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RNAi-mediated double gene knockdown and gustatory perception measurement in honey bees (*Apis mellifera*)

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Corresponding Author:	Ying Wang Arizona State University Tempe, AZ UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Arizona State University
Corresponding Author's Secondary Institution:	
First Author:	Ying Wang
First Author Secondary Information:	
Order of Authors Secondary Information:	
Abstract:	<p>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.</p> <p>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 presented two strategies to simultaneously knock down two genes (called double gene knockdown). We showed both strategies were 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.</p> <p>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.</p>
Corresponding Author E-Mail:	Ying.Wang.6@asu.edu
Order of Authors:	Ying Wang Gro V. Amdam
Author Comments:	For the part 1 of the protocol, only 'abdominal injection' is planned to be filmed. And the whole part 2 of the protocol is planned to be filmed.



Dear Editors,

We are pleased to submit our manuscript entitled “*RNAi-mediated double-gene knockdown and gustatory perception measurement in honey bees (Apis mellifera)*” to the Journal of Visualized Experiments for consideration for publication.

RNA interference (RNAi) is a powerful tool to understand gene functions in different organisms, and double stranded RNA (dsRNA) has been used successfully for RNAi in insects. However, this technique has been limited to down-regulating single gene, which cannot reveal interrelationships between genes. We developed novel methods to simultaneously down-regulate two genes in honey bees, and this method helped us to discover important interactions between two regulators, vitellogenin and juvenile hormone. This double-gene knockdown technique is easy to be applied in different insect species.

Proboscis extension responsiveness (PER) to sucrose water is a standard assay for testing the threshold of gustatory perception in honey bees, which is correlated with when honey bees transition to foraging and what they prefer to collect in the field. The PER is also used to measure the satiation and hunger level of bees after nutrition manipulation. Furthermore, the PER can be used to study learning and memory of honey bees.

Therefore, double gene-knockdown technique and PER assay are very useful methods in honey bee research. In this manuscript, we presented the protocols of the both techniques, and demonstrated how to use double knockdown approach to study gustatory perception in honey bees. We believed this manuscript is a great contribution to the society of entomology.

Sincerely,

Dr. Ying Wang

Arizona State University, School of Life Sciences

MAIN CAMPUS

COLLEGE OF LIBERAL ARTS AND SCIENCES

School of Life Sciences

PO Box 874501, Tempe, AZ 85287-4501

(480) 965-0803 Fax: (480) 965-6899



Phone: + 480-727-9439

E-mail: ying.wang.6@asu.edu

MAIN CAMPUS

COLLEGE OF LIBERAL ARTS AND SCIENCES

School of Life Sciences

PO Box 874501, Tempe, AZ 85287-4501

(480) 965-0803 Fax: (480) 965-6899

RNAi-mediated double gene knockdown and gustatory perception measurement in honey bees (*Apis mellifera*)

Authors:

Ying Wang and Gro V. Amdam

Authors: institution(s)/affiliation(s) for each author:

Ying Wang

School of Life Sciences

Arizona State University

ying.wang.6@asu.edu

Gro V. Amdam

1. School of Life Sciences

Arizona State University

2. Department of Chemistry, Biotechnology, and Food Science

University of Life Sciences, Aas, Norway

Gro.Amdam@asu.edu

Corresponding author:

Ying Wang

Keywords:

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)

Short Abstract:

In this protocol, we described two strategies that simultaneously suppress two genes (double gene knockdown) in honey bees. Then we presented how to use proboscis extension response (PER) assay to study the effect of double gene knockdown on honey bee gustatory perception.

Long 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.

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 transcripts². However, long dsRNAs have proven to be effective and specific in insects since there is a lack of this IFN³.

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 larvae⁶, whereas dsRNA injection is an effective approach for gene knockdown in honey bee embryos⁴ 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 bathed¹⁰. 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 knockdown is 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 previous study ⁹.

Protocol: Overview of the experiment (Figure 1)

Part 1: RNAi-mediated double gene knockdown

1.) DsRNA synthesis

1.1) 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/>).

1.2) DsRNA synthesis for *vg*, *usp* and *GFP*: use RiboMax T7 RNA production system from Qiagen for in vitro transcription.

1.3) DsRNA purification:

1.3.1) Denaturation and renaturation: heat the dsRNA to 85 °C for 5 minutes and let it cool down at room temperature for 1 hour.

1.3.2) DNase I treatment: add 1 µl DNase I into each dsRNA reaction and mix them by flicking the tubes. Incubate for 15 minutes at 37 °C.

1.3.3) 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 minutes at 30 °C.

1.3.4) Add 200 µl chloroform into each sample. Mix vigorously for 20 seconds. Centrifuge the samples for 15 minutes at 12,000 g at 4 °C.

1.3.5) 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 minutes at -20 °C. Centrifuge it for 10 min at 12,000 g at 4 °C.

1.3.6) Remove the supernatant and use 1000 µl 75% ethanol to wash the pellet. After centrifuging the tube for 5 minutes at 7,500 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.

2.1.) Chill newly emerged bees in a 4°C refrigerator for 1-2 minutes. 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.

2.2.) Chill the bees again in a 4°C refrigerator. Ensure the bees are completely immobile. It takes about 1-2 minutes. 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.

2.3.) Put a disposable 30G 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 seconds to be completely expelled from the syringe. After completely pushing down the plunger, leave the needle in the wound for 4-5 seconds. Observe bees for 3-5 seconds after injections. If a hemolymph droplet leaks from the wound, discard the bee.

2.4.) 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 hour 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.

2.5) 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.

2.6) Let the bees recover at room temperature for 1 hour 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 hours.

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 15-20G 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 treatment²⁴. *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 experiment²⁴. Data are analyzed by using Delta-Delta CT method²⁵.

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).

Tables and Figures:

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 \pm 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* mRNA to 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.

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 μ l dsRNA, but the mortality rapidly increased if more than 4 μ l 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* mRNA to 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 CO₂ 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 hours 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.

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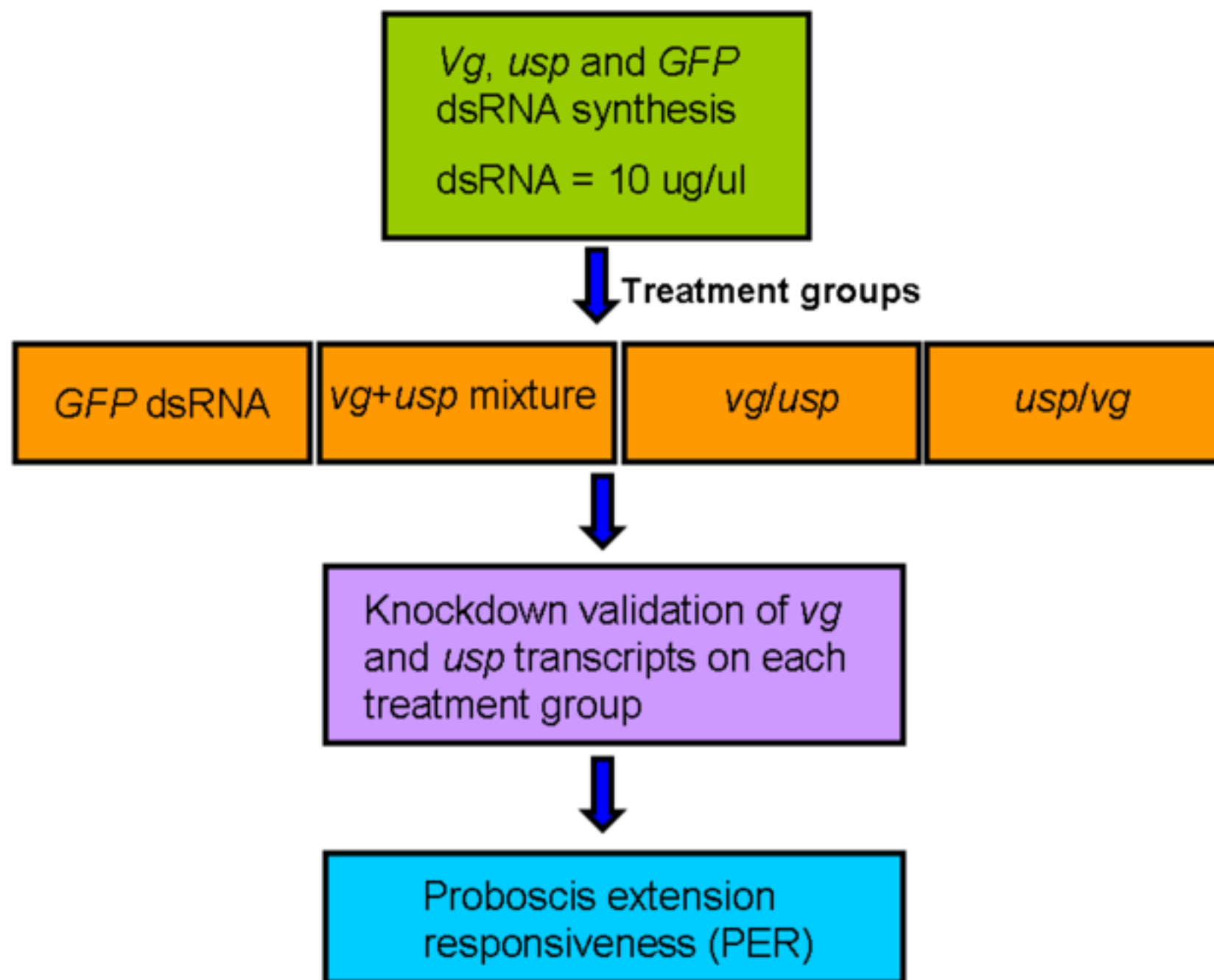


Figure 1

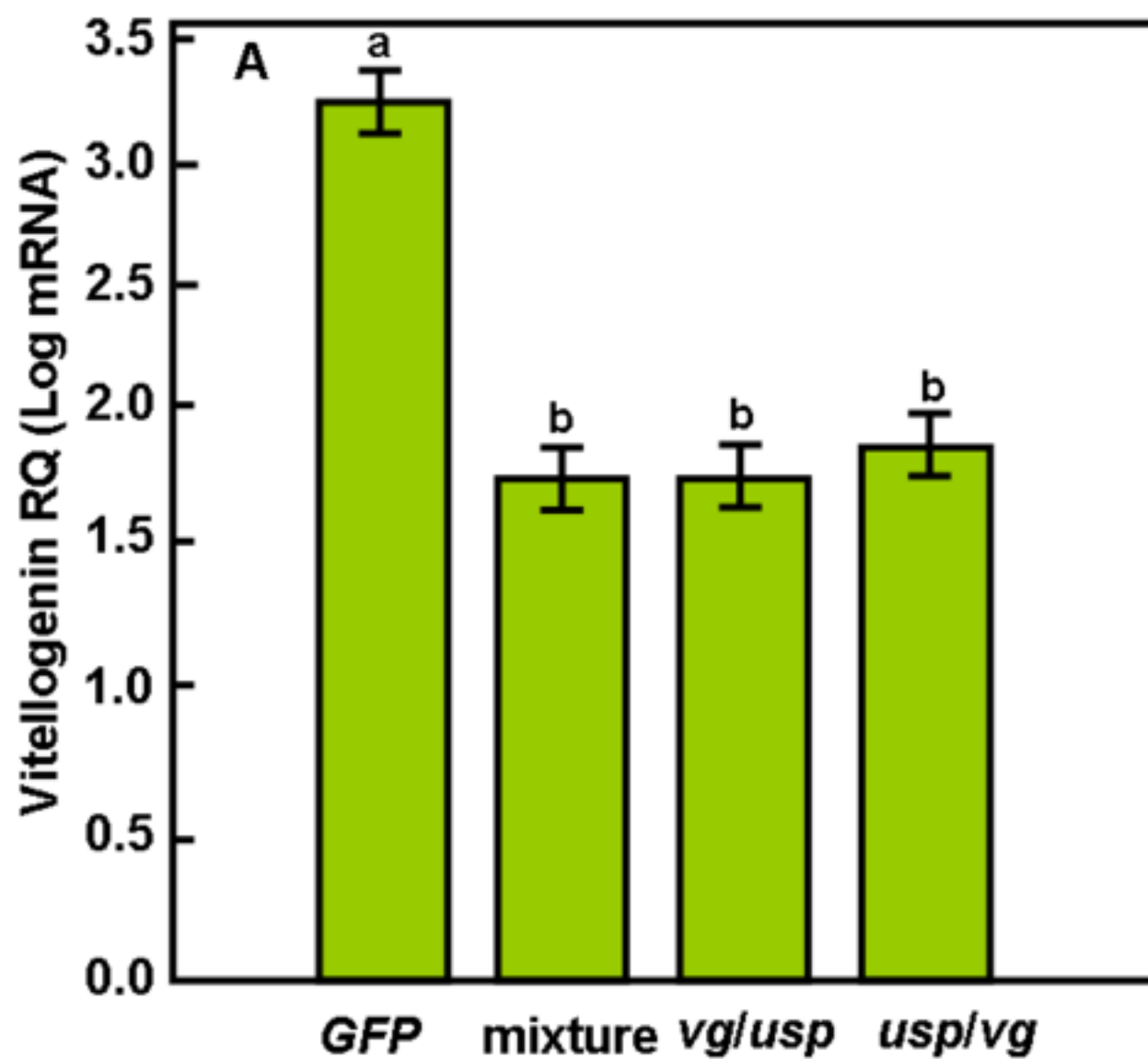


Figure 2 A

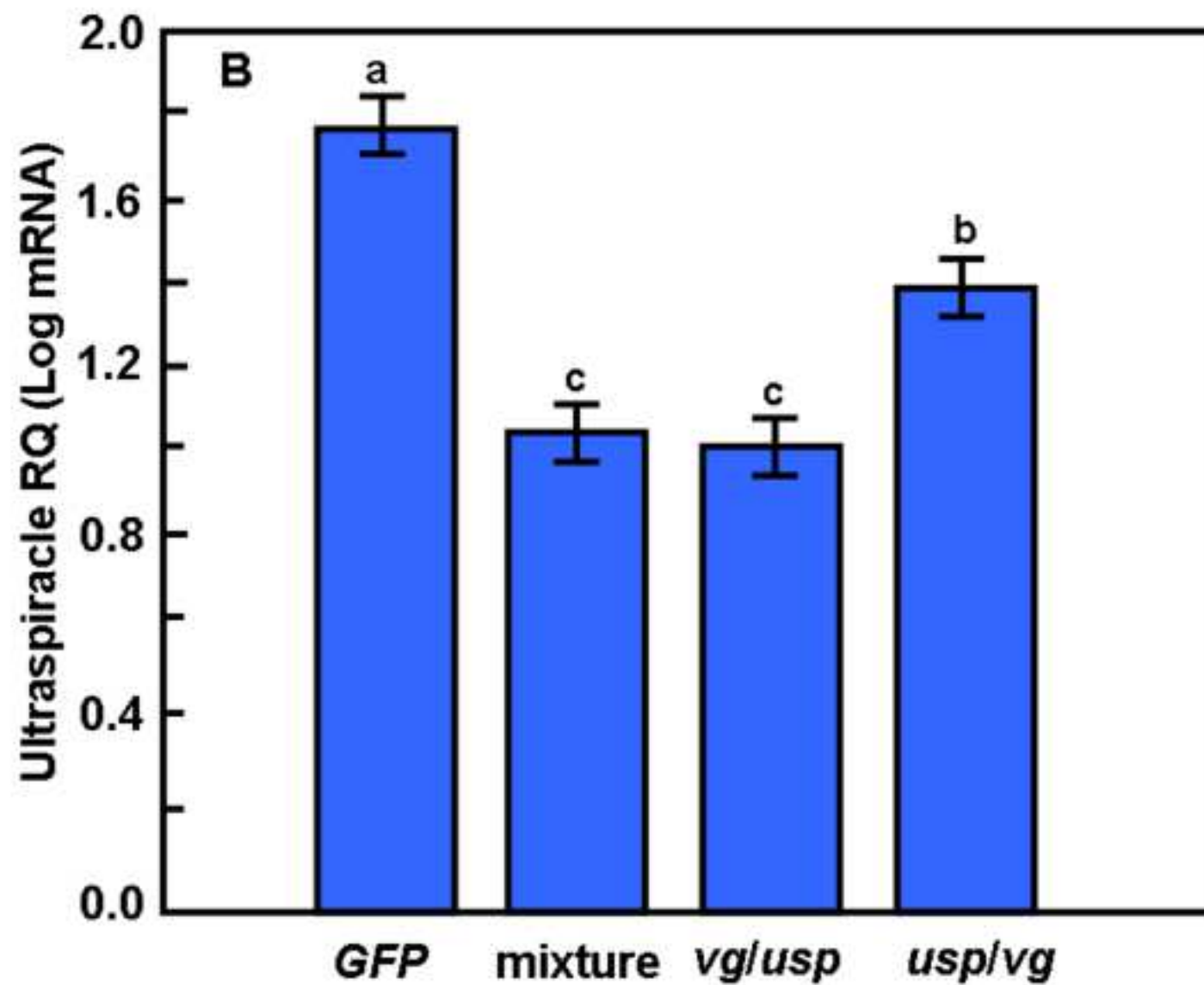


Figure 2 B

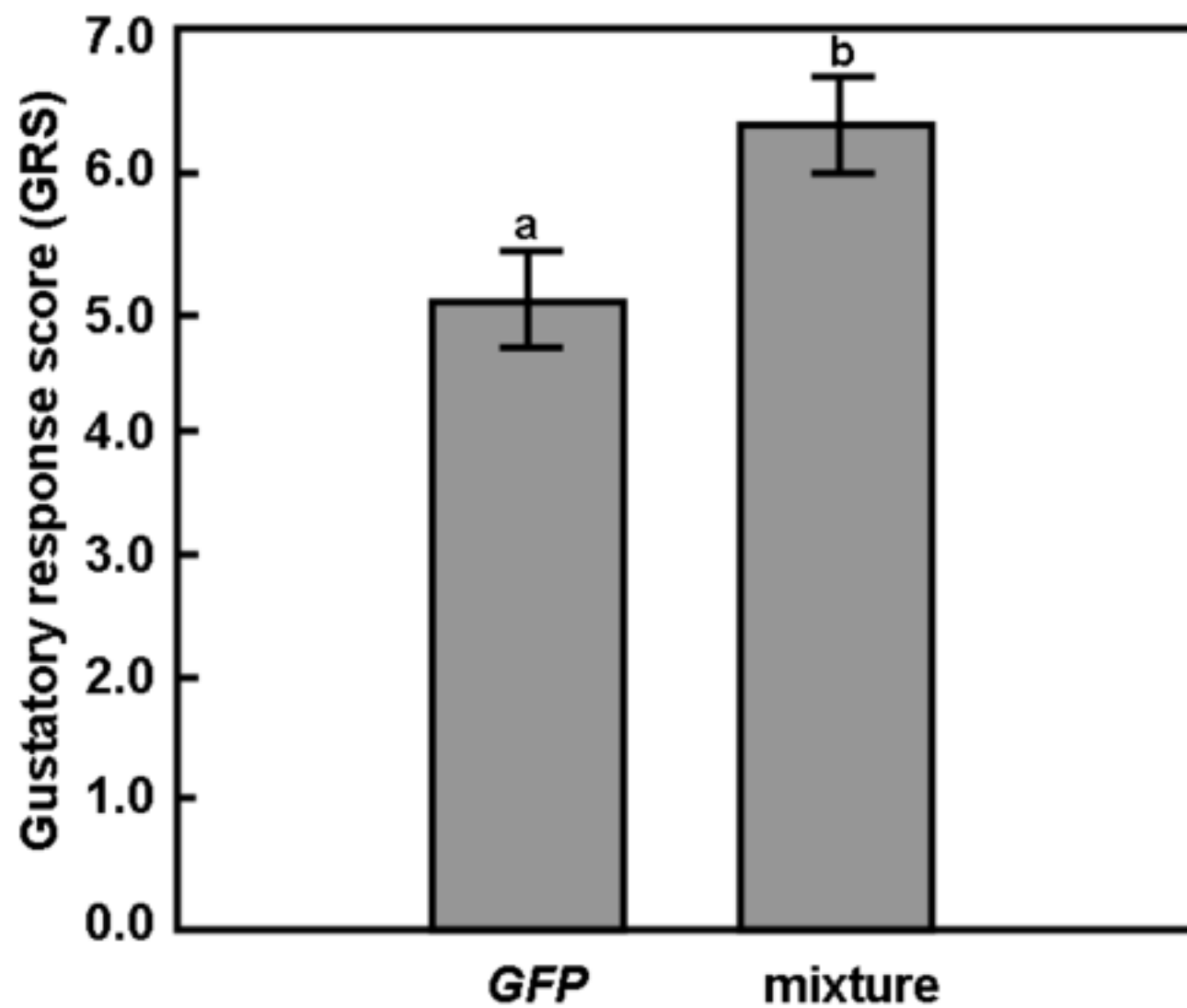


Figure 3

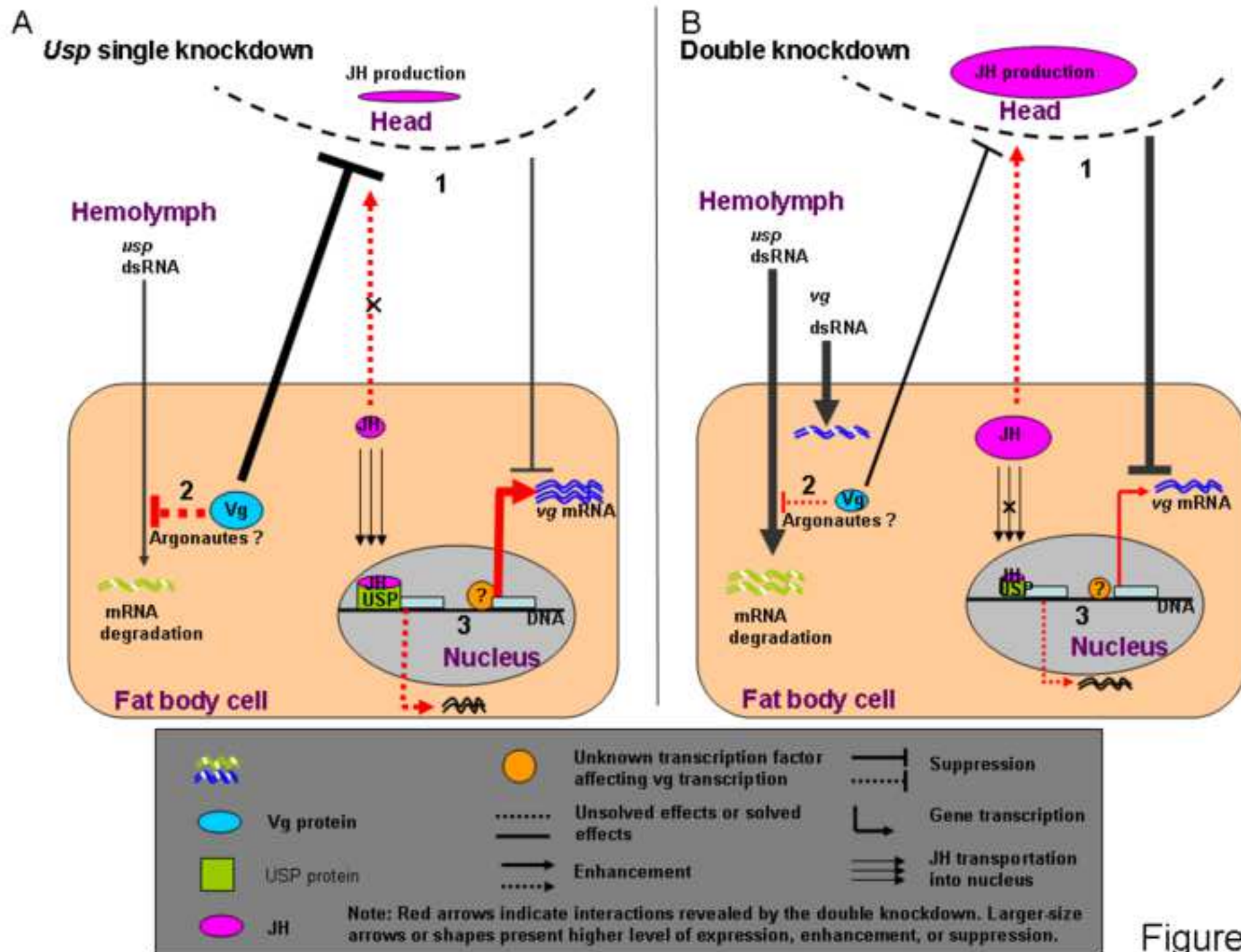


Figure 4

Name of Reagent/Material	Company	Catalog Number	Comments
GE Healthcare PCR beads in 96 well plate	GE Healthcare	27-9557-01	
QIAquick PCR purification kit	Qiagen	28104	
RiboMax T7 RNA production system	Promega	P1300	
Trizol LS	Invitrogen	10296028	
Chloroform:Isoamyl alcohol 24:1	Sigma-Aldrich	C0549	
isopropyl alcohol	Sigma-Aldrich	I9516	
Hamilton micro syringe	Hamilton	80301	
30G BD disposable needles	BD Biosciences	305106	
Sucrose	Sigma-Aldrich	84097	
1 ml Syringe	BD Biosciences	30960	
Stereo dissection microscope	Leica Microsystems	S6D	
Insect pins	Fine Science Tools	26000-25	
Wax plate			

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Author(s):

Ying Wang and Gro V. Amdam

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Xing Wang

Department:

School of Sciences, Arizona State University

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MS # (internal use):

Dear Editor,

I am thankful to receive the helpful comments from three anonymous reviewers on our manuscript (JoVE50446R1) entitled 'RNAi-mediated double-gene knockdown and gustatory perception measurement in honey bees (*Apis mellifera*)'.

I have addressed the reviewers' comments in our revision. Below is a point-by-point response that shows how I have dealt with each comment. Regarding the comments from reviewer 3, I made a conceptual figure for double *vg* and *usp* gene knockdown. I am pleased by the figure, because it exemplifies regulatory networks which can be revealed by multiple gene knockdowns. It emphasizes the importance and the power of this approach. Overall, I am very happy with the improvements that the revision has enabled us to make to the manuscript, and thank you for the helpful advice and recommendations.

With best wishes,

Ying Wang
School of Life Science,
Arizona State University
12/21/2012

Reviewer #1:

Summary:

The contribution by Wang and Amdam represents the demonstration and explanation of two unrelated methods in the honey bee: Injecting dsRNA to obtain gene knockdown and measuring gustatory perception via the proboscis extension reflex. These methods have been productively combined in another publication (Wang et al. 2012, #8 in the citation list) to obtain interesting results on the physiology of the honey bee, an emerging model species. Thus, the contribution is of significant interest to a potentially wide audience.

Minor Concerns:

1.) The text contains minor grammatical and spelling mistakes (e.g. page 3 at the

beginning of the second paragraph: " In term of behavioral development?" and should be scrutinized for language, especially the usage of "the" and "a".

The whole manuscript has been carefully checked for the grammatical and spelling mistakes.

2.) Changing the use of existing acronyms (PER commonly stands for proboscis extension reflects and the authors change it to proboscis extension responsiveness) should be avoided.

PER has been used to stand for both 'proboscis extension reflex' (Lambin et al., 2001; Vergoz et al., 2007) and 'proboscis extension response' (Matsumoto et al., ; Felsenberg et al., 2011) in honey bees. We choose to use 'proboscis extension response' since it is also used in other insects such as *Drosophila* (Shiraiwa & Carlson, 2007).

3.) The table of the reagents/materials needs to be re-formatted.

It has been re-formatted.

Reviewer #2:

Summary:

The paper tried to describe methods for using RNAi to silence two different genes and presents interesting data. It would be nice to see how they perform the injections.

Major Concerns:

I am a little concerned with the large volume of injections (2-4 ul). Did the author try to inject a higher concentration at lower volume? eg. Drying the liquid down to double the concentration so only 1 ul needs to be injected. The honey bee only has about 10-14ul total blood and 2 ul would be almost 20%.

In our experience, 4 µl of dsRNA is a safe volume for the injection. We used 3 µl dsRNA (10µg/µl) in this study, which is commonly used for dsRNA injection and has been published in our previous studies (Wang et al., 2012; Wang et al., 2010). We also found if the concentration was more than 10µg/µl, dsRNA solution became too viscous to be injected in a correct amount.

Minor Concerns:

The manuscript should be better proofread for grammatical mistakes. I listed several:

1.) Introduction paragraph 4 - Should be "in terms of"
Changed.

2.) Protocol 1.3.5 - water or chloroform?

The 'supernatant phase' in 1.3.5 includes both water and chloroform.

3.) Protocol 2.1 – thoraces

Changed.

4.) Protocol 2.2 - remove "rate" since mortality is a rate by itself.
Removed.

5.) Protocol 2.4 - not clear "was used in the use dsRNA mixture"
Changed.

6.) Protocol 2.4 - should read: "with honey supplied on the side"
Changed.

7.) Protocol 2.4 - Do you mean mesh cage instead of smash cage?
Changed.

8.) Protocol part 2, section 4 - missing "have"
Changed.

9.) Representative results - "after 6 days of dsRNA injections" should be changed to:
"at 6 days post dsRNA injections" otherwise it could be mis-interpreted as 6 days of injections...
Changed.

10.) Representative results - change 'much lower' to 'significantly lower'
Changed.

11.) Representative results - change "much higher" to significantly higher.
but $P = 0.496$ so it is not significant? but in the figure, it says A and B indicating difference.
It was a typo. It has been corrected.

12.) Figure 2 discussion - What is the mRNA relative value? Estimated copy number? How was it calculated from CT? Delta delta CT method?
I have clarified it in the revised manuscript (Page 7, line 289-291).

13.) Figure 3 discussion - Why GRS again? In other papers, Page et al called the same value (cumulated response of bee extending proboscis) as PER score. or sugar score.
GRS has been commonly used to indicate the level of gustatory perception in different studies in honey bees (Scheiner et al., 2005; Amdam et al., 2010). We choose to keep it.

14.) Figure 2a - Rename "dsRNA mixture" as "mixture" or "vg+usp". In reality, all 4 are dsRNA and the 2 lines here confused me, thinking that "mixture" was referring to all 4 different treatments, and I tried to change it as "Treatment".
Slight misalignment between numbers and tick marks on scale.
I have renamed 'dsRNA mixture' as 'mixture' in the figures.

Reviewer #3:

Summary:

This suggested video describes in general work that has been already published in June 2012 in PLoS Genetics. It describes injection and PER techniques that have been adapted from existing protocols and as such would be very useful for standardizing methods in silencing of multiple gene networks and may potentially have an impact on behavioral and pathogen related studies in honeybees.

Major Concerns:

A) In this submission and the article on which it is based, there is a lack of clarity on the JH-Vg network, which can be easily overcome by a simple diagram that describes the suggested feedback and how double silencing may potentiate and act synergistically as opposed to silencing of just one gene. Moreover, the advantage of the video is that this can be created interactively in an animated manner, which will provide clarity on why this work of multiple gene silencing is important, and how silencing of each gene effects the network and why double silencing has a synergistic effect.

We agree that a diagram not only clarifies the Vg/JH feedback loop, but exemplifies regulatory gene networks which multiple gene knockdowns can uncover. I have made a conceptual figure (Figure 4) to explain the interrelationship between *vg*, *usp* and JH, which the double gene knockdown has revealed. This figure can further emphasize the importance and the power of multiple gene knockdown approaches.

B) To support the claim that knocking down of genes effects the behavior and increases perception of sucrose, the results as presented (Fig. 3) are not so clear cut. Since foragers learn faster than young bees, the conclusions would be strengthened if associative olfactory PER would also be used for showing that bees that have their Vg-Usp silenced also learn faster to associate an odor with a sucrose reward, and that this should be added to the video.

We appreciate the reviewer's suggestion. Gustatory perception, which is evaluated by testing proboscis extension response (PER) to a concentration series of sucrose solutions, is an important behavioral predictor and connected with internal metabolisms in honey bees. Effects of *vg* and *usp* double knockdown on honey bee physiology and behavior have been studied (Wang et al., 2012) and published in PLoS Genetics in 2012. This video offers a great opportunity to provide detailed information about how to perform the double gene knockdown and PER techniques regarding the published paper. On the other hand, the published paper helps readers better understand the context in which these two techniques are used.

Associative olfactory PER is used to study learning and memory of honey bees by pairing odor stimuli and a sucrose reward. We have planned to study whether *vg* and *usp* double gene knockdown affects honey bee learning and memory in the future. Therefore, we won't include associative olfactory PER here.

Minor Concerns:

A) In section 2.3 there is a reference to the injection procedure and it is stated that "Discard bees with any hemolymph leakage", without showing how this is noted and how non-apparent leakage is monitored.

I have clarified it (Page 5, line 200-201).

B) There are some grammatical errors in the text and the references section needs to be better edited.

I have carefully corrected any errors and re-edited references.

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