Video Article

RNAi-mediated Double Gene Knockdown and Gustatory Perception Measurement in Honey Bees (*Apis mellifera*)

Ying Wang[1], Nicholas Baker[1], Gro V. Amdam[1,2]

[1]School of Life Sciences, Arizona State University

[2]Department of Chemistry, Biotechnology, and Food Science, Norwegian University of Life Sciences

Correspondence to: Ying Wang at ying.wang.6@asu.edu

URL: https://www.jove.com/video/50446
DOI: doi:10.3791/50446

Keywords: Neuroscience, Issue 77, Genetics, Behavior, Neurobiology, Molecular Biology, Chemistry, Biochemistry, biology (general), genetics (animal and plant), animal biology, RNA interference, RNAi, double stranded RNA, dsRNA, double gene knockdown, vitellogenin gene, *vg*, ultraspiracle gene, *usp*, vitellogenin protein, Vg, ultraspiracle protein, USP, green fluorescence protein, GFP, gustatory perception, proboscis extension response, PER, honey bees, *Apis mellifera*, animal model, assay

Date Published: 7/25/2013

Citation: Wang, Y., Baker, N., Amdam, G.V. RNAi-mediated Double Gene Knockdown and Gustatory Perception Measurement in Honey Bees (*Apis mellifera*). *J. Vis. Exp.* (77), e50446, doi:10.3791/50446 (2013).

Abstract

This video demonstrates novel techniques of RNA interference (RNAi) which downregulate two genes simultaneously in honey bees using double-stranded RNA (dsRNA) injections. It also presents a protocol of proboscis extension response (PER) assay for measuring gustatory perception.

RNAi-mediated gene knockdown is an effective technique downregulating target gene expression. This technique is usually used for single gene manipulation, but it has limitations to detect interactions and joint effects between genes. In the first part of this video, we present two strategies to simultaneously knock down two genes (called double gene knockdown). We show both strategies are able to effectively suppress two genes, vitellogenin (*vg*) and ultraspiracle (*usp*), which are in a regulatory feedback loop. This double gene knockdown approach can be used to dissect interrelationships between genes and can be readily applied in different insect species.

The second part of this video is a demonstration of proboscis extension response (PER) assay in honey bees after the treatment of double gene knockdown. The PER assay is a standard test for measuring gustatory perception in honey bees, which is a key predictor for how fast a honey bee's behavioral maturation is. Greater gustatory perception of nest bees indicates increased behavioral development which is often associated with an earlier age at onset of foraging and foraging specialization in pollen. In addition, PER assay can be applied to identify metabolic states of satiation or hunger in honey bees. Finally, PER assay combined with pairing different odor stimuli for conditioning the bees is also widely used for learning and memory studies in honey bees.

Video Link

The video component of this article can be found at https://www.jove.com/video/50446/

Introduction

RNA interference (RNAi) is RNA based post-transcriptional gene silencing, which occurs in a wide variety of eukaryotic organisms. The process of RNAi is triggered by endogenous or exogenous double-stranded RNA (dsRNA) precursors. The dsRNA activates the ribonuclease protein Dicer which binds and cleaves the dsRNA to small fragments (20-25 bp). Then the small fragments of the dsRNA guide a recognition and cleavage of complementary mRNAs by argonaute proteins, a catalytic component of RNA-induced silencing complex (RISC)[1]. In mammals, dsRNAs longer than 30 nt, activate an antiviral response (interferon response, IFN) which leads to nonspecific degradation of RNA transcripts [2]. However, long dsRNAs have proven to be effective and specific in insects since there is a lack of this IFN [3].

Long dsRNAs have been used for downregulation of target genes in different insect species. Honey bees are one of the pioneer insect organisms in which functions of many important genes in the development and behavior have been revealed by using dsRNA [4,5]. Several dsRNA delivery methods have been performed in honey bees: dsRNA feeding efficiently downregulates target gene expression in honey bee larvae [6], whereas dsRNA injection is an effective approach for gene knockdown in honey bee embryos [4] and adult bees [7,8].

Gene knockdown effects exhibited by applying dsRNA to insects are transient and localized. Studies have shown that both abdominal dsRNA injections and thoracic dsRNA injections effectively suppress target gene expression in abdominal fat body cells of insects [9,10]. DsRNA is injected into abdominal and thoracic cavities and fat body cells are able to take up the dsRNA from the hemolymph where the cells are bathed [10]. However, genes in other organs, such as ovaries and brains, cannot be targeted by either abdominal or thoracic injections. In order to target genes in honey bee brain, brain injection of dsRNA has also been performed, which effectively influences target gene expression in local brain areas [11]. Here, we only document abdominal dsRNA injection which is more commonly used in adult honey bees.

RNAi has been primarily used to target a single gene and has been a powerful tool to reveal the gene function. However, any gene is not isolated from others; it is in complex regulatory networks. A key to understand a biological process is to dissect how genes interact with each other, which requires simultaneous manipulations of multiple genes rather than a single gene knockdown. In mammalian cell lines, scientists have succeeded in simultaneously inhibiting two or three genes by using delivery systems [12] or multi-microRNA (miRNA) hairpin designs [13]. But in insects, multiple gene knockdowns are still untested. Here, we present different injection strategies which can achieve a double gene knockdown. We target two genes: vitellogenin (*vg*) which encodes a yolk protein precursor, and ultraspiracle (*usp*) which encodes a putative receptor for juvenile hormone (JH) and may serve as a transcription factor mediating responses to JH [14] in honey bees. Vg and JH regulate each other in a feedback loop [15] and are involved in honey bee behavioral regulation [9]. Using the double gene knockdown, we perturb both Vg and JH pathways, and discover how they jointly affect honey bee behavior and physiology and how *vg, usp* and JH interact [9].

Gustatory perception is a behavioral predictor for honey bee social behavior [16]. In terms of behavioral development, nest bees with high gustatory perception behaviorally mature fast, and usually forage early in life and prefer to collect pollen [16,17]. Although regulatory mechanisms underlying gustatory perception are still unclear, studies have shown that gustatory perception is linked to internal energy metabolisms [9], hormonal secretion [18,19] and biogenetic amine pathways [20]. Both Vg and JH are important hormonal regulators modulating gustatory perception [7,21]. In the laboratory, a variation of gustatory perception in honey bees can be evaluated by testing the proboscis extension response (PER) to different sucrose solutions. Each bee is tested by touching both her antennae with a droplet of water followed by an ascending concentration series of 0.1, 0.3, 1, 3, 10, 30% sucrose. A positive response is noted if a bee fully extends her proboscis when a droplet of water or sucrose is touched to each antenna. Based on the number of positive responses to the solutions, the gustatory perception level of each individual can be determined [16]. However, the application of the PER is not limited to measuring gustatory perception. The PER is also an effective method to test the metabolic state of bees such as satiation vs. hunger. The bees with greater responses to sucrose are hungrier in general (Wang and Amdam, unpublished data). Furthermore, the PER paradigm can also be used in associative learning and memory in honey bees. In this case bees will be trained to associate the presence of sucrose water with an odor. When the bees learn the association, only the presence of the odor can evoke a positive proboscis response without rewarding them with the sucrose [22,23]. In this video, we show how to perform PER to evaluate gustatory perception which has been connected with *vg* and *usp* double knockdown in a previous study [9].

Protocol

Part 1: RNAi-mediated double gene knockdown

1. dsRNA Synthesis

1. Design primers of dsRNAs targeting *vg, usp* and a control gene encoding green fluorescence protein (*GFP*) which is not in the honey bee genome: primers were designed using online free software Primer3 (http://frodo.wi.mit.edu/).
2. dsRNA synthesis for *vg, usp* and *GFP*: use RiboMax T7 RNA production system from Promega for *in vitro* transcription.
3. dsRNA purification:
    1. Denaturation and renaturation: heat the dsRNA to 85 °C for 5 min and let it cool down at room temperature for 1 hr.
    2. DNase I treatment: add 1 µl DNase I into each dsRNA reaction and mix them by flicking the tubes. Incubate for 15 min at 37 °C.
    3. Add 150 µl nuclease-free water and 750 µl TRizol - LS into each reaction, and gently mix them by inverting the tube. Incubate the samples for 5 min at 30 °C.
    4. Add 200 µl chloroform into each sample. Mix vigorously for 20 sec. Centrifuge the samples for 15 min at 12,000 x g at 4 °C.
    5. Transfer the supernatant phase to each new tube and add 500 µl isopropyl alcohol into each tube. Mix them by inverting the tube. Incubate the mixture for 20 min at -20 °C. Centrifuge it for 10 min at 12,000 x g at 4 °C.
    6. Remove the supernatant and use 1,000 µl 75% ethanol to wash the pellet. After centrifuging the tube for 5 min at 7,500 x g at 4 °C, air dry the dsRNA pellet, and use nuclease free water to dissolve the pellet. In order to ensure the efficacy of gene down-regulation, the dsRNA concentration should be around 9 -10 µg/µl.

2. dsRNA Abdominal Injection

For double gene knockdown, there are two strategies: 1) single injection: mix dsRNA of two genes and inject the mixture; 2) two-day injection: inject the first dsRNA targeting one gene on the first day, and inject the second dsRNA into the same bees on the second day.

1. Chill newly emerged bees in a 4 °C refrigerator for 1-2 min. When the bees are immobilized, mount 3-4 bees in parallel on Petri dishes full of solid wax with insect pins crossed between their abdomens and thoraces.
2. Chill the bees again in a 4 °C refrigerator. Ensure the bees are completely immobile. It takes about 1-2 min. The bees should look loose. If the bees become curled or contorted, it means they are chilled too long which should be avoided. Chilling too much causes high mortality, but complete immobilization is important as any movement increases the size of the wound and mortality.
3. Put a disposable 30 G needle (BD) on a Hamilton micro syringe. Take 3 µl dsRNA and make sure there is no air bubble in the syringe. The needle is inserted to a side of the abdomen to avoid damaging internal organs. Press the syringe plunger slowly to allow the dsRNA to be absorbed. Since dsRNA is very viscous, it takes 2-3 sec to be completely expelled from the syringe. After completely pushing down the plunger, leave the needle in the wound for 4-5 sec. Observe bees for 3-5 sec after injections. If a hemolymph droplet leaks from the wound, discard the bee.
4. Use different colors to mark their thoraces corresponding to different treatments. At this point if the single injection strategy was used, the bees can be placed back into a colony after 1 hr observation. However, if the two-day injection strategy was used, after being injected with the first dsRNA, the bees should be placed in a cylinder mesh cage with honey supplied on the side and be kept in an incubator at 34 °C and 80% humidity.
5. On the second day, follow steps 2.2 and 2.3 to chill the bees in the mesh cages and mount them on wax plates. Perform injections with the second dsRNA as it is described in step 2.4.

6. Let the bees recover at room temperature for 1 hr and introduce them into a colony.

Note: Knockdown effects can be detected by quantitative RT-PCR (qRT-PCR) and western blotting. In general, knockdown efficacy varies and depends on many factors. In our study, the double gene knockdown can be detected 7 days after single injections and two-day injections.

Part 2: Proboscis extension response (PER) assay

1. Collecting Bees from the Hive

On day 6 after the injections, collect treated bees from the colony without using a smoker. Collect bees in metal mesh cylinder cages. Keep 3 bees in each cage.

2. Mounting Bees for PER

Chill the bees at 4 °C until they are completely immobilized. Mount each bee vertically into a plastic tube specially designed for PER. Keep the abdomen inside the tube by using two small pieces of tape, and keep the head sticking out of the tube. Put the tubes on a rack with two rows of small plastic columns, and assign each tube (bee) with an ID number. Then put treated bees on the racks into an incubator (34 °C and 80% humidity) and keep them in the incubator for 2 hr.

3. Preparing a Series of Sucrose Water Solutions

Prepare 0%, 0.1%, 0.3%, 1%, 3%, 10% and 30% sucrose water solutions and load the solutions into syringes with 15-20 G needles.

4. PER Assay

Touch the bee's antenna with a droplet of 0% solution (water) followed by 0.1%, 0.3%, 1%, 3%, 10% and 30% sucrose water solutions. Test all bees with water first and allow at least two minutes as the interval between each solution is used. Therefore, we usually have at least 20 bees for each trial. If a bee fully extends her proboscis, a positive response is counted.

5. Data Management

Sum up the total positive responses for each individual for gustatory response score (GRS). Perform an appropriate statistical analysis on the data.

Part 3. Gene knockdown validation

Fat bodies are dissected from another set of 7-day old treated bees. A standard TRizol procedure is used for RNA extraction, which is followed by DNase I treatment [24]. *Vg* and *usp* gene expression is analyzed by a two-step qRT-PCR. *Actin* is used as a reference gene since it has stable expressions in different tissues of honey bees. Published real-time PCR primers for *vg* and *usp* gene are used in this experiment [24]. Data are analyzed by using Delta-Delta CT method [25].

Representative Results

Both the single-injection and two-day-injection strategies significantly reduced *vg* (One-way ANOVA, $p < 0.001$) (**Figure 2A**) and *usp* (One-way ANOVA, $p < 0.001$) (**Figure 2B**) transcript levels in honey bees six days after the dsRNA was injected. The suppression of *usp* transcript by using the single injection with dsRNA mixture and the two-day injection with *vg* first and *usp* second (*vg*/*usp*) was significantly lower than the two-day injection with *usp* first and *vg* second (*usp*/*vg*) (Post-hoc analysis, $p_{\text{mixture vs. } usp/vg} = 0.013$, $p_{vg/usp \text{ vs. } usp/vg} = 0.019$).

The GRS in the double knockdown bees was significantly higher than the *GFP* control bees (Student T test, $p = 0.0496$) (**Figure 3**).

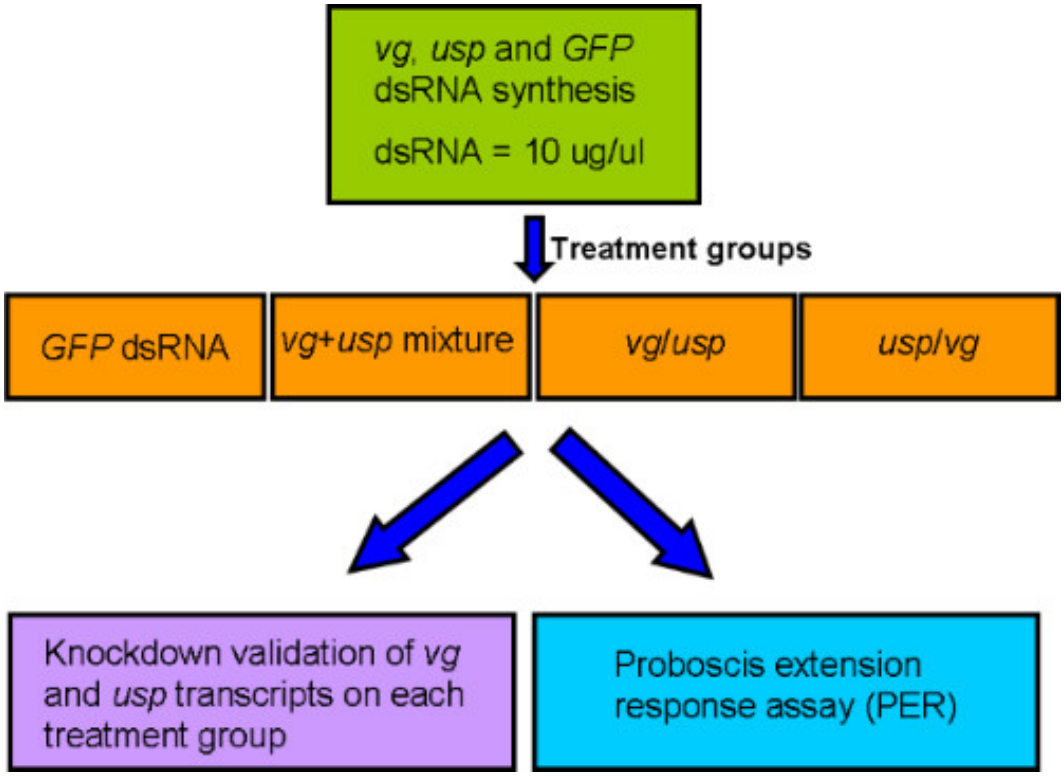

Figure 1. Flowchart of the RNA interference (RNAi)/proboscis extension response (PER) assay. Four groups of 50 newly emerged bees were injected with double-stranded RNA (dsRNA) of vitellogenin (*vg*) and ultraspiracle (*usp*) by using different combination strategies or with dsRNA of green fluorescence protein (*GFP*) gene which does not exist in the honey bee genome. The bees were placed back to a hive after the injections, and were collected after 6 days. Sixteen bees per group were collected for gene knockdown validation by using real-time PCR, and thirty per group were for proboscis extension response (PER) assay. The 'GFP dsRNA' indicates the bees injected with *GFP* dsRNA; the '*vg+usp* mixture' indicates the bees injected with *vg* and *usp* dsRNA mixture; the '*vg*/*usp*' indicates the bees injected with *vg* dsRNA on the first day, then injected with *usp* dsRNA on the second day; the '*usp*/*vg*' indicates the bees injected with *usp* dsRNA on the first day, and injected with *vg* dsRNA on the second day.

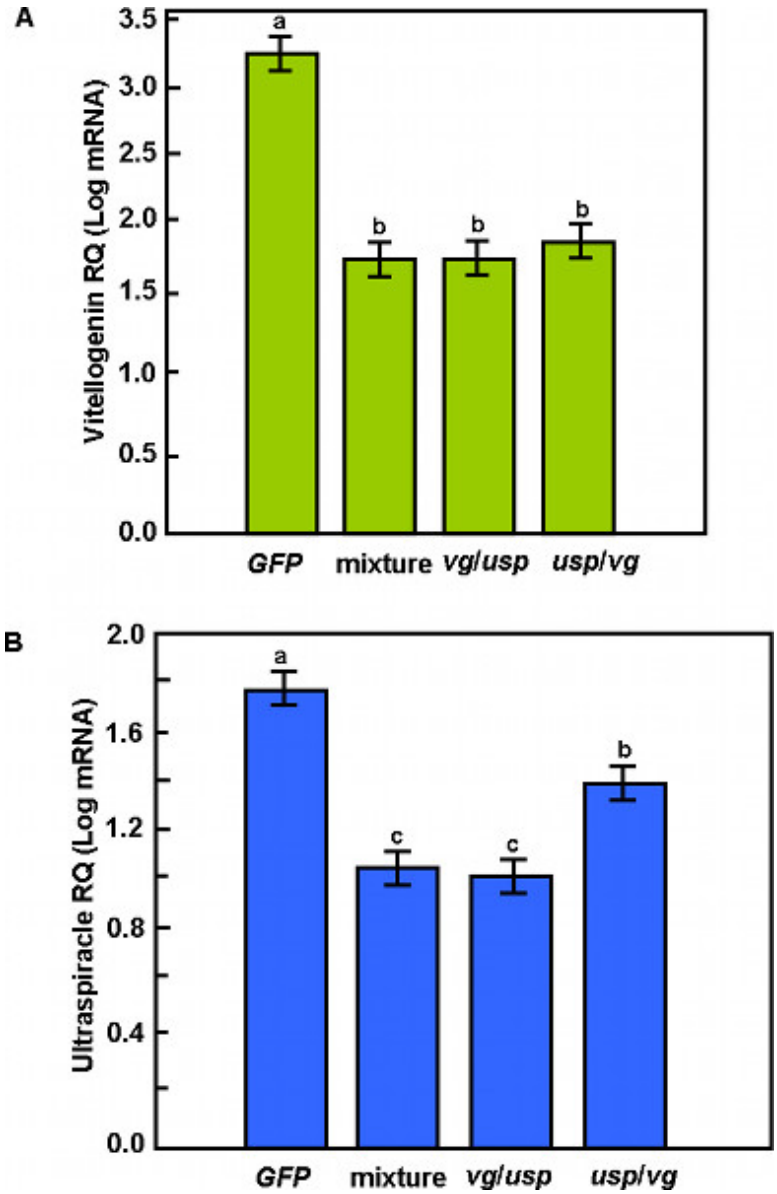

Figure 2. Double gene knockdown validation by using Real-time PCR. Log transformed mRNA relative value (mean ± s.e.m) in abdominal fat body of the treated bees. The mRNA relative value was calculated by using Delta-Delta CT method. **(A)** *Vg* expression 6 days after the dsRNA injections. The *vg* transcripts were significantly down-regulated by three double knockdown approaches (One-way ANOVA, $p < 0.001$). **(B)** *Usp* expression 6 days after the dsRNA injections. The *usp* transcripts were significantly reduced by three double knockdown approaches (One-way ANOVA, $p < 0.001$). However, the *usp* expression in the bees injected with the dsRNA mixture and *vg*/*usp* was much lower than that in the bees injected with *usp*/*vg* (Post-hoc analysis, $p_{\text{mixture vs. } usp/vg} = 0.013$, $p_{vg/usp \text{ vs. } usp/vg} = 0.019$), indicating that *vg* may be a primary regulator in the *vg* and JH regulatory loop. The 'RQ' indicates relative quantification level of the target gene transcripts. The different letters above bars denote significant differences between the treatment groups.

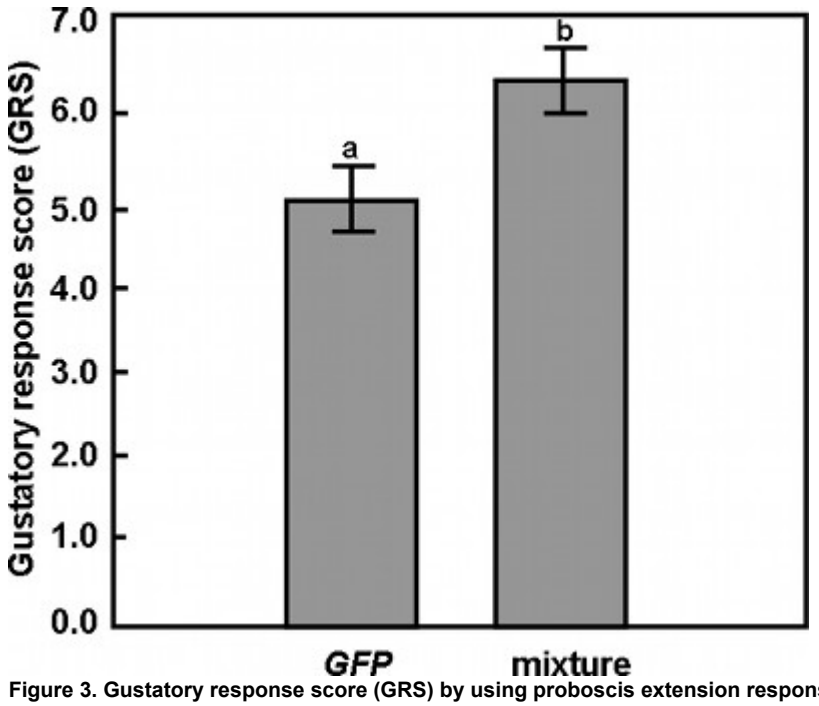

Figure 3. Gustatory response score (GRS) by using proboscis extension response (PER) assay. The bees which were injected with *vg* and *usp* dsRNA mixture had higher GRS than the *GFP* controls, indicating they were more sensitive to sucrose. The different letters above bars denote significant differences between the treatment groups (Student T test, p = 0.0496).

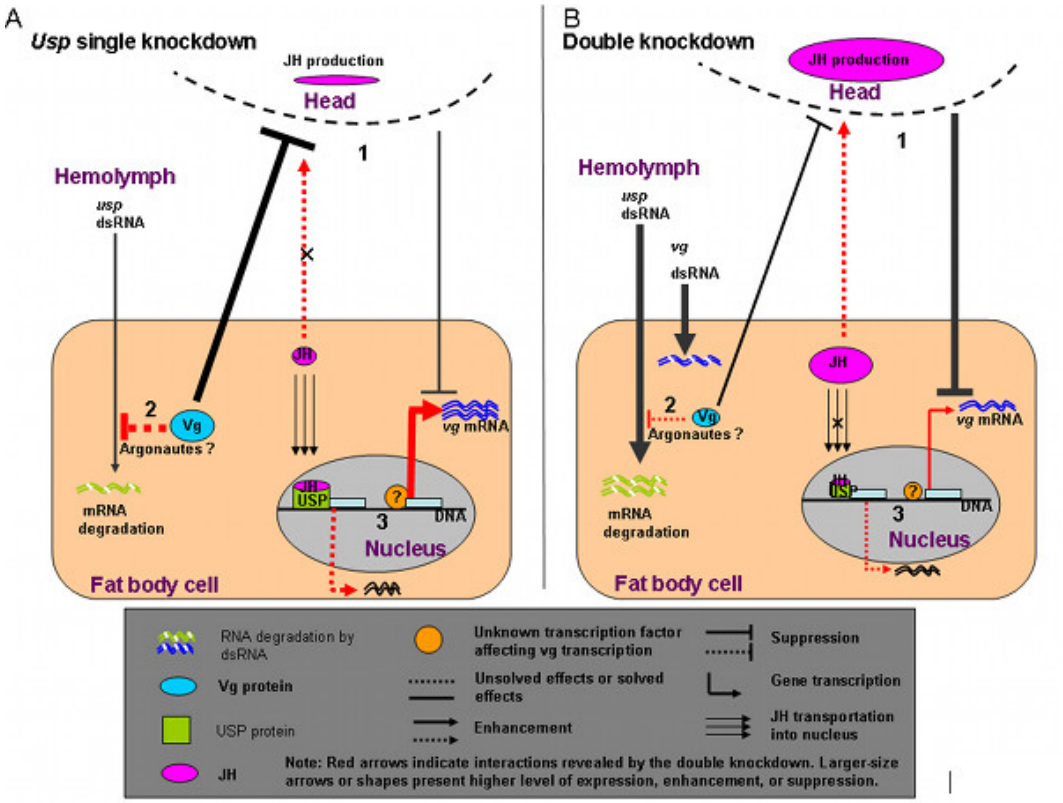

Figure 4. Possible regulatory gene networks revealed by using *vg* and *usp* double knockdown approach. Juvenile hormone (JH) is produced in the honey bee brain and secreted to the hemolymph. Vitellogenin (Vg) is a yolk protein precursor produced by fat body cells. Vg and JH are in a feedback loop regulating metabolic physiology, gustatory perception, and the age at onset of foraging and foraging preference of honey bees. Studies suggest that ultraspiracle (USP) is a putative receptor for JH and may serve as a transcription factor. Our double knockdown of *vg* and *usp* has revealed *vg* plays central roles in the relationship among *vg , usp* and JH. **1.** We found double knockdown of *vg* and *usp* caused a greater increase in JH than a *vg* single knockdown, and a *usp* single knockdown did not induce any change in JH. It suggests that Vg not only inhibits JH production, but inhibits a feedback response of JH to *usp* knockdown **(A)**. In double knockdowns, the dramatic increase of JH production results from a reduced inhibition from Vg caused by *vg* knockdown and a compensatory response to a reduced JH transduction caused by *usp* knockdown **(B)**. **2.** We found *usp* transcript abundance was more reduced by *usp* dsRNA when *vg* was primarily or simultaneously downregulated, suggesting *vg* suppresses the sensitivity of *usp* mRNAto its dsRNA. How can *vg* be involved in RNA interference (RNAi)? Vg regulates honey bee immune defense and RNAi is a part of antiviral innate immune system. Our previous study shows argonaute 3 (GB19389, *piwi*), an important component of RNA-induced silencing complex (RISC), is one of candidate genes influencing honey bee social behavior (gustatory perception, age at onset foraging and foraging preference), which is controlled by Vg/JH module. Therefore, one possibility is that Vg may suppress argonaute proteins affecting the activity of RISC **(A)**. When *vg* is downregulated, *usp* dsRNA is able to more efficiently degrade *usp* mRNA **(B)**. **3.** Our double knockdown study also reveals that *usp* does not mediate inhibitory effect of JH on Vg production in the fat body because neither single *usp* knockdown affects *vg* transcript abundance, nor double *vg* and *usp* knockdown causes further reduction of *vg* transcripts. Click here to view larger figure.

Discussion

Our study is the first effort to simultaneously knock down two genes in adult insects. Our results show that the two strategies of dsRNA injections (single injection and two-day injection) are effective for the double gene suppression, and simultaneous gene silencing of *vg* and *usp* can be tested 6 days after the dsRNA injections into bees [8,9]. However, gene knockdown efficacy varies in different insect species depending on transcript level of target gene, protein turnover rates and dsRNA uptake efficiency by cells or organs. In addition, the effect of RNAi is dose dependent. We found a newly emerged bee could well accept 4 µl dsRNA, but the mortality rapidly increased if more than 4 µl dsRNA was injected. Therefore, the two-day injection strategy may be more suitable than the single injection for an experiment which requires higher amount of injection volume.

Here, the double gene knockdown [9] we developed can be used for studying joint effects of two regulators. Additionally, the different dsRNA injection approaches presented in this video provides further tools for dissecting interrelationships between genes.

For example Vg and JH are in a feedback loop regulating honey bee physiology [9] and behavioral maturation [26,27]. Previous studies have shown that *vg* gene knockdown can increase JH level in honey bees [28], whereas topical application of JH can reduce *vg* expression [29]. In addition, studies have suggested USP is a putative receptor for JH, and may mediate responses to JH as a transcription factor [14,30]. However, how *vg* modulates JH titer and whether *usp* is involved in the regulation of *vg* by JH are still poorly understood. Using the double knockdown approach, we have discovered detailed interrelationships among *vg , usp* and JH (**Figure 4**). Firstly, our data suggest *vg* plays a central role among *vg , usp* and JH. High level of Vg not only inhibits JH production [15], but inhibits a systematic feedback response to *usp* knockdown (**Figure 4A**). In *vg* and *usp* double knockdown bees, JH production was increased more [9] than that in *vg* single knockdowns. A possible explanation is that the dramatic

increase of JH in double knockdowns results from a significant reduction in the negative effect of Vg on JH caused by *vg* knockdown and a compensatory response to a reduced JH transduction caused by *usp* knockdown (**Figure 4B**). Secondly, knockdown of *vg* first and *usp* second or simultaneous knockdown of both caused more reduction of *usp* than what a single knockdown of *usp* did. This may suggest that *vg* inhibits the sensitivity of *usp* mRNAto its dsRNA. This result is the first to link Vg with RNAi processes. Vg is involved in immune defense in honey bees [31] and RNAi is one of many antiviral systems [32] in eukaryotic organisms. Studies have shown that the RNAi process involves argonaute proteins which are conserved throughout Eukaryotes [33]. Interestingly, we previously found that argonaute 3 (GB19389, *piwi*) is one of candidate genes regulating honey bee social behavior in which Vg/JH module plays central roles [34]. Therefore, one alternative explanation is that Vg suppresses functions of argonaute proteins influencing mRNA degradation (**Figure 4A**). When *vg* is downregulated, argonautes more actively contribute to *usp* mRNA degradation caused by its dsRNA (**Figure 4B**). Finally, we found *vg* transcript abundance was not changed by *usp* knockdown, and it was reduced by *vg* and *usp* double knockdown at the similar level as that by *vg* single knockdown. These results suggest that *usp* is not involved in the inhibitive effect of JH on Vg. Overall, the double gene knockdown apparently is a powerful approach to help us understand regulatory gene network in details.

PER is a standard assay for measuring gustatory perception in honey bees, which has been used as a predictor for the development of honey bee social behavior [35]. Bees with high sensitivity to sugar usually start foraging early in life and prefer to collect pollen. Gustatory perception can also be tested for predicting the treatment effect on social behaviors before any other large scale behavior studies are performed. Additionally, we have shown that gustatory perception is correlated with internal metabolic state [9] Therefore, PER can be utilized to identify satiation and hunger levels. Furthermore, PER paired with odor conditions is employed for studying learning and memory in honey bees. Collectively, PER assay is a very useful technique for studying honey bee behavior and metabolic physiology. However, PER is sensitive to handling methods [36]. For example, bees that were anesthetized by chilling or CO_2 were more responsive to sucrose than controls [36]. PER also varies during the day and is sensitive to environmental conditions and stress. Therefore, it is very important to keep the environment and procedures consistent during the experiment. In general, we finish PER in the morning and follow the same schedule in following days. We usually start collecting bees in early morning at 8:00 am, and it takes 3 hr to get ready for PER. If PER needs to be performed across several days, it is important to finish it in a short period in case the environment changes. PER should be taken in groups and each group should include more than 20 bees because at least 2-minute interval has to be kept between different concentrations of sucrose solutions. We have done 75-100 bees per group.

Disclosures

We have nothing to disclose.

Acknowledgements

The authors would like to thank Erin Fennern for assisting experiment. This work was funded by the research Council of Norway (180504, 185306 and 191699), PEW Charitable Trust, and the National Institute on Aging (NIA P01 AG22500).

References

1. Okamura, K., Ishizuka, A., Siomi, H., & Siomi, M.C. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev.* **18**, 1655-1666 (2004).
2. Shi, Y. Mammalian RNAi for the masses. *Trends Genet.* **19**, 9-12 (2003).
3. Huvenne, H. & Smagghe, G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *J. Insect Physiol.* **56**, 227-235 (2010).
4. Beye, M., Hartel, S., Hagen, A., Hasselmann, M., & Omholt, S.W. Specific developmental gene silencing in the honey bee using a homeobox motif. *Insect Mol. Biol.* **11**, 527-532 (2002).
5. Amdam, G.V., Simoes, Z.L., Guidugli, K.R., Norberg, K., & Omholt, S.W. Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. *BMC Biotechnol.* **3**, 1 (2003).
6. Patel, A., *et al.* The making of a queen: TOR pathway governs diphenic caste development. *PLoS ONE.* **6**, e509 (2007).
7. Amdam, G.V., Norberg, K., Page, R.E., Jr., Erber, J., & Scheiner, R. Downregulation of vitellogenin gene activity increases the gustatory responsiveness of honey bee workers (*Apis mellifera*). *Behav. Brain Res.* **169**, 201-205 (2006).
8. Wang, Y., *et al.* Down-regulation of honey bee IRS gene biases behavior toward food rich in protein. *PLoS Genet.* **6**, e1000896 (2010).
9. Wang, Y., Brent, C.S., Fennern, E., & Amdam, G.V. Gustatory perception and fat body energy metabolism are jointly affected by vitellogenin and juvenile hormone in honey bees. *PLoS Genet.* **8**, e1002779 (2012).
10. Luna, B.M., Juhn, J., & James, A.A. Injection of dsRNA into Female A. aegypti Mosquitos. *J. Vis. Exp.* (5), e215, doi:10.3791/215 (2007).
11. Mussig, L., *et al.* Acute disruption of the NMDA receptor subunit NR1 in the honeybee brain selectively impairs memory formation. *J. Neurosci.* **30**, 7817-7825 (2010).
12. Stove, V., Smits, K., Naessens, E., Plum, J., & Verhasselt, B. Multiple gene knock-down by a single lentiviral vector expressing an array of short hairpin RNAs. *Electron J. Biotechnol.* **19**, 13 (2006).
13. Sun, D., Melegari, M., Sridhar, S., Rogler, C.E., & Zhu, L. Multi-miRNA hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. *Biotech.* **41**, 59-63 (2006).
14. Ament, S.A., *et al.* The transcription factor ultraspiracle influences honey bee social behavior and behavior-related gene expression. *PLoS Genet.* **8**, e1002596 (2012).
15. Amdam, G.V. & Omholt, S.W. The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. *J. Theor. Biol.* **223**, 451-464 (2003).
16. Pankiw, T. & Page, R.E. Response thresholds to sucrose predict foraging division of labor in honeybees. *Behav. Ecol. Sociobiol.* **47**, 265-267 (2000).
17. Pankiw, T., Nelson, M., Page, R.E., & Fondrk, M.K. The communal crop: modulation of sucrose response thresholds of pre-foraging honey bees with incoming nectar quality. *Behav. Ecol. Sociobiol.* **55**, 286-292 (2004).

18. Jaycox, E.R. Behavioral Changes in Worker Honey Bees (*Apis mellifera* L.) after Injection with Synthetic Juvenile Hormone (Hymenoptera: Apidae). *J. Kan. Entomol. Soc.* **49**, 165-170 (1976).
19. Jaycox, E.R., Skowronek, W., & Guynn, G. Behavioral changes in worker honey bees (*Apis mellifera*) induced by injections of a juvenile hormone mimic. *Ann. Entomol. Soc. Am.* **67**, 529-534 (1974).
20. Scheiner, R. & Pluckhahn, S., Oney, B., Blenau, W. & Erber, J. Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. *Behav. Brain Res.* **136**, 545-553 (2002).
21. Robinson, G.E. Effects of a juvenile hormone analogue on honey bee foraging behaviour and alarm pheromone production. *J. Insect. Physiol.* 277-282 (1985).
22. Giurfa, M. & Malun, D. Associative mechanosensory conditioning of the proboscis extension reflex in honeybees. *Lear Memory.* **11**, 294-302 (2004).
23. Scheiner, R., Page, R.E., Jr., & Erber, J. The effects of genotype, foraging role, and sucrose responsiveness on the tactile learning performance of honey bees (*Apis mellifera* L.). *Neurobiol. Learn Mem.* **76**, 138-150 (2001).
24. Wang, Y., *et al.* PDK1 and HR46 gene homologs tie social behavior to ovary signals. *PLoS ONE.* **4**, e4899 (2009).
25. Wang, Y., *et al.* Regulation of behaviorally associated gene networks in worker honey bee ovaries. *J. Exp. Biology.* **215**, 124-134 (2012).
26. Amdam, G.V., Csondes, A., Fondrk, M.K., & Page, R.E., Jr. Complex social behaviour derived from maternal reproductive traits. *Nature.* **439**, 76-78 (2006).
27. Amdam, G.V., Ihle, K.E., & Page, R.E. Hormones, Brain and Behavior., Pfaff, D., *et al.*, eds., Elsevier Academic Press, (2009).
28. Guidugli, K.R., *et al.* Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. *FEBS Letters.* **579**, 4961-4965 (2005).
29. Corona, M., *et al.* Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 7128-7133 (2007).
30. Sinha, S., Ling, X., Whitfield, C.W., Zhai, C., & Robinson, G.E. Genome scan for cis-regulatory DNA motifs associated with social behavior in honey bees. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 16352-16357 (2006).
31. Amdam, G.V., *et al.* Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Exp. Gerontol.* **39**, 767-773 (2004).
32. Wang, Q.C., Nie, Q.H., & Feng, Z.H. RNA interference: antiviral weapon and beyond. *World J. Gastroenterol.* **9**, 1657-1661 (2003).
33. Carmell, M.A., Xuan, Z., Zhang, M.Q., & Hannon, G.J. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev.* **16**, 2733-2742 (2002).
34. Hunt, G.J., *et al.* Behavioral genomics of honeybee foraging and nest defense. *Naturwissenschaften.* **94**, 247-267 (2007).
35. Pankiw, T., Waddington, K.D., & Page, R.E., Jr. Modulation of sucrose response thresholds in honey bees (*Apis mellifera* L.): influence of genotype, feeding, and foraging experience. *J. Comp. Physiol. A.* **187**, 293-301 (2001).
36. Pankiw, T. & Page, R.E., Jr. Effect of pheromones, hormones, and handling on sucrose response thresholds of honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A. Neuroethol. Sens. Neural. Behav. Physiol.* **189**, 675-684 (2003).

