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Jonathan C. Kershaw
Purdue University

Cordelia Running
Purdue University, crunning@purdue.edu

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Conditioning of human salivary flow using a visual cue for sour candy

Jonathan Kershaw[a]
Cordelia A Running[a]*

[a]Department of Nutrition Science and Department of Food Science, 700 W State St, Purdue University, West Lafayette IN USA

*Corresponding author: crunning@purdue.edu

Abstract

Objective: Although the “mouthwatering” to sight, smell, or thought of food is commonly accepted in food and nutrition research, the concept of mouthwatering and human salivary flow conditioning is not well accepted in salivary research. The objective of this study was to revisit whether human salivary flow could be classically conditioned to a previously neutral stimulus.

Design: Sour candy or a non-food control in opaque containers were presented to healthy participants (n=8). Simple images were consistently paired with container contents. Participants viewed the images for 15 seconds, then opened the containers and ate (candy) or did not eat (non-food control) the contents. This was repeated 14 times (7 of each stimulus). Order was semi-randomized to ensure one candy and one non-food were presented as the first two and last two stimuli. Saliva was collected with cotton dental rolls during these presentations (first two and last two) after viewing the image for 15 seconds, but before opening the container.

Results: Participants were successfully conditioned to increase salivary flow in response to the image that predicted candy, as demonstrated by greater weight of saliva in response to 1) the candy-paired image than the non-food-paired image, and 2) the candy-paired image at the end of the first visit compared with the beginning (when the image had no meaning). However, the effect was attenuated during the second visit.

Conclusions: We demonstrate classical conditioning of human salivary flow is achievable, but the effect may not persist to a second visit.

Keywords: Saliva, conditioning, sour taste

Introduction

Despite common use in lay-language, the phenomenon of “mouthwatering” in anticipation of food is contested in the scientific literature. Many in salivary research have argued that mouthwatering is not a sustainable event, at best being a very brief expression of saliva from the submandibular glands, or perhaps just an increase in human awareness of saliva that is already present in the mouth (Carpenter, 2013; Kerr, 1961). Food and nutrition research, however, maintains that mouthwatering is an inherent part of the cephalic phase response: the collection of early physiological events that prepare the oro-gastrointestinal tract for incoming food (Mattes, 2000). Thus, while salivary research contains minimal investigation of mouthwatering in recent years, food and nutrition research continues to use anticipatory or trained saliva to monitor associated responses to food, including hunger (Wooley & Wooley, 1973), desire to eat (Jansen, Stegerman, Roefs, Nederkoorn, & Havermans, 2010; Nederkoorn, Smulders, & Jansen, 2000), dietary restraint (Brunstrom, Yates, & Witcomb, 2004; Ferriday & Brunstrom, 2010; Nederkoorn & Jansen, 2002), and hedonic appeal (Proserpio, de Graaf, Laureati, Pagliarini, & Boesveldt, 2017; Ramaekers, Boesveldt, Lakemond, van Boekel, & Luning, 2013; Rogers & Hill, 1989). Reviews on the subject specific to this field can be consulted for the breadth of information available (Keesman, Aarts, Vermeent, Häfner, & Papies, 2016; Mattes, 2000; Wooley & Wooley, 1981).

This disconnect between the fields has become a particular challenge for our laboratory, which focuses on the intersections of psychology of eating, flavor sensation, and salivary biochemistry. As a consequence, we are revisiting the concept of mouthwatering in anticipation to food. In particular, we are focusing on whether salivary flow can be classically conditioned in humans. In classical conditioning, a previously neutral stimulus (e.g. a bell, the conditioned stimulus) is repeatedly associated with an unconditioned stimulus (e.g. eating food) to produce the response (e.g. salivary flow) (Pavlov, 1910). Over time, the previously neutral stimulus will cause the response to occur even in the absence of the unconditioned stimulus. If humans do indeed mouthwater in anticipation of food, then theoretically this process is trained through learning how sight or smell predicts the in-mouth sensations of food. This process is a naturally occurring classical conditioning process—the brain learns that the other sensory cues of a food predict the saliva-stimulating sensations that will occur in the mouth.

The question of whether or not humans can be classically conditioned to salivate has been asked before, with mixed results. Some data indicate conditioning is not possible in humans (Brown, 1970; Brown & Katz, 1967; Kerr, 1961; Lashley, 1916), while others show that type of stimulus, time periods between exposures, method and source of saliva collection, and other factors can vastly change the success or failure of a salivary conditioning experiment in humans (Blumberger & Glatzel, 1968; Holland & Matthews, 1970; Ilangakoon & Carpenter, 2011; White, 1978). Type of stimulus is particularly relevant to consider when comparing the literature, as both food-related, (images of food, actual food, observing others eat, etc.; used to represent previously conditioned stimuli) and non-food-related stimuli (buzzers, lights, etc.; used to study the acquisition of conditioning) have been used (Blumberger & Glatzel, 1968; Brothers & Warden, 1950; Holland & Matthews, 1970). Even when conditioning has been documented, the conditioned response can be weaker than the unconditioned response (Blumberger & Glatzel, 1968; Brothers & Warden, 1950).

Consequently, we are revisiting the concept of salivary conditioning in humans. While the concept is not particularly novel, the prevalence of two opposing views justify (indeed, they require) new data to determine whether or not this phenomenon occurs consistently in humans. We hypothesized that if we used a particularly strong salivary stimulus (sour taste), maintained an adequate time period between stimulations, and collected whole mouth saliva rather than isolating a single gland (as the equipment for collecting isolated saliva makes the experience less like normal eating), we would be able to achieve and document conditioning of salivary flow in humans. Notably, our experiment is not designed to test whether salivary glands are actively creating more saliva, but only to measure the amount of saliva that is actually expressed into the oral cavity, as that is the functional end point of interest in ingestive behavior research.

Materials & Methods

Participants between the ages of 18 and 45 were recruited from Purdue University's campus and surrounding area. Participants that had a history of taste or smell disorders; issues with too much or too little saliva; food allergies; tongue, lip, or cheek piercings; color blindness; or smoked within the past 30 days were excluded. Participants were asked whether or not they liked sour candy and how often they consumed sour candy. Written informed consent was obtained prior to beginning the study, and participants were compensated for their time. All recruiting and testing procedures were approved by the Purdue Institutional Review Board for Human Subjects Research. For all experiments, participants were instructed to drink a 500-mL bottle of water (Ice Mountain Spring Water, Nestle Waters NA) at least 1 hour prior to their appointments and to refrain from eating or drinking anything else during the hour prior to testing time. Participants were told that they would receive a series of 14 opaque cups with either two pieces of candy (sour variety, red, strawberry flavored Skittles®, Wrigley) or two pieces of a non-food control (referred to as "paper" hereafter, shown in figure 1). The "paper" was actually steel hexnuts, size 10-32, wrapped in light blue adhesive paper; these were used to aid in controlling for the sound and feel of the candies rattling in the cup when it was picked up. On the lids of each opaque container was taped one of two possible simple images (diamond or star, shown in figure 1). The images were consistently paired with either candy or paper for each participant. Participants were not explicitly told at the beginning of the experiment which image would be paired with which type of stimulus, but they were told that the image and contents pairing would be consistent. Cups were placed upside down on trays in front of the participant so they could not see the images before it was time to taste each sample. All participants completed two visits at least two days apart. Initial statistical power analysis indicated that 10 participants would be sufficient to detect an effect of conditioning on salivary flow; however, the study was stopped after 8 participants because every participant in the study showed the same pattern for the first visit, and additional testing of two more participants would not have changed the outcome. Further, analysis of the data collected indicated within-subject correlations for salivary flow were much higher than anticipated (0.93 observed, 0.75 used in power calculations).

An overview of the conditioning protocol is shown in figure 2. A total of 14 sample presentations was conducted for each participant. Half the cups contained candy and the other half paper. Sample order was semi-randomized, ensuring that samples 1 & 2 and 13 & 14 each included one candy and one paper sample. For each sample presentation, participants were instructed to swallow all saliva in his/her mouth, pick up the cup, look at the image on the lid and think about eating the contents for 15 seconds (timed by researcher). Participants were instructed and reminded not to swallow during the 15 seconds. For presentations when saliva was collected, the participant next placed two pre-weighed cotton dental rolls in the mouth and rolled them around to collect saliva (approximately 5 seconds). Participants had not seen the contents of the cup at this point, only the image on the lid. After removing the cotton dental rolls, participants removed the lid of the cup. If the cup contained candy, the participant ate the candy. The participant then rinsed with water, and a three-minute wait was imposed before repeating the process. The overall procedure is shown in figure 3.

Preliminary tests indicated that collecting saliva after every sample presentation led to mouth pain, likely because we had removed all the saliva that would buffer against the change in pH caused by the citric acid-coated candies. Because of this, we originally restricted saliva collection to samples 1 & 2, 7 & 8, and 13 & 14 (participants 1-3). Participants still noted some mouth discomfort, so we only collected saliva for samples 1, 2, 13, & 14 for participants 4-8. All data is available in the supplemental data. Participants were not told that saliva would only be collected at specific time points. Instead, they were told that we would collect saliva after some, but not all, samples.

All cotton dental rolls for saliva collection were weighed prior to use, and then again upon removal from the mouth. The initial weight of the rolls was subtracted from the final to calculate the mass of saliva generated. Saliva collection equipment (such as the Lashley cup, commonly used in salivary research) was intentionally avoided, as these methods present an artificial environment that may disrupt the natural eating experience. While simply spitting is commonly used to measure salivary "flow" in the nutrition and food science fields (Dsamou et al., 2012; Murugesh et al., 2015; Neyraud, Palicki, Schwartz, Nicklaus, & Feron, 2012; Silletti, Bult, & Stieger, 2012), we avoided this method as spitting could be altered by the subject willingness or motivation to expectorate (Running & Hayes, 2016).

Paired t-tests were used to compare saliva generated while viewing:

1) Candy image compared with paper image, visit 1, first viewing (samples 1 & 2). These points were not expected to be different, as the images meant nothing at the beginning of the test.
2) Candy image compared with paper image, last time in visit 1, first time in visit 2, and last time in visit 2. At all of these time points, we expected the candy image to stimulate more

saliva than the paper image. Respectively, the comparisons at these time points confirm whether or not conditioning was successful (last viewing visit 1); was maintained across days (first time, visit 2); and was maintained/reinforced through the end of the last visit (last time, visit 2).

3) First time compared with last time visit 1 and visit 2, for candy images. In visit 1, the last time was expected to generate more saliva than the first, if conditioning was successful. The test at visit 2 was simply to observe if people were re-conditioned, if loss of the effect was observed across days.
4) First time compared with last time visit 1 and visit 2, for paper images. These were not expected to be different, as the paper should not be training a salivary response (negative control).

Data were tested for normality using Shapiro Wilks tests. All paired datasets were normal except for the comparison of paper image to candy image at the start of visit 1 (Shapiro-Wilks $p = 0.006$). A Wilcoxon Signed Rank test was used in place of a paired t-test for this comparison. No saliva weights were directly compared across different testing days, as salivation varies from day to day and across time of day, and these were not controlled. All statistical analyses were conducted using SAS 9.4.

Results

Data on participants are shown in Table 1. Results for the paired t-tests (and Wilcoxon Signed Rank test) are shown in Table 2 and visualized in figure 4. Data indicate that increased salivary flow can be conditioned to a visual cue (more saliva for candy image at end of visit 1 compared with beginning, and more saliva for candy image compared with paper image at end of visit 1), but that the effect is not strongly maintained across days and within a second visit. Notably, one participant had the cotton dental rolls become stuck in the mouth at the first viewing of candy on visit 2 (dotted line in figure 4), which may have contributed to a higher value in that dataset. Removing that participant from the analysis results in all normally distributed data, and significant differences in visit 2 between the first and last viewing of the candy image in visit 2 (indicating that the conditioning may have restored on this visit, although it had extinguished during the time lapse from the first visit). However, there were still no significant differences between saliva generated when viewing the paper image compared with the candy image in visit 2.

Discussion

In this study, we provide evidence that human salivary flow can be classically conditioned to a previously neutral visual cue. Following conditioning, every participant in the first visit showed greater salivary flow when looking at an image they associated with sour candy compared with either the same image prior to conditioning or a different image associated with paper. On average, an additional 0.28 grams of saliva was collected over the 15 second interval, a quantity that is sufficient to be detected (Ilangakoon & Carpenter, 2011) and aid in swallowing (Lagerlöf & Dawes, 1984). The degree of response certainly varied across participants, but the direction is the same for all. However, the conditioned salivary response was not maintained by the beginning of the second day, and the strength of the conditioning appears lower in the second visit.

Previous researchers have demonstrated that the salivary response is influenced by cognitive factors (Brown, 1970; Running & Hayes, 2016). The role of psychic salivary stimulation, or the use of stimuli previously unassociated with the unconditioned response, was proposed by Pavlov (1910), and has since been supported by others (Brown, 1970; Brown & Katz, 1967;

Keesman et al., 2016; White, 1978). Additionally, mentally visualizing a food or its consumption may be important to elicit a salivary response (Keesman et al., 2016; White, 1978). In this experiment, the directions to imagine eating the contents of the cup, regardless if it contained candy or paper, may have contributed to successful conditioning. Notably, we did not ask participants in our study whether they were aware which image was linked to candy or paper by the end of the experiment, but it was quite apparent that participants were able to consciously learn the pairing. For example, while participants were required to look at the image and think about the contents every time they turned over the cup, by the end of the test when some participants opened the paper containers they would barely glance inside the cups, as they knew the contents were the paper samples. While we made sure all participants did confirm the contents for themselves, it was clear that participants knew which image was which by the end of the test (hence the reason we presented the cups upside down, to hide the images). Thus, participants were likely aware by the end of the experiment which images we expected to stimulate more salivary flow. However, this awareness of the conditioning may not be required for the effect to occur. Certainly, cognition contributes to salivary conditioning (Keesman et al., 2016), but participant awareness may not be required in all conditioning paradigms. Increased salivation has been demonstrated using an operant conditioning paradigm when participants were unaware of the reward cue (Brown & Katz, 1967), and a classically conditioned fear response has been observed independent of participant awareness (Schultz & Helmstetter, 2010).

Although others have suggested mouthwatering is an exhaustible event (Holland & Matthews, 1970; Ilangakoon & Carpenter, 2011), we observed an increase in salivary flow after repeated exposure to images associated with sour candy. While previously cited studies collected saliva at one-minute intervals (Holland & Matthews, 1970; Ilangakoon & Carpenter, 2011), we intentionally maintained a three-minute wait time between samples. Our data suggest that three minutes is sufficient for replenishment of saliva in this conditioning paradigm. Additionally, repeatedly directing the participants' focus to consuming the cup contents may also explain the observed absence of mouthwatering exhaustion, as cognitive factors like distraction can contribute to decreased salivary flow rates (Epstein, Rodefer, Wisniewski, & Caggiula, 1992).

Using actual foods and saliva collection methods that focus on keeping the consumption experience as normal as possible may be part of why our paradigm, at least during the first visit, successfully conditioned salivary flow. Earlier work in conditioning often employed stimuli and/or ingestion procedures incongruent with actual consumption experiences (Blumberger & Glatzel, 1968; Epstein et al., 1992; Holland & Matthews, 1970). Others have also suggested that the artificial laboratory setting may inhibit salivation (Drummond, 1995). Further, contextual framing influences expectoration behavior, supporting the importance of food vs. non-food expectations when conducting salivary research (Running & Hayes, 2016). Collection procedures may also alter saliva content. Pavlov (1927) noted a difference between food- and acid-stimulated saliva in dogs nearly a century ago. Others have observed a difference in amylase content depending on stimulated vs. unstimulated saliva (Brothers & Warden, 1950) or nature of the stimulus (Kemmer & Malfertheiner, 1985). As saliva flow into the mouth is considered a cephalic phase response to prepare the food and gastro-intestinal track for digestion (Mattes, 2000), the design of a protocol to best mimic the eating experience may be necessary. Such differences in design could account for the lack of observable conditioning in some prior work, if the context of the food and eating experience were violated.

We chose a sour food as the conditioning stimulus, as sour is the strongest taste stimulus for salivation; sour increases salivation even more than the hedonic aspects of the food (Dawes & Jenkins, 1964; Keesman et al., 2016; Watanabe & Dawes, 1988). The potency of an unconditioned stimulus to generate saliva has already been proposed as vital for successful conditioning (Blumberger & Glatzel, 1968). As stimuli may act as a cue to trigger previous experiences (Keesman et al., 2016; Mattes, 2000), differences in exposure to sour candy may partially explain between-subject variation, in addition to inherent biological variation among individuals and time since last meal (Horswill, Stofan, Horn, Eddy, & Murray, 2006; Humphrey & Williamson, 2001; Watanabe & Dawes, 1988). Differential responses to the sourness and hedonic appeal of the candy may also have contributed to the variation we observed, as both factors can increase salivary flow (Keesman et al., 2016; Rogers & Hill, 1989). Although we collected data on participant sour candy preferences, this study is not powered to determine if liking influenced the salivary response. Additional studies are needed to determine the contribution of hedonic appeal to conditioning of salivary flow, as the import of liking is still disputed (Mattes, 2000). However, it’s important to note that while the overall variation between subjects was large, the pattern of response to the images was consistent with a conditioning effect, at least during the first visit.

The conditioned response appears to have extinguished by visit 2 in our protocol, which could be explained by learning or habituation effects. As participants were aware that the same procedure would be repeated, cognitive factors likely influenced the response, especially as previous stimuli experiences can influence salivary flow rate (Mattes, 2000). Habituation, or a decreased response to a repeated stimulus, is another possible explanation of the discrepancy we observed between participant testing days, as others have also demonstrated greater habituation to a sour stimulus after repeated days of testing (Webb & McBurney, 1971). Further investigation is required to understand how the interaction of habituation and learning influence salivary conditioning across multiple days, and how these phenomena contribute to the anticipatory events during actual eating occasions. In addition, investigating if and how a conditioned response can be maintained is also merited, as the conditioning we observed in visit 1 did not persist across days. Potentially, the artificial environment of the laboratory and protocol could have diminished the persistence of the effect, but again, this requires further work.

Clearly, there are limitations to this work. Methods to measure salivary flow that do not interfere with the physical structures of the oral cavity and the cognitive experience of eating will inherently have experimental error in the measurements. We selected the dental rolls as the best available option due to fundamental concerns about other saliva collection techniques and the psychology of the conditioning process. We had participants roll the dentals rolls around the mouth in order to collect as much saliva as possible, however incomplete absorption of saliva to these rolls would contribute some variability. Nonetheless, the added weight of the saliva in the dental rolls will correlate with the amount of saliva in the mouth, as individuals who have more saliva will have more available for the cotton to absorb. Studies measuring flow rates using both passive drool and absorbent materials indicate similar quantities of saliva may be collected from both methods, with perhaps higher amounts collected with the absorbent materials (Beltzer et al 2010; Navazesh & Christensen 1982). Although ceiling effects may be a concern when using absorbent materials (Beltzer et al 2010), this limitation is very unlikely in our current study, as the collection period was very brief and total volume collected was not enough to overwhelm the absorbent capacity of the cotton dental rolls. Some work also notes a slightly worse test-re-test reliability of absorbent materials compared to drooling, expectorating, or suction (Navazesh & Christensen 1982), but no actual statistical analysis of differences in reliability has been conducted. Passive drool and expectoration are the most common techniques for measuring salivary flow rates, but given the documented potential influence of personality and cognition on expectorated saliva (Running & Hayes 2016), we selected cotton rolls as a more reliable measure. Clearly, all methods of salivary flow measurement have limitations. We would not recommend using any of the individual values of salivary flow in this study as diagnostic or definitive evidence of a certain rate of flow. Rather, the utility of these measurements is in the comparison, within a subject, from one time point to the next. By evaluating the results within subject, we reduce much of the inherent variability introduced by the saliva collection method. Certainly, error remains, but the purpose of the statistical analysis is to observe if the effect is greater than what would be expected due to error. In the current study, the paired analysis minimizes the between subject effects (which are large, as evidenced by the spread of saliva weights in Figure 4), and allows us to focus on what occurred within each subject. Considering the high correlation of values within-subject (0.93 in our current analysis, when looking at first to last views within a subject across all visits and sample types), we were still able to observe the effect of conditioning in visit 1 despite the noise (error) of the measurements.

Conclusions

The experiments in this study demonstrate that in an acute setting, human salivary flow can be conditioned to a previously neutral visual stimulus. However, the effect was not maintained across days under this conditioning paradigm.

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Conflicts of interest: None.

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Figures

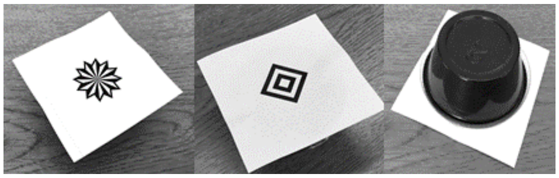


Figure 1: Images on lids and appearance of cups as seen by participants

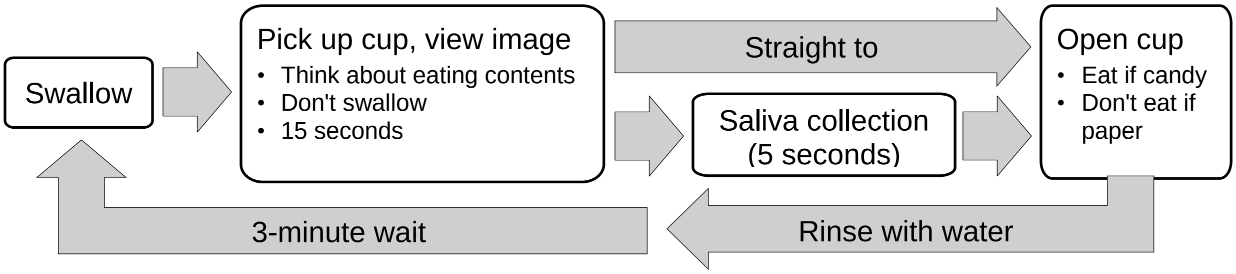
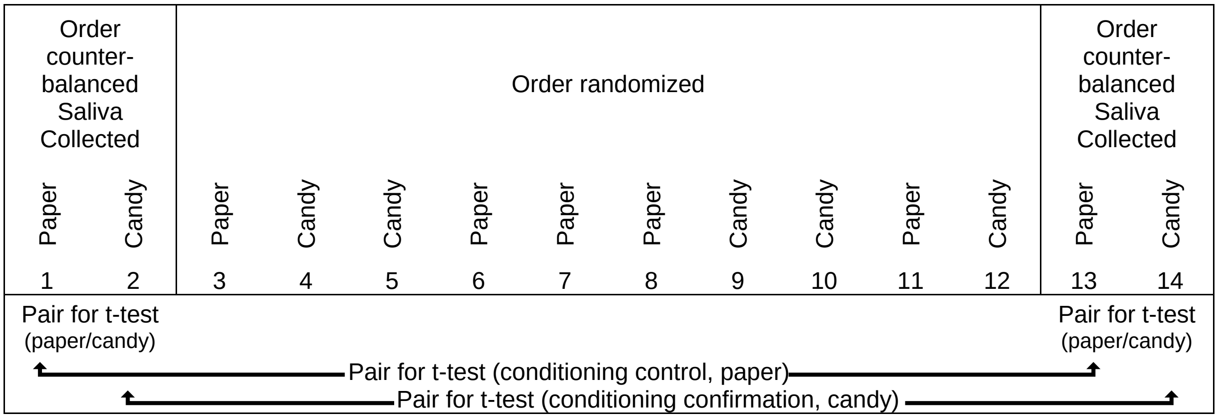


Figure 2: General protocol for each sample presentation



Order counter-balanced Saliva Collected		Order randomized										Order counter-balanced Saliva Collected	
Paper	Candy	Paper	Candy	Candy	Paper	Paper	Paper	Candy	Candy	Paper	Candy	Paper	Candy
1	2	3	4	5	6	7	8	9	10	11	12	13	14

Pair for t-test (paper/candy)

Pair for t-test (paper/candy)

Pair for t-test (conditioning control, paper)

Pair for t-test (conditioning confirmation, candy)

Figure 3: Design and planned t-tests for each visit; order of samples is an example, as the actual orders were counterbalanced and randomized as noted.

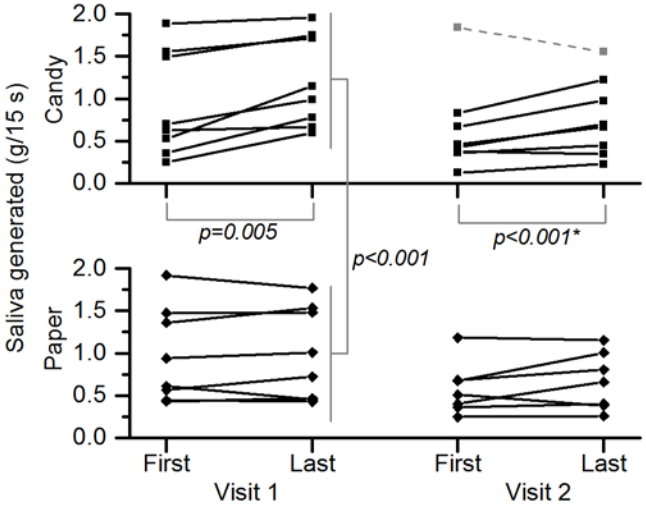


Figure 4: Saliva generated after looking at the images predicting candy or paper for 15 seconds. Each line is an individual participant. Grey dashed line is the participant whose dental rolls became stuck in the mouth while collecting saliva after the first view of candy on the Visit 2, and **p*-value does not include this participant.

Tables

Table 1: Participant characteristics

Gender (Counts)	4 Male 4 Female
Age (Range)	23 – 32
Stated liking for sour candy (in general; counts)	1 – Dislike 4 – Like 3 – No preference
Reported frequency of eating sour candy (in general; counts)	2 – Avoid sour candy 4 – Less than once per month 2 – About twice per month

Table 2: Differences in weights of saliva in grams, and statistical results

Comparison	***Mean Difference ± SD***	***p-value (t, DF)***
Visit 1: Candy image, Last – First view	**0.276 ± 0.193 g**	**0.005 (4.049, 7)**
Visit 1: Paper image, Last – First view	0.015 ± 0.123 g	0.738 (0.348, 7)
Visit 2: Candy image, Last – First view	0.130 ± 0.218 g	0.135 (1.69, 7)
Removing participant with error*	**_0.223 ± 0.062*_**	**_<0.0001 (9.51, 6)*_**
Visit 2: Paper image, Last – First view	0.099 ± 0.153 g	0.112 (1.82, 7)
Visit 1: First view, Candy image – Paper image	-0.043 ± 0.191 g	0.543 (-0.640, 7)
Visit 1: Last view, Candy image – Paper image	**0.217 ± 0.059 g**	**<0.0001 (10.4, 7)**
Visit 2: First view, Candy image– Paper image	0.012 (-0.044, 0.110)[†]	0.641 (4, 7)[†]
Removing participant with error*	_0.005 ± 0.090*_	_0.899 (0.133, 6)*_
Visit 2: Last view, Candy image – Paper image	0.118 ± 0.228 g	0.187 (1.46, 7)

Differences significant at α = 0.05 are bolded.

t: t-statistic from paired t-test; DF: Degrees of freedom

*One participant had dental rolls get stuck in the mouth when removing after viewing the candy image. Removing this participant results in the second line of results.

[†] Data not normally distributed, so median and semi-interquartile range are shown, with *p*-value from Wilcoxon Signed Rank test p-value and sign rank statistic with degrees of freedom.