DIETS: a simple and sensitive assay to measure and control the intake of complex solid foods, like high-fat diets, in *Drosophila*

Running title: measuring and controlling complex high calorie diet intake in flies

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Abstract

The fruit fly *Drosophila melanogaste*r offers a powerful model to study how diet affects the body and brain. However, existing methods for measuring their food intake often rely on dyes or tags mixed with food, which can be inaccurate due to how the flies absorb and eliminate them. Capillary-based assays like CAFE directly measure consumption, but only work with liquids and shorten fly lifespan. Additionally, capillary assays are incompatible with delivering viscous foods like high-fat diets. Even solidified high-fat diets tend to be sticky death traps for flies. Another longstanding challenge for fly researchers is that dietary restriction in flies involves diluting food, leading to compensatory feeding. To address these shortcomings, we have developed DIETS, a sensitive feeding assay that can be implemented even in low-resource settings. DIETS eliminates the need for labels and directly weighs the solid food consumed by small groups of flies over extended periods of hours to weeks. It allows us to deliver precise amounts of food to flies and implement accurate dietary restrictions. Importantly, DIETS is compatible with studying energy-dense high-fat diets. Using DIETS, we observed that, unlike a high-sugar diet, an isocaloric high-fat diet did not improve the flies' ability to withstand starvation, even though they consumed more calories and had higher fat deposition.

Keywords

Feeding assay, behavior, Drosophila, dietary restriction, (DR), food choice, high-fat diets, high-sugar diets, HFD, HSD, starvation resistance, SR

Introduction

The quality, quantity, and timing of food intake have a profound impact on the overall health, fecundity, cognition, and longevity of an organism. Food intake is influenced by internal states that reflect a lack of specific nutrients (Bjordal et al. 2014; Ribeiro and Dickson 2010; Toshima and Tanimura 2012; Prasad and Hens 2018; Steck et al. 2018; Musso, Junca, and Gordon 2021; Stafford et al. 2012; Malita et al. 2022; Morton, Meek, and Schwartz 2014), or by internal states that result from stress and pathogenesis (Crompton 1984; Yau and Potenza 2013). Food intake is also influenced by innate and learned sensory inputs, that signal the presence of nutrients (Shiu et al. 2022; Weaver et al. 2023; Das, Lin, and Waddell 2016; Oh et al. 2021; Fantino 1984). Humans and fruit flies share deep evolutionary conservation in physiology, metabolism, and nervous system function (Ugur, Chen, and Bellen 2016; Jennings 2011; Yamaguchi 2018; Diop and Bodmer 2012; Kornberg and Krasnow 2000). Precise genetic tools in flies allow the investigation of the neuro-physiological mechanisms that underlie appetite control and the myriad effects that follow the ingestion of specific foods (Lin, Senapati, and Tsao 2019; Guo, Pan, and Gong 2019; Yoshihara and Ito 2012; Owald, Lin, and Waddell 2015; Pool and Scott 2014).

For all such studies, the ability to measure, or control feeding precisely is critical. A deep understanding of the underlying biology remains incomplete without knowing how much food is consumed. Currently, there are multiple assays for estimating feeding in flies. However, they all suffer from problems that prevent their use across the board for different dietary compositions. Specifically, such shortcomings include relying on proxy measurements of feeding using dyes and other tags, using liquid-

only diets, incompatibility with longitudinal studies, and the need to sacrifice flies for measurement.

As flies consume only ~ 0.5 - 2.5 microlitres of food in a day (William W. Ja et al. 2007; Wu et al. 2020; Deshpande et al. 2014; Carvalho, Kapahi, and Benzer 2005), many fly-feeding assays rely on food tagged with dyes, radiolabeled molecules, or short oligonucleotides (Shell et al. 2018, 2021; Park, Tran, and Atkinson 2018; Wu et al. 2020; Geer, Olander, and Sharp 1970). The amount of 'tag' ingested, and eventually excreted acts as a proxy for consumption. Each tag must be carefully screened to determine whether their taste, absorption, incorporation, and excretion influence measurement, especially when physiology changes with age or genotype (Marx 2015; Deshpande et al. 2014; Shell et al. 2018, 2021). Quantification of an ingested tag requires sacrificing flies. Only the EX-Q (excreta quantification) assay avoids sacrificing flies by chasing out all ingested dye with an extended feeding period on unlabeled food. Food intake was then estimated by dye quantification from the excreta, leaving the flies alive for longitudinal experiments (Wu et al. 2020).

Ethologically, flies likely feed on liquid, solid, and semi-solid food sources. However multiple tagged and direct assays have been developed for flies solely for measuring liquid diet intake (Sellier, Reeb, and Marion-Poll 2011; Qi et al. 2015; Murphy et al. 2017; Yapici et al. 2016). Their designs are based on the CApillary FEeder Assay (CAFE), where liquid food consumed from a microcapillary is directly quantified by the drop in meniscus (William W. Ja et al. 2007). Recent reports show that feeding liquid diets over days from capillaries could nutrient-deprive flies and affect both

87 lifespan and fecundity (Park, Tran, and Atkinson 2018; Lee et al. 2008; Vigne and
88 Frelin 2010; Deshpande et al. 2014).
89
90 *Drosophila* is being widely and effectively used to gauge the effect of high-fat or
91 high-sugar diets (HFD and HSD respectively) on the physiology, metabolism,
92 nervous system function and behavior (Birse et al. 2010; Laura Palanker Musselman
93 et al. 2011; L. P. Musselman, Fink, and Baranski 2019; Van Dam et al. 2021; Vaziri
94 et al. 2020; May et al. 2019; Yang et al. 2023; Liao, Amcoff, and Nässel 2020).
95 Capillary-based liquid assays are likely incompatible with viscous, multi-ingredient
96 laboratory diets, especially liquid high-fat diets. Even when agarified, solid high-fat
97 diets tend to become sticky and trap flies on them (Diop, Birse, and Bodmer 2017),
98 especially with rising temperatures. Such problems could be the reason why very
99 few fly studies with high-fat diets have attempted to measure actual food intake,
100 especially over long periods (Shi et al. 2021; Eickelberg et al. 2022).
101
102 Here, we describe a simple, and versatile feeding assay that directly measures the
103 actual amount of solid food consumed by groups of flies. Dubbed DIETS (Direct
104 Intake Estimation and Tracking of Solid food consumption), the assay is easily
105 adaptable and inexpensive to establish even in low-resource settings. DIETS can
106 measure feeding over both short and long periods, typically from 30 minutes to 24 h.
107 Further, by periodically switching flies to fresh DIETS vials, longitudinal feeding
108 measurement can be performed over weeks. By placing an additional food cup in a
109 DIETS vial, we could also monitor short- and long- term feeding preferences in flies
110 between two distinct foods. Crucially, the small surface area of the food make DIETS
111 perfectly suited for complex high-fat diets. Flies do not stick to the food and do not

112 die even at higher temperatures. We have also modified DIETS to a flat arena setup
113 which can be combined with videography to determine the position of flies during an
114 experiment and potentially record its contact with food.
115
116 DIETS addresses a significant drawback of fly dietary restriction (DR) assays. In
117 flies, DR is mainly achieved by diluting food or nutrients (Hodge et al. 2022;
118 Partridge, Piper, and Mair 2005; Carvalho, Kapahi, and Benzer 2005; Piper and
119 Partridge 2007). However, food dilution leads to compensatory feeding and could
120 complicate the interpretation of the results (Carvalho, Kapahi, and Benzer 2005;
121 Vigne and Frelin 2010). With DIETS, we demonstrate that flies could be restricted to
122 feeding an exact amount of complex foods by offering a predetermined amount of
123 food in the cups. Hence, DIETS would be extremely useful for DR and Time
124 Restricted Feeding (TRF) protocols with complex diets.
125
126 The mechanisms by which food intake may alter fitness are not all well understood.
127 We employed DIETS to assess how two distinct calorie-dense, isocaloric food
128 sources, one rich in sugar and the other rich in saturated fats, could affect
129 food/calorie intake and contribute towards starvation resistance (SR). Surprisingly,
130 we found that while flies ingested considerably higher calories on a high-fat diet than
131 on a high-sugar diet, starvation resistance was only enhanced with the latter. The
132 size of bodily fat deposits has been strongly correlated with starvation resistance.
133 Our findings indicate that energy intake and body fat reserves do not necessarily
134 correlate positively with the flies' response to starvation.

135 **Results**

136 **Measurement of feeding by directly weighing food consumed**

137 The DIETS assay quantifies solid food consumption in flies. Consumption is directly
138 measured by weighing the amount of food offered in 4 mm diameter 'cups' to a
139 group of flies at the start of an experiment and finally weighing the amount of food
140 left over at the end of the experimental period (Fig. 1A, Fig. S1A, and Movie S1). A
141 0.75 % agar bed at the bottom of the vial minimizes evaporation and provides a
142 separate water source. A mean food weight loss from a control diet (CD; see Table
143 ST1) due to evaporation was calculated from evaporation control vials without flies
144 and was subtracted from concurrent intake measurements (Fig. 1A, Fig. S1B). We
145 typically estimated consumption in sated CS-Q flies over 2 to 24 h periods, after
146 which experiments were either terminated, or flies were transferred to fresh DIETS
147 vials for further measurements (Fig. 1A). All experiments were carried out at 25 ^{o}C
148 and 65 - 70 % relative humidity unless otherwise mentioned.

149

150 We empirically determined the size of the cups and the distance from the agar bed at
151 which loss of food by evaporation from CD was minimal. Evaporation from a 4 mm
152 diameter cup was lower than that from 6 mm or 8 mm diameter cups, and all were
153 placed 1 mm away from the agar bed (Fig. S1C). Evaporation loss from a 4 mm
154 diameter cup was minimal when placed 1 mm away from the agar bed and went up
155 considerably when the distance of the cup from the agar bed was increased up to 5
156 mm at both 18 and 25 ^{o}C. As expected, evaporation loss was higher at 25 ^{o}C than at
157 18 ^{o}C (Fig. S1D).

158

159 Intuitively, food consumption should not change significantly with small changes in
160 food cup position. We observed that the calculated CD consumption dropped if we
161 increased the distance of food cups from the agar bed from 1 mm to 3 mm, or 5 mm
162 when subtracting mean evaporation loss (Fig. S1E; left panel). However, the mean
163 consumption values remained unchanged when the mean evaporation loss was not
164 accounted for (Fig. S1E; right panel). This suggested that we were perhaps
165 overestimating evaporation loss using the evaporation control vials, especially when
166 cups were placed a little further away from the agar bed. As this overestimation of
167 evaporation loss was the minimum at 1 mm (Fig. S1D), we decided to keep cups at
168 this distance from the agar bed, unless otherwise required.
169

170 **DIETS is a sensitive and precise feeding assay**

171 To characterize the resolving power and consistency of the DIETS assay, we
172 measured feeding on CD after varying sex, group size, temperature, and food
173 dilutions. Such conditions have been reported to have affected consumption (Wu et
174 al. 2020; Klepsatel, Wildridge, and Gáliková 2019; William W. Ja et al. 2007;
175 Deshpande et al. 2014). Firstly, males and females were segregated into groups of
176 10 to 60 flies to measure food consumption over 24 h at 25 ^{o}C. Like previous reports
177 (Deshpande et al. 2014; Wu et al. 2020; Carvalho et al. 2006), the mean
178 consumption of mated females was between 40 % to 125 % more than males from
179 the same group size (Fig. 1B; see effect sizes or Δ consumption between two
180 groups). We also observed that the consumption rate per fly increased with the
181 group size and eventually plateaued with a group size of 50 for males and 25 for
182 females (Fig. 1B, Table ST2). Therefore, as reported earlier, the alteration of social
183 interactions with group size seemed to influence individual feeding behavior (Wong

184 et al. 2009). As expected, the total food consumed by a group increased with the
185 group size for both sexes and was clearly discernible with DIETS (Fig. S2A, S2B,
186 and Table ST2). In experiments with mated females, we observed extensive egg
187 deposition, mostly on the agar bed (Fig. S2C). Only ~ 2 - 4 eggs were found on a
188 food cup on average, with the number going up to ~ 8 eggs for 60 females (Fig.
189 S2D). As the mean egg weight was ~ 32 □g (data not shown), egg weight could be
190 ignored, especially if the ones on the outside of the cup were cleared before final
191 weighing. In case larvae are seen on food, we suggest switching food more
192 frequently and performing the experiments at a lower temperature if possible.
193
194 A recent study in flies has shown that an increase in temperature enhances
195 metabolic rate and feeding (Klepsatel, Wildridge, and Gáliková 2019). We also saw a
196 clear increase in CD consumption by male flies over 24 h when we raised the
197 temperature from 18 ^{o}C to 22 ^{o}C and then from 22 ^{o}C to 25 ^{o}C (Fig. 1C; see effect
198 sizes). Consumption on CD was even higher at 32 ^{o}C (Fig. S6C). We could detect
199 such changes in temperature-dependent consumption with a sample size of 12 vials
200 per condition.
201
202 Existing solid food feeding assays can detect compensatory increase or decrease in
203 feeding in flies upon diluting or concentrating food (Deshpande et al. 2014; Wu et al.
204 2020). With DIETS, we were able to discern clear differences in feeding between 1X
205 CD and 0.5X CD (~ 21 % increase) or between 1X and 2X CD (~ 56 % decrease)
206 (Fig. 1D).
207

208 To directly compare the sensitivity of DIETS with the previously reported CAFE and
209 EX-Q data (Wu et al. 2020), we measured compensatory feeding from 1X to 2X and
210 to 4X concentrations of 2.5 % sucrose - 2.5 % yeast extract (2.5 % SY: 1X) diet
211 (n=10, to match with Wu et al. 2020). We compared the standardized effect sizes in
212 Hedge's g along with its MOE (half the magnitude of 95 % CIs of the Hedge's g: see
213 Materials and Methods for further details) across assays (Fig. 1E). Between 1X and
214 2X diets, the analysis showed that DIETS and CAFE report effect sizes that are
215 close to each other (-2.59 for DIETS, and -3.12 for CAFE). However, a larger effect
216 size is seen with EX-Q (-5.51), mainly because 1X consumption measured by EX-Q
217 was elevated (Fig. 1E). Between 2X and 4X diets, all three assays report quite
218 similar effect sizes (-2.87 for DIETS, -2.39 for CAFE, and -2.98 with EX-Q). Keeping
219 in mind the small size of the data sets (n=10), the MOE was also generally
220 comparable (Fig. 1E). We concluded that DIETS was as sensitive as CAFE and EX-
221 Q and likely equally precise (compare MOEs of all DIETS data reported in Table
222 ST2).
223
224 To test whether DIETS can be used to assess longitudinal feeding, we transferred
225 groups of 50 male flies into fresh assay vials every 24 h for 4 weeks. Actual
226 consumption was assessed over the 1st, 4th, 7th, 14th, 21st, and 28th day (schema;
227 Fig. 1F). Feeding was reliably measured over the whole period, with very little
228 change in feeding when compared to the 1st-day measurement, except on the 28th
229 day when a considerable feeding decline was observed. We also observed that with
230 a large group size of 50, flies started to die around the 14th day, and by the 28th day,
231 almost 50 % of the flies had perished (Fig. 1F).
232

233 Next, to test whether flies were starving in DIETS due to a smaller food source and
234 hence eventually dying early, we compared the starvation resistance of male flies
235 (10, 25, and 50 flies/group) after being in DIETS vials or normal food vials for 1 day
236 and 7 days. We found that flies showed almost similar starvation resistance across
237 group sizes and modes of food delivery (Fig. S3A). Together, our results suggest
238 that no discernible change in survival is observed in DIETS up to 7 days. We also
239 measured feeding on CD in two other strains available in the lab, *CS-BZ,* and w^{1118},
240 and observed that *CS-BZ* had elevated feeding as compared to the other two strains
241 (Fig. S3B).
242
243 To demonstrate the suitability of DIETS with thermogenetic manipulations at 32 ^{o}C,
244 we activated Piezo-GAL4 labeled neurons using a temperature-sensitive cation
245 channel, dTRPA1 (Hamada et al. 2008) during a 30-minute feeding period at 32 °C.
246 Piezo-GAL4 labeled mechanosensory neurons densely innervate the crop and
247 anterior midgut and regulate ingestion volume by controlling the crop size (S. Min et
248 al. 2021). As reported earlier (S. Min et al. 2021), we too observed a defect in
249 feeding, in fact, a more severe one, upon artificial activation of Piezo-GAL4 neurons
250 compared to parental controls (Fig. S3C; left panel). Feeding reduction in Piezo-
251 GAL4>UAS-dTRPA1 flies was not observed at 21 °C (Fig. S3C; right panel).
252 Activation of serotonergic neurons labeled by R50H05-GAL4 induces hunger and
253 feeding in sated flies (Albin et al. 2015). We replicated these results with DIETS, with
254 increased feeding observed in R50H05-GAL4>UAS-dTRPA1 flies at 32 °C (Fig.
255 S3C; left panel), but not at 21 °C (Fig. S3C; right panel).
256
257

Short-term feeding measurements with DIETS

Next, we wanted to determine whether DIETS could be used for short-term feeding measurements. For this, we starved groups of 50 male flies on 0.75 % agar for 6 h, 12 h, or 24 h. We then measured the consumption of these differentially starved groups for 30 minutes in DIETS vials with Brilliant Blue FCF labeled CD (Fig. 2A, schema). Parallelly, flies were also processed after 30 minutes to measure consumption colorimetrically. Both techniques could detect the linear increase in consumption with increasing starvation and also detect sizable effect size differences with each step of starvation. For the sake of comparison across assay types, here too, we plotted standardized effect sizes in the form of Hegde's g (Fig. 2A). With DIETS, we had larger Hedge's g values (Fig. 2A) than with blue dye incorporation across all comparisons (feeding after 6 -vs.- 0, 12 -vs.- 6 and 24 vs. 12 h starvation times). The MOEs of these effect sizes are also comparable across the two assays (Fig. 2A; see values represented as effect sizes, MOEs; also in Table ST2). These results suggested that for short-term measurements, DIETS was more sensitive in picking differences than blue dye incorporation but equally precise.

Next, to demonstrate whether we can use DIETS for repeated short-term feeding measurements, we measured feeding every 2 h in groups of 50 male and female flies for 2 days continuously (Fig. 2B, schema). Previous studies had reported two peaks of feeding around the morning and evening light-dark transitions (May et al. 2019; Niu et al. 2021; Ro, Harvanek, and Pletcher 2014; Li et al. 2021; Xu, Zheng, and Sehgal 2008). However, we observed only a single evening feeding peak around 'lights-off' (Fig. 2B). Combining consumption measurements of the two 'peak' groups spanning the 'lights off' time and comparing them with similarly merged 'day'

283 and ‘night’ groups on either side, we could see the effect of day to night transition on
284 feeding (Fig. S4A). We also observed that otherwise, feeding levels were quite
285 similar both during the day and night. From the above experiments, we concluded
286 that DIETS is also sensitive and precise for both discrete and repeated short-term
287 food intake measurements in sated flies.
288

289 Short-term and long-term two-choice food preference with DIETS

290 We reasoned that dietary choice between two foods could also be measured if flies
291 were given access to two cups of distinct foods. To validate food preference, we
292 arranged choices between CD and CD laced with increasing concentrations (2.5 mM
293 to 15 mM) of bitter-tasting quinine (QUI), for groups of 50 males, for 24 h (Fig. 2C,
294 schema). Cups were placed 1 mm from the agar bed and diametrically opposite to
295 each other. Fully assembled DIETS choice vials were covered with white paper to
296 act as a diffuser and prevent light bias (Fig. S4B; images). In control DIETS vials,
297 flies ate equally on average from both the cups with CD. As expected, flies
298 preferentially fed from the CD cup over the CD + QUI cup, with increasing
299 concentrations of quinine (Fig. 2C). However, combined consumption of food from
300 both cups remained unchanged across quinine concentrations (Fig. S4C). Similarly,
301 we measured short-term preference over 2 h in starved flies. We observed a strong
302 preference to feed from the CD cup over the CD + 15 mM QUI food cup (Fig. 2D).
303 Total consumption stayed constant in this case, as well (Fig. S4D). Food preference
304 in DIETS can also be represented as a consumption preference index (CPI) (Fig. 2C;
305 see inset). Corresponding CPI values are shown on top of the consumption plots
306 (Fig. 2C and 2D).
307

308 **Implementing precise DR and TRF regimens with DIETS**

309 Dietary restriction (DR) in flies is usually carried out by diluting food or protein
310 components (Partridge, Piper, and Mair 2005; Bass et al. 2007; Piper and Partridge
311 2007; W. W. Ja et al. 2009; K.-J. Min and Tatar 2006; Hodge et al. 2022; Katewa et
312 al. 2012). Since we could control the amount of food we offer to flies, we decided to
313 ascertain whether we could use DIETS to achieve precise DR.
314 In the 'no DR' control groups, 56 mg of CD was offered to groups of male flies on
315 average (Fig. 3A). We had determined that this amount of food was non-limiting to a
316 group of 50 male flies (see Material and Methods for details). To implement 90 %
317 DR, the mean food offered to a group of flies was adjusted to be ~90-91% of 56 mg
318 (51 mg), on average to groups of males (Fig. 3A; labeled 'a'). We similarly aimed for
319 various degrees of DR up to 60% [Fig. 3A; (a / 56) * 100. Ultimately, achieved DR
320 was calculated based on average actual consumption in each group (Fig. 3A;
321 labeled 'b'), in proportion to the mean consumption (43 mg) in the 'no DR' group [Fig.
322 3A; (b / 43) * 100]. We found that the amount fed by groups of flies at all the DR
323 levels clustered narrowly. This was also reflected in the extremely narrow MOEs of
324 each group (Fig. 3A, see error distribution of effect sizes. Also see Table ST2). We
325 concluded that the DIETS setup can be used to restrict the amount of food
326 consumed by a group of flies within a few percentage points.
327
328 Time-restricted feeding (TRF) protocols have been imposed on flies to gauge the
329 effect on the overall health and longevity of flies (Cabrera, Young, and Axelrod 2020;
330 Gill et al. 2015; Ulgherait et al. 2021). To investigate the impact of a daily pattern of
331 feeding and fasting on food intake with DIETS, we measured CD consumption in
332 groups of 20 male flies for 24 h under three different conditions: (a) the TRF group

333 where feeding was restricted to ‘lights-on’ phase, ZT = 0 - 12, (b) An *ad libitum*
334 feeding “ALF (24)” group with food cup changed every 24 h at ZT = 0, and (c) A
335 second a*d libitum* feeding group, “ALF (12 + 12)” with food cup changed every 12 h
336 at ZT = 0 and 12. All groups were habituated as described above for 3 days to let
337 them adjust to their respective conditions. Consumption was measured for the next
338 24 h period (Fig. 3B; schema). In agreement with previous reports (Cabrera, Young,
339 and Axelrod 2020; Gill et al. 2015), we found that flies under TRF consumed similar
340 amount of food during the daytime as the ALF (24) and ALF (12 + 12) groups
341 managed to eat over a whole day (Fig. 3B). Next, upon comparing daytime feeding
342 ‘a’ with nighttime feeding ‘b’ within the ALF (12+12) group, our data revealed that
343 flies fed equally during the day and night (Fig. 3B). We concluded that DIETS can be
344 used to implement TRF protocols with solid foods and simultaneously measure
345 feeding too.
346

347 Combining consumption measurement with video tracking: DIETS-
348 arena

349 DIETS vials are not ideal for videographic analysis because of their dimensions and
350 also due to the location of the cups next to the agar bed. As a proof of concept that
351 video analysis of fly location can be combined with DIETS style measurement of
352 food consumption, DIETS-arenas were designed (Fig. S5A). First, to determine the
353 efficacy of the flat DIETS-arenas, we measured 2 h CD consumption in sated and
354 starved male flies with both DIETS vials and DIETS-arena side by side (Fig. 3C;
355 schema). We observed a similar increase in feeding in the starved groups compared
356 to the sated group with both vials and arenas (Fig. 3C, left panel). The feeding

357 baseline was higher in the DIETS-arenas compared to DIETS vials for both sated
358 and starved flies. Further investigation will be needed to determine the cause.
359 Next, to analyze the temporal dynamics of feeding with the same set of sated and
360 starved flies, we defined a $Food_{ROI}$ around the food cup in the center of the arena
361 (see Material and Methods). We found that the percentage of starved flies that
362 occupied the $Food_{ROI}$, went from 74 % to ~ 40 % in the first 20 minutes. Thereafter
363 starved flies maintained an average of ~ 44 % $Food_{ROI}$ occupancy. In contrast, fed
364 flies maintain a low and steady ~ 11 % occupancy of the $Food_{ROI}$ over 90 minutes
365 (Fig. 3C, right panel). This pattern is also reflected in temporal snapshots from two
366 representative sets, each of sated and starved flies (Fig. S5B). In conclusion, with
367 the help of video analysis from DIETS-arenas, we could visualize dynamic changes
368 in food occupancy that could be correlated with the increased feeding observed in
369 starved flies.
370

371 **Longitudinal measurements of high-fat diet (HFD) intake with DIETS**

372 High-fat diets (HFDs) for flies have been typically made by adding coconut oil as a
373 source of saturated fat to fly food (Birse et al. 2010; Diop, Birse, and Bodmer 2017).
374 HFDs are difficult to work with as the fat melts even at ~ 25 °C, rendering the food
375 sticky and lethal for flies unless care is taken (Diop, Birse, and Bodmer 2017). The
376 sticky consistency of fatty food could potentially affect feeding too. While a previous
377 study claims the use of CAFE for delivering high-fat food (Shi et al. 2021) , we
378 sought to independently verify the suitability of CAFE with 10 % sHFD made in the
379 lab. We re-confirmed earlier observations in the lab that such complex food tends to
380 separate within the CAFE capillaries, leading to gaps in the continuous supply of
381 food (Fig. S6A). We reasoned that DIETS, with its low food surface area, may

382 resolve such issues. We prepared HFDs with added 5 % to 20 % coconut oil at the
383 expense of water in the CD. We recalibrated evaporation control vials for HFDs and
384 found that HFD food cups were best placed 2 mm from the agar bed. At lesser
385 distances (1 mm), the HFD tended to accumulate moisture, leading to misleading
386 changes in weight (data not shown). Consumption was measured on the 1st, $4^{th,}$ and
387 7th day (Fig. 4A, schema). We found that consumption of diets with 5 % and 10 %
388 added coconut oil was comparable with CD throughout (Fig. 4A). As the added lipids
389 made the food more energy-rich, energy intake was increased at both these
390 conditions compared to CD (Fig. S6B). We also saw that consumption of the diet
391 with 20 % added oil was considerably reduced from all the other groups (Fig. 4A; see
392 effect sizes HFD). As a result, energy intake in the 20 % HFD group was virtually
393 similar or slightly increased to the 10 % HFD group (Fig. S6B). To determine whether
394 decreased food intake on HFDs reflects an energy limit sensing mechanism or
395 simply because higher fat concentrations are taste aversive to flies requires further
396 investigation. DIETS was also suitable for consumption measurement on HFD even
397 at 32 ^{o}C (Fig. S6C), enabling thermogenetic experiments with HFDs. Overall, we
398 concluded that DIETS was suitable for feeding measurements on HFDs over a wide
399 range of temperatures.

400

401 As reported earlier (Birse et al. 2010), upon lipophilic Oil-Red-O staining, we
402 detected increasing mid-gut fat deposition after 7 days with an increasing percentage
403 of oil in the flies' diet (Fig. S6D). This showed that HFD feeding in DIETS yielded
404 expected physiological changes. Next, to ascertain whether feeding from HFDs in
405 DIETS influenced gene expression changes in key genes, as reported earlier by
406 RNA-Seq (Stobdan et al. 2019), we performed q-PCR analysis. We extracted RNA

407 from the heads of male flies kept on either 20 % HFD or CD for 1, 4, and 7 days and
408 probed gene expression using q-PCR (see Materials and Methods). We re-analyzed
409 the raw RNA-Seq data generated previously (Stobdan et al. 2019) using DESeq2
410 (Love, Huber, and Anders 2014) and used p-adjusted < 0.05 as our threshold (Table
411 ST3). From this analysis, we selected four of the most differentially expressed genes
412 (DEGs) to analyze from our collected head RNA samples. Out of these, three were
413 upregulated, namely *takeout* (*to*), *Obp83a*, and *Cyp4e3*, and one down-regulated
414 gene, *TotA* (Fig. 4B). Our experiment revealed dynamic changes in the expression of
415 the selected genes due to HFD feeding. On day 1, the expression of these genes
416 showed variability as compared to the earlier study (Stobdan et al. 2019). However,
417 by day 4 and then on day 7, their expression was consistent with Stobdan et al.,
418 2019 (Fig. 4B). We concluded that DIETS enables us to measure feeding in a group
419 of flies and subsequently use the same animals for post-feeding analyses like gene
420 expression changes.

421

422 **A saturated high-fat diet fails to enhance starvation resistance**

423 Chronic consumption of diets rich in sugar improves starvation resistance (SR) in
424 female flies, likely due to increased bodily fat deposits (van Dam et al. 2020;
425 Chandegra et al. 2017; Lee and Jang 2014; Strilbytska et al. 2022; Baldal,
426 Brakefield, and Zwaan 2006; Ballard, Melvin, and Simpson 2008). However,
427 although sHFDs increase body fat deposits (Fig. S6D), a solitary study has studied
428 and reported a mild increase in SR upon sHFD feeding (Heinrichsen and Haddad
429 2012). As DIETS improve how sHFD is delivered to flies and likely decreases
430 aversion to food due to sticky surface texture, we wanted to re-examine the role of
431 sHFDs on SR. Hence, we provided OreR flies with an isocaloric 30 % high sugar diet

432 (HSD: ~21 % sucrose + other sugars) and 10 % sHFD (coconut oil) for 7 days.
433 Feeding was assessed on days 1, 4, and 7. Subsequently, flies were subjected to
434 starvation stress in 0.75 % agar vials and survival was ascertained every 24 h
435 (schema; Fig. 4C). We found that flies on 30 % HSD, consumed significantly less
436 food, but maintained a total energy intake comparable to flies on CD control food
437 (Fig. S6E and 4C). In contrast, flies on 10 % sHFD, consumed amounts
438 intermediated between those of flies on CD and 30 % HSD (Fig. S6E). However,
439 sHFD being energy dense, the flies consumed significantly more energy than the
440 control flies (Fig. 4C). Subsequently, we found that a 30 % HSD diet promotes
441 increased SR in female flies as reported earlier. However, 10 % sHFD-fed flies
442 showed near identical SR compared to control flies (Fig. 4D). We concluded that a
443 10% sHFD diet is unable to enhance SR in flies.

Discussion

DIETS is a direct measurement of food consumed by flies with no added dyes or tracers (Fig. 1). Therefore, physiological changes associated with genotype or age, including changes in the gut, do not influence measurement. While DIETS is compatible with group sizes varying from ~ 10 - 60 flies, we recommend that the group size should be optimized for specific experiments. When we measured food consumption in groups of 50 males, there was significant death after 2 weeks of feeding in DIETS vials (Fig. 1E). This could be due to increased stress and struggle for a small food source in larger groups, and likely to be mitigated by using a smaller group size for longitudinal measurements. Indeed, previous longevity studies have used much smaller group sizes of 10 - 15 flies (Lee et al. 2008; Metaxakis and Partridge 2013; Zanco et al. 2021; Krittika and Yadav 2020). We have shown that DIETS could be also utilized for measuring food preferences (Fig. 2). With DIETS we can easily monitor potential shifts in food preference between two, perhaps even three food sources, over days and weeks. The assay is also precise, and with proper humidity control, yields measurements with low variability (see MOEs in Table. ST2).

With regards to versatility, ease, and cost of use, only CAFE and EX-Q assays are comparable with DIETS (Fig. 1E). We show here that DIETS is as sensitive as CAFE (William W. Ja et al. 2007; Diegelmann et al. 2017) and EX-Q (Wu et al. 2020) to discern biologically relevant differences in food consumed (Fig. 1E; see effect sizes).

While CAFE directly measures liquid food intake, it has major drawbacks. Feeding long-term in CAFE significantly reduces both egg-laying and lifespan (Wong et al. 2009; Deshpande et al. 2014). Additionally, while studies have reported using CAFE

469 for HFDs (Shi et al. 2021), we show that complex diets like HFDs separate inside
470 capillaries, making it impossible to measure consumption (Fig. S6A). EX-Q is well
471 suited for longitudinal studies, for use with complex diets, for estimating food
472 preference with two compatible dyes, and possibly also for dietary restriction
473 protocols (Wu et al. 2020; Shell et al. 2021). However, any protocol requiring
474 measurement for short consecutive periods will not be possible with EX-Q because
475 of the ~ 3 h 'chase' phase on non-dyed food required to excrete out all the dye in
476 flies. Therefore, longitudinal measurements with EX-Q require an extra fly transfer
477 step and estimate daily consumption for only a 21 h period. Flies can independently
478 regulate food and water intake (Van Dam et al. 2021; Fanson, Yap, and Taylor 2012;
479 W. W. Ja et al. 2009). In DIETS vials, the agar bed provides a separate water source
480 to allow flies to meet their water needs independently from their food drive (Fig. 1A).
481 No separate water source is provided in the EX-Q assay (Wu et al. 2020). Ultimately,
482 EX-Q is still an indirect method of feeding quantification, and any change in dye
483 absorption or excretion with age or genotype would affect the measurement.
484
485 The DIETS assay is versatile and measures feeding over different time scales for
486 diverse foods, including high-fat diets that are difficult to work with (Diop, Birse, and
487 Bodmer 2017). This ability of DIETS is an especially significant feature as it allows
488 long-term experiments with HFDs with accurate fatty food intake measurement. We
489 are not aware of such studies in the current literature. One significant shortcoming of
490 DIETS is that it cannot monitor feeding individuality in single flies. Capacitance-
491 based single fly feeding assays like FLIC, flyPAD, optoPAD, or dFRAME are highly
492 sensitive and outperform DIETS in determining feeding microstructure, including
493 feeding duration and frequency of individual feeding bouts (Ro, Harvanek, and

494 Pletcher 2014; Itskov et al. 2014; Moreira et al. 2019; Niu et al. 2021; Weaver et al.
495 2023). However, these assays do not measure feeding directly and only quantify the
496 duration and frequency of fly proboscis contact with food. The contact of the
497 proboscis with food may not always correlate directly with the amount ingested and
498 may vary with age or genotype. Ultimately, these assays have much higher setup
499 and repair costs than DIETS and are not affordable for many. Here, we demonstrate
500 that direct food intake measurements can be carried out from flat and transparent
501 DIETS-arenas and combined with video-based temporal analysis of a fly's position.
502 Similar to previous studies (Niu et al. 2021; Jaime et al. 2023; Weaver et al. 2023),
503 with the right camera, lighting, and number of flies, the feeding microstructure of a
504 single 'tagged' fly in a group can also be recorded from DIETS-arenas.
505
506 The dilution of food or its protein components leads to compensatory feeding in flies
507 (Partridge, Piper, and Mair 2005; Chapman and Partridge 1996; Bass et al. 2007;
508 Mair et al. 2003). To the best of our knowledge, DIETS is the only assay that can
509 implement precise DR in flies without worrying about compensatory consumption
510 (Fig. 4A). Additionally, like CAFE, time-restricted feeding (TRF) protocols (Cabrera,
511 Young, and Axelrod 2020; Gill et al. 2015) are easily carried out with DIETS, with the
512 added advantage of being able to work with high-fat and high-sugar foods. This
513 ability to control and measure food intake will also make it possible to calibrate and
514 deliver precise amounts of drugs or infectious agents to a group of flies.
515
516 Multiple lines of evidence indicate that increased fat deposits strongly correlate with
517 increased SR in flies. Flies selected over generations for increased SR show
518 enhanced body fat deposits (Schwasinger-Schmidt, Kachman, and Harshman 2012;

519 Hardy et al. 2017; Chippindale, Chu, and Rose 1996; Harshman, Hoffmann, and
520 Clark 1999). Foods with higher carbohydrate content (or lower P: C ratio) show
521 increased bodily lipid content and increased SR (Lee and Jang 2014). As mentioned
522 above, other studies on HSD and subsequent SR support the same idea (van Dam
523 et al. 2020; Chandegra et al. 2017; Lee and Jang 2014; Strilbytska et al. 2022).
524 We tested SR in OreR flies after a week-long exposure to isocaloric HSD and sHFD.
525 We found that while HSD treatment expectedly improved SR over control, sHFD did
526 not (Fig. 4D). This was despite higher energy consumption on sHFD (Fig. 4C) and
527 likely increased fat deposit, similar to our observation for CS flies (Fig. S6D). We
528 therefore speculate that feeding on HSD versus sHFD, leads to the formation of
529 bodily fat deposits that could differ in aspects like composition, lipid droplet structure,
530 and the rate of utilization during starvation. Ultimately, one or more of these factors
531 could alter SR, and further investigations are needed to understand the exact
532 mechanisms.
533
534 Our DIETS platform will be ideal for similar studies, where precise delivery of
535 complex diets and accurate measurements of their consumption will be necessary.
536 We anticipate that the ability of DIETS to precisely estimate and control food intake,
537 its versatility, and the low cost of deployment will lead to its wide adoption.

538 ## Materials and Methods

539 **A. Fly lines and rearing**

540 We used the following fly strains for all experiments: Wild type Canton-S (CS-Q),
541 Oregon-R (OreR), CS-BZ, and w^{1118}, Piezo-GAL4 (BDSC # 59266) (S. Min et al.
542 2021), R50H05-GAL4 (BDSC # 38764) (Albin et al. 2015), and UAS-dTRPA1
543 (Hamada et al. 2008). All flies were reared on modified Bloomington's semi-defined
544 food (Laura Palanker Musselman et al. 2011) at 25 □ and 65 % humidity, with a 12
545 h:12 h light: dark cycle inside incubators. This food is referred to as the control diet
546 (CD: see Table. ST1 for ingredients). Temperature-sensitive UAS-dTRPA1 flies were
547 raised at 18 °C and transferred to 32 °C 30 minutes before the start of experiments
548 to induce transgene expression.

549

550 **B. Chemicals and other materials**

551 Food ingredients, namely brewer's yeast (Prime brand, instant dry yeast), table
552 sugar (Madhur brand, refined sugar) and cold-pressed coconut oil (Nutiva), Brilliant
553 blue FCF food grade (Vidhi Manufacturers 2490) were procured from local vendors.
554 Other components, like agar (Himedia: GRM666-500G), peptone (Himedia:
555 GRM001-500G), yeast extract powder (Himedia: RM027-500G), sucrose (MP
556 biomedicals: 194018), propionic acid (Sigma-Aldrich: P5561-1L), methyl-4-
557 hydroxybenzoate (Himedia: GRM1899-500G), $MgSO_4$ X $7H_2O$ (Sigma-Aldrich:
558 M1880), $CaCl_2$ X $2H_2O$ (Sigma-Aldrich: 223506), absolute ethanol (Merck: 64-17-5),
559 Oil-red-O (Sigma-Aldrich: 00625-100G), quinine hydrochloride dihydrate (Sigma-
560 Aldrich: Q1125-5G), Triton X-100 (MP biomedical: SKU:02194854-CF) and
561 paraformaldehyde (Sigma-Aldrich: P6148-500G), 0.22 □m syringe filter (MERK:
562 Z260479) were procured from authorized vendors. Light filters used for uniform red

563 light are 182: light red, 158: deep orange, and 810: zoom diffusion 1 (Bright Light
564 International). The following chemicals were used for RT-PCR; Trizol (Invitrogen:
565 15596026), Zymo Direct Zol kit (Zymo Research: R2051), Zymo RNA Clean and
566 Concentrator kit (Zymo Research: R1013), High-Capacity cDNA Reverse
567 Transcription kit (Thermo- Fisher Scientific: 4368814), KAPA SYBR FAST Universal
568 (Sigma-Aldrich: KK4602), QuantStudio™ 6 flex Real-Time PCR System (Applied
569 Biosystems: 4485691).
570
571 **C. Diet preparation**
572 To prepare the Control Diet (CD), ingredients (Table ST1) were weighed and mixed
573 in 60 mL reverse osmosis (RO) filtered water. The mixture was then cooked in a
574 microwave oven, with 2-3 intermittent stirring for ~ 2 minutes. The food mix was then
575 allowed to cool to 60 □ before adding methylparaben, propionic acid, $CaCl_2$, and
576 $MgSO_4$. The volume was adjusted to 100 mL by adding RO water. This mixture was
577 then thoroughly stirred with a whisk. To prepare diets with 0.25X to 2X
578 concentrations of CD (Fig.1D), we separately weighed all the ingredients for each
579 concentration. Agar concentration was maintained at 1 % for all the dilutions. To
580 prepare CD spiked with quinine (Fig. 3C), the required amounts of quinine were
581 added to the molten CD, below 60 □ and mixed thoroughly by shaking or vortexing.
582 2.5 % sucrose - 2.5 % yeast diet (1X SY diet), 5 % sucrose - 5 % yeast diet (2X SY
583 diet), and 10 % Sucrose - 10 % yeast diet (4X SY diet) were prepared as previously
584 described (Deshpande et al. 2014; Wu et al. 2020). To prepare saturated high-fat
585 diets (5 %, 10 %, and 20 % HFDs, w/v), the required amount of molten coconut oil
586 was added to cooked food and then mixed with a whisk. The added coconut oil
587 replaced an equal volume of water in the recipe. The food mix was allowed to cool to

588 60 □ and methylparaben, propionic acid, $CaCl_2$, and $MgSO_4$ were added. The food
589 was mixed thoroughly again with a whisk, and the volume was adjusted to 100 mL
590 by adding RO water. A 10 % HFD was isocaloric to 30 % high-sugar diet (HSD). 9%
591 sucrose is present in the CD and to prepare 30 % w/v HSD, an extra ~ 21 % sucrose
592 was added to replace the water in the CD. High-fat diets (HFDs) and high-sugar
593 diets (HSDs) exhibited hygroscopic properties, leading to the accumulation of
594 moisture on the food surface. We empirically determined the distance at which the
595 food cups had to be fixed from the agar bed to prevent this. Typically it was between
596 ~ 1- 5 mm.
597
598 **D. Feeding quantification by DIETS assay**
599 **i) DIETS vial preparation**
600 Standard fly vials (inner diameter: 23 mm, outer diameter: 25 mm, height: 90 mm)
601 and 200 µL microcentrifuge tube caps as food cups were assembled as follows. 7
602 mL 0.75 % agar solution was added to each vial to form an agar bed. After the agar
603 solidified, it was allowed to dry for 12 to 18 h at room temperature. If used
604 immediately, the agar layer leaks water, in which flies get trapped. An agar bed
605 reduces the weight loss from food cups due to evaporation and provides a separate
606 water source for flies to drink water. Solid food was provided in 200 µL
607 microcentrifuge tube (PCR-02-C; Axygen) caps (food cups) of 4 mm diameter. For
608 most experiments, ~ 70 µL of molten food from stored aliquots were pipetted into
609 food cups (Supplementary Fig. 1A). Once the food solidified, we noted the initial
610 weight of the cup + food. The initial weight of the food cups (cup + food) was kept
611 between a narrow range for consistency, typically ~ 125 - 135 mg for CD. Freshly
612 prepared food cups were fixed on the inside of the agar vials, normally at a distance

613 of 1 mm from the agar, with a small square of double-sided tape. Evaporation control
614 vials were prepared identically.

615 **ii) Food consumption and calorie intake measurement**

616 Flies were collected over the first day after eclosion, transferred to new food bottles,
617 and allowed to age for one more day. Lines that carried transgenic constructs were
618 aged for ~ 8 - 10 days after initial collection. On the 3rd day (~ 9th - 11th day for
619 transgenic lines), flies were segregated by sex under CO_2 anesthesia. Male or
620 female groups of 50 flies were placed in prepared DIETS vials and habituated for two
621 further days before actual consumption measurement. Flies were transferred to fresh
622 DIETS vials once after 24 h during the habituation process (Fig. 1A).

623 Fly groups were then transferred to DIETS vials with pre-weighed food cups for
624 feeding measurements. Evaporation control vials with no flies was maintained
625 alongside (Fig. 1A and Supplementary Fig. 1A). Viscosity of a specific food
626 influences the rate of evaporation. We also observed that certain viscous foods, like
627 foods with high fat and high sugar, in cups placed at 1 mm from the agar bed tended
628 to absorb moisture. Conversely, in diluted, less viscous foods, evaporation loss
629 increased. Therefore, with each new diet, we empirically determined the position of
630 the food cups from the agar bed to prevent moisture accumulation or loss by
631 evaporation in evaporation control vials below 10 % of the total food consumed. The
632 humidity level of the incubator could also be adjusted to keep evaporation below the
633 desired level. Most experiments were carried out at 25 □ and 65 % humidity.

634 Flies were transferred to fresh DIETS vials at the end of the period of feeding
635 measurements. The reduction in food cup weight (food consumed + evaporation
636 loss) was determined. The mean value of evaporation-mediated loss in weight (from

637 4 - 6 control vials) was deducted from the reduction in food cup weight to derive the
638 total food consumed by a group of flies. This value was usually plotted as an
639 average consumption per fly over time (e.g., mg/fly/day) or sometimes as total
640 consumption for a group of flies (mg/day).

641 Energy intake (in Fig. 4C and S6B) was calculated from the amount of food
642 consumed on CD and HFD, using the calorie values given in (Laura Palanker
643 Musselman et al. 2011) and represented as calorie/fly/day. The caloric value of the
644 Nutiva coconut oil was taken from the product label.

645 **iii) Feeding measurement in starved flies with DIETS and blue dye uptake**
646 Food intake in starved flies was measured by DIETS assay, and intake was parallelly
647 validated using a spectrophotometric estimation of Brilliant blue FCF dye. To
648 measure feeding in starved flies, 2 - 3 day old male flies were habituated in DIETS
649 vials for 2 days. For starvation, flies were kept in 0.75 % agar vials for specified
650 times. Flies were then transferred to DIETS vials with pre-weighed 1 % Brilliant blue
651 FCF 1 dye-labeled CD and allowed to feed for 30 minutes. Flies were homogenized
652 in 500 µl of PBST (1x PBS + 1 % Triton X-100) using a hand-held homogenizer
653 (BenchTop Lab Systems: BT-MT-13K). Homogenates were centrifuged at 4,000 g
654 for 5 minutes, and 100 µl of cleared supernatant was loaded into a 96-well plate.
655 Sample absorbance was measured at 630 nm on a multi-mode plate reader
656 (SPECTRAmax 250, Molecular Devices). Absorbance from a control set of flies fed
657 with food without dye was used to determine the baseline absorbance of fly lysates
658 (blank).
659
660

661 **iv) Round-the-clock circadian feeding measurements**

662 2 - 3 day old male and female flies were habituated in DIETS vials for two days.
663 Feeding measurement was initiated at 12 noon [Zeitgeber time (ZT) = 3.5; our light-
664 on time, or ZT = 0, was 8:30 AM] in fresh vials. Flies were then transferred to fresh
665 DIETS vials every 2 h for the next 50 h, and continuous measurements were taken.
666 The first 2 h feeding measurement was discarded, as it showed spiked feeding, likely
667 due to the fact that they had been transferred to fresh food after 24 h. Fly transfers
668 during the 12 h dark phase were carried out in red light.

669

670 **v) Two food choice feeding measurements with DIETS**

671 To measure food preference over varying time scales, two cups with distinct foods
672 were placed 1 mm from the agar bed, diametrically opposite each other, inside
673 DIETS vials. During the experiment, the two cups were kept parallel to the tray,
674 facing each other. To avoid a bias due to uneven lighting, individual vials were
675 wrapped in an 8.5 * 8.5 cm white printing paper sheet, acting as a light diffuser (Fig.
676 S3A). Before measuring feeding from two food sources, flies were habituated in
677 DIETS choice set up, with two CD cups for 48 h. Flies were starved, if the
678 experiment demanded, after the habituation step. A consumption preference index
679 (CPI) was also calculated as $CPI_{Food\ A}$ = [(feeding from Cup A - feeding from Cup B) /
680 total consumption from both the food cups].

681

682 **vi) DR protocol with DIETS**

683 We have achieved DR in flies by offering a fixed amount of diets in food cups to a
684 group of flies for 24 h, after 2 days of habituation on the CD diet. As a 'NO DR'
685 control (*ad-libitum* feeding), an average of 56 mg of CD was offered to groups of 50

686 male flies. This value was derived from summing the mean consumption of 50 male
687 flies (~ 39 mg: Fig. S2A), mean evaporation from food cup (~ 4 mg: Fig. S1C), and
688 mean leftover food (~ 10-13 mg: data not shown). To implement increasing degrees
689 of DR (~ 90 % to 60 %), we gradually reduced the mean food offered in cups by ~ 3
690 to 5 mg (labeled 'a'; Fig. 4A). Targeted DR % was calculated with respect to 56 mg
691 offered in the 'no DR' group. Based on actual food consumed (labeled 'b'; Fig. 4A),
692 the achieved DR % was calculated with respect to 43 mg, the mean food consumed
693 by flies in the 'no DR' group (Fig. 4A).
694
695 **vii) TRF feeding protocol with DIETS**
696 Groups of 20 male flies each were split into three groups. Two *ad libitum* feeding
697 groups, where flies were switched to fresh food vials either every 24 h [ALF (24h)] or
698 every 12 h [ALF (12+12)] were set. A third time-restricted feeding (TRF) group was
699 offered food only during the 'lights on' phase (ZT = 0 - 12). TRF flies were switched
700 on DIETS vials with no cups during the 'lights off' phase (ZT = 12 - 24). All groups
701 were habituated similarly to their experimental conditions (schema; Fig. 4B). Food
702 consumption was measured whenever flies were switched to fresh vials as indicated
703 (schema, Fig. 4B).
704
705 **E. Starvation resistance assay**
706 To compare the starvation resistance of flies, we allowed flies in varying group sizes
707 (10, 25 and 50 males) to feed from DIETS vial and regular food vial for 1 day and 7
708 days, following two days of usual habituation. For 7 days of feeding in DIETS vials,
709 flies were transferred to new DIETS vials every 24 h, while normal vials were
710 changed on alternative days. Flies were then transferred to 0.75 - 1 % agar

711 starvation vials to measure starvation resistance. Dead flies were counted every 24
712 h. The survival time of individual flies was recorded after week-long feeding on HSD
713 or sHFD. The starvation resistance of flies was computed by the Kaplan Meier plot
714 using graphpad prism.
715
716 **F. Gut lipid Oil-Red-O staining**
717 For midgut-deposited fat droplet staining, the fly's abdomen was dissected in 1X
718 PBS with fine forceps (55-INOX, FST) to remove the gut. Gut tissues were fixed in 4
719 % paraformaldehyde (prepared from pre-dissolved solution) (Sigma-Aldrich: P6148-
720 500G) for 20 minutes at room temperature on a rotary shaker. Dissected guts are
721 then rinsed twice for 10 minutes each with 1X PBS to remove the fixative. Fixed guts
722 were then incubated in Oil-Red-O stain for 30 minutes. Oil-Red-O stains were
723 prepared by dissolving 10 mg Oil-Red-O (powder) in 10 mL isopropanol (0.1 % Oil-
724 Red-O). A 6:4 ratio of 0.1 % Oil-Red-O in isopropanol to 4 mL distilled water was
725 prepared and passed through a 0.22 □m syringe filter (Merck: Z260479) before use.
726 The stained gut tissues were rinsed twice in distilled water for 10 minutes each.
727 Stained guts were then mounted on a glass slide, and images were captured using
728 an upright Nikon fluorescent microscope (Nikon Eclipse 80i) with a 20X objective
729 (Plan Fluor 20X, 0.50 NA Ph1 DLL ∞/0.17 WD 2.1).
730
731 **G. RNA extraction and qPCR**
732 After habituation on CD for 2 days, male flies were transferred on either 20 % HFD
733 or CD for 1, 4, and 7 days in DIETS vials and then flash-frozen and stored at −80 °C.
734 For RNA extraction, 30 flies were vortex-decapitated, and heads were collected on
735 dry ice, lysed in Trizol (Invitrogen: 15596026), and total RNA was extracted using a

736 Zymo Direct Zol kit (Cat No: R2051), and RNA was purified using a Zymo RNA
737 Clean and Concentrator kit (Cat No: R1013) as per the manufacturer's instructions.
738 After RNA extraction, cDNA was prepared using a High-Capacity cDNA Reverse
739 Transcription kit (Thermo-Fisher Scientific, 4368814). qPCR was performed using
740 KAPA SYBR FAST Universal (Sigma Aldrich, KK4602) on a QuantStudio™ 6 Flex
741 Real-Time PCR System (Applied Biosystems, 4485691). Fold change for each gene
742 was determined using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

743 To obtain a comprehensive list of DEGs upon exposure to sHFD, we re-analysed the
744 raw data generated by a previous study (Stobdan et al. 2019) using DESeq2 (Love,
745 Huber, and Anders 2014) and used p-adjusted value of <0.05 as cut-off. Full analysis
746 results are included in Table ST3. From this analysis, we selected top three of the
747 most upregulated genes- *takeout*, *Obp83a*, and *Cyp4e3*, and one downregulated
748 gene-*TotA*. The primers used were as follows: For RPL32 (endogenous control:
749 FBgn0002626): RPL32-F: CGCCACCAGTCGGATCGATAT and RPL32-R:
750 CATGTGGCGGGTGCGCTTGTT. For TotA (FBgn0028396), TotA-F:
751 CTCTTATGTGCTTTGCACTGCTG and TotA-R: TTTTGGAGTCATCGTCCTGGG.
752 For Cyp4e3 (FBgn0015035), Cyp4e3-F: CCCGATGTCCAGAGGAAATTGT and
753 Cyp4e3-R: GCGCTGCGCCTCCTTAATA. For takeout (FBgn0039298), to-F:
754 CAGCGGAACTGGACAGAGTAAC and to-R: GCGCCTTGTCCCCATTGAACA. For
755 Obp83a (FBgn0011281), Obp83a-F: CGAGGACGAGAAGCTCAAATGC and
756 Obp83a-R: CGGATGGACGCAGCCCTTGG.

757

758

759

760 **H. DIETS-arena and video analysis**

761 We developed a flat arena setup, DIETS-arena, with an easily replaceable 3D
762 printed food cup (transparent PLA material) in the center to enable temporal and
763 spatial analysis of flies along with food consumption measurement. Each arena
764 measured 6.5* 6.5 * 0.4 cm, and the whole setup comprised of six arenas (Fig. S6A;
765 left panel). This design was adapted from a previous study (Charroux and Royet
766 2020). 0 - 4 day old male flies were sorted under CO_2 anesthesia in groups of 15 -
767 20 and habituated in DIETS vial with CD for 2 days. Arenas were cleaned with warm
768 water and wiped clean thoroughly with a microfibre cloth. 500 □L of 1 % agar was
769 pipetted down in a ring form at a distance of 1 cm away from the edge of the central
770 food cup (Fig. S6A; left panel). The agar was left to dry for 1 h, and subsequently,
771 the arenas were covered with a transparent acrylic sheet. The initial weight of the
772 food cup with CD was weighed and was immediately placed into the slot provided at
773 the center of the arena to prevent evaporation. To transfer flies, the whole setup was
774 inverted. Food cups from individual arenas were taken out one at a time, flies were
775 transferred from a vial to the arena via a custom-designed funnel, and food cups
776 were placed back immediately to prevent escape. The arena was then placed in the
777 image capture setup (Fig. S6A; right panel). Arenas without flies were maintained
778 alongside as evaporation controls. This experiment was initiated at ZT = 1. Images
779 were captured via Arducam HQ IR-CUT camera with a 6 mm CS lens (Cat. 1251599,
780 from robu.in), connected to a Raspberry Pi 4B. The Libcamera package was used to
781 capture time-lapse images at 0.2 fps. The captured images were then undistorted
782 using the Computer Vision Toolbox in MATLAB R2022b. These images were later
783 compressed into videos of 10 fps using ImageJ (version 1.53t). A previously

784 published MATLAB code (Charroux and Royet 2020) was modified to quantify the
785 number of flies in a defined area.
786
787 To determine the number of flies on or near the food cup, we defined a circular zone
788 around the cup ($Food_{ROI}$), at a distance of 90 pixels from the center of the food cup.
789 This figure was a summation of the longest male body length (40 pixels: data not
790 shown) and the food cup radius (50 pixels; see Fig. S6A). As it sometimes proved
791 difficult to tell flies apart in the $Food_{ROI}$, especially when many were present, we
792 applied an inverted logic to determine the number of flies in each captured frame
793 outside the $Food_{ROI}$ in each arena. This ultimately allowed us to calculate the %
794 occupancy of $Food_{ROI}$ per frame as :
795 *% occupancy of* $Food_{ROI}$ *= {(Total number of flies in an arena - number of flies*
796 *detected outside* $Food_{ROI}$*)/Total number of flies in an arena} * 100*
797
798 **I. Data plotting and statistics**
799 GraphPad Prism version 8.3.1 was used for most main data plotting. Almost all data
800 are represented as scatter plots with error bars representing the mean with a 95 %
801 confidence interval (CI). Starvation resistance was plotted as Kaplan-Meier graph.
802 Estimation statistics, including effect sizes, the margin of error (MOE), and relevant
803 comparisons between groups performed for each experiment are reported in
804 Supplementary Table 2 (ST2). For all figures, unless mentioned, the effect size is
805 calculated as the mean difference between the two groups. This represents the
806 magnitude of difference between the two groups. The effect size is represented as
807 the dot in the spread. The two black lines that extend from the effect size dot denote
808 the 95% CI and are a measure of the precision of the data. To generate the CI,

809 around 5000 bias-corrected and accelerated (BCa) bootstrap resampling was done
810 and the effect size of each resample was plotted to generate the CI arms. The
811 magnitude of one arm of the confidence interval width represents the MOE. The
812 effect size for Fig. 1E and 2A was measured by the standardized mean difference,
813 Hedge's g. Hedge's g is the difference in mean of two groups, standardized over the
814 pooled standard deviation of the two groups, with a correction applied for small (~
815 less than 20) group sizes (Turner and Bernard 2006). All estimation/delta plots were
816 generated using an online website: www.estimationstats.com (Ho et al. 2019). We
817 extracted data for EX-Q and CAFE from (Wu et al. 2020) , Fig. 3C, using
818 WebPlotDigitizer (automeris.io/WebPlotDigitizer).

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Contributions

G.D. and M.R.T. and P.C. conceived the design of the DIETS assay. G.D. conceived the DIETS-arenas assay. S.S. built the DIETS-arenas and modified the code for DIETS-arena from work cited. Initial trials and design refinement was done by G.D, M.R.T, P.C, S.S, and B.P. Experiments on starvation resistance following HSD or sHFD were carried out by B.P. qPCR experiments were done by R.S.P.Y performed, and supervised by P.A. Both R.S.P.Y and P.A analyzed the data. All experiments, data analysis, and plotting in the manuscript were performed by G.D, P.A, M.R.T, P.C, B.P., S.S, and R.S.P.Y. Statistical analysis was performed by B.P and S.S. G.D directed the research and wrote the manuscript with inputs from all.

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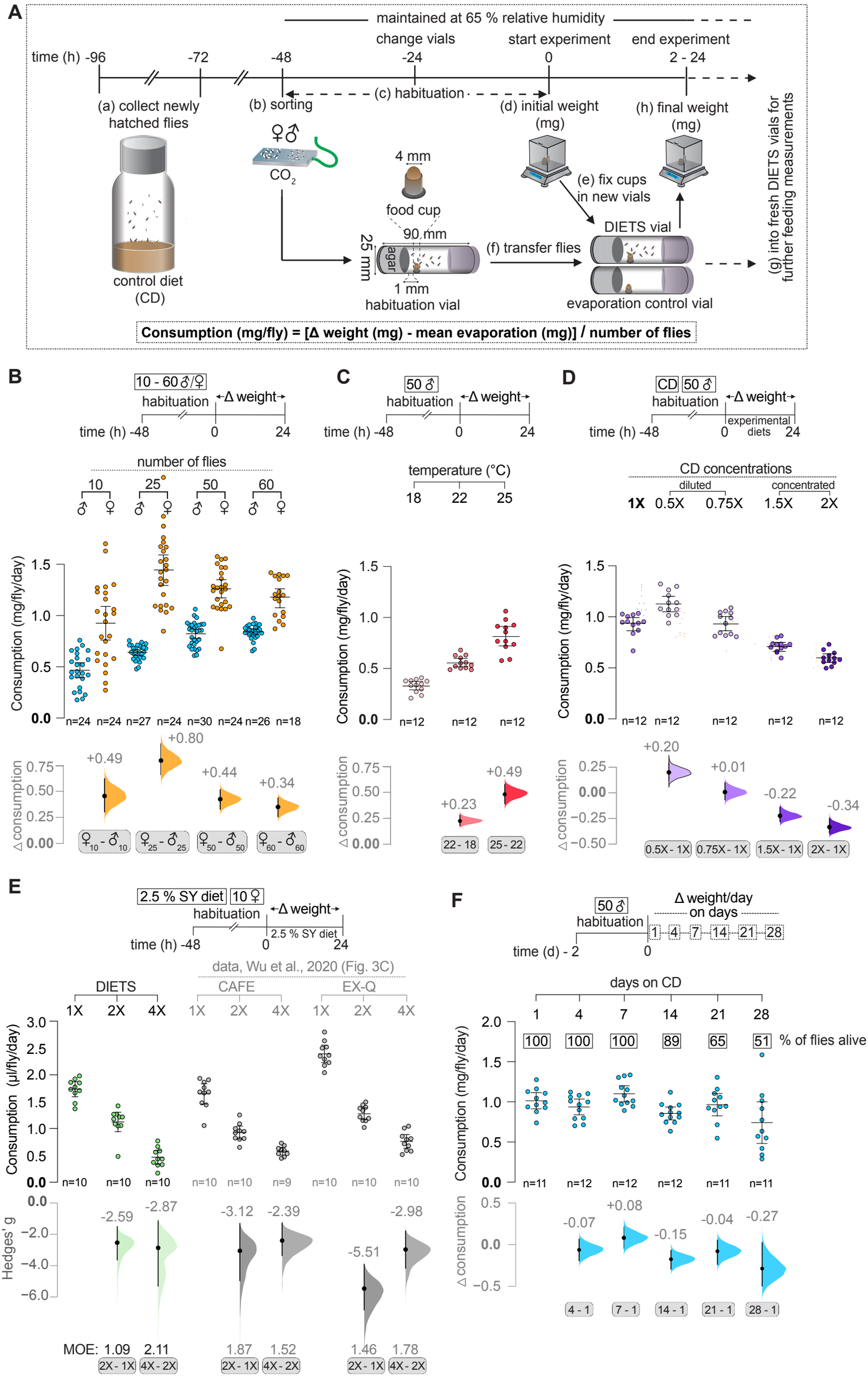
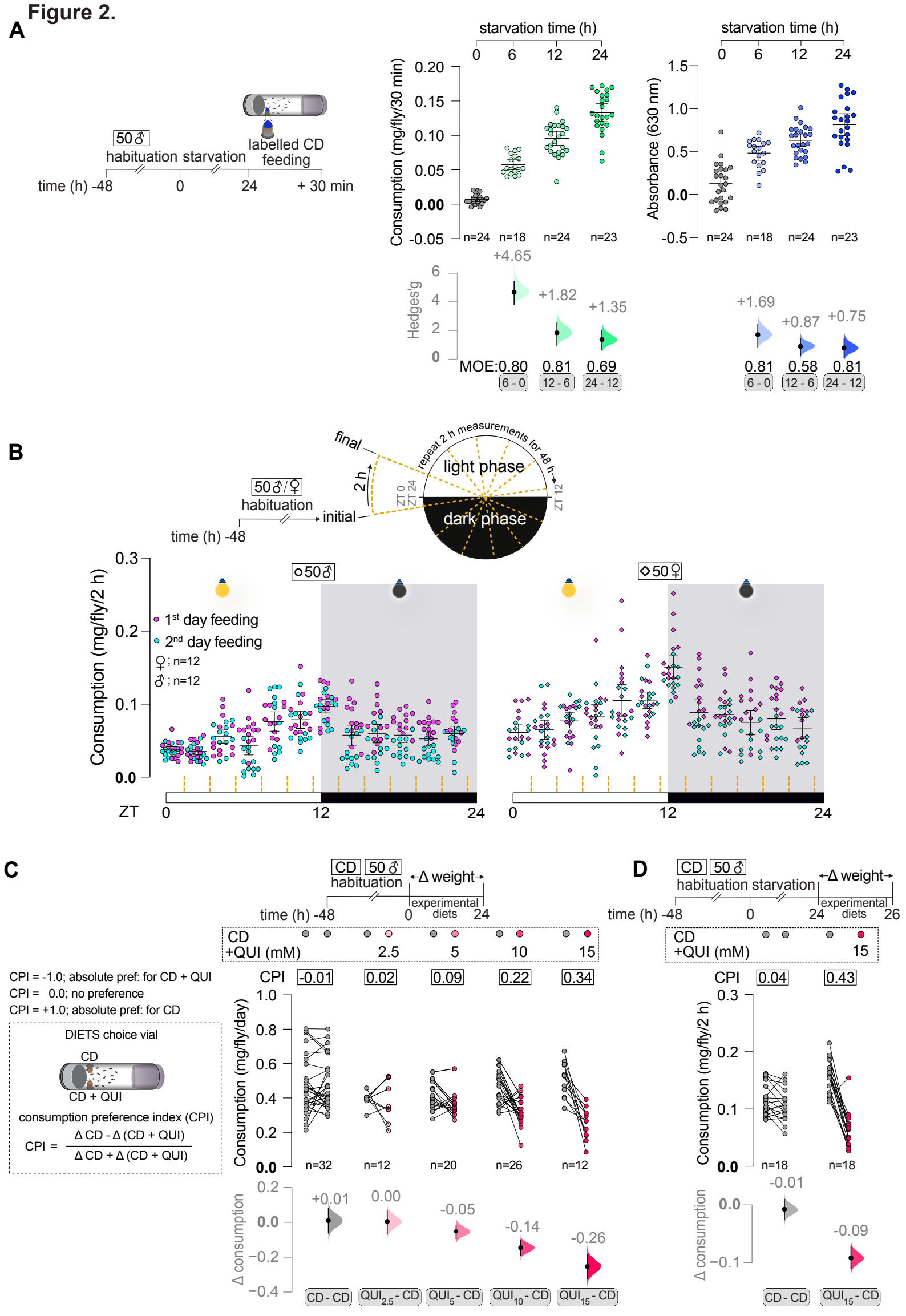
Figure 1.


A
maintained at 65 % relative humidity
change vials
start experiment
end experiment
time (h) -96
-72
-48
-24
0
2 - 24
(a) collect newly hatched flies
(b) sorting
(c) habituation
(d) initial weight (mg)
(h) final weight (mg)
CO2
4 mm
food cup
90 mm
25 mm
agar
1 mm
habituation vial
(e) fix cups in new vials
DIETS vial
(f) transfer flies
evaporation control vial
(g) into fresh DIETS vials for further feeding measurements
control diet (CD)
Consumption (mg/fly) = [Δ weight (mg) - mean evaporation (mg)] / number of flies
B
10 - 60 ♂/♀
habituation
Δ weight
time (h) -48
0
24
number of flies
10
25
50
60
Consumption (mg/fly/day)
n=24 n=24 n=27 n=24 n=30 n=24 n=26 n=18
Δ consumption
+0.49
+0.80
+0.44
+0.34
♀10 - ♂10
♀25 - ♂25
♀50 - ♂50
♀60 - ♂60
C
50 ♂
habituation
Δ weight
time (h) -48
0
24
temperature (°C)
18
22
25
Consumption (mg/fly/day)
n=12 n=12 n=12
Δ consumption
+0.23
+0.49
22 - 18
25 - 22
D
CD
50 ♂
habituation
Δ weight
time (h) -48
0
experimental diets
24
CD concentrations
diluted
concentrated
1X
0.5X
0.75X
1.5X
2X
Consumption (mg/fly/day)
n=12 n=12 n=12 n=12 n=12
Δ consumption
+0.20
+0.01
-0.22
-0.34
0.5X - 1X
0.75X - 1X
1.5X - 1X
2X - 1X
E
2.5 % SY diet
10 ♀
habituation
Δ weight
2.5 % SY diet
time (h) -48
0
24
data, Wu et al., 2020 (Fig. 3C)
DIETS
CAFE
EX-Q
1X
2X
4X
Consumption (µl/fly/day)
n=10 n=10 n=10 n=10 n=10 n=9 n=10 n=10 n=10
Hedges' g
-2.59
-2.87
-3.12
-2.39
-5.51
-2.98
MOE: 1.09
2.11
1.87
1.52
1.46
1.78
2X - 1X
4X - 2X
F
50 ♂
habituation
Δ weight/day on days
1
4
7
14
21
28
time (d) - 2
0
days on CD
% of flies alive
100
100
100
89
65
51
Consumption (mg/fly/day)
n=11 n=12 n=12 n=12 n=11 n=11
Δ consumption
-0.07
+0.08
-0.15
-0.04
-0.27
4 - 1
7 - 1
14 - 1
21 - 1
28 - 1

1168 **Figure 1. DIETS (Direct Intake Estimation and longitudinal Tracking of Solid**
1169 **food consumption) is a sensitive assay that can measure food consumption in**
1170 **flies over days.**
1171 **(A)** Schematic outline of the DIETS assay. **(B)** Comparison of 24 h control diet (CD)
1172 consumption in males and females of increasing group sizes. Females consistently
1173 eat a greater amount of food than males, regardless of the group size. Mean
1174 consumption by both sexes increased with group size. **(C)** CD consumption by
1175 groups of 50 male flies was measured over 24 h at different temperatures. An
1176 increase in the amount of food consumed was observed with an increase in
1177 temperature. **(D)** Feeding by groups of 50 male flies on varying concentrations of CD
1178 was measured. Flies show compensatory feeding upon diluting or concentrating CD.
1179 **(E)** Comparing the sensitivity of DIETS, CAFE, and EX-Q assays in resolving food
1180 intake difference between 1X, 2X, and 4X concentrations of 2.5 % sucrose - 2.5 %
1181 yeast extract (2.5 % SY) diet. DIETS show comparable resolution to CAFE and EX-
1182 Q. **(F)** Longitudinal measurement of CD consumption over days 1, 4, 7, 14, 21, and
1183 28 for 50 male flies. Consumption mostly remains unaltered, except for a dip in
1184 feeding by the end of 28 days, possibly due to the mortality observed. Flies start
1185 dying between the 7th and the 14th day. Scatter plots of raw data with mean ± 95 %
1186 CI are shown. Δ 'effect size' plots below each raw data graph depict mean
1187 differences (black dots; values labeled) between relevant groups or standardized
1188 mean difference, hedge's g for Fig. 1E. The 95 % CI (black lines) and distribution of
1189 the mean differences (curve), generated are also shown. See Materials and Methods
1190 for further details.

Figure 2.

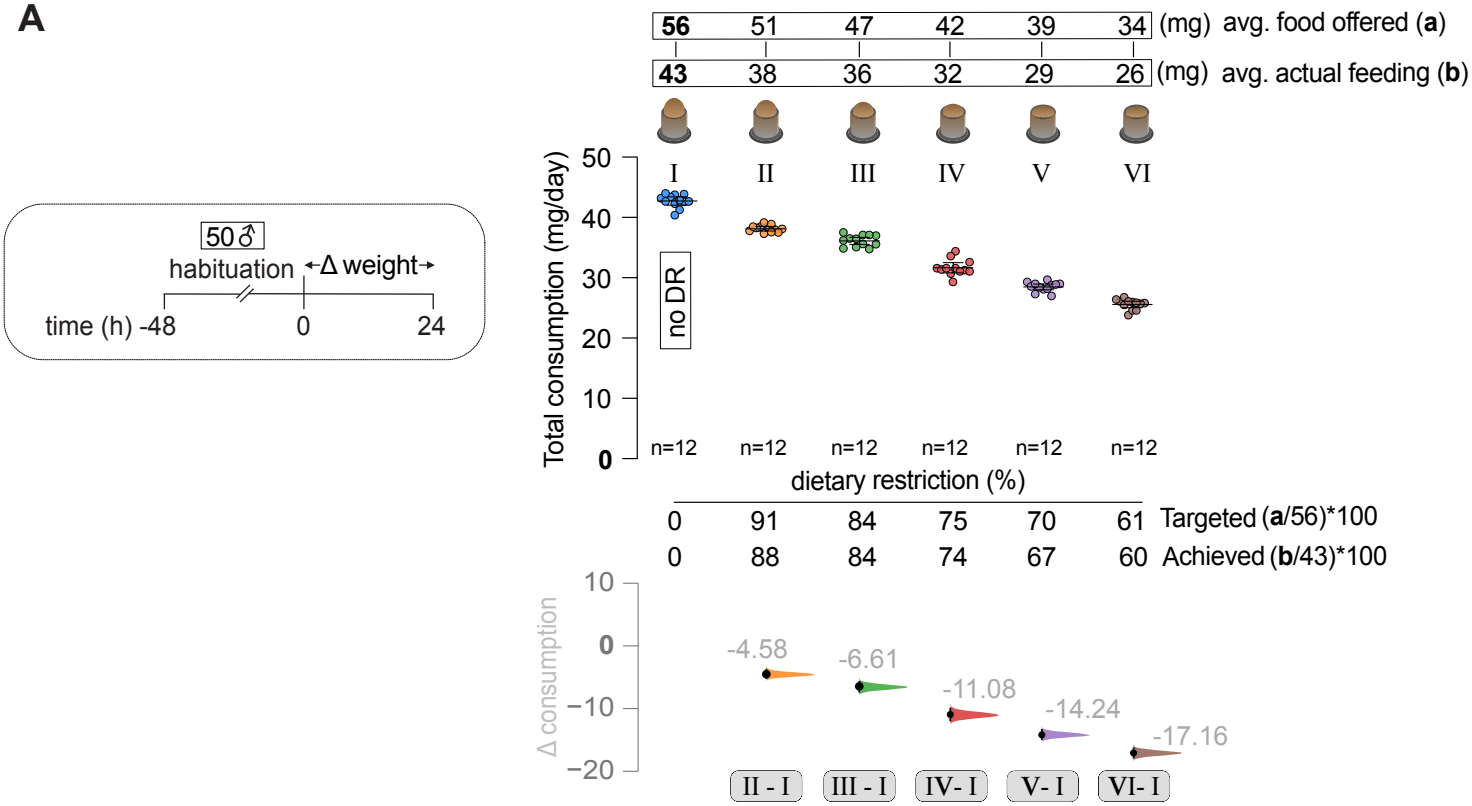

A
50♂
habituation
starvation
labelled CD feeding
time (h) -48
0
24
+ 30 min
starvation time (h)
0 6 12 24
Consumption (mg/fly/30 min)
0.20
0.15
0.10
0.05
0.00
-0.05
n=24 n=18 n=24 n=23
Absorbance (630 nm)
1.5
1.0
0.5
0.0
-0.5
n=24 n=18 n=24 n=23
Hedges'g
+4.65
+1.82
+1.35
MOE:0.80 0.81 0.69
6 - 0
12 - 6
24 - 12
+1.69
+0.87
+0.75
0.81 0.58 0.81
6 - 0
12 - 6
24 - 12
B
final
repeat 2 h measurements for 48 h
light phase
dark phase
2 h
ZT 0
ZT 24
ZT 12
50♂/♀
habituation
initial
time (h) -48
Consumption (mg/fly/2 h)
0.3
0.2
0.1
0.0
1st day feeding
2nd day feeding
♀; n=12
♂; n=12
50♂
50♀
ZT
0
12
24
C
CD
50♂
habituation
Δ weight
experimental diets
time (h) -48
0
24
CD +QUI (mM)
2.5
5
10
15
CPI = -1.0; absolute pref: for CD + QUI
CPI = 0.0; no preference
CPI = +1.0; absolute pref: for CD
DIETS choice vial
CD
CD + QUI
consumption preference index (CPI)
CPI = Δ CD - Δ (CD + QUI) / Δ CD + Δ (CD + QUI)
CPI
-0.01
0.02
0.09
0.22
0.34
Consumption (mg/fly/day)
1.0
0.8
0.6
0.4
0.2
0.0
n=32 n=12 n=20 n=26 n=12
Δ consumption
0.2
0.0
−0.2
−0.4
+0.01
0.00
-0.05
-0.14
-0.26
CD - CD
QUI2.5 - CD
QUI5 - CD
QUI10 - CD
QUI15 - CD
D
CD
50♂
habituation
starvation
Δ weight
experimental diets
time (h) -48
0
24
26
CD +QUI (mM)
15
CPI
0.04
0.43
Consumption (mg/fly/2 h)
0.3
0.2
0.1
0.0
n=18 n=18
Δ consumption
0.0
−0.1
-0.01
-0.09
CD - CD
QUI15 - CD

1191 **Figure 2. DIETS for short-term feeding measurements and food choice.**

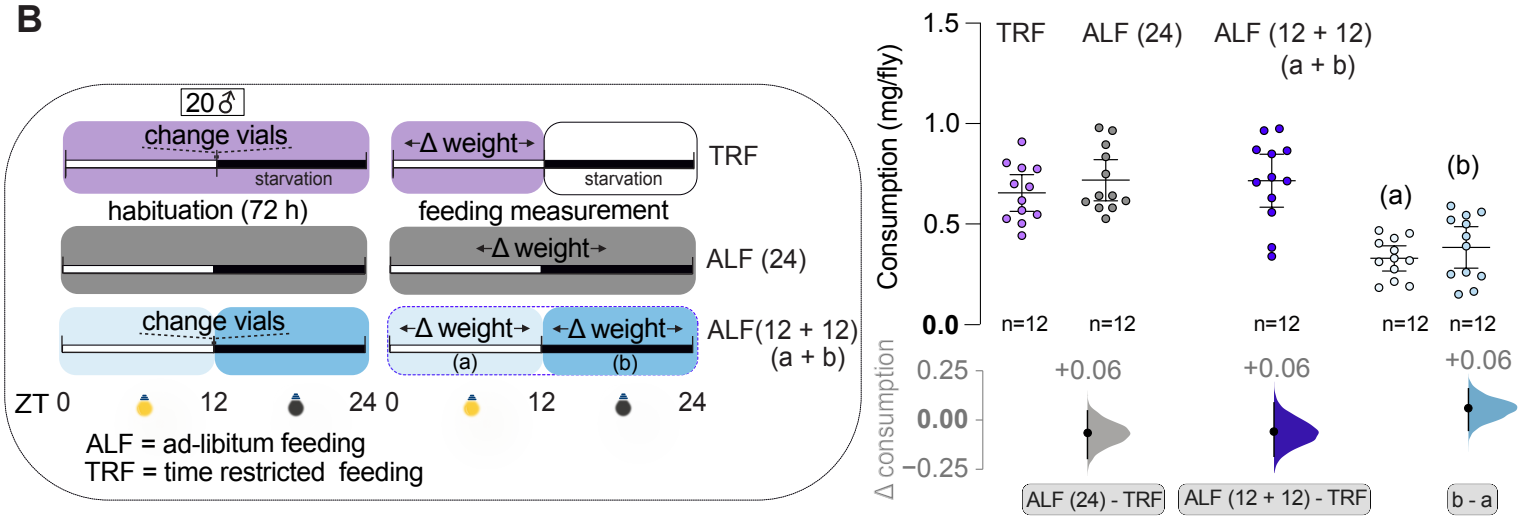
1192 **(A)** Comparing the sensitivity of DIETS with blue dye uptake in measuring short-term
1193 feeding. 30 minutes of feeding on CD supplemented with Brilliant blue FCF in
1194 differentially starved male flies was measured using DIETS. Flies showed an
1195 increase in feeding with increased starvation (left panel). A simultaneous dye
1196 accumulation measurement was conducted by crushing the flies after feeding (right
1197 panel). Hedge's g is effect size, in this case, the difference in mean of two groups,
1198 standardized over the pooled standard deviation of the two groups. DIETS assay
1199 yields larger 'g' values compared to blue dye estimation for comparison of feeding
1200 between the same groups. **(B)** Continuous 2 h feeding measurements in sated male
1201 and female flies measured over 2 days. Both sexes show a distinct evening feeding
1202 peak just around the lights-off time at ZT = 11.5-12.5. **(C)** 24 h, food preferences
1203 between CD and CD mixed with quinine. Flies show an increasing dose-dependent
1204 aversion towards CD adulterated with quinine. **(D)** 2 h food preferences between CD
1205 and CD adulterated with 15 mM quinine in 24 h starved flies. Flies showed a strong
1206 aversion towards quinine-laced food. Scatter plots of raw data with mean ± 95 % CI
1207 are shown. Δ 'effect size' plots below each raw data graph depict mean differences
1208 (black dots; values labeled), between the two relevant groups being compared. The
1209 95 % CI (black lines) and distribution of the mean differences (curve) generated by
1210 bootstrap resampling of the data are also shown. In (A) standardized effect size,
1211 Hedge's g has been plotted, and MOEs have been reported. See Materials and
1212 Methods for further details.

Figure 3.

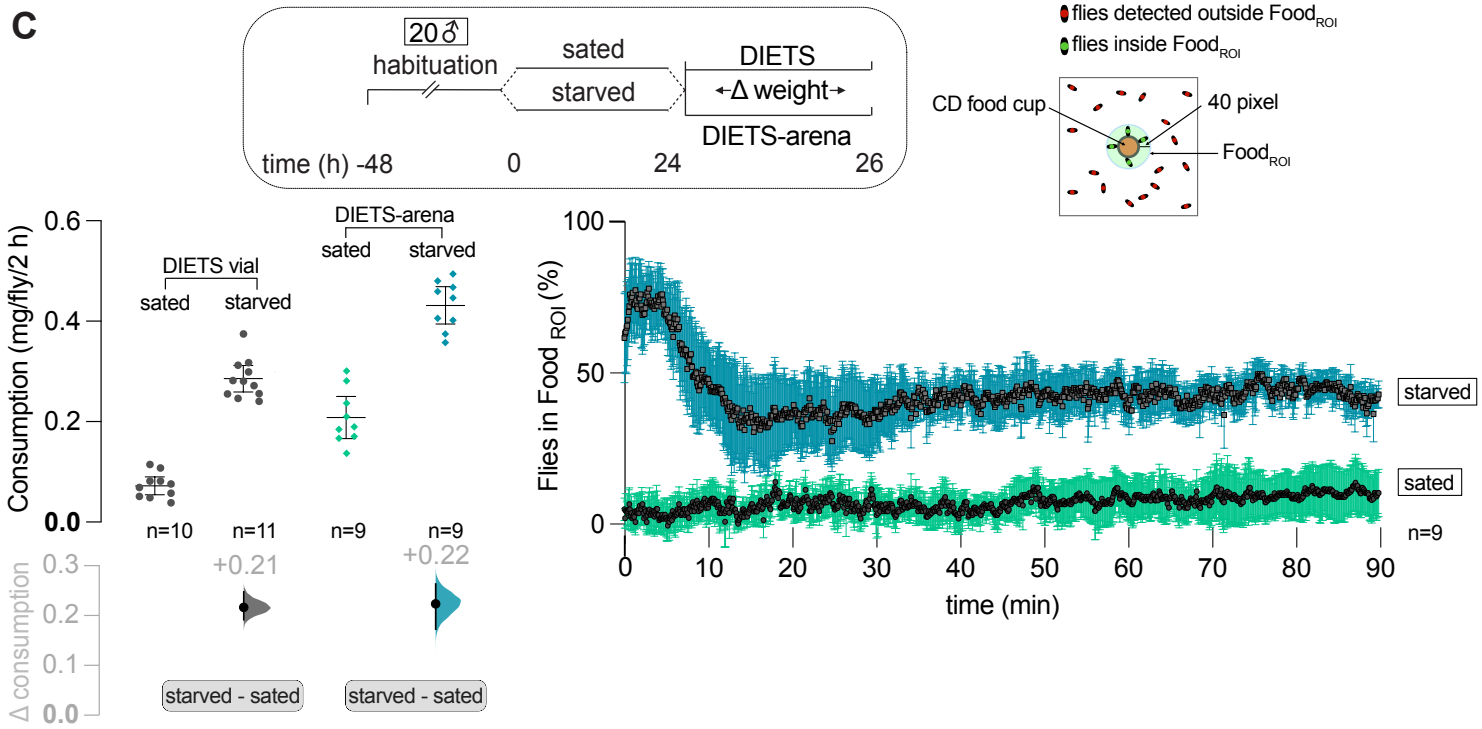
A


50♂
habituation
Δ weight
time (h) -48
0
24
56 51 47 42 39 34 (mg) avg. food offered (a)
43 38 36 32 29 26 (mg) avg. actual feeding (b)
I II III IV V VI
no DR
Total consumption (mg/day)
n=12 n=12 n=12 n=12 n=12 n=12
dietary restriction (%)
0 91 84 75 70 61 Targeted (a/56)*100
0 88 84 74 67 60 Achieved (b/43)*100
Δ consumption
-4.58
-6.61
-11.08
-14.24
-17.16
II - I
III - I
IV- I
V- I
VI- I

B

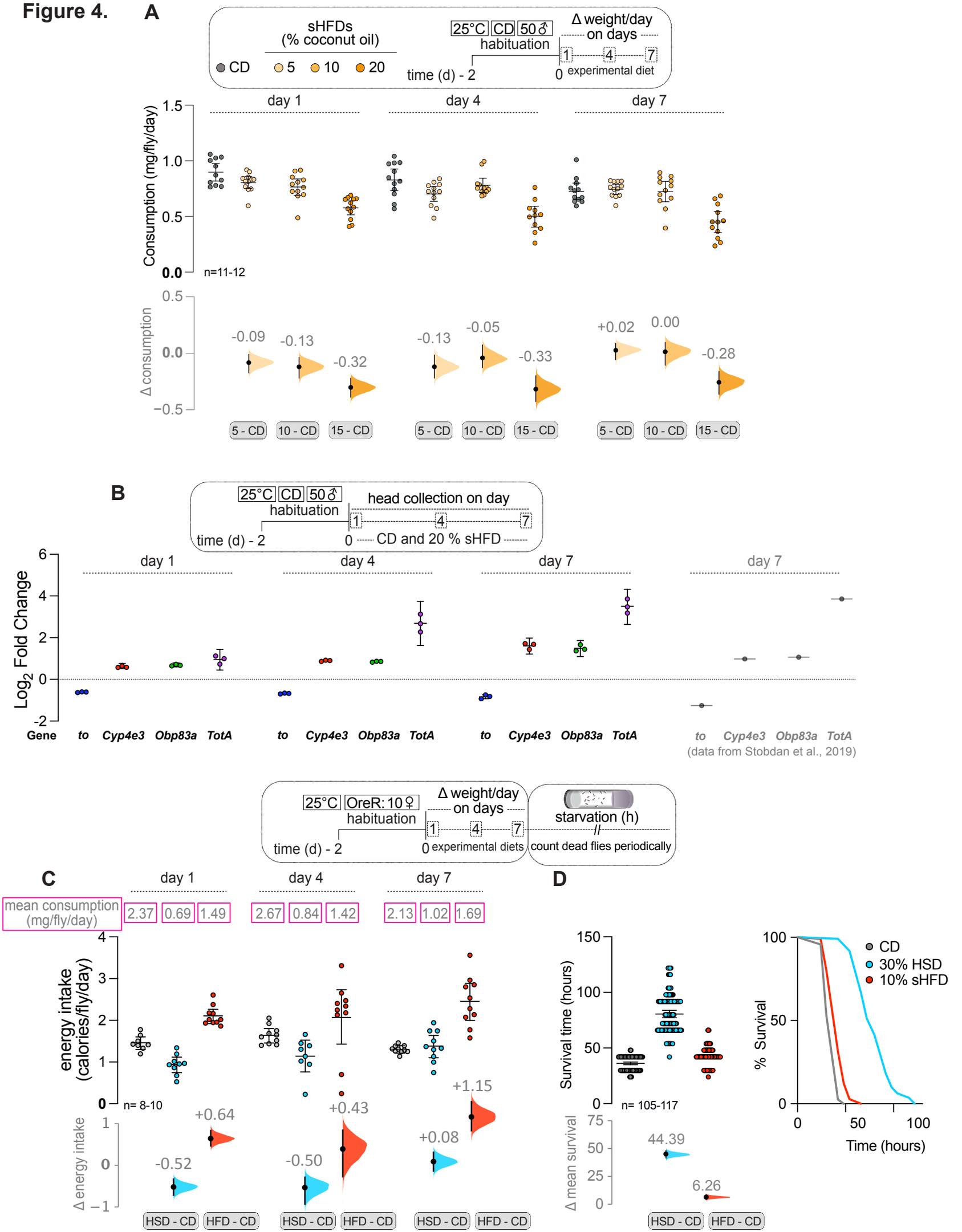

20♂
change vials
starvation
habituation (72 h)
Δ weight
starvation
feeding measurement
TRF
Δ weight
ALF (24)
change vials
Δ weight
Δ weight
(a)
(b)
ALF(12 + 12)
(a + b)
ZT 0 12 24 0 12 24
ALF = ad-libitum feeding
TRF = time restricted feeding
Consumption (mg/fly)
TRF
ALF (24)
ALF (12 + 12)
(a + b)
(a)
(b)
n=12 n=12 n=12 n=12 n=12
Δ consumption
+0.06
+0.06
+0.06
ALF (24) - TRF
ALF (12 + 12) - TRF
b - a

C


20♂
habituation
sated
starved
DIETS
Δ weight
DIETS-arena
time (h) -48
0
24
26
flies detected outside Food ROI
flies inside Food ROI
CD food cup
40 pixel
Food ROI
Consumption (mg/fly/2 h)
DIETS vial
sated
starved
DIETS-arena
sated
starved
n=10 n=11 n=9 n=9
Δ consumption
+0.21
+0.22
starved - sated
starved - sated
Flies in Food ROI (%)
starved
sated
n=9
time (min)

1237 **Figure 4. DIETS is well suited to study longitudinal feeding on high-fat diets**
1238 **and associated transcriptional and physiological changes.**
1239 **(A)** In a longitudinal feeding study, flies were fed 5%, 10% and 20% saturated high-
1240 fat diets made with coconut oil (sHFD), and feeding measurements were conducted
1241 on days 1, 4, and 7. While consumption remained mostly constant up to 10 % extra
1242 coconut oil in the diet, feeding decreased noticeably with the addition of 20 %
1243 coconut oil. **(B)** Differential expression of *takeout* (*to*)*, Cytochrome P450-4e3*
1244 (*Cyp4e3*), *Odorant-binding protein 83a* (*Obp83a*), and *Turandot A* (*TotA*) was
1245 examined by qPCR from heads of flies that have been fed with 20 % sHFD for 1, 4,
1246 and 7 days, with respect to control flies fed CD. These genes were earlier found to
1247 be some of the most differentially expressed genes from an earlier study with a
1248 similar design. The direction and magnitude of differential expression of the above
1249 candidate genes on day 4 and 7 were comparable to that in day 7 samples from the
1250 previous study (Stobdan et al. 2019), as shown. **(C)** Energy intake was determined
1251 over 24 h periods on days 1, 4, and 7 in groups of flies fed 30 % HSD or isocaloric
1252 10 % sHFD. The corresponding mean feeding values are represented above inside
1253 pink boxes. Flies fed on 10 % sHFD showed increased energy intake compared to
1254 the CD and HSD fed groups on all days. **(D)** The same flies were transferred to 0.75
1255 % agar vials after 7 days of dietary treatment and the number of dead flies recorded
1256 every 6 - 12 hours. Survival time (left panel), and percent survival under starvation
1257 (right panel) of flies are shown. While HSD treatment increases starvation
1258 resistance, sHFD treatment does not. Scatter plots of raw data with mean ± 95 % CI
1259 are shown. For Figure 4C starvation resistance was plotted as Kaplan-Meier graph.
1260 Δ 'effect size' plots below each raw data graph depict mean differences (black dots;
1261 values labeled), between the two relevant groups being compared. The 95 % CI

Figure 4.


A
sHFDs
(% coconut oil)
CD
5
10
20
25°C
CD
50♂
habituation
Δ weight/day
on days
1
4
7
time (d) - 2
0
experimental diet
day 1
day 4
day 7
Consumption (mg/fly/day)
1.5
1.0
0.5
0.0
n=11-12
Δ consumption
0.5
0.0
−0.5
-0.09
-0.13
-0.32
-0.13
-0.05
-0.33
+0.02
0.00
-0.28
5 - CD
10 - CD
15 - CD
B
25°C
CD
50♂
habituation
head collection on day
time (d) - 2
CD and 20 % sHFD
Log2 Fold Change
6
4
2
0
-2
Gene
to
Cyp4e3
Obp83a
TotA
(data from Stobdan et al., 2019)
25°C
OreR: 10♀
habituation
Δ weight/day
on days
starvation (h)
count dead flies periodically
C
mean consumption
(mg/fly/day)
2.37
0.69
1.49
2.67
0.84
1.42
2.13
1.02
1.69
energy intake
(calories/fly/day)
4
3
2
1
0
n= 8-10
Δ energy intake
1
0
−1
-0.52
+0.64
-0.50
+0.43
+0.08
+1.15
HSD - CD
HFD - CD
D
Survival time (hours)
150
100
50
0
n= 105-117
Δ mean survival
75
50
25
0
44.39
6.26
% Survival
100
50
0
Time (hours)
CD
30% HSD
10% sHFD

1237 **Figure 4. DIETS is well suited to study longitudinal feeding on high-fat diets**
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1248 similar design. The direction and magnitude of differential expression of the above
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1254 the CD and HSD fed groups on all days. **(D)** The same flies were transferred to 0.75
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1259 are shown. For Figure 4C starvation resistance was plotted as Kaplan-Meier graph.
1260 Δ 'effect size' plots below each raw data graph depict mean differences (black dots;
1261 values labeled), between the two relevant groups being compared. The 95 % CI

(black lines) and distribution of the mean differences (curve) generated by bootstrap resampling of the data are also shown. See Materials and Methods for further details.