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Brain regions and molecular pathways responding to food reward type and value in honey bees

M. S. McNeill[†,‡,§,1], K. M. Kapheim[*,†,‡,¶,1], A. Brockmann[†,], T. A. W. McGill[†] and G. E. Robinson[†,‡,§]**

[†]Department of Entomology, [‡]Carl R. Woese Institute for Genomic Biology, [§]Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, IL, [¶]Department of Biology, Utah State University, Logan, UT, USA, and [**]National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

[1] *These authors contributed equally to this work.*

**Corresponding author: K. M. Kapheim, Department of Biology, Utah State University, 5305 Old Main Hill, Logan, UT 84341, USA. E-mail: karen.kapheim@usu.edu*

The ability of honey bees to evaluate differences in food type and value is crucial for colony success, but these assessments are made by individuals who bring food to the hive, eating little, if any, of it themselves. We tested the hypothesis that responses to food type (pollen or nectar) and value involve different subsets of brain regions, and genes responsive to food. mRNA *in situ* hybridization of *c-jun* revealed that brain regions responsive to differences in food type were mostly different from regions responsive to differences in food value, except those dorsal and lateral to the mushroom body calyces, which responded to all three. Transcriptomic profiles of the mushroom bodies generated by RNA sequencing gave the following results: (1) responses to differences in food type or value included a subset of molecular pathways involved in the response to food reward; (2) genes responsive to food reward, food type and food value were enriched for (the Gene Ontology categories) mitochondrial and endoplasmic reticulum activity; (3) genes responsive to only food and food type were enriched for regulation of transcription and translation; and (4) genes responsive to only food and food value were enriched for regulation of neuronal signaling. These results reveal how activities necessary for colony survival are channeled through the reward system of individual honey bees.

Keywords: *Apis mellifera*, dopamine, ecology, honey bee, *in situ* hybridization, mushroom bodies, reward system, RNAseq, social behavior, WGCNA

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Brain regions involved in reward processing are collectively known as the 'reward system', and they are involved in orchestrating behavioral responses to various rewarding stimuli such as food and reproductive activity. The reward system was first described in mammals (Olds & Milner 1954) and has since been studied primarily in the context of behaviors linked to individual benefit. There is evidence from primates that reward systems also are involved in the regulation of cooperative behavior (De Waal *et al.* 2008). Honey bees and other social insects are well known for many intricately coordinated cooperative behaviors, but it is not known how reward systems are involved in regulating these behaviors.

An animal's response to reward is complex, inducing changes in internal state that activate behavioral outputs. How neuroanatomical and neuromolecular features of each of these aspects interact as part of the entire reward response process is largely unknown in insects. Several insect brain regions are known to respond strongly to sucrose ingestion. These include the antennal lobes, gnathal ganglion (formerly known as the subesophageal ganglion), lateral protocerebrum, mushroom bodies and antennal mechanosensory and motor centers (Giurfa & Sandoz 2012; Waddell 2010). Recently, the rind (*i.e.*, somata-containing regions) lateral to the gnathal ganglion (formerly known as the lateral subesophageal ganglion), rind lateral to the lateral calyx (dorsal optic lobe), rind dorsolateral to the lobula (dorsal optic lobe), rind ventrolateral to the lobula (ventral optic lobe), and rind dorsal to the superior posterior slope (dorsal posterior protocerebrum) also were shown to be responsive to sucrose (McNeill & Robinson 2015). For some of these regions, direct connections have been experimentally shown between sensory processing and behavioral output (Erber *et al.* 1980; Hammer & Menzel 1998; Komischke *et al.* 2005). Recent studies have suggested that rewarding and aversive sensory stimuli are integrated in the mushroom bodies, and mushroom body output neurons carry the combination of higher order valence and internal state information required for correctly selecting a behavioral output (Aso *et al.* 2014a,b; Giurfa 2013; Menzel 2014; Strube-Bloss *et al.* 2011).

Molecular analyses of insect reward have revealed the involvement of a number of key molecules. These include the neuromodulators dopamine and octopamine (Søvik *et al.* 2015; Waddell 2013); though, it remains unclear how information carried by octopamine (OA) and dopamine (DA) is translated into the complex network of molecular pathways that also mediate reward. In addition, several neuropeptides including neuropeptide F (Brockmann *et al.* 2009; Shohat-Ophir *et al.* 2012), a variety of intracellular signaling molecules including protein kinase G and protein kinase A (Kaun & Sokolowski 2009; Scheiner *et al.* 2003; Thamm & Scheiner 2014), and the manganese transporter, malvolio

(Ben-Shahar *et al.* 2004) have been implicated in reward processing. Most of these findings are based on laboratory assays that involve ingestion and digestion of food for individual sustenance. However, most of the food collected by foraging honey bees in nature is not digested; it is instead brought back to the hive to share with other colony members (Blatt & Roces 2001).

Individual honey bee foragers collect food for their entire colony, and a major challenge is to collect enough food from flowers (their sole source of food) to sustain them through seasonal variation and lengthy periods of floral dearth. To maximize colony performance, individual foragers must be attuned to changing colony needs for food type (e.g. carbohydrates obtained from nectar or protein and lipids obtained from pollen) and be able to recognize especially valuable floral resources that provide for these needs as efficiently as possible (Seeley 1995). Evidence that honey bees evaluate such differences comes from studies of the honey bee dance language; returning foragers communicate information on the distance, direction, quality and type of resources they have collected to hive mates via highly stereotyped sounds and movements (Seeley 1995; von Frisch 1967).

Studies of mammals have revealed that assessments of food type and quality involve subcomponents of a more general reward system (Cromwell & Schultz 2003; Lardeux *et al.* 2009), but whether reward responses are organized this way in other taxa is unknown. Immediate early genes (IEGs) as indicators of neuronal activation and comparative transcriptomics are effective methods for probing whether independently evolved processes are derived from similar neuromolecular systems (Rittschof *et al.* 2014; Vilpoux *et al.* 2009), and we used both in this study to address this issue.

We hypothesized that the reward mechanisms involved in selfish behavior also are involved in the regulation of social foraging behavior in honey bees. We predicted that responses to differences in food type and food value, which are essential components of the honey bee social foraging system, involve subcomponents of a more generalized food reward system. To explore this prediction, we studied the similarities and differences in neuroanatomical and transcriptional pathways associated with honey bee responses to food, food type and food value (Fig. 1).

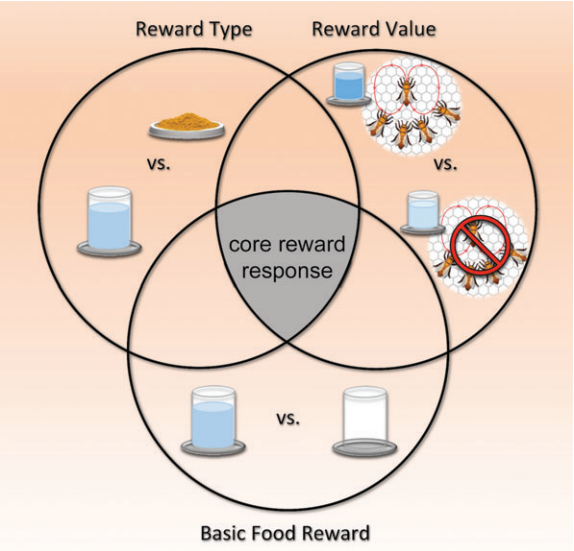


Figure 1: Experimental design to identify neuroanatomical and molecular components of responses to food reward in honey bees. Background shading indicates the continuum from more general to more specific reward responses (from light to dark shading). The more general response to reward includes the response to food compared to no food. More specific responses include responses to differences in reward type (pollen vs. nectar) and differences in reward value (high vs. low sugar concentration). Bees in Experiment 1 encountered a feeder on their first foraging trip of the day that either contained artificial nectar or was empty; bees were sampled that did or did not receive this food reward. Bees in Experiment 2 were sampled after they collected either pollen or artificial nectar. Bees in Experiment 3 were allowed to forage for artificial nectar with different sugar concentrations; the high concentration elicited a 'dance' upon return to the beehive and the low concentration did not. The decision of whether to dance or not reflects the bees' assessment of food quality, and it is part of a complex communication system that also symbolically gives information on the distance and direction of the floral resource. Each of these experiments was designed to isolate the response to different aspects of the reward response, but all three include a core response.

Materials and methods

Bees

Female forager bees were collected from colonies derived from naturally mated queens maintained according to standard methods at the University of Illinois Bee Research Facility (Urbana, IL). The colonies were typical of North American populations of *Apis mellifera*, which are hybrids of various European-derived subspecies, mostly *A. m. ligustica*. Different individual colonies were used in each of the three behavioral experiments described below. Colonies for Experiments 1–3 were located in a large outdoor, screened enclosure ($6 \times 20 \times 3\ m^3$) to precisely control their access to food. Additional bees were collected from one colony to detect the presence of octopaminergic and dopaminergic neuronal activation. Bees from Experiments 2–3 were used for both RNAseq and mRNA *in situ* hybridization (ISH); bees from Experiment 1 were only used for RNAseq.

Behaviors

Experiment 1: response to sucrose food reward

We collected bees ($N = 20$) from one colony (adult population ca. 10 000 bees) in September 2012 for this experiment. We scheduled feeding times so that the colony learned to anticipate the availability of artificial nectar (sucrose syrup, 30% w/v) inside the enclosure on each of 10 training days from 1200 h until sundown or until the feeder was emptied by the bees. This sucrose concentration was high enough to cause a large number of bees to anticipate the food reward (Moore & Rankin 1983; Naeger *et al.* 2011; von Frisch 1967).

At the start of every collection day, we made sure that a large number of bees left their hive in search of the food, and then we closed the entrance to prevent additional bees from exiting. This helped ensure that the collected bees had discovered the feeder on their own, rather than being recruited to it by successful foragers via dance language. As a control, we presented an empty feeder inside the enclosure for 9–14 min, and collected bees that landed

on it, presumably anticipating collecting sucrose syrup. These bees performed search and flight behaviors but did not receive a reward. Following collection of control bees, we presented a feeder with 30% w/v sucrose syrup for 6–12 min. We collected bees that landed, ingested the sucrose syrup and started to take off to return to their colony. All collections were made by placing a ventilated plastic cage above the bees, and allowing them to walk into it on their own. The caged bees were then returned to their colony for 60 min to allow experience-dependent changes in brain gene expression to occur prior to being sacrificed. The caged bees were separated from the other bees in their colony by a screen that prevented any food sharing. We collected the same number of reward-receiving and control bees each day. Collections occurred at the same time every day (1100–1130 h) to minimize circadian effects on behavior and gene expression (Naeger *et al.* 2011). All bees ($N = 20$) were flash frozen in liquid nitrogen in the field to capture behaviorally relevant patterns of brain gene expression, and stored at −80°C until prepared for RNAseq analysis. The results from Experiment 1 are referred to as 'response to food' (Fig. 1).

Experiment 2: response to differences in food type

Methods were the same as in Experiment 1, except for the following. First, two colonies were used (one at a time), and the sucrose concentration varied slightly by colony. Sucrose concentrations (26% or 39% w/v) were chosen empirically, based on the minimum concentration sufficient to cause a large number of bees to anticipate the presentation of the food source. Second, we also provided a feeder with ground bee pollen (Betterbee Inc., Greenwich, NY, U.S.A.) so that bees could choose which food type they preferred. Sucrose syrup and pollen were provided at two separate feeders inside the enclosure on each day from 1200 h until sundown (1930–2000 h) or until the feeders were empty. Bees were allowed to land at either feeder and gather the resource of their choice (Fig. 1). Bees generally specialize in gathering either nectar or pollen (Page 2013), and we rarely observed bees foraging at both feeders. Collections for Experiment 2 were made in August 2010 from both colonies, for both RNAseq and ISH. Bees for RNAseq ($N = 20$) were collected as in Experiment 1. Bees for ISH ($N = 19$) were anesthetized on wet ice and stored in 4% paraformaldehyde at −20°C until analysis.

Experiment 3: response to differences in food value

Methods were the same as in Experiment 1 except for the following. The two bee colonies (containing adult populations of ca. 8000 bees) used for this experiment were each housed in glass-walled observation hives, which allowed us to observe whether a focal bee danced or not upon returning from the feeder. Dance provides a unique, natural and quantifiable readout of a bee's assessment of the value of collected food rewards; individuals only dance if the reward is of sufficient value, based on an assessment of the quantity, concentration and distance of the resource from the hive, and the level of colony need (Seeley 1995; von Frisch 1967). We varied the sucrose concentration in the feeder to generate either high- (23 or 58% w/v) or low- (10 or 23% w/v) value rewards. Reward value was determined empirically for each colony; most bees returned to the hive and danced for the high value reward, while most bees did not dance for the low value reward (Fig. 1).

One complication associated with this method is that the stimulus varied between dancers and non-dancers; this absolute difference in reward may also be driving the differences in brain gene expression in addition to differences in relative reward assessment. An alternative would have been to sample bees that danced or did not dance for the same sucrose concentration (Barron *et al.* 2007b), but this would have led to concerns that inter-individual differences in response may be related to a variety of factors and not just reward assessment. Of the two, we believe that the method used was preferable for capturing the transcriptomic response to differences in reward value.

The low concentration feeder was presented first on each collection day. Bees arriving at the feeder were marked on the abdomen with paint (Testors Corporation, Rockford, IL, U.S.A.) so we could easily identify them upon return to the hive. Dancing bees were those that performed ≥8 'round dance' circuits, which indicates robust dancing (Barron *et al.* 2007a,b). Non-dancers were those that did not perform any dance circuits within approximately 20 seconds after returning to the hive (Seeley & Tovey 1994) and then walked beyond the 'dance floor', an area near the hive entrance where most dances occur.

One pair of sucrose feeders was available from 1000 h to 1130 h daily for collections for RNAseq, and a second pair of feeders was available from 1330 h to 1445 h daily for collections for ISH. Bees from both colonies were used for both types of analysis, as in Experiment 2 (RNAseq, $N = 20$; ISH, $N = 14$).

mRNA in situ hybridization

Overview

We relied principally on the IEG *c-jun* as a marker for neuronal activation. Rapid change in IEG expression has been a useful marker of neuronal activity in vertebrates (Clayton 2000; Guzowski *et al.* 2005), and increasingly, in honey bees (Alaux & Robinson 2007; Fujita *et al.* 2013; Lutz & Robinson 2013; Ugajin *et al.* 2012, 2013). A recent analysis of regions in the brain responsive to ingested sucrose in the laboratory used *c-jun* ISH (McNeill & Robinson 2015).

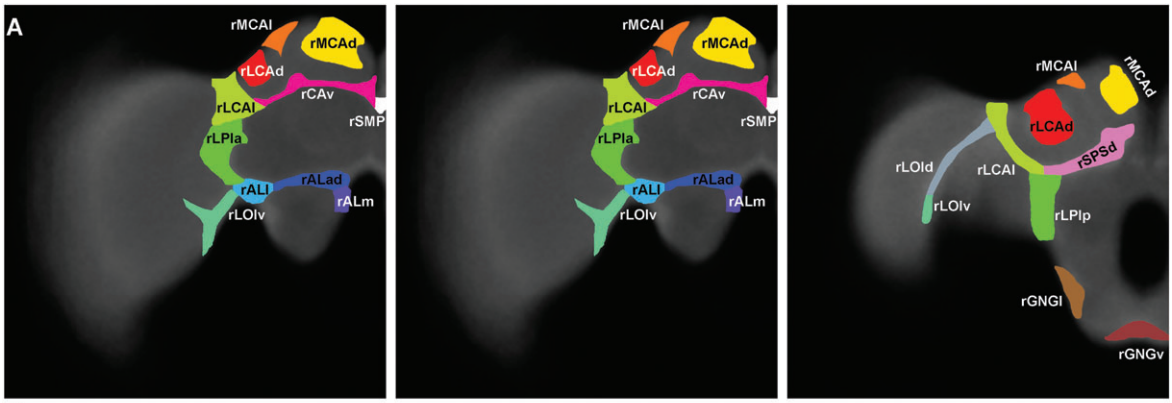
We supplemented results from *c-jun* mRNA ISH with an mRNA ISH-based analysis of genes required for octopaminergic or dopaminergic neuronal activity. OA and DA are implicated in the honey bee's responsiveness to food rewards (Barron *et al.* 2007b; Hammer & Menzel 1998; Lagisz *et al.* 2015). We focused on neurons that express *tyrosine hydroxylase* (*th)* and *tyramine beta hydroxylase* (*tbh)*, which encode the rate-limiting enzymes in DA and OA biosynthesis, respectively (Friggi-Grelin *et al.* 2003; Roeder 2005). We assumed that DA or OA neurons responded to food or differences in food type or food value if we detected *th* or *tbh* and differential *c-jun* expression in the same, spatially normalized voxel. We collected returning foragers from a typical colony located outside of the research facility between June and August 2011.

Analysis methods

In situ hybridization was conducted with a previously published protocol that labels targeted mRNA molecules expressed in whole-mount brains with 48 DNA probes, each bound directly to a fluorophore (McNeill & Robinson 2015; Table S1, Supporting Information). Probes bind in series along the mRNA transcript. Brains collected in Experiments 2 and 3 were incubated with probes targeting *c-jun,* and all additional brains were incubated with probes targeting both *th* and *tbh*. Optimal hybridization temperatures were determined empirically; *c-jun*: 34°C or *th*/*tbh*: 37°C, respectively. Brains were imaged using a Zeiss LSM 700 confocal microscope (Carl Zeiss Microimaging Inc., Oberkochen, Germany), resulting in a stack of optical sections representing a single brain image. Brain images were manually re-oriented for consistency (AMIRA software, Visage Imaging, version: 5.3.0), and then spatially normalized against a previously published honey bee brain model to allow for voxel comparisons between individuals (McNeill & Robinson 2015) using AUTOMATIC REGISTRATION TOOLBOX software (version: 2011.09.22; Ardekani *et al.* 2005). The previously published model (McNeill & Robinson 2015) was developed based on an older honey bee brain model (HBSconfocal; http://www.neurobiologie.fu-berlin.de/default.html; Rybak *et al.* 2010). We developed the new model to improve spatial normalization of brain images with very bright somata-containing regions. To allow for an easier comparison to past results using the honey bee brain model, we present some of our results using each model as a backdrop.

We developed new names for most somata-containing brain regions in the style of the Insect Brain Name Working group (Ito *et al.* 2014). The aim of the Insect Brain Name Working group is to make brain region names more uniform across insect species. We compare our newly developed names with those we used in a previous publication (McNeill & Robinson 2015; Fig. 2).

Statistical analyses were performed with a Student's *T*-test (3dttest++, AFNI software; version AFNI_2011_12_21_1014; Cox 1996) on each voxel to compare gene expression intensity between relevant behavioral groups. We combined all brain images (3dTcat) in each experiment, and estimated the smoothness of the somata-containing



B

New Region Abbreviation	New Region Name	McNeill&Robinson 2015 Region Abbreviation	McNeill&Robinson 2015 Region Name	Food	Type	Value
rAMMCl	rind lateral to the antennal mechanosensory and motor center	AMMC	antennal mechanosensory and motor center			
rLPla	rind lateroanterior to the lateral protocerebrum	IP	lateral protocerebrum			
rLPlp	rind lateroposterior to the lateral protocerebrum	IP	lateral protocerebrum			
rCAv	rind ventral to the calyx	aP	anterior protocerebrum	C3		
rALad	rind anterodorsal to the antennal lobe	dAL	dorsal antennal lobe		C1	
rLCAl	rind lateral to the lateral calyx	dOL	dorsal optic lobe	C3	C3	
rLOld	rind laterodorsal to the lobula	dOL	dorsal optic lobe			
rSPSd	rind dorsal to the superior posterior slope	dpP	dorsal posterior protocerebrum			S_P
rALl	rind lateral to the antennal lobe	lAL	lateral antennal lobe			
rLCAd	rind dorsal to the lateral calyx	lMB	lateral mushroom body			
rSMP	rind of the superior medial protocerebrum	PI	pars intercerebralis			
rGNGl	rind lateral to the gnathal ganglion	lSEG	lateral subesophageal ganglion	G6a/b & $S_{3/5/6}$		
rGNGv	rind ventral to the gnathal ganglion	vSEG	ventral subesophageal ganglion			
rALm	rind medial to the antennal lobe	mAL	medial antennal lobe		G3a	
rMCAd	rind dorsal to the medial calyx	mMB	medial mushroom body			
rMCAl	rind lateral to the medial calyx	mdP	medial dorsal protocerebrum			
rLOlv	rind lateroventral to the lobula	vOL	ventral optic lobe	S_L		

Figure 2: Honey bee brain region names and activation patterns. (A) Cartoon drawing of most somata-containing regions of the honey bee brain. The three optical sections from McNeill and Robinson (2015) were taken at intervals from the anterior (left) to posterior (right). New abbreviations were added, which correspond to brain region names based upon recommendations made by the Insect Brain Name Working Group (Ito *et al.* 2014). Full names are listed in (B). (B) Brain regions responsive to food reward (McNeill & Robinson 2015), differences in food type (sucrose syrup, $n = 10$; pollen, $n = 9$), or differences in food value (high value, $n = 8$; low value, $n = 6$). Shaded boxes indicate brain regions with differential *c-jun* expression. Differential *c-jun* expression spatially corresponded with some dopaminergic (yellow) or octopaminergic (magenta) neurons. We describe activation of five previously described DA-expressing populations (C_1, C_3, S_L, S_P, $S_3/S_5/S_6$) and two previously described OA-expressing populations (G3a/b and G6a/b; Lehman *et al.* 2006; Sinakevitch *et al.* 2005). We represent G3a/b, G6a/b and $S_3/S_5/S_6$ as a single populations because we are unable to distinguish between them. Brain activation patterns correspond to images shown in Fig. S4.

brain regions using 3dFWHMx with 2difMAD and demed options (AFNI software). Smoothness corresponded to the relative difference in signal intensity between adjacent voxels, and variations in smoothness are likely a byproduct of tissue and image processing. Highly smooth brain images indicate the intensities of adjacent voxels are similar and may co-vary. A Monte Carlo simulation (3dClustSim, AFNI software) used the smoothness estimate to predict that clusters of 79 and 92 or more significant voxels (output of the Student's *T*-test analysis, $P < 0.05$) would not differentially express *c-jun* by chance (family-wise error of $\alpha < 0.1$) for Experiments 2 and 3, respectively. Somata-containing brain regions were described using published anatomical designations (Kiya *et al.* 2007; McNeill & Robinson 2015; Mobbs 1982). We describe responsive brain regions as those that contain voxel clusters, which in turn represent the underlying responsive neuronal somata. We removed voxel clusters lateral to the medulla from our analysis because the remaining optic pigment may have altered the brain spatial normalization for this region, causing an artifactual signal.

RNA sequencing

Brain dissections

We used an established protocol (Lutz & Robinson 2013) to dissect a region of the brain enriched for mushroom body tissue; we obtained 63.8–702.6 (median = 412.2) ng of total RNA from each sample ($N = 60$).

cDNA library preparation and sequencing

After all bees were collected for each experiment, we paired bees collected from the same colony on the same day in different treatment groups. Pairs were then processed together during each step. In Experiment 1, the treatment groups were food vs. no food; Experiment 2, pollen vs. sucrose; and Experiment 3, bees that danced vs. bees that did not dance. Poly-adenylated RNA was enriched from the total RNA pool using Oligo(dT)25 DynaBeads (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.), fragmented, and converted to complementary DNA (cDNA; NEXTflex Directional RNA-Seq Kit dUTP Based instructions, Bioo Scientific Corporation, Austin, TX, U.S.A.). Average fragment sizes were estimated with a BioAnalyzer High sensitivity DNA assay (Agilent Technologies, Santa Clara, CA, U.S.A.); fragments were an average of 356 (Experiment 1), 348 (Experiment 2) and 351 (Experiment 3) base pairs (bp). complementary DNA libraries were labeled with NEXTflex RNA-Seq Barcodes for multiplexing during sequencing. The final cDNA library concentration was estimated using a Qubit (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.), and then eight libraries were pooled. The total concentration of all adapter-ligated fragments within assembled library pools

was confirmed using KAPA Library Quantification kits (Kapa Biosystems, Wilmington, MA, U.S.A.). The pooled libraries were sequenced on a HiSeq 2000 (Illumina Inc., San Diego, CA, U.S.A.) at the W.M. Keck Center for Comparative and Functional Genomics within the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. The average number of 100 bp, paired-end reads was 24.7 (Experiment 1), 25.6 (Experiment 2) and 25.3 (Experiment 3) million per sample.

Data analysis

Reads were trimmed to remove adapter and low quality sequence using TRIMMOMATIC (v0.30), and mapped to the v4.5 build of the honey bee genome (Elsik *et al.* 2014) using TOPHAT (v2.0.8). An average of 68.89% (Experiment 1), 72.48% (Experiment 2) and 73.20% (Experiment 3) reads mapped to the genome. We counted reads mapping to genes (Official Gene Set 3.2) using HTSEQ (v0.54). Of all sequenced reads, an average of 59.9% (Experiment 1), 57.66% (Experiment 2) and 48.25% (Experiment 3) mapped to genes.

We used EDGER (v3.4.2) running in R for Mac (v3.0.2) to model variance in gene expression. Only genes expressed with at least one read count per million in two samples were analyzed. We normalized differences in the abundance of read counts mapped to genes between samples using the trimmed mean of M-values (TMM) normalization method (Robinson & Oshlack 2010). Each experiment was fit with a general linear model (GLM) with two or three factors: pair, treatment and estimated library concentration. Estimated library concentration was included when it explained some of the variance in the read counts (Experiments 1 and 2, but not 3). We estimated the variance in gene expression using common, trended and tagwise dispersion models sequentially (McCarthy *et al.* 2012), and we chose a prior degrees of freedom value that best fit all genes to the tagwise dispersion model, similar to a previously described method (McCarthy *et al.* 2012).

We removed samples from further analyses that were likely to bias the results. We removed one pair of samples in Experiment 1 because it was an outlier on a multidimensional scaling plot (McLoughlin *et al.* 2014). We removed one pair of samples in Experiment 2 because the cDNA libraries were below the minimum recommended concentration for sequencing, which may have induced a biased expression profile.

Four of the genes responsive to food (GB47718, GB54611, GB43247 and GB48020) are more highly expressed in hypopharyngeal glands than brain ($\log_2$ fold difference > 4; Rittschof *et al.* 2014). Because the hypopharyngeal glands may contaminate the dissected brain tissue, differences in these genes may represent artifacts of the dissection process. However, we kept these genes in the dataset because they made up a very small percentage of the overall transcriptional response, and they were not detected in bees used in Experiments 2 or 3. It is unlikely that the inclusion of these genes affected our overall conclusions.

Fold change comparisons

We compared the expression changes induced in each experiment by quantifying the similarity of each gene list based on rank-ordered log fold change. We calculated a similarity score for differential expression between each experiment using the R package 'OrderedList' (Yang *et al.* 2006). In this program, similarity score is calculated based on a weighted sum of the overlap in gene lists ordered by log fold change at each rank, with more weight given to the top ranks. Top ranks correspond to genes with large expression differences between treatment groups within an experiment. The optimal weighting parameter is derived based on maximum separation of the distribution of observed scores and scores derived from random lists. The significance of the similarity score was evaluated by computing an empirical *P* value for the median observed score, based on the set of random scores. We initially analyzed overlap among the filtered and normalized gene lists, and then we repeated the analysis with only those genes differentially expressed (false discovery rate [FDR] < 0.1) in Experiment 1. Per the recommendation of the OrderedList documentation, we considered samples within our previously described treatment pairs to be 'unpaired' for these analyses because gene expression measurements were taken from unique individuals.

Weighted gene co-expression network analysis

For each Experiment, $\log_2$ transformed, TMM-normalized expression values for genes were imported into WEIGHTED GENE CO-EXPRESSION NETWORK ANALYSIS (WGCNA; Zhang & Horvath 2005; v1.34 running in R for Linux, v3.0.0). As above, analyzes were restricted to genes differentially expressed in response to food in Experiment 1. Variation in gene expression between sample pairs or due to variation in input library size was removed using removeBatchEffects (LIMMA package, v3.16.8). Unsigned modules containing at least 30 co-expressed genes were formed using deepSplit = 3; similarly co-expressed modules (Pearson Correlation coefficients > 0.9) were merged. In each experiment, a small proportion of genes was not assigned to any module (Experiment 1: 12, 2: 62, 3: 9). The eigengene of some modules was significantly correlated with treatment (FDR < 0.1) in all three experiments.

Gene ontology

Gene ontology (GO) annotation terms for each gene were obtained from the corresponding INTERPRO entry (v36.0; Apweiler *et al.* 2001), identified based on a search including Pfam, PRINTS, PROSITE, ProDom and SMART databases. Enriched GO terms were identified among module hub genes for each WGCNA module correlated with treatment using GOstats v2.28.0 in R for Mac. We defined hub genes as genes within each module that were significantly correlated with treatment (either negative or positive, depending on the eigengene correlation to the treatment) and significantly and positively correlated with the module eigengene ($P < 0.05$). Prior work indicated that hub genes best represent network modules (Langfelder *et al.* 2011).

Gene ontology term comparisons

Gene Ontology terms significantly enriched in treatment-responsive modules were described in 2-dimensional semantic space using REVIGO with SimRel and 0.9 allowed similarity options (http://revigo.irb.hr/; Supek *et al.* 2011). All enriched GO terms were analyzed by REVIGO simultaneously to maintain relative semantic similarity distances. REVIGO simplifies lists of GO terms to retain only those terms that are the most unique. Graphs representing unique terms were generated in R for Mac.

Results

Brain regions responsive to food, food type and food value

We compared brain regions responsive to differences in food type or food value with those we previously reported to be responsive to a general food reward, ingestion of sucrose syrup in the laboratory (McNeill & Robinson 2015). Responsive regions in both the current study and McNeill and Robinson (2015) were identified by differential *c-jun* expression. The previously reported response to sucrose was extensive, involving 20 voxel clusters distributed across ten different brain regions. Only 2.9% of neuronal somata previously found to be responsive to sucrose (McNeill & Robinson 2015) responded to differences in food type in the present study. These somata were distributed across five brain regions (rind lateral to the lateral calyx, rind lateral to the antennal lobe, rind dorsal to the medial calyx, rind dorsal to the lateral calyx, and rind lateroanterior to the lateral protocerebrum; Figs. 2,3,S2,S4). Even fewer (0.8%) neuronal somata responsive to sucrose responded to food value. These somata were

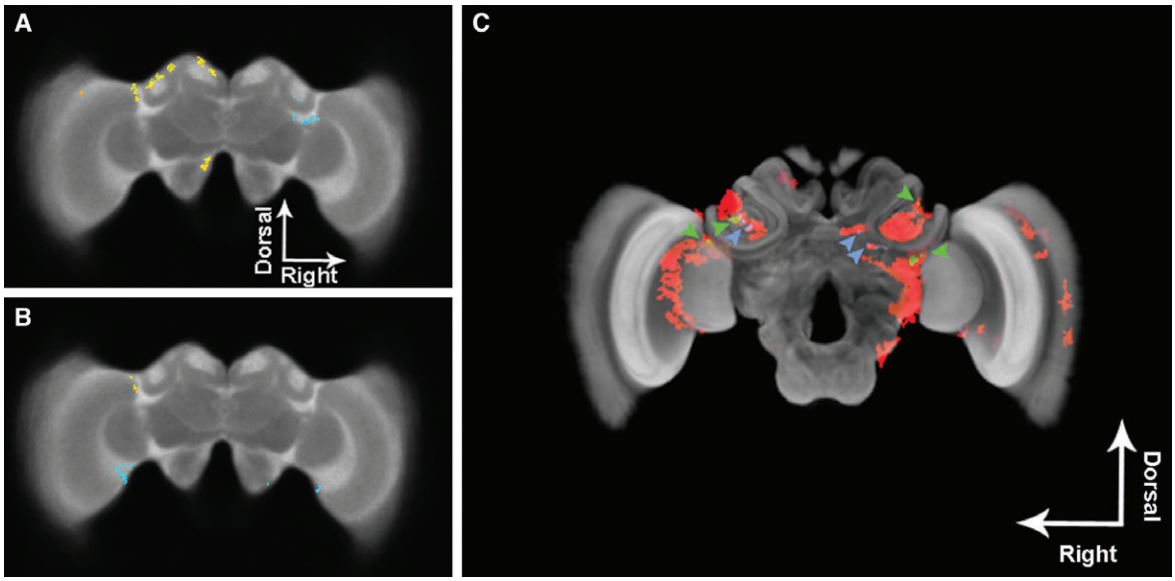


Figure 3: Brain regions responsive to food, differences in food type and differences in food value. (A and B) Optical section of regions differently responsive to food types (from Fig. S2) or values (from Fig. S3). Higher (orange colors) or lower (blue colors) *c-jun* expression occurs in a region-specific manner in bees collecting sucrose syrup ($n = 10$) minus pollen ($n = 9$, A) or high value ($n = 8$) minus low value ($n = 6$) sucrose syrup (B). More saturated heat map colors correspond to smaller P values below the indicated threshold (Student's T-test; A, threshold T statistic = 2.10; B, threshold T statistic = 2.179). Orientation in A corresponds to panels (A and B). (C) Posterior view of a three-dimensional representation of brain regions responsive to food (red) and regions also responsive to food type (green) or food value (blue). All regions responsive to food are shown in red (McNeill & Robinson 2015). Regions containing neuronal somata commonly responsive to both food and food type are indicated with green arrowheads. Regions containing neuronal somata responsive to both food and food value are indicated with blue arrowheads. Responsive voxels are presented on the previously published honey bee standard brain model background (HBSconfocal; http://www.neurobiologie.fu-berlin.de/default.html; Rybak *et al.* 2010). Brain orientation presented in C is only for panel (C). Brain regions responsive to each stimulus, including those regions not containing neuronal somata responsive to multiple stimuli, are summarized in Fig. 2.

also distributed across six different brain regions (rind lateral to the lateral calyx, rind laterodorsal to the lobula, rind dorsal to the medial calyx, rind dorsal to the lateral calyx, rind lateral to the gnathal ganglion, and rind lateroventral to the lobula; Figs. 2,3,S3,S4). Three brain regions (rind lateral to the lateral calyx, rind dorsal to the medial calyx, and rind dorsal to the lateral calyx) contained somata that responded to food, food type and food value (Figs. 2,S4).

Some regions of the brain were uniquely responsive to food, food type or food value (Fig. 2). The rind lateroposterior to the lateral protocerebrum and rind ventral to the calyx were only responsive to food; the rind anteriodorsal to the antennal lobe, rind medial to the antennal lobe, and rind lateral to the medial calyx were only responsive to differences in food type; and the rind lateral to the antennal mechanosensory and motor center was only responsive to differences in food value.

Similarly, most dopaminergic and octopaminergic neuron populations (described in Farris 2008; Lehman *et al.* 2006; Schafer & Rehder 1989; Sinakevitch *et al.* 2005 and Fig. S4) were uniquely responsive to food, food type or food value (Figs. 2,S4). The exception was the C3 dopaminergic neuron population in the rind lateral to the lateral calyx, which responded to both food and food type. By contrast, the C3 dopaminergic neuron population in the rind ventral to the calyx only responded to food. S_L and $S_3/S_5/S_6$ dopaminergic and G6a/b octopaminergic neuron populations also only responded to food. C1 dopaminergic and G3a/b octopaminergic neurons only responded to food type, and S_P dopaminergic neurons only responded to food value.

Though we did not design our experiments to test for lateralized brain activity, we nonetheless observed differential expression of *c-jun* on only one side of the brain for some brain regions. For example, we detected a response to differences in food type in the anterior portion of the left rind dorsal to the medial calyx, but not the right, and we detected a response to differences in food value in the posterior portion of the right, but not the left, rind dorsal to the lateral calyx (Fig. S4). Evidence for lateralization in the bee brain is inconsistent – it is observed in some circumstances (Anfora *et al.* 2010; Frasnelli *et al.* 2010a; Letzkus *et al.* 2006; McNeill & Robinson 2015; Rigosi *et al.* 2015), but not in others (Hammer *et al.* 2009; Kiya *et al.* 2007).

Mushroom body transcriptional responses to food, food type and food value

Transcriptomic analysis was performed on samples that were primarily composed of mushroom body somata and calyx

tissue (Fig. S1). The mushroom body region of the insect brain is well known for multi-modal sensory information integration and reward memory processing (Giurfa 2013). In addition, our neuroanatomical analysis revealed that mushroom body subregions are responsive to food, differences in food type and differences in food value (Figs. 2,3,S4), supporting the idea that transcriptional changes in the mushroom bodies are an important component of reward processing.

We compared the lists of genes detected in our experiments with genes detected in previous studies of gene expression in the honey bee mushroom bodies (Lutz & Robinson 2013; Sen Sarma *et al.* 2010). The Sen Sarma et al. and Lutz and Robinson studies detected the same set of 8924 genes, and were performed on the same microarray platform. With RNAseq we detected 7703 (86.3%) of these genes plus an additional 2942 genes in Experiment 1; 7648 (85.7%) plus an additional 2974 genes in Experiment 2; and 7722 (86.5%) plus an additional 2982 genes in Experiment 3. These results reflect our expectations, as the microarray platform used in Sen Sarma et al. and Lutz and Robinson was designed from an earlier version of the honey bee genome, which had fewer predicted genes. Additionally, RNAseq permits detection of genes beyond those represented on a microarray. These comparisons indicate that genes expressed in the honey bee mushroom bodies were broadly represented in our experiments.

We detected 1190 genes differentially expressed in the mushroom bodies in response to food (FDR < 0.1, Table S2). Only five and three genes were differentially expressed in response to differences in food type and food value, respectively. However, we observed significant overlaps in the fold change-ranked list of genes for each experiment when based on just the set of 1190 genes responsive to food reward (food reward and food type weighted overlap score = 390.7, $P = 0.05$; food reward and food value weighted overlap score = 6846.7, $P = 0.05$), and marginally significant overlaps when based on all genes (food reward and food type weighted overlap score = 8139.0, $P = 0.06$; food reward and food value weighted overlap score = 24 519.2, $P = 0.08$). These results suggest that some of the genes in the mushroom bodies that responded to food reward also responded to differences in food type or value, but the magnitude of the responses was smaller.

We also assessed the possibility that differences in effect size of the experimental treatments contributed to the large differences in the magnitude of the transcriptomic responses (i.e., number of differentially expressed genes) between Experiment 1 and Experiments 2 or 3. A power analysis (Busby *et al.* 2013) determined that we had 77%, 60% and 70% power to detect >70% of genes showing a two-fold difference in expression in Experiments 1, 2 and 3, respectively. Our power to detect differences in the second two experiments was lower than in the first, despite the similar sample size. This may reflect a smaller effect of food type and food value on brain gene expression, compared to the consumption of food. This is plausible given that the former two likely reflect different aspects of an appetitive reward.

We used WGCNA (Zhang & Horvath 2005) to identify key molecular pathways involved in the mushroom body response to food and to determine whether these pathways also were involved in the response to differences in food type or value. We identified modules of co-expressed genes separately within each experiment, using only the 1190 genes responsive to food reward. Many of the modules of co-expressed genes were significantly correlated with one or more of the three aspects of food reward we studied. Genes differentially expressed in response to food in Experiment 1 formed three co-expression modules (containing 521, 539 and 118 genes), and the eigengene of each of these modules was significantly correlated with the response to food. There were twelve modules in Experiment 2 and seven in Experiment 3. Of these, the eigengenes of three modules (containing 288 genes) from Experiment 2 were correlated with differences in food type, and the eigengenes of four modules (718 genes) from Experiment 3 were correlated with differences in food value (Table S3). In general, gene modules responsive to differences in food type or value were formed of distinct subsets of the overall response to food (Figs. 4,S5).

Gene Ontology enrichment analyses of the WGCNA modules gave the following functional insights. There were common molecular pathways involved in the response to food, food type and food value (Fig. 5, Table S4). Co-expression modules responsive to food, food type and food value were significantly enriched (hypergeometric test, $P < 0.05$) for GO terms related to mitochondria/endoplasmic reticulum, nucleoside biosynthesis and nucleic acid binding. Also, co-expression modules responsive to stimuli in Experiments 2 and 3 (Table S4) were enriched for mostly different GO terms, but enriched terms were generally also enriched within co-expression modules responsive to food in Experiment 1. Modules responsive to both food and food type were enriched for genes associated with regulation of transcription and translation. Modules responsive to both food and food value included genes related to neurotransmitter and neuropeptide signaling, such as the *dopamine/ecdysteriod receptor*, *serotonin receptor 2*, *allatostatin receptor* and *glutamate-gated chloride channel* (*GluClalpha*).

Discussion

We have identified similarities in the neuroanatomical and neurogenomic architecture of reward-based behavior in insects and mammals. Brain regions and gene networks responsive to differences in food type or value in honey bees form a mixture of distinct and common subsets of the brain regions and gene networks responsive to a more general food stimulus, which parallel findings from studies of rodents (Cromwell & Schultz 2003; Lardeux *et al.* 2009). These results suggest that the observed responses to food type and value involve representations of reward.

It previously was not known whether reward information is encoded in a highly distributed fashion in the honey bee brain, as most studies have focused either on the activation of specific brain regions or a very small set of neurons. There is, however, some evidence from studies of *Drosophila* suggesting that food reward information is encoded by the distribution pattern of activated neurons, best captured by a systems-level analysis of brain activation (Campbell *et al.*

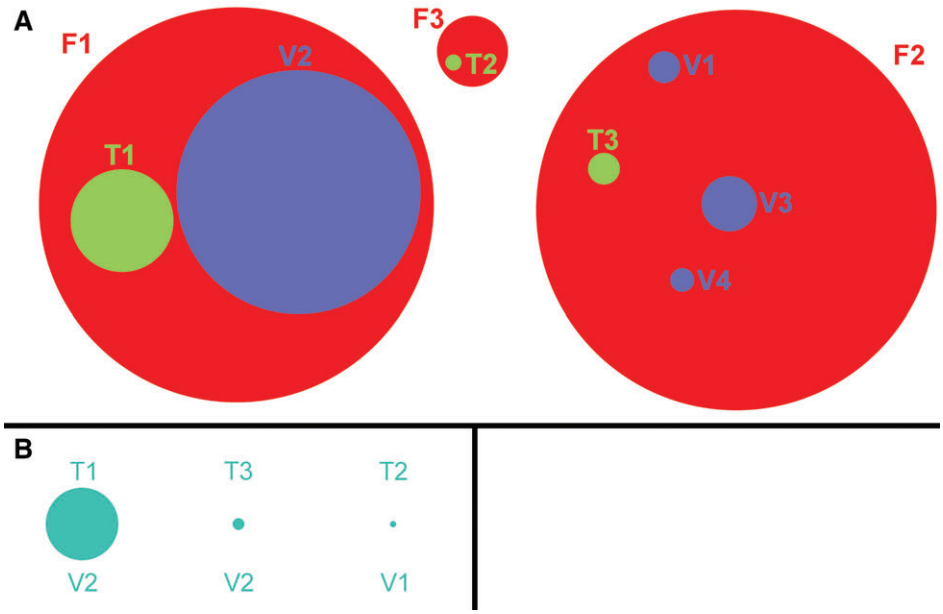


Figure 4: Genes in modules responsive to food, differences in food type and differences in food value. Circles are proportionally sized to the number of represented genes, and labels represent the experiment (F = food, T = type and V = value) and module number. (A) Circles represent genes in modules responsive to a food reward (red), and the common genes in modules responsive to differences in food type (green) or food value (blue). (B) Teal circles represent the number of genes common to modules responsive to differences in food type and food value. For both (A) and (B), only module overlaps with a significant number of common genes are shown (hypergeometric test, FDR < 0.05). Responsive modules are the subset of all formed modules with eigengenes significantly correlated with treatment (listed in Table S3, compared in Fig. S5).

2013; Huetteroth *et al.* 2015). This is similar to our general understanding of highly distributed memory engrams in the brain (Mayford 2014). Results from other studies, however, have reported evidence for a centralized reward system in *Drosophila* (Aso *et al.* 2014a,b). Our results, correlative in nature, are consistent with an anatomically distributed activation pattern of reward-related responses. Our study focused entirely on food rewards, so it is not known whether neurons identified in this study are also activated by non-food reward stimuli.

We detected some parts of the honey bee brain that were responsive to differences in food type or value, but not to food itself. These parts may contain neurons that are involved in some of the unique aspects of food-associated behaviors in honey bees. For example, bees collecting pollen store it in specialized structures on their hind legs, and bees that experience a high-value food reward communicate it to nest mates via the dance language. Further investigation of these locations in the brain may provide new insights into the regulation of reward responses.

While our study did not explicitly aim to examine brain lateralization, our findings support the idea that some brain regions are involved unilaterally in processing information about food, food types and food values. Rigosi *et al.* (2015) suggested that unilateral brain activation might allow for greater information processing because more unique computations can be calculated in parallel. However, the notion of unilateral honey bee brain activation remains controversial; some researchers have found supportive evidence (Anfora *et al.* 2010; Frasnelli *et al.* 2010a,b; Letzkus *et al.* 2006; Rigosi *et al.* 2015) while others have not (Hammer *et al.* 2009; Kiya *et al.* 2007). Our results are not definitive on this issue; we cannot precisely measure the relative amount of contralateral brain activation because the brain model was not constructed to have bilaterally consistent spatial coordinates.

It has been widely thought that OA and DA play opposing roles in insect behavior, mediating reward and punishment, respectively (Schwaerzel *et al.* 2003; Unoki *et al.* 2005). However, two studies of *Drosophila* demonstrated that octopaminergic signals are passed through certain dopaminergic neurons to mediate sugar reward (Burke *et al.* 2012; Liu *et al.* 2012). Recently it was reported that different dopaminergic neurons in the *Drosophila* brain play parallel roles to mediate short or long term memory (Huetteroth *et al.* 2015; Yamagata *et al.* 2015), sweet and nutritional value (Huetteroth *et al.* 2015), and sugar and water rewards (Burke *et al.* 2012; Lin *et al.* 2014). In addition, new evidence supports a role for DA in mediating reward behaviors in honey bees (Lagisz *et al.* 2015). While our methods were unable to distinguish activation of individual somata, clusters of responsive somata sometimes included our DA or OA markers in locations known to contain small numbers of dopaminergic or octopaminergic neurons. These results also implicate activation of dopaminergic neurons in mediating food reward, differences in reward type and differences

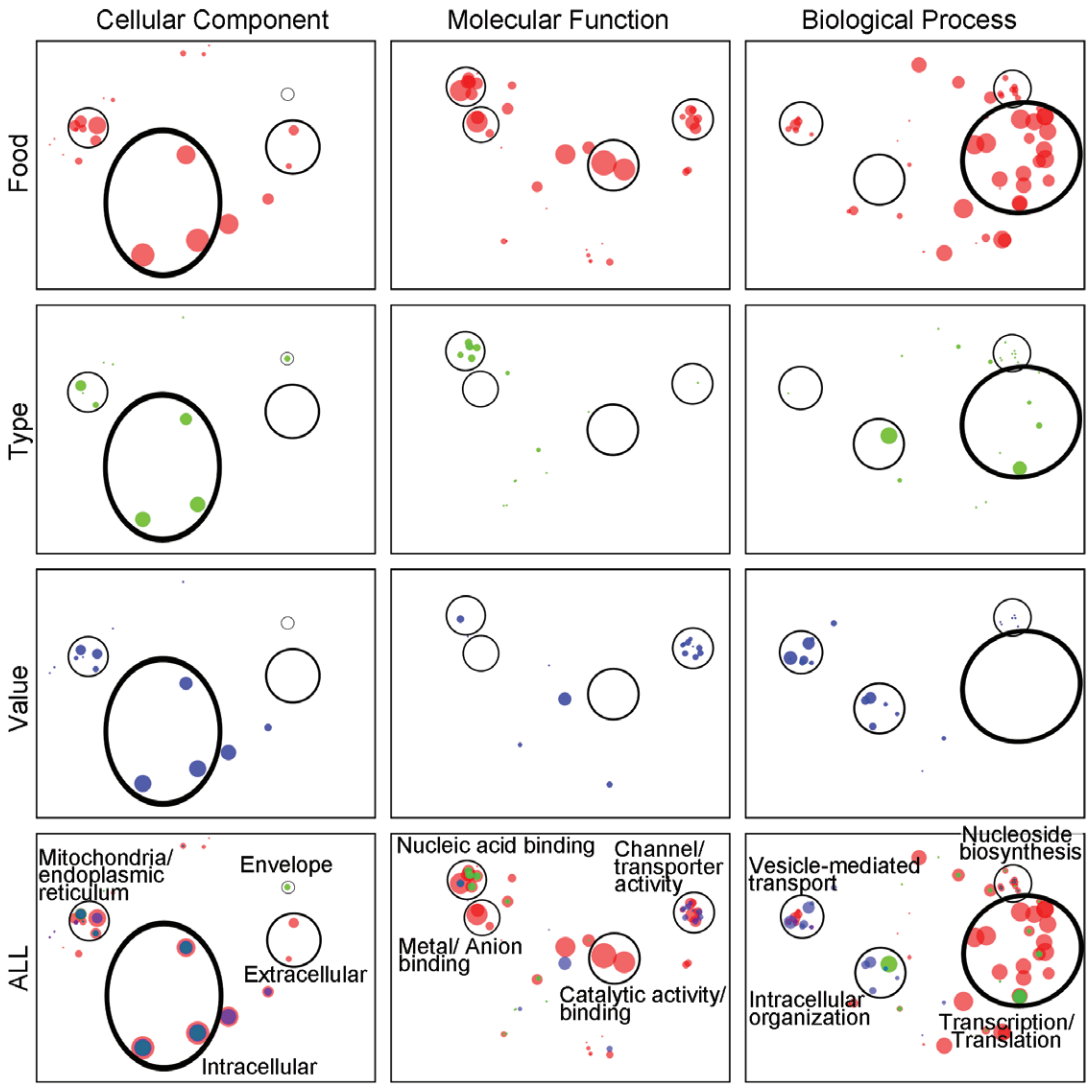


Figure 5: GO terms enriched in modules correlated with treatment and plotted in 2-dimensional semantic space. Each column of graphs represents a different ontology. Specific GO terms enriched in modules correlated with the response to food (red), differences in food type (green), or differences in food value (blue) are plotted on the same axis coordinates individually (top three rows) or together (bottom row). Semantically similar GO terms are circled. The same circles are drawn on all graphs within each column.

in reward value. Functional analyses of the relevant neuronal populations are necessary to confirm these findings, especially at the single cell level.

We were surprised to find that the VUMmx1 neuron, which has been implicated in mediating reward-related behaviors in the honey bee (Hammer 1993), did not respond to food or differences in food type or value in our study. One possible explanation is that our marker of neuronal activation, *c-jun*, does not respond to neuronal activity in the VUMmx1 neuron. In studies of the mammalian brain, multiple IEGs together describe a larger set of responsive neurons than any single gene (Grimm & Tischmeyer 1997). Alternatively, it is possible that the 3-dimensional size of the *c-jun* response in the VUMmx1 neuron was below our limit of statistical detection, unlike for the observed clusters of dopaminergic and octopaminergic cells. A third possibility is that specific octopaminergic neurons are differently involved in food reward information processing depending on whether the animal is starved or satiated, consistent with recent descriptions of specific dopaminergic neuron populations in *Drosophila* (Huetteroth *et al.* 2015).

Brain transcriptional responses to reward are largely unknown for insects. Previous studies with rodents have shown that changes in diet or acute drug rewards elicit transcriptional changes in the brain (Piechota *et al.* 2010), but only one previous study has addressed similar questions in honey bees (Naeger *et al.* 2011). Different groups of honey bees from the same colony trained to forage at different

times and locations showed distinct transcriptomic profiles at the level of the whole brain, suggesting food rewards are likely associated with a molecular signature in the brain (Naeger *et al.* 2011). Although our ISH results support an anatomically distributed response to reward, the rinds dorsal to the mushroom body calyces were differentially responsive to multiple aspects of reward (Fig. 2). The mushroom bodies are also a key area of integration of multiple stimuli and play an important role in cognition (Aso *et al.* 2014b; Farris 2008). Other brain regions are likely to also play an important role in reward response, and future research should investigate the molecular pathways activated by various reward components in these regions.

Our results suggest that assessment of food type and food value involves mostly different molecular pathways in the mushroom bodies, at least when measured 60 min after reward exposure. The response to differences in food value in honey bee mushroom bodies was enriched for genes involved in cell–cell signaling, neuropeptide pathways, and hormone pathways. These results suggest that the early stages of processing reward quality involve fast-acting responders, such as neuropeptides, neurohormones and the intracellular signaling cascades they release. The response we measured at 60 min is likely part of a tuning of these systems for future responses. By contrast, the response to differences in food type was enriched for genes involved in the regulation of transcription and translation. These results suggest that the early stages of processing reward type are dominated by activation of gene regulatory networks and protein synthesis, which may lead to longer-lasting transcriptional cascades than for assessment of food type. Perhaps these differences reflect ecological variation that is associated with honey bee foraging. Bees must adjust rapidly to variation in food value, as the quality of floral resources changes quickly, even over the period of a day (Richter & Waddington 1993). By contrast, individual differences in the tendency to collect nectar or pollen have a strong inherited component and are stable over the lifetime (Page 2013; Pankiw & Page 2000).

We detected conserved elements of the transcriptomic response to food across diverse taxa. It is not known whether this reflects evolutionary conservation of the reward system across distantly related taxa or independently evolved reward systems that are derived from similar neuromolecular systems (Rittschof *et al.* 2014; Vilpoux *et al.* 2009). We highlight the supporting evidence with the following three examples.

First, we detected changes in the expression of genes involved in RNA regulation, which also are differentially expressed in response to the consumption of yeast in *Drosophila* (Gershman *et al.* 2007). It is intriguing that both bees and flies share this response to food because unlike flies, bees do not immediately digest the foods they collect while foraging. This suggests that a transcriptomic cascade is likely triggered by aspects of food collection independent of digestion.

Second, we detected changes in the expression of genes involved in the TOR pathway. Nutritional state and drug rewards, such as those involved in addiction, are regulated through the TOR pathway in both insects and mammals (Bailey *et al.* 2012; Cota *et al.* 2006; Wu & Brown 2006). The TOR protein functions similarly in insects and mammals (Oldham 2011), and a recent study of the mammalian reward system revealed it regulates the mitochondria-associated endoplasmic reticulum membrane, which facilitates the transfer of lipids and calcium (Betz *et al.* 2013). The TOR pathway itself was not enriched among genes responding to reward in our experiments, but three TOR pathway genes, *foxo*, *rictor* and *akt1*, were in modules that were responsive to one or more aspects of reward. Moreover, the mitochondria and endoplasmic reticulum were enriched among gene modules responsive to all aspects of reward, and these cellular components are the center of TOR activity. These results suggest that the TOR pathway may play a conserved role in the response to reward, in both insects and mammals.

Third, we detected changes in the expression of genes involved in dopaminergic pathways. Dopaminergic pathways are known to reside at the core of reward processing in mammals and insects (Perry & Barron 2013), and in our study *dopamine N-acetyltransferase* and *dopamine/ecdysteroid receptor* were in modules that were responsive to food, food type or food value. These two genes do not have mammalian orthologs, but they are key components of DA signaling in insects (Yamamoto & Seto 2014). DA signaling in the nucleus accumbens is also known to partially regulate food valuation in mammals (de Araujo 2011). A recently published study found an association between DA and reward in honey bees (Lagisz *et al.* 2015), and both our transcriptomic and ISH results support a role for dopaminergic signaling in processing food value. These results suggest that certain subpopulations of dopaminergic neurons and dopamine synthesis pathways participate in reward signaling in honey bees, as in fruit flies and mammals.

Our interpretation of some of these results is based on the assumption that honey bees do not digest the sucrose solution they collect, but some evidence suggests honey bees can digest food while in flight (Blatt & Roces 2001). For pollen, the situation is even more clear; as stated above, bees transport pollen in specialized structures on their hind legs, and bees collecting pollen were unlikely to have collected sucrose in our assays because we did not observe bees flying between the sucrose and pollen feeders. If digestion of the sucrose syrup was an important effect in our study, we would expect similar brain activity and transcriptional signals in Experiments 1 (bees that collected sucrose vs. no food) and Experiment 2 (bees that collected sucrose vs. pollen) because both comparisons included groups of bees that either did or did not ingest the sucrose solution. We did not detect strong similarities between these two experiments, and thus we conclude that digestion of the ingested sucrose food reward was unlikely to have played a strong role in our results.

In summary, our findings indicate that the neuroanatomical and neuromolecular architectures of reward-based behavior in bees share some common features and specific molecular substrates with mammals. Honey bees collect food for their colony rather than for themselves, indicating that social evolution has relied on elements of reward processing that function at the level of the individual in order to build a social reward system.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1: Brain region dissected for RNA sequencing. Blue dashed line indicates the dissected region containing mostly mushroom body (MB) tissue, outlined in black dashed lines.

Figure S2: Brain regions differently activated in bees collecting different food types. Eleven voxel clusters in eight brain regions were responsive (Student's *T*-Test, threshold T statistic = 2.10; sucrose syrup, $n = 10$; pollen, $n = 9$). Heat map color intensity corresponds to the P value at each voxel between bees gathering each resource. Orange voxels represent higher expression in bees gathering sucrose syrup, and blue voxels represent higher expression in bees gathering pollen. One section is shown every 22.5 μm. ME, medulla; MB, mushroom body; AL, antennal lobe; OL, optic lobe; GG, gnathal ganglion. Scale bar = 500 μm.

Figure S3: Brain regions differently activated in bees collecting different food values. Eleven voxel clusters in seven brain regions were responsive (Student's *T*-Test, threshold T statistic = 2.179; high value, $n = 8$; low value, $n = 6$). Heat map color intensity corresponds to the P value at each voxel between bees gathering each resource. Orange voxels represent higher expression in bees gathering food of high value, and blue voxels represent higher expression in bees gathering food of low value. One section is shown every 22.5 μm. ME, medulla; MB, mushroom body; AL, antennal lobe; OL, optic lobe; GG, gnathal ganglion. Scale bar = 500 μm.

Figure S4: Brain regions responsive to food reward and/or differences in food type or value. Voxel clusters differentially expressing *c-jun* in response to food (red; food reward, McNeill & Robinson 2015), different food types (green; sucrose syrup, $n = 10$; pollen, $n = 9$), food values (blue; high value, $n = 8$; low value, $n = 6$) are represented by masks. Green and blue masks correspond to statistical differences presented in Figs. S2 and S3, respectively. Arrowheads indicate voxels responsive to food and different food types (orange), food and different food values (purple), or different food types and values (light blue). Cartoon drawings of somata represent hand tracings of spatially normalized markers for DA (*th*; yellow) and OA (*tbh*; magenta) expressing neurons. We detected seven previously described DA-expressing populations (generated from one spatially normalized brain image, C_1, C_3, S_L, S_P, $S_3/S_5/S_6$; Schafer & Rehder 1989) and three previous described OA-expressing populations (G3a/b, G6a/b, VUM; Lehman *et al.* 2006; Sinakevitch *et al.* 2005; images generated from the mean intensity value of 3 spatially normalized brain images). We represent $S_3/S_5/S_6$, G3a/b and G6a/b as single populations because we are unable to distinguish between them. S_4 and S_7 are posterior to the last optical section and were not responsive to rewards (not shown). The C3 and S_L groups are labeled twice to describe their 3-dimensional nature. Scale bar = 500 μm. AL, antennal lobe; OL, optic lobe; MB, mushroom body; rCAv, rind ventral to the calyx; rLCAl, rind lateral to the lateral calyx.

Figure S5: Modules of co-expressed genes in bees responding to food ($n = 9$ per group), differences in food type ($n = 9$ per group), or differences in food value ($n = 10$ per group). Module numbers correspond to those described in Fig. 4 and Table S3. Red shading represents significant overlap of member genes (FDR < 0.05). Modules correlated with treatment in each experiment are highlighted in yellow (FDR < 0.1). (a) Numerical values represent FDR-corrected hypergeometric P values for each module pairwise comparison. (b) Numerical values represent the number of genes shared between indicated modules.

Table S1: DNA sequences of probe sets used to reveal mRNA expression patterns.

Table S2: Descriptive statistics for each gene in bees responding to food, differences in food type or differences in food value.

Table S3: Gene co-expression modules correlated with the response to food ($n = 9$ per group), differences in food type ($n = 9$ per group), or differences in food value ($n = 10$ per group). 'No module' indicates the genes were not assigned to a module by WGNCA.

Table S4: GO terms enriched in treatment-responsive modules.