Taste triggers a homeostatic temperature control in hungry flies

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SUMMARY

Hungry animals consistently show a desire to obtain food. Even a brief sensory detection of food can trigger bursts of physiological and behavioral changes. However, the underlying mechanisms by which the sensation of food triggers the acute behavioral response remain elusive. We have previously shown in *Drosophila* that hunger drives a preference for low temperature. Because *Drosophila* is a small ectotherm, a preference for low temperature implies a low body temperature and a low metabolic rate. Here, we show that taste sensing triggers a switch from a low to a high temperature preference in hungry flies. We show that taste stimulation by artificial sweeteners or optogenetics triggers an acute warm preference, but is not sufficient to reach the fed state. Instead, nutrient intake is required to reach the fed state. The data suggest that starvation recovery is controlled by two components: taste-evoked and nutrient-induced warm preferences, and that taste and nutrient quality play distinct roles in starvation recovery. Animals are motivated to eat based on time of day or hunger. We found that clock genes and hunger signals profoundly control the taste-evoked warm preferences. Thus, our data suggest that the taste-evoked response is one of the critical layers of regulatory mechanisms representing internal energy homeostasis and metabolism.

INTRODUCTION

Animals are constantly sensing environmental stimuli and changing their behavior or physiology based on their internal state [1-7]. Hungry animals are strongly attracted to food. Immediately after seeing, smelling, or chewing food, even without absorbing nutrients, a burst of physiological changes is suddenly initiated in the body. These responses are known in mammals as the cephalic phase response (CPR) [8]. For example, a flood of saliva and gastrointestinal secretions prepares hungry animals to digest food [9-13]. Starvation results in lower body temperatures, and chewing food triggers a rapid increase in heat production, demonstrating CPR in thermogenesis [14-16]. However, the underlying mechanisms of how the sensation of food without nutrients triggers the behavioral response remain unclear.

To address this question, we used a relatively simple and versatile model organism, *Drosophila melanogaster*. Flies exhibit robust temperature preference behavior [17,18]. Due to the low mass of small ectotherms, the source of temperature comes from the environment. Therefore, their body temperatures are close to the ambient temperature [19,20]. For temperature regulation, animals are not simply passive receivers of ambient temperature. Instead, they actively choose an ambient temperature based on their internal state. For example, we have shown that preferred temperature increases during the daytime and decreases during the night time, exhibiting circadian rhythms of temperature preference (temperature preference rhythms: TPR) [21]. Because their surrounding temperature is very close to their body temperature, TPR leads to body temperature rhythms (BTR) that is very similar to mammalian BTR [22,23]. Another example is the starvation. Hunger stress forces flies to change their behavior and physiological response [24,25]. We previously showed that the hungry flies prefer a lower temperature [26]. The flies in a lower environmental temperature has been shown to have a lower metabolic rate, and the flies in a higher environmental temperature

72 has been shown to have a higher metabolic rate [26-28]. Therefore, hungry flies choose a lower
73 temperature and therefore, their metabolic rate is lower. Similarly, in mammals, starvation causes
74 a lower body temperature, hypothermia [6]. In mammals, body temperature is controlled by the
75 balance between heat loss and heat production. The starved mammals have been shown a lower
76 heat production [5-7]. Therefore, both flies and mammals, the starvation causes a low body
77 temperature.

78 The flies exhibit robust feeding behaviors [29,30] and molecular and neural mechanisms of
79 taste are well documented [2,31-36]. Therefore, we focused on taste and temperature regulation and
80 asked how the taste cue triggers a robust behavioral recovery of temperature preference in starving
81 flies. We show in hungry flies that taste without nutrients induces a switch from a low to a high
82 temperature preference. While taste leads to a warmer temperature preference, nutrient intake
83 causes the flies to prefer an even warmer temperature. This nutrient-induced warm preference
84 results in a complete recovery from starvation. Thus, taste-evoked warm preference is different
85 from nutrient-induced warm preference and potentially similar physiology as CPR. Therefore,
86 when animals emerge from starvation, they use a two-step approach to recovery: taste-evoked and
87 nutrient-induced warm preference. While a rapid component is elicited by food taste alone, a
88 slower component requires nutrient intake.

89 Animals are motivated to eat based on their internal state, such as time of day or degree of
90 hunger. The circadian clock drives daily feeding rhythms [37-39] and anticipates meal timing. Daily
91 feeding timing influences energy homeostasis and metabolism [40,41]. Circadian clocks control
92 feeding behavior in part via orexigenic peptidergic/hormonal regulation such as neuropeptide Y
93 (NPY) and agouti-related peptide (AgRP) neurons, which are critical for regulating feeding and
94 metabolism [39,42]. Feeding-fasting cycles modulate peripheral organs in liver, gut, pancreas, and so

on [43], suggesting that circadian clocks, peptidergic/hormonal signals, and peripheral organs are organically coordinated to enable animals maintain their internal states constantly. We found that clock genes and hunger signals are strongly required for taste-evoked warm preference. The data suggest that taste-evoked response is an indispensable physiological response that represents internal state. Taken together, our data shed new light on the role of the taste-evoked response and highlight a crucial aspect of our understanding of feeding state and energy homeostasis.

122 **RESULTS**

123 **Food detection triggers a warm preference.**

124 To investigate how food detection influences temperature preference behavior in *Drosophila* [17,18],
125 the *white*^{1118} (*w*^{1118}) control flies were fed fly food containing carbohydrate, protein, and fat
126 sources (Fig. 1A and S1) and tested in temperature preference behavioral assays (Fig. S1) [26,44].
127 The flies are released into a chamber set to a temperature range of 16-34°C and subsequently
128 accumulate at their preferred temperature (Tp) at 25.2±0.2 °C within 30 minutes (min) (Fig. 1B:
129 fed, white bar). On the other hand, when *w*^{1118} flies were starved overnight with water only, they
130 preferred 21.7±0.3 °C (Fig. 1A and 1B: overnight starvation (STV), gray bar). Thus, starvation
131 leads to a lower Tp. As we have previously reported, starvation strongly influences temperature
132 preference [26].

133 To examine how starved flies recover from lower Tp, they were offered fly food for 5 min,
134 10 min, 30 min, and 1 hr (Fig. 1A). Immediately after the flies were refed, the temperature
135 preference behavior assay was examined. The assay takes 30 min from the time the flies are placed
136 in the apparatus until the final choice is made. For example, in the case of 5 min refed flies, it took
137 a total of 35 min from the start of refeeding to the end of the assay. After 10 min, 30 min, or 1 hr
138 of fly food refeeding, starved flies preferred a temperature similar to that of fed flies (Fig. 1B, 1F,
139 and S2: statistics shown as red stars, Table S1), suggesting a full recovery from starvation. On the
140 other hand, refeeding after 5 min resulted in a warmer temperature than the starved flies.
141 Nevertheless, Tp did not reach that of the fed flies (Fig. 1B, 1F, and S2). Therefore, refeeding the
142 flies for 5 min resulted in a partial recovery from the starved state (Fig. 1B, 1F, and S2, statistics
143 shown as green stars, Table S1). Thus, our data suggest that food intake triggers a warm preference
144 in starved flies.

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147 **Sucralose refeeding promotes a warm preference.**

148 While only 5 min of refeeding fly food caused hungry flies to prefer a slightly warmer temperature,
149 10 min of refeeding caused hungry flies prefer a similarly warmer temperature as the fed flies (Fig.
150 1B, 1F, and S2). Therefore, we hypothesized that food-sensing cues might be important for the
151 warm preference. Sucralose is an artificial sweetener that activates sweet taste neurons in
152 *Drosophila* [45] and modulates taste behaviors such as the proboscis extension reflex [46-49].
153 Importantly, sucralose is a non-metabolizable sugar and has no calories. Therefore, to investigate
154 how food-sensing cues are involved in warm preference, we examined how sucralose refeeding
155 changes the temperature preference of starved flies. After starved flies were refed sucralose for 10
156 min or 1 hr (Fig. 1A), they preferred a warmer temperature; however, Tp was halfway between
157 Tps of fed and starved flies (Fig. 1C, F and S2: blue, Table S1). Thus, sucralose ingestion induces
158 a warm preference but shows a partial recovery of Tp from the starved state.

159 While refeeding the flies with food resulted in a full recovery of Tp from the starved state,
160 refeeding them with sucralose resulted in only a partial recovery of Tp. Therefore, starved flies
161 may use both taste cues and nutrients to fully recover Tp from the starved state. To evaluate this
162 possibility, we used glucose, which contains both sweetness (gustatory cues) and nutrients (i.e.,
163 metabolizable sugars) (Fig. 1A), and tested glucose refeeding for 5 min, 10 min, and 1 hr. We
164 found that 10 min or 1 hr glucose refeeding resulted in full recovery of Tp from the starved state
165 (Fig. 1D, 1F and S2: statistics shown in red NS, Table S1) and was significantly different from
166 starved flies (Fig. 1D, 1F and S2: statistics shown in green stars, Table S1). Thus, our data showed

167 that sucralose refeeding induced partial recovery and glucose refeeding induced full recovery from
168 the starved state.

169 It is still possible that the starved flies consumed glucose faster than sucralose during the
170 first 10 min, which could result in a different warming preference. To rule out this possibility, we
171 examined how often starved flies touched glucose, sucralose, or water during the 30 min using the
172 Fly Liquid-food Interaction Counter (FLIC) system [50]. The FLIC system assays allow us to monitor
173 how much interaction between the fly and the liquid food reflects feeding episodes. We found that
174 starved flies touched glucose and sucralose food at similar frequencies and more frequently than
175 water during the 30 min test period (Fig. 1G, Table S1). The data suggest that flies are likely to
176 feed on glucose and sucralose at similar rates. Therefore, we concluded that the differential effect
177 of sucralose and glucose refeeding on temperature preference was not due to differences in feeding
178 rate.

179 Furthermore, to confirm that sweet taste is more important than sugar structure, we instead
180 used fructose, another simple sugar that contains sweetness and nutrients. Glucose and fructose
181 are monosaccharides and a member of hexose and pentose, respectively. We tested fructose
182 refeeding for 10 min and 1 hr. We found that 10 min of fructose refeeding resulted in full recovery
183 of Tp from the starved state (Fig. 1E, 1F and S2: statistics shown in red NS, Table S1), and 10 min
184 and 1 hr fructose refeeding were significantly different from starved flies (Fig. 1E, 1F and S2:
185 statistics shown in green stars, Table S1). The data suggest that sweet taste is more important than
186 the structures of the sugar compounds.

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189 **Activation of sweet taste neurons leads to warm preference.**

190 To determine how taste elicits a warm preference, we focused on the sweet gustatory receptors
191 (Grs), which detect sweet taste. We used sweet Gr mutants and asked whether sweet Grs are
192 involved in taste-evoked warm preference. Two different sweet Gr mutants, *Gr5a*$^{-/-}$*; Gr64a*$^{-/-}$ and
193 *Gr5a*$^{-/-}$*;;Gr61a*$^{-/-}$*, Gr64a-f*$^{-/-}$, are known to reduce sugar sensitivity compared to the control
194 [47,48,51,52]. We found that sweet Gr mutant flies exhibited a normal starvation response in which the
195 Tp of starved flies was lower than that of fed flies (Fig. 2A and 2B, white and gray bars, statistics
196 shown as green stars, Table S1). However, starved sweet Gr mutant flies did not increase Tp after
197 10 min sucralose refeeding (Fig. 2A and 2B, blue bars, statistics shown as green and red stars,
198 Table S1). These data suggest that sweet Grs are involved in taste-evoked warm preference.

199 Sweet Grs are expressed in the sweet Gr-expressing neurons (GRNs) located in the
200 proboscis and forelegs [52,53]. To determine whether sweet GRNs are involved in taste-evoked warm
201 preference, we silenced all sweet GRNs. We expressed the inwardly rectifying K^+ channel Kir2.1
202 (*uas-Kir*) [54] using *Gr64f-Gal4*, which is expressed in all sweet GRNs in the proboscis and forelegs
203 [47,52,53]. Inactivation of all sweet GRNs showed a normal starvation response (Fig. 2C, white and
204 gray bars, statistics shown as green stars). However, flies silencing all sweet GRNs failed to show
205 a warm preference after 10 min of sucralose refeeding (Fig. 2C, blue bar, statistics shown as green
206 and red stars, Table S1). This phenotype was similar to the data obtained with the sweet Gr mutant
207 strains (Fig. 2A and 2B). On the other hand, control flies (*Gr64f-Gal4/+* and *uas-Kir/+*) showed a
208 normal starvation response and a taste-evoked warm preference (Fig. 2D and 2E, gray and blue
209 bars, respectively, statistics shown as green and red stars, Table S1). Thus, our data indicate that
210 sweet GRNs are required for taste-evoked warm preference.

211 To further investigate whether activation of sweet GRNs induces a warm preference, we
212 used the optogenetic approach, a red light sensitive channelrhodopsin, CsChrimson [55,56]. Starved
213 flies were given water containing 0.8 mM all-trans-retinal (ATR), the chromophore required for
214 CsChrimson activation. These flies were not fed sucralose; instead, gustatory neurons in starved
215 flies were excited by red light pulses (flashing on and off at 10 Hz) for 10 min (Fig. 2F). In this
216 case, although the flies were not refed, the gustatory neurons were artificially excited by
217 CsChrimson activation so that we could evaluate the effect of excitation of sweet GRNs on taste-
218 evoked warm preference.

219 CsChrimson was expressed in sweet GRNs in the proboscis and legs (all sweet GRNs)
220 using *Gr64f-Gal4*. These flies showed a normal starvation response (Fig. 2G-2J, white and gray
221 bars, statistics shown as green and red stars, Table S1). Excitation of all sweet GRNs by red light
222 pulses elicited a warm preference, and Tp was intermediate between fed and starved flies,
223 suggesting partial recovery (Fig. 2G, yellow bar, statistics shown as green and red stars, Fig. S2B
224 Table S1). However, neither excitation of Gr5a- (Fig. 2H) nor Gr64a-expressing neurons (Fig. 2I)
225 induced a warm preference (yellow bars, Table S1). While *Gr64a-Gal4* is expressed only in the
226 legs, *Gr5a-Gal4* is expressed in the proboscis and legs, but does not cover all sweet GRNs like
227 *Gr64f-Gal4* [52,53]. Notably, control flies (*UAS-CsChrimson/+*) did not show a warm preference to
228 red light pulses with ATR application (Fig. 2J). Taken together, our data suggest that excitation of
229 all sweet GRNs results in a warm preference.

230 We next asked whether the sweet Grs contribute to the nutrient-induced warm preference.
231 We found that all these starved flies did not increase Tp after 10 min of glucose intake (Fig. 2A-
232 2C, green bars, statistics shown as green and red stars, Table S1). All control flies showed normal
233 responses to 10 min of glucose refeeding (Fig. 2D and 2E, green bars, Table S1). The data suggest

234 that the sweet Grs which we tested are potentially expressed in tissues/neurons required for internal
235 nutrient sensing. Notably, we found that flies increased Tp after 10 min of refeeding with fly food
236 containing carbohydrate, fat, and protein (Fig. 2A-2C, orange bars, statistics shown as green and
237 red stars, Table S1). All control flies showed normal responses to 10 min of fly food intake (Fig.
238 2D and 2E, orange bars, Table S1). The data suggest that gustatory neurons are required for warm
239 preference in carbohydrate refeeding, but not for other nutrients such as fat or protein (see
240 Discussion). Because flies have sensory neurons that detect fatty acids [57-59] or amino acids [60-63],
241 these neurons may drive the response to fly food intake. This is likely why the sweet-insensitive
242 flies can still recover after eating fly food (Fig 2A-2C, orange bars).

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245 **The temperature-sensing neurons are involved in taste-evoked warm preference.**

246 The warm-sensing neurons, anterior cells (ACs), and the cold-sensing *R11F02-Gal4*-expressing
247 neurons control temperature preference behavior [26,44,64]. Small ectotherms such as *Drosophila* set
248 their Tp to avoid noxious temperatures using temperature information from cold- and warm-
249 sensing neurons [17,18,44]. We have previously shown that starved flies choose a lower Tp, the so-
250 called hunger-driven lower Tp [26]. ACs control the hunger-driven lower Tp, but cold-sensing
251 *R11F02-Gal4*-expressing neurons do not [26]. ACs express transient receptor potential A1 (TrpA1),
252 which responds to a warm temperature >25 °C [44,65]. The set point of ACs in fed flies, which is ~25
253 °C, is lowered in starved flies. Therefore, the lower set point of ACs corresponds to the lower Tp
254 in starved flies.

255 First, we asked whether ACs are involved in taste-evoked warm preference. Because the
256 ACs are important for the hunger-driven lower Tp [26], the AC-silenced flies did not show a
257 significant difference in Tp between fed and starved conditions for only one overnight of starvation
258 [26]. Therefore, we first extended the starvation time to two overnights so that the AC-silenced flies
259 showed a significant difference in Tp between fed and starved conditions (Fig. 3A, white and gray
260 bars, statistics shown as green and red stars, Table S1). Importantly, longer periods of starvation
261 do not affect the ability of w^{1118} flies to recover (Fig. S3).

262 To examine whether ACs regulate taste-evoked warm preference, we refed sucralose to
263 AC-silenced flies for 10 min. We found that the Tp of the refed flies was still similar to that of the
264 starved flies (Fig. 3A, blue bar, statistics shown as green and red stars, Table S1), indicating that
265 sucralose refeeding could not restore Tp and that ACs are involved in taste-evoked warm
266 preference. Significantly, even when the AC-silenced flies were starved for two overnights, they
267 were able to recover Tp to the normal food for 10 min and to glucose for 1 hr (Fig. 3A, orange and
268 green bars), suggesting that the starved AC-silenced flies were still capable of recovery.

269 Other temperature-sensing neurons involved in temperature preference behavior are cold-
270 sensing *R11F02-Gal4*-expressing neurons [26,64]. To determine whether *R11F02-Gal4*-expressing
271 neurons are involved in taste-evoked warm preference, we silenced *R11F02-Gal4*-expressing
272 neurons using *uas-Kir*. We found that the flies showed a significant difference in Tp between fed
273 and starved conditions, but the flies did not show a warm preference upon sucralose refeeding (Fig.
274 3B, gray and blue bars, statistics shown as green and red stars, Table S1). As controls, $TrpA1^{SH}$-
275 *Gal4/+*, *R11F02-Gal4/+* and *uas-Kir/+* flies showed normal starvation response and taste-evoked
276 warm preference (Fig. 3C-3E).

277 To further ensure the results, we used optogenetics to artificially excite warm and cold
278 neurons with *TrpA1*^{SH}*-Gal4* and *R11F02-Gal4*, respectively, by red light pulses for 10 min. We
279 compared the Tp of starved flies with and without ATR under red light. Tp of starved flies with
280 ATR was significantly increased (Fig. 3F and 3G, yellow bars, statistics shown as green and red
281 stars, Table S1) compared to those without ATR (Fig. 3F and 3G, gray bars, Table S1). Therefore,
282 these data indicate that ACs and *R11F02-Gal4*-expressing neurons are required for taste-evoked
283 warm preference.

284 Next, we asked whether temperature-sensitive neurons contribute to the nutrient-induced
285 warm preference. We used the warm- or cold neuron silenced flies (*TrpA1*^{SH}*-Gal4* or *R11F02-*
286 *Gal4>uas-Kir*) and found that all these starved flies did not increase Tp after 10 min glucose intake
287 (Fig. 3A and 3B, green bars, Table S1), but increased Tp after 10 min refeeding with fly food
288 containing carbohydrate, fat, and protein (Fig. 3A and 3B, orange bars, Table S1). Notably, AC-
289 silenced flies increased Tp after 1 hr glucose intake (Fig. 3A, green bars, Table S1). All control
290 flies showed normal responses to both 10 min glucose refeeding and fly food intake (Fig. 3C-3E,
291 green bars, Table S1). The data suggest that temperature-sensing neurons are required for warm
292 preference in carbohydrate refeeding, but not in other foods such as fat or protein (see Discussion).

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295 **Olfaction is possibly involved in a warm preference for hungry flies.**

296 We also investigated the potential effects of olfaction. We used mutants of the odorant receptor co-
297 receptor, *Orco* (*Orco*^{1}), which has an olfactory defect [66]. We found that the flies showed a
298 significant difference in Tp between fed and starved conditions, but the flies did not show a warm

299 preference upon sucralose refeeding (Fig. S4A and S4C, gray and blue bars, statistics shown as
300 green and red stars, Table S1). We found that all of these starved flies increased Tp after 10 min of
301 glucose or fly food intake and showed a full recovery after 1 hr of glucose intake (Fig. S4B and
302 S4C, orange and green bars, statistics shown as green and red stars, Table S1). The data suggest
303 that olfaction may be involved in the warm preference in sucralose refeeding.

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306 **Internal state influences taste-evoked warm preference in hungry flies.**

307 Internal state strongly influences motivation to feed. However, how internal state influences
308 starving animals to exhibit a food response remains unclear. Hunger represents the food-deficient
309 state in the body, which induces the release of hunger signals such as neuropeptide Y (NPY). NPY
310 promotes foraging and feeding behavior in mammals and flies [67]. While intracerebroventricular
311 injection of NPY induces the cephalic phase response (CPR) [68], injection of NPY antagonists
312 suppresses CPR in dogs, suggesting that NPY is a regulator of CPR in mammals [69]. Therefore, we
313 first focused on neuropeptide F (NPF) and small neuropeptide F (sNPF), which are the *Drosophila*
314 homologue and orthologue of mammalian NPY, respectively [67], and asked whether they are
315 involved in taste-evoked warm preference. In *NPF* mutant ($NPF^{-/-}$) or *sNPF* hypomorph (*sNPF*
316 *hypo*) mutant, we found that the Tp of fed and starved flies were significantly different, showing a
317 normal starved response (Fig. 4A and 4B: white and gray bars, statistics shown as green and red
318 stars, Table S1). However, they failed to show a taste-evoked warm preference after 10 min of
319 sucralose refeeding (Fig. 4A and 4B: blue bars, statistics shown as green and red stars, Table S1).
320 Thus, NPF and sNPF are required for taste-evoked warm preference after sucralose refeeding.

321 We next asked whether NPF and sNPF are involved in the nutrient-induced warm preference
322 during glucose refeeding. We found that starved *NPF*$^{-/-}$ mutants significantly increased Tp after 1
323 hr of glucose refeeding (Fig. 4A, dark green bar, statistics shown as green and red stars, Table S1).
324 We also found that starved *sNPF hypo* mutants significantly increased Tp after 10 min and 1 hr of
325 glucose refeeding (Fig. 4B, green and dark green bars, statistics shown as green and red stars, Table
326 S1). In addition, both *NPF*$^{-/-}$ and *sNPF hypo* mutants increased Tp after 10 min of fly food
327 refeeding (Fig. 4A and 4B, orange bars, statistics shown as green and red stars, Table S1). However,
328 when these flies were fed both glucose and fly food, they did not reach the same Tp as the fed flies.
329 These data suggest that NPF and sNPF also play a role in the modulation of nutrient-induced warm
330 preference.
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333 **Factors involving hunger regulate taste-evoked warm preference.**
334 Based on the above results, we hypothesized that hunger signals might be involved in taste-evoked
335 warm preference. To test this hypothesis, we focused on several factors involved in the hunger
336 state. The major hunger signals [24], diuretic hormone 44 (DH44), and adipokinetic hormone (AKH)
337 are the mammalian corticotropin-releasing hormone homologue [70] and the functional glucagon
338 homologue, respectively [71,72]. We found that DH44- or AKH-expressing neuron silenced flies
339 failed to show a taste-evoked warm preference after sucralose refeeding. However, they were able
340 to increase Tp after 10 min glucose or fly food refeeding (Fig. 4D and 4F, green and orange bars,
341 statistics shown as green and red stars, Table S1), suggesting a normal nutrient-induced warm
342 preference. The control flies (*Dh44-Gal4/+*, *Akh-Gal4/+*, and *UAS-Kir/+*) showed a normal warm
343 preference to sucralose (Fig. 4C, 4E and 4J, blue bars, statistics shown as green and red stars, Table

344 S1), glucose (Fig. 4C, 4E and 4J, green bars, statistics shown as green and red stars, Table S1), and
345 normal fly food refeeding (Fig. 4C, 4E and 4J, orange bars, statistics shown as green and red stars,
346 Table S1). The data suggest that DH44 or AKH neurons are required for taste-evoked warm
347 preference, but not for nutrient-induced warm preference.

348 Insulin-like peptide 6 (Ilp6) is a homologue of mammalian insulin-like growth factor 1
349 (IGF1), and *ilp6* mRNA expression is increased in starved flies [73-75]. Because Ilp6 is important for
350 the hunger-driven lower Tp [26], the *ilp6* mutants did not show a significant difference in Tp between
351 fed and starved conditions for only one overnight starvation. Therefore, we first extended the
352 starvation time to three overnights. We found that the *ilp6* loss-of-function (*ilp6 LOF*) mutant
353 failed to show a taste-evoked warm preference after sucralose refeeding (Fig. 4G, blue bars,
354 statistics shown as green and red stars, Table S1), but did show a nutrient-induced warm preference
355 after glucose refeeding (Fig. 4G, green bars, statistics shown as green and red stars, Table S1).
356 These data suggest that Ilp6 is required for taste-evoked warm preference but not for nutrient-
357 induced warm preference after glucose or fly food refeeding.

358 Unpaired3 (Upd3) is a *Drosophila* cytokine that is upregulated under nutritional stress [76].
359 We found that the *upd3* mutants failed to show a taste-evoked warm preference after sucralose
360 refeeding (Fig. 4H, blue bar, statistics shown as green and red stars, Table S1), but showed a
361 nutrient-induced warm preference after glucose or normal food refeeding (Fig. 4H, green and
362 orange bars, statistics shown as green and red stars, Table S1). These data suggest that Upd3 is
363 required for taste-evoked warm preference, but not for nutrient-induced warm preference after
364 glucose or normal food refeeding.

365 We also examined the role of the satiety factor Unpaired2 (Upd2), a functional leptin
366 homologue in flies [77], in taste-evoked warm preference. The *upd2* mutants failed to show a warm

367 preference after refeeding of sucralose, glucose or fly food (Fig. 4I, blue, green and orange bars,
368 statistics shown as green and red stars, Table S1). Thus, our data suggest that factors involved in
369 the hunger state are required for taste-evoked warm preference.

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371

372 **Flies show a taste-evoked warm preference at all times of the day.**

373 Animals anticipate feeding schedules at a time of day that is tightly controlled by the circadian
374 clock [41]. Flies show a rhythmic feeding pattern: one peak in the morning [50] or two peaks in the
375 morning and evening [78]. Because food cues induce a warm preference, we wondered whether
376 feeding rhythm and taste-evoked warm preference are coordinated. If so, they should show a
377 parallel phenotype.

378 Since flies exhibit one of the circadian outputs, the temperature preference rhythm (TPR)
379 [21], Tp gradually increases during the day and peaks in the evening. First, we tested starvation
380 responses at Zeitgeber time (ZT)1-3, 4-6, 7-9, and 10-12 under light and dark (LD) conditions,
381 with flies being offered only water for 24 h prior to the experiments at each time point. We found
382 that both w^{1118} and *yellow*^{1} *white*^{1} ($y^{1}w^{1}$) flies had higher Tp in the fed state and lower Tp in the
383 starved state at all times of the day (Fig. 5A and 5B: black and gray lines; Table S1). Next, we
384 refed sucralose to starved flies at all time points tested and examined taste-evoked warm preference.
385 While starved $y^{1}w^{1}$ flies showed a taste-evoked warm preference at all time points (Fig. 5B1, gray
386 and blue lines, Table S1), starved w^{1118} flies showed a significant taste-evoked warm preference at
387 ZT4-6 and 10-12 (Fig. 5A1, gray and blue lines, Table S1).

388 Because starved w^{1118} flies showed an advanced phase shift of TPR with a peak at ZT7-9
389 (Fig. 5A, gray), it is likely that the highest Tp simply masks the taste-evoked warm preference at
390 ZT7-9. We also focused on nutrient- (carbohydrate-) induced warm preference. Starved w^{1118} and
391 y^1w^1 flies successfully increased Tp after 10 min of glucose refeeding (Fig. 5A2 and B2, green line,
392 Table S1). Glucose refeeding for 1 hr resulted in Tp similar to that of fed w^{1118} flies (Fig. 5A3, dark
393 green line). Because the feeding rhythm peaks in the morning or morning/evening [50,78], our data
394 suggest that the feeding rhythm and taste-evoked warm preference do not occur in parallel.

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397 **Circadian clock genes are required for taste-evoked warm preference, but not for nutrient-**
398 **induced warm preference.**

399 We asked whether the circadian clock is involved in taste-evoked warm preference. We used clock
400 gene null mutants, *period*^{01} (*per*^{01}) and *timeless*^{01} (*tim*^{01}). Although they showed significant
401 starvation responses (Fig. 5C and 5D, black and gray lines, Table S1), neither starved *per*^{01} nor
402 *tim*^{01} mutants could show taste-evoked warm preference upon sucralose refeeding (Fig. 5C1 and
403 5D1, blue lines, Table S1). Nevertheless, they fully recovered upon glucose refeeding in LD at any
404 time of day (Fig. 5C2 and 5D2, green lines, Table S1). Therefore, our data suggest that clock genes
405 are required for taste-evoked warm preference, but not for nutrient-induced warm preference.

406 However, starved *per*^{01} and *tim*^{01} mutants may eat sucralose less frequently than glucose,
407 which could result in a failure to show a taste-evoked warm preference. Therefore, we examined
408 how often starved *per*^{01} and *tim*^{01} mutants touched glucose, sucralose, or water during the 30 min
409 using FLIC assays [50] (Fig. S5A-S5C). Interestingly, starved *per*^{01} and *tim*^{01} mutants touched water

410 significantly more often than glucose or sucralose (Fig. S5B and S5C, Table S1). Although starved
411 *tim*^{01} flies touched glucose slightly more than sucralose for only 10 min, this phenotype is not
412 consistent with *per*^{01} and *w*^{1118} flies. However, these mutants still showed a similar Tp pattern for
413 sucralose and glucose refeeding (Fig. 5C and 5D). The results suggest that although the *tim*^{01} flies
414 can eat sufficient amount of sucralose over glucose, their food intake does not affect the Tp
415 behavioral phenotype. Thus, we conclude that in *per*^{01} and *w*^{1118} flies, the differential response
416 between taste-evoked and nutrient-induced warm preferences is not due to feeding rate.

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430 **DISCUSSION**

431 When animals are hungry, sensory detection of food (sight, smell, or chewing) initiates digestion
432 even before the food enters the stomach. The food-evoked responses are also observed in
433 thermogenesis, heart rate, and respiratory rate in mammals [14,15,79]. These responses are referred to
434 as the cephalic phase response (CPR) and contribute to the physiological regulation of digestion,
435 nutrient homeostasis, and daily energy homeostasis [11]. While starved flies show a cold preference,
436 we show here that the food cue, such as the excitation of gustatory neurons, triggers a warm
437 preference, and the nutritional value triggers an even higher warm preference. Thus, when flies
438 exit the starvation state, they use a two-step approach to recovery, taste-evoked and nutrient-
439 induced warm preferences. The taste-evoked warm preference in *Drosophila* may be a
440 physiological response potentially equivalent to CPR in mammals. Furthermore, we found that
441 internal needs, controlled by hunger signals and circadian clock genes, influence taste-evoked
442 warm preference. Thus, we propose that the taste-evoked response plays an important role in
443 recovery and represents another layer of regulation of energy homeostasis.

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446 **Tp is determined by the taste cue.**

447 Starved flies increase Tp in response to a nutrient-free taste cue (Fig. 1C and 1F), resulting in a
448 taste-evoked warm preference. We showed that silencing of ACs or cold neurons caused a loss of
449 taste-evoked warm preference (Fig. 3A-3E), and that excitation of ACs or cold neurons induced a
450 taste-evoked warm preference (Fig. 3F and 3G). The data suggest that both warm and cold neurons
451 are important for taste-evoked warm preference: while ACs are required for the hunger-driven

452 lower Tp [26], both ACs and cold neurons are likely to be important for this taste-evoked warm
453 preference.

454 The hunger-driven lower Tp is a slower response because starvation gradually lowers their
455 Tp [26]. In contrast, the taste-evoked warm preference is a rapid response. Once ACs and cold
456 neurons are directly or indirectly activated, starved flies quickly move to a warmer area. This is
457 interesting because even when ACs and cold neurons are activated by warm and cold, respectively,
458 the activation of these neurons causes a warm preference. Given that the sensory detection of food
459 (sight, smell, or chewing food) triggers CPR, the activation of these sensory neurons may induce
460 CPR. Tp in sucralose-refed hungry flies is between that of fed and starved flies (Figs. 2 and 3),
461 making it difficult to detect the smaller temperature differences using the calcium imaging
462 experiments. Therefore, we speculate that both ACs and cold neurons may facilitate rapid recovery
463 from starvation so that flies can quickly return to their preferred temperature - body temperature -
464 to a normal state.

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467 **Internal state influences taste-evoked warm preference.**

468 We show that mutants of genes involved in hunger and the circadian clock fail to show taste-
469 evoked warm preference, suggesting that hunger and clock genes are important for taste-evoked
470 warm preference. At a certain time of day, animals are hungry for food [50,78]. Thus, the hungry state
471 acts as a gatekeeper, opening the gate of the circuits when hungry flies detect the food information
472 that leads to taste-evoked warm preference (Figs. 4 and S6, blue arrow). While most of the hunger
473 signals we focused on are important for taste-evoked warm preference, some hunger signals are

474 also required for both taste-evoked and nutrient-induced warm preferences (Fig. 4). Notably,
475 sensory signals contribute to both taste-evoked and nutrient-induced warm preferences (Figs. 2, 3
476 and S6, blue and green arrows). Thus, taste-evoked warm preference and nutrient-induced warm
477 preference differ at the internal state level, but not at the sensory level. This idea is analogous to
478 appetitive memory formation. Sweet taste and nutrients regulate the different layers of the memory
479 formation process. Recent evidence suggests that the rewarding process can be subdivided; the
480 sweet taste is for short-term memory and the nutrient is for long-term memory [80-82]. The data
481 suggest that the taste-evoked response functions differently from the nutrient-induced response.
482 Therefore, taste sensation is not just the precursor to nutrient sensing/absorption, but plays an
483 essential role in the rapid initiation of a taste-evoked behavior that would help the animal survive.

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486 **How do hunger signals or clock genes contribute to taste-evoked warm preference?**

487 The hunger signaling hormones/peptides studied in this project are important for taste modulation.
488 For example, mammalian NPY and its *Drosophila* homolog NPF modulate the output of taste
489 signals [49,83,84]. The AKH receptor is expressed in a subset of gustatory neurons that may modulate
490 taste information for carbohydrate metabolism [85]. Therefore, the hunger signals are likely to
491 modulate the downstream of the sensory neurons, which may result in a taste-evoked warm
492 preference.

493 Circadian clock genes control and coordinate the expression of many clock-controlled
494 genes in the body [86-89]. Therefore, we expect that the absence of clock genes will disrupt the
495 molecular and neural networks of homeostasis, including metabolism, that are essential for animal

496 life. For example, taste neurons express clock genes, and impaired clock function in taste neurons
497 disrupts daily rhythms in feeding behavior [90]. Temperature-sensing neurons transmit hot or cold
498 temperature information to central clock neurons [91-95]. Therefore, the disrupted central clock in
499 clock mutants may respond imprecisely to temperature signals. There are many possible reasons
500 why the lack of clock gene expression in the brain is likely to cause abnormal taste-evoked warm
501 preference.

502 In addition, hunger signals may contribute to the regulation of circadian output. DH44 is
503 located in the dorsomedial region of the fly brain, the pars intercerebralis (PI), and DH44-
504 expressing neurons play a role in the output pathway of the central clock [96,97]. Insulin-producing
505 cells (IPCs) are also located in addition to DH44-expressing neurons [98-101]. IPCs receive a variety
506 of information, including circadian [97,102] and metabolic signals [73-75,77]. and then transduce the
507 signals downstream to release Ilps. Both Upd2 and Ilp6, which are expressed in the fat body
508 respond to metabolic states and remotely regulate Ilp expression [73-75,77]. Insect fat body is
509 analogous to the fat tissues and liver in the vertebrates [103,104]. Therefore, each hunger signal may
510 have its specific function for taste-evoked warm preference. Further studies are needed to describe
511 the entire process.

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514 **Taste-evoked warm preference may be CPR in flies.**

515 The introduction of food into the body disrupts the internal milieu, so CPR is a necessary process
516 that helps animals prepare for digestion. Specifically, in mammals, taste leads to an immediate
517 increase in body temperature and metabolic rate. Starvation results in lower body temperatures,

518 and chewing food, even before it enters the stomach, triggers a rapid increase in heat production,
519 demonstrating CPR in thermogenesis [14,15].

520 Starved flies have a lower Tp [26]. Because *Drosophila* is a small ectotherm, the lower Tp
521 indicates a lower body temperature [19,20]. Even when the flies do not receive food, the sweet taste
522 and the excitation of sweet neurons induce starved flies to show a warm preference, which
523 eventually leads to a warmer body temperature. In fact, CPR is known to be influenced by smell
524 as well in mammals [9]. We have shown in flies that olfactory mutants fail to show a warm preference
525 when refed sucralose (Fig. S4). Starvation leads to lower body temperatures, and food cues,
526 including taste and odor, rapidly induce a rise in body temperature before food enters the body.
527 Thus, the taste-evoked warm preference in *Drosophila* may be a physiological response equivalent
528 to one of the CPRs observed in mammals.

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531 **CPR is essential because both starved mammals and starved flies must rapidly regulate their**
532 **body temperature to survive.**

533 As soon as starved flies taste food, the sensory signals trigger CPR. They can move to a warmer
534 place to prepare to raise their body temperature (Fig. S6, blue arrows). CPR may allow flies to
535 choose a more hospitable place to restore their physiological state and allow for a higher
536 metabolism, and eventually move on to the next step, such as foraging and actively seeking a mate
537 before competitors arrive. Thus, CPR may be a strategy for the fly's survival. Similarly, starvation
538 or malnutrition in mammals leads to lower body temperatures [6,7,105], and biting food triggers heat
539 production, which is CPR [14-16]. Thus, while starvation in both flies and mammals leads to lower
540 body temperatures, food cues initiate CPR by increasing body temperature and nutrient intake,

541 resulting in full recovery from starvation. Our data suggest that *Drosophila* CPR may be a
542 physiological response equivalent to CPR observed in other animals. Thus, *Drosophila* may shed
543 new light on the regulation of CPR and provide a deeper understanding of the relationship between
544 CPR and metabolism.

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Contributions

F.N.H and Y.U designed the experiments. Y.U, E.N., T.N. and J.S. performed the temperature preference behavioral assays and data analysis. Y.U., S.H.S., S.S.U. and J.C. performed the feeding experiments and the data analysis. F.N.H and Y.U wrote the manuscript.

Declaration of Interest

The authors declare no competing interests

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928 **FIGURE**

929 **Figure 1. Hungry flies switch from cold to warm preference upon food detection.**

930 **(A)** A schematic diagram of the experimental conditions. Flies were reared on fly food.
931 Emerging flies were kept on fly food for one or two days (orange) and then starved with water-
932 absorbing paper. Starvation was applied for 1-3 overnights (ON). The duration of starvation was
933 chosen when the flies showed a statistically significant difference in Tp between fed and starved
934 conditions (see "Starvation conditions" in Materials and methods for details). Starved flies were
935 refed with either fly food (orange bar), 2 mL sucralose (blue), glucose solution (green), or
936 fructose solution (purple) absorbed paper and then examined for temperature preference (Tp)
937 behavioral assays. Details are provided in the experimental procedures.

938 **(B-E)** Comparison of preferred temperature (Tp) of *white*^{1118} (w^{1118}) control flies between fed
939 (white bar), starved (STV; gray bar), and refed (orange, blue, or green bar) states. Starvation was
940 applied for 1ON. Starved flies were refed with fly food (orange bar) for 5, 10, 30, or 60 min (1
941 hr) (B), 2.8 mM sucralose solution (blue bar) for 10 min or 1 hr (C), 2.8 mM (equivalent to 5%)
942 glucose solution (green bar) for 5 min, 10 min, or 1 hr (D), or 2.8mM fructose solution (purple
943 bar) for 10 min or 1 hr (E). Behavioral experiments were performed at the specific time points,
944 Zeitgeber time (ZT) 4-7. ZT0 and ZT12 are light on and light off, respectively. Dots on each bar
945 indicate individual Tp in the assays. Numbers in italics indicate the number of trials. The
946 Shapiro-Wilk test was performed to test for normality. One-way ANOVA was performed to
947 compare Tp between each refeeding condition. Red or green stars indicate Tukey's post hoc test
948 comparing differences between experimental and fed or starved conditions, respectively. Data
949 are presented as mean Tp with SEM. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. ****$p < 0.0001$. NS
950 indicates no significance.

951 **(F)** Comparison of Tp among the following conditions in w^{1118} (control) flies: fed (white bar),
952 starved overnight (O/N) (water or STV; gray bar), refed with fly food (fly food; orange bar),
953 sucralose (blue bars), glucose (green bars), or fructose (purple bars). The same data are used in
954 Fig. 1B-E. A distribution of temperature preference in each condition is shown in Fig. S2A. The
955 Shapiro-Wilk test was performed to test for normality. One-way ANOVA was performed to
956 compare Tp between each refeeding condition. Red (or green) stars indicate Tukey's post hoc test
957 comparing differences between experimental and fed (or starved) conditions, respectively. *p <
958 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. NS indicates not significant.
959 **(G)** Feeding assay: The number of touches to water (gray), 2.8 mM (equivalent to 5%) glucose
960 (green), or 2.8 mM sucralose in each solution (blue) was examined using w^{1118} flies starved for
961 24 hrs. Water, glucose and sucralose were tested individually in the separate experiments. A
962 cumulative number of touches to water or sugar solution for 0-30 min was plotted. Two-way
963 ANOVA was used for multiple comparisons. Blue and green stars show Fisher's LSD post hoc
964 test comparing sucralose (blue stars) or glucose (green stars) solution feeding to water drinking.
965 All data shown are means with SEM. *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001.
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967
968 **Figure 2. Gustatory neurons are essential for taste-evoked warm preference.**
969 **(A-E)** Comparison of preferred temperature (Tp) of flies between fed (white bar), starved (STV;
970 gray bar), and refed (orange, blue, green, or dark green bar) states. Flies were starved for 2
971 overnights (ON) except for *Gr5a*$^{-/-}$*;;Gr61a*$^{-/-}$*, Gr64a-f*$^{-/-}$ (1.5ON). Starved flies were refed with
972 fly food for 10 min (fly food; orange bars), sucralose for 10 min (blue bars), or glucose for 10
973 min (green bars), or 1 hr (dark green bars).

974 **(F)** Schematic of the optogenetic activation assay.

975 **(G-J)** Comparison of Tp of flies between fed (white bar), starved (STV; gray bar), and starved
976 with all-trans-retinal (ATR; yellow bars), which is the chromophore required for CsChrimson
977 activation. Gustatory neurons in starved flies were excited by red light pulses (flashing on and
978 off at 10 Hz) for 10 min. Starvation was performed for 2ON. Averaged fly distributions of flies
979 in the temperature gradient for *Gr64fGal4>CsChrimson* were shown in Fig. S2B. Behavioral
980 experiments were performed on ZT4-7. Dots on each bar indicate individual Tp in assays.
981 Numbers in italics indicate the number of trials. The Shapiro-Wilk test was used to test for
982 normality. One-way ANOVA was used for statistical analysis. Red or green stars indicate Tukey's
983 post hoc test compared between each experiment and the fed (red) or starved (green) condition.
984 All data presented are means with SEM. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. ****$p < 0.0001$.
985 NS indicates not significant.

986

987

988 **Figure 3. Both warm and cold temperature sensing neurons are involved in taste-evoked**
989 **warm preference.**

990 **(A-E)** Comparison of preferred temperature (Tp) of flies between fed (white bar), starved (STV;
991 gray bar), and refed (orange, blue, green, or dark green bar) conditions. Starvation was applied
992 for 2 overnights (ON). Starved flies were refed with fly food for 10 min (orange bar), sucralose
993 for 10 min (blue bar), or glucose for 10 min (green bar) or 1 hr (dark green bar). The Shapiro-
994 Wilk test was used to test for normality. One-way ANOVA or Kruskal-Wallis test was used for
995 statistical analysis. Red or green stars indicate Tukey's post hoc test or Dunn's test compared
996 between each experiment and the fed (red) or starved (green) condition, respectively.

997 **(F and G)** Comparison of Tp between starved (STV; gray bar) and all-trans-retinal (ATR;
998 yellow bar) starved flies. Starvation was performed for 2ON. Warm neurons (F) or cold neurons
999 (G) in starved flies expressed CsChrimson, which was excited by red light pulses for 10 min. The
1000 Shapiro-Wilk test was performed to test for normality. Student's t-test or Kolmogorov-Smirnov
1001 test was used for statistical analysis. (G) *tubGal80^{ts}; R11F02-Gal4>uas-CsChrimson* flies were
1002 reared at 18°C, and emerged adults were collected and stored at 29°C. See Materials and
1003 Methods for details. These behavioral experiments were performed on ZT4-7. The dots on each
1004 bar indicate individual Tp in the assays. Numbers in italics indicate the number of experiments.
1005 All data shown are means with SEM. *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. NS
1006
1007
1008 **Figure 4. Hunger signals are involved in taste-evoked warm preference.**
1009 Comparison of preferred temperature (Tp) of flies between fed (white bar), starved (STV; gray
1010 bar), and refed states (orange, blue, green, or dark green). Flies were starved for 2 overnights
1011 (ON) except for *ilp6* mutant flies (3ON). Starved flies were refed with fly food (orange bar),
1012 sucralose (blue bar), or glucose (green bar) for 10 min. These behavioral experiments were
1013 conducted on ZT4-7. Dots on each bar indicate individual Tp in the assays. Numbers in italics
1014 indicate the number of trials. Shapiro-Wilk test was performed for normality test. One-way
1015 ANOVA was used for statistical analysis. Red or green stars indicate Tukey's post hoc test
1016 comparing between each experiment to the fed (red) or starved (green) condition, respectively.
1017 All data presented are means with SEM. *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001.
1018 NS indicates not significant.
1019

1020

1021 **Figure 5. Clock genes are involved in taste-evoked warm preference.**

1022 **(A-D)** Comparison of preferred temperature (Tp) of flies between fed (white circles), starved

1023 (STV; gray triangles), and refed (blue, green, or dark green squares) states. Flies were starved for

1024 24 hrs. Starved flies were refed with sucralose (blue squares, A1-D1) or glucose for 10 min

1025 (green squares, A2-D2) or 1 hr (dark green squares, A3). After the 10 min or 1 hr refeeding, the

1026 temperature preference behavior assays were performed immediately at ZT1-3, ZT4-6, ZT7-9,

1027 and ZT10-12 in LD. The Shapiro-Wilk test was performed to test for normality. One-way

1028 ANOVA or Kruskal-Wallis test was used for statistical analysis. Stars indicate Tukey's post hoc

1029 test or Dunn's test compared between each experiment and the starved condition at the same time

1030 point. All data presented are means with SEM. *$p<0.05$. **$p<0.01$. ***$p<0.001$. ****$p < 0.0001$.

1031 **(E)** Comparison of Tp during the daytime in each feeding state. One-way ANOVA or Kruskal-

1032 Wallis test was used for statistical analysis between ZT1-3 and ZT7-9 or ZT10-12, respectively.

1033 R and AR indicate rhythmic and arrhythmic, respectively, during the daytime.

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1036 **Figure S1. *Drosophila* temperature preference behavior assay (Related to Fig. 1)**

1037 (A) Three different feeding conditions, fed, starved and refed, were used in this study.

1038 (B) A temperature gradient of 17-33°C in air is established in the chamber between the aluminum

1039 metal plate and the Plexiglas cover. Flies are applied through the hole into the chamber. Once the

1040 flies are applied, they spread out in the chamber and then gradually accumulate at the specific

1041 temperature ranges within 30 min, which is called the preferred temperature (Tp).

1042 (C) One of the representative results of Tp experiments (top, normal (fed flies); bottom,
1043 starvation (starved flies)). Since their body temperature is close to the temperature of their
1044 surrounding microenvironment, their body temperature is determined by measuring their Tp.

1045

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1047 **Figure S2. The distribution of temperature preference in each experimental condition**
1048 **(Related to Fig. 1 and 2)**

1049 Comparison of the distribution of temperature preference in each feeding condition in w^{1118}
1050 control flies (A) and the *Gr64fGal4>CsChrimson* flies (B). Heat maps were generated using the
1051 Conditional formatting tool in Microsoft Excel. The averaged percentages of flies settling on the
1052 apparatus within each one-degree temperature interval were used to create the heat maps. Each
1053 parameter to draw them using Conditional formatting tool is as follows: Minimum Value: 0,
1054 Midpoint Value: 15%, and Maximum Value: 60% for w^{1118}. Minimum Value: 0, Midpoint Value:
1055 10%, and Maximum Value: 45% for *Gr64fGal4>CsChrimson*.

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1057

1058 **Figure S3. Duration of starvation is unlikely to affect the ability to recover (Related to Fig.**
1059 **3)**

1060 Comparison of preferred temperature (Tp) of w^{1118} control flies between fed (white bar), starved
1061 (STV; gray bar), and refed conditions (blue or green bar). Flies were starved for overnight
1062 (STV1ON; gray bar) or 1.5 ON (STV1.5ON; dark gray bar), i.e. starved for 18-21 hrs or 26-29
1063 hrs, respectively. Starved flies were refed with sucralose for 10 min (blue bar) or glucose for 10

min (green bar). These behavioral experiments were performed on ZT4-7. Dots on each bar indicate individual Tp in the assays. Numbers in italics indicate the number of trials. Shapiro-Wilk test was performed to test for normality. One-way ANOVA or Kruskal-Wallis test was used for statistical analysis. Red or green stars indicate Tukey's post hoc test or Dunn's test comparing differences between experimental and fed or starved conditions, respectively. All data shown are means with sem. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. ****$p < 0.0001$. NS indicates no significance. The same data from Fed, STV1ON, STV1ON+refed sucralose for 10 min and STV1ON+refed sucralose for 10 min in Fig. 1B-F are used in Fig. S3.

Figure S4. *Orco* is involved in the taste-evoked warm preference.

Comparison of the preferred temperature (Tp) of *Orco*^{1} olfaction-deficient mutant flies between fed (white bar), starved (STV; gray bar), and refed (blue or green bar). *Orco*^{1} flies were starved for 2 overnights (ON). Starved flies were refed with sucralose for 10 min (blue bar; A) or glucose for 10 min (green bar; B). Pooled data of both sucralose- and glucose-refeeding bioassay data are shown in C. The Shapiro-Wilk test was performed to test for normality. One-way ANOVA or Kruskal-Wallis test was used for statistical analysis. Red or green stars indicate Tukey's post hoc test or Dunn's test compared between each experiment and the fed (red) or starved (green) condition, respectively.

1085 **Figure S5. Number of touches to water, sucralose and glucose using *yw*, *per*^{01} and *tim*^{01}**

1086 **flies. (Related to Figures 1 and 5)**

1087 Feeding assay: The number of touches to water (gray), 2.8 mM (equivalent to 5%) glucose

1088 (green), or 2.8 mM sucralose solution (blue) was examined using *yw*, *per*^{01}, or *tim*^{01} flies starved

1089 for 24 hrs. Cumulative number of touches for 0-30 min was plotted. Two-way ANOVA was used

1090 for multiple comparisons. Blue and green stars show Fisher's LSD post hoc test comparing

1091 sucralose (blue stars) or glucose (green stars) solution feeding to water drinking. Black stars

1092 show the statistical difference between sucralose and glucose refeeding. All data presented are

1093 means with SEM. *$p < 0.05$. *F*$p < 0.01$. ***$p < 0.001$. ****$p < 0.0001$.

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1096 **Figure S6. MODEL**

1097 **A schematic diagram of the recovery of preferred temperature (Tp) by taste-evoked warm**

1098 **preference (blue arrows) and nutrient-induced warm preference (green arrows).**

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1108 **Materials and Methods**

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1110 All flies were reared under 12 hr-light/12 hr-dark (LD) cycles at 25°C and 60-70% humidity in
1111 an incubator (DRoS33SD, Powers Scientific Inc.) with an electric timer (light on: 8am; light off:
1112 8pm). The light intensity was 1000-1400 lux. All flies were reared on custom fly food recipe,
1113 with the following composition per 1 L of food: 6.0 g sucrose, 7.3 g agar, 44.6 g cornmeal, 22.3
1114 g yeast and 16.3 mL molasses, as previously described [26]. *white*^{1118} (w^{1118}) and *yellow*^{1} *white*^{1}
1115 (y^1w^1) flies were used as control flies. *EP5Δ; Gr64a*^{1} (*Gr5a*$^{-/-}$*; Gr64a*$^{-/-}$) was kindly provided by
1116 Dr. Anupama Dahanukar [47]. *R1; Gr5a-LexA; +; ΔGr61a, ΔGr64a-f* (*Gr5a*$^{-/-}$*; Gr61a*$^{-/-}$*, Gr64a-f*$^{-/-}$)
1117 were kindly provided by Dr. Hubert Amrein [51,52]. *Dh44-Gal4* was kindly provided by Dr. Greg
1118 Suh [48]. *TrpA1*^{SH}*-gal4* was kindly provided by Dr. Paul A. Garrity [44]. Other fly lines were
1119 provided by Bloomington Drosophila stock center and Vienna Drosophila Stock Center.

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1122 **Temperature preference behavioral assay**

1123 The temperature preference behavior assays were examined using a temperatures gradient, set
1124 from 16-34°C and were performed for 30 min in an environmental room maintained at 25°C
1125 /60%-70% humidity, as previously described [26]. Because starved flies showed a lower preferred
1126 temperature (Tp), the temperature regulation was lower than usual (Umezaki et al. 2018 Current
1127 Biology). We prepared a total of 40-50 flies (male and female flies were mixed) for fed condition
1128 experiments and 90-100 flies for overnight(s) starved and refed condition experiments for one
1129 trial. Flies were never reused in subsequent trials. In the starved and refed experiments, we
1130 prepared twice the number of flies needed for a trial because almost half of them died from

1131 starvation stress. Others climbed on the wall and ceiling (starved flies are usually hyperactive [106],
1132 even though we applied slippery coating chemicals (PTFE; Cat# 665800, Sigma or byFormica
1133 PTFE Plus, https://byformica.com/products/fluon-plus-ptfe-escape-prevention-coating) to the
1134 Plexiglass covers.

1135 Behavioral assays were performed for 30 min at Zeitgeber time (ZT) 4-7 (light on and
1136 light off are defined as ZT0 and ZT12, respectively), and starvation was initiated at ZT9-10
1137 (starved for 1, 2 or 3 O/N are 18-21 hr, 42-45 hr or 66-69 hr, respectively). As for STV1.5 for
1138 Fig. S3, starvation was initiated at ZT1-2 (26-29 hr). For the starvation assays, the collected flies
1139 were maintained on our fly food for at least one day and then transferred to plastic vials
1140 containing 3 mL of distilled water, which was absorbed by a Kimwipe paper. For refeeding
1141 experiments, starved flies were transferred to plastic vials containing 2 mL of 2.8 mM sugar
1142 solution (sucralose water/glucose water). Sugar solution is absorbed by half the size of a
1143 Kimwipe. The details of the starvation period are described in the following section (see
1144 "Starvation Condition").

1145 After the 30-min behavioral assay, the number of flies whose bodies were completely
1146 located on the aluminum plate was counted. Flies whose bodies were partially or completely
1147 located on the walls of the Plexiglass cover were not included in the data analysis. The
1148 percentage of flies within each one-degree temperature interval on the apparatus was calculated
1149 by dividing the number of flies within each one-degree interval by the total number of flies on
1150 the apparatus. The location of each one-degree interval was determined by measuring the
1151 temperature at 6 different points on the bottom of the apparatus. Data points were plotted as the
1152 percentage of flies within a one-degree temperature interval. The weighted mean of Tp was
1153 calculated by summing the product of the percentage of flies within a one-degree temperature

1154 interval and the corresponding temperature (e.g., fractional number of flies x 17.5°C + fractional
1155 number of flies x 18.5°C +…… fractional number of flies x 32.5°C). If the SEM of the
1156 averaged Tp was not < 0.3 after the five trials, additional trials were performed approximately 10
1157 times until the SEM was < 0.3.

1158 Microsoft Excel (Home tab>Conditional formatting tool>3-color scales and data bars)
1159 was used to create heat maps to show the distribution of flies in each experimental condition.
1160 The averaged percentages of flies that settled on the apparatus within each one-degree
1161 temperature interval were used to create the heat maps. Each scale value is as follows; minimum
1162 value: 0, midpoint value: 15%, and maximum value: 60% for w^{1118}. Minimum value: 0, middle
1163 value: 10%, and maximum value: 45% for Gr64fGal4>CsChrimson.

1164
1165

1166 **Starvation conditions and recovery**

1167 Most of the flies were starved for 2 overnights (O/N). Because some flies (e.g. *ilp6* mutants)
1168 show starvation resistance and seem to be still healthy even after 2 O/N of starvation. We had to
1169 starve them for 3 O/N to show a significant difference in Tp between fed and starved flies. On
1170 the other hand, some flies (e.g., w^{1118} flies) are very sick after 3 O/N of starvation, in which case
1171 we only had to starve them for 1 day. Therefore, the starvation conditions we used for this
1172 manuscript are from 1 to 3 O/N.

1173 First, we confirmed the starvation period by focusing on Tp which resulted in a
1174 statistically significant Tp difference between fed and starved flies; as mentioned above, flies
1175 prefer lower temperatures when starvation is prolonged [26]. Therefore, when Tp was not
1176 statistically different between fed and starved flies, we extended the starvation period from 1 to 3

1177 O/N. Importantly, we show in Fig. S3 that the duration of starvation does not affect the recovery
1178 effect. Furthermore, w^{1118} flies cannot survive 42-49 or 66-69 hours of starvation.
1179
1180
1181 **Temperature preference rhythm (TPR) assay**
1182 For the TPR assays, we performed temperature preference behavior assays in different time
1183 windows during the daytime (zeitgeber time (ZT) or circadian time (CT) 1-3, 4-6, 7-9, and 10-
1184 12) as described previously [21,107]. Because starvation duration directly affects flies' Tp [26]
1185 starvation was initiated at each time window to adjust the starvation duration at each time point,
1186 which means flies were starved for 24 hrs or 48 hrs but not 1 or 2 O/N. Each behavioral assay
1187 was not examined during these time periods (ZT or CT0-1 and 11.5-12) because of large
1188 phenotype variation around light on and light off.
1189 Furthermore, insulin levels were shown to peak at 10 min and gradually decline [108]. Also,
1190 how quickly the flies can consume food is unclear. These factors may influence temperature
1191 preference behavior. Therefore, to minimize these effects, we decided to test the temperature
1192 preference behavioral assay immediately after the flies had eaten the food.
1193
1194
1195 **Optogenetic activation**
1196 For the optogenetic activation of the target neurons for behavioral assays, the red-light-sensitive
1197 channelrodopsin, *UAS-CsChrimson*, was crossed with each Gal4 driver. Flies were reared on fly
1198 food at 25°C and 60-70% humidity under LD cycles in an incubator (DRoS33SD, Powers
1199 Scientific Inc.) with an electric timer. After the flies emerged, adult flies were collected and

1200 maintained on fly food for 1-2 days. The next day, flies were transferred to water with or without
1201 0.8 mM all-trans retinal (ATR; #R2500, Sigma) diluted in dimethyl sulfoxide (DMSO; #472301,
1202 Sigma) for 2 O/N. To activate flies expressing UAS-CsChrimson crossed with Gal4 drivers, we
1203 used a 627-nm red light-emitting diode (LED) equipped with a pulsed photoillumination system
1204 (10 Hz, 0.08 mWmm-2). Flies were exposed to pulsed red light for 10 min, which corresponds to
1205 the refeeding period. This photoillumination system was used in an incubator (Sanyo Scientific,
1206 MIR-154) and followed by temperature preference behavioral assays.

1207 The *R11F02-Gal4>uas-CsChrimson* flies do not develop into adults and die in the pupal
1208 stage. Therefore, the Gal4/Gal80ts system was used to restrict *uas-CsChrimson* expression. The
1209 *Gal80^{ts}* is a temperature sensitive allele of *Gal80* that causes *Gal4* inhibition at 18°C and
1210 activation at 29°C [109].The *tubGal80^{ts}; R11F02-Gal4>uas-CsChrimson* flies were reared on fly
1211 food at 18°C. Emerging adult flies were collected and kept on fly food at 29°C. The next day,
1212 flies were transferred to water (starved condition) with or without 0.8 mM ATR for 2 O/N.
1213 Starved flies with or without ATR application were exposed to pulsed red light for 10 min
1214 (equivalent to the refeeding period) and then immediately loaded into the behavioral apparatus
1215 for behavioral assays to measure their Tp. All flies were treated with ATR after they had fully
1216 developed into the adults. This means that Gal4-expressing cells were activated by red light via
1217 CsChrimson only at adult stages.

1218

1219

1220 **Feeding assay**

1221 To measure individual fly feeding, we used the Fly Liquid Interaction Counter (FLIC) system [50].
1222 Groups of 1-2 day old male and female flies were starved for 24 hours starting between ZT4-5

1223 (12-1pm). Individual flies were then loaded into FLIC monitors. Flies were acclimated to the
1224 monitors for 30 min with access to water in the feeding wells. At the start of the feeding study,
1225 water was replaced with 2.8 mM sucralose or 2.8 mM glucose solution (equivalent to 5%
1226 glucose concentration) and the number of licks (touches) was recorded for 30 min. Water,
1227 sucralose, or glucose water was administered individually in separate experiments. Assays were
1228 performed on ZT4-7. To account for the potential confounding effect of startle response when
1229 food is changed, wells where water was replaced with new water were used as a control. FLIC
1230 raw data were analyzed using the FLIC R code master (Pletcher Lab, available at
1231 https://github.com/PletcherLab/FLIC_R_Code). Lick counts were obtained and summed in 5-
1232 min window bins, while cumulative licks were obtained by successively summing the licks in
1233 these bins.

Fig. 1

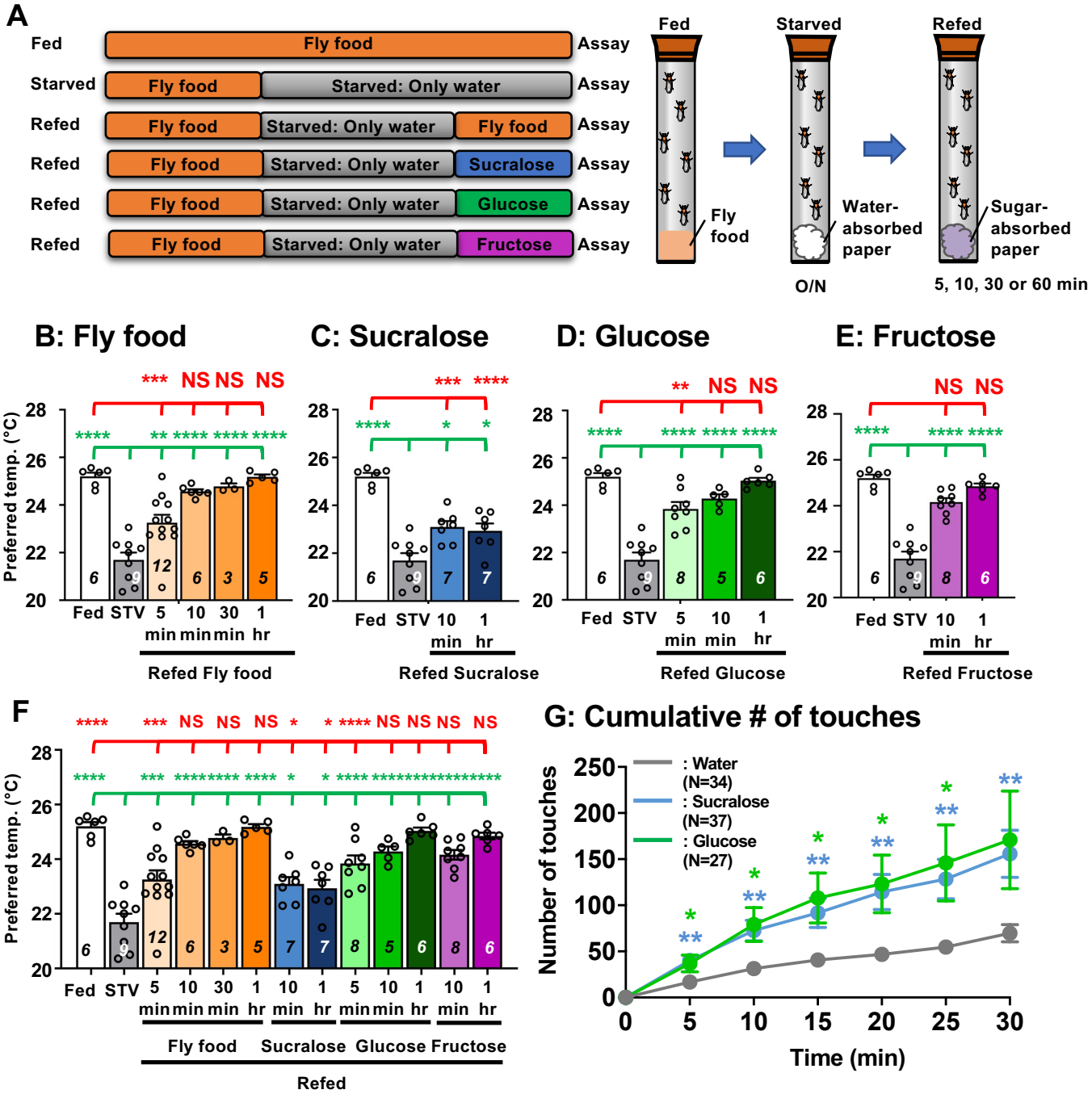

A
Fed
Fly food
Assay
Starved
Fly food
Starved: Only water
Assay
Refed
Fly food
Starved: Only water
Fly food
Assay
Refed
Fly food
Starved: Only water
Sucralose
Assay
Refed
Fly food
Starved: Only water
Glucose
Assay
Refed
Fly food
Starved: Only water
Fructose
Assay
Fed
Starved
Refed
Fly food
Water-absorbed paper
Sugar-absorbed paper
O/N
5, 10, 30 or 60 min
B: Fly food
C: Sucralose
D: Glucose
E: Fructose
Preferred temp. (°C)
Fed STV 5 min 10 min 30 min 1 hr
Refed Fly food
Fed STV 10 min 1 hr
Refed Sucralose
Refed Glucose
Refed Fructose
F
Fly food
Sucralose
Glucose
Fructose
Refed
G: Cumulative # of touches
: Water (N=34)
: Sucralose (N=37)
: Glucose (N=27)
Number of touches
Time (min)

Fig. 2

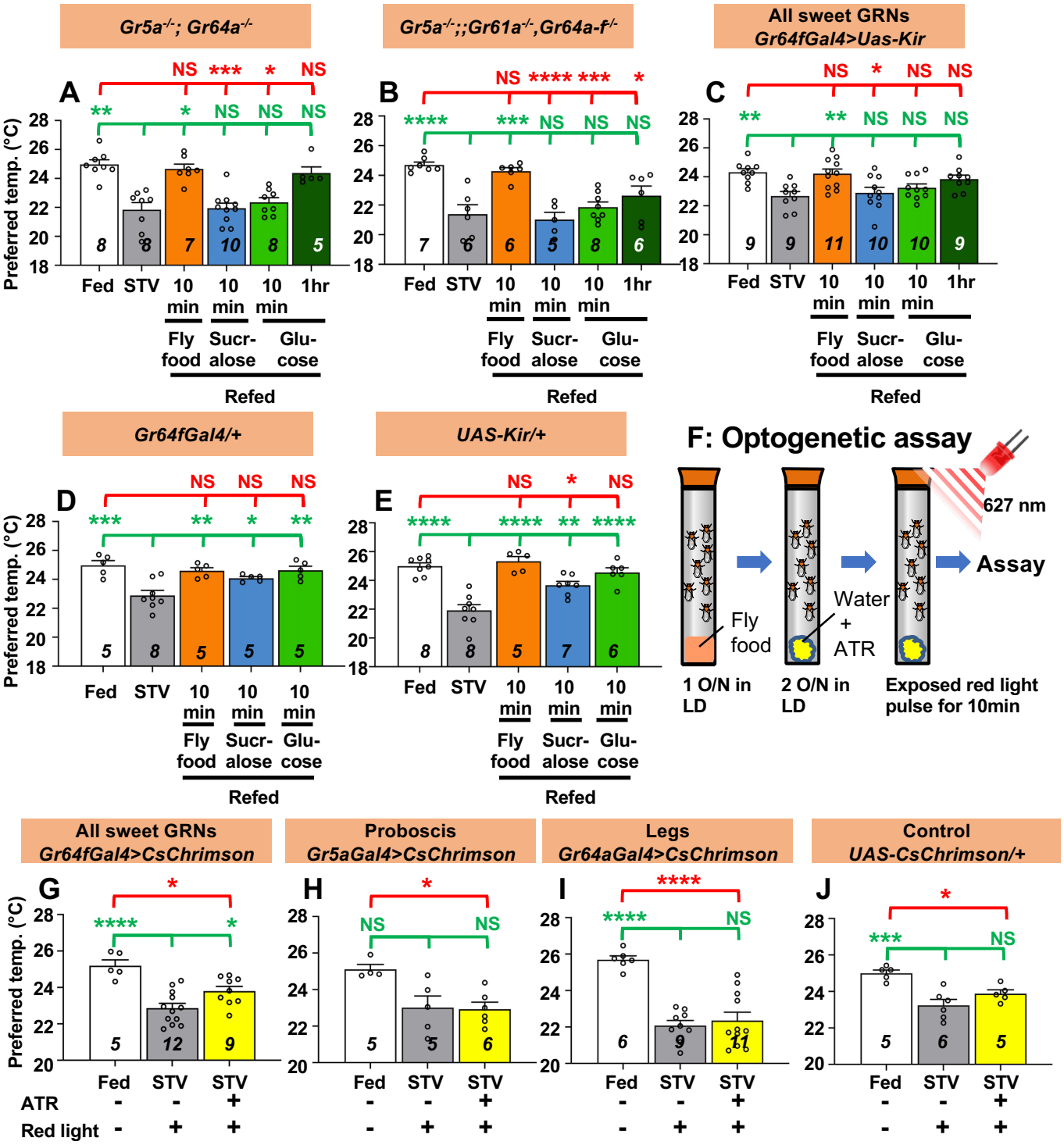

Gr5a-/-; Gr64a-/-
Gr5a-/-;;Gr61a-/-,Gr64a-f-/-
All sweet GRNs
Gr64fGal4>Uas-Kir
A
B
C
Preferred temp. (°C)
Fed
STV
10 min
1hr
Fly food
Sucr-alose
Glu-cose
Refed
Gr64fGal4/+
UAS-Kir/+
D
E
F: Optogenetic assay
627 nm
Assay
Fly food
Water + ATR
1 O/N in LD
2 O/N in LD
Exposed red light pulse for 10min
All sweet GRNs
Gr64fGal4>CsChrimson
Proboscis
Gr5aGal4>CsChrimson
Legs
Gr64aGal4>CsChrimson
Control
UAS-CsChrimson/+
G
H
I
J
ATR
Red light

Fig. 3

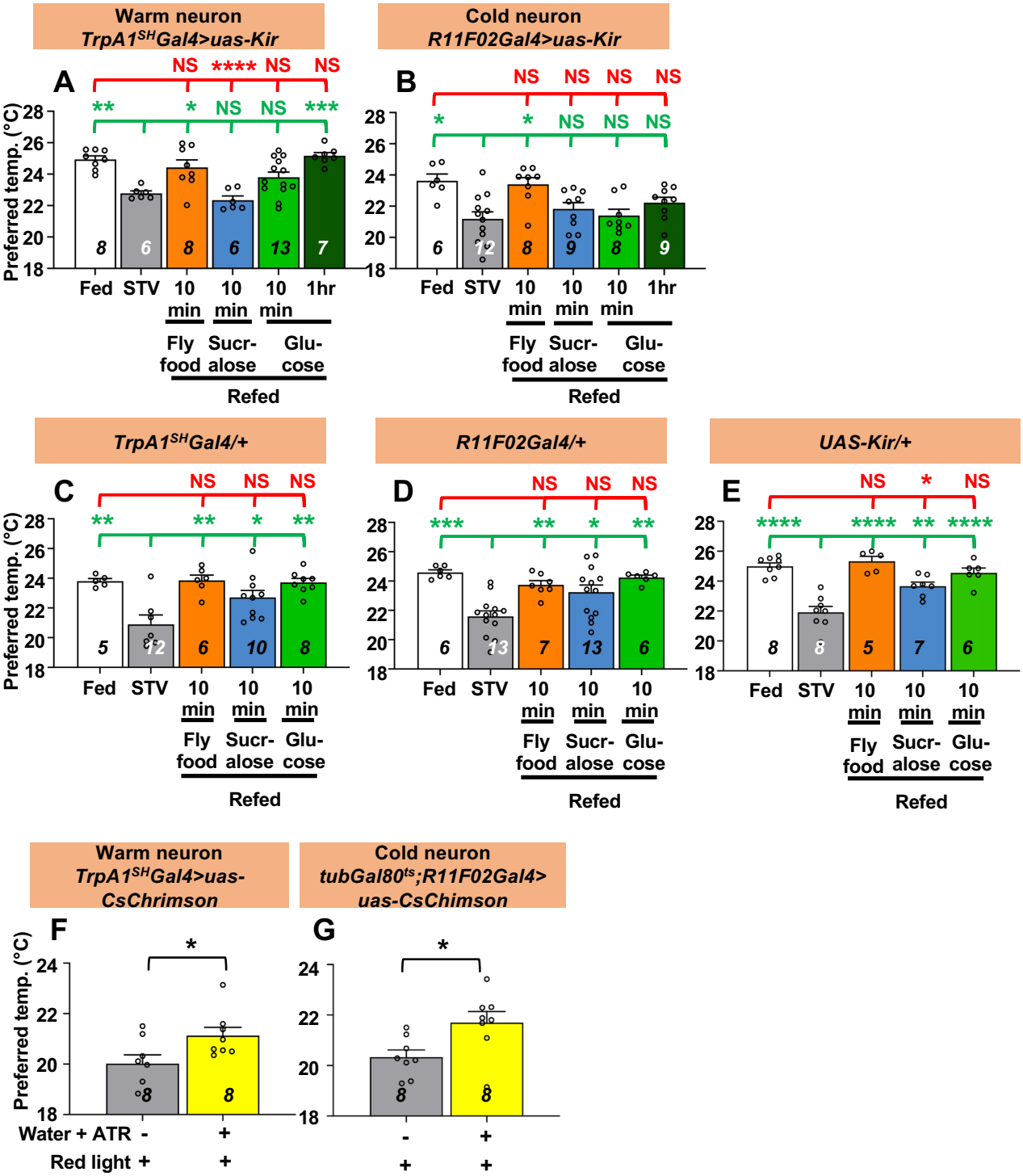

Warm neuron
TrpA1SHGal4>uas-Kir
Cold neuron
R11F02Gal4>uas-Kir
A
B
Preferred temp. (°C)
Fed
STV
10 min
10 min
10 min
1hr
Fly food
Sucr-alose
Glu-cose
Refed
TrpA1SHGal4/+
R11F02Gal4/+
UAS-Kir/+
C
D
E
Warm neuron
TrpA1SHGal4>uas-CsChrimson
Cold neuron
tubGal80ts;R11F02Gal4>uas-CsChimson
F
G
Water + ATR
Red light

Fig. 4

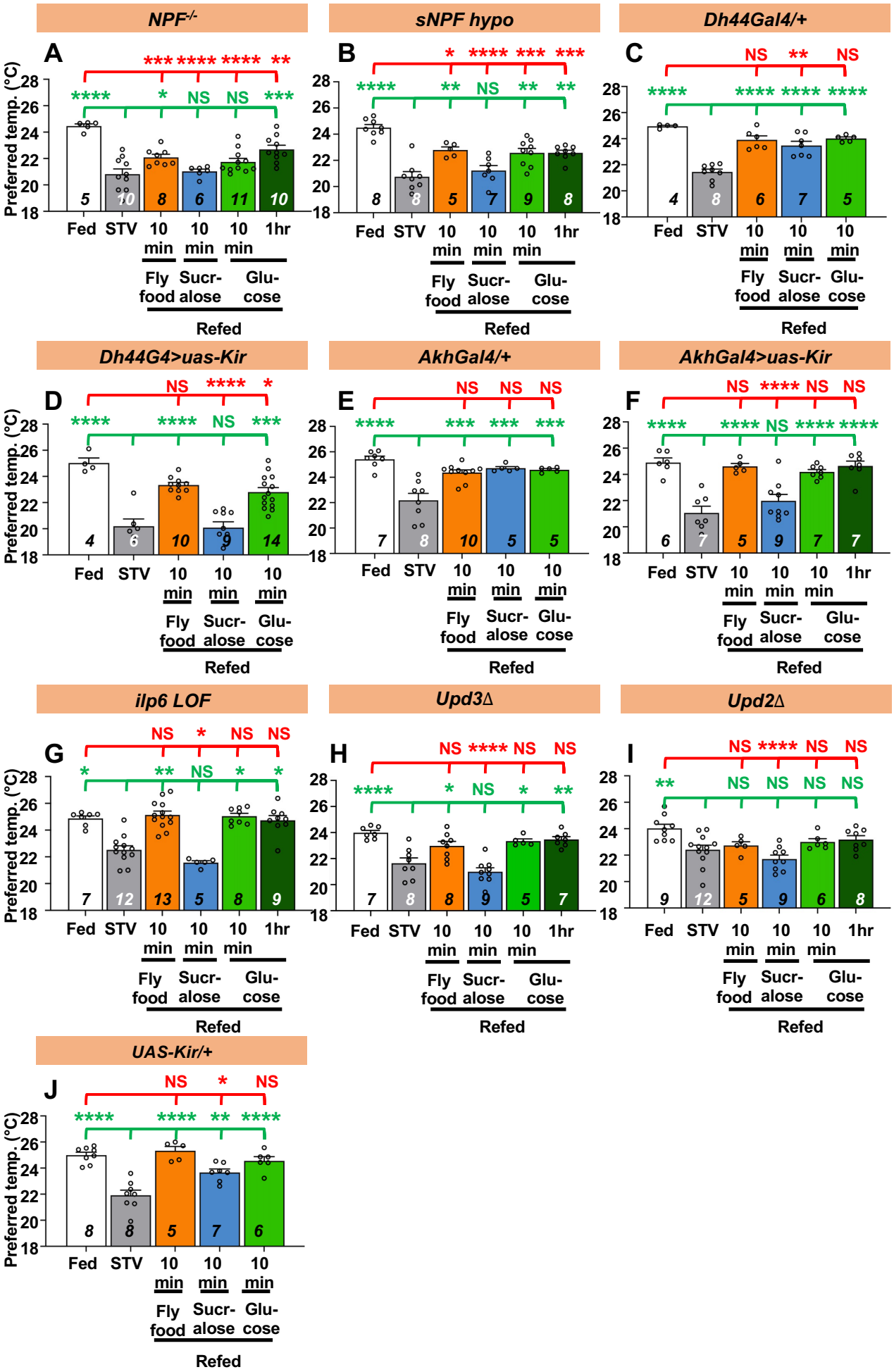
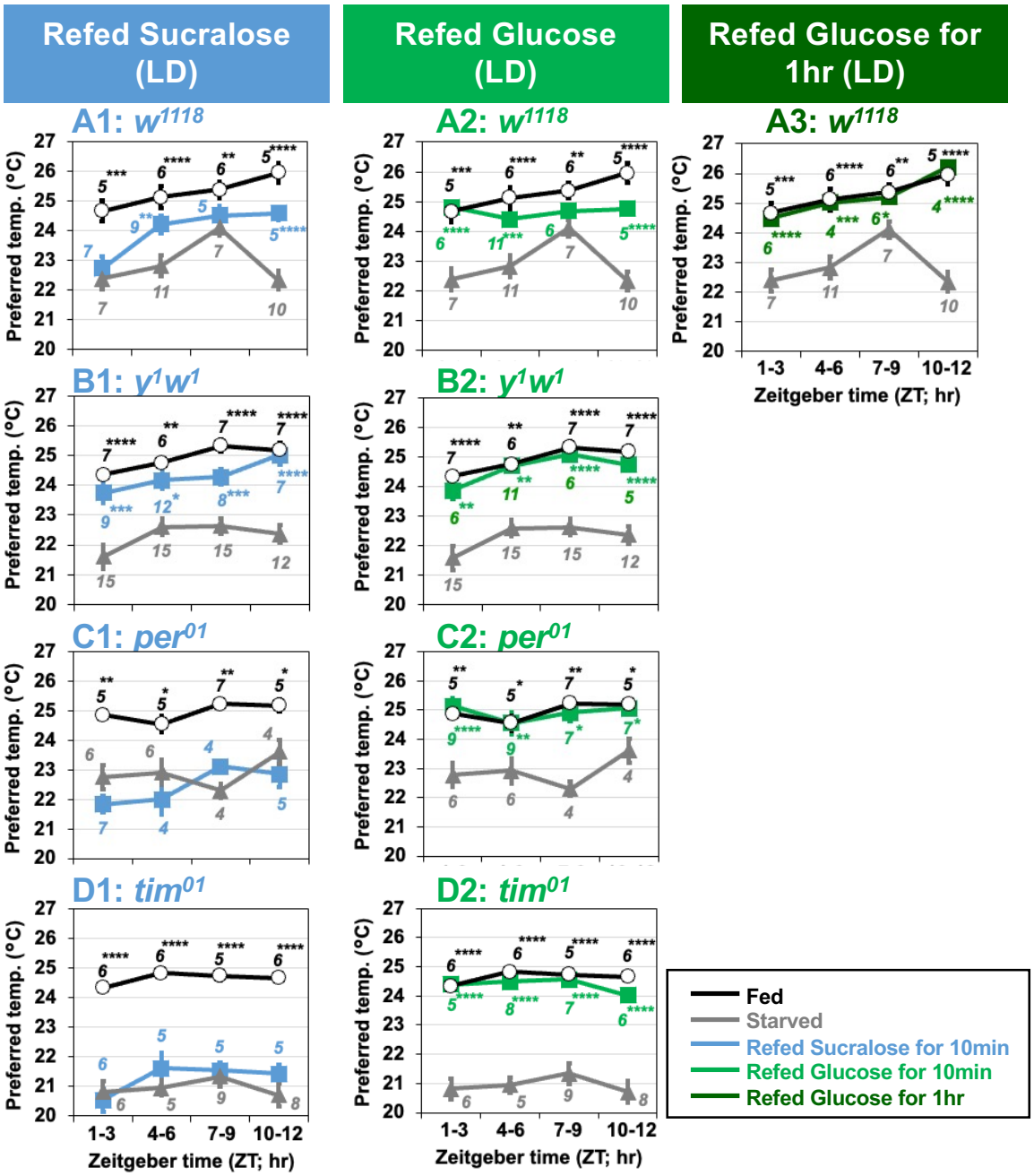

NPF-/-
A
*** **** **** **
**** * NS NS ***
Preferred temp. (°C)
28
26
24
22
20
18
5 10 8 6 11 10
Fed STV 10 min 10 min 10 min 1hr
Fly food Sucr-alose Glu-cose
Refed
sNPF hypo
B
* **** *** ***
**** ** NS ** **
8 8 5 7 9 8
Dh44Gal4/+
C
NS ** NS
**** **** **** ****
4 8 6 7 5
Dh44G4>uas-Kir
D
NS **** *
**** **** NS ***
4 6 10 9 14
AkhGal4/+
E
NS NS NS
**** *** *** ***
7 8 10 5 5
AkhGal4>uas-Kir
F
NS **** NS NS
**** **** NS **** ****
6 7 5 9 7 7
ilp6 LOF
G
NS * NS NS
* ** NS * *
7 12 13 5 8 9
Upd3Δ
H
NS **** NS NS
**** * NS * **
7 8 8 9 5 7
Upd2Δ
I
NS **** NS NS
** NS NS NS NS
9 12 5 9 6 8
UAS-Kir/+
J
NS * NS
**** **** ** ****
8 8 5 7 6

Fig. 5



E

LD	Fed	Starvation	Refed Sucralose	Refed Glucose	Refed Glucose for 1hr
A: w[1118]	R	R	R	AR	R
B: y[1]w[1]	R	AR	AR	R	
C: per[01]	AR	AR	AR	AR	
D: tim[01]	AR	AR	AR	AR	

Fig. S1

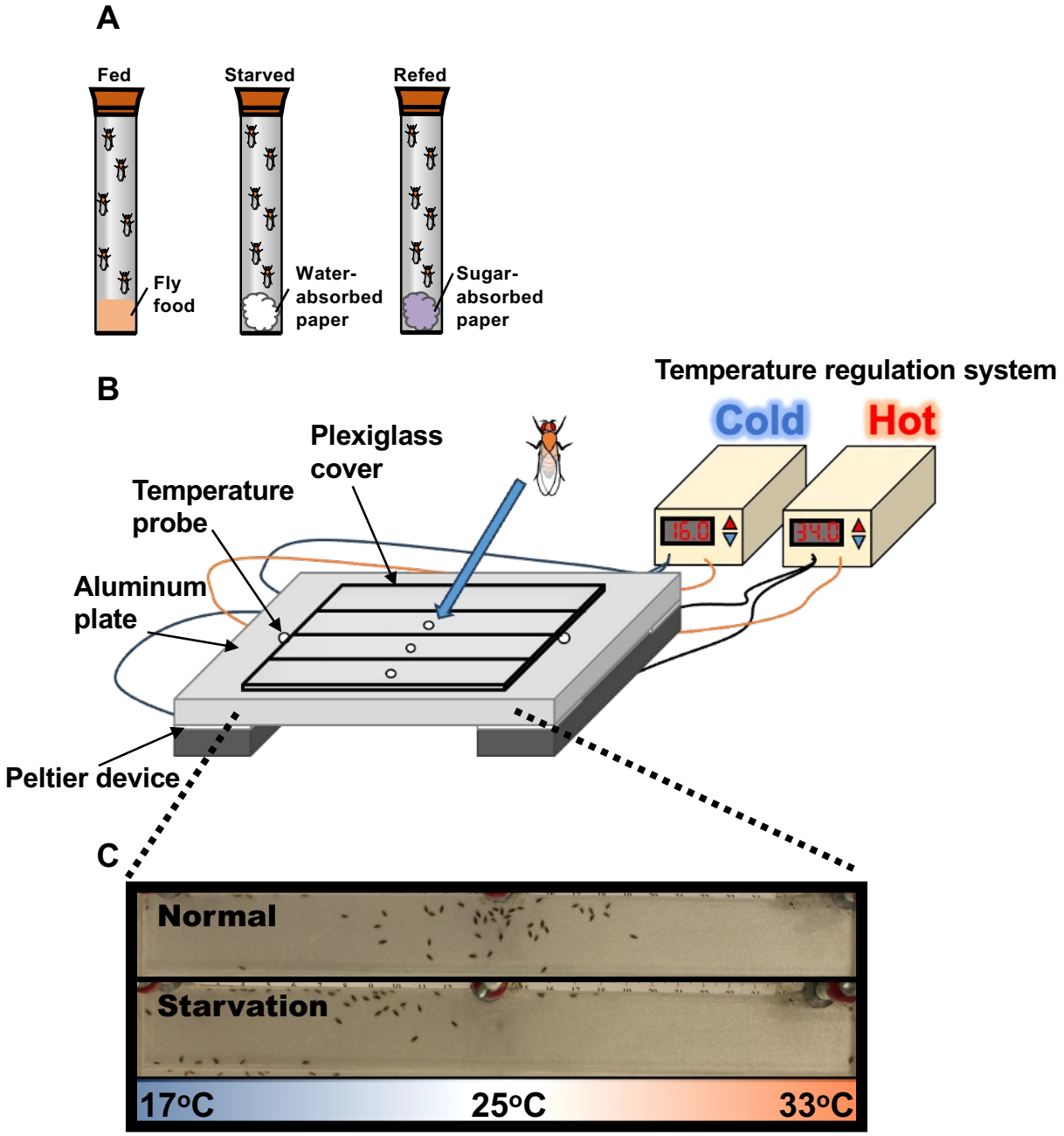

A
Fed
Starved
Refed
Fly food
Water-absorbed paper
Sugar-absorbed paper
B
Temperature regulation system
Cold
Hot
Plexiglass cover
Temperature probe
Aluminum plate
Peltier device
C
Normal
Starvation
17°C
25°C
33°C

Fig. S2

A: *w*[1118]

	Temperature gradient (℃)															
Feeding condition	17-18	18-19	19-20	20-21	21-22	22-23	23-24	24-25	25-26	26-27	27-28	28-29	29-30	30-31	31-32	32-33
Fed	0%	0%	0%	3%	1%	4%	3%	19%	51%	17%	1%	0%	0%	0%	0%	1%
STV	15%	8%	7%	13%	12%	13%	11%	11%	4%	4%	3%	0%	0%	0%	0%	0%
Refed fly food for 5min	6%	6%	6%	5%	10%	8%	12%	18%	15%	8%	4%	1%	1%	0%	1%	0%
Refed fly food for 10min	1%	2%	2%	2%	3%	3%	10%	33%	31%	8%	2%	1%	1%	1%	0%	0%
Refed fly food for 30min	0%	0%	0%	0%	2%	5%	13%	41%	25%	11%	0%	1%	0%	1%	0%	0%
Refed fly food for 1hr	0%	0%	0%	2%	1%	6%	7%	25%	31%	20%	4%	1%	0%	1%	1%	0%
Refed Sucralose for 10min	2%	0%	1%	9%	19%	17%	19%	20%	7%	3%	2%	1%	0%	0%	0%	0%
Refed Sucralose for 1hr	5%	5%	8%	6%	9%	7%	23%	17%	11%	5%	1%	1%	1%	0%	0%	0%
Refed Glucose for 5min	3%	1%	2%	7%	8%	12%	16%	27%	14%	5%	3%	1%	0%	1%	0%	0%
Refed Glucose for 10min	1%	0%	3%	2%	5%	10%	16%	23%	26%	9%	1%	2%	0%	1%	0%	0%
Refed Glucose for 1hr	1%	0%	1%	1%	1%	4%	9%	23%	43%	14%	3%	1%	1%	0%	0%	0%

B: *Gr64fGal4>uas-CsChrimson*

	Temperature gradient (℃)															
Feeding condition	17-18	18-19	19-20	20-21	21-22	22-23	23-24	24-25	25-26	26-27	27-28	28-29	29-30	30-31	31-32	32-33
Fed	0%	2%	1%	1%	1%	5%	8%	10%	43%	25%	5%	0%	0%	0%	0%	0%
STV without ATR and red light exposure for 10min	7%	6%	5%	11%	5%	10%	15%	13%	19%	8%	0%	0%	0%	0%	0%	0%
STV with ATR and red light exposure for 10min	2%	4%	3%	4%	4%	10%	16%	25%	22%	6%	2%	0%	0%	1%	0%	0%

Fig. S3

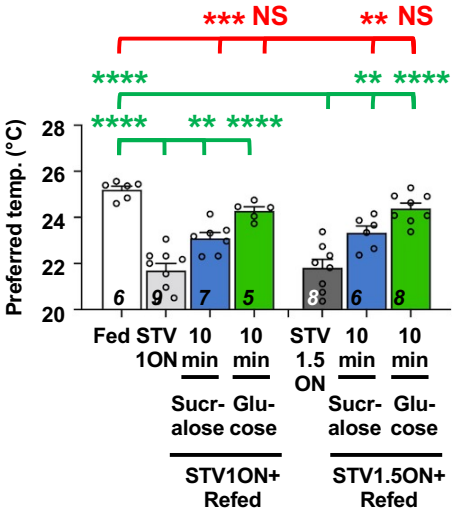

*** NS
** NS
****
** ****
**** ** ****
Preferred temp. (°C)
28
26
24
22
20
6
9
7
5
8
6
8
Fed
STV 1ON
10 min
10 min
STV 1.5 ON
10 min
10 min
Sucr-alose
Glu-cose
Sucr-alose
Glu-cose
STV1ON+ Refed
STV1.5ON+ Refed

Fig. S4

Orco1

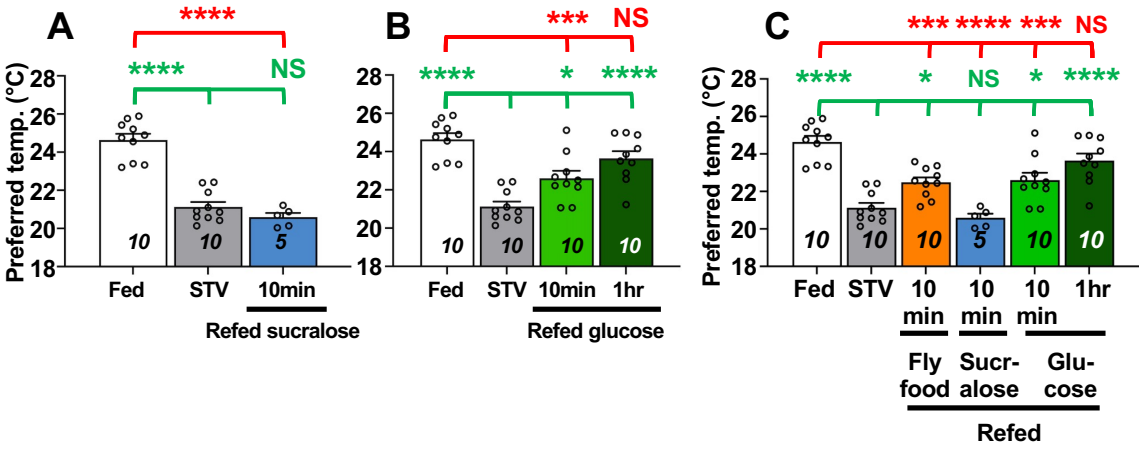

A
B
C
Preferred temp. (°C)
28
26
24
22
20
18
****
NS
***
*
Fed
STV
10min
1hr
10 min
Refed sucralose
Refed glucose
Fly food
Sucr-alose
Glu-cose
Refed

Fig. S5

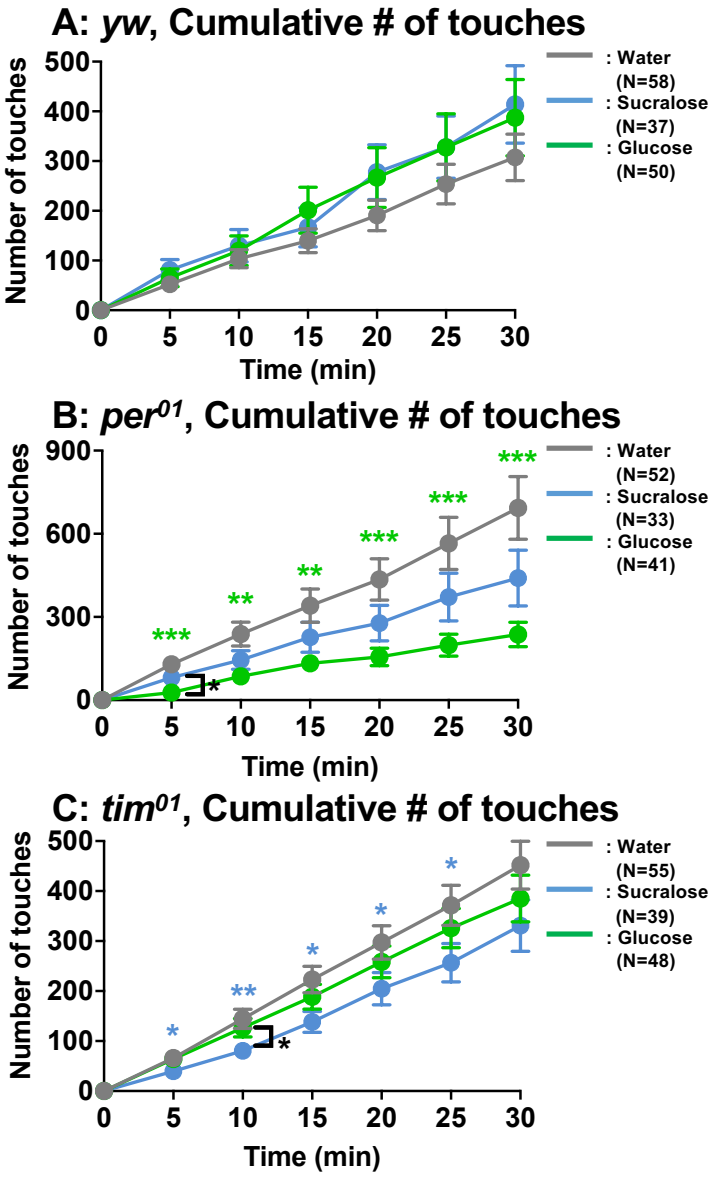
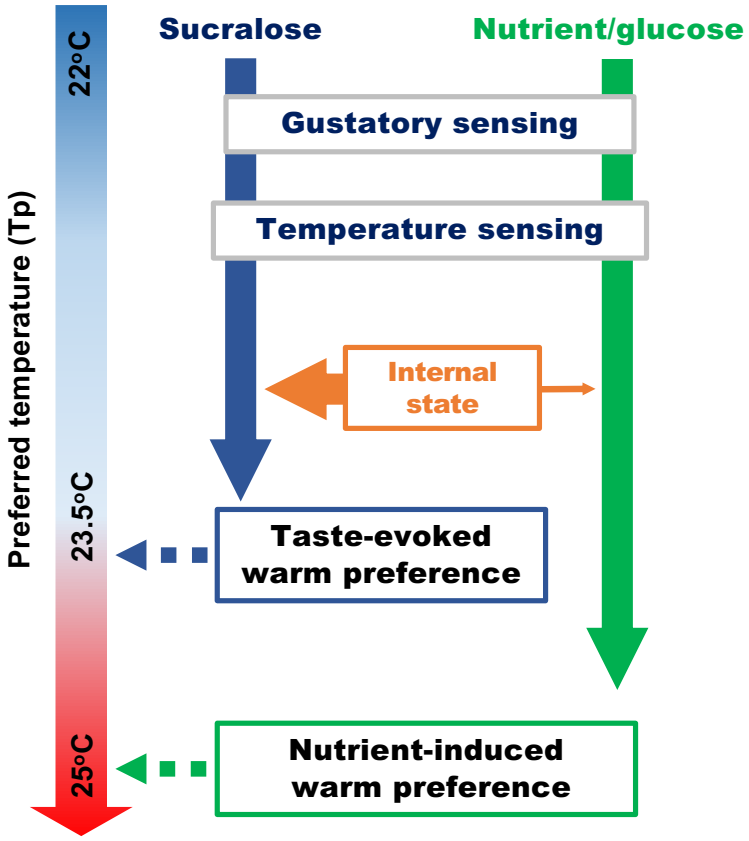

A: *yw*, Cumulative # of touches
Number of touches
Time (min)
: Water (N=58)
: Sucralose (N=37)
: Glucose (N=50)
B: *per*^{01}, Cumulative # of touches
Number of touches
Time (min)
: Water (N=52)
: Sucralose (N=33)
: Glucose (N=41)
C: *tim*^{01}, Cumulative # of touches
Number of touches
Time (min)
: Water (N=55)
: Sucralose (N=39)
: Glucose (N=48)

Fig. S6


Sucralose
Nutrient/glucose
Gustatory sensing
Temperature sensing
Internal state
Taste-evoked warm preference
Nutrient-induced warm preference
Preferred temperature (Tp)
22°C
23.5°C
25°C