Behavioral dissection of hunger states in *Drosophila*

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Abstract

Hunger is a motivational drive that promotes feeding, and it can be generated by the physiological need to consume nutrients as well as the hedonic properties of food. Brain circuits and mechanisms that regulate feeding have been described, but which of these contribute to the generation of motive forces that drive feeding is unclear. Here, we describe our first efforts at behaviorally and neuronally distinguishing hedonic from homeostatic hunger states in *Drosophila melanogaster* and propose that this system can be used as a model to dissect the molecular mechanisms that underlie feeding motivation. We visually identify and quantify behaviors exhibited by hungry flies and find that increased feeding duration is a behavioral signature of hedonic feeding motivation. Using a genetically-encoded marker of neuronal activity, we find that the mushroom body (MB) lobes are activated by hedonic food environments, and we use optogenetic inhibition to implicate a PAM>α'/β' MB circuit in hedonic feeding motivation. The identification of discrete hunger states in flies and the development of behavioral assays to measure them offers a framework to begin dissecting the molecular and circuit mechanisms that generate motivational states in the brain.

Introduction

Hedonist philosophers believed that pleasure is the supreme goal of life and that behavior is determined by the pursuit of it (Aristotle et al., 2004). Hunger for tasty delicacies, for example, is a particularly potent motivator of behavior, as it can be promoted not only by a physiological need to consume nutrients, but also by the hedonic properties of food. Due to its dichotomous nature, defining behavioral and neural correlates of hunger is a challenge that has perplexed scientists for over a century. Early physiologists wondered how to explain hunger and where it might originate, and they eventually described hunger as a dull ache or gnawing sensation arising from the stomach or abdominal region (Cannon & Washburn, 1993). This historical description has aided the discovery of hormones like ghrelin, insulin, and others that communicate information from the periphery to the brain and have roles in controlling feeding in response to energetic or nutritional needs, a state we now refer to as homeostatic hunger. In the decades since these discoveries, it has become clear that peripheral signals and others are integrated in the central nervous system to promote changes in behavior and reward signaling, which we interpret as indicators of hunger, but the nature of the motivational brain systems that drive these outputs remains less straight forward to identify and quantify.

Homeostatic feeding, or “need-based” intake, is defined as food intake that is necessary to ensure caloric and nutritional needs are met, and it has been quantified by measuring bulk food intake. Such measures may be of total caloric intake or consumption of specific nutrients, but it is unclear whether these measures are representative of hunger that is driven by energetic need, nutrient deficiency, or some other motivation, which has led to variation in the way measures of homeostatic hunger are reported and interpreted (for review of mammalian brain circuits that modulate feeding, see (Alcantara et al., 2022; Andermann & Lowell, 2017; Saper et al., 2002)). However, the observation that most animals, including humans, regulate food intake around a protein target and the identification of discrete neuronal populations that regulate

protein-specific feeding in model systems suggest that levels of homeostatic hunger may be primarily determined by the amount of protein an animal requires and thus, in recent years, protein intake has become a common method for measuring need-based hunger (Gosby et al., 2011; Liu et al., 2017; Ro et al., 2016; Simpson et al., 2003; Weaver et al., 2022).

Our understanding of hunger has continued to evolve, due in part to the observation that the pleasurable effects of food also stimulate feeding independently of physiological need, a state referred to as hedonic hunger (Lowe & Butryn, 2007). Hedonic, or “need-free”, intake is thought to be evoked by the incentive salience or tastiness of food, and thus, the sweetness of the food environment is predicted to play a larger role (Lowe & Butryn, 2007). Indeed, hedonic hunger is likely the predominant driver of feeding in modern human environments where food is readily available and energy deprivation is seldom experienced. Hedonic responses to various food or environmental manipulations have been described as either “liking” responses (relating to the pleasure of a rewarding experience) or “wanting” responses (relating to the motivation to seek a reward), which arise from different neuronal substrates (reviewed in (Berridge, 2009)). Accordingly, “liking” is measured in rats by tracking facial responses in response to sweet tastants, while “wanting” is measured by tracking latency to search for and consume a tasty food treat or by using the novelty suppressed feeding assay that attempts to measure an animal’s ability to overcome an aversive environment to consume a food reward (Arcego et al., 2020; Pecina et al., 2006). While observations like these have greatly aided our ability to identify hedonic hunger states in mammals, much less is known about their neuronal and mechanistic origins relative to homeostatic hunger.

Although we now understand that hunger stratifies into two distinct states, one driven by need and the other by pleasure, we have a limited understanding of their behavioral and neuronal distinctions and connections. In mammals, brain circuits that modulate homeostatic or hedonic

appetites have been identified. The well-described AGRP neurons in the arcuate nucleus of the hypothalamus, for example, are critical components of a circuit for homeostatic feeding (for review, see (Sternson & Eiselt, 2017)). They express receptors for peripherally released hormones that signal energetic state, are more active upon food deprivation, and transmit a negative valence signal that drives animals to find and consume food when the need arises (Betley et al., 2015; Denis et al., 2015; Willesen et al., 1999; Zigman et al., 2006). In addition to systems that seem to motivate homeostatic feeding, distinct circuits have also been associated with hedonic feeding including the monoaminergic populations that express serotonin and/or dopamine, and the mesolimbic dopamine pathway (Ahn et al., 2022; Altherr et al., 2021; Denis et al., 2015; Ghiglieri et al., 1997; Rossi & Stuber, 2018). While there is evidence to suggest that both branches of hunger are modulated by homeostatic state and hedonic feedback (e.g., (Denis et al., 2015; Krashes et al., 2011; Zigman et al., 2006)), the mechanistic origins that determine how various hunger drives work together and independently to generate feeding responses have yet to be fully described.

Simpler model systems, such as the fruit fly, *Drosophila melanogaster*, have proven valuable for insights into the neural circuitry that modulates feeding, but the nature of their hunger drives is unknown. Whether flies experience hedonic hunger, for example, is unclear, although it seems likely that they do; hungry flies learn to associate odors with a sucrose reward and when fed a high-sugar diet they overeat and become obese (Huetteroth et al., 2015; May et al., 2019). Strikingly, these behaviors are all thought to occur through mechanisms involving dopaminergic neurons in the fly brain which, when artificially activated, promote foraging and food intake (Tsao et al., 2018). Honeybees have also exhibited signatures of food wanting, a component of hedonic hunger, which manifests through activation of dopaminergic populations in the brain that are reflective of reward expectation (Huang et al., 2022).

130 To better understand the different motive forces that drive feeding, it seems clear that there is a
131 need to move beyond measures of food intake or foraging behavior, which have been useful for
132 identifying external and internal factors that change the way an animal eats, in order to dissect
133 when and to what degree animals may be experiencing different types of hunger. Here we
134 describe our first efforts to address this need by generating clear behavioral definitions of
135 hunger states in *Drosophila* with the hope of providing an attractive system for defining the
136 molecular characteristics of their independent roles and the relationships between them, which
137 has not yet been possible in mammalian systems. We identify a repertoire of feeding micro-
138 behaviors in the fly and use them to argue that *Drosophila* experience both homeostatic and
139 hedonic hunger. We identify neural correlates of hedonic hunger in specific lobes of the
140 *Drosophila* mushroom body and show that they are required for hedonic feeding. Finally, we
141 develop a framework to examine the relationships between hedonic and homeostatic hunger
142 states and present evidence for the existence of a system that acts as a potentiator of the
143 relationship between them. Translating findings about the nature of feeding drives and the
144 relationships among them could have far reaching implications for obesity and energy balance
145 research and other behaviors influenced by motivational drives, including drug addiction and
146 eating disorders.
147
148 **Results**
149 We set out to complement and enrich traditional measures of feeding by identifying new metrics
150 that might be useful for classifying different hunger states. Fly feeding behavior is typically
151 described as changes in volumetric food intake, which is difficult to accurately quantify at the
152 individual level due to the small amount of food ingested (Deshpande et al., 2014; Shell et al.,
153 2021; Shell et al., 2018), or by using automated systems that detect physical interactions with
154 food sources (Itskov et al., 2014; Ro et al., 2014). These automated devices have revealed
155 rhythmic patterns of feeding behavior in flies that mimic feeding strategies in other animals,

156 suggesting that the behaviors are highly organized and centrally regulated. They are, however,
157 unable to distinguish different stereotypical actions that a freely-moving fly might exhibit when
158 interacting with a food source. Detailed behavioral characterizations of this sort have proven to
159 be useful read-outs of neural states in other contexts and have the potential to inform our
160 understanding of the motivational states that drive feeding (Chen et al., 2002; Ning et al., 2022).
161 Much like human infants, for example, facial expressions and tongue movements in rats in
162 response to appetitive tastants have been used to identify hedonic reactions and the brain
163 systems that mediate them (Berridge, 1996).
164
165 To identify nuances in individual fly feeding, we developed video systems for manual and high-
166 throughput annotation of what flies do when they eat. In all cases, we recorded freely-moving
167 flies placed in feeding chambers of the Fly Liquid Interaction Counter (FLIC) system, which is
168 designed to continuously record individual flies' interactions with food sources (Ro et al., 2014).
169 We began by simply recording flies in the FLIC and manually curating each video in its entirety
170 (Fig. 1A). With the hope of observing the greatest variety of behaviors, we collected
171 observations from flies ranging from 5-20 days old, from female and male sated and starved
172 flies, from flies housed singly or in groups of up to three, and from flies interacting with 5% or
173 10% sucrose food. We observed 30 flies and nearly 300 "events", which were demarcated by
174 intervals greater than one second during which the fly was not in physical contact with the food.
175 Events were easy to identify, and different behavioral characteristics associated with events
176 were easy to distinguish from each other (Slooten, 1994). Rare events (observed in fewer than
177 5% of flies) were not considered further, and events during which sight of the fly was out of
178 focus or obscured (typically 1-2 events per fly) were ignored. Based on the remaining events,
179 we created a library of user-defined behaviors that captured common, distinct, and definable
180 interactions of flies with their food (Table 1).

Table 1. Library of Feeding Micro-Behaviors

Event Type	*Code*	*Behavior Description*
Other	G	Groom
	UC	Unknown contact
Interaction	H	Front legs touch food
	FF	Hind legs touch food
Fast feeding	F	Single movement of proboscis into food to feed for 1-3 seconds
	FH	Meets requirements for F and H
Long feeding	L	Proboscis continuously in food while feeding for > 4 seconds
	LH	Meets requirements for L and H
	LQ	Proboscis moves in and out quickly while feeding for > 4 seconds

We classified behaviors into four event categories: long feeding events, fast feeding events, interaction events, and other (Table 1). The first event type comprised fast feeding events (termed “F” or “FH”) during which we observed contact of the proboscis with the food for less than four seconds. These types of events were common (occurring in ~95% of flies, Fig. 1B, 1C) and were sometimes simultaneous with the interaction of a fly’s legs with the food. Long feeding events (termed “L”, “LH”, or “LQ”) were also identified, during which time we observed contact of the proboscis with the food for more than four seconds. Long events were less common than F-type events and could also include simultaneous interaction of legs with food. Long feeding events were either a continuous and sustained interaction of the proboscis with the food (L, occurring in ~85% of flies, Fig. 1B, 1C) or, less commonly, a continuous interaction during which the proboscis moved quickly in and out of the food (LQ, occurring in ~60% of flies, Fig. 1B, 1C). A fourth type of behavior was termed an interaction event (“H” or “FF”) during which the fly interacted with the food only using its legs; no proboscis contact was observed. These putative tasting interactions often occurred in a “pitter-patter” motion, as if the fly were “playing” with their food, and they occurred in greater than 90% of flies (Fig. 1B, 1C). Lastly, we

occasionally observed other non-feeding events (“G” or “UC”) that, nevertheless, often occurred just before or after feeding (occurring in ~60% of flies, Fig. 1B, 1C). These tended to be various grooming behaviors, including using the forelegs to rub the head after interacting with food.

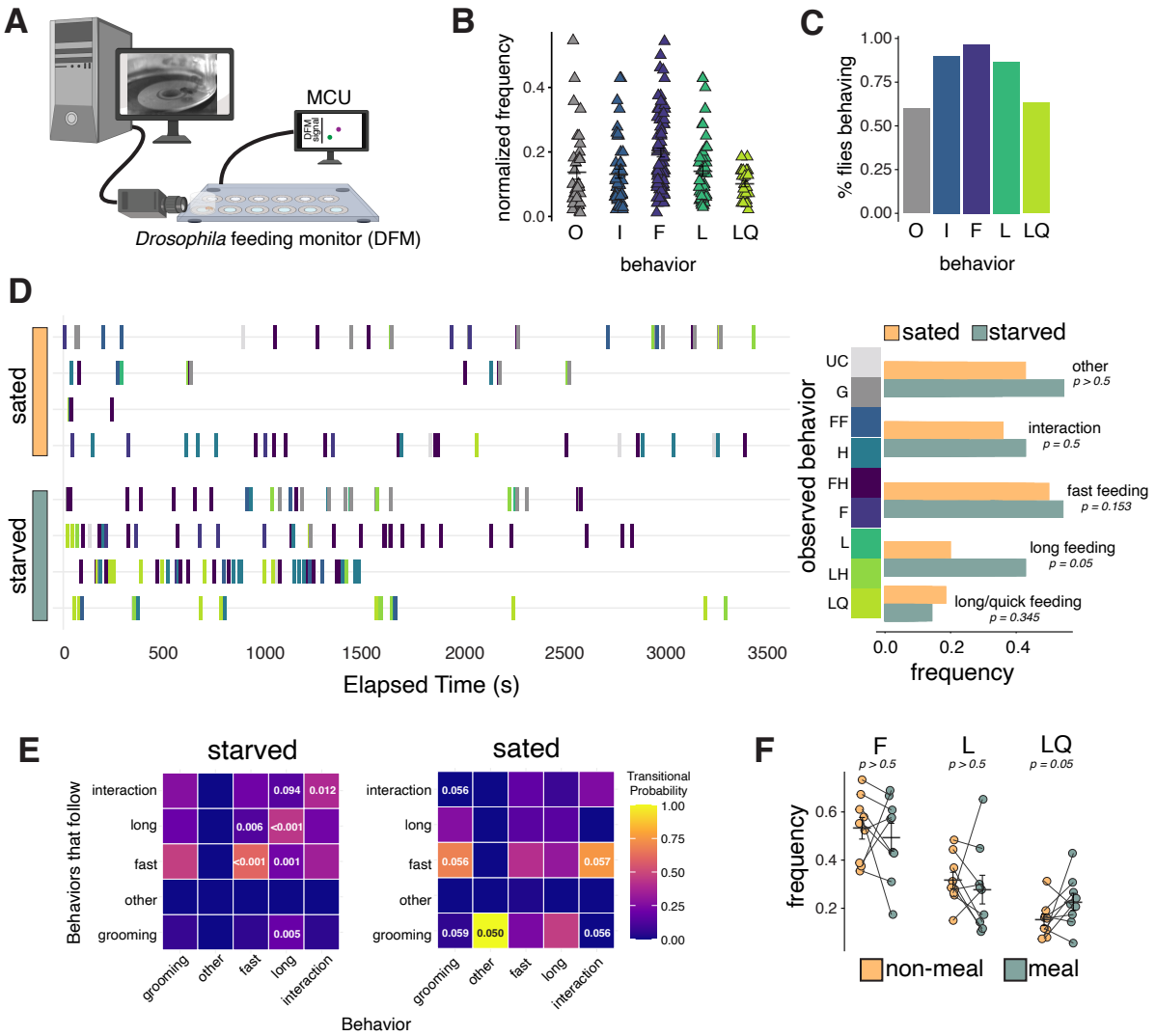


Figure 1. Characterization of feeding micro-behaviors in flies. (A) Cartoon schematic of setup for filming flies on the FLIC. Frame capture is triggered by interactions of flies with the food source. **(B)** Frequency of all observed feeding micro-behaviors: other (O), interaction (I), fast (F), long (L), long/quick (LQ). Each data point represents the total number of times an event occurred normalized to all observed events for each fly. **(C)** Percentage of flies that engaged in each feeding micro-behavior. (**D)** Representative temporal plots of sated and starved fly feeding micro-behaviors (left panel) and frequency of each behavior (right panel, two-tailed t-tests). Each row in left panel represents one fly. **(E)** Heat maps of starved (left) and sated (right) transitional probabilities of observed behaviors (transitional probabilities are generated by dividing the observed count for each event pair by the total occurrences of the given event. P-values are determined based on Z-scores, as described in (Blumstein & Daniel, 2007)). **(F)** Frequency of each behavior during meal (7AM/PM-10AM/PM) vs. non-meal times of day (one-tailed t-tests).

We next asked whether our behavioral classification could be used to distinguish hungry from sated flies. Recognizing a need to significantly increase the number of observed feeding events to provide sufficient statistical power to detect differences in behavioral tendencies, we designed a direct communication link between our custom DTrack video software and the FLIC system to trigger video capture only when there was physical contact between a fly and the food source, together with 5s windows before and after the contact. This allowed us to create video compilations of feeding behaviors for fully fed or starved (24hr) flies over several hours or days. Over 500 distinct events in these compilations were then manually scored using our classification of feeding behaviors, and comparisons were made between fully-fed and starved flies for each behavioral category in Table 1. We observed that starved flies exhibited a significantly greater frequency of feeding behaviors overall, as expected (sated event frequency = 0.606 events/minute compared to starved event frequency = 0.788 events/minute, $p = 0.061$, data not shown), and that the relative frequency with which L-type feeding events occurred was increased while the relative frequency with which F feeding events occurred was decreased (Fig. 1D, quantified at right). The observation that starved flies exhibit more frequent long-duration events is consistent with recent reports that starved flies increase “sips per burst” – a measure that is comparable to our definition of L and LQ events (Itskov et al., 2014).

Qualitative aspects of the relationships between individual behaviors - that is, the architecture that results from sequences of behaviors - can reveal complexities that may also distinguish between different neural states (Casarrubea et al., 2019; Casarrubea et al., 2015; Flavell et al., 2022). Thus, we asked whether certain sequential patterns in fly feeding behavior appear repeatedly and whether they are affected by starvation. To do this, we used classic sequence analysis to map the probability that one event follows another in a sequence more often than what is expected to occur by chance alone - an approach that has been useful for identifying

240 stereotyped patterns or structures of behaviors that tend to occur together (Ning et al., 2022;
241 Slooten, 1994). We observed that hungry flies, whom we had starved for 24 hours, but not sated
242 flies, followed F feeding events with additional F feeding events (transitional probability = 0.6 in
243 starved flies with p-value <0.001, compared to a non-significant transitional probability = 0.55 in
244 fully-fed flies) and L-type feeding events with additional L-type feeding events (transitional
245 probability = 0.439 in starved flies with p-value <0.001 compared to non-significant transitional
246 probability = 0.1538 in fully-fed flies) more often than would be expected by chance (Fig. 1E). In
247 contrast, fully-fed flies were likely to engage in F-type events after grooming or interaction
248 events and were more likely to groom following most other events, which may be reflective of a
249 less goal-oriented behavioral state compared to that of starved flies (Fig. 1E).
250
251 Having established that long-duration events occur more frequently and in a predictable
252 sequence in starved flies, we sought to replicate this finding in flies during their normal circadian
253 "meal-time", when they are presumably physiologically hungry but not yet starved. When
254 monitored over several days on the FLIC, flies exhibited circadian meal patterns that
255 corresponded to the light cycle, with one peak in the morning when lights turn on and a second
256 peak in the evening when lights turn off (Fig. 1 – Fig. Supp. 1). We obtained 24hr video
257 compilations of nearly 700 feeding events in the FLIC and used the flies' endogenous feeding
258 rhythm to designate "meal" and "non-meal" times of day. We manually scored the videos during
259 each time for fast, long, and long/quick events. We found that during mealtimes flies exhibited a
260 significantly greater frequency of LQ events, while the frequencies of F and L events were
261 statistically indistinguishable between times of day (Fig 1F). Thus, both starved flies and flies
262 during their circadian meal-time exhibit an increase in the total number of feeding events
263 together with an increase in the relative frequency of long feeding events.

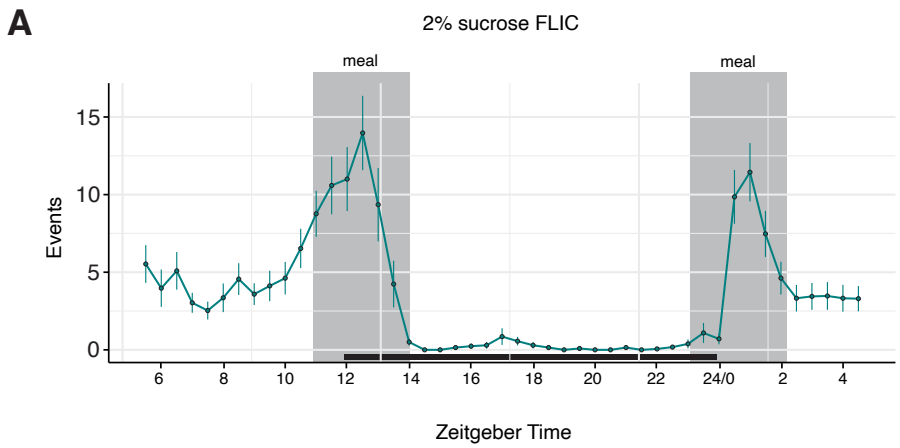


Figure 1 – Figure Supplement 1. Flies exhibit distinct morning and evening feeding peaks. (A) Representative temporal plot of food interactions on 2% sucrose FLIC from female *Canton-S* flies monitored over 24 hrs. Each data point represents the binned average number of events from a population of 38 flies in the previous 30-minute interval. Shaded regions indicate the times of day designated as "meal-times".

The FLIC generates several quantitative signal measures when flies interact with food, and we asked whether any of these numerical metrics might substitute for direct visual observation and distinguish among fast feeding events, long feeding events, and interactions. To determine how signal measures change in accordance with a fly's behavior, we scored 15 video compilations (resulting in 332 independent events) of flies interacting on the FLIC for F-type, L-type, or I-type events, and we matched each visually identified behavior to its corresponding FLIC signal data (e.g., Figs. 2A-2C). We evaluated differences in the characteristics of signal intensity for each event (i.e., its variance, minimum, maximum, average, and total) as well as the duration of the signal from each event. We found that only signal duration effectively distinguished long events from both fast events and interactions (Fig. 2D-2I). Thus, we established that longer signal durations from the FLIC are representative of L-type feeding events. We also noted that maximum signal intensity and the variance of the signal can distinguish interactions from both types of feeding events, which may be broadly useful to distinguish non-feeding interactions (i.e., tasting events) from feeding events (Ro et al., 2014).

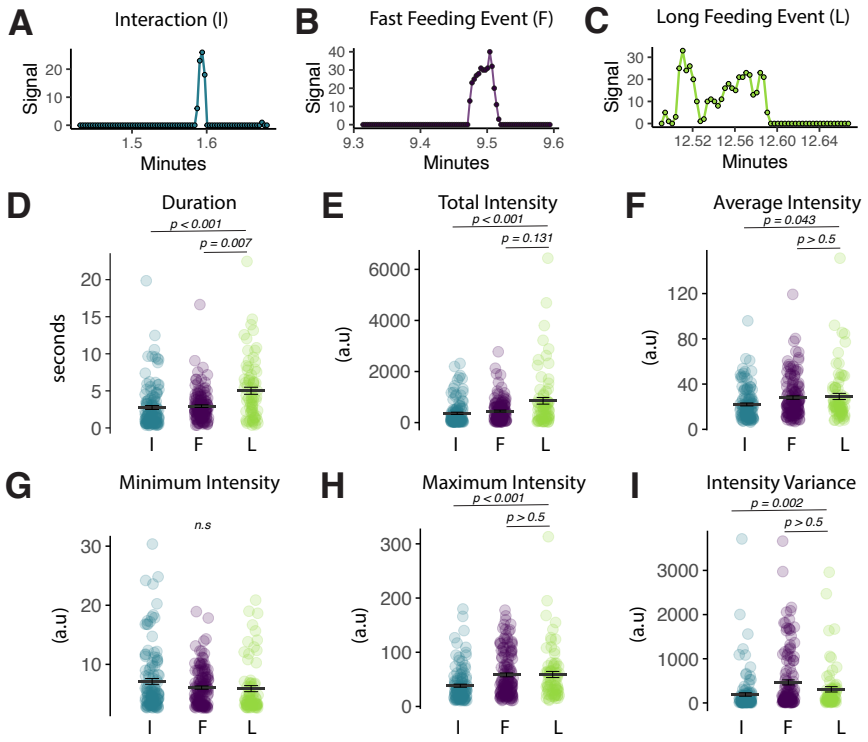


Figure 2. FLIC signal characteristics distinguish between event-types. (A-C) Representative FLIC signal generated during a visually identified interaction (I), fast feeding (F), or long feeding (L) event. **(D-I)** Signal characteristics generated by the FLIC during visually identified I, F, or L events. Each data point represents one event from one fly (one-way ANOVA with Tukey's post-hoc).

Development of an assay to identify homeostatic and hedonic feeding

Having established that hungry flies increase the duration of their feeding events as well as the total number of events (i.e., Figure 3A, top panel), we next asked if we might be able to identify distinct hunger drives in flies and, if so, whether our behavioral and quantitative metrics would distinguish between them. Recognizing that the original assays used for behavioral observation, in which flies were exposed to a single, homogenous food source, were likely insufficient to distinguish different types of feeding drives, we designed two types of food choice environments that we might expect would distinguish homeostatic from hedonic feeding. Homeostatic feeding drive in flies is thought to be measured by the extent to which flies choose to consume a yeast-containing food over one consisting of sucrose only, which we will refer to hereafter as a yeast

303 preference (Liu et al., 2017; Ro et al., 2016). Therefore, for measures of homeostatic drive, we
304 exposed flies to a choice of 2% sucrose vs. [2% yeast + 1% sucrose], and we focused our
305 analysis on the flies' relative interactions with the yeast containing food. To measure hedonic
306 drive, on the other hand, we incorporated theorized requirements to experimentally measure
307 hedonic hunger in mammals: animals must be energy replete and they must be tested on highly
308 palatable food (Lowe & Butryn, 2007). We therefore exposed flies to a "hedonic choice"
309 environment which mimicked the "homeostatic choice" environment except that the 2% sucrose
310 food was replaced with sweeter 20% sucrose, and we focused our analysis on comparing
311 interactions with the sucrose food between the food environments (e.g., Fig. 3A bottom panel).
312 This design ensured flies remained sated by access to yeast while also allowing feeding on the
313 highly palatable 20% sucrose solution.
314

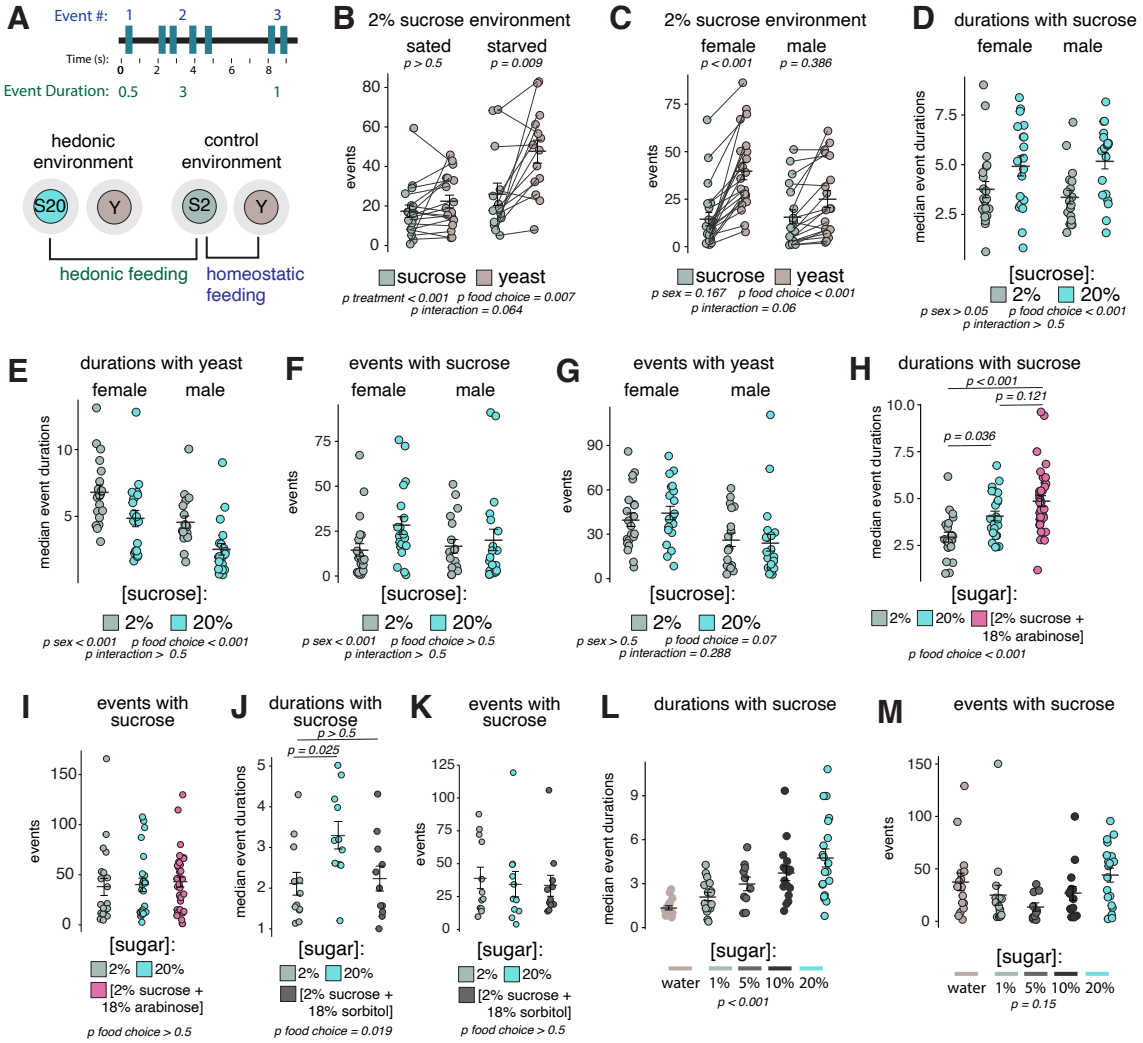
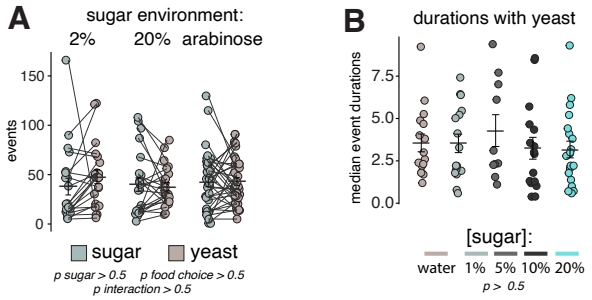


Figure 3. Feeding event durations are increased by hedonic food environments. (A) Cartoon schematic of event number and event duration (top). Cartoon schematic of hedonic and control food choice environments, and comparisons used to define metrics for homeostatic and hedonic feeding (bottom). **(B)** Total number of events with sucrose or yeast in the control food choice environment from sated or 24-hr starved female *Canton-S* flies (two-way ANOVA with Tukey's post-hoc). **(C)** Total number of events with sucrose or yeast in the control food choice environment from sated female and male *Canton-S* flies (two-way ANOVA with Tukey's post-hoc). **(D-E)** Median event durations with sucrose (D) or yeast (E) in the control vs. hedonic food choice environments from sated female and male *Canton-S* flies (two-way ANOVA). **(F-G)** Total number of events with sucrose (F) or yeast (G) in the control vs. hedonic food choice environments from sated female and male *Canton-S* flies (two-way ANOVA). **(H-I)** Median event durations (H) and total number of events (I) with sugar in the indicated food choice environments from sated female *Canton-S* flies (one-way ANOVA with Tukey's post-hoc). **(J-K)** Median event durations (J) and total number of events (K) with sugar in the indicated food choice environment from sated female *Canton-S* flies (one-way ANOVA with Tukey's post-hoc). **(L-M)** Median event durations (L) and total number of events (M) with sucrose in the indicated food choice environments (one-way ANOVA).

332 We observed that the 2% sucrose environment elicited an increased yeast preference in starved
333 compared to fully-fed female flies (Fig. 3B), reinforcing the notion that the total number of events
334 with yeast vs. sucrose can be used as a relative measure of homeostatic hunger. We also
335 observed an increase in yeast preference in fully-fed female compared to male flies (Fig. 3C),
336 which may be reflective of a known difference in protein requirements, and therefore
337 homeostatic hunger levels, between male and female flies. We next evaluated female and male
338 fully-fed flies in the 20% sucrose food environment compared to the 2% sucrose environment,
339 which elicited increased event durations with 20% sucrose compared to the 2% sucrose food
340 (Fig. 3D) and also elicited decreased event durations with yeast food (Fig. 3E). The total
341 number of events on sucrose or yeast wells was statistically indistinguishable between food
342 environments (Fig. 3F, 3G), suggesting that modulation of event duration, but not total number
343 of events, may be a feature of hedonic food environments. We concluded that the measurable
344 increase in sucrose event durations in the hedonic food environment may reflect increases in a
345 hedonic hunger drive while total number of events on yeast vs. sucrose food may reflect
346 homeostatic state.
347
348 To challenge our measure of hedonic feeding, we reasoned that if increased event durations
349 were indeed a result of hedonic hunger, then sweetness alone should be sufficient to increase
350 event durations without modulating measures of homeostatic feeding. We tested two additional
351 food choice environments where we altered either the sweetness or caloric content of the sugar
352 choice. Sweetness was manipulated by using the non-metabolizable sweetener arabinose ([2%
353 sucrose + 18% arabinose], which mimics the caloric and nutritive content of the 2% sucrose but
354 contains the sweetness of the 20% sucrose choice. Caloric content was manipulated using the
355 nutritive, but not sweet, sugar alcohol, sorbitol ([2% sucrose + 18% sorbitol], which mimics the
356 caloric content of the 20% sucrose but contains the sweetness of the 2% sucrose (Burke &
357 Waddell, 2011; Hassett, 1948). We observed that the sweet arabinose food environment elicited

358 increased event durations that were comparable to flies in the 20% sucrose environment, with
359 no statistical difference in total event number (Figs. 3H, 3I, Fig. 3 – Fig. Supp. 1A) and that the
360 caloric but not sweet sorbitol food environment was not sufficient to increase event durations or
361 total event number (Figs. 3J, 3K). Finally, we tested several additional food choice environments
362 containing a range of sucrose concentrations and observed that event durations were shortest
363 when the sucrose choice contained only water or 1% sucrose and increased linearly as the
364 sucrose concentration increased, while event durations with yeast food and total number of
365 sucrose events were not statistically different (Fig. 3L, 3M, Fig. 3 – Fig. Supp. 1B). Thus, sweet
366 taste was sufficient to evoke longer feeding event durations, while calories alone were not,
367 indicating that this metric obtained from our hedonic food choice paradigm may reliably quantify
368 hedonic feeding.



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370 **Figure 3 – Figure Supplement 1. Hedonic food environments do not elicit increases in**
371 **total number of events or event durations with yeast. (A)** Total number of events with sugar
372 or yeast in indicated food choice environments from sated female *Canton-S* flies (two-way
373 ANOVA). **(B)** Median event durations with yeast in the indicated food choice environments from
374 sated female *Canton-S* flies (one-way ANOVA).
375

376 Whether and how homeostatic and hedonic feeding mechanisms interact or overlap is
377 controversial, and thus, we asked whether our metrics of homeostatic and hedonic hunger are
378 correlated among individual flies. To obtain flies with a range of homeostatic needs, we used
379 fully-fed and starved flies to obtain feeding measures in our control or hedonic food choice
380 environments. Starvation elicited an increase in yeast interactions compared to 2% sucrose

381 (Fig. 4A) that was more pronounced in flies starved for 48 vs. 24 hours (Fig. 4B), which is
382 consistent with our expectation for increased homeostatic drive. We found that starvation
383 seemed to marginally increase differences in sucrose event durations in hedonic vs. control
384 environments, indicating that homeostatic hunger may enhance hedonic feeding (Fig. 4C). For
385 each fly, we next calculated metrics for homeostatic drive (# interactions with yeast food) as well
386 as hedonic drive (sucrose event durations) and plotted their relationship along the x- and y-
387 axes, respectively. We reasoned that if the circuits or programs that drive hedonic and
388 homeostatic feeding were completely independent, then we should observe a slope close to
389 zero. A significantly positive or negative slope, however, would suggest that increasing
390 physiological need might also modulate hedonic hunger, or vice versa. We observed a
391 significantly positive correlation between our measures of homeostatic and hedonic feeding
392 (Fig. 4D), suggesting that increasing physiological need might also potentiate hedonic hunger,
393 or vice versa, which is consistent with the observation in mammals that hunger increases the
394 reward value of food (Fulton, 2010). Interestingly, we also noted that separating regression lines
395 from flies in the control vs. hedonic food environments revealed parallel lines whose slopes
396 were not significantly different but were separated by a shift along the y-axis, indicating again
397 that a sweeter food environment promotes longer event durations but may not modulate the
398 relationship between hunger drives. These findings indicate that homeostatic state is a
399 potentiator of hedonic feeding and establish that inferences of hunger states and measures of
400 feeding should include considerations of each component independently as well as their
401 interaction.
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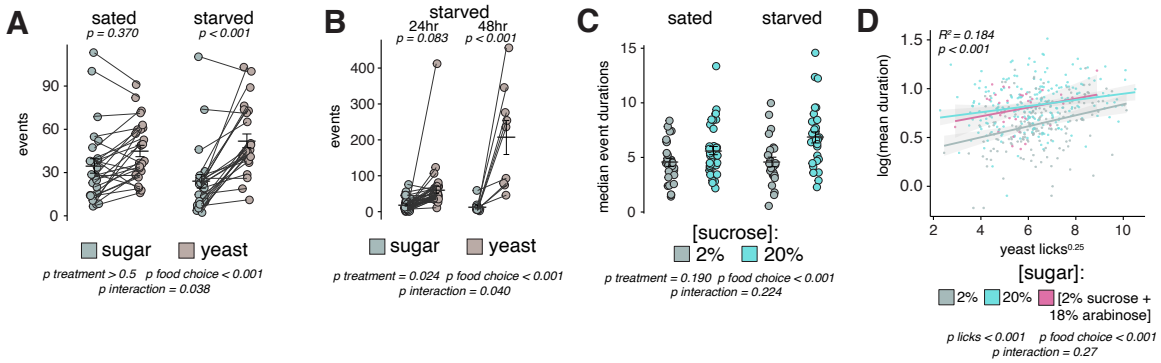

Figure 4. Hedonic feeding is modulated by homeostatic hunger. (A) Total number of events with sucrose or yeast in the control food choice environment from sated or 24-hr starved female *Canton-S* flies (two-way ANOVA with Tukey's post-hoc). **(B)** Total number of events with sucrose or yeast in the control food choice environment from 24-hr vs. 48-hr starved female *Canton-S* flies (two-way ANOVA with Tukey's post-hoc). **(C)** Median event durations with sucrose in the control vs. hedonic food environments from sated vs. 24-hr starved female *Canton-S* flies (two-way ANOVA). **(D)** Linear regression of interactions with yeast (licks) versus mean event durations with indicated sugar. Each data point represents one fly (yeast licks are expressed as a transformation to the 0.25 power and event durations are expressed as a log transformation to achieve normality, call = lm(SucroseDurations~YeastLicks*FoodEnvironment), two-way ANOVA, adjusted R^2=0.1835).

Identification of neural correlates of hedonic hunger

A good deal is known about neural circuits associated with homeostatic hunger in *Drosophila*, and we wondered whether our behavioral and quantitative classification metrics could aid in identification of representative neural correlates of a hedonic hunger state. We began by conducting an unbiased screen for neurons that respond to hedonic food environments using the genetically-encoded calcium indicator CaMPARI driven by a pan-neuronal GAL4 driver (Nsyb-GAL4). CaMPARI is a green fluorescent protein that irreversibly photoconverts to red when flies are simultaneously exposed to increases in intracellular calcium and UV light provided by the experimenter (Edwards et al., 2020; Fosque et al., 2015). We placed groups of 3 naïve, fully-fed flies in the hedonic food choice environment or the control food environment and allowed them to habituate for 1 hour. Flies in each chamber were then exposed to cycles of UV light (10s on/10s off) for a total of 1 hour to label neuronal populations that were differentially active when flies are in the hedonic vs. the control food environment. Brains were dissected and

430 imaged immediately, and the ratio of red:green fluorescent protein was analyzed, with a higher
431 ratio indicating increased neuronal activity. We observed that the mushroom body had a higher
432 ratio of red:green fluorescence when flies were in the hedonic food environment, indicating that
433 this population was more active (Fig. 5A).
434
435 To validate the findings of our unbiased screen and as a direct test of mushroom body activity in
436 hedonic food environments, we restricted the expression of CaMPARI to the mushroom body
437 using the *238Y* enhancer-trap line (Yang et al., 1995). We observed that the hedonic food
438 environment increased red:green fluorescence in the horizontal mushroom body lobes
439 compared to the control food environment (Fig. 5B). The *Drosophila* mushroom body is a well-
440 described structure known to be involved in reinforcement and motivational control and is
441 innervated by dopaminergic populations that are thought to encode reinforcing properties of
442 food, including relaying information about sweet taste (Aso et al., 2009; Aso et al., 2014; Das et
443 al., 2016). Thus, our observation that mushroom body neurons are activated in hedonic food
444 environments is consistent with prior knowledge of their roles in motivation and reward
445 (Landayan et al., 2018).

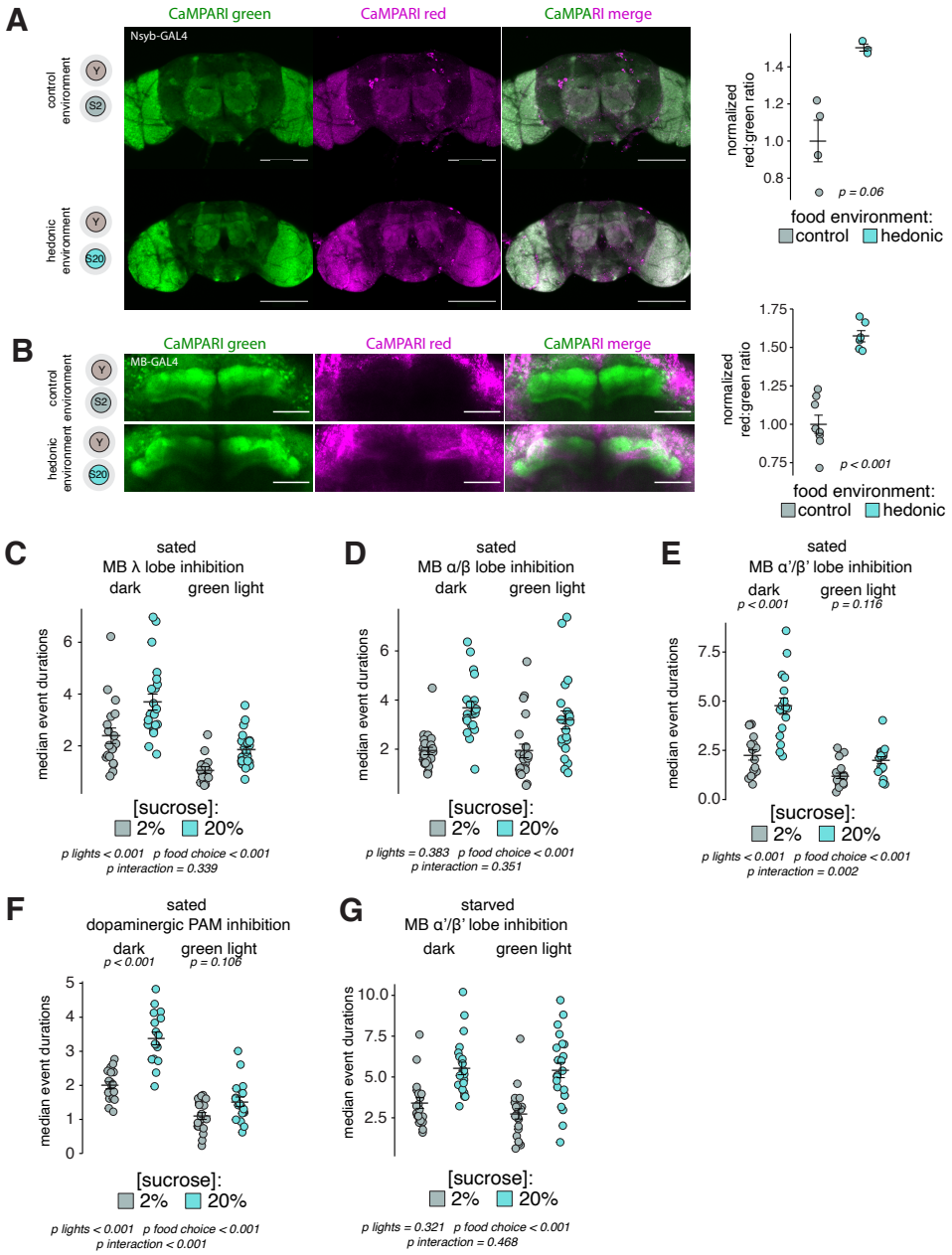

Figure 5. Hedonic hunger requires distinct mushroom body lobes. (A) Representative maximum intensity projections from brains of flies expressing *Nsyb-GAL4>UAS-CaMPARI* exposed to control or hedonic food environments, quantified at right (scale bar = 100 uM, two-tailed t-test). **(B)** Representative maximum intensity projections from brains of flies expressing *MB238Y-GAL4>UAS-CaMPARI*, quantified at right (scale bar = 10 uM, one-tailed t-test). **(C-E)** Median event durations with sucrose in control vs. hedonic food environments from sated female flies expressing *UAS-GtACR* driven by either (C) *MB131B-GAL4* (γ(d);γ(m)), (D) *MB008B-GAL4* (α/β(c);α/β(p);α/β(s)), or (E) *MB461B-GAL4* (α'/β'(ap);α'β'(m)) (two-way ANOVA with Tukey's post-hoc). **(F)** Median event durations with sucrose in control vs. hedonic food environments from sated female flies expressing *PAM-GAL4>UAS-GtACR* (two-way ANOVA with Tukey's post-hoc). **(G)** Median event durations with sucrose in control vs. hedonic food environments from 24-hr starved female flies expressing *MB461B-GAL4>UAS-GtACR* (two-way ANOVA).

To determine whether mushroom body neurons are involved in hedonic hunger beyond correlative increases in activity, we tested for their requirement in hedonic and homeostatic feeding. We used the genetically-encoded optogenetic tool GtACR to selectively inhibit mushroom body neurons in our food choice assay. GtACR is a channelrhodopsin that is activated upon green light exposure and increases chloride conductance, resulting in hyperpolarization and silencing of neuronal populations (Mohammad et al., 2017). We expressed GtACR in either the γ, α/β, or α'/β' lobes of the mushroom body (Aso et al., 2014) and found that communication from the γ and α/β lobes is dispensable for both hedonic and homeostatic feeding (Fig. 5C, 5D, Fig.5 – Fig. Supp. 1A, 1B). In contrast, signaling specifically from the α'/β' lobes was required for hedonic, but not homeostatic, feeding (Fig. 5E, Fig. 5 – Fig. Supp. 1C). The mushroom body horizonal lobes are innervated by dopaminergic neurons from the PAM cluster, which were also required for hedonic feeding (Fig. 5F) (Mao & Davis, 2009). These results indicate that a distinct PAM to α'/β' mushroom body circuit regulates hedonic feeding, which is consistent with known descriptions of anatomical and functional segregation of mushroom body lobes (Aso et al., 2014; Landayan et al., 2018).

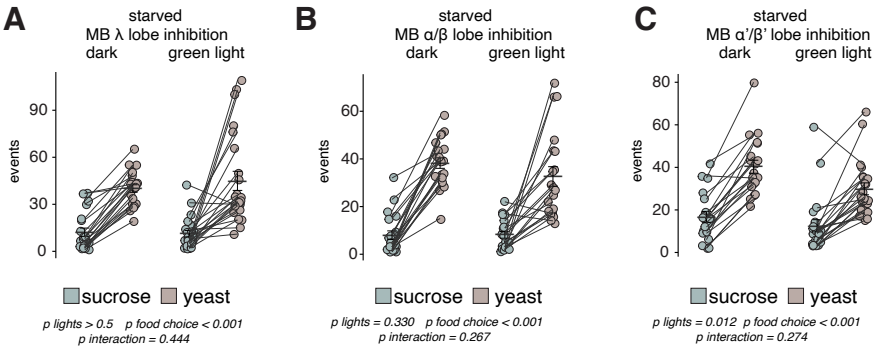

Figure 5 – Figure Supplement 1. Mushroom body lobes are not required for homeostatic hunger. (A-C) Total number of events with sucrose or yeast in the control food choice environment from 24-hr starved female flies expressing *UAS-GtACR* driven by either (A) *MB131B-GAL4* (γ(d);γ(m)), (B) *MB008B-GAL4* (α/β(c);α/β(p);α/β(s)), or (C) *MB461B-GAL4* (α'/β'(ap);α'β'(m)) (two-way ANOVA).

482 We next sought to determine whether communication from the α'/β' lobe was required for the
483 potentiating effects of homeostatic hunger on hedonic feeding. We reasoned that if the α'/β' lobe
484 also functions as a potentiator between the two hunger states, then inhibition of these neurons
485 would be expected to prevent increases in hedonic feeding when flies are starved. Instead, we
486 observed that starvation restored hedonic feeding in flies whose α'/β' lobes were inhibited (Fig.
487 5G), suggesting that potentiation of hedonic feeding by homeostatic state involves the
488 recruitment of additional and independent circuits for hedonic feeding.
489

490 **Discussion**

491 Animals exquisitely regulate feeding according to internal needs, but they are also subject to
492 their desires. Thus, food intake only sometimes occurs in response to homeostatic hunger and
493 can also occur in response to emotional stress, social environment, illness, and pursuit of
494 reward (Siemian et al., 2021; Xu et al., 2019). Because animals eat for many reasons other than
495 hunger, food intake alone may be a poor indicator of hunger states and may obscure
496 investigations into the motivational forces that drive behavior. Here, we observed the feeding
497 micro-behaviors of flies and developed a behavioral classification system to assist in the
498 identification of feeding drives. We established that flies exhibit behavioral signatures of two
499 hunger states, homeostatic and hedonic, and we used them to identify neurocircuits that are
500 required for hedonic, but not homeostatic, feeding. We also showed that homeostatic and
501 hedonic feeding are both independent and overlapping depending on environmental context,
502 and we developed a simple interaction framework that researchers can use to investigate these
503 relationships in detail. The development of this paradigm presents entry-points into the
504 discovery of new homeostatic and hedonic hunger circuits in flies, better classification of feeding
505 circuits which have already been described, and dissection of mechanistic relationships among
506 them.

507

508 The *Drosophila* MB supports a wide variety of behaviors and functions, in part due to its
509 complexity and heterogeneity in anatomical structure (Aso et al., 2009). The lobes of the MB are
510 formed by the interconnected axons of ~2000 Kenyon cells and 34 MB output neurons, and they
511 receive modulatory input from ~20 dopaminergic cell types (Aso et al., 2014). The MB lobes
512 have been anatomically and functionally divided into three classes: γ, α/β, and α'/β', which have
513 been further divided into functionally distinct compartments (Aso et al., 2014; Crittenden et al.,
514 1998; Tanaka et al., 2008). Our data suggest that compartments of the α'/β' MB lobes, but not
515 the γ or α/β lobes, are required for hedonic feeding. Our experiments isolated a discrete location
516 within the functionally heterogenous mushroom body and yet, the α'/β' lobe still contains more
517 than 350 Kenyon cells that are divided into middle (m) and anterior-posterior (ap) compartments
518 (Aso et al., 2014). Future investigations that utilize existing and emerging intersectional
519 technologies will be useful for discerning the specific cells within the α'/β' MB lobes that promote
520 hedonic feeding, as well as their modulatory inputs and outputs, and for evaluation of the cell-
521 intrinsic mechanisms that determine how hedonic hunger is encoded.

522

523 Connections from dopaminergic populations in the PAM cluster with MB-intrinsic Kenyon cells
524 have been implicated in appetitive learning, signaling reward in response to sugar, motivation to
525 overcome environmental stress, and promoting wakefulness (Burke et al., 2012; Haynes et al.,
526 2015; Hermanns et al., 2022; Liu et al., 2012). PAM projections to Kenyon cell axons in the β'
527 compartment of the MB respond to sugar by increasing calcium transients, which has led to the
528 idea that these synapses transmit information about sweet taste (Liu et al., 2012; May et al.,
529 2020). Interestingly, these responses are increased 2-fold by starvation, suggesting that they
530 scale in response to environmental context or degree of need (Liu et al., 2012), and artificial
531 activation of PAM neurons also increases foraging behavior (Tsao et al., 2018). Our finding that

532 the PAM>α'/β' circuit is required for promoting hedonic hunger builds upon a rich body of
533 literature, and together these observations offer a model that may unite the many described
534 functions of this node. One possibility is that this circuit acts as a permissive or motive force that
535 gates or fuels downstream mechanisms involved in behavioral responses to environmental
536 stimuli. Indeed, learning, reward, and wakefulness are all processes that might benefit from or
537 require a force that sustains them. This view offers an attractive entry into understanding the
538 seat of motivation in the fly brain.
539
540 Decades of research on the neural control of feeding have resulted in the identification of
541 mammalian neurocircuits involved in the control of feeding, and technological advances have
542 allowed researchers to manipulate them with an increasing degree of specificity. We are,
543 however, approaching the limits of what we can learn from manipulations and technologies of
544 these sorts and thus, complimenting them with discoveries from model systems in which more
545 specific manipulations can be tested may accelerate our ability to understand how the brain
546 generates motivated states. The hedonic circuits we have identified here in flies, for example,
547 can be immediately investigated at multiple levels to assess the neuronal and molecular
548 mechanisms that determine their role in feeding, including spatiotemporal manipulation of cell-
549 signaling pathways, electrophysiological recordings in live animals in response to various food
550 environments or energetic deficits, and optogenetic manipulation paired with close behavioral
551 monitoring. These types of experiments would fulfill several main goals in the study of the neural
552 control of appetite regulation (Ahn et al., 2022) and, despite differences in the precise identity of
553 feeding neurocircuits between flies and mammals, discoveries about foundational principles and
554 conserved signaling pathways may inform and direct mammalian studies.
555

556 **Materials and Methods**

557 **Fly stocks and husbandry**

558 Fly stocks were maintained on a standard cornmeal-based larval growth medium and in a
559 controlled environment (21C, 60% humidity) with a 12:12hr light:dark cycle. We controlled the
560 developmental larval density by manually aliquoting 32 ul of collected eggs into individual
561 bottles containing 25-50mL of food at 25C. For FLIC experiments, following eclosion mixed sex
562 flies were kept on cornmeal medium at 18C until they were 7-14 days old. One day prior to the
563 experiments, flies were sorted by sex and transferred onto either SY10 medium (10% [w/v]
564 sucrose and 10% [w/v] yeast) or wet starved (1 Kimwipe with 2mL of H20) at 25C. Experiments
565 that used flies starved for 48 hours were sorted to starvation vials and kept at 25C two days
566 prior to the experiments. We used mated female or male flies that were between 7-14 days old
567 for all experiments. The following stocks were used for experiments: *Canton-S, UAS-CaMPARI*
568 (BDSC #68762)*, MB238Y-GAL4* (BDSC #81009)*, MB131B-GAL4* (BDSC #68265)*, MB008B-*
569 *GAL4* (BDSC #68291)*, MB461B-GAL4* (BDSC #68327)*, GMR58E02-GAL4* (BDSC #41347)*,*
570 and *LexAop2-CsChrimson;UAS-CaMPARI2;GMR57C10-GAL4* (BDSC #81089) were obtained
571 from the Bloomington *Drosophila* Stock Center. *UAS-GtACR* was provided by M. Dus
572 (University of Michigan, MI).

573

574 **Video Recording and Scoring**

575 The video recording setup for initial behavioral characterizations (Fig. 1B, 1C, Supp. Table 1)
576 consisted of the Fly Liquid-Food Counter (FLIC), a custom single-well enclosure, and a light to
577 illuminate the recording area. A FLIR Grasshopper camera (GS3-U3-28S5C-C) fitted with a
578 Fujinon varifocal lens (MFR #DV3.4x3.8SA-1) was positioned against the lid to record the FLIC
579 well and the surrounding enclosed area. Video recording and FLIC data collection were started
580 simultaneously, and video length was dependent on the maximum number of frames that could
581 be stored. This setup recorded continuous video of the fly and saved this footage unaltered. For

582 experiments in Figures 1D-1F, we developed a direct communication link between our custom
583 DTrack video software and the FLIC system to trigger video capture only when there was
584 physical contact between a fly and the food source, together with 5s windows before and after
585 the contact (see Fig. 1A).
586
587 Videos of sated or 24-hour wet-starved male and female Canton-S flies were recorded. A single
588 fly was aspirated into the FLIC, which contained 5% or 10% sucrose food solution in 4 mg/l
589 $MgCl_2$. Videos were recorded at various times of day between 10AM and 7PM and had an
590 average length of about 51 minutes. Videos collected for circadian analysis were recorded over
591 a 24-hour period, and "meal-times" were defined as 7AM-10AM and 7PM-10PM. All videos were
592 recorded on the lab bench. Manual, frame-by-frame annotation was used to classify user-
593 defined behaviors. Experimenters were blinded to the treatment for video scoring. Data was
594 analyzed using the open-source JWatcher software and R studio.
595
596 **Fly Liquid-Food Interaction Counter (FLIC) assays**
597 Flies were tested on the Fly Liquid-Food Interaction Counter (FLIC) system as previously
598 described (Ro et al., 2014) (Sable Systems International). FLIC *Drosophila* Feeding Monitors
599 (DFMs, Sable Systems International, model DFMV3) were used in the two-choice configuration
600 and each chamber was loaded with food solutions containing a sugar choice (1%-20% sucrose,
601 [18% arabinose + 2% sucrose], or [18% sorbitol + 2% sucrose]) and a yeast choice ([2% bacto
602 yeast extract + 1% sucrose]) in 4 mg/l $MgCl_2$. Flies were briefly anesthetized on ice and
603 aspirated into the DFM chambers, ensuring that each DFM received flies from all treatments.
604 We began recording immediately after loading flies (generally, loading all DFMs requires <10
605 minutes) and measured FLIC interactions for 6 hours. Each FLIC experiment contains pooled
606 data from two independent replicates. FLIC data were analyzed using custom R code, which is
607 available at https://github.com/PletcherLab/FLIC_R_Code. Default thresholds were used for

608 analysis except for the following: minimum feeding threshold = 10, tasting threshold = (0,10).
609 Animals that did not participate (i.e. returned zero values), or whose DFM returned an unstable
610 baseline signal, were excluded from analysis.
611
612 **Optogenetic FLIC Assays**
613 Flies expressing UAS-GtACR in desired neuronal populations were reared as described above.
614 When 7-14 days old, flies were sorted to cornmeal medium containing 200 μm all-*trans*-retinal
615 (from a stock solution of 100 mM ATR in 100% ethanol) and kept in the dark for 1-4 days at
616 18C. Flies were shifted to 25C for 24 hours before the experiment and flipped to either SY10
617 media containing 800 μm all-*trans*-retinal or starvation vials. Custom FLIC lids were fitted with
618 530 nm LEDs (Luxeon). Custom hardware and firmware were designed to allow the
619 experimenter to control LED intensity and a range of light stimulus parameters. We used a
620 stimulus frequency of 40 Hz and a pulse width of 800 ms for the entire duration of the 6 hour
621 FLIC experiment.
622
623 **CaMPARI**
624 CaMPARI photoconversion was carried out according to previously established protocols with
625 some modifications (Edwards et al., 2020). A 405 nM LED (Thorlabs M405L3) was connected to
626 a 1.2A LED driver (Thor Labs LEDD1B). Custom hardware and firmware were designed to allow
627 the experimenter to control LED intensity and a range of light stimulus parameters. Female flies
628 expressing *UAS-CaMPARI* were reared as described above. Custom choice MOCAPS were
629 filled with fresh SY10% on one side and either 2% or 20% sucrose in 2% bacto agar on the
630 other side. Groups of 5 flies were briefly anesthetized on ice and aspirated into the appropriate
631 food environments. A custom clear cap was placed on top of the mocap to enclose the flies. The
632 405 nM LED was placed on top (~1/4 inch above the food surface) and the flies were allowed to

633 habituate to the environment for 1 hour. After habituation, flies received UV light stimulation for
634 1 hour (cycling 10s on and 10s off, 1.0A). Immediately following light stimulation, flies were
635 protected from light and brains were dissected as previously described (Wu & Luo, 2006).
636 Brains were mounted between a glass microscope slide and a #1.5 cover glass separated by a
637 custom bridge in VECTASHIELD Antifade Mounting Medium (Vector Laboratories). Samples
638 were imaged on a Nikon A1 Confocal Microscope with a 20X air objective using 488 nm and
639 561 nm laser lines. Z-stacks were collected at a 1-2 μm step. All treatments were mounted
640 under the same cover slip. Image processing was performed using ImageJ (NIH), and the
641 experimenter was blinded to treatment. Slices containing the ROI were identified and collapsed
642 into an average intensity projection. ROIs were measured in the red and green channel after
643 background subtraction, and a ratio of red:green fluorescence is reported.
644
645 **Statistics**
646 For comparisons involving only one level, we used Student's t-test to detect significant
647 differences between two treatments or one-way ANOVA followed by Tukey's post-hoc test after
648 verifying normality and equality of variances. T-tests were two-tailed during initial
649 characterization experiments, or one-tailed in future experiments where the predicted direction
650 of change was known. For comparisons involving more than one level, we used two-way
651 ANOVA to detect significant interactions between the levels and followed up with Tukey's post-
652 hoc when significance was detected ($p<0.05$). In cases where experimental replicates were
653 pooled, a two-way ANOVA with blocking for experiment was performed to ensure non-
654 significant experimental effects. For all dot and bar plots, error bars represent the SEM. All
655 statistical tests and graphing were performed using R. Specific details of statistical analyses are
656 presented in the figure legends.
657

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Author contributions:

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Methodology: KJW, SDP

Investigation: KJW, SR, RR, TC, RAH

Visualization: KJW

Funding acquisition: KJW, SDP

Project administration: KJW, SDP

Supervision: KJW, SDP

Writing – original draft: KJW, SDP

Writing – review & editing: KJW, SDP

Competing interests: The senior author (S.D.P) is a share holder in the company, Flidea, which has developed technology related to the FLIC feeding system. **Data and materials availability:** All data generated during this study are provided with the manuscript. FLIC data were analyzed using custom R code, which is available at https://github.com/PletcherLab/FLIC_R_Code.

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