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Post-fasting olfactory, transcriptional, and feeding responses in Drosophila

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ABSTRACT

The sensation of hunger after a period of fasting and of satiety after eating is crucial to behavioral regulation of food intake, but the biological mechanisms regulating these sensations are incompletely understood. We studied the behavioral and physiological adaptations to fasting in the vinegar fly (*Drosophila melanogaster*). Here we show that both male and female flies increased their rate of food intake transiently in the post-fasted state. Although the basal feeding rate was higher in females than males, the magnitude of the post-fasting feeding response was the same in both sexes. Flies returned to a stable baseline feeding rate within 12 h after return to food for males and 24 h for females. This modulation in feeding was accompanied by a significant increase in the size of the crop organ of the digestive system, suggesting that fasted flies responded both by increasing their food intake and storing reserve food in their crop. Flies demonstrated increased behavioral attraction to an attractive odor when food-deprived. Expression profiling of head, body, and chemosensory tissues by microarray analysis revealed 415 genes regulated by fasting after 24 h and 723 genes after 48 h, with downregulated genes outnumbering upregulated genes in each tissue and fasting time point. These transcriptional changes showed rich temporal dynamics and affected genes across multiple functional gene ontology categories. These observations suggest that a coordinated transcriptional response to internal physiological state may regulate both ingestive behaviors and chemosensory perception of food.

1. Introduction

To ensure survival, animals including humans must regulate their behavioral response to environmental stimuli according to their internal state. For feeding behavior, nutritional status is a key determinant of how food is perceived and in the decision on whether and how much to eat. Behavioral and imaging studies in vertebrates suggest that sensory modalities, such as vision and taste, are modified by nutritional status. For example, normal-weight human subjects rated visual food stimuli as significantly more pleasant when they had been fasted than under normal eating conditions [1]. Functional magnetic resonance imaging studies revealed that fasted human subjects showed stronger activity in taste areas of the insula and adjacent dorsolateral prefrontal cortex as well as in visual areas of the inferior occipito-temporal cortex [1], while subjects showed significantly decreased activity in taste centers after two days of overeating [2]. Another study demonstrated that rodents showed a significant increase in olfactory sensitivity after fasting [3], suggesting a role for the olfactory system in modification of eating behavior according to nutritional status. However, the genes and neuronal circuits underlying these changes in sensory perception of food in different nutritional states remain poorly understood in any animal.

Classical studies in the blowfly, *Phormia regina*, by Dethier and colleagues elucidated the features and modulation of insect feeding behavior [4]. Many of these behaviors are conserved in the vinegar fly *Drosophila melanogaster* [5], a convenient genetic model organism for studying the effect of genes on behavior [6]. Previous studies in *Drosophila* looked at proxy measures for food intake, such as frequency of extension of the proboscis towards sucrose, rate of defecation, and accumulation of dyed food in the crop, a food storage organ [5,7], and showed phenotypic changes that were dependent on the nutritional state of the fly. These studies indicated that flies are capable of regulating their feeding behavior, but the assays did not allow for precise quantification of the effect of hunger on food intake.

Like mammals, *Drosophila* regulate their feeding behavior according to nutritional status [8]. Many of the known genetic modulators of nutrient sensing in mammals are conserved in *Drosophila*. For example, insulin signaling is a key component of the physiological response to nutritional status in both mammals and flies [9]. Neuropeptide

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regulators of feeding behavior are also conserved in *Drosophila*, including fly homologues of neuromedin U and neuropeptide Y [10–13]. Neural signaling mediated by short neuropeptide F (sNPF) was recently shown to increased olfactory-mediated food seeking behavior after fasting in the fly [14]. In this study, fasting was shown to induce increased sNPF receptor signaling, which in turn increased pre-synaptic activity in *Drosophila* olfactory neurons [14].

To identify additional genes that may regulate responses to fasting, we quantified changes in *Drosophila* feeding behavior after fasting. We adapted a previously described behavioral assay, the CAFE [15], for quantitative studies of post-fasting food intake. Flies that were food-deprived subsequently consumed more food, and at a faster rate, than flies that were fed *ad libitum*. We next examined the temporal dynamics of this hunger-driven increase in food intake and found evidence for a post-fasting feeding response in both male and female flies. We also found that male flies showed an increased response to an attractive odor after fasting without any changes in peripheral olfactory sensitivity. Finally, we carried out whole-genome transcriptional profiling of four tissue types—head, body, antenna, and proboscis/maxillary palp—to reveal several hundred genes whose levels were significantly modulated by fasting.

2. Methods

2.1. Drosophila stocks

Drosophila stocks were maintained on conventional cornmeal–agar–molasses medium under a 12 h light:12 h dark cycle at 25 °C, where lights on corresponded to 9 am. The Canton-S strain was used as wild type control for behavior, electrophysiology, and for the microarray experiments.

2.2. CAFE assay

Capillary feeder (CAFE) assays were modified from Ja et al. [15]. The CAFE chamber consisted of an empty wide polystyrene vial (Fisher AS-519) with a cotton acetate plug moistened with deionized water at the bottom of the vial for humidity. The top of the vial was plugged with a size 5.5 one-hole black rubber stopper (VWR product number 59581-265) into which a 200 µl pipette tip was inserted. A 5 µl glass capillary (VWR 53432-706) was inserted through the pipette tip, which was trimmed by a razor blade to an opening sufficient to fit the glass capillary, and food was delivered through the glass capillary. Liquid food consisted of 10% (w:v) sucrose (Fisher), 5% (w:v) yeast extract (Fisher BP1422-500), unless otherwise noted, with 40 µl of green food coloring (McCormick) added to every 800 µl of food to enhance visibility of the liquid meniscus in the capillary. Fasted flies had access to a capillary filled with deionized water. Flies were allowed free access to food in the CAFE for two days prior to the first measurement. Five male flies were used in each CAFE but data were plotted as µl food consumed per fly. CAFE assays were carried out under a 12 h light:12 h dark cycle at 25 °C and 70% relative humidity. The phase relationship of the CAFE assay to the light:dark cycle was such that time zero of the CAFE corresponded to lights on or Zeitgeber time (ZT) 0.

The *increase in intake per hour* (IIPH) was calculated as $IIPH = (C_{h_k} - C_{h_{k-1}})/(h_k - h_{k-1})$, where $h_2 = 6$, $h_4 = 12$, $h_5 = 24$ and C_{h_k} is the cumulative food consumption at time h_k. Temporal patterns of the CAFE experimental data were modeled using linear mixed-model effects with a random intercept for each sample and appropriate fixed effects factors. An autoregressive process of order 1 was added to model the autocorrelation structure of the time data. In the case of the variable IIPH, the correlation structure used was compound symmetric. In all cases, the best model was selected based on the AIC/BIC criterion. In the case of feeding response to 5% sucrose and 5% yeast in male flies (Fig. 1B), a model with fixed effect Fasting Status (Pre-fast/Post-fast) and Hours was considered. To model the response to 10% sucrose and 5% yeast (Fig. 1C), a factor Sex (Female/Male) was added to the model, in addition to Fasting Status and Hours. To model the Increase in consumption after feeding (Post-fasting minus Pre-fasting) (Fig. 1D), the factors Hours and Sex were considered as fixed effect. In the case of food consumption on fed and fasted flies over multiple days (Fig. 2B) a model with fixed effect Group (Group 1/Group 2) and Hours was considered for each Day. The same fixed factors were considered while modeling IIPH (Figs. 1E and 2C).

2.3. Crop measurements

Flies were reared as described earlier and placed in a CAFE assay three days post adult eclosion. After three days of continuous access to liquid food (10% sucrose plus 5% yeast extract), male flies were either fed or fasted in the CAFE for 24 h. Following this experimental period, a capillary containing 10% sucrose plus 5% yeast plus 0.02% fluorescein isothiocyanate (FITC; Sigma-Aldrich) was introduced into the CAFE and flies were permitted to feed for 3 h. Flies were then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) + 0.1% Triton for 1 h, then washed for 20 min, three times, in PBS. Dissected crops were visualized with a Zeiss LSM510 confocal microscope to visualize the size of the crop in the two treatment groups. Different confocal settings were used to visualize the crops to obtain the best quality images for quantifying crop diameter. Crop diameter was measured by placing the digital scale bar available in the LSM510 confocal software across the widest portion of the crop in each sample.

2.4. Olfactory trap assay

Flies (aged 3–5 days post eclosion) were fed or fasted for 0, 12, 24, or 48 h before being placed in a two-choice behavior chamber modified from the one described by Ditzen et al. [16]. Two round holes (2.2 cm diameter) were punched along the midline into a 15 cm Petri dish at equal distance (3 cm) from the rim. Rigid cellulose acetate plugs perforated with pipet tips (1 ml) connected the Petri dish with two empty wide polystyrene vial (Fisher AS-519) with a wet cotton acetate plug at the bottom of each vial for humidity. Each trap contained a small piece of filter paper (approximately 3 cm × 0.5 cm; Fisher Scientific 09-795F) with 10 µl of either deionized water or 3-methyl-thio-1-propanol (Sigma-Aldrich; CAS 505-10-2). Flies were lightly anesthetized with carbon dioxide and placed into the large Petri dish, from which they could enter either trap. After 24 h, the number of flies that entered either trap was counted. This number was divided by the total number of flies that was placed in the Petri dish at the start of the experiment, and the percentage of flies entering each trap was thus calculated. 30–50 male flies were used in each trap, with at least five replicates used for each comparison. Significance was assessed using a two-way ANOVA with the factors Hours and Trap (Odor/Water).

2.5. Single sensillum recordings

Flies were aged for 5 days post eclosion before recordings. One group was fasted for 24 h on wet cotton, and one group was given free access to fly food before recording. Single sensillum extracellular recordings of male flies were performed as described [16,17]. Briefly, activity of olfactory sensory neurons was recorded using a 10x AC probe connected to an IDAC-4 amplifier. 3-methyl-thio-1-propanol (Sigma-Aldrich; CAS 505-10-2) was obtained at high purity and diluted (v/v) in paraffin oil as specified in Fig. 3C–D. One filter paper strip (3 × 50 mm) imbued with 30 µl of the desired odor dilution was inserted into a glass Pasteur pipette. Charcoal purified air was delivered by a CS-55 stimulus air controller (Syntech, Kirchzarten, Germany) through the pipette to the fly antennae for 1 s. Prior to recordings, air was forced through the pipette for 1–3 s to remove dead space in the

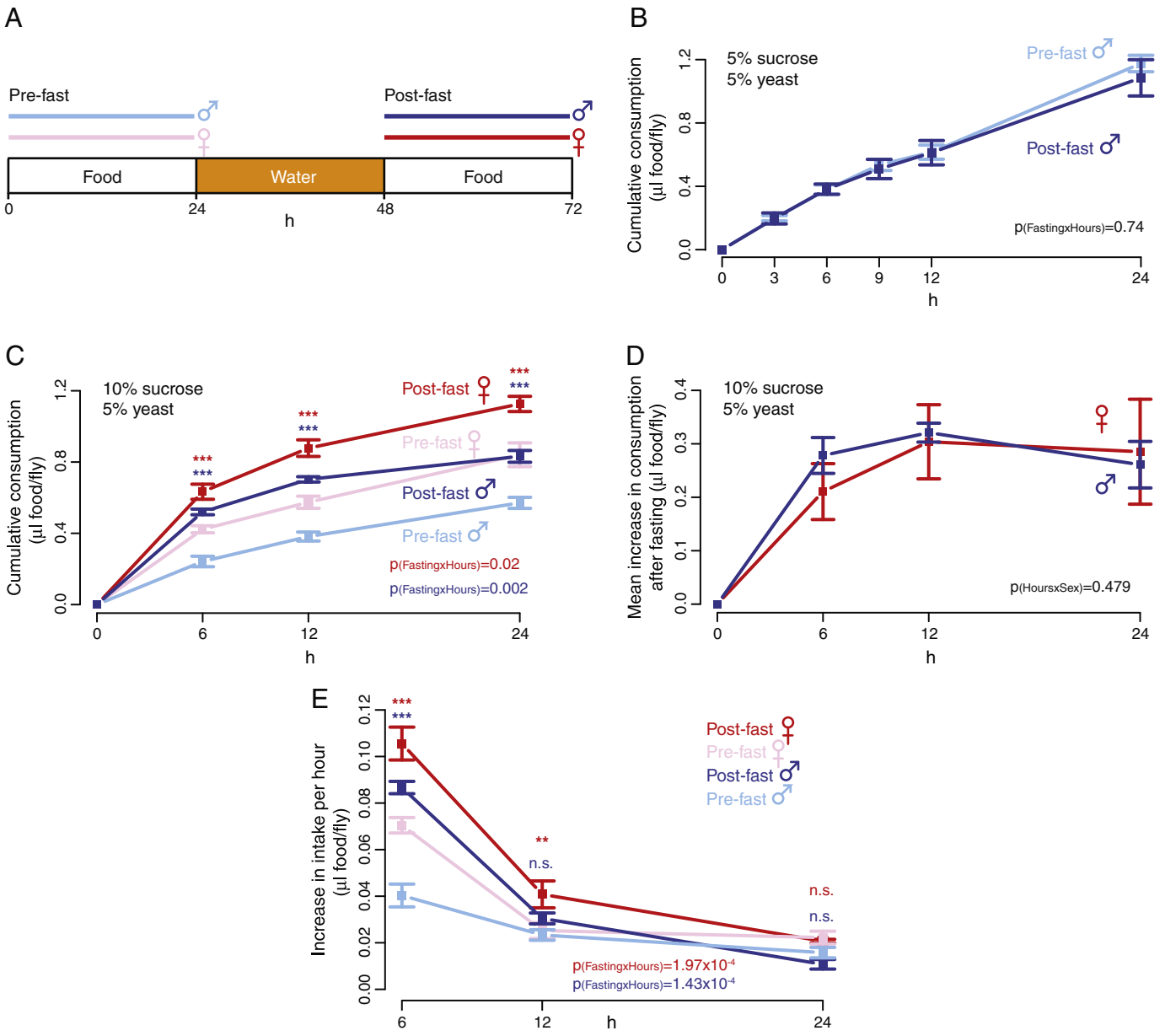

Fig. 1. Optimizing the CAFE to measure post-fasting feeding response. (A) Timeline of feeding assay to measure post-fasting feeding response. Both the sex of the fly and the feeding state are color-coded. (B) Food intake over 24 h in a CAFE assay of male flies that were fed a mixture of 5% sucrose and 5% yeast n = 6–10 CAFE assays. (C) Food intake over 24 h in a CAFE assay of female and male flies that were fed a mixture of 10% sucrose plus 5% yeast. n = 10 CAFE assays. D) Mean increase in food consumption after fasting for male and female flies derived from Fig. 1C. (E) Increase in food intake per hour for male and female flies before and after fasting derived from Fig. 1C. All data in (B–E) are plotted as mean ± SEM. Statistical comparisons in (C) and (E) were between a given sex post-fasting vs. pre-fasting and are color-coded (female: red; male: blue). n.s. $p > 005$; $^{**}p < 0.01$; $^{***}p < 0.001$.

odor delivery system. The odor was allowed to equilibrate in gas phase for at least 15 s before application. Each odorant pipette was used at most three times and no more than three sensilla were tested per animal. The ab5 sensillum was identified by its size, location, and responsiveness to its preferred odorants [18].

Data were collected using Autospike (Syntech) and analyzed by custom spike sorting algorithms [16]. Since spikes from the A and B cells cannot be differentiated in the ab5 sensillum, spikes from these neurons were grouped together for analysis. Based on previous data, the ab5A neuron does not respond to 3-methyl-thio-1-propanol, a selective agonist for the ab5B neuron. Spike trains were grouped into 200 ms bins and responses were calculated by subtracting the average spontaneous activity 15 s before odor application from the activity during the first 600 ms after odor delivery. Dose–response curves were fitted in Origin-Pro 8.0 using the Hill equation and were compared for significance with an F-test.

2.6. Microarray analysis

Changes in *D. melanogaster* gene expression in fasting flies 24 h and 48 h after withdrawal from feeding were assessed using microarray analysis with whole genome arrays from Affymetrix (*Drosophila* 2.0). Four tissue groups were collected from flies that had been fasted for 0 h, 24 h, and 48 h: head (minus chemosensory organs), antenna, maxillary palp plus proboscis, and body (minus the head). Typically five but at least three biological replicates were collected from each tissue group per time point.

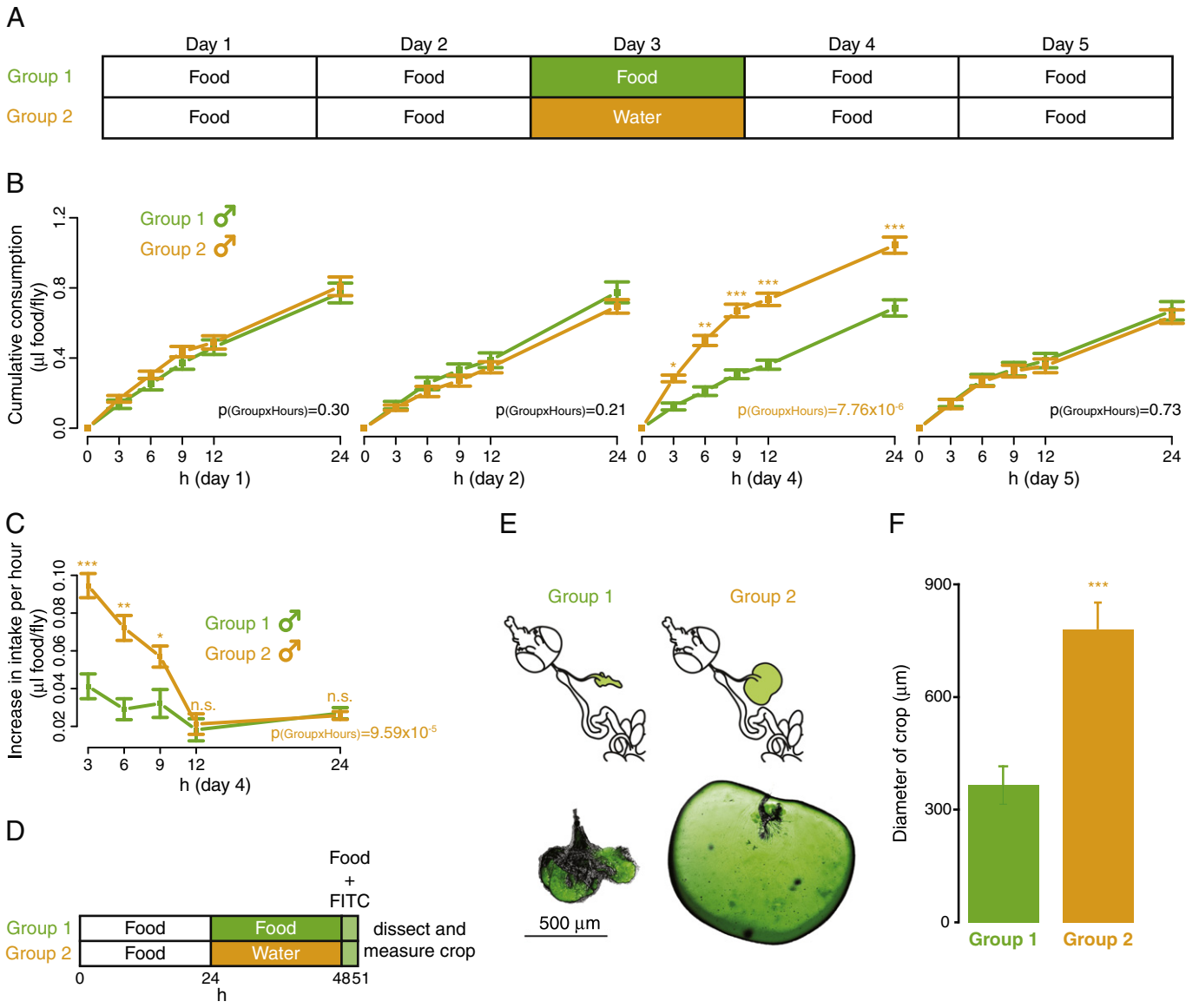

Fig. 2. Food consumption of fed and fasted flies over multiple days in the CAFE. (A) Schematic of CAFE experiment across 5 experimental days. (B) Food intake per fly is plotted at 3, 6, 9, 12, and 24 h after the start of the assay and is calculated as µl food consumed per fly. n = 10 CAFE assays. (C) Increase in food intake per hour calculated from data for day 4 in Fig. 2B. (D) Schematic of crop measurement experiments. (E) (Top) Schematic diagram of insect gastrointestinal system, showing the difference in relative size between an empty and a full crop. Reproduced with permission from Melcher et al. [11]. (Bottom) Representative confocal images of crops from fasted or fed flies. Scale bar = 500 m. (F) Crop diameter measured in fasted and fed flies (***$p<0.001$; n = 9 crops). All data in (B, C, F) are plotted as mean ± SEM.

Male flies (aged 3 days post eclosion) were separated into 3 groups (Fig. 4A) and either kept on fly food or fasted. The first group was fasted immediately and dissected 24 h later. The second group was fasted immediately and dissected 48 h later, and the third group was continuously fed and dissected 72 h later. This third continuously fed group comprised the 0 h fasted set of animals (Fig. 4A). The flies were kept at constant temperature and humidity (25°C, 70% humidity) under a 12 h light:12 h dark cycle, and all dissections were carried out at from about 11 am–1 pm (ZT2-4) to control for circadian effects on gene expression. We staggered the three groups according to this schedule because the dissections are time-consuming and it was not possible to dissect all three groups on the same day, while also staying within this small range of ZT.

RNA was extracted using the RNEasy kit (Qiagen) and RNA quality was assessed by visual inspection of electropherograms produced by the Agilent 2100 Bioanalyzer, using the Pico analyzer assay. cDNA was synthesized, linearly amplified, and labeled using the commercially available Ovation kit. Probe production and array hybridization was carried out by the Rockefeller Gene Array Core Facility.

Microarray analysis was conducted in R (www.r-project.org) using Bioconductor packages (www.bioconductor.org). Harshlight [19] was used to evaluate the quality control (QC) of the images along with classical Affymetrix QC measures. Expression values were obtained by using a GCRMA algorithm. Probesets with SD<0.1 and expression values (in $\log_2$-scale) smaller than 3 in all samples were excluded from the analysis. In our experimental design, observations for each tissue and time were derived within 6 different pools of flies. To identify which genes significantly changed with fasting in each fly tissue, a repeated measures ANOVA model with factors [tissue] (Antenna/Body/Head/Palp and Proboscis), [time] (0, 24, 48 h fasting) and block factor [pool] was considered. Model estimation and hypothesis testing was carried out using the *limma* framework

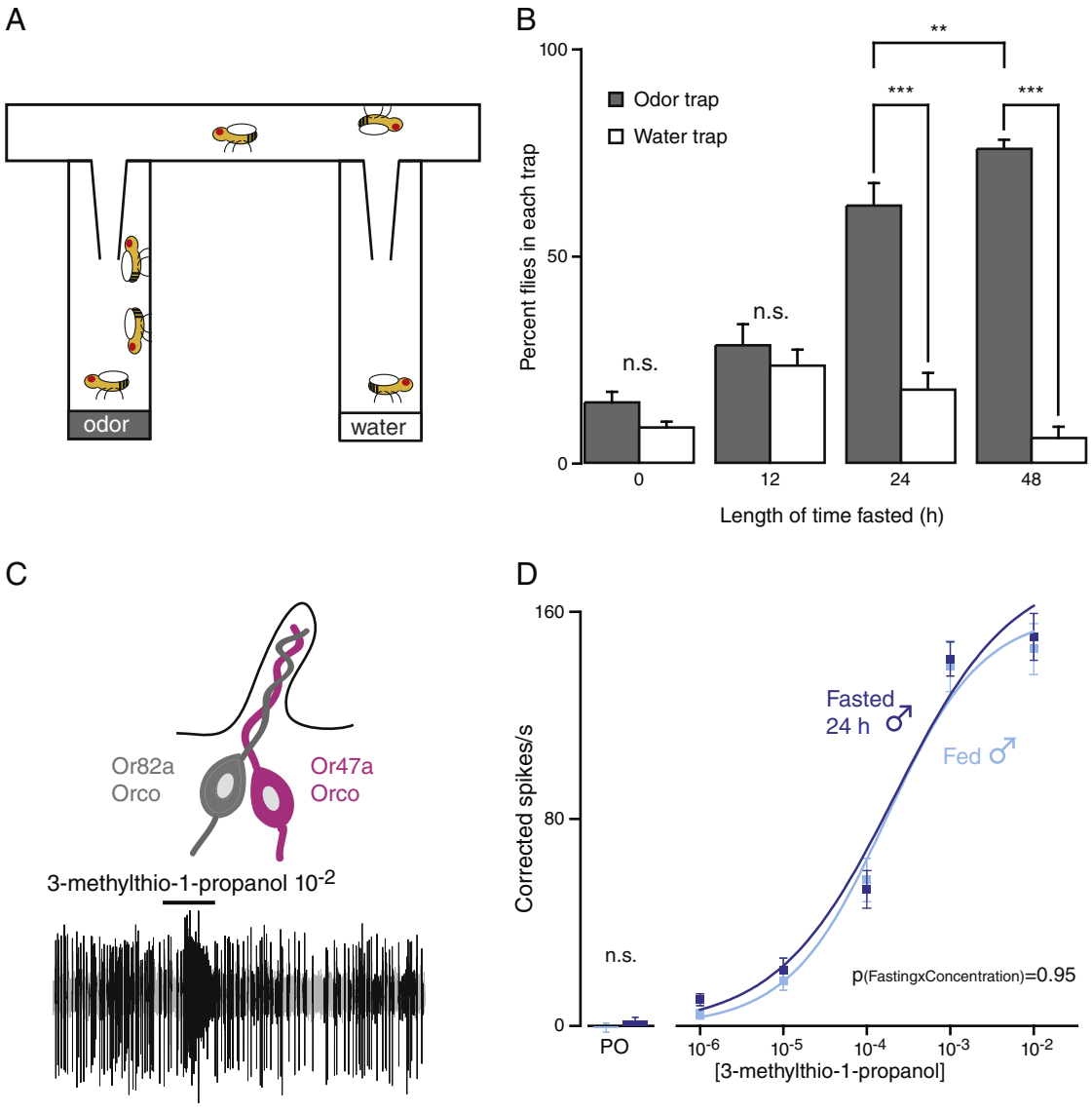

Fig. 3. Attraction to odor increases with fasting. (A) Schematic of two-choice olfactory trap assay to test attraction to odor after fasting. (B) Per cent flies entering either the odor trap containing 3-methylthio-1-propanol (gray bars) or the water trap (white bars). n = 6-16 olfactory traps per time point. **p<0.01; ***p<0.001. (C) (Top) Schematic of *Drosophila* ab5 single sensillum recordings. (Bottom) A representative trace of the response of ab5 sensillum to 3-methylthio-1-propanol, where the horizontal black bar represents the 1 s period of odor delivery. (D) Dose–response curves of *Or47a* ab5B olfactory sensory neuron responses to 3-methylthio-1-propanol in fed and fasted flies. n = 9 sensilla. All data in (B, E) are plotted as mean ± SEM.

[20]. For each tissue, the moderated *t*-test [21] was used to compare the differences with baseline. P-values were adjusted for multiple hypotheses using the Benjamini–Hochberg procedure, which controls the False Discovery Rate (FDR). Genes with FDR<0.01 and fold change larger than 4 were considered differentially expressed genes. Hierarchical clustering with Pearson correlation and average agglomeration method was used to find patterns of behavior of genes with significant change at any time point. The optimal number of clusters was calculated using simulations using the *pvclust* package,

Complete lists of differentially expressed genes regulated by fasting in all four tissues samples are available as Excel files in the Supplementary Materials (Supplementary Tables 1–4). Raw microarray data are available in the NCBI GEO Gene Expression Omnibus (Accession number GSE27927).

Functional annotation and analysis of gene ontology enrichment in Tables 2, 3 was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) program, freely available through the NIH [22].

3. Results

3.1. Flies displayed a robust post-fasting feeding response

We asked whether food intake behavior in flies was affected by feeding status, and if so, whether we could precisely quantify the temporal dynamics of food intake after fasting. Our goal was to establish a robust and precise assay to measure food intake behavior in post-fasted flies to aid in future genetic studies. We used the CAFE apparatus and liquid food developed in the Benzer laboratory, in which flies are fed 5% sucrose plus 5% yeast [15]. Three day-old male flies were placed in the CAFE and were fed *ad libitum* for 2 days while adapting to the CAFE. We then measured food intake at regular intervals before and after 24 h of fasting to compare food intake behavior in fed versus post-fasted nutritional states (Fig. 1A). We found that when flies were fed 5% sucrose plus 5% yeast, there was no change in food intake in the post-fasted state [p(Fasting × Hours) = 0.74; Fig. 1B]. However, when flies were fed a diet of 10% sucrose plus 5%

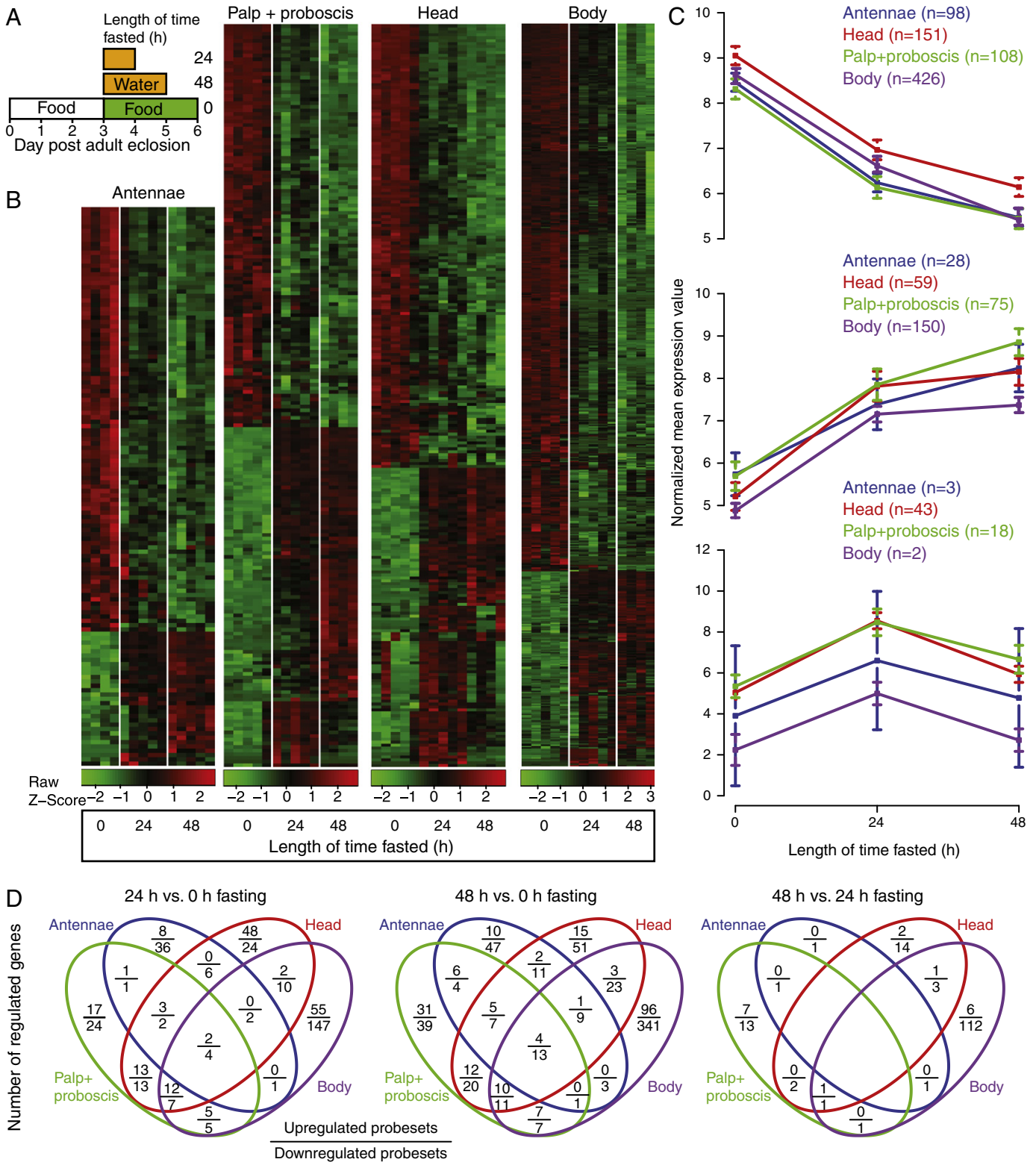

Fig. 4. Microarray identifies genes regulated by fasting in *Drosophila*. (A)Time line of sample preparation for the microarray experiments. (B) Heat maps of biological replicates of Affymetrix gene expression profiles from four different *Drosophila* tissues at 0, 24, and 48 h of fasting. Raw Z-scores are displayed with the color scale at the bottom of each heat map with downregulated genes in green and upregulated genes in red. (C) Dynamics of mean expression level of all probe-sets in all four tissues that are downregulated (upper panel), upregulated (middle panel), or show a peak at 24 h fasting (bottom panel). Tissues are color-coded and numbers of probesets in each plot are indicated at the right. (D) Venn diagram of probe-sets up-regulated (upper number) or down-regulated (lower number) in four different tissue groups at different times of fasting.

yeast, both male and female flies showed a significant increase in food intake in the post-fasted state [p(Fasting × Hours) = 0.02 for male flies; p(Fasting × Hours) = 0.002 for female flies] (Fig. 1C). Although females consumed a greater amount of food than males both before and after fasting, male and female flies displayed the same increase in rate of food consumption after fasting [p(Hours × Sex) = 0.479);

Table 1
Number of probesets (genes) differentially expressed after fasting.

	24 h fasting		48 h fasting	
	Upregulated	Downregulated	Upregulated	Downregulated
Antennae	14 (14)	52 (51)	28 (27)	95 (93)
Palp + proboscis	53 (49)	56 (53)	75 (66)	102 (93)
Head	80 (72)	68 (65)	52 (50)	145 (136)
Body	76 (72)	176 (167)	121 (108)	408 (384)
All tissues	166 (152)	282 (265)	202 (179)	587 (547)
All differentially expressed	448 (415)		786 (723)	

(FDR<0.01, fold-change>4).

Fig. 1D]. The absolute difference in post-fasting food intake between male and female flies could be entirely attributed to their baseline differences in pre-fasting food intake (Fig. 1C). We explored the temporal dynamics of the return to baseline feeding after fasting and found that while male flies returned to baseline levels by 12 h, female flies showed slightly elevated feeding at 12 h and a return to baseline by 24 h (Fig. 1E).

To further explore post-fasting feeding dynamics in flies fed 10% sucrose plus 5% yeast, we measured food intake in male flies at smaller regular intervals over the course of five days, with the third day consisting of access to food (Group 1) or water only (Group 2) (Fig. 2A). Flies that were given continuous access to food did not vary their food intake over the course of the entire 5 day experiment [$p(\text{Days} \times \text{Hours} = 0.1903$; Fig. 2B). However, flies that were fasted on day 3 (Group 2) showed a significant increase in food intake on day 4 [$p = 7.76 \times 10^{-6}$; Fig. 2B]. We measured the effect of fasting on the rate of food consumption and found that flies increased their rate of food intake immediately after 24 h of fasting, and the rate of food consumption returned to pre-fasting levels after 12 h of reintroduction to food (Fig. 2C). Therefore, the CAFE assay using 10% sucrose plus 5% yeast is a robust method for measuring the magnitude and temporal dynamics of post-fasting food intake behavior.

3.2. Fasted flies stored more food in their crop than flies that had free access to food

Insects possess a food-storage organ, the crop, which is small and deflated under *ad libitum* feeding conditions. When flies are food deprived, however, subsequent food intake leads to qualitatively larger crops, suggesting that there is an increase in meal volume following fasting [5]. We set out to quantify the increased size of the crop in post-fasted flies. We placed flies in the CAFE, with Group 1 having free access to food, and Group 2 being fasted for 24 h (Fig. 2D). Both groups were then fed fluorescein-labeled food for 3 h before their crops were dissected and measured (Fig. 2D and E). We found that fasted flies had a crop diameter that was twice that of flies that had continuous, free access to food (Fig. 2F). This is consistent with the result in Fig. 2B showing that post-fasted flies consumed significantly more food than flies fed *ad libitum* in the first 3 h after reintroduction to food.

3.3. Behavioral attraction to an odor increased with fasting

Since virtually all organisms modulate food-seeking behavior based on nutritional status, we asked whether fasting affects olfactory-driven responses to attractive odor in *Drosophila*. Flies show attractive and repulsive responses to different odors [23], including attraction to the attractive odor 3-methylthio-1-propanol, which is a ligand for the odorant receptor Or47a [16]. To measure the effect of nutritional status on attraction to this odor, we used a two-choice olfactory trap assay (Fig. 3A) in which flies may enter either a trap containing the odor or one containing water alone. Flies that were fasted for 24 or 48 h entered the odor trap significantly more often than flies that had not been fasted or were fasted for only 12 h ($p = 6.88 \times 10^{-13}$ for 24 h; $p = 2.22 \times 10^{-16}$ for 48 h; Fig. 3B). Moreover this difference increased with increased fasting. Flies that had been fasted for 48 h were significantly more attracted to odor than those that had been fasted for 24 h ($p = 0.0033$; Fig. 3B). Therefore, behavioral attraction to an odor was strongly modulated by nutritional status, with increased food deprivation leading to increased attraction to a food-like odor.

This change in olfactory response could be due to increased sensitivity of peripheral olfactory sensory neurons, or to changes in central nervous system responses in the fasted state. To distinguish

Table 2
Genes down-regulated after 24 h fasting.

Gene ontology	# of genes			
	Head	Body	Antenna	Palp + proboscis
Defense response	16	13	10	7
Immune response	13	10	9	6
Innate immune response	11	9	7	4
Oxidation reduction	16	44	12	
Response to bacterium	9	7	6	
Fatty acid metabolic process	4	7		
Antibacterial humoral response	5		3	
Antimicrobial humoral response	6		4	
Cell killing	2		2	
Defense response to bacterium	7		4	
Defense response to fungus	3		3	
Humoral immune response	6		4	
Killing of cells of another organism	2		2	
Response to fungus	3		3	
Aminoglycan catabolic process	3			
Aminoglycan metabolic process	5			
Carbohydrate catabolic process	4			
Glycosaminoglycan catabolic process	3			
Glycosaminoglycan metabolic process	3			
Peptidoglycan catabolic process	3			
Peptidoglycan metabolic process	3			
Polysaccharide catabolic process	3			
Polysaccharide metabolic process	5			
4-hydroxyproline metabolic process		4		
Anion transport		7		
Carboxylic acid biosynthetic process		6		
Cellular amino acid derivative metabolic process		6		
Cellular lipid catabolic process		4		
Fatty acid biosynthetic process		5		
Hormone metabolic process		6		
Juvenile hormone metabolic process		3		
Lipid catabolic process		5		
Melanin biosynthetic process		3		
Melanin metabolic process		4		
Organic acid biosynthetic process		6		
Organic anion transport		5		
Peptidyl-proline hydroxylation to 4-hydroxy-L-proline		4		
Peptidyl-proline modification		4		
Proteolysis		61		
Regulation of hormone levels		6		
Response to UV		3		
Secondary metabolic process		10		
Sesquiterpene metabolic process		3		
Sesquiterpenoid metabolic process		3		
Terpene metabolic process		3		
Terpenoid metabolic process		3		
Amine biosynthetic process			3	
Response to pheromone			3	
Cognition				7
Sensory perception				7
Sensory perception of Chemical stimulus				7
Sensory perception of smell				4

(p<0.05).

Table 3
Genes up-regulated after 24 h fasting.

Gene ontology	# of genes			
	Head	Body	Antenna	Palp + proboscis
De novo' IMP biosynthetic process	2	2		2
IMP biosynthetic process	3	3		3
IMP metabolic process	3	3		3
Nucleoside monophosphate biosynthetic process	3	4		3
Purine nucleoside monophosphate biosynthetic process	3	4		3
Purine nucleoside monophosphate metabolic process	3	4		3
Purine ribonucleoside monophosphate biosynthetic process	3	4		3
Purine ribonucleoside monophosphate metabolic process	3	4		3
Ribonucleoside monophosphate biosynthetic process	3	4		3
Ribonucleoside monophosphate metabolic process	3	4		3
Nucleoside monophosphate metabolic process	3	4		3
Nucleotide biosynthetic process	4	5		
Purine nucleotide biosynthetic process	4	5		
Purine nucleotide metabolic process	4	5		
Purine ribonucleotide biosynthetic process	4	5		
Purine ribonucleotide metabolic process	4	5		
Ribonucleotide biosynthetic process	4	5		
Ribonucleotide metabolic process	4	5		
Long-chain fatty acid metabolic process	2			
Nucleoside monophosphate metabolic process	3			
Regulation of tube length, open tracheal system	2			
Microtubule-based movement		4		
Nucleobase, nucleoside and nucleotide biosynthetic process		5		
Nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process		5		
Phototransduction		3		
Response to extracellular stimulus			2	
Response to nutrient levels			2	
Glycine metabolic process				2

($p<0.05$).

these two hypotheses, we recorded sensitivity to odor in the peripheral olfactory system and asked whether neuronal response to 3-methylthio-1-propanol is increased in flies that have been food deprived. The antenna, the principal olfactory organ of the fly, is covered by sensory sensilla that house the dendrites of olfactory sensory neurons (Fig. 3C). Previous work by Hallem et al. [18] showed that 3-methylthio-1-propanol is primarily detected by olfactory sensory neurons expressing Or47a, which are housed in the ab5 class of olfactory sensilla. We therefore performed extracellular single sensillum recordings in the ab5 sensillum in response to 3-methylthio-1-propanol (Fig. 3C). We measured responses of the Or47a neuron to 3-methylthio-1-propanol, across a range of odor concentrations in fed and in 24 h fasted flies. Electrical activity of Or47a neurons was not significantly different in fed versus fasted flies (F-test, $p = 0.95$; Fig. 3D). This suggests that the increased behavioral attraction to 3-methylthio-1-propanol in fasted flies may be caused by central adaptations affecting olfactory sensitivity, as was recently shown for the Or42b circuit in *Drosophila* [14]. We cannot exclude the possibility that OSNs other than those expressing Or47a also respond to 3-methylthio-1-propanol.

3.4. Microarray expression profiling identified genes whose expression levels are regulated by nutritional status

Having established a robust assay to measure post-fasting feeding responses in flies, we set out to catalogue genes whose expression is modulated by fasting. Some of these genes may represent novel pathways that regulate feeding behavior and the states of hunger and satiety in the fly. Previous studies examined transcriptional changes that were dependent on nutritional status in whole larvae [24] and in adult head tissue [25] and we sought to extend the list of candidate genes by screening adult body, head, and the adult head chemosensory organs comprising the antenna, maxillary palp, and proboscis.

We used a microarray approach to identify gene expression changes after 24 and 48 h of fasting and compared this to expression in fed flies (Fig. 4A). We carried out microarray analysis of fly head, body, and chemosensory organs (Fig. 4B; see Supplementary Tables 1–4 for detailed information for all genes that are differentially regulated by fasting). Across all tissues examined, 415 genes were differentially expressed after 24 h fasting and 723 genes after 48 h fasting (Fig. 4; Table 1; Supplementary Tables 1–4).

As expected, previously described genes whose expression is sensitive to nutritional status were positive hits in our array. A previous study of transcription in the adult fly head found that *fit*, *CG8147*, and *Obp99b* were among the most highly regulated transcripts in food-deprived adult flies [25]. These genes were identified through our arrays as highly regulated by nutritional status. We also found that *sugarbabe*, a zinc finger protein previously found to be regulated by sugar intake in larvae, was regulated by food intake in adult head tissue [24].

Of genes that were regulated by fasting, 18 were shown by the Pankratz lab to be regulated by feeding in larvae [24]. Meunier et al. [26] previously showed that *takeout*, a putative juvenile hormone-like binding protein, shows increased expression with fasting. In our array analysis, we found that *takeout* was significantly regulated in three tissues (head, body, palp + proboscis) after either 24 h of 48 h of fasting when compared with baseline (Supplementary Tables 1, 2, 4). While we did find *takeout* to be highly expressed in the antenna [mean expression values: 14.47 (Antennae), 13.04 (Head), 13.63 (Palp + proboscis) and 10.8 (Body)], its expression was not significantly modulated by 24 or 48 h of fasting in antennae (Supplementary Table S3).

We next compared our array results to those obtained by Fujikawa et al. [25] and found a reasonable correspondence between our results and the previously published work. When we compared our head microarray results to those from Fujikawa et al. [25], 4 upregulated and 16 downregulated genes were present in both studies. This number is small because of the more stringent p-values and larger fold-change applied in our study ($p<0.01$ and 4-fold change). If we apply a less stringent statistical cut-off ($p<0.05$ and 2-fold change), a comparison

of our study and Fujikawa et al. [25] finds 12 upregulated and 28-downregulated genes in common between the two studies. When we used the methodological approach presented by Suárez-Fariñas et al. [27] to look more globally at microarray data in different studies, we found that the Fujikawa list does correlate with our study. If we sort all of our genes by fold change at 24 h of fasting, genes upregulated in Fujikawa et al. ranked at the top of our gene list (Enrichment score ES = 0.6, $p<10^{-4}$), whereas downregulated genes were at the bottom (ES = −0.9, $p<10^{-4}$). Therefore, our study and that published by Fujikawa et al. [25] reach similar overall conclusions for genes regulated by fasting in the fly head. Disagreement among lists of differentially expressed genes is fairly common [28] and can be caused by lab effect, sample size, and/or choice of statistical analysis and in this case the strain of flies used. In conclusion, our study expands this microarray analysis beyond the head as presented in Fujikawa et al. [25] to the body, the antennae, and maxillary palp/proboscis.

Our array analysis detected transcripts that were previously shown to be regulated by feeding state. One exception is that we failed to find any regulation of the sNPFR1 gene, which encodes a feeding-related neuropeptide receptor, in any of the tissue types sampled (data not shown). A recent paper reported strong up-regulation of the sNPFR1 gene in fly antennae after fasting [14]. However, this study examined transcriptional changes after 4 h of fasting, whereas our work studied later stages of fasting. The discordant results may be explained by different temporal profiles of regulation of this receptor gene.

qPCR analysis of three representative genes (*sugarbabe*, *takeout*, *fit*) validated the fasting-induced down-regulation in head gene expression seen in the microarray (data not shown). Gene expression changes could be clustered into three different temporal dynamics (Fig. 4C). Most genes in the four tissues showed decreased levels of expression after 24 h, which was further decreased after 48 h of fasting (Fig. 4C, top). Conversely, a smaller number of genes showing increased expression after 24 h of fasting that continued to increase at 48 h of fasting (Fig. 4C, middle). Finally, a small number of genes showed increased expression after 24 h, but decreased expression after 48 h of fasting (Fig. 4C, bottom).

Many more genes were downregulated (n = 265 at 24 h; n = 547 at 48 h) than upregulated (n = 152 at 24 h; n = 179 at 48 h) across all four tissue types (Fig. 4D; Table 1). By comparing genes that were regulated by feeding status in the four different tissues, we found that each tissue group has its own unique panel of feeding-regulated genes. Few (0 to 13) probe-sets were regulated by feeding in all four tissue groups and also displayed the same temporal dynamics of gene expression after fasting (Fig. 4D).

Certain classes of genes were enriched in our microarray study and this varied with tissue type. Only three categories of genes showed coordinate downregulation in all tissues: defense response, immune response, and innate immune response genes (Table 2). This may reflect a reallocation of the fly's genetic resources away from host defense during the fasted state. Genes involved in protein, carbohydrate, and lipid metabolism were all down-regulated in the fly body after 24 h of fasting (Table 1), most likely as a genetic strategy to conserve energy. In contrast, genes involved in nucleotide biosynthesis were the dominant category upregulated after fasting (Table 2).

4. Discussion

The ability to regulate food-seeking behavior in response to nutritional status is central to animal survival. Using flies, we showed that nutritionally-deprived animals display a modified perception of food stimuli, such that they are more attracted to food. In addition, fasted animals consumed food in larger quantities than satiated animals, a finding that is comparable to the same phenomenon in mammals. We have begun to address the question of how nutrition modulates behavior by establishing robust and quantitative post-fasting behavioral assays in *Drosophila* vinegar flies, and by uncovering genes that are regulated by fasting, some of which may represent genetic mediators of post-fasting behavior.

The development of the CAFE assay by the Benzer lab was a significant advance over past tools used to measure food intake, since it allows for precise, short-term measurements of food intake in real-time [15]. However, the CAFE as described by Ja et al. [15] did not allow for measurement of post-fasting feeding responses because flies fed 5% sucrose plus 5% yeast did not modulate their food intake after fasting. We aimed to define robust, quantitative measures of post-fasting feeding behavior. We found that flies fed a higher concentration of sucrose did show a post-fasting feeding response. In the 24 h following fasting, animals consumed a significantly larger amount of food than under *ad libitum* conditions. Thus, we conclude that a CAFE assay using 10% sucrose plus 5% yeast is suitable for measuring post-fasting food intake, whereas a CAFE assay with 5% sucrose plus 5% yeast produced animals in a constant-state of low level hunger that continuously ingest a maximum amount of food. This conclusion is reinforced by the fact that flies that were fed 10% sucrose plus 5% yeast consumed less food under *ad libitum* conditions than flies that were fed 5% sucrose plus 5% yeast.

There is a debate in the literature over whether the concentration of sucrose in the diet influences *Drosophila* food intake. One study found that flies increase the volume of food consumed as the percent of sucrose in the diet increases [5] . More recently, Carvalho et al. found the opposite result, that flies ingest a smaller volume of food when the concentration of sucrose increases [8]. Our results support the latter finding [8].

In addition to increased consumption in the CAFE, flies also modify their post-fasting behavior through changes in olfactory responsiveness. We showed that fasted flies showed increased behavioral responses to an attractive odor than satiated flies, suggesting that the olfactory system is affected by nutritional status. This phenomenon has been previously documented in the nematode *C. elegans*. Worms normally react to the smell of octanol by initiating backwards movement, but in the absence of food the animal significantly slowed its behavioral response to this odorant [29]. Starvation also increased olfactory adaptation in worms to some odorants, and animals recovered from this effect by re-feeding [30]. In the case of the worm, serotonin is suspected to act as the mediator of a hunger signal, since administration of serotonin mimics feeding in olfactory behavior assays [29,30]. In flies, serotonin enhances sensitivity of the antennal lobe projection neurons under certain conditions [31]. Perhaps serotonin, or a similar secreted molecule, regulates 3-methyl-thio-1-propanol sensitivity in post-starved flies as well. The two-choice olfactory trap assay may be useful for testing whether serotonin or other mutants show an abnormal olfactory response when fasted.

Flies were previously shown to have increased gustatory sensitivity to sugar in the fasted state [26]. This increase in sensitivity was found by electrophysiological recording of taste sensilla on the fly labellum [26], demonstrating that fasted flies have an increase in taste receptor activity in the peripheral gustatory system. We reasoned that increased olfactory attraction to 3-methyl-thio-1-propanol might also be mediated by an increase in activity in peripheral sensory neurons. However, our electrophysiological recordings did not support this hypothesis, since we found no difference in Or47a responses to the odor in the fed or fasted state. While we cannot rule out that olfactory neurons other than those expressing Or47a respond to 3-methylthio-1-propanol, our negative data suggest that increased behavioral attraction in the fasted state may be caused by a more central mechanism, such as increased activity in the antennal lobe or higher brain structures. Indeed, previous work in rodents showed that olfactory bulb activity in response to food odor was modulated by feeding state [32]. A recent paper in the fly described an elegant mechanism by which insulin signaling modulates the expression of sNPFR1, leading to pre-synaptic enhancement of odor responses

selectively in the fasted state [14]. This in turn increased food-seeking behavior in fasted animals.

By carrying out gene expression analysis in flies during satiated and starved states, we were able to identify genes whose expression levels are related to nutritional status. While some of these genes belong to gene classes that one would expect to be controlled by feeding (such as those involved in nutrient metabolism), others are uncharacterized or have an assigned function not obviously related to feeding or behavior. Flies represent an ideal organism in which to conduct further genetic studies to uncover the role of these uncharacterized genes in regulating feeding behavior. The relative ease of conducting RNAi knockdown experiments in *Drosophila*, coupled with a quantitative post-feeding assay, opens the door to future reverse-genetics experiments to identify novel regulators of post-fasting feeding behavior.

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Contributors

S.F.F. designed and carried out all the experiments in the paper with the exception of the single sensillum recordings in Fig. 3C–D, carried out by M.P., and the CAFE experiments in Fig. 1B–E, carried out by C.E.C. M. S.-F. carried out the statistical analysis of all the CAFE, olfactory trap, microarray and qPCR data. L.B.V. and S.F.F. together with M.S-F. conceived and directed the project, interpreted the results, and wrote the paper.

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