

# Distance-Responsive Genes Found in Dancing Honey Bees

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## Summary

We report that regions of the honey bee brain involved in visual processing and learning and memory show a specific genomic response to distance information. These results were obtained with an established method that separates effects of perceived distance from effects of actual distance flown. Individuals forced to shift from a short to perceived long distance to reach a feeding site showed gene

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expression differences in the optic lobes and mushroom bodies relative to individuals that continued to perceive a short distance, even though they all flew the same distance. Bioinformatic analyses suggest that the genomic response to distance information involves learning and memory systems associated with well-known signaling pathways, synaptic remodeling, transcription factors and protein metabolism. By demonstrating distance-sensitive brain gene expression, our findings also dramatically extend the emerging paradigm of the genome as a dynamic regulator of behavior, that is particularly responsive to stimuli important in social life.

## **Introduction**

Animals may need to know distances for many reasons, but in honey bees, distance is socially recalled and shared with colony members through dance communication (von Frisch 1967). Honey bees use this communication system to recruit nestmates to attractive food sources by providing information about distance, direction, and quality (Dyer 2002), especially in environments where food is patchily distributed (Dornhaus & Chittka 2004). Behavioral experiments demonstrate that bees measure distance via image motion on the eye (optic flow) (Esch & Burns 1995), but neural representations of distance are not known, for any animal species that measures distance. Molecular analyses have the potential to suggest biological processes that might be involved in brain calculations of distance.

Honey bees may forage over long distances (up to 12 km) and therefore need to be sensitive to distance information (Seeley 1995). Foraging distances greater than 100 m are encoded by a series of abdominal “waggle” movements whose duration is correlated with the vector distance of the food source from the hive and whose orientation indicates the direction. Foraging distances less than 100 m are represented by “round” dances that do not provide distance information.

To begin to explore the molecular bases of distance measurement, we used microarray analysis to determine whether there are distance responsive genes in the bee brain, i.e., genes whose expression changes in response to differences in the distance flown from hive to food resource. This study was motivated both by findings of gene activation in the brain in response to highly specific naturally

occurring environmental stimuli, such as bird song (Clayton 2000), and by evidence for foraging-related effects on brain gene expression in honey bees (Whitfield *et al.* 2003, 2006). Transcriptomic analysis provided the opportunity to indirectly measure aspects of brain activity in association with distance estimation in naturalistic, free flying, conditions, which is not yet routine for measurements of brain electrical activity (Fee & Leonardo 2001). Bioinformatic analysis was then used to infer biological processes associated with distance responsiveness, as reflected in the patterns of gene expression.

Studying molecular representations of distance during natural flight risks confounding effects of an individual's perception of distance and effects of differential energy expenditure resulting from differences in distance flown. To avoid this problem, we used an established method that separates effects of perceived distance from effects of actual distance flown (Srinivasan *et al.* 2000).

## **Materials and methods**

### ***Behavioral manipulations***

We trained bees to fly to a feeding station through a narrow, 1.8 m long tunnel with interchangeable visual patterns on the walls (Srinivasan *et al.* 2000). Since bees measure distance from optic flow, they can be tricked into perceiving a long (*L*) distance when flying past a vertical striped pattern (generating high amounts of image motion, similar to a visually dense landscape), and a short (*S*) distance when flying past a horizontal striped pattern (similar to a visually sparse landscape). We sampled three groups of bees from the same colony, all who had flown the same distance but perceived that they were flying different distances (Fig. 1).

On Days 1 and 2 of the experiment, "*S*→*L*" bees flew a short distance and on Day 3 they were forced to perceive flying a long distance. This mimics the common natural condition of a decline in the profitability of a floral patch forcing bees to change foraging location (Seeley 1983). As a control, "*S*→*S*" bees flew a short distance and then were sham-manipulated to continue perceiving a short flight distance. We also attempted to collect "*L*→*S*" bees for microarray analysis. *L*→*S* bees initially perceived a long flight distance on Days 1 and 2 and then were forced to perceive flying a short distance

on Day 3. It was harder to train  $L \rightarrow S$  bees than  $S \rightarrow L$  bees, and relatively few  $L \rightarrow S$  bees passed the behavioral performance threshold for selection for microarray analysis (see below and Table S1). They were thus reserved only for subsequent follow-up with quantitative PCR, and no  $L \rightarrow L$  bees were collected.

Thirty minutes after exposure to the novel or control distance, bees were captured in liquid nitrogen. All focal bees ( $N = 21 S \rightarrow S$ ;  $22 S \rightarrow L$ ; and  $13 L \rightarrow S$  bees) were removed from their glass-walled observation hive and placed into liquid nitrogen. We sampled two brain regions: the optic lobes and mushroom bodies, because previous studies implicate them in distance information processing (Bausenwein & Fischbach 1992; Ehmer & Gronenberg 2002).

Videotape analysis of dance behavior for each focal bee was used to select the top performing individuals ( $N=5/\text{group}$ ) for microarray analysis. To validate the effects of the flight experiences, we used the bees' own dance language to assess how far (they indicated that) they had flown. This was done on Day 2: the  $L$  distance was expected to provoke waggle dances and the  $S$  distance, round dances. Threshold for selection was  $> 60\%$  "correct" dances for either short or long distances (Srinivasan *et al.* 2000) (Table S1). Dissection of frozen brain regions and mRNA preparation and amplification was as in Sen-Sarma *et al.* (2009).

### ***Microarray analysis***

Using a paired "loop" design, we compared one  $S \rightarrow L$  and one  $S \rightarrow S$  individual on each honey bee "whole genome" microarray (Alaux *et al.* 2009), either samples from mushroom bodies (MB) or optic lobes (OL). Each pair was matched as best as possible for number of trips made through the tunnel on Day 2.

Forty arrays were used, 8 arrays for each of the five pairs of bees, with each sample (OL or MB) used on two arrays (dye swap to control for possible differential effects of Cy5 and Cy3 dyes on hybridization intensities). Oligonucleotide spots flagged with "-100" by Axon GenePix 6.0 (Molecular Devices, Sunnyvale, CA) were removed from the analysis, and the remaining data filtered using the

median of control elements on the microarray as the threshold. LOWESS normalization was carried out on the log<sub>2</sub> transformed intensities, which were adjusted for global dye and microarray effects. Spots representing 9478 transcripts (out of 13,440) passed these quality control filters. Data were analyzed with a mixed-effects ANOVA model fitted using SAS Proc MIXED (SAS Institute, Cary, NC.). The model included the fixed effects of dye, distance (L, S), trip (1, 2, 3), brain region (MB, OL), corresponding first and second-order interactions, and the random effects of bee and microarray. Results are reported for, and subsequent correlation analysis carried out on, only those genes that were detected on every array. All probability values were corrected for multiple testing using FDR calculations.

Gene expression correlation was performed in R on the log<sub>2</sub>-transformed individual bee and brain region estimates of 8200 genes that were not distance responsive, using Spearman's rank correlation test. Distance-correlated genes were defined as genes that are correlated ( $p < 0.05$ , Benjamini-Hochberg corrected) with a distance-responsive gene in a Spearman's rank correlation test performed across all samples obtained from  $S \rightarrow S$  and  $S \rightarrow L$  bees ( $N = 5$  individuals/group  $\times$  2 brain regions = 20 samples). GO categories were assigned to distance-responsive genes using FUNC (Prüfer et al., 2007) with the following settings: hypergeometric test, *Drosophila melanogaster* GO annotation (31 October 2009), only for categories that had  $\geq 5$  genes per GO category. Only distance-correlated genes that have a 1:1 *Drosophila melanogaster* ortholog were used for this analysis. Only GO categories with  $p < 0.001$  before "refinement" (removal of GO terms significant solely because they are "parent" categories) and  $p < 0.05$  after refinement were reported. Principal Component Analysis of log<sub>2</sub>-transformed estimates of the 29 annotated distance-responsive genes for the 10 individuals  $\times$  two brain regions was carried out in JMP (SAS Institute).

### ***Real-time quantitative PCR***

Quantitative PCR (qPCR) was performed as in Corona *et al.* (2005) with an ABI Prism HT7900 sequence detection system (Hayward, CA), Perfecta Sybr Green (Quanta Biosciences, Gaithersburg, MD) and normalized to a standard curve with known quantities of bee genomic DNA (ABI User

Bulletin #2). *Rootcap protein* (*Arabidopsis*) was used as a spiking control. *Armc4* and *GB18933* expression levels were analyzed with a non-parametric ANOVA (Kruskal Wallis rank sum test with a one-way Chi-square approximation) performed in JMP (SAS Institute) to test for effects of distance on gene expression. Due to technical problems, very little experimental material was available for qPCR, so we analyzed only two genes shown to be differentially expressed in microarray analysis.

## Results and discussion

A total of 29 annotated genes and 23 unannotated transcripts (out of 9478 transcripts that passed quality control analysis on the microarray) were differentially regulated (ANOVA, FDR < 0.05) between  $S \rightarrow L$  and  $S \rightarrow S$  bees, either in the optic lobes, mushroom bodies, or both (Table 1). By contrast, 1817 transcripts were differentially regulated between the two brain regions at the same FDR, comparable to a previous study of brain region-differences in a different set of foraging bees (Sen-Sarma *et al.* 2009).

Despite only 29 annotated genes showing differential expression between  $S \rightarrow L$  and  $S \rightarrow S$  bees, Principal Component Analysis (PCA) of expression profiles clearly distinguished the two distance groups. The first two principal components accounted for 63% of the variance between individuals (Fig. 2A).

Only half of the 29 genes showed regional expression biases in response to a change in perceived distance and PCA also revealed similar effects of distance on gene expression in both brain regions. This result is interesting because the two brain regions have different functions; the optic lobes process visual input from the eyes and the mushroom bodies carry out higher order processing of multimodal sensory input from multiple parts of the brain. The similarity of expression profiles suggests that processing distance information engages similar molecular pathways in the two brain regions; coordinated gene expression during dance communication also has been reported for motor-related brain regions (Sen Sarma *et al.* 2009). In addition, the fact that the mushroom bodies showed a genomic response to distance suggests that the effects of distance on the bee brain are not solely related to stimulus

perception, but also engage molecular pathways involved in distance-related memories. This hypothesis should be tested in future studies.

To probe the transcriptional network(s) associated with distance responsiveness, we enlarged our small list of distance responsive genes using an established method (Nowick *et al.* 2009) for identifying genes whose expression levels were significantly correlated with the distance-responsive genes (Spearman's rho, Benjamini-Hochberg corrected  $p < 0.05$ ) but were not themselves significantly distance responsive. We used this method to highlight functional categories represented by groups of "distance-correlated" genes. Gene Ontology (Ashburner *et al.* 2000) (GO) enrichment analysis revealed significant overrepresentation of genes associated with GO terms related to synaptic plasticity, cell signaling, locomotion, circadian rhythm, gene expression, protein synthesis and turnover, and energy production (Table S3).

Genes involved in Wnt signaling, implicated in learning and memory (Chen *et al.* 2006), also were shown to be overrepresented, and were correlated with the distance-responsive genes *Orb2*, *Apisimin* and *CYP6A51*, a member of the cytochrome p450 family that so far only has been found in bees and other hymenopterans (Oakeshott *et al.* 2010) (Table S3). *Orb2* is a cytoplasmic polyadenylation element binding (CPEB) protein required for long-term retention of courtship memory in *Drosophila* (Keleman *et al.* 2007). In addition, one of the distance-responsive genes upregulated in  $S \rightarrow L$  bees was *dunce*, a cAMP dependent phosphodiesterase involved in learning and memory (Davis 1996).

We used qPCR to compare the expression of two of the 29 distance-responsive genes. *Armc4* (*Armadillo repeat containing 4*) had shown the biggest response with microarray analysis, > 20 fold higher expression in  $S \rightarrow L$  relative to  $S \rightarrow S$  bees in both brain regions. *Armc4* is a member of the Armadillo family of proteins, which also have been implicated in neuronal plasticity (Maguschak & Ressler 2008). qPCR revealed a significant effect of distance on *Armc4* expression (Fig 2B), upregulated in  $S \rightarrow L$  relative to  $S \rightarrow S$  bees, as with microarrays. In addition, *Armc4* was upregulated in  $S \rightarrow L$  relative to  $L \rightarrow S$  bees. *GB18933*, a neurotransmitter-gated ion channel gene, also showed similar results for  $S \rightarrow S$  and  $S \rightarrow L$  bees in both microarray and qPCR analysis (Fig 2B) but in contrast to *Armc4*, *GB18933* was

upregulated in both  $S \rightarrow L$  and  $L \rightarrow S$  bees. Extrapolating from just these two genes, it appears that some genes (e.g., *GB18933*) respond generally to novelty or any change in distance, while other genes (e.g., *Armc4*) respond only to more specific changes in distance.

From these results alone it is not possible to know whether some of the genes identified here are involved in the retrieval of distance information during dance communication. Foragers need to remember distances; they share distance information during bouts of dance communication that occur up to one hour after a successful foraging trip and they themselves often visit the same floral patches over the course of one or more days (Dyer 2002; von Frisch 1967). The mushroom bodies have been implicated in spatial learning (Mizunami *et al.* 1998) and motor control (Serway *et al.* 2009), and are especially well studied with respect to odor memory (Davis 2005). Just odor exposure alone in the hive can trigger some bees to return to a familiar food source (Reinhard *et al.* 2004). Our results provide a starting point for studying how animals measure and remember distance (Wehner 2003).

Our results indicate that the responsiveness of the genome to social information extends to inputs that require the formation of quantitative representations in the brain. Previous studies identified effects of stimuli that are novel or represent categorical information (Clayton 2000; Robinson *et al.* 2008). One challenge in behavioral genomics is to elucidate how brain genomic responses lead to adaptive behavior. Distance measurement joins a growing number of naturally occurring and experimentally accessible behavioral traits that will help us solve this important problem.

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## Figure Legends

**Fig 1.** Experimental design exploited perception of distance by optic flow on the retina to “trick” bees into perceiving either a long or a short distance, while actually flying the same distance. This allowed us to measure the effects of distance information on brain gene expression while separating effects of perceived distance from effects of actual distance flown.

**Fig 2.** Perception of different distances during foraging flight causes expression changes in a small number of genes in the optic lobes (OL) and mushroom bodies (MB). (A) Principal Component Analysis of distance responsive gene expression in “short→long” ( $S \rightarrow L$ ) and “short→short” ( $S \rightarrow S$ ) bees nevertheless reveal clear separation of the two distance groups and similar effects of distance on gene expression in both brain regions. (B) qPCR analysis revealed different types of distance effects. *Armc4* was responsive to “specific” distance (see text) while *GB18933* was responsive to “novel” distance (Mean + SE, Kruskal-Wallis test,  $p < 0.001$ ).  $N=5$  bees/group.  $S \rightarrow S$  and  $S \rightarrow L$  bees also were used in microarray analysis;  $L \rightarrow S$  bees were only used for qPCR.

**Table 1.** Distance-responsive genes in honey bee mushroom bodies and optic lobes.

| <b>Oligo</b> | <b>Fly ortholog</b>                               | <b>Fold Difference (S→L vs. S→S)</b> | <b>P value (post-hoc T test)</b> |
|--------------|---|--------------------------------------|----------------------------------|
| AM11638      | <i>Armc4</i>                                      | 21.33                                | 3.1E-34                          |
| AM02392      | -   | 14.30                                | 1.7E-29                          |
| AM04113      | -   | 6.02                                 | 2.4E-18                          |
| AM06519      | <i>ATP-binding cassette sub-family A member 3</i> | 3.43                                 | 6.6E-11                          |
| AM01836      | -   | 3.21                                 | 4.3E-10                          |
| AM11490      | -   | 3.21                                 | 4.3E-10                          |
| AM01437      | -   | 2.74                                 | 3.7E-08                          |
| AM12055      | <i>Heat shock protein Hsp70Ab</i>                 | 2.59                                 | 1.6E-07                          |
| AM00691      | -   | 2.48                                 | 5.0E-07                          |
| AM12020      | <i>Apisimin</i>                                   | 2.43                                 | 8.4E-07                          |
| AM11706      | <i>Golden Goal</i>                                | 2.35                                 | 2.0E-06                          |
| AM02265      | -   | 2.32                                 | 2.7E-06                          |
| AM02467      | -   | 2.32                                 | 2.7E-06                          |
| AM00690      | -   | 2.25                                 | 5.7E-06                          |
| AM00417R     | -   | 2.24                                 | 6.3E-06                          |
| AM02832      | <i>Invadolysin</i>                                | 2.23                                 | 6.9E-06                          |
| AM10558      | -   | 2.21                                 | 8.5E-06                          |
| AM03481      | <i>CYP342A1</i>                                   | 2.17                                 | 1.3E-05                          |
| AM00658      | -   | 2.14                                 | 1.8E-05                          |
| AM04874      | -   | 2.13                                 | 2.1E-05                          |
| AM12444      | <i>Starvin</i>                                    | 2.07                                 | 4.0E-05                          |
| AM01177      | -   | 2.05                                 | 5.1E-05                          |
| AM00480      | -   | 2.01                                 | 7.6E-05                          |
| AM00851      | -   | 1.99                                 | 9.2E-05                          |
| AM11471      | -   | 1.99                                 | 9.2E-05                          |
| AM00691R     | -   | 1.95                                 | 1.5E-04                          |
| AM11661      | -   | 1.93                                 | 1.8E-04                          |
| AM10016      | -   | 1.93                                 | 1.8E-04                          |
| AM07900      | <i>Dunce</i>                                      | 1.90                                 | 2.5E-04                          |
| AM08417      | <i>Orb2</i>                                       | 1.89                                 | 2.9E-04                          |
| AM01970      | -   | 1.88                                 | 3.2E-04                          |
| AM00974      | <i>Protein silver</i>                             | 0.53                                 | 2.9E-04                          |
| AM02929      | <i>Gr64f</i>                                      | 0.52                                 | 2.2E-04                          |
| AM01666      | -   | 0.51                                 | 1.4E-04                          |
| AM09062      | -   | 0.51                                 | 1.2E-04                          |
| AM09476      | <i>CYP6AS1</i>                                    | 0.50                                 | 7.2E-05                          |
| AM00963R     | -   | 0.48                                 | 3.8E-05                          |

|          |                   |      |         |
|----------|-------------------|------|---------|
| AM12674  | -                 | 0.45 | 9.6E-06 |
| AM00464  | -                 | 0.45 | 7.5E-06 |
| AM01400  | -                 | 0.45 | 6.2E-06 |
| AM05793  | -                 | 0.44 | 5.6E-06 |
| AM10261  | -                 | 0.44 | 5.1E-06 |
| AM00464R | -                 | 0.44 | 5.0E-06 |
| AM12749  | -                 | 0.43 | 3.2E-06 |
| AM12887R | -                 | 0.43 | 2.3E-06 |
| AM02118  | -                 | 0.38 | 1.3E-07 |
| AM00770  | -                 | 0.37 | 5.9E-08 |
| AM00523  | -                 | 0.35 | 9.5E-09 |
| AM07337  | -                 | 0.33 | 3.1E-09 |
| AM00360  | <i>Apidaecin1</i> | 0.24 | 1.5E-13 |
| AM00353  | <i>Apidaecin2</i> | 0.23 | 3.3E-14 |
| AM00356  | <i>Apidaecin1</i> | 0.22 | 2.1E-14 |
| AM00351  | <i>Apidaecin2</i> | 0.22 | 7.5E-15 |
| AM00357  | <i>Apidaecin1</i> | 0.21 | 4.1E-15 |
| AM00354  | <i>Apidaecin1</i> | 0.20 | 1.2E-15 |
| AM00359  | -                 | 0.20 | 5.5E-16 |
| AM00352  | <i>Apidaecin2</i> | 0.20 | 5.5E-16 |
| AM06814  | -                 | 0.17 | 3.0E-18 |

**Supplementary Table 1.** Selection of bees for molecular analyses based on behavioral performance. S→L and S→S bees were used for microarray analysis and S→L, S→S and L→S bees were used for qPCR analysis. Results of dance behavior analysis were used to select individuals. We analyzed dance behavior on Day 2, when bees were flying through a tunnel with horizontal stripes (S→L and S→S groups) or vertical stripes (L→S). S→L and S→S groups were both expected to perceive flying a “short” distance and thus dance predominantly round dances while L→S were expected to perceive flying a “long” distance and thus dance predominantly direction-oriented waggle dances. All bees for microarray analysis showed > 60% correct dances. Bees in the L→S group were more variable and used only for qPCR.

| <b>Distance</b>   |              |                         |                          |  |
|-------------------|--------------|-------------------------|--------------------------|--|
| <b>Experience</b> | <b>Bee #</b> | <b>Round dances (%)</b> | <b>Waggle dances (%)</b> |  |
| S→L               | 1            | 86                      | 14                       |  |
| S→L               | 2            | 100                     | 0                        |  |
| S→L               | 3            | 85                      | 15                       |  |
| S→L               | 4            | 100                     | 0                        |  |
| S→L               | 5            | 100                     | 0                        |  |
| S→S               | 6            | 83                      | 17                       |  |
| S→S               | 7            | 89                      | 11                       |  |
| S→S               | 8            | 100                     | 0                        |  |
| S→S               | 9            | 80                      | 20                       |  |
| S→S               | 10           | 94                      | 6                        |  |
| L→S               | 11           | 16                      | 84                       |  |
| L→S               | 12           | 45                      | 55                       |  |
| L→S               | 13           | 38                      | 63                       |  |
| L→S               | 14           | 38                      | 63                       |  |
| L→S               | 15           | 63                      | 38                       |  |

Figure 1

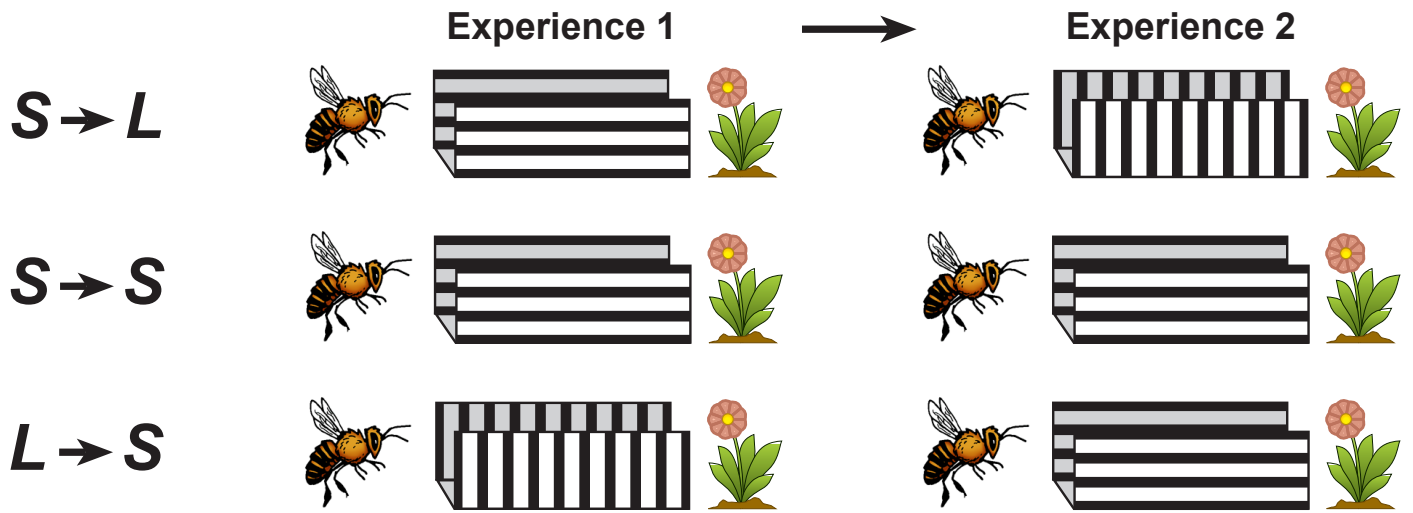


Figure 2A

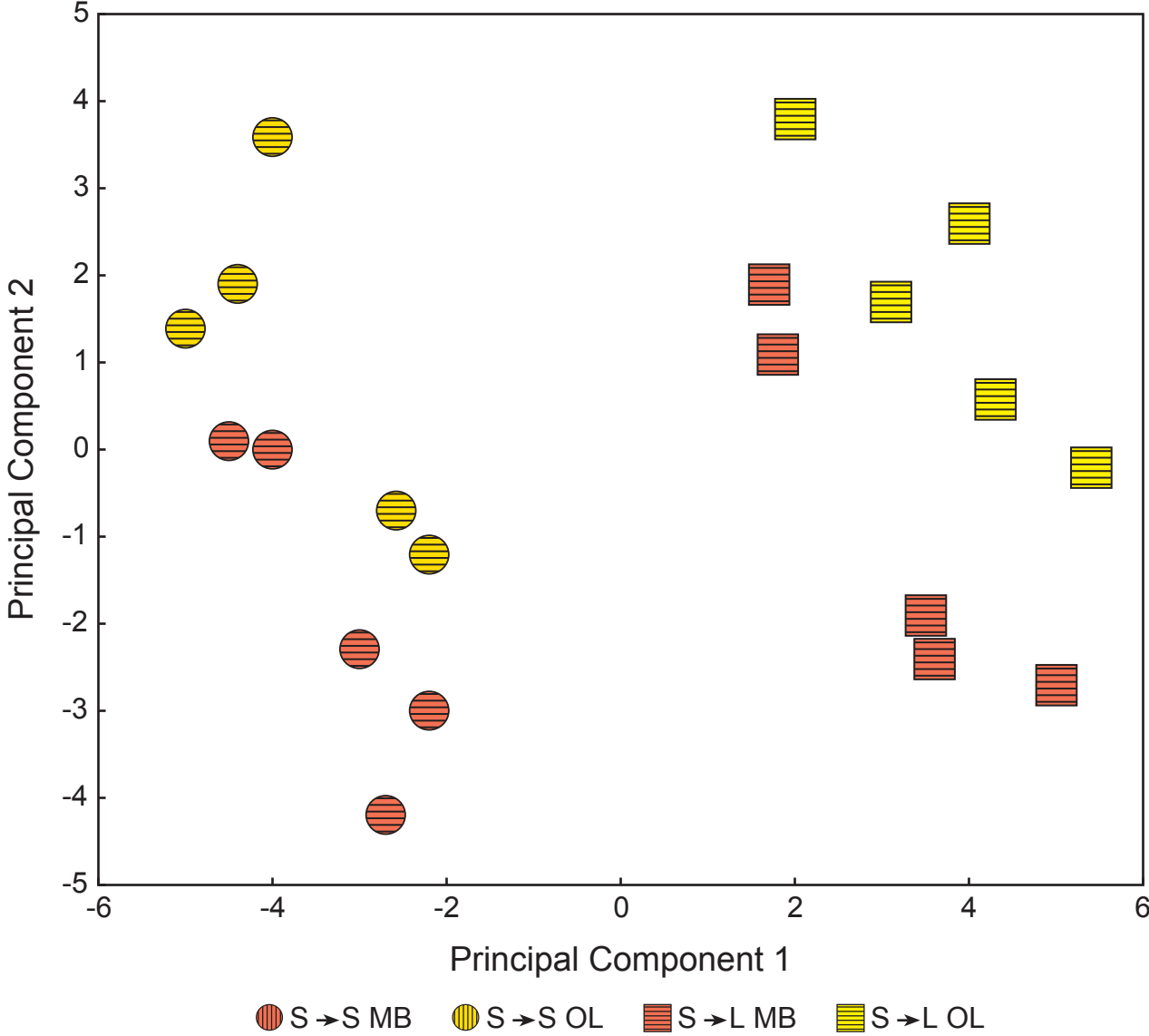


Figure 2B

