Published in final edited form as:

Learn Motiv. 2018 February ; 61: 85–96. doi:10.1016/j.lmot.2017.07.001.

Examining the influence of CS duration and US density on cue-potentiated feeding through analyses of licking microstructure

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Abstract

In the current study, groups of mice were trained with either short (20 s) or long (120 s) conditioned stimulus (CS) durations associated with different rates of sucrose unconditioned stimulus (US) delivery, to examine whether different behavioral forms of cue-potentiated feeding in sated mice would be evoked. In training mice received presentations of an auditory CS for 20 s during which a sucrose US was delivered at a density of 1/9 s (Group-20-s). A second group of mice received an auditory CS for 120 s and a US density of 1/49 s (Group-120-s). During training, a shorter CS duration and higher rate of US delivery resulted in greater acquisition of food cup responding, and during the test stage Group-20-s mice also displayed higher CS evoked lick rates, though all mice showed cue-potentiated feeding. An analysis of licking microstructure also revealed that Group-120-s mice displayed CS evoked licking behavior that reflected an increase in the perceived palatability of the sucrose US. These findings are discussed with respect to the influence of CS interval and US density on associatively activated sensory and affective representations of a US, and contrast mediated effects resulting from presentation of excitatory and inhibitory conditioned stimuli.

1. Introduction

Associative learning studies examine the nature of representations that underlie the formation of associations between conditioned stimuli (CSs) and unconditioned stimuli (USs). It is acknowledged that USs can contain multiple complex events that can be readily distinguished from one another, such as the sensory (e.g., taste and smell) and affective features (e.g., the intrinsic affective value obtained from consuming an appetitive outcome) (e.g., Delamater & Oakeshott, 2007; Hall, 2001; Konorski, 1967; Wagner and Brandon, 1989). Indeed, numerous studies (Betts, Brandon & Wagner, 1996; Corbit & Balleine, 2005; Dickinson & Balleine, 2001; Holland, 2004; Lennartz & Weinberger, 1992; Saddoris, Gallagher & Holland, 2005) and theories of learning (Konorski, 1967; Wagner & Brandon, 1989) suggest that CSs are capable of entering into these distinct associations with the US.

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Furthermore, CSs paired with food can later potentiate consumption of that food, as shown in cue-potentiated feeding (CPF) studies (Johnson, 2013; Weingarten, 1983; Zamble, 1973). In CPF, food-deprived rodents are trained to associate a conditioned stimulus (CS) with the delivery of an appetitive reinforcer, such as a sucrose solution or pellet (Dailey, Moran, Holland & Johnson, 2016; Holland & Gallagher, 2003; Galarce, McDannald, & Holland, 2010). Consumption of the reinforcer is subsequently potentiated by presenting the CS while the animal is food sated, an effect that is thought to reflect the acquisition of value by the CS appropriate to the unconditioned stimulus (US), leading to eating of it in a sated state. This may occur by enhancing incentive motivation (Holland, Hatfield & Gallagher, 2001) and/or through more detailed effects on the specific properties of the US (Galarce, Crombag & Holland, 2007; Johnson, 2013; Sherwood, Holland, Adamantidis & Johnson, 2015). However, the mechanisms underlying the expression of CPF and its effects over the sensory and affective features of reinforcement have not been well characterized.

Konorski (1967) distinguished between learning about detailed specific sensory features relative to more general properties of reinforcement, with each being associated with their own dissociable consummatory and preparatory responses, respectively. According to this account, given that sensory experiences typically occur over short durations (e.g., taste), any CSs that are present would be expected to enter into these associations within these shorter timeframes. By comparison, temporally more persistent motivational drive states that occur over longer time frames, such as the slow-temporal kinetics that mediate hunger would therefore more readily enter into associations with longer duration intervals (Konorski, 1967). Delamater and Holland (2008) devised a number of experiments to examine the effects of CS-US interval and US density on learning about these putative dissociable event representations. However, in contrast to Konorski's speculations, CSs trained with shorter duration CS-US intervals (or with an increased density of US occurrence within the CS) failed to elicit conditions that should have favored the display of sensory-specific responses. In Delamater and Holland's (2008) study (Experiment 4), rats were trained to associate two different CSs with different appetitive reinforcers (i.e., CS1 – US1; CS2 – US2). Rats were subsequently tested for CPF—those trained with a CS-US interval of 20 s displayed reinforcer-selective CPF, such that intake rates above periods when no stimuli were presented occurred only when the presented CS was congruent with the available US. On the other hand, rats trained with a CS-US interval of 2 s failed to show any evidence for sensory-specific CPF (Delamater & Holland, 2008).

As acknowledged by Delamater and Holland, it is possible that the failure to observe specific sensory effects consistent with Konorski's (1967) account may have reflected the response measures used in their study (e.g., overall intake of the US), which may have lacked the fidelity to uncover sensory-specific associations. The analysis of licking microstructure may be profitable in this regard as it allows the opportunity to distinguish between patterns of intake that are driven by sensory-evaluative and/or motivational variables. Thus, as rodents engage in consumption of a fluid they display stereotyped rhythmic tongue movements (Stellar & Hill, 1952) where the majority of interlick intervals (ILIs) fall <250 ms and reflect continuous licking bursts under the control of a putative central pattern generator (Davis & Smith, 1992; Wiesenfeld et al., 1977). Although overall intake is typically used to study the degree of acceptance or rejection of a consumed solution, this

measure is the aggregate of numerous behavioral variables, which include the evaluation of taste stimuli, and the post-ingestive motivational factors that result from the accumulation (or absence) of nutrients in the gastrointestinal (GI) tract (Davis & Levine, 1977). It is possible to distinguish between evaluative and motivational variables controlling intake behavior by examining the temporal distribution of pauses in an otherwise steady stream of licking (Johnson, 2017). Thus, the average number of licks (burst size) that occur within each burst of licking reflects a measure of evaluation of the consumed solution (Smith & Davis, 1992; Spector et al., 1998). This measure displays a monotonic relationship with increases in the concentration of a palatable sucrose solution (Smith & Davis, 1992; Spector et al., 1998), whereas the introduction of bitterness through quinine adulteration leads to a monotonic decrease in this measure as the sucrose-quinine solution becomes less palatable (Hsiao & Fan, 1993). It is important to note that burst size does not simply reflect the overall intake of a solution, which will typically display an inverted-U-shaped function, where maximum consumption is observed with intermediate (rather than the highest) concentrations of palatable tastants (Stellar, 1960). Moreover, when fluid is prevented from reaching the GI tract via an esophageal fistula (i.e., sham feeding; Hull, Livingston, Rouse, & Barker, 1951), the average number of licks that occur within each burst is unchanged relative to normal real feeding conditions (Davis & Smith, 1992). By contrast, the number of bursts after each pause interval is significantly elevated in sham feeding conditions (Davis & Smith, 1992; Davis, Smith, Singh, & McCann, 2000) and likely reflects the heightened motivational status of the animal due to the elimination of post-ingestive inhibition (Smith, 2001). In addition, burst number has been shown to display a non-monotonic relationship with the concentration of a palatable tastant (Davis & Smith, 1992). More recently, this measure has also been suggested to underlie a reward-oriented response (D'Aquila, 2010; D'Aquila, Rossi Rizzi & Galistu, 2011). Within this account, each time a rodent contacts the reward, the evaluation of it updates and leads to a subsequent reattribution of incentive motivational properties to exogenous reward-related stimuli, which can be tracked by burst number (D'Aquila, 2010). While the merits of these accounts will be described in the general discussion, the precise and quantitative recording of individual licks offers insights into physiologically and behaviorally distinct measures that may reflect different features of US-elicited responses, and thus be more readily observed when CSs that evoke distinct representations are presented. To date, a number of associative learning studies have implemented licking microstructure analyses to examine alterations in associatively activated event representations (Austen et al., 2016; Baird, John & Nguyen, 2008; Dwyer, 2008; Dwyer et al., 2009; Lin, Arthurs, Amodeo & Reilly, 2012; Monk et al., 2014; Myers & Sclafani, 2001a). The experiment reported below provides further evidence of the strength in using these analyses to determine the complex events underlying associations between CSs and event representations.

In the current study, groups of mice were trained with either short (20 s) or long (120 s) CS durations associated with different rates of US delivery and followed by tests of CPF (Zamble, 1973; Weingarten, 1983; Johnson, 2013). An analysis of licking microstructure was used to distinguish between feeding behavior based on the evaluation of reward or the motivation to initiate contact with it. Specifically, in this study the duration of the CS and the rate of US occurrence were manipulated (Delameter & Holland, 2008; Gibbon & Balsam,

1981; Leenartz & Weinberger, 1992). If the shorter duration 20 s CS associated with a higher rate of US occurrence (1/9 s) is critical to differentially encoding highly specific than more general components of reinforcement, at test we might expect that this CS should more readily reflect licking behavior that is related to an increase in the evaluative taste properties of the US (i.e., sensory encoding). By comparison, a longer duration 120 s CS that is associated with a sparser rate of US occurrence (1/49 s) should in training more readily enter into associations with the general features of reinforcement. Thus, at test feeding should be dependent on more preparatory features of behavior that would likely facilitate the initiation of meal intake—with licking microstructure this may be reflected by increases in the number of bursts initiated.

2. Materials and Methods

2.1 Subjects

Male C57Bl/6J mice (n=32) were obtained from Jackson Labs (Bar Harbor, ME) at 12 weeks of age and housed in cages under a 12 h light/dark cycle (lights on at 07:00–19:00 h). One mouse was euthanized shortly after being received due to injuries resulting from littermate aggression. Following one week of acclimatization and handling, mice were food deprived to 90% of their free feeding weight by limiting access to a single meal, which the mice typically received after the completion of behavioral training and testing. Behavior took place between the hours of 9AM and 1PM each day for consecutive days until the study was complete. Animal procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and under the auspices of the Michigan State University Animal Care and Use Committee.

2.2 Apparatus

Behavioral testing took place in four individual conditioning chambers (24 × 20 × 18 cm, LWH) with aluminum front and back walls, clear polycarbonate sides and a floor made of stainless steel rods spaced 0.5 cm apart (Med Associates, St Albans, VT, USA). Each conditioning chamber contained a food cup on the right-side and within it a food well, which could house a maximum of 50 µl of liquid and was delivered by a syringe pump that was assigned for each chamber and located on a table adjacent to the conditioning chambers. A vacuum was attached to one of the lines, and was connected to the food well. When active the vacuum could exhaust away any fluid from the food well. Fiber optics were used to introduce a light beam that crossed the diameter of the food well. Licks were recorded upon breaking the light beam that passed through the fluid bolus (Johnson et al., 2010). Stimuli were delivered from a speaker mounted in the center of the rear wall. The stimuli were a white noise and 1500 Hz tone, each set at approximately 10 dB above background noise (≈65 dB). A magazine clicker was positioned on the outside of the conditioning chamber, directly above the food cup. A camera was positioned on the outside wall opposite the entrance of the food cup, which allowed for recording of mouse behavior in the food cup and confirming the availability of the sucrose US in the food well. A red house-light outside of the chamber was lit when the test chambers were in use. Testing took place in a dark room with only a red light on. An IBM-compatible computer equipped with Med-PC software (Med Associates) controlled and recorded all stimuli and responses, while a second

computer recorded digital video images (AVerMedia, Fremont, CA) obtained from the food cup.

2.3 Procedure

Mice were run in two separate cohorts and once mice in each cohort reached their target weight, the following day each mouse was assigned a conditioning chamber and remained in that chamber for the duration of each training and testing session. Mice first received single daily magazine training sessions for two days, where a 10% sucrose (w/v) reward was immediately available in the food well. Upon entering the food magazine the mouse was free to consume its reward, initiating the first of 16 trials. During each trial, 50 μ l of sucrose was made available and was contemporaneously delivered with the presentation of the magazine clicker. Following 10 s, any remaining fluid was exhausted out of the food well by brief (0.1 s) activation of the vacuum. The intertrial interval (ITI) between reward deliveries varied randomly on a random time 120 s schedule with a full session of 16 trials taking approximately 30–45 min. At the conclusion of the second day of the magazine training, successful food cup training was determined by confirming that each mouse spent a minimum of 10 s in the food well while the sucrose US was available.

Following food cup training, mice were equally divided into two groups: Group-20-s and Group-120-s prior (based on comparable food cup training) and received 10 Pavlovian conditioning sessions, each lasting approximately 40 min with one session occurring each day. Each session contained 5 tone CS and 5 noise CS presentations, separated by a variable ITI of 90 s. For half the mice in each group, sucrose was delivered during tone presentations but not white noise, whereas for the remaining mice in each group the stimulus-outcome contingencies were reversed. For mice in Group-20-s, presentation of either the tone or noise was divided into four 5 s epochs (Figure 1a). For the reinforced CS+ trials, during each trial 50 μ l of a 10% sucrose reward was delivered twice during two of the four epochs, with the constraint that by the end of each session, for the first 5 s epoch sucrose delivery could occur on a maximum of two out of the ten trials (Table 1). Thus, CS+ responses from the three trials where sucrose was not delivered during the first 5 s epoch were used as the measure of conditioning. The reward schedule for the specific delivery of sucrose during each trial was changed every two to three days. Thus, in Group-20-s, across training mice received a mean US density of 1/9 s (Table 1). Any reward not consumed was removed at the end of the trial via the vacuum. During a CS– trial, no reward was delivered. For mice in Group-120-s, presentation of the stimuli was also divided into four epochs, however these epochs were 30 s in length (Figure 1b). For the reinforced CS+ trials, 50 μ l of a 10% sucrose reward was delivered at the beginning of two of the four epochs. A similar reward delivery constraint was adopted to that described for Group-20-s mice, with CS+ responses during the first 5 s of epoch 1 on trials when sucrose was not available serving as the measure of conditioning. However, for Group-120-s mice, the mean US density across training was 1/49 s (Table 1). For data presentation, CS– responses during the first 5 s of epoch 1, from three of the five trials (chosen at random) were used.

On completion of the Pavlovian conditioning sessions, subjects were given 2 days (3 nights) of *ad-libitum* access to their standard laboratory diet, with the goal of restoring subjects to at

least 100% of their original baseline weight. Mice were then tested for CPF with the CS+ and CS−, with each cue tested individually on separate days, and the order of tests fully counterbalanced between groups of mice. The two potentiated feeding test sessions examined the effects of each stimulus on consumption: the sucrose reward was available for consumption at all times for both tests. At the start of the session, 50 µl of sucrose was available in the food well and additional 25 µl deliveries occurred every 20 licks as mice consumed the liquid. This ensured that sucrose reward was consistently available to the mice and the amount consumed was entirely dependent on the number of licks each mouse made. To confirm that the food well was consistently replete with sucrose, the experimenter monitored the video display throughout each test session. For mice in Group-20-s, the test session began with a 2 min baseline period, which was followed by 2 min ITI followed by one of 4 test trials during which the tone or noise stimulus was presented for 20 s (Figure 1b). Each subsequent trial was followed by a fixed 2 min ITI. Mice in Group-120-s received an identical test session with one exception that during the 4 test trials the tone or noise was presented for 120 s (Figure 1b).

2.4 Data analysis

For training, the percent time in the food cup data were analyzed using a mixed three-way ANOVA with a between subject variable of group (Group-20-s, Group-120-s), and within-subject variables of cue (CS+, CS−) and session (1–10). Separate group × session ANOVA's for each cue were implemented, which were designed to examine differences in CS responding between the groups, as were separate cue × session ANOVA's designed to examine within-group responding for each cue across the training sessions. For the test stage licking data, group × test (CS+, CS−) × period (CS, ITI) ANOVA's were implemented. In addition, separate group × period (CS, ITI) ANOVA's for each CS test were conducted to determine the effects of each separate stimulus on sucrose consumption between the groups. A test (CS+, CS−) × period (CS, ITI) ANOVA for each group was also implemented in order to examine whether differences in the pattern of licking would be observed within the two groups of mice. To examine the nature of significant interactions, tests of simple main effects were employed. For the analysis of licking microstructure, the average number of licks occurring prior to a pause >1s were calculated for the CS and ITI periods for each test, as were the number of bursts occurring after each 1 s pause interval (Spector et al., 1998). Finally, the licking data in the initial 2 min period prior to the presentation of CSs were analyzed by group × test ANOVA's.

3. Results

3.1 Training

During the test stage, three mice from Group-20-s failed to show any licking for sucrose during either the CS+ or CS−, and were thus excluded from the study. For the remaining mice, Pavlovian acquisition revealed that the percent of time in the food cup was greater as the sessions progressed for mice in Group-20-s relative to Group-120-s mice, whereas both groups of mice showed comparable negligible responding to the CS− (Figure 2). A mixed three-way group × cue × session ANOVA revealed a main effect of group ($F(1,26) = 228.35$, $p < 0.001$), cue ($F(1,26) = 1427.52$, $p < 0.001$), and session ($F(9,234) = 33.05$, $p < 0.001$). In

addition, significant interactions were revealed between cue and group ($F(1,26) = 419.13$, $p < 0.001$), session and group ($F(9,234) = 27.25$, $p < 0.001$), cue and session ($F(9,234) = 31.84$, $p < 0.001$), and a significant three-way interaction ($F(9,234) = 23.49$, $p < 0.001$). To examine the nature of this latter interaction, subsequent cue $\times$ session ANOVA's for each group revealed for both Group-20-s and Group-120-s mice that CS+ percent time responding differed significantly from CS- for all sessions (smallest F-value; Group-20-s session 1, $F(1,11) = 8.21$, $p = 0.02$). Separate session $\times$ group ANOVA's for each cue also revealed for CS+ a significant main effect of group ($F(1,26) = 327.57$, $p < 0.001$), session ($F(9,234) = 40.81$, $p < 0.001$) and a significant interaction between the two variables ($F(9,234) = 31.74$, $p < 0.001$), with individual contrasts for each session revealing significant group differences in conditioned responding from session 2 onwards (smallest F-value, session 2, $F(1,26) = 31.01$, $p < 0.001$). The same analysis conducted for CS- responding revealed no effect of group, session or interaction (F 's < 1 ; p 's > 0.44), with no differences between the groups in responding during any session (Largest F-value; session 5, $F(1,26) = 3.40$, $p = 0.08$). Thus, all mice readily acquired the Pavlovian discrimination, though food cup responding was significantly greater for the shorter duration CS associated with higher density US exposure.

3.2 Test: Food cup responding

During the test stage, Group-20-s mice showing the highest amount of responding during the CS+ (percent time in food cup $\pm$ SEM; Group-20-s 22.21 ± 2.78 ; Group-120-s 6.68 ± 0.57) compared to the CS- (Group-20-s 5.3 ± 1.32 ; Group-120-s 4.13 ± 0.62). Group $\times$ test ANOVA revealed a main effect of group ($F(1,26) = 28.42$, $p < 0.001$), test ($F(1,26) = 60.82$, $p < 0.001$) and a significant interaction between the two variables ($F(1,26) = 33.11$, $p < 0.001$). Tests of simple main effects revealed significantly greater food cup responding in Group-20-s relative to Group-120-s mice during the CS+ ($F(1,26) = 38.93$, $p < 0.001$) but not CS- ($F < 1$). In addition, Group-20-s ($F(1,26) = 80.35$, $p < 0.001$) but not Group-120-s mice ($F(1,26) = 2.43$, $p = 0.13$) displayed greater food cup responding during CS+ relative to CS-.

3.3 Test: Lick/min data

The data of primary interest are shown in Figure 3. The mean lick rate during the CPF test (Figure 3a) for each CS and the respective ITI revealed that lick rate was highest for Group-20-s mice during the CS+ relative to the CS-, ITI, and compared to Group-120-s mice. A group $\times$ test $\times$ period ANOVA on the lick rate data revealed a main effect of group ($F(1,26) = 11.54$, $p < 0.01$), test ($F(1,26) = 10.57$, $p < 0.01$), and period ($F(1,26) = 16.76$, $p < 0.001$). In addition, significant test $\times$ group ($F(1,26) = 12.18$, $p < 0.01$), period $\times$ group ($F(1,26) = 16.76$, $p < 0.001$), test $\times$ period ($F(1,26) = 43.29$, $p < 0.001$) and a test $\times$ period $\times$ group interaction ($F(1,26) = 12.22$, $p < 0.01$) was revealed. For the CS+ test, a separate group $\times$ period ANOVA revealed a main effect of group ($F(1,26) = 16.45$, $p < 0.001$), period ($F(1,26) = 31.33$, $p < 0.001$) and a significant interaction between the two variables ($F(1,26) = 13.32$, $p < 0.01$). To examine the nature of the significant interaction, tests of simple main effects revealed significant elevation in lick rate for Group-20-s compared to Group-120-s mice during the CS+ ($F(1,26) = 15.97$, $p < 0.001$) but not ITI ($F(1,26) = 1.30$, $p = 0.26$). A similar analysis adopted for the CS- data revealed a significant main effect of test only ($F(1,26) = 4.69$, $p = 0.03$).

Separate test $\times$ period ANOVA's for each group revealed for Group-20-s mice a significant main effect of test ($F(1,11) = 12.71, p < 0.001$), period ($F(1,11) = 11.38, p < 0.01$) and interaction between the two variables ($F(1,11) = 23.62, p < 0.001$). Tests of simple main effects revealed significant elevation in lick rate during the CS+ relative to CS- ($F(1,11) = 19.00, p < 0.01$). In addition, lick rate was elevated during the CS+ compared to the ITI ($F(1,11) = 17.19, p = 0.001$), whereas it was comparable for the CS- relative to its corresponding ITI period ($F(1,11) = 1.07, p = 0.32$). Finally, the analysis also revealed that the lick rate during ITI for the CS- test was significantly elevated compared to the ITI period for the CS+ test ($F(1,11) = 7.92, p = 0.01$). For Group-120-s mice, no main effect of test ($F < 1$), period ($F(1,15) = 1.88, p = 0.18$), but a significant test $\times$ period interaction was noted ($F(1,15) = 15.66, p = 0.001$). This interaction reflected a significant elevation in lick rate during the CS+ relative to the CS- ($F(1,15) = 8.12, p = 0.01$). In addition, lick rate was elevated during the CS+ test compared to the matched ITI period ($F(1,15) = 16.04, p = 0.001$), whereas for the CS- test there was a trend for elevated lick rate during the ITI compared to the CS period ($F(1,15) = 4.47, p = 0.05$). Finally, there was also a trend for elevated lick rate for the ITI during the CS- relative to the ITI for the CS+ test ($F(1,15) = 4.43, p = 0.05$).

To provide some insight into whether differences in the sampling duration of the CSs might account for differences in lick rate between the groups, the rate of licking during the initial 20 s segment during each CS presentation for Group-120-s was examined. The lick rate during this sampling period for the CS+ was 30.18 ± 4.97 , and for the CS- was 9.42 ± 2.86 . Thus, this revealed a similar pattern of lick responding to that seen when the total CS duration was examined. ANOVA revealed for Group-120-s compared to Group-20-s mice a reduced lick rate during the CS+ ($F(1,26) = 9.99, p < 0.01$) but not CS- ($F(1,26) = 4.48, p = 0.09$). In addition, lick rate was significantly greater during the CS+ compared to the CS- in Group-120-s when the initial 20 s period was analyzed ($F(1,15) = 15.35, p = 0.001$).

3.4 Test: Burst size

To characterize differences in licking behavior resulting from presentation of CSs that differed in CS duration and US density, mean burst size was examined (Figure 3b). A group $\times$ test $\times$ period ANOVA analysis of these data revealed a significant main effect of group ($F(1,26) = 4.42, p < 0.05$), and a significant test $\times$ period ($F(1,26) = 43.28, p < 0.001$) interaction. A separate group $\times$ period ANOVA for the CS+ test revealed a significant group $\times$ period interaction ($F(1,26) = 4.47, p < 0.05$) due to a significant elevation in burst size during the CS+ relative to the ITI for Group-120-s ($F(1,26) = 9.76, p < 0.01$) but not Group-20-s mice ($F < 1$). In addition, burst size was significantly larger for Group-120-s mice during the CS+ relative to Group-20-s mice ($F(1,26) = 5.53, p < 0.05$), though the mean burst size was comparable when their respective ITI periods were compared ($F < 1$). A similar analysis adopted for the CS- data revealed a main effect of group ($F(1,26) = 4.52, p < 0.05$), period ($F(1,26) = 10.60, p < 0.01$), but no significant interaction ($F < 1$). A test $\times$ period ANOVA to examine burst size responding for Group-20-s mice revealed a main effect of period ($F(1,11) = 8.83, p = 0.01$), and a tendency for a test $\times$ period interaction ($F(1,11) = 3.33, p = 0.08$) due to an elevation in burst size during the CS+ compared to the CS- ($F(1,11) = 4.99, p < 0.05$), but no differences between the corresponding ITI periods ($F < 1$). Furthermore, a significant reduction in burst size occurred during the CS- relative to its

corresponding ITI ($F(1,11) = 10.08$, $p < 0.01$), whereas this measure was similar during the CS+ and the ITI ($F < 1$). Finally, for Group-120-s, the test $\times$ period ANOVA revealed no main effects of test or period (F 's < 1 ; p 's > 0.37), however a significant interaction was noted ($F(1,15) = 11.41$, $p < 0.01$). Test of simple main effects revealed elevated burst size during the CS+ compared to the CS- periods ($F(1,15) = 5.01$, $p < 0.05$), and a significant differences in this measure when comparing the ITI's from the two respective tests ($F(1,15) = 7.78$, $p = 0.01$), which reflected a significant elevation in this measure during the ITI for the CS- compared to the CS+ test. Collectively, these findings suggest that consumption of the sucrose solution during presentation of the CS+ relative to CS- period was in part driven by an increase in burst size irrespective of the CS duration and density of US delivery in training. However, for Group-20-s mice, burst size was comparable during the CS+ and ITI, whereas it was significantly reduced for the CS- relative to its corresponding ITI period. On the other hand, for Group-120-s mice bursts of licking were elevated during the CS+ relative to the ITI, whereas a reduction in this measure and corresponding increase was revealed for the CS- and ITI periods, respectively.

3.5 Test: Burst number

In general, the number of bursts after a pause > 1000 ms were greater for each group during the CS+ compared to the CS- periods. In addition, for Group-20-s mice burst number was lower during the CS- compared to the corresponding ITI, whereas for Group-120-s mice higher burst number was revealed during the CS+ compared to their ITI period (Figure 3c). The group $\times$ test $\times$ period ANOVA revealed a main effect of period, and a significant period $\times$ group ($F(1,26) = 7.67$, $p = 0.01$) and test $\times$ period ($F(1,26) = 22.75$, $p < 0.001$) interactions. The group $\times$ period ANOVA for the CS+ test revealed no main effect of group ($F < 1$), period ($F(1,26) = 1.5$, $p = 0.23$), nor interaction ($F(1,26) = 2.78$, $p = 0.11$). However, for the CS- test this analysis revealed a significant period $\times$ group interaction ($F(1,26) = 7.31$, $p = 0.01$) due to a significant reduction in burst number during the CS- compared to the ITI for Group-20-s ($F(1,26) = 21.21$, $p < 0.001$) but not Group-120-s mice ($F(1,26) = 1.41$, $p = 0.24$). In addition, while the number of bursts during the CS- were similar between the groups ($F(1,26) = 2.54$, $p = 0.12$), during this test Group-20-s showed elevated burst number during the ITI relative to Group-120-s mice ($F(1,26) = 4.71$, $p < 0.05$). The test $\times$ period ANOVA revealed for Group-20-s mice a significant test $\times$ period interaction ($F(1,11) = 9.17$, $p = 0.01$) due to significant elevation of burst number during the CS+ compared to the CS- ($F(1,11) = 10.8$, $p < 0.01$) and an elevation during the ITI for the CS- compared to the CS+ test ($F(1,11) = 4.95$, $p < 0.05$). Furthermore, during presentation of the CS+, the number of bursts were similar to the ITI ($F < 1$), whereas they were significantly elevated during the ITI compared to the CS- ($F(1,11) = 14.52$, $p < 0.01$). The test $\times$ period ANOVA for Group-120-s mice revealed a significant test $\times$ period ANOVA ($F(1,15) = 15.08$, $p = 0.001$), which reflected a tendency for an increase in burst number during the CS+ compared to the CS- ($F(1,15) = 4.17$, $p = 0.05$), with a similar burst number revealed when comparing the ITI responses during these tests ($F(1,15) = 1.03$, $p = 0.32$). In addition, the number of bursts were elevated for the CS+ compared to the respective ITI period for this test ($F(1,15) = 7.34$, $p = 0.01$), whereas the number of bursts were similar during the CS- and the ITI ($F(1,15) = 2.12$, $p = 0.16$).

3.6 Test: Baseline data

To confirm that prior to any CS presentation, the pattern of licking between the groups was comparable, the number of licks during the initial 2-min period prior to the 1st CS presentation was examined (Figure 3). Collectively, the total licks and the measures of licking microstructure were comparable between groups and across tests, with no effects of group, test or interactions revealed for any measure (F 's<1; p 's>0.31).

4. General Discussion

In the current study, at test mice trained with a 20 s CS and a high rate of US delivery (Group-20-s mice) also displayed greater food cup behavior and a higher rate of licking compared to mice trained with a 120 s CS and a lower rate of US delivery (Group-120-s mice). This effect on lick rate was independent of the sampling duration, as Group-120-s mice displayed a similar lower rate of licking during the initial 20 s periods of each 120 s CS, as they did through its entire presentation. Although differences in lick rate during the CS+ trials were revealed, both groups of mice showed enhanced lick rate during the CS+, relative to the ITI and the CS-. Licking microstructure analysis revealed that while both groups displayed higher burst size during the CS+ relative to CS-, Group-120-s displayed a higher burst size compared to Group-20-s mice, and only in Group-120-s was this measure elevated during the CS+ compared to the corresponding ITI period. In addition, both groups of mice displayed a reduction in burst size during the CS- when compared to the ITI period, however for Group-20-s mice burst size was similar in the ITI for both CS+ and CS- tests, whereas in Group-120-s mice the ITI burst size was elevated during the CS- relative to CS+ test. With respect to the number of bursts initiated, this measure was generally elevated during the CS+ irrespective of training contingencies. For Group-20-s mice, in the CS+ test burst number was similar during the CS relative to the ITI, whereas for the CS- test burst number was significantly reduced relative to the ITI. Moreover, the ITI burst number during the CS- test was elevated in Group-20-s compared to Group-120-s mice, though this latter group showed tentative evidence of an increase in this measure during the CS+ relative to the ITI. Finally, for conditioned approach behavior, during training a shorter CS duration and higher rate of US delivery resulted in greater acquisition. Although a similar pattern of food cup responding during the CPF test was revealed, as is the case with CPF studies (Galarce, Crombag & Holland, 2007; Johnson, 2013; Sherwood, Holland, Adamantidis & Johnson, 2015), conditioned approach behavior during the test was confounded by the unrestricted access to the US.

Konorski (1967) suggested that shorter duration CSs should more readily enter into associations with the sensory properties of a US. In doing so, highly specific sensory components of the US could be associatively activated by the CS and lead to the elicitation of consummatory responses. To the extent that the average bursts of licking that occur during the intake of a fluid reflect its palatability, one might anticipate the type of sensory-specific responding evoked by a shorter duration CS could reflect changes in the specific taste properties, which would be revealed by an increased mean size of licking bursts (Davis & Smith, 1992; Spector et al., 1998; Smith, 2001; Johnson, 2017). On the other hand, Konorski also suggested that more diffuse motivational drive states that occur over longer

time frames, such as the slow-temporal kinetics that modulate hunger levels, would enter into associations with longer duration intervals. According to Konorski (1967), any associations with these features of the US would be expected to conditionally evoke preparatory responses. With respect to feeding behaviors, this would likely facilitate meal initiation and would be revealed through observing the frequency of engaging in novel bouts of licking behavior—i.e., burst number. Consistent with our previous findings (Sherwood et al., 2015), mice trained with a 20 s CS and a high US density displayed an elevation in lick rate during the CS+ compared to the CS–, which was in part accounted for by an increase in the average bursts of licking during CS+ evoked consumption. We have previously taken this to suggest the expression of CPF based on CS+ evoked increase in the perceived palatability of the sucrose US (Sherwood et al., 2015), however the additional examination of licking microstructure during the ITI in the current study questions this account. Thus, during the ITI the average bursts of licking were comparable to the CS+, whereas for the CS– test, mean burst size was reduced relative to the ITI. In other words, the difference in burst size during the CSs may have reflected a reduction in stimulus palatability produced by presentation of the CS–, not an elevation produced by the CS+. While the nature of this effect awaits further characterization (e.g., via summation and retardation tests; Rescorla, 1969), it is tempting to speculate that the CS– may have been functioning in a manner that evoked conditioned inhibitory properties (Hoffman, 1968; Konorski, 1948; Szwejkowska & Konorski, 1959; Trapold & Fairlie, 1965), inhibiting activation of the US representation (Rescorla & Holland, 1977; Hall, 2001) via detailed reinforcer-specific inhibitory associations (Delameter et al., 2003). Accordingly, during CPF this may have been revealed by the suppression of the specific taste features of the US.

Though the above findings question whether a 20 s CS and higher rate of US delivery functions to evoke CPF by influencing the detailed sensory features of the US, it was notable that Group-120-s mice showed more convincing evidence of this form of CPF. Thus, in contrast to Konorski's (1967) account, mice trained with a longer duration CS and a lower US density displayed at test CS+ evoked increases in burst size relative to mice trained with a shorter duration CS-US interval. Moreover, in Group-120-s mice burst size was elevated during the CS+ compared to the corresponding ITI period for that test, and when compared to the CS– period. In addition, as with Group-20-s mice, presentation of the CS– appeared to reduce the taste properties of the sucrose, as on its termination an increase in the burst size was revealed during the ITI. As mentioned above, this latter effect may reflect outcome-specific inhibitory effects of the CS– (Delameter et al., 2003; Holland, 1989). However, unlike the effects observed in Group-20-s, burst size in Group-120-s mice during the ITI for the CS+ and CS– tests were significantly different from one another, which may reflect contrast-mediated effects on ITI burst size, produced by the termination of excitatory and inhibitory CSs. Contrast effects in general appear to at least partly influence stimulus palatability (Flaherty, 1982) and have been revealed to do so under anticipatory (Wright, Gilmour & Dwyer, 2013), successive (Austen & Sanderson, 2016) and simultaneous (Dwyer, Lydall & Hayward, 2011) contrast conditions. Dwyer, Lydall and Hayward (2011) tested rats using a discrete-trial contrast procedure in which a bottle was first extended on the left side of the cage for 60 s (sample bottle), which was then retracted and a bottle of the right side of the cage was then made available for 60 s (test bottle). Although the test bottle

was always 8% sucrose, the sample bottle was 2, 8 or 32% sucrose. Through this approach, both negative (32%→8%) and positive (2%→8%) contrast effects could be compared to control trials (8%→8%). The consumption of the 8% test solution was decreased when it was preceded by the 32% sucrose, whereas test consumption increased when it followed the 2% solution. Notably, these changes in overall consumption were accompanied by corresponding decreases and increases in the average bursts of licking for negative and positive contrasts effects, respectively (Dwyer et al., 2011). To further explore the possibility of CS evoked contrast-mediated effects, future studies employing nonassociative unpaired control groups would be useful to establish whether the presumed alterations of sensory evaluative processing during the ITI reflect CS-evoked contrast effects that are either positive or negative in nature.

With respect to burst number, while both groups showed an elevation during the CS+ compared to CS- presentation, only for mice trained with the longer duration CS was this measure elevated during CS+ compared to ITI. This may suggest that CSs of longer duration and lower US density engage more diffuse general properties of reinforcement and accordingly elicit preparatory behaviors during CPF (Konorski, 1967) in a manner that might enhance the likelihood of initiating feeding behaviors. One caveat of this interpretation is the failure to observe significant elevation in CS+ evoked burst number for Group-120-s compared to Group-20-s mice. In this latter group of mice, CS- reduced burst number relative to the ITI, though no differences in CS- burst number were revealed between the groups.

The current findings suggest that relatively longer CS durations and lower rates of US delivery can evoke feeding based on the specific properties of a US. Delamater and Holland (2008) also observed a similar pattern though in their multiple outcome design, CS-US intervals of 20 s promoted sensory-specific responding, whereas 2 s CS-US intervals failed to do so. In their study, the capacity for a CS to evoke responding based on the specific features of reinforcers was demonstrated mainly using instrumental actions, thus it was possible that the response measures that were used may have been preparatory in nature (Delamater & Holland, 2008); thus reducing the possibility to observe the influence of sensory-specific associations on consummatory responses, which Konorski (1967) assumed would occur with presentation of shorter durations CSs. Nevertheless, in the current study the use of licking microstructure to more readily characterize sensory-specific (taste) responses that are consummatory in nature (i.e., mean burst size) also failed to reveal any observations that were consistent with Konorski's account. While the current findings suggest that the durations chosen in Group-20-s in the current study were suboptimal for eliciting CPF based on the sensory relative to more motivational features of reinforcement, a 20 s CS is capable of evoking reinforcer-specific CPF (Delamater & Holland, 2008). However, compared to the single outcome design used in the current study, the use of a multiple outcome designs (Delamater & Holland, 2008) might especially encourage associations between CSs and detailed sensory properties of USs (Colwill & Rescorla, 1985; Johnson et al., 2009; Holland, 2004; Ostlund & Balleine, 2008), which could more broadly reveal sensory-specific effects, including at shorter CS-US intervals.

An alternative account of the current findings can be described using Scalar Expectancy Theory (SET) (Gibbon, 1977; Gibbon & Balsam, 1981), which accurately predicts the performance of Group-20-s mice during training and in their overall lick rate during CPF. Within this account animals generate expectancies of the reinforcer during the CS (T) and the experimental context (I). In the current study, the I/T ratio for Group-20-s mice would be significantly higher than that for Group-120-s mice. Accordingly, SET would predict that expectancy of the reinforcer during the CS would be greater for Group-20-s mice, which would lead to greater conditioning. More generally, numerous studies on interval timing suggest that it is more accurate with shorter than longer durations (Lejeune & Wearden, 2006) with the idea that timing accuracy may lead to more conditioned responding, which would account for the stronger conditioning in Group-20-s. Although time is a significant variable in associative learning and in establishing conditioned responding, it is notable that food cup approach is not necessarily predictive of CPF (Delamater & Holland, 2008).

While the current findings suggest that CS duration and US density manipulations can evoke different behavioral forms of CPF, it is important to recognize that a number of caveats of this study exist. First, it is possible that differences in test conditions may have prevented potential group differences in CPF from being observed. For instance, in Group-20-s mice the reduced sampling period for the CS+ compared to the ITI and Group-120-s mice may have prevented the opportunity to observe CPF via the detailed sensory features of the US. In licking microstructure it is typical to provide access periods of longer time frames than those reported here (e.g., Spector et al., 1998; Johnson et al., 2010), therefore it is currently unknown whether the durations used in the current test (particularly in Group-20-s mice) provided ample opportunity for the mice to express differences in overall lick rate that could reveal increases in bursts of licking (e.g., relative to the ITI period). However, it is notable that a comparison using this measure between the CS+ and CS- in Group-20-s mice did reveal differences during these cues, suggesting that it is possible to observe burst size differences during CPF when these brief temporal windows are used. Moreover, other studies have also used brief sampling periods to reveal learning-dependent increases in the perceived palatability of a stimulus (Austen et al., 2016; Sherwood et al., 2015). With respect to burst number, it is unlikely that the relatively small volume of fluid ingested during training or testing contributed significantly to post-ingestive inhibitory feedback (Davis & Smith, 1992; Spector et al., 1998), thus questioning the nature of this response as it relates to ingestive behavior in the current study. Second, it is unclear the extent to which the reported effects reflect some interaction between CS duration and the mean interval between US presentations, or unintended effects resulting from these training contingencies. For instance, the longer duration interval between US deliveries in Group-120-s mice could more readily facilitate inhibition of delay (Rescorla, 1967), which could account for the diminished rates of conditioned approach behavior during training and reduced overall lick rate at test. Thus, in order to determine the conditions that produce differences in licking behavior during CPF testing, it would be important to systematically and separately manipulate CS duration, CS-US interval, US density and the proportion of time that the US is available during the CS. While the effects of each of these variables on producing different behavioral forms of CPF remain unknown, when rats were tested with the same CS duration and different densities of US exposure (Delamater & Holland, 2008), increases in

US density (Experiment 3) produced comparable negligible effects as did manipulations of CS-US interval (Experiments 1 & 2) on selective Pavlovian-instrumental-transfer. Third, while mean burst size reflects stimulus palatability (Smith, 2001), it has been suggested that this measure is only strictly reflective of palatability during initial stages of consumption, prior to post-ingestive inhibition. For example, forebrain infusion of the anorexigenic melanocortin MC3/4-R agonist MTII was found to reduce the duration of licking bursts across 120 min glucose intake tests, however this was not observed when the first three bursts of the meal were examined (Williams et al., 2002). In other words, the suppressive effects of melanocortin activation on this measure of palatability were only evident following the accumulation of fluid in the GI tract. Forth, although burst number has traditionally been thought to reflect a general influence of GI inhibition on motivation state (Davis & Smith, 1992; Spector et al., 1998; Smith, 2001; Johnson, 2017), more recently this measure has also been suggested to reflect a reward-oriented response evoked by the attribution of incentive motivational properties to a reward-related stimulus (D'Aquila, 2010; D'Aquila, Rossi Rizzi & Galistu, 2011). D'Aquila (2010) proposed that dopamine D1-like receptor stimulation leads to the activation of reward-associated responses that are revealed through burst number. These responses are updated via a dopamine D2-like receptor evaluation process that occurs as consummatory contact with the reward proceeds (i.e., burst size). Consistent with this account, inactivation of D2 receptors leads to a reduction in the mean size of licking bursts and a within-session decrement in burst number (D'Aquila, 2010; Galistu A, D'Aquila, 2013). We recently provided additional pharmacological support showing that lateral ventricle infusions of the D2 receptor antagonist eticlopride in mice disrupted consumption by specifically reducing the number of bursts (Robles & Johnson, 2016). Thus, within this account, the attribution of incentive properties to food stimuli and food-related cues can facilitate food-seeking behavior, such that the frequency with which an animal initiates a new burst of licking behavior reflects motivational features of feeding behavior (D'Aquila, 2010). However, findings from our laboratory suggest that burst initiation can be dissociated from other more typical measures of food-seeking, such as conditioned approach to the food source (Sherwood et al., 2015). In this study, deletion of the Melanin Concentrating Hormone receptor, MCH-1R, in knock-out mice disrupted CPF by reducing both the mean size and number of licking bursts, even though both knock-out and wild-type mice showed comparable elevated food cup entries to the CS+ relative to the CS- (Sherwood et al., 2015). This capacity for a CS+ to evoke conditioned approach behavior, which can be dissociated from its capacity to initiate bursts of licking is problematic for the account proposed by D'Aquila (2010).

4.1 Conclusion

In contrast to the expectation that shorter duration CSs should evoke sensory-specific feeding behaviors, whereas longer duration CSs should evoke feeding based on the more general features of reinforcement, the current study revealed that relatively longer duration CSs associated with a more sparse US delivery readily promoted feeding based on an alteration in the specific properties of the US. On the other hand, perhaps consistent with Konorski's (1967) account there was tentative evidence that longer duration CSs more readily promoted feeding within a session, based on an increase in burst initiation, which could reflect an enhancement via preparatory response strategies. In addition, comparison of

licking behavior during the ITI's revealed possible contrast-mediated effects produced by excitatory and inhibitory CSs. These findings were revealed by an analysis of licking microstructure, which provides an automated, precise and quantifiable approach to examine the variables controlling ingestive behavior (Davis & Smith, 1992; Spector et al., 1998; Smith, 2001). When applied to the study of learning, licking microstructure analyses can be used to uncover the development, maintenance and topography of responses associated with distinct associatively activated event representations (Austen et al., 2016; Lin, Arthurs, Amodeo & Reilly, 2012; Monk et al., 2014; Dwyer, 2008; Dwyer et al., 2009; Myers & Sclafani, 2001a). The current experiments provide additional evidence of the strength in adopting these analyses to determine the nature of associations between elements of learning. Moreover, with the advent of techniques such as optogenetics that allow targeted cellular manipulations with millisecond precision in awake behaving transgenic mice, combining the temporal resolution afforded by licking microstructure offers the opportunity for readily tractable behavioral readout following manipulations of brain circuitry within discrete temporal episodes of a single test session.

Acknowledgments

This research was partly supported by a Pilot and Feasibility Grant from the Michigan Diabetes Research Center (NIH Grant 2P30-DK020572), and internal startup funds provided by the Department of Psychology at Michigan State University. I wish to thank Ryan Gifford for his assistance with behavioral running.

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Figure 1.

Training and testing contingencies. **(a)** In training during CS+ trials Group-20-s mice received a 20 s CS+, whereas Group-120-s received a 120 s CS+. For each group, the CS+ was divided into four equal separate epochs. **(b)** During the test stage mice received separate test for the CS+ and CS-. For each test mice received a baseline period, followed by CS+ or CS- trials separated by a fixed ITI.

Figure 2. Food cup responding during the training stage. Data reflect the percent time spent in the food cup during the CS+ and CS- (relative to the preceding 5 s pre-CS period) in mice trained with a short (20 s) or long (120 s) CS.

Figure 3.

Cue-potentiated feeding test data for Group-20-s (left panels) and Group-120-s mice (right panels). **(a)** The average lick rate during the CS+ and CS- tests when mice were presented with the CS (closed bars) and during the ITI (open bars). **(b,c)** The analysis of licking microstructure during CS+ and CS- tests. The **(b)** average bursts of licking, and **(c)** the number of bursts during the CS (closed bars) and ITI (open bars). * indicates significant test $\times$ period interaction ($p < 0.01$), # indicates significant group $\times$ period interaction ($p < 0.05$).

Figure 4. Lick responses during the initial 2 min period prior to 1st CS trial. No differences in **(a)** total licks, **(b)** burst size, or **(c)** burst number were seen

Table 1

Training contingencies for CS presentation and US delivery in Groups-20-s and Group-120-s. Mice in Group-20-s were presented with a 20 s auditory CS +, which was divided into four separate 5 s epochs. Mice in Group-120-s were presented with a 120 s auditory CS+, which was divided into four separate 30 s epochs. The US delivery point (in seconds) is specified for each epoch. Within each session, mice received 5 CS+ trials and the number of US deliveries per session and within each epoch is described.

		Epoch 1	Epoch 2	Epoch 3	Epoch 4
Group-20-S	CS	0–5 s	6–10 s	11–15 s	16–20 s
	US	1 s	6s	11 s	16s
Group-120-S	CS	0–30 s	31–60 s	61–90 s	91–120 s
	US	1 s	31 s	61 s	91 s
US deliveries/session		2	3	2	3