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From Microarrays to Behavior: Genes Controlled by Feeding State in Mosquitoes and Flies

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FROM MICROARRAYS TO BEHAVIOR:

GENES CONTROLLED BY FEEDING STATE IN MOSQUITOES AND FLIES

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Across many species, animals carefully regulate their food intake according to their energy needs. They are able to do so through the ability to sense hunger or satiety cues. In vertebrates, these signals are released by the gastrointestinal tract and by adipose tissue, and reach feeding centers in the brain, where they stimulate the release of peptides that modulate feeding behavior (Benarroch, 2010; Berthoud, 2008). Although many of these neuronal populations have been identified in rodent models, the neural circuitry behind behavioral modification of food intake remains largely unknown.

Insects like the blowfly and the locust have classically been used to describe basic features of feeding behavior (Bernays and Chapman, 1974; Dethier, 1976). These animals, as well as vinegar flies and mosquitoes have been shown to modify their feeding behavior according to their internal nutritional status (Edgecomb et al., 1994; Takken et al., 2001). Thus they are good models for examining the question of how this modulation of behavior occurs. Moreover, *Drosophila melanogaster* has been used to study mechanisms of complex behaviors to great effect, and there are ample genetic tools available to study feeding behavior in this organism (Vosshall, 2007).

We set out to identify genes that regulate feeding behavior according to nutritional status. *Anopheles gambiae* mosquitoes were previously shown to display reduced host-seeking behavior for forty-eight hours after taking a blood-meal (Takken et al., 2001). We used whole genome microarrays to look for genes that are regulated in olfactory tissue by blood-feeding, and that therefore might function to modify olfactory driven host-seeking behavior according to nutritional state. We found that two odorant receptor genes are significantly regulated by blood-feeding. These are therefore candidate receptors for ligands that are important for host-seeking.

We then extended our studies to *Drosophila* with the goal of identifying novel regulators of post-fasting feeding behavior. First we defined two stereotypical post-fasting behaviors in flies: increased attraction to food odor, and increased consumption of liquid food. We then looked for candidate genes that regulate these behaviors by looking for transcripts that are regulated by fasting and found that 247 genes in the head are significantly regulated by nutritional status. Finally, we carried out a targeted genetic screen using RNA interference against these candidate genes. We looked for flies that show a defective post-fasting food intake response, and found eleven genes that cause such a behavioral disruption. These genes may represent novel regulators of hunger and satiety in insects, laying the groundwork for future studies of modification of feeding behavior.

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3MTP	3-methyl-thio-1-propanol
AGRP	agouti-related protein
CAFE	capillary feeding assay
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CNS	central nervous system
DILP	*Drosophila* insulin-like peptide
EGFR	epidermal growth factor receptor
FDR	false discovery rate
fMRI	functional magnetic resonance imaging
GPROR	OR in *Anopheles gambiae*
NPF	neuropeptide F
NPY	neuropeptide Y
OBP	odorant binding protein
ODE	odorant degrading enzyme
OR	odorant receptor
OSN	olfactory sensory neuron
POMC	pro-opiomelanocortin
PVR	poliovirus receptor
RIN-1	ras-interacting protein 1
RT-PCR	reverse-transcriptase polymerase chain reaction
sNPF	short neuropeptide F
TOR	target of rapamycin

1 Introduction

1.1 What is feeding behavior?

All organisms require food to survive. However, normal eating is discontinuous, with periods of feeding alternating with periods of non-feeding activities, such as mating or grooming, or sleep (Wiepkema, 1971). Thus feeding competes with other required activities, and animals must prioritize the relative importance of feeding at any given moment to other activities that are required for survival. Normal feeding behavior, or the ability for animals to regulate food-seeking and, consequently, food ingestion, is displayed by virtually all animals.

Patterns of eating vary across species, with some animals, like the giant silkworm, not eating at all as adults, and others, like the locust, eating multiple meals per day (Dethier, 1976). Some animals' food intake is limited by the availability of prey, while others have constant access to food. However, all animals (except perhaps filter feeding fish) experience periods of food intake and periods of abstinence from food; they must regulate their food intake according to their internal nutritional status (de Krom et al., 2009; Uher et al., 2006). In humans, when the drive to eat is no longer tied to the body's need for energy, obesity or eating disorders such as anorexia nervosa can ensue. Therefore, investigations of normal and mutant feeding behavior in model organisms might yield insight into mechanism of human disease.

Under *ad libitum* feeding conditions, animals have constant access to food. It can be useful to study feeding behavior under these conditions because one can observe the frequency and duration of feeding intervals in the absence of a need to "hunt" or seek food (Wiepkema, 1971). Simply put, by giving an animal free access to food, it becomes easier to see whether the animal chooses to eat or to engage in other activities. When flies or rats are observed under *ad libitum* feeding conditions, they consume food in discrete episodes of feeding, or "meals" (Ja et al., 2007; Le Magnen and Tallon, 1966). Thus, to study feeding behavior, one can look at factors that contribute to meal size or meal frequency. The amount of food ingested in a single meal may be influenced by a number of factors, including caloric content of the food (Carvalho et al., 2005; Edgecomb et al., 1994), length of the non-feeding interval (Wiepkema, 1971), energy expenditure during the non-feeding period (de Krom et al., 2009), and food deprivation.

1.2 Neurogenetics of feeding behavior

Feeding behavior is controlled by the interaction between environmental stimuli ,such as the presence of palatable food; nutritional status; energy expenditure; and genetically encoded heritable factors (Rankinen and Bouchard, 2006). These complex signals must be integrated in the brain so that the animal can perform an appropriate behavioral output: to eat or not to eat.

Long periods of non-feeding intervals or food deprivation lead to hunger. Hunger is a subjective feeling that increases during a fasting period and is relieved by feeding (Uher et al., 2006). It is felt more strongly by women than men (Uher et al., 2006), can adversely affect mood (Uher et al., 2006), and most significantly, it leads to increased food consumption. Hunger is relieved by food consumption, which leads to satiety, and therefore to decreased food intake. In vertebrates, much is known about how hunger and satiety are sensed and acted upon by the central nervous system, as reviewed below.

1.2.1 Humoral signals of hunger and satiety in vertebrates

As food enters the gastrointestinal tracts, several signals are generated in the gut which relay nutritional status to the brain. These gut signals are crucial for the control of appetite and the regulation of energy balance. Most known sensory signals originating in the gut are negative regulators of appetite in that they are only activated in the presence of nutrients in the gastrointestinal tract (Berthoud, 2008). The one known exception is ghrelin, the first gut hormone found to increase appetite (Wren et al., 2000), and which may be involved in eating disorders (Monteleone et al., 2008; Takaya et al., 2008). Ghrelin, cholecystokinin (CCK), Glucagon like peptide-1, and peptide YY are gut hormones that can powerfully influence the control of food intake and regulation of energy balance. Other hormones and peptides, such as serotonin, may also be involved. These hormones are released from enteroendocrine cells in the gut mucosa, and can

act on the brain both through the circulation and through primary afferent neurons that terminate in the nucleus of the solitary tract (Benarroch, 2010). More research is needed to know exactly what mechanism is used by each relevant hormone.

In addition to signals arising from the stomach and bowels, leptin and insulin have anorexigenic effects on long term feeding. Leptin is synthesized in adipose tissue in response to fat content (Considine et al., 1996; Zhang et al., 1994). When injected in rodents, leptin inhibits feeding (Jacob et al., 1997; Satoh et al., 1998). Insulin, produced in the pancreas, is similar to leptin in that it also circulates in levels proportional to fat stores, and it also inhibits feeding and leads to increased weight loss (Baura et al., 1993). Circulating messengers like leptin and insulin can reach their receptors in the hypothalamus by diffusing across the third ventricle to the arcuate nucleus (Elmquist et al., 1998).

1.2.2 Mechano- and chemosensory signals of satiety from the stomach

The stomach is a highly innervated organ that, through mechanosensors on vagal afferent nerves, can sense distention because of food intake. However, little is known about the molecular identity of these stretch receptors and of their signal transduction mechanism (Fox, 2006). In addition to its ability to sense stretch, the stomach has also been recently found to contain chemosensors that detect the presence of sweet or bitter foods (Rozengurt, 2006; Sutherland et al.,

2007). This intriguing finding greatly expands our understanding of the concept of "taste" and, more generally, of the role of the alimentary canal in processing food.

1.2.3 Hypothalamic control of food intake

In mammals, the primary brain region that receives hunger and satiety signals and that, in turn, regulates homeostatic food intake is the hypothalamus. Some of the key neuronal populations reside in the arcuate nucleus. They can be divided into two basic groups that have opposite effects on food intake, and that interact with one another. The first group, consisting of neurons in the lateral portion of the arcuate nucleus, contains pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) synthesizing neurons (Kristensen et al., 1998). Leptin is the prototypical hormonal stimulator of POMC/CART neurons (Cheung et al., 1997; Thornton et al., 1997). These neurons send anorexigenic signals that reduce food intake and increase energy expenditure.

The second group consists of neurons in the medial portion of the arcuate nucleus. These neurons are inhibited by leptin and are activated by ghrelin, and they synthesize Neuropeptide-Y (NPY) and Agouti-related protein (AGRP), in addition to GABA (Broberger et al., 1998). Activation of these neurons has an orexigenic effect through numerous pathways. NPY itself seems to inhibit POMC neuron activity (Dietrich and Horvath, 2009). It also acts on NPY receptors in other hypothalamic areas to increase food intake and decrease energy

expenditure.

1.2.4 Mechanosensory signals of hunger and satiety in invertebrates

Early work by Dethier and others asked whether insects, like vertebrates, sense stretch or fullness of parts of the gastrointestinal tract, and whether this sensation contributes to termination of a feeding episode (Dethier, 1976). Their results, and later work by others, showed that, like mammals, insects can sense fullness in the gastrointestinal tract, and this sensation contributes to termination of food intake.

Specifically, Dethier found that distention of the foregut, but not the midgut or the hindgut, inhibited chemosensory input from taste neurons on the fly labellum, which would normally cause the fly to begin eating (Dethier, 1976). He postulated that the mechanism by which the foregut overrides chemosensory input is via a stretch receptor. Although the molecular identity of this receptor was never found, Gelperin conducted nerve resection experiments in the blowfly to show that there are at least two nerves which branch off the ventral nerve cord that sense stretch, or fullness, in the fly gut. First, he found that there are two neurons in a branch of the recurrent nerve that are activated by expansion of the foregut (Gelperin, 1967). He later found branches of the main abdominal nerve which surround but do not innervate the crop, and which display electrical activity when the crop distends, thus stretching the abdominal nerve (Dethier, 1976).

In addition to this early work in the blowfly, other experiments have shown that a stretch mechanism for sensing satiety also exists in several mosquito species (Hocking, 1971). For example, by giving mosquitoes a saline enema, Klowden found that abdominal distention is sufficient to terminate blood-feeding in the Yellow Fever mosquito *Aedes aegypti* (Klowden and Lea, 1979a).

1.2.5 The role of hemolymph in controlling feeding in insects

While the ventral nerve cord can relay information to the insect brain about short term food intake, circulating hemolymph provides longer term information on nutritional status (Simpson and Raubenheimer, 1993). Hemolymph, the insect equivalent of blood, contains the nutrients that are absorbed from food, as well as peptides and hormones that reach their targets through endocrine effects. As such, it contains valuable information for an insect regarding what and when it has eaten.

One important feature of hemolymph is that its osmolality is an indicator of the amount of solute circulating, and thus a rough indication of nutritional status. In experiments on *Locusta migratoria*, Bernays and Chapman found that meal size reduced proportionally with increased osmotic pressure in the hemolymph (Bernays and Chapman, 1974). However, osmotic pressure is only a rough indicator of how much has been ingested, and later evidence showed that circulating levels of specific macromolecules such as amino acids and sugars provide nutrient-specific information to insects (Simpson and Raubenheimer,

1993). More recent work has confirmed that flies have two distinct types of "hunger," one for protein and one for carbohydrate. The transcriptional and behavioral profile of animals in these two hunger states has been shown to be distinct (Zinke et al., 2002), and adults that are specifically starved of protein show an increased preference for yeast, a source of dietary amino acids (Ribeiro and Dickson, 2010).

Although the mechanism by which hemolymph influences feeding remains unknown, the best understood mechanism is of modulation of peripheral taste responsiveness by circulating amino acids and sugars. Experiments in the locust demonstrated that artificially raising the profile of amino acids in the hemolymph of an insect fed on a low-protein diet to that of a locust fed on a high-protein food, through injection, led to marked reduction in responsiveness of taste sensilla on the maxillary palps to stimulation with an amino acid mix (Simpson and Simpson, 1992). The same injection had no effect on the peripheral response to sugar (Simpson and Simpson, 1992).

1.2.6 Genetics of feeding behavior in flies

Since *Drosophila*, like mammals, regulate their feeding behavior according to nutritional status, and because of the ease of their genetic manipulation, they represent an inviting model for elucidating the neurogenetics of feeding behavior. Importantly, many of the known genetic modulators of nutrient sensing in mammals are conserved in *Drosophila*.

1.2.6.1 Insulin, DILPs, and TOR

Insulin signaling is a key component of the physiological response to nutritional status in both mammals and flies (Teleman, 2010). *Drosophila* contain seven insulin-like peptides (called *Drosophila* insulin-like peptides, or DILPs), which are homologues of mammalian insulin and insulin-like growth factors, and they express one insulin receptor (Teleman, 2010). DILPs are released from neurosecretory cells in response to amino acid intake. They act on the insulin receptor, which signals through a conserved PI3 kinase and leads to growth of the larva in response to nutritional conditions (Oldham and Hafen, 2003). Insulin may be a link between nutritional status and transcription in flies, since insulin targets the FOXO transcription factor, which regulates some gene expression in response to starvation in larvae (Teleman et al., 2008). Behavioral studies reveal that overexpression of DILPs in neurons leads to defective post-fasting food intake in larvae (Wu et al., 2005).

The target of rapamycin (TOR) signaling pathway is a downstream target of insulin/PI3K signaling. Some of the transcriptional effects of starvation have been found to be mediated through the TOR pathway (Li et al., 2010). For example, TOR signaling controls ribosomal RNA transcription in larvae (Grewal et al., 2007). A null allele of TOR phenocopies amino acid starvation and leads to growth arrest in early larval development (Zhang et al., 2000). TOR has therefore been suggested as a signal of amino acid reserves. Recent work by

Vargas et al. showed that flies with activated S6K, an upstream activator of TOR, phenocopy amino acid deprivation (Vargas et al., 2010), while Ribeiro and Dickson find that both inhibition and activation of neuronal TOR/S6K signaling stimulates yeast feeding. These results leave open the question of how TOR signals nutritional status (Ribeiro and Dickson, 2010).

1.2.6.2 Neuropeptides and *takeout*

Neuropeptide regulators of feeding behavior are also conserved in *Drosophila*. For example, *hugin*, a fly homologue of neuromedin was recently characterized (Melcher et al., 2006; Melcher and Pankratz, 2005). In rats, administration of neuromedin U suppresses food intake (Howard et al., 2000). In fly larvae, overexpression of the *hugin* neuropeptide gene leads to reduced feeding and smaller larvae (Melcher and Pankratz, 2005).

The neuropeptide Y system is also conserved in flies. The fly homologue of NPY, Neuropeptide-F (NPF), is expressed in both the midgut and the central nervous system (CNS) (Brown et al., 1999). sNPFs are arthropod neuropeptides that are structurally similar, but shorter in length than NPF (Veenstra et al., 2008). In *Drosophila*, sNPF seems to be expressed only in the brain (Lee et al., 2004), but its receptor is found in the crop and in the hindgut and midgut (Mertens et al., 2002).

In mammals, neuropeptide Y is a potent stimulator of feeding when administered centrally (Stanley and Leibowitz, 1985). NPF also plays an

orexigenic role in flies (Lee et al., 2004; Wu et al., 2003). NPF seems to modulate feeding behavior in larvae while sNPF may play a role in adult feeding. *Drosophila* larvae normally wander away from food after five days of continuous eating. *NPF* is expressed in the larval brain during the continuous feeding period, but not during the wandering stage, suggesting a role for this peptide in mediating this feeding behavior (Wu et al., 2003). Moreover, gain of function larval NPF mutants display prolonged feeding, while loss of function larvae show premature wandering behavior (Wu et al., 2003). In adult flies, overexpression of the short form of the neuropeptide, sNPF, leads to overeating (Lee et al., 2004).

Another protein that seems to play a role in feeding behavior in flies is Takeout. Takeout is a putative juvenile hormone binding protein whose levels of mRNA increase with starvation, but its mechanism of influencing feeding is unknown (Meunier et al., 2007; Sarov-Blat et al., 2000). Interestingly, *takeout* is expressed in the head as well as in the cardia and the crop, two structures related to feeding.

1.3 Regulation of feeding by modulation of sensory input

In addition to metabolic signals of energy needs and environmental cues such as the presence of food, the smell and taste of food related stimuli also regulate the decision whether or not to eat. Sensory neurons that express olfactory and gustatory receptors transmit signals to the brain, where they are integrated with other metabolic signals that regulate feeding behavior. Cortical

integration of these signals primarily occurs in the insula and in the orbitofrontal cortex (Critchley and Rolls, 1996; Rolls, 2008). As described below, behavioral and imaging studies suggest that sensory modalities, such as taste and olfaction, are modified by metabolic signals of nutritional status, such that food becomes more attractive to hungry animals than to non-hungry animals.

1.3.1 The effect of nutritional status on gustation

One way in which nutritional status affects food consumption is through modulation of sensory responses to food. For example, taste is affected by hunger. Functional magnetic resonance imaging (fMRI) studies reveal that fasted subjects show stronger activity in taste areas of the insula and adjacent dorsolateral prefrontal cortex (Uher et al., 2006). Other studies show significantly decreased activity in brain taste centers after two days of overfeeding (Cornier et al., 2009). In monkeys that are fed to satiety, responses of neurons to the taste of the glucose were significantly decreased, and were accompanied by a change in behavior from acceptance to rejection of glucose (Rolls et al., 1989).

Some evidence suggests that metabolic signals alter taste sensitivity in the peripheral nervous system in addition to their action on higher brain centers. For example, the leptin receptor is expressed in peripheral taste cells (Shigemura et al., 2004), and exogenous administration of leptin suppresses responses of peripheral taste nerves to sweet substances (Kawai et al., 2000) and decreases behavioral responses to sugars (Shigemura et al., 2004). In flies,

electrophysiological recordings of taste neurons in fasted flies reveal increased sensitivity to sugar in peripheral taste neurons (Meunier et al., 2007). These results suggest that modification of this sensory modality occurs not just in higher brain centers, but also in peripheral sensory systems.

Hunger might also modulate taste sensation by decreasing the threshold for consumption of unpalatable foods. In human behavioral experiments, fasted subjects place less importance on tasty foods, as compared to immediately available foods (Hoefling and Strack, 2010). This corresponds with the observation that fasted subjects are less disgusted by the sight of unpalatable food (Hoefling et al., 2009).

1.3.2 The effect of fasting on olfaction

The sense of smell is also significantly modulated by internal nutritional status. When tested in a behavior assay, rodents show a significant increase in olfactory sensitivity after fasting, such that they can detect lower concentrations of a conditioned odor when they have been fasted (Aime et al., 2007). A similar phenomenon has been documented in the nematode *C. elegans*. The worm normally reacts to the smell of octanol by initiating backwards movement. In the absence of food, the animal is significantly slower in its behavioral response to this odorant (Chao et al., 2004). Starvation also increases olfactory adaptation in worms to some odorants, and animals can recover from this effect by re-feeding (Colbert and Bargmann, 1997). 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 (Chao et al., 2004; Colbert and Bargmann, 1997).

Fasting may also lead to increased food consumption by altering the perception of olfactory stimuli that are related to food. For example, when human subjects are asked to rate the pleasantness of a non-food odor and a food odor in the fasted and in satiated states, they rate the food odor as more pleasant when they are fasted as compared to when they are satiated. However the same is not true of their response to a non-food odor (Albrecht et al., 2009). Similarly, when rats are presented with odor under food-deprived or satiated state, food-deprived rats exhibit significantly increased activity in the olfactory bulb compared to satiated rats when presented with food odor, but not when presented with a control odor (Pager et al., 1972).

While there are many descriptions of changes in feeding behavior according to nutritional status, the mechanisms by which hunger and satiety influence behavior remain largely unknown. Although the main feeding centers of the mammalian brain have been identified, we still do not know the neural circuitry that underlies nutrition-dependent changes in sensory and behavioral response to food.

Insects are inviting organism by which to further our understanding of the neurogenetics behind nutritional control of feeding behavior. Classical work in the blowfly and in the locust established that insects display stereotyped feeding

behaviors and that, importantly, they modulate feeding according to nutritional status. *Drosophila*, in particular, has been an excellent model for probing human diseases because of its genetic similarity to higher organisms and the ease of behavioral and genetic studies. However, it has been underutilized as a tool to probe the mechanism by which nutritional status affects food-seeking. Although we do not yet know the central feeding centers of the fly brain, nor do we know the main signals of hunger and satiety in the fly, we have the genetic and behavioral tools to answer such questions in this organism. Finally, by studying feeding behavior in insects, we may gain an opportunity to slow disease transmission by gaining ways to interrupt feeding in blood-feeding insects such as the malaria mosquito.

The remainder of this dissertation will focus on investigations into specific insect behaviors that are modulated by nutritional status. First, I will discuss our studies of transcriptional regulation of blood-feeding behavior in *Anopheles gambiae*, since this is a unique feeding behavior that is dependent on nutritional status. The remainder of this work will focus on post-fasting feeding behavior in *Drosophila*. I will describe our studies of olfactory response to an attractive odor in flies that have been fasted or satiated. Then, I will discuss my examination of post-fasting food intake, as well as our efforts to use this robust behavior as the basis for a targeted screen to identify novel genetic modulators of feeding behavior.

2 Transcriptional regulation of blood-feeding behavior in *Anopheles gambiae*

2.1 Introduction

2.1.1 The role of insect vector ecology in addressing neglected tropical diseases

A number of the world's most devastating diseases are spread by the bite of a mosquito. These include filariasis, Japanese encephalitis, dengue, yellow fever, and malaria. Human malaria itself causes 1-3 million deaths annually, making it the third largest infectious killer in the world (WHO, 1999). The disease can be caused by one of several different *Plasmodium* species, but all of these parasites are invariably spread by the bite of an *Anopheles* mosquito (Nighorn and Hildebrand, 2002). Consequently, any effective malaria control policy should and often does include a component targeted toward controlling the mosquito vector. At present, vector control strategies primarily consist of the use of bednets to prevent insect bites, insecticide spraying in certain areas, and alteration of the native environment to make it less conducive to mosquito breeding (Klempner et al, 2007). An important new target for interruption of vector transmission of the parasite is through manipulation of the mosquito's olfactory system, since *Anopheles* females find the source of their blood meal through olfactory cues given off by their host.

2.1.2 Olfactory driven behavior in mosquitoes

The olfactory-driven behavior of malaria mosquitoes has been described in detail by field researchers over the last few decades. It is well known that female mosquitoes prefer plant and nectar odors in the first 3-5 days of life, and then switch preferences to host odors (Takken and Knols, 1999). This corresponds with the typical female feeding pattern of eating only nectar during the first 3-5 days of life, and then taking a first blood meal and mating during days 5-7 (Figure 1).

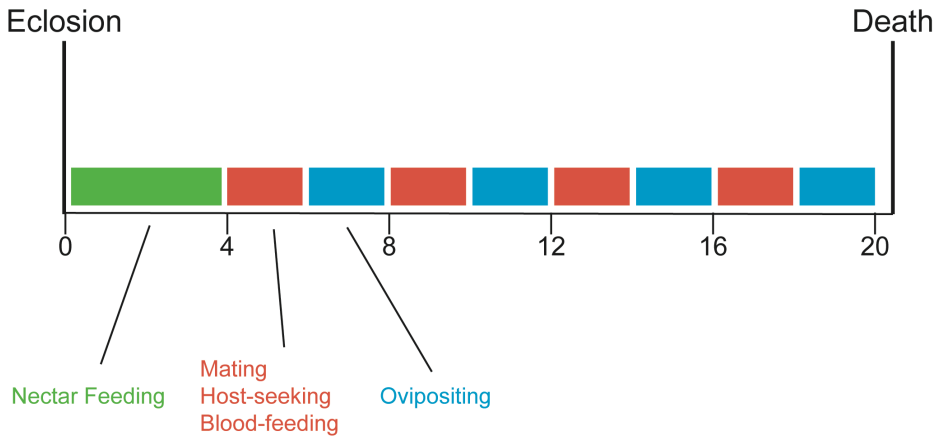

Figure 1 Olfactory-driven behaviors in female *Anopheles gambiae.* Female mosquitoes rely on nectar for nutrients during the first 3-5 days of life, and then obtain nutrients almost exclusively from blood. Blood-feeding and ovipositing are olfactory-driven behaviors that do not occur simultaneously.

Experiments dating to the 1940s have shown that human odors drive host-seeking in malaria mosquitoes. In one field study, a tent occupied by humans attracted significantly more *Anopheles gambiae* than did an unoccupied tent

(Mukabana et al., 2002). Other studies have attempted to control for the physical presence of a host by presenting host odors in the absence of a physical host—who also emits heat and alters convection currents, and could thus be attracting mosquitoes. In these studies, airborne human volatiles were collected and presented to mosquitoes through a sampling device, and it was shown that mosquitoes are attracted to these odors from a distance (Davis and Bowen, 1994; Mboera et al., 2000). Other field studies have shown that some people are more attractive to mosquitoes than others, and that this differential attractiveness is entirely odor-mediated (Mukabana et al., 2004).

The two classic human-derived odors that are attractive to mosquitoes are lactic acid and carbon dioxide, though most lactic acid studies have been conducted in *Aedes aegypti*. Other compounds that are present in human emanations and that have been shown to attract anopheline mosquitoes include acetone, 1-octen-3-ol, and fatty acids of the type that are produced by bacteria residing on human skin. Limburger cheese odor has also been found to be strongly attractive to *Anopheles* mosquitoes, which is not surprising as the odor is reminiscent of human feet (de Jong and Knols, 1995). The behavioral activity of many of these odorants has been complemented by limited electrophysiological recordings of whole antennae and of single sensilla (Qiu et al., 2006; Takken et al., 2001). These electrophysiological studies confirm that mosquito neurons respond to human-derived odors when these neurons are subject to extracellular recording in the presence of odor.

Behavioral studies show that there is a change in olfactory driven behavior in female mosquitoes following a blood meal. In studies of *Aedes aegypti*, Klowden and colleagues described a two-stage inhibition of host-seeking after a vitellogenic blood meal. First, after a sufficient amount of blood is ingested, abdominal stretch receptors send neural signals that mediate a behavioral change (Klowden and Lea, 1979a). Then, approximately twenty-four hours later, the distention is alleviated and an unidentified factor in the hemolymph inhibits host-seeking until oviposition occurs (Klowden and Lea, 1979b). Transferring the hemolymph from a blood-fed mosquito to a non-blood-fed mosquito mimics the inhibitory effect on host-seeking that occurs after a blood-meal.

This second form of host-seeking inhibition is also observed in *Anopheles gambiae*. Takken describes blood-feeding behavior in five-day old mosquitoes that were given a human blood meal and subsequently exposed to human hand odor in a dual-choice olfactometer (Takken et al., 2001). At twenty-four hours after blood-feeding, fewer than 2% of the mosquitoes responded to human odors, whereas control (sugar-fed) mosquitoes consistently respond to human odors in the same apparatus (Figure 2). This suggests that there is an unknown mechanism which dampens the mosquito's attraction to human host odor for 24 to 48 hours after blood-feeding.

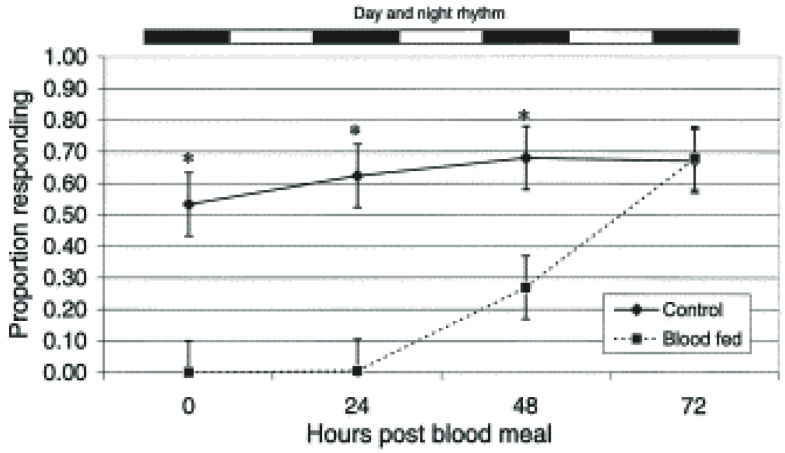

Figure 2 Host-seeking is suppressed in mosquitoes for approximately 48 hours after blood-feeding.

Control mosquitoes were fed only sugar and did not have access to a blood meal. The x-axis represents the proportion of mosquitoes flying upwind toward a human-odor baited trap in an olfactometer. (Adapted from Takken et al., 2001).

Following the behavior assay, the mosquitoes' ovaries were dissected and revealed a correlation between gonotrophic stage and response to human odorants. Mosquitoes that were in less developed gonotrophic stages did not respond to host odors, while those that had fully developed ovaries or had recently oviposited were far more responsive to human odorants (Takken et al., 2001). This implied that host-seeking is inhibited while oocytes are developing, and is restored once the mosquito has laid her eggs and is ready for another blood meal. Electrophysiological studies support the theory that there is a change in olfactory sensitivity at the level of the olfactory sensory neuron. These studies conclude that certain classes of neurons respond to specific odors only after the

mosquito blood feeds, while others neurons respond to other odors only before the blood meal (Qiu et al., 2006).

2.1.3 Organization of the mosquito olfactory system

Insects detect odors through a family of highly diverse odorant receptors (ORs). These are divergent seven transmembrane domain proteins whose signal transduction mechanism is controversial (Benton et al., 2006; Buck and Axel, 1991; Clyne et al., 1999; Vosshall et al., 1999). ORs are expressed on the dendritic surface of olfactory sensory neurons (OSNs), which are present primarily in the antenna and maxillary palp, the two head appendages that are the insect's primary olfactory organs (Vosshall et al., 1999). There are approximately 1200 such OSNs in the *Drosophila* antenna (Stocker, 1994) and each expresses typically one but occasionally up to three ligand-binding ORs and the Or83b co-receptor (Couto et al.; Fishilevich and Vosshall, 2005).

Like the *Drosophila* antenna, the mosquito antenna is covered by special sensory hairs called sensilla (Figure 3). Four different types of sensilla have been identified in female *Anopheles* and two of these sensilla types, the trichoid and the grooved-peg sensilla, house the majority of olfactory neurons. The outer surface of each sensillum is covered by a highly perforated cuticle that allows for entry of odor molecules. Insect sensilla are hollow and are filled with a fluid called sensillar lymph, the content of which is regulated by non-neuronal support cells that secrete ions and proteins into the sensillar shaft. One such class of proteins,

the odorant binding proteins (OBPs), may act to solubilize odor molecules and present them to a sensory dendrite (Smith, 1996).

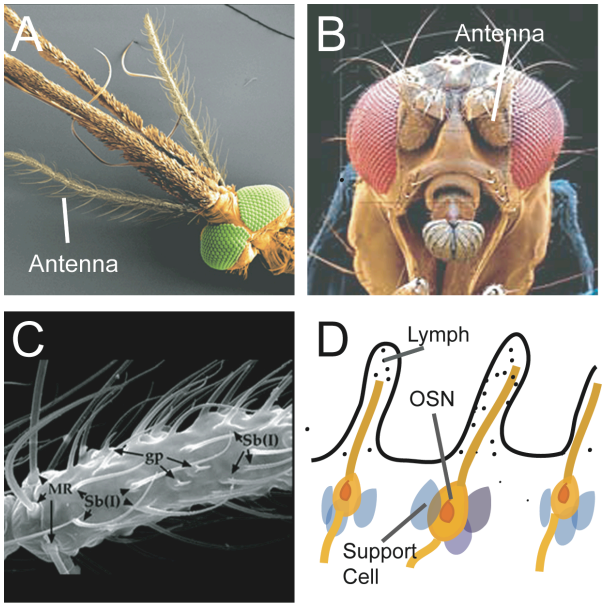

Figure 3 Primary olfactory organ for adult *Anopheles gambiae* and *Drosophila melanogaster.*

A, Head of an *Anopheles* adult female. **B,** Head of an adult *Drosophila.* **C,** Scanning electron microscope close-up of female *Anopheles* antenna. Individual sensilla are labeled with arrows. **D,** Schematic of insect chemosensory sensilla. Olfactory Sensory Neurons (OSNs) expressing an OR are at the base of the sensillum, surrounded by support cells which contribute proteins and ions to the surrounding lymph. Each OSN has a sensory dendrite that projects to the tip of the sensillum, where it interacts with odorants. For simplicity, each sensillum is shown housing one OSN, whereas true insect sensilla typically contain dendrites from one to four OSNs.

Once an odorant interacts with an OR, downstream signals lead to action potentials that send an odor-stimulated signal to the antennal lobe of the insect brain. In the *Drosophila* olfactory system, OSNs that express the same receptor

project to the same glomerulus in the insect antennal lobe (Couto et al., 2005; Fishilevich and Vosshall, 2005; Laissue et al., 1999).

The *Anopheles gambiae* genome contains seventy-nine ORs (Hill et al., 2002). These genes form a diverse family, with some members showing as little as 8% sequence homology to one another. Among *Anopheles* ORs, about half are more than fifty percent divergent from all *Drosophila* odorant receptors, leading researchers to believe that it is these divergent ORs that may underlie specific host preference for mosquitoes. One receptor, *GPROR7,* was found to be orthologous to the *Drosophila* Or83b, with these genes sharing 78% sequence identity (Hill et al., 2002; Jones et al., 2005; Pitts et al., 2004). This finding is significant because *Drosophila* Or83b is expressed in most olfactory receptor neurons in the fly, is essential for olfaction *in vivo*, and has recently been found to couple with another OR at the dendritic membrane of the OSN (Benton et al., 2006; Vosshall et al., 2000). Furthermore, work from our laboratory showed that *GPROR7* can functionally rescue Or83b mutant flies (Jones et al., 2005). Thus GPROR7 is likely vital to a functional olfactory system in the mosquito, possibly acting as a co-receptor.

2.1.4 Odorant receptors and blood-feeding behavior

A previous study identified one *Anopheles* odorant receptor, *GPROR1*, that is absent from female antennae after blood feeding through non-quantitative RT-PCR (Fox et al., 2001). This OR was subsequently found to be tuned to 4-

methyl-phenol, a component of human sweat (Hallem et al., 2004b). While these studies suggest that GPROR1 is important for mediating attraction to human host odor, and that its expression levels are regulated by blood-feeding status, further studies must be done to determine to characterize this phenomenon. Notably, I have been unable to replicate these RT-PCR results using our own tissue samples. Furthermore, microarray analysis (Section 2.3.1) does not confirm that *GPROR1* is down-regulated after blood-feeding. Thus, while previous studies suggest that ORs may mediate behavioral changes after blood feeding, further work must be done to identify and characterize the specific ORs that are important for behavioral changes following blood-feeding.

2.2 Materials and Methods

2.2.1 Isolation of Mosquito RNA

To quantify gene expression levels in antennae from female mosquitoes at different feeding states, it was necessary to isolate sufficient genetic material for use on a commercial gene chip. Mosquitoes were provided by The Malaria Research and Reference Reagent Resource Center (MR4) at the Center for Disease Control (CDC). Mosquitoes were dissected and approximately 100 antennae were collected for each microarray experiment. RNA quality was assessed by visual inspection of electropherograms produced by the Agilent 2100 Bioanalyzer, using the Pico analyzer assay (Figure 4). In each electropherogram, abundant 18S and 28S ribosomal RNA peaks were evident,

and the height of the 28S ribosomal peak was used as an indicator of RNA quality. In such plots, the higher the peak, the more intact the RNA sample is. Similar RNA quality was seen for samples taken from mosquito antenna and from non-olfactory body tissue, despite the large differences in quantity of RNA between these two types of samples. We conclude that our methods for obtaining RNA from dissected mosquito antennae are robust.

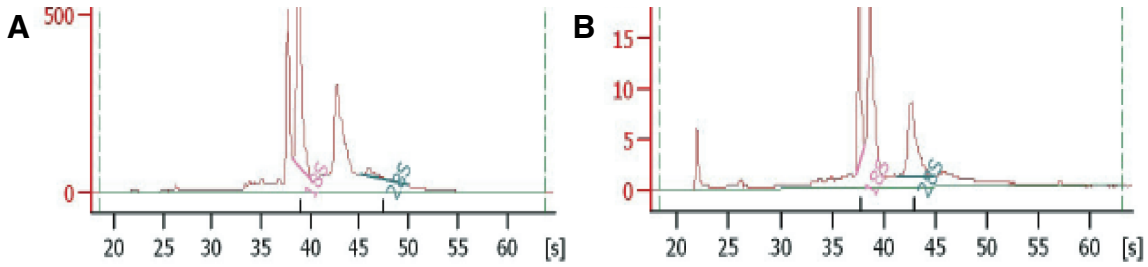

Figure 4 High-quality RNA obtained from *Anopheles* antennae and bodies.

Total RNA samples from **A,** 8 mosquito bodies (minus olfactory tissue) and **B,** 120 antennae, were loaded on an Agilent BioAnalyzer using the Pico LabChip, which can detect as little as 200pg RNA. Peaks represent ribosomal RNA.

2.2.2 Microarray and Quality Control

Changes in *A. gambiae* gene expression 24 hours after a blood-meal were assessed using microarray analysis with whole *Anopheles* genome Affymetrix microarrays. At least four biological replicates were collected from each of four experimental groups: antenna from females before a blood meal and from

females twenty-four hours after a blood meal, and non-olfactory body tissue from the same females. RNA was extracted and cDNA was synthesized, linearly amplified, and labeled using the commercially available Ovation kit (Nugen). Probe production and array hybridization was carried out by the Rockefeller Gene Array Core Facility, which hybridized probes from the above RNA samples to Affymetrix gene arrays that combine on one chip approximately 14,900 genes from *A. gambiae* with 4,300 genes from the *Plasmodium falciparum* genome.

In collaboration with Dr. Mayte Suárez-Fariñas (The Rockefeller University), the microarray data were analyzed to extract regulated genes with high statistical support. We chose to focus on genes that had a fold change greater than 2 and a false discovery rate (FDR) less than 0.2. The AffyQCReport R package of BioConductor was used to generate the initial quality control analysis. Average background levels and percent present calls were found to be similar across all chips. The level correlation of signal intensities showed between 87% and 99% similarity between chips, indicating that the samples were similar to one another in quality and in overall gene expression.

2.3 Results

2.3.1 Transcriptional changes correspond with behavioral changes after blood-feeding

We looked for genes whose levels of expression change depending on blood-feeding status. We were particularly interested in genes that are regulated

by blood-feeding exclusively in the antenna, since these represent candidate genes for regulating olfactory drive behavior. A total of 1301 probe sets showed at least two-fold variation in signal intensity between mosquitoes before and after a blood-meal. Of these, 58 showed changes in expression exclusively in antennae, with 36 genes showing up-regulation after a blood-meal, and 22 genes showing down-regulation. These genes represent promising candidates for further analysis (Figure 5).

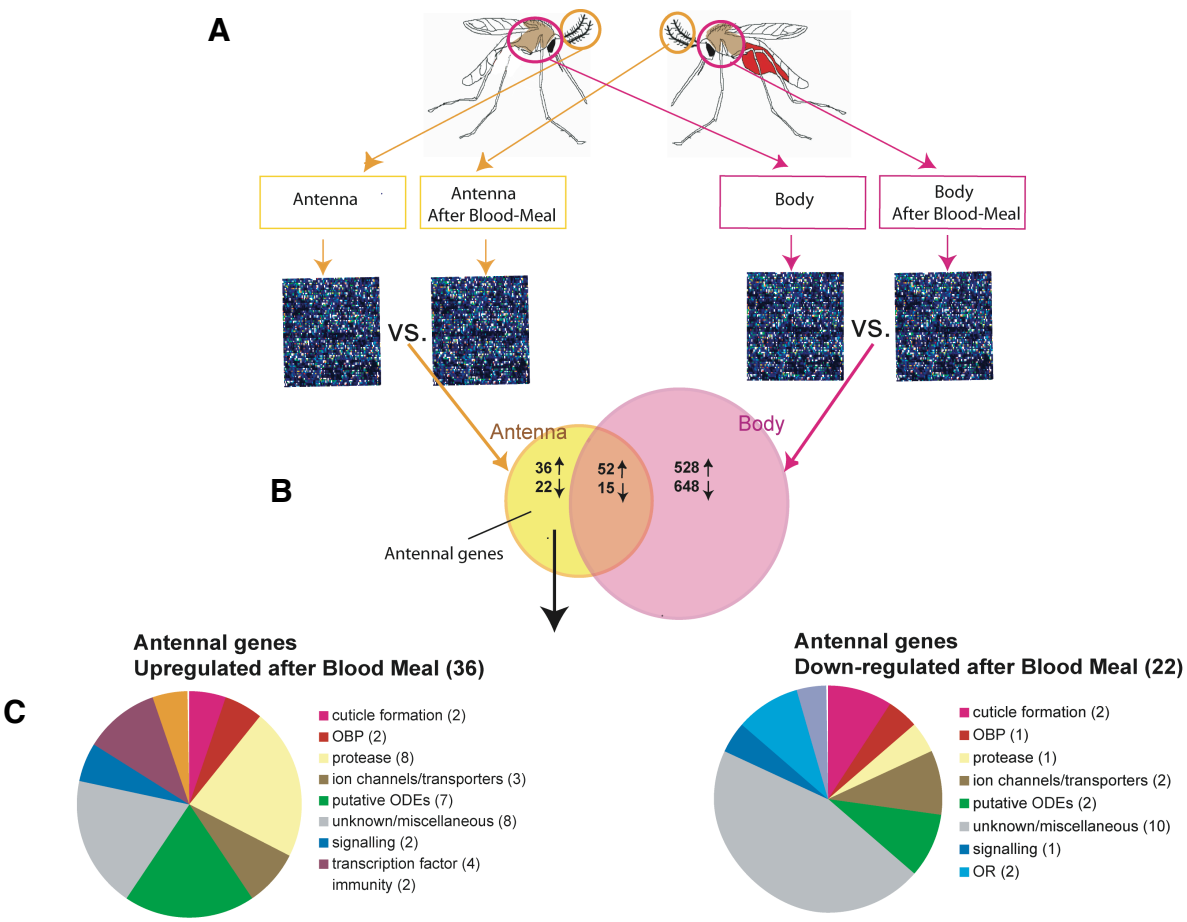

Figure 5 A microarray analysis of gene expression in mosquito antenna and non-olfactory body tissue identified genes that are regulated by blood feeding.

A, Mosquitoes before and after a blood-meal were dissected for tissue to be used in a microarray. **B,** Summary of microarray results comparing gene expression before and after blood-feeding in antenna (yellow) and body (pink). Numbers indicate genes that are up or down-regulated following a blood-meal in mosquito tissue. **C,** Pie-chart of protein classes encoded by genes that are up- or down-

regulated specifically in mosquito antenna. OBP= odorant binding protein. ODE= odorant degrading enzyme.

2.3.2 Identification of odorant receptor genes that are regulated by blood feeding

We wanted to know whether odorant receptor gene expression was affected by blood-feeding status. Two out of the fifty-eight genes that were identified as regulated by blood-feeding exclusively in the antenna are ORs. These genes, *GPROR56/57* and *GPROR69*, were approximately four-fold and three-fold down-regulated, respectively, after blood-feeding. GPROR56/57 are two closely related odorant receptors that both hybridize to the same Affymetrix probe. My hypothesis is that these receptors recognize a component of human host odor and that their expression levels modulate olfactory sensitivity, ultimately contributing to blood feeding behaviors. Recent studies by the Carlson and Zwiebel groups found odors that activate most *Anopheles* odorant receptors, including GPROR56 and GPROR57 (but not GPROR69) (Carey et al., 2010; Wang et al., 2010). These receptors were activated by a number of odors, which draw from diverse classes of ligands, including aromatics, alcohols, terpines and esters. Some of these odors, especially the aromatic compounds, could be of interest in host-seeking.

2.4 Conclusion

Understanding the genetic basis for mosquito feeding behavior is vital to developing targeted new strategies to combat vector-borne diseases. Using

knowledge and tools developed from previous studies of the *Drosophila* olfactory system, we looked at gene expression in malaria mosquitoes and found many transcripts that are regulated by feeding state. This information should form the basis for future studies of the roles of specific genes in regulating blood-feeding behavior.

3 Post-fasting feeding behavior in *Drosophila melanogaster*

3.1 Introduction

For organisms to survive, they must regulate their behavioral response to environmental stimuli according to their internal state. In the case of the malaria mosquito, the animal behaves differently toward attractive human odor depending on the mosquito's blood-feeding status. In non-blood feeding animals, such as the vinegar fly, the animal's nutritional status is a key determinant in how food is perceived and in the decision on whether and how much to eat. However, the neuronal and genetic bases of the regulation of feeding behavior remain poorly understood. The genetic tools available for studies in *Drosophila* make this an enticing animal model for studies of this phenomenon,

Classical studies in the blowfly, *Phormia regina*, described basic features of insect feeding behavior, with many of these behaviors subsequently shown to be conserved in *Drosophila* (Dethier, 1976; Edgecomb et al., 1994). Previous studies in *Drosophila* looked at proxy measures for food intake, such as the fly's willingness to extend its proboscis towards sucrose, rate of defecation, and accumulation of food in the crop, a food storage organ, (Edgecomb et al., 1994; Wong et al., 2009), and showed phenotypic changes that were dependent on the fly's nutritional state. 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, and thus they could not investigate the

genetic or neuronal underpinnings of feeding behavior. The development of the capillary feeder, CAFE, assay by the Benzer lab was a significant improvement over past tools used to measure food intake, since it allows for precise, short-term measurements of food intake in real-time (Ja et al., 2007).

The ability to regulate food-seeking behavior in response to nutritional status is central to an organism's survival. Nutritionally-deprived animals show modified perception of food stimuli, such that they are more sensitive to or more attracted to food stimuli. At the same time, animals in the fasted state consume food in larger quantities than do satiated animals. How this process is regulated genetically remains unknown. We have begun to address this question by establishing robust and quantitative post-fasting behavioral assays in the vinegar fly. This opens the door to a neuron-specific genetic screen to identify novel regulators of eating behavior.

3.2 Methods

3.2.1 *Drosophila* stocks

Drosophila stocks were maintained on conventional cornmeal-agar-molasses medium under a 12 hour light:12 hour dark cycle at 25°C. The *Canton-S* strain was used as wild type control. RNAi experiments were carried out using a driver strain obtained from the Dickson Lab: w; UAS-dicer-2; Elav-Gal4. UAS-Takeout-RNAi was generated at the Vienna *Drosophila* RNAi Center.

3.2.2 Olfactory trap assay

Flies were fed on conventional fly food or fasted with access to only water for 12, 24, or 48 hours before being placed in a two-choice behavior chamber modeled after one described by Ditzen et al (Ditzen et al., 2008). Each trap was humidified and contained a small piece of filter paper with 10μl of either water or odor (3-methyl-thio-1-propanol). Flies could enter one of the two traps through a small plastic pipette tip inserted into top of each vial. After 24 h, the flies were scored by calculating the percent of flies in each trap. 30-50 male flies were used in each trap, with at least five replicates used for each comparison. Significance was assessed using the Mann-Whitney U test.

3.2.3 Single sensillum recordings

Wild type flies were aged for five days before recordings. One group was fasted for 24 h on wet cotton, and one group was given free access to food before recording. Single sensillum extracellular recordings of male flies were performed as described (Pellegrino et al., 2010). Briefly, activity of olfactory sensory neurons was recorded using a 10x AC probe connected to an IDAC-4 amplifier. Odorants were obtained from Sigma-Aldrich at high purity and diluted (v/v) in paraffin oil as specified in the figures. One filter paper strip (3 x 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. 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 (Hallem and Carlson, 2006).

Data were collected using Autospike (Syntech) and analyzed by custom-made spike sorting algorithms. Since spikes from the A and B cells can not be differentiated in the ab5 sensillum, spikes from these neurons were grouped together for analysis. Based on previous data, the ab5a sensillum does not respond to 3MT1P(D'Ettorre and Heinze, 2005; Ditzen et al., 2008; Hallem et al., 2004a). Spike trains were grouped in 200 ms bins and responses were calculated by subtracting the average spontaneous activity of 15 s before odor application from the activity during the first 500 ms (excitatory odorants) or 1s (inhibitory odorants) after odor delivery. Curves were fit in Origin-Pro 8.0 using the Hill equation and were compared for significance with an F-test.

3.2.4 CAFE assay

Capillary feeding assays were modified from the design proposed by Ja et al. (Ja et al., 2007). The CAFE chamber consists of an empty wide polystyrene

vial (Fisher AS-519) with a wet cotton acetate plug at the bottom of the CAFE, for humidity. The top of the CAFE is plugged with a size 5.5 one-hole black rubber stopper (VWR product number 59581-265) into which is inserted a cut pipette tip. A 5 μl glass capillary (VWR 53432-706) is inserted through the pipette tip, and food is delivered through the glass capillary. Liquid food consists of 10% sucrose, 5% yeast, unless otherwise noted, with 40 μl of McCormick green food coloring added to every 800 μl of food. Food intake is calculated by measuring the depression of the meniscus of food in the capillary. For the statistical comparisons between cumulative food consumption, two-way ANOVA was applied. 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. Data shown is consumption per fly.

3.2.5 Crop measurements

Flies were reared as described above. After three days of continuous access to 10% sucrose plus 5% yeast, flies were either fed or fasted for 24 hours. Following this experimental period, flies were fed food consisting of 10% sucrose plus 5% yeast plus 0.02% FITC for three hours. Flies were then fixed in PBS plus 0.1% Triton for twenty minutes, then washed in PBS three times, for thirty minutes each. Crops were then dissected and were visualized with a Zeiss LSM510 confocal microscope.

3.3 Results

3.3.1 Behavioral attraction to an odor increases with fasting

Since virtually all organisms modulate food-seeking behavior based on nutritional status, we asked whether fasting affects olfactory-driven responses to food in *Drosophila*. Flies show attractive and repulsive responses to different odors (Keller and Vosshall, 2007), including attraction to the food-like odor 3-methylthio-1-propanol (3MTP), for which the corresponding olfactory receptor is known (Ditzen et al., 2008). To measure the effect of nutritional status on attraction to this odor, we used a two-choice olfactory trap assay (Figure 6A) in which flies may enter a trap containing the odor or one containing water alone.

Flies that were fasted for 24 or 48 hours enter the odor trap significantly more often than flies that have not been fasted or have been fasted for 12 hours (Figure 6B). Therefore, behavioral attraction to an odor is strongly modulated by nutritional status, with increased food deprivation leading to increased attraction to a food-like odor.

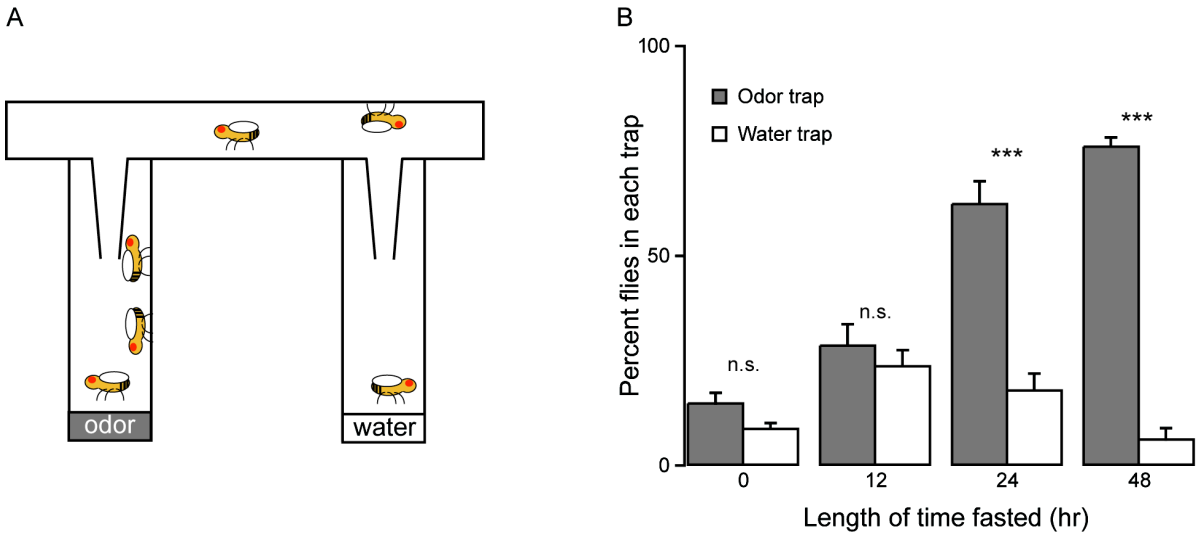

Figure 6 Attraction to food odor increases with extended fasting

(A) Schematic of two-choice olfactory trap assay to test attraction to a food odor. Approximately thirty flies were placed in the top chamber and allowed to enter either a chamber containing a food odor (3-methylthio-1-propanol) or water. Flies in each trap were counted after 24 hours.
(B) Flies fasted for 24 or 48 hours are significantly more attracted to odor than water. Data shown are mean±s.e.m. ***$p<0.001$. n= 6-16 traps per time point.

This change in olfactory response can be due to increased sensitivity to food odor in peripheral olfactory sensory neurons, or to changes in central nervous system responses in the fasted state. To distinguish between these two possibilities, we looked at sensitivity to food odor in the peripheral olfactory system and asked whether neuronal response to 3MTP is increased in flies that have been food deprived.

The fly's main olfactory organ, the antenna, is covered by sensory sensilla that house the dendrites of olfactory sensory neurons (Figure 7A). Previous work

by Hallem and Carlson (Hallem and Carlson, 2006) showed that 3MTP is primarily detected by olfactory sensory neurons expressing OR47a, which is housed in the ab5 sensillum. We therefore performed extracellular single sensillum recordings in the ab5 sensillum in response to 3MTP (Figure 7B).

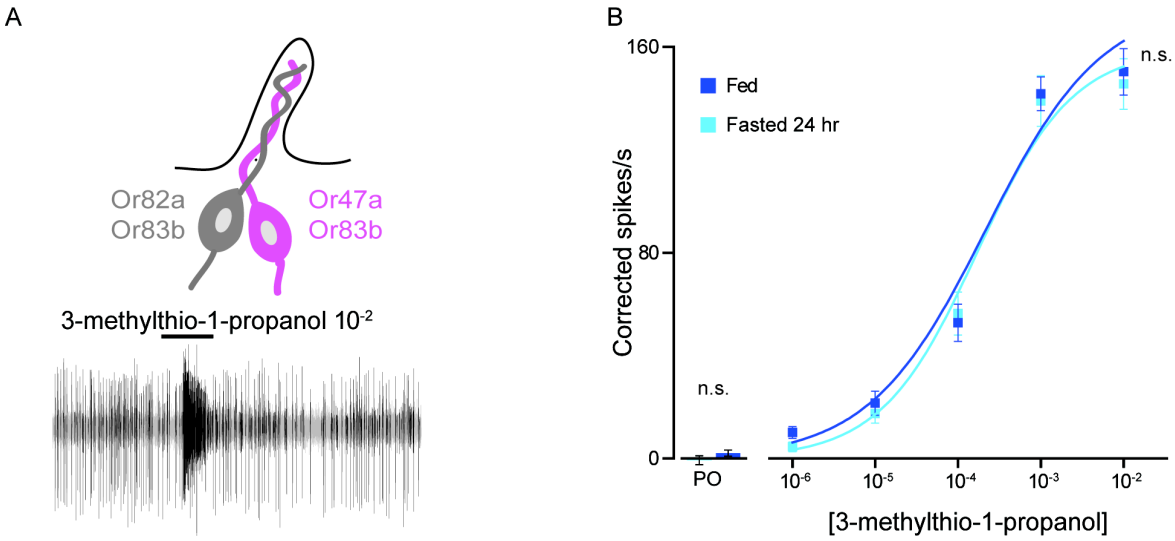

Figure 7 Single sensillum recording of ab5 sensillum in response to an attractive odor

(A) Schematic of *Drosophila* single sensillum recordings. Horizontal black bar represents period of odor delivery. Shown is a representative trace of the response of ab5 sensillum to 3MTP.
(B) Dose response curves of Or47a olfactory sensory neuron responses to 3-methylthio-1-propanol in fed and fasted flies. There is no significant difference between fed and fasted flies in response to the odor. Data shown are mean±s.e.m. n= 9 sensilla.

We measured responses of the OR47a neuron to 3MTP across a range of odor concentrations in fed and in 24-hour fasted flies. Evoked activity of OR47a

neurons as measured by spikes per second was not significantly different in fasted versus satiated flies (Figure 7B).

3.3.2 Flies display a robust post-fasting feeding response

In addition to olfactory-driven behavior, food intake behavior is also affected by feeding status, although in *Drosophila* the mechanisms behind this influence are unknown (Edgecomb et al., 1994). We wanted to establish a robust and precise assay to measure food intake behavior in post-fasted flies to aid in future genetic studies. We began our studies using the CAFE apparatus and liquid food developed in the Benzer laboratory, in which flies are fed 5% sucrose plus 5% yeast (Ja et al., 2007).

Three day old male flies were placed in the CAFE and were fed *ad libitum* for 2 days in order to acclimate to the CAFE. We then measured food intake at regular intervals before and after 24 hours of fasting to compare food intake behavior in fed versus post-fasted nutritional states (Figure 8A). We found that when flies are fed 5% sucrose plus 5% yeast, there was no change in the amount of food intake in the post-fasted state (Figure 8B). However, when flies were fed a diet of 10% sucrose plus 5% yeast, they showed a significant increase in food intake in the post-fasted state (Figure 8C). This effect was seen in both male and female flies, although females consume significantly more food than males (Figure 8C).

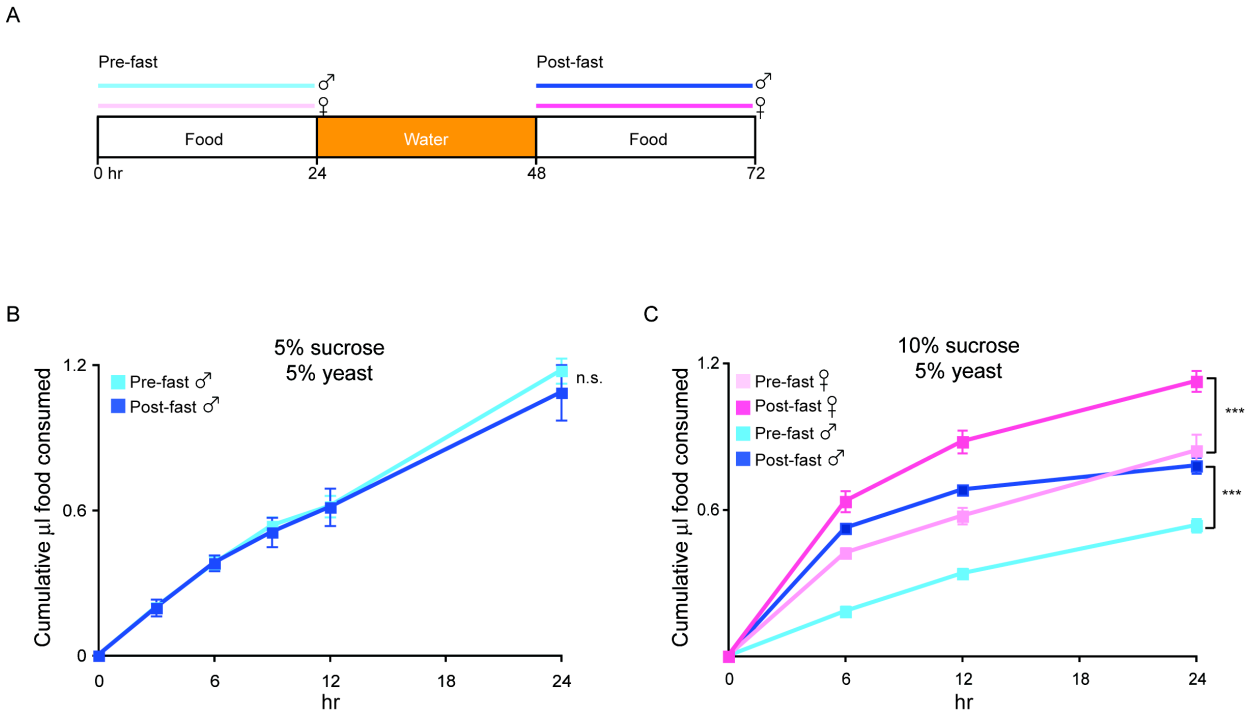

Figure 8 Flies show a robust post-fasting feeding response

(A) Timeline of feeding assay to measure post-fasting feeding response.

(B) Flies that were fed a mixture of 5% sucrose and 5% yeast did not increase food intake after fasting. n = 6-10 CAFEs.

(C) Males and females fed 10% sucrose plus 5% yeast both showed an increase in food intake after 24 hours of food deprivation. ***p<0.001. n = 10 CAFEs.

To further explore post-fasting feeding dynamics in flies fed 10% sucrose plus 5% yeast, we measured food intake in male flies at regular intervals over the course of five days, with the third day consisting of access to food (Group 1) or water only (Group 2) (Figure 9). We found that the flies that were fasted (Group 2) on day 3 showed a significant increase in food intake on day 4 (Figure 9). This same group of flies returned to pre-fast levels of feeding on day 5, after 24 hours

of re-feeding (Figure 9). Flies that were given continuous access to food did not vary their food intake over the course of the experiment (Figure 9). Therefore, the CAFE assay using 10% sucrose plus 5% yeast is a robust method for measuring post-fasting food intake behavior.

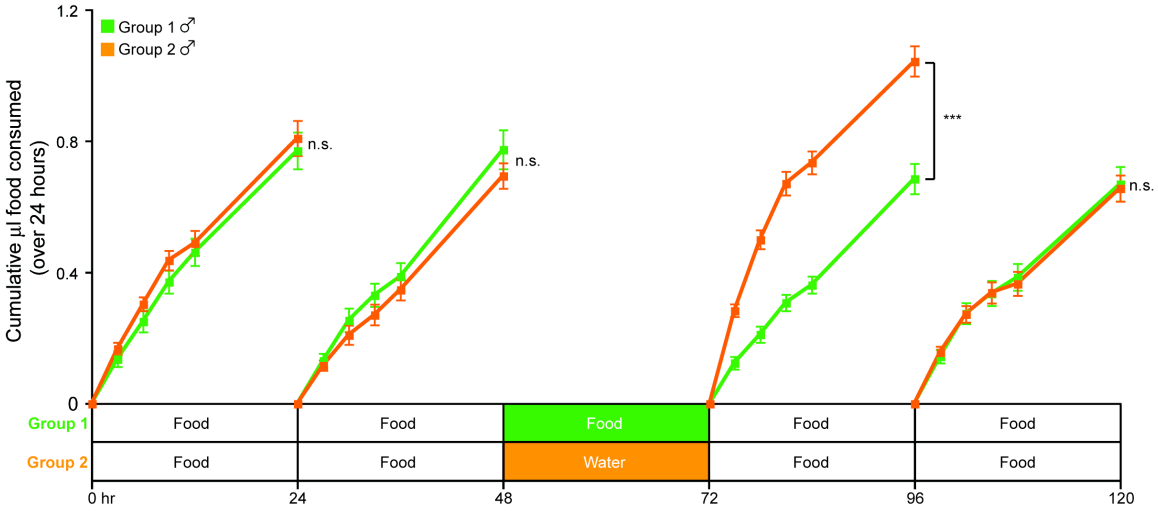

Figure 9 Food consumption of fed and fasted flies over multiple days in the CAFE.

Both groups were treated identically except for the time period from 48 to 72 hours, when Group 1 was fed freely while Group 2 was fasted. Food is 10% sucrose, 5% yeast. After being fasted on the third day, Group 2 flies showed an increase in food intake that goes back to baseline on the second day post-fasting. ***$p<0.001$. n = 10 CAFEs.

3.3.3 Fasted flies store more food in their crop than flies that have free access to food

Insects possess a unique food-storage organ, the crop, which is empty 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 (Edgecomb et al., 1994). We quantified 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 hours. Both groups were then fed fluorescein labeled food for three hours before their crops were dissected and measured (Figure 10). We found that flies that are post-fasted have crops that were more than twice as wide as flies that had continuous, free access to food (Figure 10). This is consistent with our previous result showing that post-fasted flies consumed significantly more food than flies fed *ad libitum*.

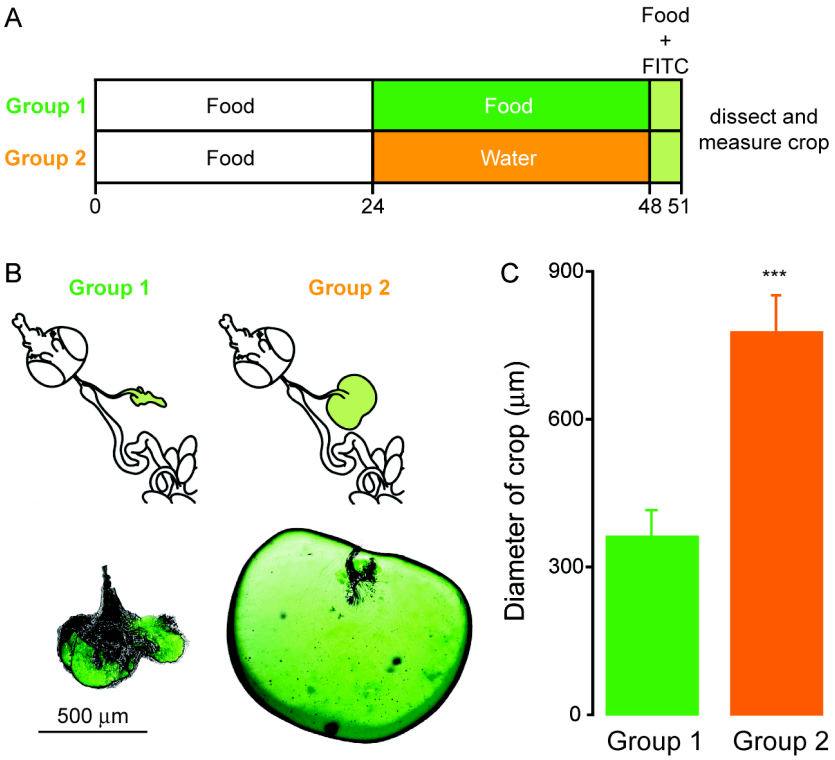

Figure 10 Post-fasted flies store more food in their crops than flies fed *ad libitum*

(A) Timeline of crop dissection experiments. Both groups were given free access to food for twenty-four hours. Group 1 continued to have free access to food while Group 2 was fasted for twenty-four hours. Both groups were then fed liquid food with 0.02% FITC for three hours prior to crop dissection.

(B))(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. (Melcher and Pankratz, 2005). (bottom) Confocal images of crops from post-fast or free-fed flies. Scale bar=500 mm.

(C) Post-fasted flies that are given access to food have substantially larger crops than flies that have had continuous access to food. t-test, ***p<0.001. n = 9 crops.

3.4 Conclusion

Our electrophysiological results suggest that the increased behavioral attraction to food odor in the fasted state may be caused by a more central mechanism, such as increased activity in the antennal lobe or higher brain structures, rather than increased peripheral sensitivity in sensory dendrites. Indeed, previous work on vertebrates showed changes in activity in olfactory brain centers in response to food odor that are dependent on feeding state (Pager et al., 1972). Further studies in the fly may elucidate which brain centers are hypersensitive in the fasted state, and the mechanism behind this change in sensitivity.

In our initial CAFE experiments, we found that flies that are fed 5% sucrose, 5% yeast do not increase food intake after fasting. These results indicate that the CAFE as described by Ja et al. (Ja et al., 2007) 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 found that flies fed a higher concentration of sucrose, 10% sucrose plus 5% yeast, do show a post-fasting feeding response. In the 24 hours following fasting, they consume a significantly larger amount of food than they do under *ad libitum* conditions. Thus, we conclude that the CAFE using 10% sucrose plus 5% yeast is a good assay for measuring post-fasting food intake. Furthermore, we conclude that flies fed 5% sucrose plus 5% yeast are in a constant-state of low level hunger, and are continuously eating a maximum amount of food. This is reinforced by the fact that

flies that are fed 10% sucrose plus 5% yeast consume less under *ad libitum* conditions than flies that are fed 5% sucrose plus 5% yeast.

There is a debate in the literature over whether the concentration of sucrose in the flies' diet influences food intake. One study found that flies increase the volume of food consumed as the percent of sucrose in the flies' diet increases (Edgecomb et al., 1994). More recently, Carvalho et al. found the opposite result, that flies ingest a smaller volume of food when the concentration of sucrose increases (Carvalho et al., 2005). Our results support the latter finding, since our flies consume more food when given 5% sucrose plus 5% yeast than when they are given 10% sucrose plus 5% yeast.

Our examination of feeding behavior in wild-type *Drosophila* showed a quantifiable increase in food intake under specific feeding conditions. This lays the groundwork for a genetic screen to find mutants that do not regulate food intake according to nutritional status, with the goal of identifying novel genetic regulators of feeding behavior.

4 A targeted genetic screen to identify novel regulators of feeding behavior in *Drosophila melanogaster*

4.1 Introduction

Most of the known regulators of feeding in *Drosophila* have been shown to act in larvae but not in adults. Therefore, there remain unidentified genes involved in feeding regulation. Having established a robust way to measure post-fasting food intake in flies, we set out to identify novel genes that regulate feeding behavior. We used a microarray approach to screen for the effect of fasting on gene expression. The goal is to identify genes that underlie the post-fasting olfactory and food intake phenotypes described in previous sections. We looked for functional significance of these genes through a targeted RNAi based screen for behavioral mutants.

4.1.1 Known genes whose transcriptional regulation modifies feeding behavior in *Drosophila*

Although more is known about genetic modifiers of food intake in vertebrates, recent studies have uncovered genes that influence feeding in flies (Lee et al., 2004; Melcher et al., 2007; Wu et al., 2003). While NPF mutants seem to display a feeding phenotype only in larvae (Wu et al., 2003), sNPF mutants show abnormal feeding in the larval and the adult stages (Lee et al.,

2004). Overexpression of sNPF in neurons causes adults flies to consume significantly more blue food as detected by visualizing blue dye in the abdomen than control flies, while RNAi knockdown of the peptide leads to fewer flies consuming blue food than in control genotypes (Lee et al., 2004). These experiments were conducted under non-fasting conditions.

In addition to sNPF, another gene that may regulate feeding in adults in *takeout.* Takeout is a member of a large family of putative small molecule binding proteins that is similar to juvenile hormone binding protein (Sarov-Blat et al., 2000). Meunier et al. find that, under *ad libitum* conditions, *takeout* mutant adult flies are hyperphagic (Meunier et al., 2007). Intriguingly, they also found that *takeout* mutants show a significantly reduced intake in feeding post-fasting (Meunier et al., 2007). Expression studies of *takeout* remain cursory, and it is unknown if the feeding effect of *takeout* mutants is due to loss of activity in the fat body, where *takeout* is known to be expressed, or in other tissues, such as the brain or the antenna, where there is weaker evidence of *takeout* expression (Dauwalder et al., 2002; Sarov-Blat et al., 2000; So et al., 2000).

Finally, Al-Anzi recently identified two classes of neurons in the fly brain that regulated overall fat deposition in the fly (Al-Anzi et al., 2009). These two neuronal populations, identified by expression of c673a-Gal4 and fruitless-Gal4, may represent part of an as-yet unknown central feeding center in the fly brain When c673a neurons are silenced, adult flies consume significantly more food

than control flies. However, the specific genes that operate in these neurons to control fat storage remain unidentified.

4.1.2 Expected transcripts that are modified by nutritional status

Previous studies have examined transcriptional changes that are dependent on nutritional status in whole larvae (Zinke et al., 2002) and in adult head tissue (Fujikawa et al., 2009). These studies as well as previous work in vertebrates (Yamamoto et al., 2009) have shown that transcriptional changes do occur in response to food intake. The study of larval transcription identified a novel gene, *sugarbabe,* as the most highly regulated gene when larvae are allowed access to sugar only (Zinke et al., 2002). Fujikawa's study of transcription on the head found that *fit*, *CG8147*, and *Obp99b* are among the most highly regulated transcripts in food-deprived adult flies (Fujikawa et al., 2009). Thus one might expect to find these genes in an independent examination of feeding-regulated transcripts.

Finally, since food intake initiates metabolic changes in the fly, a study of the effect of feeding on transcription ought to yield genes involved in lipid, carbohydrate, and protein metabolism.

4.1.3 RNAi in *Drosophila*

RNAi in *Drosophila* is achieved through expression of a double-stranded hairpin consisting of an inverted repeat of a fragment of a gene. The expressed

hairpin is then processed by cell endogenous machinery, and often also an exogenous dicer protein, into approximately 19-nucleotide fragments which then target the gene of interest (Dietzl et al., 2007). The RNA hairpin is expressed via the Gal4-UAS system, with UAS-RNAi for the gene of interest integrated into the genome of a mutant fly. Thus it is possible to drive expression of the RNAi in select tissues, using tissue-specific Gal4 drivers. Moreover, resource centers such as the Vienna *Drosophila* Research Council (Vienna) and *Drosophila* RNAi Screening Center (Boston) have made it possible to carry out large scale RNAi screens through their construction of libraries of integrated UAS-RNAi hairpins.

One of the main concerns that arises from use of RNAi to knock-down gene expression is the potential for off-target effects. Since the dsRNA hairpin is digested into smaller fragments, there is a chance that one or more of these fragments could have sequence homology to an off target gene (Perrimon and Mathey-Prevot, 2007). Certain features of the RNAi hairpin, such as the presence of 6 or more contiguous trinucleotides CA[AGCT] (or CAN repeats), are more likely to lead to off target effects (Ma et al., 2006). A newer RNAi library generated by VDRC contains 10 or more of these problematic repeats in less than 1% of it's RNAi lines (Dietzl et al., 2007). However, since it is difficult to determine whether an observed phenotype is due to targeted or off target effects, it is useful to test multiple hairpins for genes of interest.

4.2 Methods

4.2.1 Fly stocks

Wild-type Canton-S flies were used for microarray experiments. For RNAi experiments, the following driver, obtained from the Dickson Lab, was used: w; uas-dicer-2; Elav-Gal-4. UAS- RNAi flies were generated at the Vienna *Drosophila* RNAi Center.

4.2.2 Microarray

Changes in *D. melanogaster* gene expression in fasting flies 24 and 48 hours after 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, 24, and 48 hours: head (minus chemosensory organs), antenna, palp and proboscis, and body (minus the head). Five biological replicates were collected from each tissue group per time point.

Male Canton-S flies were used in all experiments. Male flies were aged for 2-3 days, and were then separated into 3 groups. The first group was fasted and dissected 24 hours later. The second group was fasted and dissected 48 hours later, and the third group was fasted and dissected 72 hours later. The flies were kept at constant temperature and humidity (25 degrees, 70% humidity), and all dissection were carried out at the same time of day to avoid circadian effects on gene expression.

RNA was extracted using the RNEasy kit (Qiagen) and 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.

In collaboration with Mayte Suárez-Fariñas (The Rockefeller University), the microarray data were analyzed to extract regulated genes with high statistical support. We focused on genes that had a fold change greater than 4 and a false discovery rate (FDR) less than 0.01. Quality control was as described above (section 2.2.2).

4.2.3 CAFE based screen

Flies were generated for behavioral testing by crossing Elav-Gal4, UAS-dicer driver virgin females to UAS-RNAi males. Virgin females were acquired by heat-shocking the driver line, which contains a heat-shock-hid element on the y chromosome that kills >99% of male progeny while sparing females. Six females laid eggs for two days before being discarded, to prevent crowding of the larvae. The progeny of this cross were aged for 2-3 days, after which male flies were separated. Five male flies were placed in each CAFE and were given two days of *ad libitum* access to food, to recover from carbon dioxide anesthesia and to acclimate to the CAFE.

Each genotype was observed for five consecutive days in the CAFE. For the first two days, the flies acclimated to the CAFE with free access to food. On

the third day, flies were given food and food intake was measured at 6 hours and again at 24 hours. Food intake was determined by manually measuring the depression of the meniscus as a result of feeding. On the fourth day, flies were deprived of food. On the fifth day, flies were fed again, and food intake was measured at six hours. Flies were fed 10% sucrose plus 5% yeast, and capillaries were switched at least once every 24 hours. CAFEs were constructed and food intake measured as described in 3.2.4.

4.3 Results

4.3.1 Microarray analysis to identify candidate genes that may regulate feeding behavior

To determine whch genes may influence post-fasting feeding behavior in flies, we looked for genes that had changes in gene expression following 24 or 48 hours of fasting. We initially focused on genes whose expression was regulated in head RNA, because we were interested in central nervous system hunger regulators. In an analysis of head RNA, 247 genes were significantly regulated by feeding state. As expected, genes that are known to have expression levels affected by nutritional status were positive hits in our array: *fit*, *Obp99b*, *takeout* and *sugarbabe.* Thus the array, under our conditions, was able to detect transcripts that were previously shown to have a relationship with feeding (Fujikawa et al., 2009; Zinke et al., 2002).

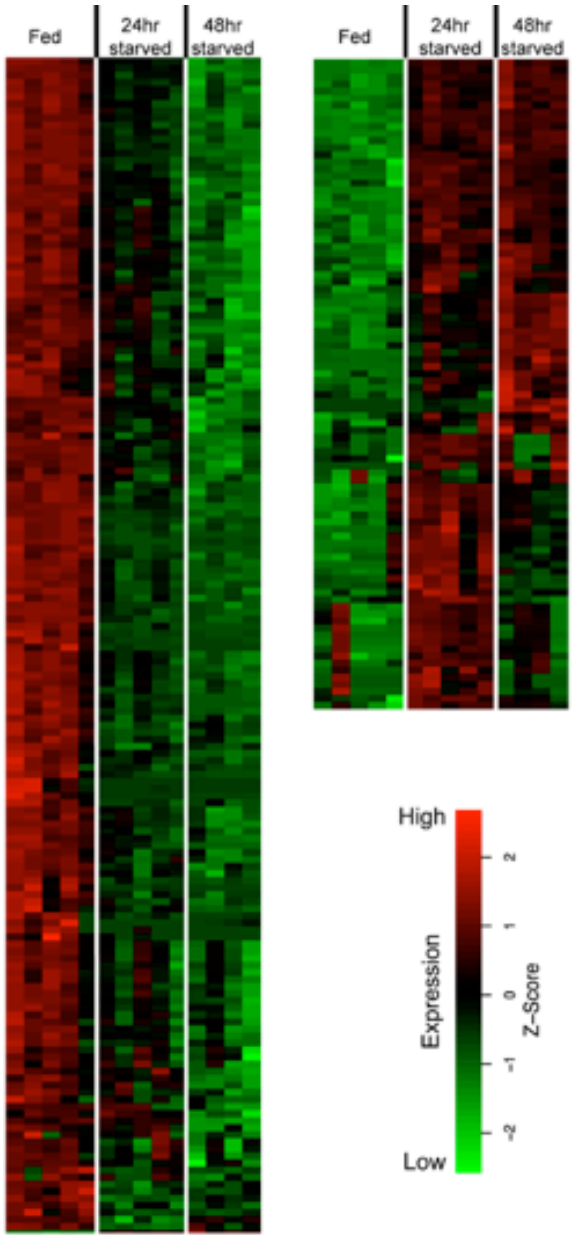

Figure 11 Gene expression in the head is regulated by feeding state

Shown are relative levels of gene expression for all genes that showed changes in expression in the head after 24 or 48 hours of fasting. Each vertical column is a biological replicate. White bars separate data from before fasting, after 24 hours of fasting, and after 48 hours of fasting. Most genes show decreased expression after 24 and 48 hours of fasting.

A standard approach to assess the relevance of a microarray study, or to look for interesting genes in large gene lists, is to look for enrichment of genes in the microarray results list, as compared to all genes in the genome (Dennis et al., 2003). We used the Database for Annotation, Visualization, and Integrated Discovery (DAVID) program, provided free by the NIH, to look for enriched genes in our study (Huang da et al., 2009). Our results, shown in Table 1, show that certain classes of genes are enriched in our list of genes that are regulated by feeding. We find that genes related to immune function and genes related to lipid metabolism, two systems that are closely associated with food intake, have expression levels regulated by nutritional status (Chandra, 1997).

Table 1

Functional groups of genes regulated by feeding in *Drosophila* head tissue			
	Gene Function	# of genes	enrichment P-value
1	immune response	19	0.0002
2	lipid metabolism	19	0.0007
3	metabolism	14	0.005
4	cuticle structure	10	0.007
5	metal ion binding	49	0.03
6	chemosensation	19	0.09

Of the 247 head genes that are affected by nutritional status, 151 genes showed decreased expression after fasting, while the remainder showed increased expression after 24 hours of fasting. We reasoned that genes with

expression levels that increase upon fasting might be candidate hunger signals, while genes whose expression levels decrease with fasting might be candidate satiety signals. We also found 74 out of 247 genes that represent non-annotated transcripts with no known function. Of the previously annotated genes, 10 are putative or known transcription factors, 12 are putative or known receptors, and 18 have been shown by the Pankratz lab to be regulated by feeding in larvae (Zinke et al., 2002).

We selected a subset of genes from the microarray results for behavioral analysis. Most of these genes were found to be regulated by feeding status exclusively in the head, while some were found to be regulated in the body (Figure 12). We obtained RNAi lines for these 174 genes for use in a CAFE-based behavior screen to look for novel regulators of post-fasting feeding behavior.

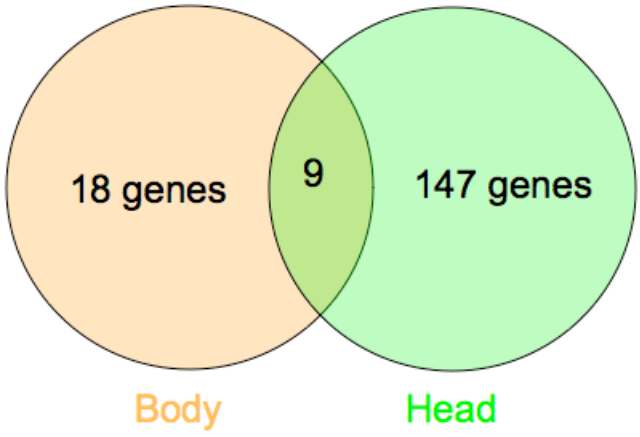

Figure 12 Genes were selected for an RNAi screen to look for feeding behavior mutants. Genes were selected based on putative function, protein prediction, or because they were novel genes with no known function. 147 genes were exclusively regulated by feeding status in the fly head.

4.3.2 A proof-of-principle experiment shows that the six-hour CAFE can identify genes that control feeding behavior

Our studies in wild-type *Drosophila* (section 3.3.2) indicated that post-fasted flies consumed significantly more food in six hours than pre-fasted flies, and that this difference was quantifiable using the CAFE. Takeout, a putative hormone binding protein, was previously shown to control feeding in adult flies. We wondered whether flies with altered expression of a known feeding related gene, *takeout*, would behave differently from control flies in the CAFE. Using a neuron specific driver (Elav-Gal4) to drive expression of RNAi against *takeout* in neurons and measured absolute food intake over six hours of feeding, once before and once after 24 hours of fasting (Figure 13A).

We found that control flies (Elav-Gal4 alone and UAS-takeout-RNAi alone) increased consumption by 75-92% (0.16-0.19 microliters) after fasting (Figure 13B). However, flies that have decreased neuronal expression levels of *takeout* did not increase their food consumption after fasting. We conclude that pan-neuronal knockdown of *takeout* abolishes the post-fasting increase in food intake in *Drosophila*. This experiment establishes the CAFE as an assay capable of identifying genetic modifiers of food intake behavior.

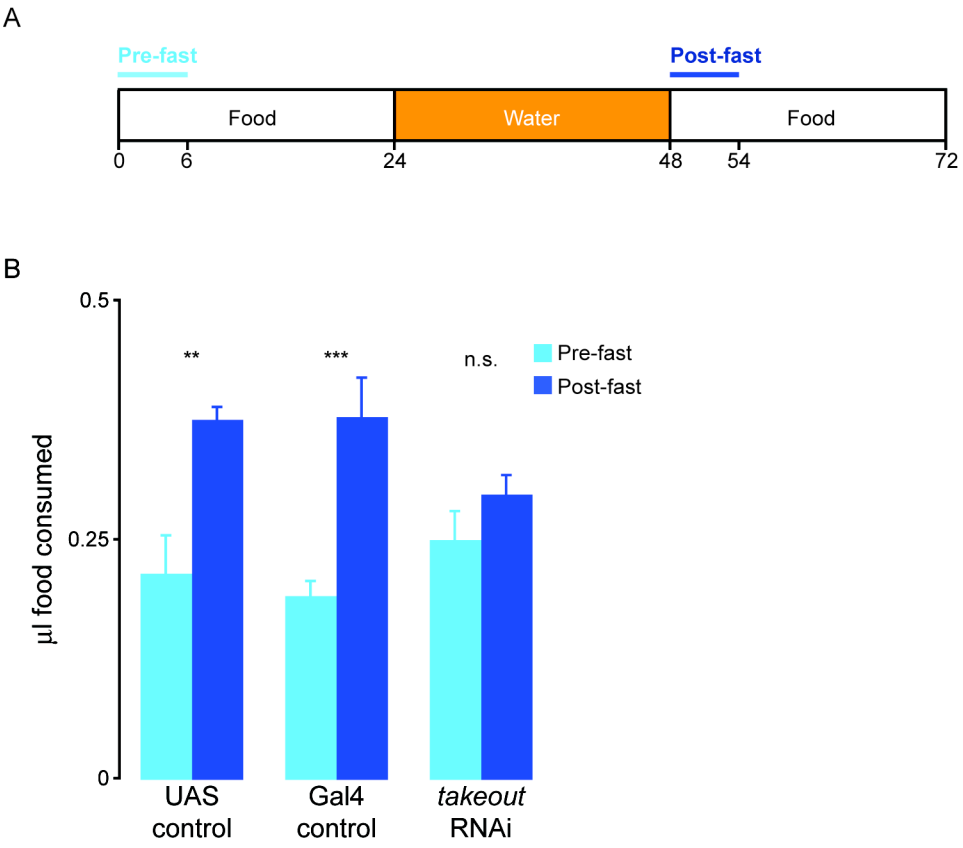

Figure 13. Six hour feeding measurements can identify feeding mutants

(A) Timeline of feeding measurements to identify flies defective in post-fasting feeding response. Food intake was measured at 6hr and 54hr to determine pre-fast and post-fast food consumption.

(B) Flies that have decreased takeout expression in neurons show defective post-fasting food intake. Pre-fasting food intake is not significantly different in the mutant flies, compared to controls (light blue bars). **p<.01, ***p<.001. n = 7-10 CAFEs.

4.3.3 A targeted screen to identify novel regulators of feeding behavior

4.3.3.1 RNAi lines chosen for screening

We next asked if genes identified in our microarray screen are involved in feeding behavior by carrying out a pilot RNAi screen. A total of 395 RNAi lines representing 162 feeding candidate genes were obtained from the Vienna *Drosophila* Resource Center (VDRC) for use in the screen. These genes were selected from the complete list of genes generated by our microarray on the basis of the following criteria: all known/putative transcription factors (10 genes); receptors (12 genes); intracellular signaling molecules (2 genes); Novel genes (74 genes); secreted proteins. Eight genes overlapped with a previous study of fat-body specific transcripts (Jiang et al., 2005), and 18 were previously found to be regulated by nutritional status in larvae (Zinke et al., 2002). In addition, the majority of the genes we tested (147) were regulated by feeding only in head tissue, while the remainder were regulated by feeding in the body alone, or in the body and the head.

4.3.3.2 Genes that may influence the post-fasting response in food intake

We compared food intake after six hours of feeding before and after fasting, and looked for RNAi genotypes that had a significantly decreased post-fasting food intake when compared to control flies. RNAi lines were screened under the control of a pan-neuronal driver, Elav-Gal4. Control flies (Elav-Gal4 x W1118) have had a post-fast food increase of approximately 0.25 μl. This means

they consumed 0.25 μl more after fasting as compared to before fasting (Figure 14, black bar). As expected, flies did not increase food intake after a mock fast in which they experienced the same CAFE conditions as fasted flies, but received liquid food while other flies were being fasted (Figure 14, grey bar).

337 RNAi lines were tested in the primary screen. Of these, 59 (18%) showed a significantly different post-fasting food intake response than controls. This corresponds to a False Discovery Rate (FDR) of 0.3. Since we are primarily interested in genes that reduce the post-fasting feeding response, I focused on the mutants that had significantly *lower* increase in food intake after fasting, as compared to control flies. 37 lines fit this criterion. I re-tested 18 of these lines, and 11 remained positive hits (61% of re-tested lines). These 11 RNAi lines represent 11 distinct genes. I then tested multiple hairpins and RNAi insertions (when available) for these 11 genes (Figure 12). Two of the genes (*CG6129* and *CG17032*) that we found to have a mutant post-fasting response were previously found to have transcript levels regulated by nutritional status (Fujikawa et al., 2009).

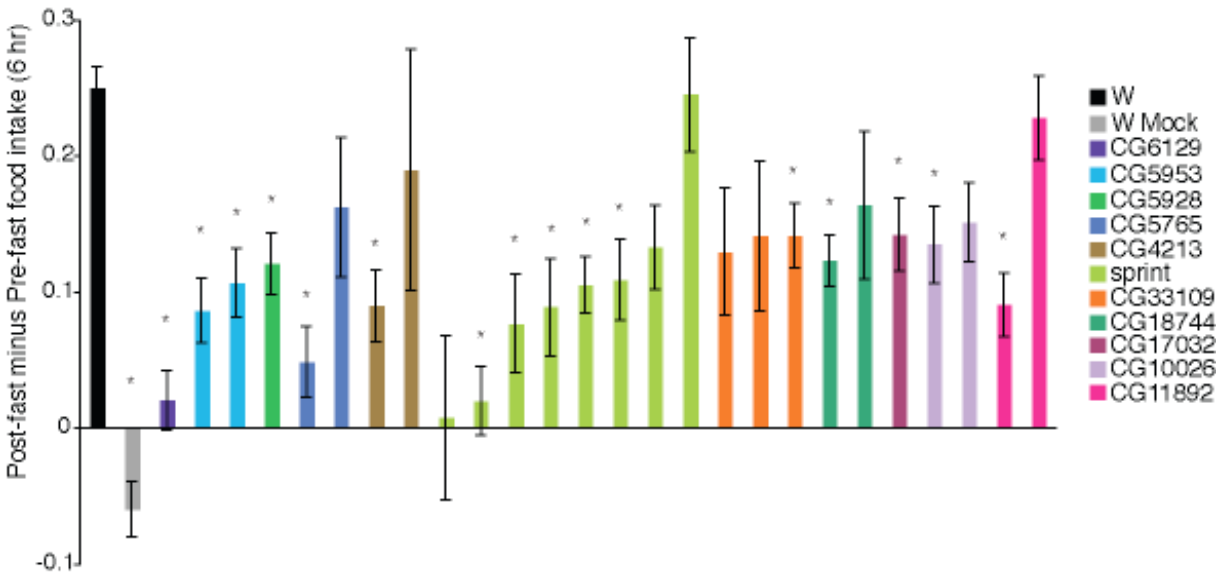

Figure 14. Post-fasting food intake in control and RNAi flies.

11 genes showed significantly reduced post-fasting food intake. Control flies (black bar) increase food intake by 0.25 ml. For 2 genes (*CG5953* and *sprint*) results were confirmed by multiple hairpins that were significantly different from control. *p<0.05, t-test with Bonferroni correction for multiple testing. n = 5-25 CAFEs per genotype, with 5 flies per CAFE.

We next asked whether these 11 genes were essential for normal feeding, or if disrupting them had a specific effect on the post-fasting response of food intake. We looked at normal, *ad libitum* eating over 24 hours in the CAFE, before fasting, and found that these RNAi genotypes do not consume significantly more or less than control flies under normal, non-fasting conditions (Figure 15). Therefore we concluded that these 11 genes do not grossly affect normal eating, and that their effect is specific to the post-fasting increase in food intake.

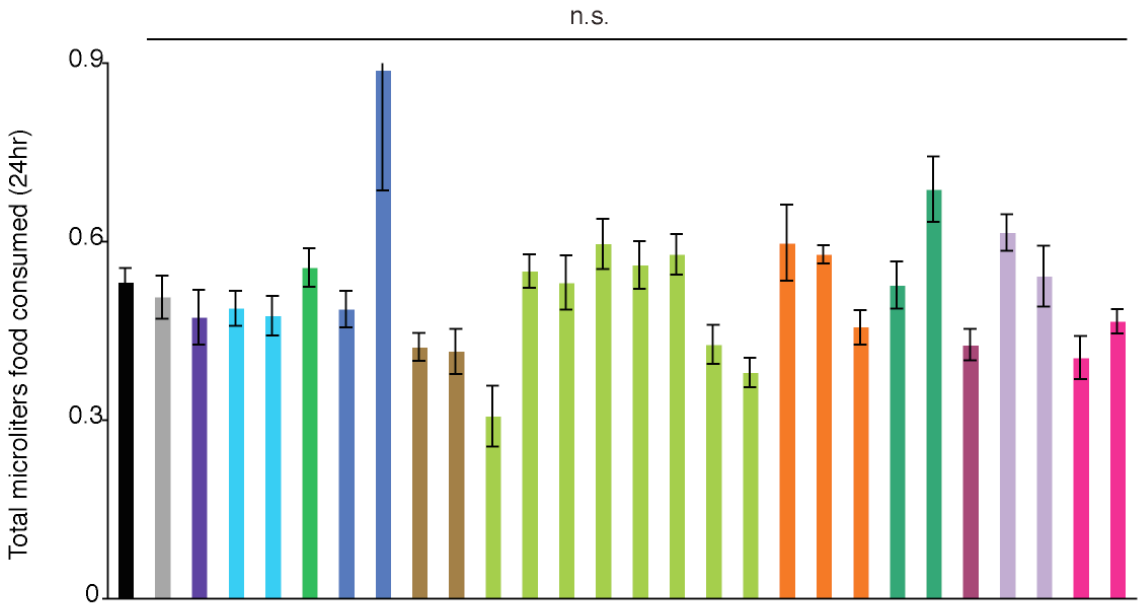

Figure 15. RNAi disruption of post-fasting food intake does not affect normal eating under *ad libitum* conditions.

Shown is total food consumption, per fly, over 24 hours for flies with RNAi knock-down of eleven candidate genes. Colors refer to the same genes as in figure 11. Multiple bars per color signify different hairpins or hairpin-insertions for each gene, where available. RNAi genotypes do not consume significantly more food than control flies. n.s., not significant, t-test with Bonferroni correction for multiple comparisons. n = 5-25 CAFEs per genotype, with 5 flies per CAFE.

Although we screened the known adult-feeding related gene *snpf*, this does not appear as a positive hit in our screen. This may be because *snpf* mutants significantly increase food intake within 10 minutes of feeding after a fast (Lee et al., 2004), and we measured food intake after 6 hours. Therefore any increase during the first ten minutes may be "diluted" by the larger total

consumption in six hours. We also did not find an overall increase in food intake in snpf-RNAi flies, which is not concordant with results described in Lee et al. (Lee et al., 2004). One possible reason for this discrepancy might be that we are using a different pan-neuronal driver than was used in previous snpf experiments.

The eleven genes we have thus far identified are varied in their known and putative function (Table 2). Four are insect-specific genes with no known function. Others have invertebrate and vertebrate homologues, but their role in feeding is unknown and is difficult to predict based on predicted protein domains. CG5953 contains a DNA binding domain and may represent a novel transcription factor that, like *sugarbabe,* is active based on nutritional status.

Table 2

Gene	Protein features	Homology	Fold change Head (24hr fasted)	Fold change Body (24hr fasted)
CG6129*	limited Drosophila studies show CG6129 only in chordontal neurons	49% rootletin (mouse)	-4	
CG5953	DNA binding domain	insect specific	5	3
CG5928	no known domains	insect specific		-5
CG5765	mucin (part of protective matrix)	55% proteoglycan 4 (mouse)		-7
CG4213	no known domains	insect specific	2.5	2
sprint	Ras-activated Rab5 guanine exchange factor.	51% RIN-1 (human)	10	6
CG33109	no known domains	insect specific	9	
CG18744	no known domains	44% arrestin domain containing (human)		-8
CG17032*	glutamine/asparagine (Q/N)-rich ('prion') domain	C. elegans: pqn-75	-2.5	-2
CG10026	SEC14 domain: lipid binding	51% clavesin 1-retinaldehyde binding protein (human)	-5	
CG11892	choline kinase domain	C. elegans PK-C like proteins		-5

Two candidates, *CG6129* and *sprint*, are most interesting because of their homology to known vertebrate genes, and we focused the remainder of our study on these two genes. When we test the UAS-RNAi alone for hairpins against these genes, we find that they do not show a significantly different behavior from controls (Figure 16). Therefore we conclude that the RNAi phenotype we see for these two genes is not due to an insertional effect of the UAS-RNAi construct.

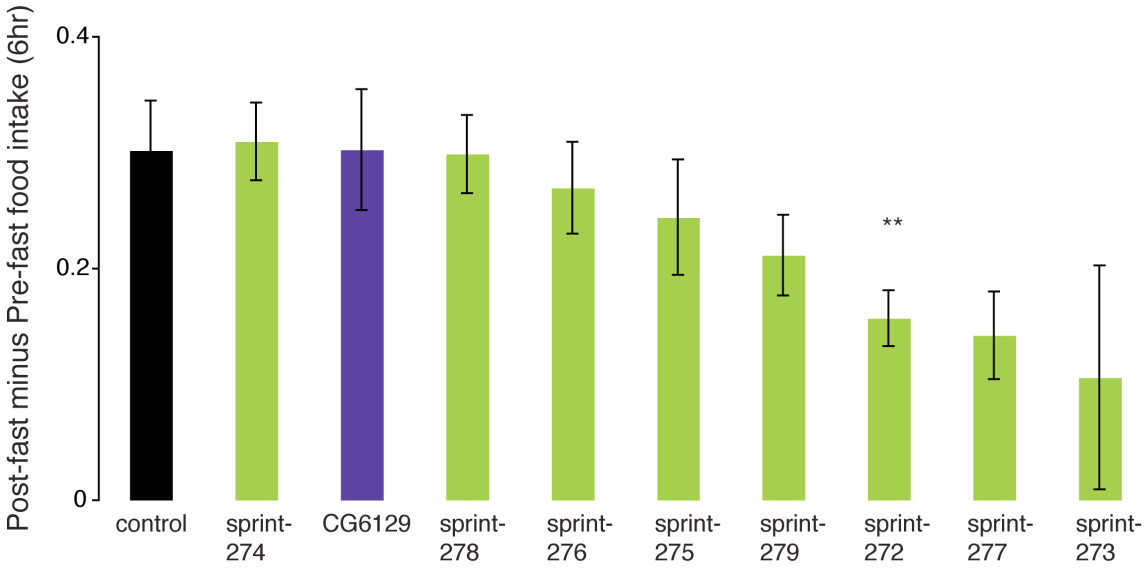

Figure 16. UAS-RNAi control data for *CG6129* and *sprint* RNAi lines.

UAS-RNAi lines were tested alone, without being driven by a promoter-Gal4. With the exception of UAS-RNAi-sprint line 272, none showed a significantly different post-fasting feeding phenotype from control. ** $p<0.01$ t-test with Bonferroni correction.

4.3.3.3 CG6129 and sprint are conserved genes that may regulate post-fasting food intake

CG6129 is the *Drosophila* homologue (49% protein similarity, 27% identity) to the vertebrate gene *rootletin*. Rootletin is a component of the ciliary rootlet, which is a cytoskeleton feature of some ciliated cells. The exact function of the rootlet is now known. However, in some ciliated cells it couples closely with the mitochondria (Olsson, 1962), suggesting a role beyond simple structural support. In mammalian cells, rootletin helps maintain centrosome cohesion at various points in the cell cycle (Bahe et al., 2005). Rootletin has been most extensively studied in mouse photoreceptor cells, which are large, ciliated, and have a prominent rootlet (Yang et al., 2002). Mice that are mutant for the *rootletin* gene have photoreceptor cells that are completely devoid of a rootlet (Yang et al., 2005). Moreover, these mice show susceptibility to lung disease, and their photoreceptors degenerate early, suggesting that rootletin protein and the rootlet are essential for normal ciliary function over the life of a ciliated cell (Yang et al., 2005).

In flies, the only known ciliated cells are type-I sensory neurons, and sperm (Han et al., 2003). Photoreceptor cells are non-ciliated in *Drosophila*. Primary cilia are located at dendritic ends of sensory neurons, where they capture and transmit signals from environmental mechanical or chemical stimuli (Dubruille et al., 2002). Laurencon et al. found expression of a CG6129::GFP

fusion in ciliated chordotonal neurons in *Drosophila* (Laurencon et al., 2007). Although cilia have not directly been implicated in feeding behavior, their presence on sensory neurons, including those that detect stretch, makes them an appealing candidate for further study of feeding regulation.

Another interesting candidate from our behavioral screen is *sprint*, a *Drosophila* homologue of *RIN-1*, Ras-interacting protein (51% protein similarity, including a conserved SH2 domain). In mammals, RIN-1 binds to activated Ras, a membrane-associated G-protein that couples with receptor and non-receptor tyrosine kinases (Han et al., 1997). RIN-1 can bind to and activate ABL kinases, which are ubiquitously expressed and mediate cytoskeletal remodeling (Hu et al., 2005). RIN-1 can also activate the GTPase Rab5, thereby promoting RAB5-mediated endocytosis of cell-surface receptors (Barbieri et al., 2003). All of this suggests that RIN-1 may function in multiple capacities to affect receptor endocytosis and cellular remodeling.

In addition to its role in receptor trafficking, RIN-1 might also affect neuronal plasticity. In mice, RIN-1 expression turns on in adults, and the highest levels of expression are found in the mouse forebrain (Dhaka et al., 2003). A role for RIN-1 in synaptic plasticity has been suggested (Bliss et al., 2010), as behavioral and electrophysiological evidence shows that RIN-1 knock-out mice display elevated amygdala function in response to stimulation (Dhaka et al., 2003).

The sprint locus in *Drosophila* is complex and large (90kb), and is

predicted to encode at least two distinct transcripts (Szabo et al., 2001). In addition to their localization in the embryonic midgut and in the embryonic CNS (Szabo et al., 2001) *sprint* gene products are localized to migrating border cells. *Sprint* has been shown to have role in promoting receptor endocytosis in border cells, (Jekely et al., 2005), a small cluster of cells that directionally migrate toward oocytes during *Drosophila* oogenesis (Rorth, 2002). The cells contain two receptor tyrosine kinases, PVR and EGFR, which can sense chemical cues that are released by the oocytes. Upon stimulation by ligand, the receptors initiate a cascade that includes recruitment of cytoskeleton molecules to help the border cells move toward the target oocytes. *Sprint* helps maintain the spatial localization of the receptor tyrosine kinase signal by promoting endocytosis of the receptors during the migration process (Jekely et al., 2005). One hypothesis for how sprint affects feeding behavior, then, is through a possible role in endocytosis of a receptor for a hunger or a satiety signal.

4.4 Conclusion

By conducting a microarray, we were able to identify 247 transcripts that are regulated by nutritional status. This enabled us to undertake a targeted screen for potential regulators of post-fasting food intake, a feeding behavior that is tied to nutritional status. Our screen uncovered several novel and interesting candidate genes that are ripe for further mechanistic studies.

5 Implications of the current study and prospects for future research

This dissertation examines two distinct feeding modalities: blood-feeding behavior in mosquitoes and post-fasting feeding behavior in vinegar flies. The first occurs only in female mosquitoes, which do not need blood for their own survival but only to complete the maturation of their eggs, while the latter is essential for survival of flies. These two types of insect feeding have behavioral features in common. First, in both cases, classical studies suggest that the animal undergoes a behavioral switch after it has taken in a meal such that it is less interested in eating again for a certain period of time. Second, both the mosquito and the fly seem to display a change in their response to external stimuli, such as the presence of food, after food intake. And in both cases, the genes and circuits behind the observed behaviors are largely unknown.

We hypothesized that changes in feeding behavior that are dependent on nutritional status might be influenced by corresponding changes in gene expression. This does not exclude the possibility that protein modifications also influence the specific behaviors we are studying, and indeed it is likely that both types of genetic regulation occur. However, by focusing our efforts on microarray analysis and genetic screening, we identified possible transcriptional regulators of feeding behavior.

In the mosquito, we focused on transcriptional changes that occur in the antenna, since we were looking for novel regulators of olfactory-driven feeding behavior in this animal. By looking at transcriptional changes in olfactory tissue

and comparing it to transcriptional changes in the body as a whole, we were able to identify genes that are regulated by blood-feeding only in the antenna, and that are not just general, body-wide effects of blood-feeding.

By looking at changes in antennal gene expression based on blood-feeding status, we hypothesized that we would uncover new molecules that receive or transduce an odor signal. Our study of gene expression in post-blood fed mosquitoes is the first to look at transcriptional changes in olfactory-specific tissue that are dependent on feeding behavior.

We identified two odorant receptors, GPROR56/57 and GPROR69, whose expression levels are regulated by blood-feeding. Since the mosquito is no longer attracted to host odor after blood-feeding, it may respond by down-regulating expression of receptors that are necessary to sense host odor. Therefore, we hypothesized that since expression levels of these two receptors goes down after blood-feeding, these receptors may recognize host odors. A recent study by the Carlson and Zwiebel groups found odors that activate most *Anopheles* odorant receptors, including GPROR56/57 but not GPROR69 (Wang et al., 2010). Of the six odorants that were found to elicit a strong response in GPROR56/57, none are known to be a component of human emanations. Perhaps there are other odorants, not tested in this study, that elicit a response in this odorant receptor, and which are important in helping the mosquito locate an animal host. Alternatively, these odorant receptors may not be important for finding a host, but for another behavior that occurs coincidentally with host-seeking, such as mating.

Further work should be carried out to identify new ligands that activate GPROR56/57 and GPROR69. Moreover, with new genetic tools available in the mosquito, it should be possible to knock-out or to down-regulate these receptors and observe the effects on host-seeking in mutant animals.

Our initial results in *Anopheles gambiae* were important early steps in establishing the mosquito as a viable organism for studies of molecular effects downstream of blood-feeding. Follow-up work should continue in *Aedes aegypti*, a mosquito species that is easier to rear in the laboratory, that displays cyclical blood-feeding behavior, and for which a complete genome is available. To begin, the question of whether OR expression or trafficking is modulated by nutritional status remains an open one, and warrants further study. Our work in the mosquito shows that gene expression of at least two ORs is influenced by blood-feeding status. The next step would be to determine whether this change in gene expression corresponds to a selective increase or decrease in sensitivity to odor in neurons that house these ORs. Single sensillum recordings of mosquito antenna should be done to answer this question. Since our lab and others have established robust *Aedes aegypti* colonies, it will be possible to extend this technique from *Drosophila* to *Aedes* and to record from neurons that express these odorant receptors, once the neurons have been identified through localization studies.

In addition to our results showing that two odorant receptor genes are regulated by blood-feeding, our microarray experiments in mosquito antennae

also identified a member of the *takeout* family as being expressed in the antenna and regulated by blood-feeding status. *Takeout* was previously found to be regulated by feeding in *Drosophila*, and it is also expressed in the *Drosophila* antenna. *Takeout* shows sequence homology to Juvenile Hormone Binding Protein, and it is predicted to contain a signal peptide sequence. Therefore, members of the takeout family of proteins might function by binding to juvenile hormone, or, they may be binding to a different class of molecules. One possibility is that takeout family proteins might bind directly to odors and act as odorant binding proteins. A study of one member of the takeout family in *Phormia regina* found that the protein is secreted into the sensillar lymph by support cells in the blowfly's antenna (Fujikawa et al., 2006). Our preliminary experiments show that members of this gene family are expressed in support cells in olfactory sensilla in *Drosophila* (data not shown). This supports the idea that takeout family proteins may act as odorant binding proteins.

Although it is not known how or if takeout proteins act in the olfactory system, the fact that members of this gene family are regulated by nutritional status in both mosquitoes and flies, and that they are expressed in the mosquito and fly antenna, suggests an important role for this uncharacterized family in regulating the olfactory system in relation to feeding status. Takeout might therefore be a clue to a mechanistic link between regulation of blood-feeding in mosquitoes and regulation of eating in flies. It suggests that, in both systems, a binding protein is produced in response to nutritional status, and circulates in

hemolymph to act of tissues that are important for feeding. This hypothesis should be further examined by looking at the entire takeout family in both *Drosophila* and *Anopheles*.

The question remains, in both *Drosophila* and in *Anopheles*, of how circulating hemolymph alters feeding behavior. A simple but untested hypothesis is that neurosecretory cells in the insect gut release hormones or peptides in response to food intake, which circulate in the hemolymph and act on the brain or on peripheral sensory organs to control feeding. Klowden began to address this question in studies of hemolymph transfer from blood fed to non-blood fed mosquitoes (Klowden and Lea, 1979b). He found that mosquito hemolymph recipients acted as though they, too had taken a blood-meal, and altered their behavioral response to host-stimuli (Klowden and Lea, 1979b). In that case, he was able to narrow the source of the secreted hormone or peptide to the mosquito ovaries. A similar phenomenon may be occurring in *Drosophila* after food intake, whereby the gut or the fat body releases a satiety signal that circulates to reach the CNS.

Our microarray studies in *Drosophila* identified a neuropeptide receptor, Gonadotropin-releasing hormone receptor (GRHR), as well as a putative neuropeptide, CG13056, that are regulated by fasting in the fly. Perhaps this neuropeptide is secreted in the hemolyph in relation to feeding status, and acts on the brain to affect behavior. Likewise, GRHR may be a candidate for a neuropeptide receptor that acts in the brain to affect feeding. Future studies

should also attempt to isolate neuropeptides that are present in hemolymph in different nutritional states, perhaps through mass spectrometry.

Our results in mosquitoes and in flies indicate that the mechanism behind nutritional dependent changes in olfactory sensitivity differs between *Anopheles* and *Drosophila*. In *Anopheles*, we found indirect evidence that the peripheral olfactory system was altered by nutritional status, because certain ORs, expressed in peripheral sensory neurons, are regulated by feeding. This suggests that changes in the amount of particular odorant receptors at the sensory dendrite affect behavior. On the other hand, our electrophysiology studies in *Drosophila* suggest that peripheral sensitivity to odor remains unaltered by nutritional status, and that the change in olfactory attraction to odor is not due to a change in odor sensitivity in the periphery.

Our results in *Drosophila* suggest that it is more likely that behavioral changes tied to feeding state are induced by changes in parts of the olfactory system that are further downstream from sensory dendrites. How could this be? One possibility is that the antennal lobe, or a higher brain center involved in decision-making, globally becomes more excitable in fasted animals, leading to increased behavioral attraction to odor in the fasted state. Another possibility is that only certain glomeruli in the antennal lobe have enhanced activity in the fasted state. In either of these cases, the neurons might be more excitable due to a circulating neuropeptide that signals nutritional state. Another possibility is that peripheral sensory neurons *do* have increased activity in the fasted state, but

that the increased activity is due to a receptor at the pre-synaptic, axonal end of the neuron rather than at sensory dendrites. Olfactory sensory neurons project to the antennal lobe, thus a circulating peptide might act on receptors localized to the presynaptic terminal of the OSN axon to amplify the neuronal signal as it is passed to projection neurons in the antennal lobe.

Further studies in both insects should continue to probe the question of which parts of the olfactory system are susceptible to modulation by hunger or satiety signals. To begin, our studies did not exhaustively examine all possible sensilla that respond to 3MTP. Before ruling out sensory dendrite as the source of increased sensitivity to this odor in fasted animals, further electrophysiological studies must be carried out on all sensilla that respond to this odor. Next, the antennal lobe should be imaged in response to odor in the fed and in the fasted state. Genetic tools are available in *Drosophila* to produce flies with genetically encoded fluorescent indicator in antennal lobe neurons. These flies can then be examined under two photon calcium imaging to measure projection neuron responses to odor in flies that are fed or fasted. This can be a starting point to look for CNS changes in response to odor in different nutritional states.

The second part of this thesis quantitatively examined feeding behavior in post-fasted flies and found that disruption of at least eleven genes leads to a significant decrease in the normal post-fasting food intake response. The precision with which we are able to quantify the increase in post-fasting food intake in flies highlights the benefits of using *Drosophila* as a model system for

feeding behavior. Moreover, the ease of gene expression analysis and genetic screens in this species allowed us to find novel candidates for regulators of feeding behavior that would not have been identified otherwise.

Two genes that were not previously known to be associated with feeding behavior, and which present intriguing connections between other cellular processes and feeding, are *CG6129* and *sprint*. *CG6129*, a *rootletin* homologue, is an exciting candidate for further study. Although there is no clearly understood connection between cilia dysfunction and feeding behavior, some diseases of cilia, or ciliopathies, lead to obesity, which strongly suggests a link between cilia and body-weight regulation in mammals (Sen Gupta et al., 2009). Bardet-Biedl Syndrome and Alstrom Syndrome are two human ciliopathies in which patients often show obesity in addition to other symptoms resulting from dysfunctional cilia. In these two diseases, the obesity may be caused by hyperphagia, as demonstrated by mouse models of these diseases (Arsov et al., 2006; Rahmouni et al., 2008). Although these are relatively rare genetic diseases, they suggest a strong link between cilia and obesity. This opens up a new avenue for researching the origins of obesity in the general population, which we can begin by studying the link between cilia and feeding behavior in simple animal models.

One way in which defective cilia may lead to abnormal feeding behavior is through a disruption in normal signaling cascades that depend on the cilia for localization of receptors. Without functional cilia, receptors that normally localize to ciliary membranes (including several GPCRs and possibly some serotonin

receptors) cannot traffic properly. Therefore, resulting behavior or physiological defects in ciliopathies are often due to disrupted signaling pathways (Veland et al., 2009). In the case of feeding behavior, one hypothesis is that cilia may be important for mechanosensory neurons on the gut to detect stretch from food intake, and that disrupted cilia are on sensory neurons that are particularly important for sensing hunger or satiety can lead to defective eating behavior. In this case, mechanosensors would be unable to detect stretch, and the animal would not properly sense food intake.

Another novel potential regulator of feeding behavior that we uncovered through microarray analysis and subsequent RNAi screening is *sprint*. *Sprint* was previously shown to function in *Drosophila* by playing a role in endocytosis of receptor tyrosine kinases in migrating border cells of the embryo. We hypothesize that*Sprint* might likewise affect feeding behavior through its role in receptor endocytosis. *Sprint* might regulate feeding behavior in normal flies through endocytosis of a receptor that mediates a hunger or satiety signal. Under this scenario, *sprint* RNAi mutants would fail to be properly endocytose the (as yet unknown) receptor, leading to disruptive signaling activity in the cell. The insulin receptor is one such candidate for a receptor that might be regulated in this way. It is already known that the insulin receptor undergoes endocytosis upon insulin stimulation (Khan et al., 1989). Furthermore, RIN-1, the mammalian homologue of sprint, is involved with endocytosis of the insulin receptor in cell culture (Hunker et al., 2006), so it is conceivable that the role is conserved in

Drosophila sprint. Further experiments are needed to show if an interaction between *sprint* and the insulin receptor occurs in adult *Drosophila.*

Alternatively, *sprint* may modify feeding behavior through its role in shaping neurons. In the adult mouse brain, RIN-1 helps shape synaptic connections through selective endocytosis of Eph receptors, which are important for interactions between neighboring cells (Deininger et al., 2008), and which are conserved in *Drosophila*. The *sprint* RNAi mutants we generated may have defective synaptic wiring in neuronal circuits that are important for the post-fasting feeding response. If this is true then localization studies of *sprint* in adult brains will be highly useful in identifying neuronal populations that drive feeding behavior.

Although this dissertation focuses on feeding behavior in two simple organisms, the mosquito and the fly, a biomedical scientist's ultimate goal is to better the human condition through discoveries that shed new light on human disease. In this case, the simple feeding behaviors of insects are relevant to two different types of human disease: vector-borne illnesses such as malaria, and metabolic disorders such as obesity.

Insect-borne diseases have a profound impact on world health both through human diseases such as malaria and yellow-fever, and through the devastation of crops that are important for food production. In both cases, it is the insect's feeding behavior that ultimately leads to infection and spread of

disease. By understanding the genes that are involved with regulating feeding behavior in mosquitoes, we may uncover novel targets for drugs that can confuse the mosquito's normal drive for blood-feeding. More specifically, by uncovering one or two odorant receptors that are especially important for host-seeking, we can target these receptors in a small molecule screen that looks for inhibitors of mosquito OR function.

Studying feeding behavior in insects, and especially in the genetically tractable fly, can also help us uncover new genetic pathways that are involved in diseases related to food intake. Obesity continues to be an important and increasing threat to global health. Disorders of food intake have largely been studied in humans and in rodent models, and this has been a worthwhile approach, as such studies have uncovered some of the central regulator of appetite in humans, including leptin and NPY. However, simple organisms such as the fly are emerging as powerful models for uncovering and understanding new genes that are important for feeding regulation. Our unbiased microarray in *Drosophila* illustrates the utility of using flies to find genetic pathways related to obesity. We found that a component of the ciliary rootlet is important for normal feeding regulation. We can therefore use *Drosophila* to understand the mysterious connection between cilia and food intake, which was previously recognized through disorders like Bardet-Biedl, but which remains poorly understood. Further studies of feeding behavior in *Drosophila* should reveal

other conserved genes that regulate appetite, and which may lead to novel therapeutics for obesity.

In 1976, Vincent Dethier, a pioneer in the study of insect feeding wrote: “Since no evidence has been found that an endogenous neural center drives feeding behavior, the peripheral chemosensory receptors…constitute the sole source of excitatory input that initiates and drives feeding” (Dethier, 1976). In the thirty-four years since Dethier wrote *The Hungry Fly*, *Drosophila* has been used to understand complex behaviors such as courtship, aggression, and olfactory learning (Vosshall, 2007). The time is ripe to extend the tools available to *Drosophila* geneticists to locate the endogenous neural centers that drive feeding. This is crucial to our understanding of an essential component of animal behavior, and the knowledge we gain in an insect model has the potential to aid in future studies of feeding in disease vectors as well as in humans.

Publications

In Preparation:

Farhadian, S.F., Cho, C.E., Pellegrino, M.P., and Vosshall, L.B. (2010). Post-fasting feeding and olfactory responses in *Drosophila.*

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