Direct Intake Estimation and Longitudinal Tracking of Solid-food Consumption (DIETS) in *Drosophila*

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Abstract

In *Drosophila*, an organism much used for dietary studies, comprehending the regulation of feeding and the effect of diet requires accurate measurement of food intake. Although flies are normally housed on agar-based foods, direct estimation of solid feeding has been thought infeasible due to the minute amounts they consume. Moreover, it has been technically challenging to precisely restrict the amount of food flies consume during an experiment. Here, we report a label-free, sensitive, and precise assay, DIETS (Direct Intake Estimation and Tracking of Solid food consumption), that is compatible with videography. In DIETS, food consumed by a group of flies, from a small cup is directly weighed over hours to days. Dietary choice is measured in the assay by weighing food intake from two cups with distinct diets. DIETS enables the quantification of a high-fat diet intake over days. Notably, by limiting food in the cups, we could precisely implement targeted dietary restrictions in flies without diluting nutrients or causing compensatory feeding.

Keywords

Feeding assay, behavior, Drosophila, dietary restriction (DR), time-restricted feeding (TRF), food choice, high-fat diets, DIETS

Introduction

The quality, quantity and timing of food intake have profound influences on organismal and cellular level physiology of all living beings, leading to either overall well-being or a pathology. Food can affect neurons, neural plasticity, and brain-body communication to ultimately alter hunger, satiety, and feeding (Gómez-Pinilla 2008; Kang et al. 2022; Ekstrand et al. 2021; Tulloch et al. 2015; Spencer et al. 2017; Oriach et al. 2016; Chaix et al. 2019; Flanagan et al. 2020; Fadó et al. 2022). Due to deep evolutionary conservation in physiology, metabolism, nervous system function, together with a well-annotated genome, and excellent genetic tools, flies are being used to rapidly advance our understanding of the mechanisms underlying the effects of high-calorie diets, dietary restriction (DR), and time-restricted feeding (TRF) regimens in health and disease (Zanco et al. 2021; Hodge et al. 2022; Kapahi, Kaeberlein, and Hansen 2017; Piper and Partridge 2007; Cabrera, Young, and Axelrod 2020; Gill et al. 2015; Livelo et al. 2023). Precisely estimating and controlling food consumption is essential for both designing good experiments and for a complete mechanistic understanding of how the quantity of food affects physiology and behavior in *Drosophila*.

Fruit flies have been reported to consume only ~ 0.5 - 2.5 microlitres of food in a day, depending on the diet (William W. Ja et al. 2007; Qi et al. 2015; Wu et al. 2020; Deshpande et al. 2014). As a result, many fly-feeding assays are based on ingestion and excretion of dyes, radiolabeled molecules or short oligonucleotide tags mixed with food (Carvalho, Kapahi, and Benzer 2005; Shell et al. 2021, 2018; Park, Tran, and Atkinson 2018; Wu et al. 2020; Tanimura, Isono, and Yamamoto 1988; Geer, Olander,

and Sharp 1970). Most label-based assays are indirect and require sacrificing flies to quantify the amount of the label in the fly as a proxy for consumption. Labels could influence feeding by the virtue of possessing an appetitive or aversive taste. Their absorption or excretion can also be influenced by changes in physiology or age. Radioactive labels are expensive, hazardous, and they have to be incorporated into macromolecules to be detected. Such incorporation itself is subject to be altered by age and diet (Marx 2015; Deshpande et al. 2014; Shell et al. 2021, 2018). Since the amount of label in the flies tends to plateau after a while due to excretion (Deshpande et al. 2014), in a recently developed solid-food assay Con-Ex, both ingested and egested dye are colorimetrically measured for food-intake measurement (Shell et al. 2018). In the EX-Q assay, all dye ingested with labeled solid food is excreted out upon subsequent feeding with unlabeled food, and food intake is estimated by dye present only in the excreta, leaving the flies alive for longitudinal experiments (Wu et al. 2020).

Multiple label-free, direct intake measuring liquid feeding assays have also been developed for flies (Ro, Harvanek, and Pletcher 2014; Itskov et al. 2014; William W. Ja et al. 2007; Qi et al. 2015; Murphy et al. 2017; Yapici et al. 2016). Assays like Capillary Feeder Assay, CAFE (William W. Ja et al. 2007), MultiCAFE with choice between foods in multiple capillaries (Sellier, Reeb, and Marion-Poll 2011), Expresso, a sensitive automated version of CAFE (Yapici et al. 2016), and Activity Recording CAFE, ARC (Murphy et al. 2017), directly measure liquid food consumed from capillaries. One major drawback of these assays is that viscous, multi-ingredient laboratory diets, including liquid high-fat diets, simply separate into its components inside capillaries. Assays for

single flies, like optoPAD, flyPAD, and FLIC are also label-free assays that provide fine temporal resolution data on how a fly touches liquid/solid food with its proboscis. However, these assays use contact time with food as a proxy for feeding and do not record actual food intake (Moreira et al. 2019; Ro, Harvanek, and Pletcher 2014; Itskov et al. 2014). Another notable drawback of single fly assays is that it is unable to record how social interactions in a group could influence behavior, including feeding (Wong et al. 2009; Jezovit, Alwash, and Levine 2021).

The ability to quantify and control consumption over a period is also necessary for DR (Mair, Piper, and Partridge 2005; Piper and Bartke 2008) and time restricted feeding (TRF) protocols (Gill et al. 2015). In flies, DR is mainly achieved by diluting food or nutrients (Partridge, Piper, and Mair 2005; Mair, Piper, and Partridge 2005). However, food dilution leads to compensatory feeding and could complicate the interpretation of the results (Piper and Bartke 2008; Mair, Piper, and Partridge 2005; W. W. Ja et al. 2009; Carvalho, Kapahi, and Benzer 2005). Overall, the exact amount of food offered to a group of flies has rarely been controlled.

Till date, no single fly feeding assay has been reported to both directly measure food intake from complex solid diets and to control the amount of food delivered. Here, we describe a simple, versatile, and adaptable feeding assay that directly measures the actual amount of solid food consumed by groups of flies. Dubbed as DIETS (Direct Intake Estimation and Tracking of Solid food consumption), the assay can measure feeding over both short and long periods, typically from 30 minutes to 24 h. Further, by

periodically switching flies to fresh DIETS vials, longitudinal feeding measurement can be performed over days and weeks. By placing an additional food cup in a DIETS vial, we could also monitor short- and long-term feeding preferences in flies. The small surface area of the food in cups makes it possible to measure intake from dense and sticky complex diets like oily, high-fat diets, as flies do not get stuck to the food even at higher temperatures (~ 25 - 32 ℃). As flies are not sacrificed for intake measurement, consumption measurement from DIETS can be easily combined with metabolic, biochemical, physiological, and other behavioral measurements of the flies' internal states. Additionally, we demonstrated that flies could be restricted to feeding an exact amount of food by offering a predetermined amount of food in the cups. Hence, DIETS could be used for DR protocols and also TRF protocols. Finally, we also provide evidence that consumption measurements can be combined with videography to determine the temporal position of flies by modifying the assay to flat arenas.

Results

Measurement of feeding by directly weighing food consumed

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

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

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

DIETS is a sensitive and precise feeding assay

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

Figure 1.

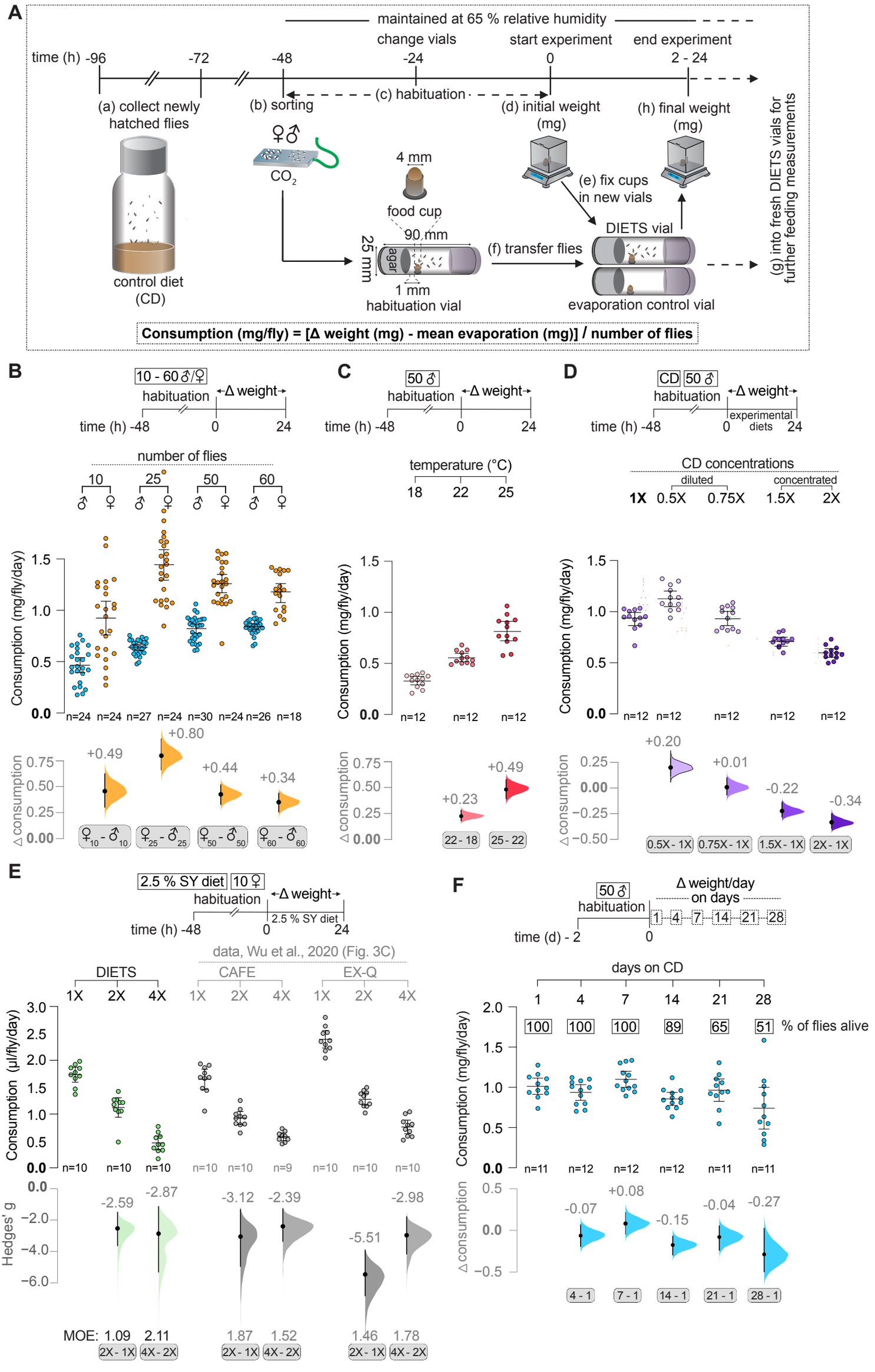
A
maintained at 65 % relative humidity
time (h) -96
-72
-48
-24
0
2 - 24
change vials
start experiment
end experiment
(a) collect newly hatched flies
(b) sorting
(c) habituation
(d) initial weight (mg)
(h) final weight (mg)
CO2
4 mm
food cup
90 mm
25 mm
agar
1 mm
habituation vial
control diet (CD)
(e) fix cups in new vials
DIETS vial
(f) transfer flies
evaporation control vial
(g) into fresh DIETS vials for further feeding measurements
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 1X 2X 4X 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
1 4 7 14 21 28
100 100 100 89 65 51 % of flies alive
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

Figure 1. DIETS (Direct Intake Estimation and longitudinal Tracking of Solid food consumption) is a sensitive assay that can measure food consumption in flies over days.

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

interactions with group size seemed to influence individual feeding behavior (Wong et al. 2009). As expected, the total food consumed by a group increased with the group size for both sexes and was clearly discernible with DIETS (Fig. S2A, S2B and Table ST2). In experiments with mated females, we observed extensive egg deposition, mostly on the agar bed (Fig. S2C). Only ~ 2 - 4 eggs were found on a food cup on average, with the number going up to ~ 8 eggs for 60 females (Fig. S2D). As the mean egg weight was ~ 32 μg (data not shown), egg weight could be ignored, especially if the ones on the outside of the cup were cleared before final weighing. In case larvae are seen on food, we suggest switching food more frequently and to perform the experiments at a lower temperature if possible. As feeding seemed to plateau at a group size of 50, and the margin of error (MOE) in assessing consumption in males was generally smaller (Table ST2; see entries for Fig. 1), we decided to perform all subsequent experiments with 50 male flies.

A recent study in flies has shown that an increase in temperature enhances metabolic rate and feeding (Klepsatel, Wildridge, and Gáliková 2019). We also saw a clear increase in CD consumption by male flies over 24 h when we raised the temperature from 18 ^{o}C to 22 ^{o}C and then from 22 ^{o}C to 25 ^{o}C (Fig. 1C; see effect sizes). Consumption on CD was even higher at 32 ^{o}C (Fig. S5B). We could detect such changes in temperature-dependent consumption with a sample size of 12 vials per condition.

Existing solid food feeding assays can detect compensatory increase or decrease in feeding in flies upon diluting or concentrating food (Deshpande et al. 2014; Wu et al. 2020). With DIETS, we were able to discern clear differences in feeding between 1X CD and 0.5X CD (~ 21 % increase) or between 1X and 2X CD (~ 56 % decrease) (Fig. 1D).

To directly compare the sensitivity of DIETS with the previously reported CAFE and EX-Q data (Wu et al. 2020), we measured compensatory feeding from 1X to 2X and to 4X concentrations of 2.5 % sucrose - 2.5 % yeast extract (2.5 % SY: 1X) diet (n=10, to match with Wu et al. 2020). We compared the standardized effect sizes in Hedge's g (see Materials and Methods for details) along with its 95 % MOE across assays (Fig. 1E). Between 1X and 2X diets, the analysis showed that DIETS and CAFE report effect sizes that are close to each other (-2.59 for DIETS, and -3.12 for CAFE). However, a larger effect size is seen with EX-Q (-5.51), mainly because 1X consumption measured by EX-Q was elevated (Fig.1E). Between 2X and 4X diets, all three assays report quite similar effect sizes (-2.87 for DIETS, -2.39 for CAFE, and -2.98 with EX-Q). Keeping in mind the small size of the data sets (n=10), the MOE or half the magnitude of 95 % CIs of the Hedge's g was also generally comparable (Fig. 1E). We concluded that DIETS was as sensitive as CAFE and EX-Q and likely equally precise (compare MOEs of all DIETS data reported in Table ST2).

To test whether DIETS can be used to assess longitudinal feeding, we transferred groups of 50 male flies into fresh assay vials every 24 h for 4 weeks. Actual consumption was assessed over the 1st, 4th and 7th, 14th, 21st, and 28th day (schema; Fig.

1F). Feeding was reliably measured over the whole period, with very little change in feeding when compared to the 1st day measurement, except on the 28th day when a considerable feeding decline was observed. We also observed that with a large group size of 50, flies started to die around the 14th day, and by the 28th day, almost 50 % of flies had perished.

Next, to test whether flies were starving in DIETS due to a smaller food source and hence eventually dying early, we compared the starvation resistance of male flies (10, 25, and 50 flies/group) after being in DIETS vials or normal food vials for 1 day and 7 days. We found that flies showed almost similar starvation resistance across group sizes and modes of food delivery (Fig. S3A). Together, our results suggest that no discernible change in survival is observed in DIETS up to 7 days. We also measured feeding on CD in two other strains in the lab, *CS-BZ* and w^{1118}, and observed that *CS-BZ* had elevated feeding as compared to the other two strains (Fig. S3B).

To demonstrate the suitability of DIETS with thermogenetic manipulations at 32 ^{o}C, we activated Piezo-GAL4 labeled neurons using a temperature-sensitive cation channel, dTRPA1 (Hamada et al. 2008) during a 30 minutes feeding period at 32 °C. Piezo-GAL4 labeled mechanosensory neurons densely innervate the crop and anterior midgut and regulate ingestion volume by controlling the crop size (S. Min et al. 2021). As reported earlier (S. Min et al. 2021), we too observed a defect in feeding, in fact, a more severe one, upon artificial activation of Piezo-GAL4 neurons compared to parental controls. Feeding reduction in Piezo-GAL4>UAS-dTRPA1 flies was not observed at 21 °C (Fig.

S3C; right panel). Activation of serotonergic neurons labeled by R50H05-GAL4 induces hunger and feeding in sated flies (Albin et al. 2015). We replicated these results with DIETS, with increased feeding observed in R50H05-GAL4>UAS-dTRPA1 flies at 32 °C, but not at 21 °C (Fig. S3C; right panel).

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.

Figure 2.

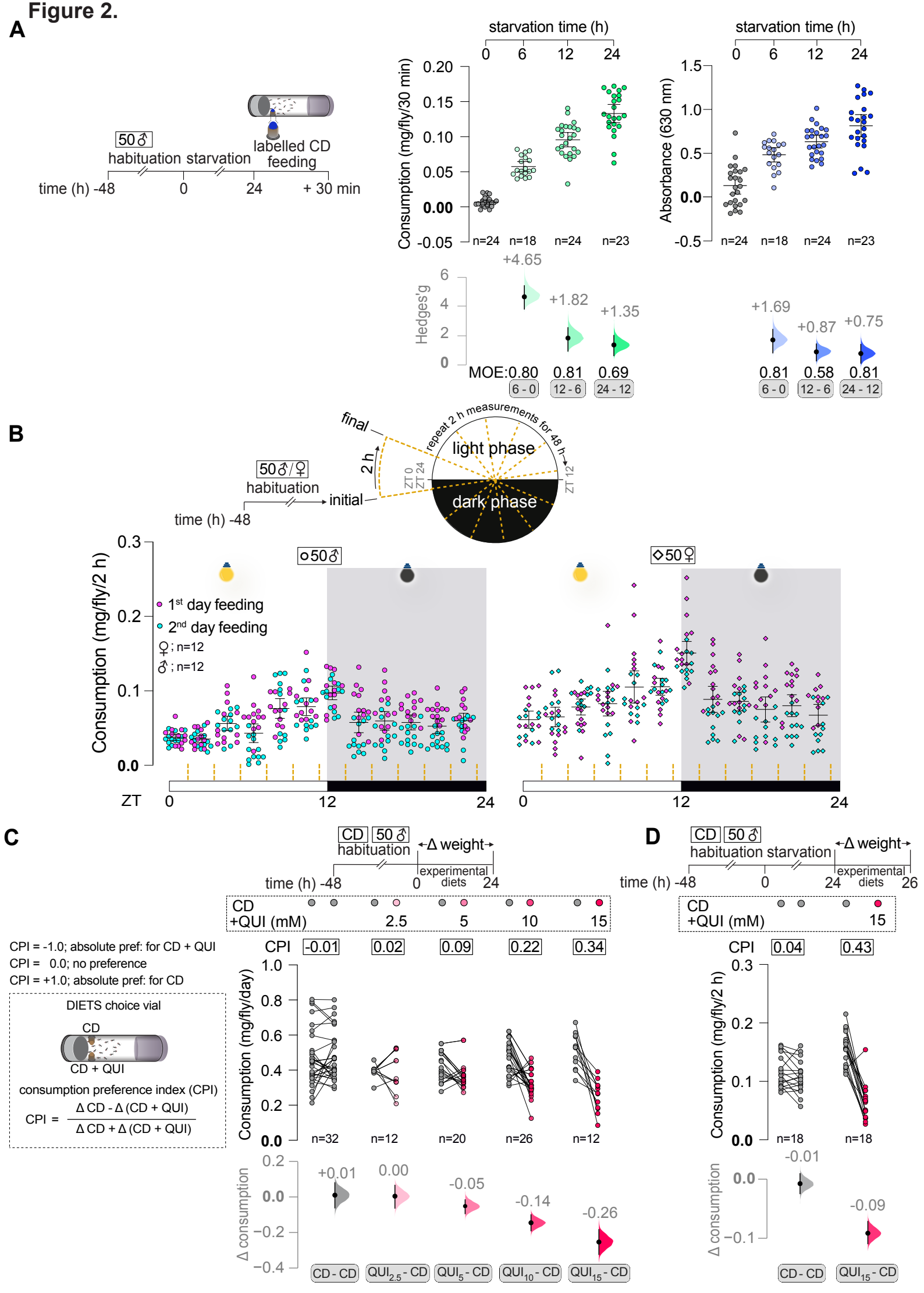
A
50♂
habituation
starvation
labelled CD feeding
time (h) -48
0
24
+ 30 min
starvation time (h)
0 6 12 24
Consumption (mg/fly/30 min)
0.20
0.15
0.10
0.05
0.00
-0.05
n=24 n=18 n=24 n=23
Absorbance (630 nm)
1.5
1.0
0.5
0.0
-0.5
n=24 n=18 n=24 n=23
Hedges'g
+4.65
+1.82
+1.35
MOE:0.80 0.81 0.69
6 - 0
12 - 6
24 - 12
+1.69
+0.87
+0.75
0.81 0.58 0.81
6 - 0
12 - 6
24 - 12
B
50♂/♀
habituation
time (h) -48
initial
final
2 h
repeat 2 h measurements for 48 h
light phase
dark phase
ZT 0
ZT 24
ZT 12
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

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

(A) Comparing sensitivity of DIETS with blue dye uptake in measuring short-term feeding. 30 minutes feeding on CD supplemented with Brilliant blue FCF in differentially starved male flies was measured using DIETS. Flies showed an increase in feeding with increased starvation (left panel). A simultaneous dye accumulation measurement was conducted by crushing the flies after feeding (right panel). Hedge's g is effect size, in this case, the difference in mean of two groups, standardized over the pooled standard deviation of the two groups. DIETS assay yields larger 'g' values compared to blue dye estimation for comparison of feeding between the same groups. **(B)** Continuous 2 h feeding measurements in sated male and female flies measured over 2 days. Both sexes show a distinct evening feeding peak just around the lights-off time at ZT = 11.5-12.5. **(C)** 24 h, food preferences between CD and CD mixed with quinine. Flies show an increasing dose-dependent aversion towards CD adulterated with quinine. **(D)** 2 h food preferences between CD and CD adulterated with 15 mM quinine in 24 h starved flies. Flies showed a strong aversion towards quinine-laced food. Δ 'effect size' plots below each raw data graph depict mean differences (black dots; 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. In (A) standardized effect size, Hedge's g has been plotted, and MOEs have been reported. See Materials and Methods for further details.

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

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

We reasoned that dietary choice between two foods could also be measured if flies were given access to two cups with distinct food. To validate food preference, we arranged choices between CD and CD laced with increasing concentrations (2.5 mM to 15 mM) of bitter-tasting quinine (QUI), for groups of 50 males, for 24 h (Fig. 2C, schema). Cups were placed 1 mm from the agar bed and diametrically opposite to each other. Fully assembled DIETS choice vials were covered with white paper to act as a diffuser and prevent light bias (Fig. S4B; images). In control DIETS vials, flies ate equally on average from both the cups with CD. As expected, flies preferentially fed

from the CD cup over the CD + QUI cup, with increasing concentrations of quinine (Fig. 2C). However, combined consumption of food from both cups remained unchanged across quinine concentrations (Fig. S4C). Similarly, we measured short-term preference over 2 h in starved flies. We observed a strong preference to feed from the CD cup over the CD + 15 mM QUI food cup (Fig. 2D). Total consumption stayed constant in this case, as well (Fig. S4D). Food preference in DIETS can also be represented as a consumption preference index (CPI) (Fig. 2C; see inset). Corresponding CPI values are shown on top of the consumption plots (Fig. 2C and 2D).

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

High-fat diets (HFDs) for flies have been typically made by adding coconut oil as a source of saturated fat to fly food (Birse et al. 2010; Diop, Birse, and Bodmer 2017). HFDs are difficult to work with as the fat melts even at ~ 25 °C, rendering the food sticky and lethal for flies unless care is taken (Diop, Birse, and Bodmer 2017). The change in the consistency of food could potentially affect feeding too. We reasoned that DIETS, with its low food surface area, may resolve such issues. We prepared HFDs with added 5 % to 20 % coconut oil at the expense of water in the mix. We recalibrated evaporation control vials for HFDs and found that HFD food cups were best placed 2 mm from the agar bed. At lesser distances (1 mm), the HFD tended to accumulate moisture, leading to misleading changes in weight (data not shown). Consumption was measured on the 1st, 4th and 7th day (Fig. 3A, schema). We found that consumption of diets with 5 % and 10 % added coconut oil was comparable with CD throughout (Fig. 3A). As the added lipids made the food more calorie-rich, calorie intake was increased at both these

Figure 3.

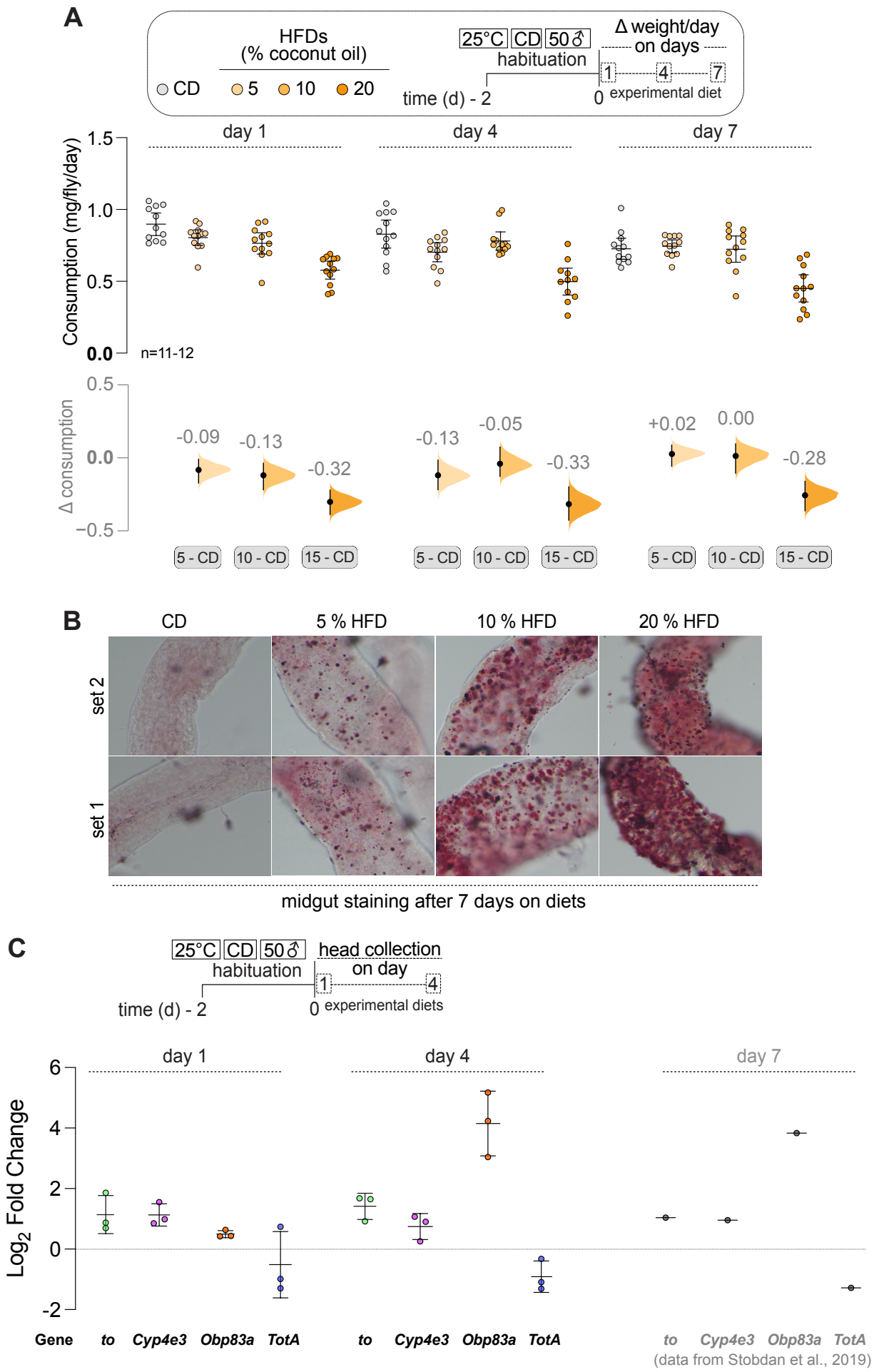
A
HFDs
(% coconut oil)
CD
5
10
20
25°C
CD
50♂
habituation
Δ weight/day
on days
1
4
7
time (d) - 2
0
experimental diet
day 1
day 4
day 7
Consumption (mg/fly/day)
1.5
1.0
0.5
0.0
n=11-12
Δ consumption
0.5
0.0
−0.5
-0.09
-0.13
-0.32
-0.13
-0.05
-0.33
+0.02
0.00
-0.28
5 - CD
10 - CD
15 - CD
B
CD
5 % HFD
10 % HFD
20 % HFD
set 2
set 1
midgut staining after 7 days on diets
C
25°C
CD
50♂
head collection
on day
habituation
1
4
time (d) - 2
0
experimental diets
day 1
day 4
day 7
Log2 Fold Change
6
4
2
0
-2
Gene
to
Cyp4e3
Obp83a
TotA
(data from Stobdan et al., 2019)

Figure 3. DIETS is well suited to study longitudinal feeding on high-fat diets and associated physiological and transcriptional changes.

(A) In a longitudinal feeding study, flies were fed a high-fat diet (HFD), and feeding measurements were conducted on days 1, 4, and 7. While consumption remained mostly constant up to 10 % extra coconut oil in the diet, feeding decreased noticeably with the addition of 20 % coconut oil. **(B)** Oil-Red-O staining of lipid droplets was performed on the midgut of flies fed different HFDs for 7 days. Fat deposition increased with % of coconut oil in the food. **(C)** Differential expression of *takeout* (*to*)*, Cytochrome P450-4e3* (*Cyp4e3*), *Odorant-binding protein 83a* (*Obp83a*), and *Turandot A* (*TotA*) was examined by qPCR from heads of flies that have been fed with 20 % HFD for 1 and 4 days, with respect to control flies fed CD. These genes were earlier found to be some of the most differentially expressed genes from a study with similar design (Stobdan et al. 2019). The direction and magnitude of differential expression of the above candidate genes on day 4 was comparable to that in day 7 samples from the previous study, as shown. Δ 'effect size' plots below each raw data graph depict mean differences (black dots; 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.

conditions compared to CD (Fig. S5A). We also saw that consumption of the diet with 20 % added oil was considerably reduced from all the other groups (Fig. 3A; see effect sizes HFD). As a result, calorie intake in the 20 % HFD group was virtually similar to the 10 % HFD group, except on the 1st day (Fig. S5A). To understand whether this reflects a calorie limit sensing mechanism or simply because higher fat concentrations are taste aversive to flies requires further investigation. DIETS was also suitable for consumption measurement on HFD even at 32 ^{o}C, enabling thermogenetic experiments with HFDs (Fig. S5B). Overall, we concluded that DIETS was suitable for feeding measurements on HFDs over a wide range of temperatures.

As reported earlier (Birse et al. 2010), upon lipophilic Oil-Red-O staining, we detected increasing mid-gut fat deposition after 7 days with an increasing percentage of oil in the flies' diet (Fig. 3B). This showed that HFD feeding in DIETS yielded expected physiological changes. Further, to ascertain whether feeding from HFDs in DIETS influenced gene expression changes in key genes, as reported earlier by RNA-Seq (Stobdan et al. 2019), we performed q-PCR analysis. We extracted RNA from the heads of male flies kept on either 20 % HFD or CD for 1 and 4 days and probed gene expression using qPCR (see Materials and Methods). We re-analysed the raw RNA-Seq data generated previously (Stobdan et al. 2019) using DESeq2 (Love, Huber, and Anders 2014) and used p-adjusted < 0.05 as our threshold. From this analysis, we selected four of the most differentially expressed genes (DEGs) to analyze from our collected head RNA samples. Out of these, three were upregulated, namely takeout, Obp83a, and Cyp4e3, and one down-regulated gene, TotA (Fig. 3C). Our assay revealed dynamic changes in the expression of the selected genes due to HFD feeding.

On day 1, the expression of these genes showed variability as compared to the earlier study (Stobdan et al. 2019). However, by day 4, their expression was more consistent with Stobdan et al., 2019 (Fig. 3C). Note here that RNA-Seq analysis in Stobdan et al., 2019 was carried out from flies that were exposed to HFD for 7 days. Our 4 day data trended towards the earlier study, in spite of minor differences in diet composition and methods used for gene expression analysis. We concluded that DIETS would be well suited for measuring gene expression changes after dietary manipulations.

Implement precise DR and TRF regimens with DIETS

Dietary restriction (DR) in flies is usually carried out by diluting food (Partridge, Piper, and Mair 2005; Bass et al. 2007; Piper and Partridge 2007; W. W. Ja et al. 2009; K.-J. Min and Tatar 2006). Since we could control the amount of food we offer to flies, we decided to ascertain whether we could use DIETS to achieve precise DR. In the 'no DR' control groups, ~ 56 mg of CD was offered to flies (see Material and Methods for details). To implement ~ 90 % to 60 % DR, we gradually reduced the mean food offered, 'a' in cups (Fig. 4A). Targeted % DR was calculated as (a / 56) * 100. In the same 'no DR' group, mean consumption was 43 mg (Fig. 4A). Actually achieved % DR was calculated as (b / 43) * 100, where 'b' was the mean consumption in the DR groups (Fig. 4A). We found that the amount fed by groups of flies at all the DR levels clustered narrowly. This was also reflected in the extremely narrow MOEs of each group (Fig. 4A, see error distribution of effect sizes. Also see Table ST2). We concluded that the DIETS setup can be used to restrict the amount of food consumed by a group of flies within a few percentage points.

Figure 4.

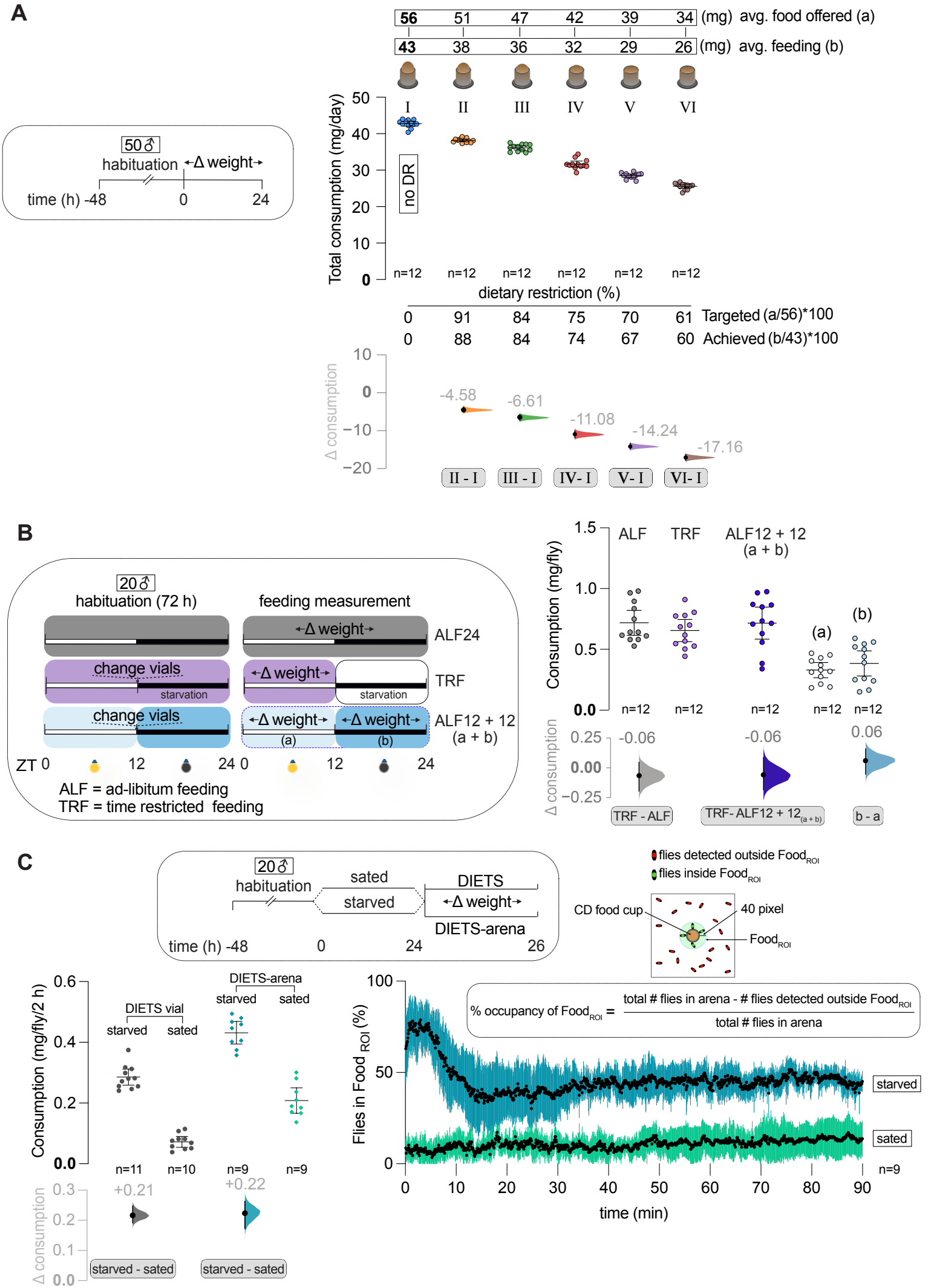
A
50♂
habituation
←Δ weight→
time (h) -48
0
24
56 51 47 42 39 34 (mg) avg. food offered (a)
43 38 36 32 29 26 (mg) avg. feeding (b)
I II III IV V VI
no DR
Total consumption (mg/day)
n=12 n=12 n=12 n=12 n=12 n=12
dietary restriction (%)
0 91 84 75 70 61 Targeted (a/56)*100
0 88 84 74 67 60 Achieved (b/43)*100
Δ consumption
-4.58
-6.61
-11.08
-14.24
-17.16
II - I
III - I
IV- I
V- I
VI- I
B
20♂
habituation (72 h)
feeding measurement
←Δ weight→
ALF24
change vials
starvation
TRF
ALF12 + 12
(a + b)
(a)
(b)
ZT 0 12 24 0 12 24
ALF = ad-libitum feeding
TRF = time restricted feeding
Consumption (mg/fly)
ALF
TRF
ALF12 + 12
(a + b)
n=12 n=12 n=12 n=12 n=12
-0.06
-0.06
0.06
TRF - ALF
TRF- ALF12 + 12(a + b)
b - a
C
20♂
habituation
sated
starved
DIETS
←Δ weight→
DIETS-arena
time (h) -48
0
24
26
flies detected outside FoodROI
flies inside FoodROI
CD food cup
40 pixel
FoodROI
Consumption (mg/fly/2 h)
DIETS vial
starved
sated
DIETS-arena
starved
sated
n=11 n=10 n=9 n=9
+0.21
+0.22
starved - sated
starved - sated
% occupancy of FoodROI = total # flies in arena - # flies detected outside FoodROI / total # flies in arena
Flies in Food ROI (%)
starved
sated
n=9
time (min)

Figure 4. Precise DR, TRF, and temporal analysis of feeding with DIETS.

(A) Precise DR was implemented by offering a predetermined amount of food in cups to groups of 50 flies. Consumption was measured over a 24 h period. Targeted and achieved DR was calculated based on food offered and consumed in the 'no DR' group. The groups are labeled with roman numerals I to VI **(B)** Flies restricted to feeding only during the day feed almost equal amounts of food as the control *ad libitum* fed groups. CD consumption was measured over 24 h under three conditions: Two *ad libitum* groups, where food was changed once every 24 h (ALF) or every 12 h at night to day and day to night transitions (ALF12+12). The time-restricted feeding (TRF) groups were only given access to food for 12 h during the lights-on period. All groups were habituated for 3 days under respective conditions before measuring feeding **(C)** Weighing food consumption works equally well in flat arenas (left panel). Starved flies feed more than sated flies over a 2 h period, and the difference in their feeding can be quantified equally well in vials and in flat arenas. Starved flies show higher occupancy of food than sated flies over a 2 h period (right panel). Food occupancy of the same groups of starved and sated flies were quantified from DIETS-arenas by image analysis. Images of DIETS-arenas were captured once every 5 s. The number of flies outside the defined FoodROI was determined, and the total number of flies being known, the number of flies inside FoodROI was calculated as a percentage. Δ 'effect size' plots below each raw data graph depict mean differences (black dots; 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.

Recently, time restricted feeding (TRF) protocols have been imposed on flies to gauge the effect on the overall health and longevity of flies (Cabrera, Young, and Axelrod 2020; Gill et al. 2015; Ulgherait et al. 2021). To investigate the impact of a daily pattern of feeding and fasting on food intake with DIETS, we measured CD consumption in groups of 20 male flies for 24 h under three different conditions: (a) A control *ad libitum* feeding (ALF) group with food cup changed every 24 h at ZT = 0, (b) A second control a*d libitum* feeding group with food cup changed every 12 h (ALF12+12), and (c) the experimental group where feeding was restricted to 'lights-on' phase, ZT = 0 - 12 (TRF). During the 'lights off' period, the TRF groups remained in DIETS vials with no food cup. All groups were habituated as described above for 3 days to let them adjust to their respective conditions. Consumption was measured for the next 24 h period (Fig. 4B; schema). In agreement with previous reports (Cabrera, Young, and Axelrod 2020; Gill et al. 2015), we found that flies under TRF consumed the same amount of food during the daytime as the control groups managed to eat over a whole day (Fig. 4B). Our data also revealed that flies fed equally during the day and night (Fig. 4B; ALF12+12 group, compare daytime feeding 'a' with nightime feeding 'b'). We concluded that DIETS can be used to implement TRF protocols with solid foods and simultaneously measure feeding too.

Combining consumption measurement with video tracking: DIETS-arena

DIETS vials are not ideal for videographic analysis because of their dimensions and also due to the location of the cups next to the agar bed. As a proof of concept that video analysis of fly location can be combined with DIETS style measurement of food

consumption, DIETS-arenas were designed (Fig. S6A). First, to determine the efficacy of the flat DIETS-arenas, we measured 2 h CD consumption in sated and starved male flies with both DIETS vials and DIETS-arena side by side (Fig. 4C; schema). We observed similar increase in feeding in the starved groups compared to the sated group with both vials and arenas (Fig. 4C, left panel). The feeding baseline was higher in the DIETS-arenas compared to DIETS vials for both sated and starved flies. Further investigation will be needed to determine the cause.

Next, to analyse the temporal dynamics of feeding with the same set of sated and starved flies, we defined a $Food_{ROI}$ around the food cup in the center of the arena (see Material and Methods). We found that the percentage of starved flies that occupied the $Food_{ROI}$, went from 74 % to ~ 40 % in the first 20 minutes. Thereafter starved flies maintained an average of ~ 44 % $Food_{ROI}$ occupancy. In contrast, fed flies maintain a low and steady ~ 11 % occupancy of the $Food_{ROI}$ over 90 minutes (Fig. 4C, right panel). This pattern is also reflected in temporal snapshots from two representative sets, each of sated and starved flies (Fig. S6B). In conclusion, with the help of video analysis from DIETS-arenas, we could visualise dynamic changes in food occupancy that could be correlated with the increased feeding observed in starved flies.

Discussion

DIETS is a direct measurement of food consumed by flies with no added dyes or tracers (Fig. 1). Therefore, age or genotype related physiological changes, including changes in the gut, will not influence measurement. We show here that DIETS is as sensitive as CAFE (William W. Ja et al. 2007; Diegelmann et al. 2017) and EX-Q assays (Wu et al. 2020) to discern biologically relevant differences in food consumed over long and short periods (Fig. 1E and Fig. 2A; see effect sizes). The assay is also precise, and with proper humidity control, yields measurements with low variability (see MOEs in Table. ST2). One major drawback of DIETS is that it cannot be used for studying feeding individuality in single flies.

DIETS is versatile and can be used to measure feeding over different time scales for diverse foods, including high-fat diets that are difficult to work with (Diop, Birse, and Bodmer 2017). This is an especially important feature of DIETS because, to our knowledge, there is no other report of longitudinal consumption measurement on high-fat diets. Flies can independently regulate food and water intake (Van Dam et al. 2021; Fanson, Yap, and Taylor 2012; W. W. Ja et al. 2009). In DIETS vials, the agar bed provides a separate water source so that flies can meet their water needs independently from their food drive (Fig. 1A). We have shown that DIETS could be also utilised for measuring food preference (Fig. 2). Food preferences with DIETS can be easily extended over days, to monitor how preferences may shift over time.

While DIETS can be used for group sizes varying from ~ 10 - 60 flies, we recommend that group size be optimized for specific experiments. We observed that when measuring the consumption of groups of 50 males, there was significant death after 2 weeks of feeding in DIETS vials (Fig. 1E). Such increase in death might be due to increased struggle for a small food source in larger groups, and could be mitigated by using smaller group size for longitudinal measurements. Indeed, previous longevity studies have used much smaller group sizes of 10 - 15 flies (Piper and Partridge 2016; Lee et al. 2008; Metaxakis and Partridge 2013; Zanco et al. 2021; Krittika and Yadav 2020).

In terms of versatility, ease and cost of use, only CAFE and EX-Q assays come closest to DIETS (Fig. 1E). While CAFE directly measures food intake, it has significant drawbacks. Feeding long-term in CAFE significantly reduces both egg-laying and lifespan (Wong et al. 2009; Deshpande et al. 2014). Further, consumption of complex lab diets, including fat supplemented diets, is not possible with CAFE (M.T, and B. P; personal communication). We show that DIETS is suitable for the consumption of high-fat diets (Fig. 4A) and high-sugar diets (data not shown), even at higher temperatures (Fig. S5B). EX-Q is well suited for longitudinal studies, for use with complex diets, for estimating food preference with two compatible dyes and also possibly for dietary restriction protocols (Wu et al. 2020; Shell et al. 2021). However, any protocol that requires measurement for short consecutive periods will not be possible with EX-Q because of the ~ 3 h ‘chase’ phase on non-dyed food required to excrete out all the dye in flies. Importantly, EX-Q is still an indirect method of feeding

quantification and any change in the way the dye is absorbed or excreted with age or genotype would affect the measurement. EX-Q in its current form is also incompatible with video analysis of fly behavior.

Capacitance based single fly feeding assays like FLIC/flyPAD/optoPAD/dFRAME are highly sensitive and outperform DIETS in determining feeding microstructure, including feeding duration and frequency of individual feeding bouts (Ro, Harvanek, and Pletcher 2014; Itskov et al. 2014; Moreira et al. 2019; Niu et al. 2021; Weaver et al. 2023). However, these assays too, do not measure feeding directly. They are also unable to monitor consumption from multiple flies and hence are not suitable when studying the influence of group dynamics on feeding. Here, we demonstrate that direct food weight measurements can be carried out from flat and transparent DIETS-arenas and can be combined with video-based temporal analysis of a fly's position. Similar to previous studies (Niu et al. 2021; Jaime et al. 2023), with the right camera, lighting, and number of flies, the feeding microstructure of a single 'tagged' fly in a group can be recorded from DIETS-arenas too.

With DIETS, we were also able to continuously monitor consumption over short periods for 2 days by transferring flies to fresh vials every two hours (Fig. 2B). However, flies are frequently disturbed during transfers in such cases. This could be avoided by switching food cups via a hole drilled on the side of DIETS vials without disturbing the flies. This design has been adapted for the DIETS-arenas (Fig. S6A). Amongst existing assays, such short-term repeated measurements can only be derived with Expresso (Yapici et

al. 2016) and FLIC/flyPAD/optoPAD (Itskov et al. 2014; Moreira et al. 2019) platforms that measure single fly feeding and have much higher setup cost than DIETS. Interestingly, unlike earlier studies, we observed only a single evening feeding peak and also sustained levels of feeding at night (Fig. 2B and Fig. 4B). We speculate that perhaps the prominent night-time feeding could be an outcome of group effect, as we use 50 flies (Fig. 2B) or 20 flies (Fig. 4B) for our experiments.

Dilution of food or nutrient content of food leads to compensatory feeding (Partridge, Piper, and Mair 2005; Chapman and Partridge 1996; Bass et al. 2007; Mair et al. 2003). To the best of our knowledge, DIETS is the only assay that can implement precise DR in flies without worrying about compensatory consumption (Fig. 4A). Additionally, like CAFE, TRF protocols (Cabrera, Young, and Axelrod 2020; Gill et al. 2015) are easily carried out with DIETS, with the added advantage of being able to work with high-fat and high-sugar foods. In conclusion, we anticipate that the ability of DIETS to easily measure and control diverse facets of consumption behavior will lead to its wide adoption.

Materials and Methods

A. Fly lines and rearing

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

B. Chemicals and other materials

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

vendors. Light filters used for uniform red light are 182: light red, 158: deep orange and 810: zoom difusion 1 (Bright light international). The following chemicals were used for RT-PCR; Trizol (Invitrogen: 15596026), Zymo Direct Zol kit (Zymo Research: R2051), Zymo RNA Clean and Concentrator kit (Zymo Research: R1013), High-Capacity cDNA Reverse Transcription kit (Thermo- Fisher Scientific: 4368814), KAPA SYBR FAST Universal (Sigma-Aldrich: KK4602), QuantStudio™ 6 flex Real-Time PCR System (Applied Biosystems: 4485691).

C. Diet preparation

To prepare the Control Diet (CD), ingredients (Table ST1) were weighed and mixed in 60 mL reverse osmosis (RO) filtered water. The mixture was then cooked in a microwave oven, with 2-3 intermittent stirring for ~ 2 minutes. The food mix was then allowed to cool to 60 ℃ before adding methylparaben, propionic acid, $CaCl_2$, and $MgSO_4$. The volume was adjusted to 100 mL by adding RO water. This mixture was then thoroughly stirred with a whisk. To prepare diets with 0.25X to 2X concentrations of CD (Fig.1D), we separately weighed all the ingredients for each concentration. Agar concentration was maintained at 1 % for all the dilutions. To prepare CD spiked with quinine (Fig. 3C), the required amounts of qunine was added to molten CD, below 60 ℃ and mixed thoroughly by shaking or vortexing. 2.5 % sucrose - 2.5 % yeast diet (1X SY diet), 5 % Surcore 5 % Yeast diet (2X SY diet) and 10 % Sucrose 10 % Yeast diet (4X diet) were prepared as previously stated (Deshpande et al. 2014; Wu et al. 2020). To prepare saturated high-fat diets (HFD), a required amount of molten coconut oil was added to cooked food and then mixed with a whisk. The food mix was allowed to cool to

60 ℃ and methylparaben, propionic acid, $CaCl_2$, and $MgSO_4$ was added. The food was mixed thoroughly again with a whisk, and the volume was adjusted to 100 mL by adding RO water.

D. Feeding quantification by DIETS assay

i) DIETS vial preparation

Standard fly vials (inner diameter: 23 mm, outer diameter: 25 mm, height: 90 mm) and 200 µL microcentrifuge tube caps as food cups were assembled as follows. 7 mL 0.75 % agar solution was added to each vial to form an agar bed. After the agar solidified, it was allowed to dry for 12 to 18 h at room temperature. If used immediately, the agar layer leaks water, in which flies get trapped. An agar bed reduces the weight loss from food cups due to evaporation and provides a separate water source for flies to drink water. Solid food was provided in 200 µL microcentrifuge tube (PCR-02-C; Axygen) caps (food cups) of 4 mm diameter. For most experiments, ~ 70 µL of molten food from stored aliquots were pipetted into food cups (Supplementary Fig. 1A). Once the food solidified, we noted the initial weight of cup + food. The initial weight of the food cups (cup + food) was kept between a narrow range for consistency, typically ~ 125 - 135 mg for CD. Freshly prepared food cups were fixed on the inside of the agar vials, normally at a distance of 1 mm from the agar, with a small square of double-sided tape. Evaporation control vials were prepared identically.

ii) Food consumption and calorie intake measurement

Flies were collected over the first day after eclosion, and transferred to new food bottles

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

Fly groups were then transferred to DIETS vials with pre-weighed food cups for feeding measurements. Evaporation control vials with no flies were maintained alongside (Fig. 1A and Supplementary Fig. 1A). Viscosity of a specific food influences the rate of evaporation. We also observed that certain viscous foods, like foods with high-fat and high sugar, in cups placed at 1 mm from the agar bed tended to absorb moisture. Conversely, in diluted, less viscous foods, evaporation loss increased. Therefore, with each new diet, we empirically determined the position of the food cups from the agar bed to prevent moisture accumulation or loss by evaporation in evaporation control vials below 10 % of the total food consumed. The humidity level of the incubator could also be adjusted to keep evaporation below the desired level. DIETS vials were kept horizontally in a tray inside the incubator, with the food cups facing upward (Fig. S1A). Most experiments were carried out at 25 ℃ and 65 % humidity.

Flies were transferred to fresh DIETS vials at the end of the period of feeding measurements. The reduction in food cup weight (food consumed + evaporation loss) was determined. The mean value of evaporation mediated loss in weight (from 4 - 6 control vials) was deducted from the reduction in food cup weight to derive the total food consumed by a group of flies. This value was usually plotted as an average

consumption per fly over time (e.g., mg/fly/day) or sometimes as total consumption for a group of flies (mg/day).

Calorie intake (in Fig. 3B) was calculated from the amount of food consumed on CD and HFD, using the calorie values given in (Musselman et al. 2011). The caloric value of the Nutiva coconut oil was taken from the product label.

iii) Feeding measurement in starved flies with DIETS and blue dye uptake

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

iv) Round the clock circadian feeding measurements

2 - 3 day old male and female flies were habituated in DIETS vials for two days. Feeding measurement was initiated at 12 noon [Zeitgeber time (ZT) = 3.5; our light-on time, or ZT = 0, was 8:30 AM] in fresh vials. Flies were then transferred to fresh DIETS

vials every 2 h for the next 50 h, and continuous measurements were taken. The first 2 h feeding measurement was discarded, as it showed spiked in feeding, likely due to the fact that they had been transferred to fresh food after 24 h. Fly transfers during the 12 h dark phase were carried out in red light.

v) Two food choice feeding measurements with DIETS

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

vi) DR protocol with DIETS

We have achieved DR in flies by offering a fixed amount of diets in food cups to a group of flies for 24 h, after 2 days of habituation on the CD diet. As a 'NO DR' control (*ad-libitum* feeding), an average of 56 mg of CD was offered to groups of 50 male flies. This value was derived from summing the mean consumption of 50 male flies (~ 39 mg: Fig. S2A), mean evaporation from food cup (~ 4 mg: Fig. S1C), and mean leftover food

(~ 10-13 mg: data not shown). To implement increasing degrees of DR (~ 90 % to 60 %), we gradually reduced the mean food offered in cups by ~ 3 to 5 mg (labeled ‘a’; Fig. 4A). Targeted DR % was calculated with respect to 56 mg offered in the ‘no DR’ group. Based on actual food consumed (labeled ‘b’; Fig. 4A), the achieved DR % was calculated with respect to 43 mg, the mean food consumed by flies in the ‘no DR’ group (Fig. 4A).

vii) TRF feeding protocol with DIETS

Groups of 20 male flies each were split into three groups. Two *ad libitum* feeding groups, where flies were switched to fresh food vials either every 24 h (ALF) or every 12 h (ALF12+12) were set. A third time-restricted feeding (TRF) group was offered food only during the ‘lights on' phase (ZT = 0 - 12). TRF flies were switched on DIETS vials with no cups during the ‘lights off’ phase (ZT = 12 - 24). All groups were habituated similar to their experimental conditions (schema; Fig. 4B). Food consumption was measured whenever flies were switched to fresh vials as indicated (schema, Fig. 4B).

E. Starvation sensitivity assay

To compare the starvation sensitivity of flies, we allowed flies in varying group sizes (10, 25 and 50 males) to feed from DIETS vial and regular food vial for 1 day and 7 days, following two days of usual habituation. For 7 days feeding in DIETS vials, flies were transferred to new DIETS vials every 24 h, while normal vials were changed on alternative days. Flies were then transferred to 1 % agar starvation vials to measure starvation sensitivity. Agar vials were changed every 24 h, and dead flies were counted.

F. Gut lipid Oil-Red-O staining

For midgut-deposited fat droplet staining, the fly's abdomen was dissected in 1X PBS with fine forceps (55-INOX, FST) to remove the gut. Gut tissues were fixed in 4 % paraformaldehyde (prepared from pre-dissolved solution) (Sigma-Aldrich: P6148-500G) for 20 minutes at room temperature on a rotary shaker. Dissected guts are then rinsed twice for 10 minutes each with 1X PBS to remove the fixative. Fixed guts were then incubated in Oil-Red-O stain for 30 minutes. Oil-Red-O stains were prepared by dissolving 10 mg Oil-Red-O (powder) in 10 mL isopropanol (0.1 % Oil-Red-O). A 6:4 ratio of 0.1 % Oil-Red-O in isopropanol to 4 mL distilled water was prepared and passed through a 0.22 μm syringe filter (Merck: Z260479) before use. The stained gut tissues were rinsed twice in distilled water for 10 minutes each. Stained guts were then mounted on a glass slide, and images were captured using an upright Nikon fluorescent microscope (Nikon Eclipse 80i) with a 20X objective (Plan Fluor 20X, 0.50 NA Ph1 DLL ∞/0.17 WD 2.1).

G. RNA extraction and qPCR

After habituation on CD for 2 days, male flies were transferred on either 20 % HFD or CD for 1 and 4 days in DIETS vials and then flash-frozen and stored at −80 °C. For RNA extraction, 30 flies were vortex-decapitated, and heads were collected on dry ice, lysed in Trizol (Invitrogen: 15596026), and total RNA was extracted using a Zymo Direct Zol kit (Cat No: R2051), and RNA was purified using a Zymo RNA Clean and Concentrator kit (Cat No: R1013) as per the manufacturer's instructions.

After RNA extraction, cDNA was prepared using a High-Capacity cDNA Reverse Transcription kit (Thermo- Fisher Scientific, 4368814). qPCR was performed using KAPA SYBR FAST Universal (Sigma Aldrich, KK4602) on a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, 4485691). Fold change for each gene was determined using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). The primers used were as follows: For RPL32 (endogenous control: FBgn0002626): RPL32-F: CGCCACCAGTCGGATCGATAT and RPL32-R: CATGTGGCGGGTGCGCTTGTT. For TotA (FBgn0028396), TotA-F: CTCTTATGTGCTTTGCACTGCTG and TotA-R: TTTTGGAGTCATCGTCCTGGG. For Cyp4e3 (FBgn0015035), Cyp4e3-F: CCCGATGTCCAGAGGAAATTGT and Cyp4e3-R: GCGCTGCGCCTCCTTAATA. For takeout (FBgn0039298), to-F: CAGCGGAACTGGACAGAGTAAC and to-R: GCGCCTTGTCCCCATTGAACA. For Obp83a (FBgn0011281), Obp83a-F: CGAGGACGAGAAGCTCAAATGC and Obp83a-R: CGGATGGACGCAGCCCTTGG.

H. DIETS-arena and video analysis

We developed a flat arena setup, DIETS-arena, with an easily replaceable 3D printed food cup (transparent PLA material) in the centre to enable temporal and spatial analysis of flies along with food consumption measurement. Each arena measured 6.5* 6.5 * 0.4 cm, and the whole setup comprised of six arenas (Fig. S6A; left panel). This design was adapted from a previous study (Charroux and Royet 2020). 0 - 4 day old male flies were sorted under CO_2 anesthesia in groups of 15 - 20 and habituated in DIETS vial with CD for 2 days. Arenas were cleaned with warm water and wiped clean thoroughly with a microfibre cloth. 500 μL of 1 % agar was pipetted down in a ring-form at a distance of 1 cm away from the edge of the central food cup (Fig. S6A; left panel) .

The agar was left to dry for 1 h, and subsequently, arenas were covered with a transparent acrylic sheet. The initial weight of the food cup with CD was weighed and was immediately placed into the slot provided at the centre of the arena to prevent evaporation. To transfer flies, the whole setup was inverted. Food cups of individual arenas were taken out one at a time, flies were transferred from a vial to the arena via a custom-designed funnel, and food cups were placed back immediately to prevent escape. The arena was then placed in the image capture setup (Fig. S6A; right panel). Arenas without flies were maintained alongside as evaporation controls. This experiment was initiated at ZT = 1. Images were captured via Arducam HQ IR-CUT camera with a 6 mm CS lens (Cat. 1251599,from robu.in), connected to a Raspberry Pi 4B. The Libcamera package was used to capture time-lapse images at 0.2 fps. The captured images were then undistorted using the Computer Vision Toolbox in MATLAB R2022b. These images were later compressed into videos of 10 fps using ImageJ (version 1.53t). A previously published MATLAB code (Charroux and Royet 2020) was modified to quantify the number of flies in a defined area.

To determine the number of flies on or near the food cup, we defined a circular zone around the cup ($Food_{ROI}$), at a distance of 90 pixels from the centre of the food cup. This figure was a summation of the longest male body length (40 pixels: data not shown) and the food cup radius (50 pixels; see Fig. S6A). As it sometimes proved difficult to tell flies apart in the $Food_{ROI}$, especially when many were present, we applied an inverted logic to determine the number of flies in each captured frame outside the $Food_{ROI}$ in each arena. This ultimately allowed us to calculate the % occupancy of $Food_{ROI}$ per frame as :

% occupancy of $Food_{ROI}$ *= {(Total number of flies in an arena - number of flies detected outside* $Food_{ROI}$*)/Total number of flies in an arena} * 100*

I. Data plotting and statistics

GraphPad Prism version 8.3.1 was used for all the statistical analyses performed. All data points are represented as a scatter plot with error bars representing the mean with a 95 % confidence interval (CI). Estimation statistics, including effect sizes, margin of error (MOE) and relevant comparisons between groups performed for each experiment are reported in Supplemental Table 2 (ST2). For all figures, unless mentioned, effect size is calculated as the mean difference between two groups. This represents the magnitude of difference between two groups. The effect size is represented as the dot in the spread. The two black lines that extend from the effect size dot denote the 95% CI and is a measure of precision of the data. To generate the CI, around 5000 bias-corrected and accelerated (BCa) bootstrap resampling was done and the effect size of each resample was plotted to generate the CI arms. The magnitude of one arm of the confidence interval width represents the MOE. The effect size for Fig. 1E and 2A was measured by the standardized mean difference, Hedge's g. Hedge's g is the difference in mean of two groups, standardized over the pooled standard deviation of the two groups, with a correction applied for small (~ less than 20) group sizes (Turner and Bernard 2006). All estimation / delta plots were generated using an online website: www.estimationstats.com (Ho et al. 2019). We extracted data for EX-Q and CAFE from (Wu et al. 2020)0, Fig. 3C, using WebPlotDigitizer (automeris.io/WebPlotDigitizer).

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Contributions

G.D. and M.R.T. and P.C. conceived the design of DIETS assay. G.D. conceived the DIETS-arenas assay. S.S. built the DIETS-arenas and developed/managed/modified the code for DIET-arena. Initial trials and design refinement was done by G.D, M.R.T, P.C, S.S and B.P. R.S.P.Y performed qPCR experiments and analysed the data with PA. All experiments, data analysis and plotting in the manuscript was performed by G.D, 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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