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研究課題名（和文）Genetic control of honeybee dance

研究課題名（英文）Genetic control of honeybee dance

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研究成果の概要（和文）：本研究はミツバチのダンス言語をモデルとし複雑な形質の遺伝的構築の理解を目的とする。コロニー中の個々のミツバチの行動を追跡するソフトウェアの開発は大きく進展した。しかし、本研究で用いたミツバチ品種が2シーズン交配せず、沖縄の気候に適応できないことがわかったため、追跡ソフトをダンス言語のリンケージ解析とは別の研究で活用するとともに、ミツバチの行動学や生理学研究のための遺伝子発現抑制実験(RNAi)へと軸足を移した。今後、これらの研究を通して得られた研究ツールを用い、本ミツバチ品種に適した地域の共同研究者とミツバチの交配を行うことで、当初の計画通りダンス言語の遺伝的構築の解析に取り組む予定である。

研究成果の概要（英文）：Understanding how genes make new phenotypes has been an ongoing challenge, despite the availability of genome-wide data. This project aimed to further our knowledge of the genetic organization of complex traits using they honey bee dance language a model. We made significant progress on developing the tracking software. Unfortunately, the geographic race of honey bees we proposed for the study did not perform well in the Okinawan climate. After two seasons of failed crosses we pivoted towards other applications of this technology and other experimental perturbations of gene expression (RNAi) for the study of bee behavior and physiology. The experimental toolkit developed in the course of this grant has great potential, and we are applying it to a range of other studies. Using the developed tools, we will also continue to work on the genetic architecture of dance, as originally planned, but conducting the crosses with a collaborator located in a more suitable climate.

研究分野：　生態・環境

キーワード：　animal behavior　social insects　machine learning

１．研究開始当初の背景

Despite remarkable technological advances in the field of genome sequencing, the genetic underpinnings underlying most complex traits, and how these mechanisms lead to the evolution of new phenotypes remains poorly known. Finding genes responsible for observable phenotypes, and with particular relevance to ecology and evolution remains a key goal. This is particularly true for behavioral traits, which are often difficult to accurately phenotype and are context-dependent in expression.

２．研究の目的

The main challenges of this research are to find behavioral traits under defined genetic control, and to have high-throughput means of capturing and quantifying the behavior. Therefore, goals of this project were two-fold: (1) to identify traits under suitable genetic control and (2) to develop a system capable of tracking the behaviors of individual bees over long periods of time.

Our initial idea was to use the honey bee dance language as a model, and use linkage mapping to identify loci of interest. However, despite two seasons of attempting to construct the requisite crosses, we were unable to do so, because one of the geographic races we used was imported from Europe and did not thrive in the subtropical climate of Okinawa. As a result, we continued development of the visual tracking tools, and focused on a different phenotype (reproductive division of labor) for study of genetic control using a different technique (RNAi).

Computer vision objectives

Image-based dense object tracking is of broad interest in the monitoring of crowd movement as well as the study of collective behavior in biological systems. Automated recognition of individuals in a dense group based on video recording would allow for the efficient implementation of monitoring and tracking frameworks with no additional manual labeling or tracking devices, which are often either impractical or invasive. The challenges in image based dense object recognition include occlusions and variability in viewpoints and individual appearance. However, recent progress in convolutional neural networks (CNNs). For image segmentation, scene analysis, and object detection represent promising developments towards dense object detection and tracking. We apply these tools to a classical unsolved problem in behavioral ecology, the identification of individual organisms in a honeybee hive.

Phenotypic control objectives

Specifically, we tested the hypothesis that reproductive division of labor in the honey bee exapted an ancient regulatory framework used to specify sex in insects, controlled by the Doublesex transcription factor. While the regulatory mechanisms underlying reproductive division of labor have been well-studied, we hypothesized that a more upstream regulatory mechanism may exist, based on the fact that key components of caste differentiation (ovarian development and chemical signaling) are controlled by the Doublesex pathway in other insects.

３．研究の方法

Computer vision

We used solution integrating the fully convolutional neural network U-Net with a recurrent component for accurate object detection in a video sequence. Image data was generated from high-resolution video recordings of a custom-designed observation beehive in which a honeybee colony was placed on one side of a beehive comb, covered with transparent glass and illuminated with infrared light which is imperceptible to the bees. The movement of bees was recorded at speeds up to 70 fps to minimize blur due to movement (Fig. 1).

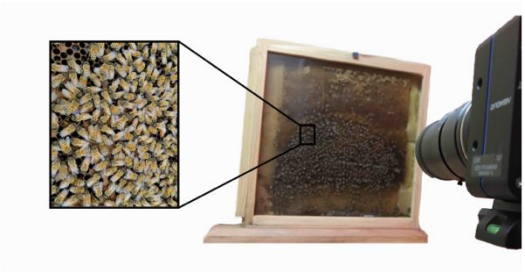


Fig. 1. Overview of behavioral data acquisition setup.

To generate training data we used custom JavaScript interface for manual annotation of bee locations and orientations in the images. Through the interface the user defines a bee position

and orientation by dragging, dropping, and rotating a bee symbol in an image. We used this interface to generate a labeled image set through Amazon Mechanical Turk.

Phenotypic control

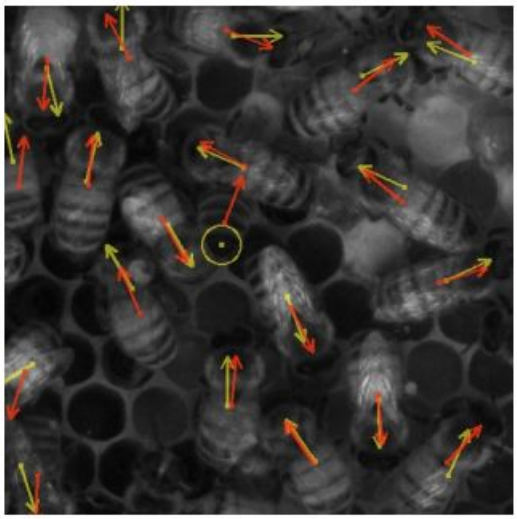
We targeted the female-specific F2 isoform of Dsx (NCBI ID NM_001134935.1), which differs from the other Dsx isoform that is expressed in both sexes (NCBI ID NM_001134936). The experiments were performed at the Okinawa Institute of Science and Technology apiary using Italian bees. We dissected surviving experimental bees and kept heads at -80°C for further pheromonal analysis. To quantify ovary activation, we scored dissected ovaries on a standard scale from 0 to 3: 0 being used for underdeveloped ovaries (without distinguishable oocytes), 1 for enlarged ovaries, 2 for ovaries containing small oocytes, and 3 for developed ovaries with eggs. Heads were transferred into borosilicate glass tubes, inserted into a 2-mL Eppendorf tubes containing 400 µL of chloroform and 5 µL of internal standard solution (composed of 1 mg of octanoic acid, 1 mg of tetradecane in 4 mL dichloromethane). The concentration of mandibular gland components was then assessed by GC/MS. From each treatment group, we selected 10 honey bees (20 total, 10 honey bees with lower and 10 bees with higher ovary activation scores) for RNA-seq analysis, sequencing data on the HiSeq 4000 machine. We employed two alternative approaches for differential gene expression analysis: a more traditional pipeline (RSEM) using read mapping and an alignment-free method (Kallisto). We used edgeR for differential gene expression of RSEM data, and Sleuth for Kallisto data. They gave the same results for Dsx and Vg levels, and we proceeded with the former pipeline for weighted gene co-expression analysis (WGCNA) to identify the Dsx-responsive module.

4．研究成果

Computer vision

We were able to successfully identify the position and orientation of unmarked bees. The best performing algorithm had error rates similar to those of humans, as estimated by re-labeling tasks on Amazon Mechanical Turk. Overall, bees could be predicted with a true positive rate of 0.96, and a false positive rate of 0.14. Furthermore, we could predict body orientation with an average error of just 12 degrees (Fig. 2)

Fig. 2. Predicted and measured body positions and orientation of unmarked bees. Measured orientation is in yellow and predicted directed axis in red.

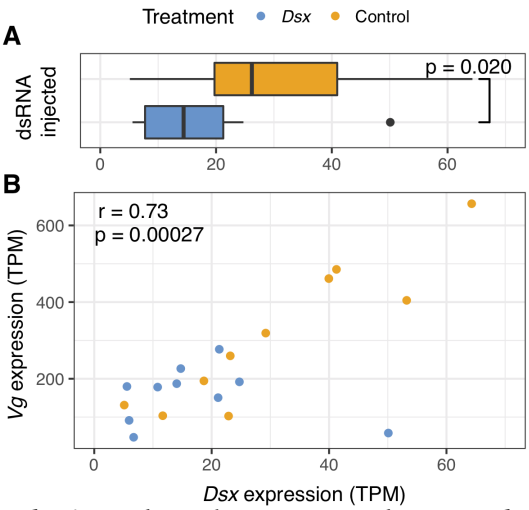


To the best of my knowledge this is the first time bees could be tracked across the entire colony without tags, opening perspectives for the study of whole-hive organization.

Phenotypic control

We sequenced twenty libraries split evenly among treatment and control groups on an Illumina HiSeq 2500 sequencer at the OIST Sequencing Center (SQC). The experiment yielded 3.5×107 ± 4.7×106 (s.d.) RSEM-mapped single-end reads per library. The overall fit of observed vs. expected spike-in controls transcripts explained 87% of the variance, indicating adequate technical performance. The fit did not increase at subsequent abundance cutoffs; therefore, we used data for downstream analyses without additional filtration. We detected significantly lower Dsx expression in RNAi-treated worker abdomens using both the RSEM/edgeR and Kallisto/Sleuth pipelines (Fig. 3A). Furthermore, only the expression of the targeted female-specific isoform of Dsx was lower. Levels of Vg were also significantly lower in bees where Dsx was knocked down, and were strongly correlated with those of Dsx, as predicted (Fig. 3B).

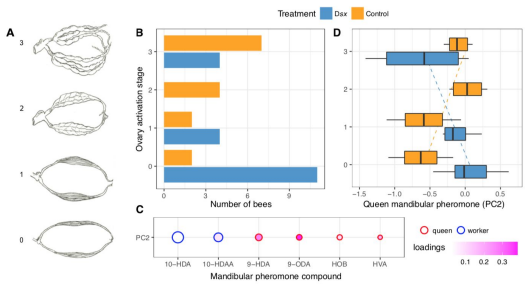
Fig. 3. Dsx knockdown confirms the regulatory link between Dsx and Vg in honey bees. (A) RNA-seq confirmed that mean expression levels of Dsx were reduced 43.3% relative to GFP-injected controls (measured in Transcripts Per Million (TPM) mapped reads). (B) As predicted, expression of Dsx and Vg were tightly linked overall, and Vg expression levels were significantly lower in Dsx-knockdown bees (one-tailed p = 0.0082). Vg is an egg yolk precursor, and its levels in workers are strongly socially repressed in queenright



colonies. These data support the central role of Dsx in honey bee physiology, in part by direct control of Vg, a key gene involved in coordinating diverse aspects of social organization.

Pheromonal profile and ovary activation are often linked, but the specific relationship between pheromone profile composition and ovary activation stage is unknown. Therefore we simultaneously analyzed both phenotypic responses via a cumulative link principal component regression model and ovary activation stage as the response variable. To account for correlations among mandibular pheromone compounds, first two principal components were used as predictors, along with Dsx knockdown treatment, and interactions (Fig. 4). Dsx knockdown resulted in lower ovary development when compared to the non-target control gene (Z = 3.1, one-tailed p = 0.0011) and more worker-like scores on the PC2 axis (Z=-2.2, one-tailed p=0.015). Furthermore, there was a significant interaction between these two explanatory variables (Z = 2.3, p = 0.021), which meant that Dsx-knockdown disrupted the relationship between ovary activation level and the amount of pheromone produced. This can be seen in Fig. 4, where control workers show the typical positive association between ovary activation and queen-like mandibular pheromone production, and Dsx-knockdown workers show the opposite pattern.

Fig. 4.Dsx knockdown decreases ovary development and production of queen-like mandibular gland components. (A) Worker ovary activation stage. Stage 0 represents underdeveloped ovaries typical of young workers. Stage 1 represents enlarged ovaries. Stage 2 represents ovaries that have begun to develop, containing small oocytes, and Stage 3 denotes developed ovaries with eggs. (B) Ovary activation was significantly lower in Dsx-injected samples vs. controls, indicating that Dsx controls ovary development, as predicted (Z = 3.1, one-tailed p = 0.0011). (C) Higher values along the second principal component axis corresponded to more queen-like mandibular gland profiles (red circle outlines) (26, 70). Circle size represents the relative total amount of each component. (D) Dsx knockdown affected the relationship between ovary activation stage and levels of queen-like mandibular pheromones.



Weighted gene co-expression analysis (WGCNA) takes advantage of correlations between gene expression patterns of genes across libraries to identify ‘modules’ of genes showing similar expression profiles. WGCNA has been shown to reconstruct protein-protein interaction networks with reasonable accuracy, based solely on gene expression data. Therefore, WGCNA allowed us to examine the gene regulatory network surrounding Dsx. In particular we took advantage of the concept of ‘module membership’, which is the

overall connectedness of a gene to other members of the same network. More important genes tend to have greater membership.
WGCNA identified a network containing Dsx and 966 other genes, out of a total of 13,811. Genes in this network were generally over-expressed in control bees, and their module membership was strongly correlated with the log2 fold-count of control vs. treated gene expression, i.e., genes most involved in the network showed the greatest responses to Dsx knockdown. This module also contained Vg, as would be expected, due to its tight regulation by Dsx. It also contained another well-known gene that was experimentally shown to affect caste, the DNA methyltransferase Dnmt3. Within the co-expression module, Dsx was part of a tightly connected network of transcription factors and other regulatory proteins. Overall, the module was enriched for gene ontology terms associated with regulation, particularly of transcription, and signaling. Genes in this module were upregulated in control bees, and genes that were more tightly connected to other genes in the module were more likely to respond to Dsx knockdown. Therefore, sensitivity to Dsx knockdown is a core property of this module.

While primary mechanisms of insect sex determination are diverse, they always converge on the Dsx pathway, which involves the regulation of various sexually dimorphic traits. Insects have co-opted Dsx for a variety of different functions, ranging from sexual ornamentation in beetles, to butterfly wing patterning. Importantly, Dsx regulates ovary development and the production of sex pheromones. Consequently, the Dsx pathway provides an ancient gene regulatory framework coupling two building blocks of eusociality - differential fertility and pheromonal signaling. We thus hypothesized that it has also been co-opted during the evolution of eusociality. Experimental knockdown of Dsx confirmed that it regulates ovary activation, likely by regulating the egg yolk precursor Vg (Fig.3), and in pheromonal signaling in adult workers (Fig. 4). We identified a Dsx-responsive gene co-expression module that is enriched in gene ontology terms associated with biological regulation. This module is also preserved in larval gene expression data, and is specifically associated with the queen-destined developmental trajectory. These data suggest that in honey bees, reproductive division of labor evolved by taking advantage of existing sex-specific developmental regulatory networks.

Social evolution may have taken advantage of a ‘genetic toolkit’ consisting of ancient gene regulatory networks that have been re-wired for social living. Although many studies have provided support for this hypothesis, the components comprising this toolkit and how they interact remain unclear. Putative genes in the genetic toolkit that have been identified to date are enriched in members of nutrition and juvenile hormone-signalling pathways, all of which act downstream of Dsx during development. For example, Vg is a key toolkit genes, and under its direct Dsx control. Other genes that have been experimentally shown to affect caste, such as Dnmt3 and Mrjp1 also responded to Dsx knockdown suggesting that they interact with it either directly or indirectly. Studies on horned beetles have shown that Dsx interacts with nutritional levels to produce alternative phenotypes, i.e., the size of horns. This likely also happens in honey bees, since Vg is sensitive to nutritional state in a variety of insects, including honey bees. Furthermore, Dsx can indirectly control other aspects of colony function via Vg, which has many coordinating effects on social organization.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

Because of the delay due to failed crosses, our first publication has just been accepted this year as a conference paper in computer vision, and the first phenotypic paper is under review. We are conducting a range of additional studies combining both approaches during this field season, and they should lead to additional publications.

〔雑誌論文〕（計　　1 件）

① Mariana Velasque,Lijun Qiu, Alexander S. Mikheyev. The Doublesex sex

determination pathway regulates reproductive division of labor in honey bees,biorxiv preprint (in review)
Doi: https://doi.org/10.1101/314492

〔学会発表〕（計　　1件）

① Katarzyna Bozek, Laetitia Hebert, Alexander S. Mikheyev, Greg J. Stephens. Towards Dense Object Tracking in a 2D Honeybee Hive The IEEE Conference on Computer Vision and Pattern Recognition (CVPR), 2018, pp. 4185-4193

６．研究組織

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