (11). In the current study, we applied a very small amount of fatty acid (22.5 kcal), which was not enough to trigger major changes in the LF/HF ratio. However, LF/HF ratio decreased in positive emotion after placebo, whereas it increased in positive emotion after fatty acid. McCraty *et al*. (37) reported that healthy male and female volunteers had increased LF/HF ratio during positive emotion induction. However, they did not report participants' nutritional state. It is also noteworthy that the major component of the LF/HF ratio, the high frequency component (HF), is sensitive to breathing (22), and healthy volunteers reportedly had decreased respiratory activities during positive emotion induction, and therefore increased HF (13). Moreover, as described above, the fatty acid induced vagal withdrawal. This effect may have overtaken the effect of breathing on HF during positive emotion induction, and furthermore, increased the LF/HF ratio during positive emotion after fatty acid. Unfortunately, we did not measure breathing rate in the current study. Therefore, we were not able to address the role of respiration on the emotion-by-nutrient interaction effect on LF/HF ratio in the current study. It is noteworthy that we performed the HRV measurement in the current study for explorative purposes. Further investigation in animal models will be necessary.

We did not find any effect of nutrient or emotion, nor their interaction, on food intake (21). The milkshake drinking task was performed at the end of each visit, when the effects of the nutrient infusion and/or emotion induction may already have had faded away. It is also noteworthy that the nutrient signal (2.5g lauric acid) in the current study was very subtle. Although we have observed an increase in anorexigenic

443 hormones after the fatty acid infusion, the effect may not have been strong enough to
444 impact on appetite related sensations, nor on hedonic food intake.

445 There were a few limitations to our study. First, we have studied a relatively small
446 sample size, albeit identical to our previous study with negative emotion (2). Besides,
447 a within-subject design with Latin-Square was applied to further reduce variance, and
448 for the same purpose, only women were recruited to avoid confounding sex
449 differences although this comes at the expense of compromising generalizability of
450 our findings to both sexes. Third, we induced either positive or neutral emotion in an
451 experimental environment, which does not necessarily apply to a real world situation.

452 In conclusion, we found that the subliminal nutrient signal triggered by intragastric
453 fatty acid infusion did not influence participants' appetite-related sensations or
454 emotional state ratings. However, positive emotion induction suppressed
455 octanoylated ghrelin release. The anorexigenic hormones, including CCK and GLP1
456 responded to the nutrient, but not to the emotion inductions. Moreover, both positive
457 emotion and subliminal fatty acid decreased cardiac vagal efferent tone. Further, the
458 fatty acid reversed the effect of positive emotion on sympathovagal balance. We
459 provided, for the first time to our knowledge, evidence that positive emotion induction
460 inhibits release of the orexigenic hormone, ghrelin, in its activated form, in healthy
461 women. Ghrelin is the most important orexigenic hormone in humans, with clinical
462 significance in obesity and eating disorders. Our novel findings linking positive
463 emotion and ghrelin secretion, although in need of confirmation, may provide first
464 new insights in the intricate link between feeding and emotions in health as well as
465 the abovementioned disorders, and, more broadly, affective disorders.

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Conflict of Interests

The authors have no conflict of interests to declare for this article.

Authors' Contributions

DZ, NW, JT and LVO designed the research; LB generated the random allocation sequence; LB enrolled participants; LB assigned participants to interventions; DZ, LB, JB and JI conducted research; DZ and LVO analyzed data and performed statistical analysis; DZ and LVO wrote the manuscript. LVO had primary responsibility for the final content. All authors read and approved the final manuscript.

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Tables

Table 1. Results of linear mixed model analysis on the effects of nutrient infusion and emotion induction on appetite-related sensations.

	Hunger			Prospective food consumption			Satiety			Fullness		
	df	F	p	df	F	p	Df	F	p	df	F	p
nutrient	1,40	0.59	.45	1,40	0.13	.72	1,40	0.02	.89	1,40	0.00	.80
emotion	1,40	1.03	.32	1,40	2.16	.15	1,40	0.23	.64	1,40	1.02	.21
emotion-by-nutrient	1,40	2.24	.14	1,40	1.82	.18	1,40	0.05	.83	1,40	0.53	.88
time	4,135	2.53	.060	4,135	1.04	.38	4,134	0.94	.39	4,135	5.32	*.002*
time-by-nutrient	4,135	1.90	.13	4,135	1.31	.27	4,134	1.17	.42	4,135	3.49	*.018*
time-by-emotion	4,135	0.34	.79	4,135	1.41	.31	4,134	0.85	.32	4,135	0.58	.47

df: degrees of freedom. significant effects in italic

Table 2. Results of linear mixed model analysis on the effects of nutrient infusion and emotion induction on emotional valence, arousal, and dominance ratings.

	Valence			Arousal			Dominance		
	df	F	p	df	F	p	df	F	p
nutrient	1,30	3.80	.06	1,135	2.36	.13	1,30	1.91	.18
emotion	1,30	16.49	*<.001*	1,135	7.70	*.006*	1,30	6.22	*.018*
emotion-by-nutrient	1,30	0.29	.59	1,135	1.39	.24	1,30	0.47	.50
Main effect of time	3,105	7.65	*<.001*	3,135	5.68	*.001*	3,105	8.54	*<.001*
time-by-nutrient	3,105	0.57	.63	3,135	0.13	.48	3,105	0.46	.71
time-by-emotion	3,105	0.41	.75	3,135	0.83	.88	3,105	0.33	.80

df: degrees of freedom. significant effects in italic

Table 3. Results of linear mixed model analysis on the effects of nutrient infusion and emotion induction on emotional valence, arousal, and dominance ratings.

	Ghrelin			CCK			GLP1			PYY		
	df	F	p	df	F	p	df	F	P	df	F	P
nutrient	1,40	1.14	.29	1,133	69.30	*<.001*	1,40	8.90	*.0048*	1,39	0.58	.45
emotion	1,40	5.91	*.020*	1,133	0.28	.60	1,40	1.63	.21	1,39	0.02	.89
emotion-by-nutrient	1,40	2.89	.097	1,133	0.07	.79	1,40	0.21	.65	1,39	2.67	.11
time	3,134	0.07	.98	3,133	1.89	.13	3,135	9.44	*<.001*	3,132	2.90	*.038*
time-by-nutrient	3,134	1.68	.17	3,133	0.94	.42	3,135	4.04	*.0087*	3,132	1.78	.15
time-by-emotion	3,134	3.51	*.017*	3,133	0.31	.81	3,135	1.21	.31	3,132	2.82	*.041*

The plasma octanoylated ghrelin, cholecystokinin (CCK), glucagon like peptide 1 (GLP1) and peptide YY (PYY) concentration were the dependent variables. df: degrees of freedom. significant effects in italic

Figure legends

Figure 1. Schematic overview of the study procedure for each visit. *HRV: heart rate variability. *During the milkshake drinking task, only the classical music was applied.*

Figure 2. Appetite-related sensations, including (A) hunger, (B) prospective food consumption, (C) satiety and (D) fullness, were not different between nutrient nor emotion conditions.

Figure 3. Emotional (A) valence and (B) arousal, and (C) dominance measured by self-assessment manikin increased in positive emotion induction. *The increase of the emotional states were independent of time.*

Figure 4. (A) Octanoylated ghrelin level decreased after positive emotion compared to neutral emotion at t=20-40min (*p_{Holm}<0.05), but there was no difference between fatty acid and placebo. (B) Cholecystokinin (CCK) increased after fatty acid infusion. (C) Glucagon-like peptide 1 (GLP1) level increased after fatty acid compared to placebo at t=30-40 min (*p_{Holm}<0.05). (D) Peptide YY (PYY) levels did not differ after fatty acid compared to placebo. *There was no nutrient-by-emotion interaction in any of the hormones.*

Figure 5. (A) Both positive emotion and fatty acid attenuated root mean square of successive differences (RMSSD) compared to control. However, there was no emotion-by-nutrient interaction. (B) Low frequency to high frequency (LF/HF) ratio decreased in positive emotion induction after placebo. The effect of positive emotion on LF/HF ratio was reversed after fatty acid.