Expression of AmGR10 of the Gustatory Receptor Family in Honey Bee Is Correlated with Nursing Behavior

Yisilahaiti Paerhati1a, Shinichi Ishiguro2, Risa Ueda-Matsuo2, Ping Yang1ab, Tetsuro Yamashita1, Kikukatsu Ito1, Hideaki Maekawa3, Hiroko Tani4, Koichi Suzuki2*

1 The United Graduate School of Agricultural Sciences, Iwate University, Morioka, Iwate, Japan, 2 Organization for Research Promotion, Iwate University, Morioka, Iwate, Japan, 3 Biochemical Science Association, Shinagawa-ku, Tokyo, Japan, 4 Institute for Bee Products and Health Science, Yamada Apiculture Center, Inc., Kagamino, Okayama, Japan

a Current address: Scientific Affairs Center, ARKRAY Marketing, Inc., Kamigyo-ku, Kyoto, Japan
b Current address: Honzo Pharmaceutical Co., Ltd., Shizuoka, Shizuoka, Japan
* koichi@iwate-u.ac.jp

Abstract
We investigated the association between the expression of a gene encoding gustatory receptor (G10) and division of labor in the honey bee, *Apis mellifera*. Among 10 GR genes encoding proteins 15% ~ 99% amino acid identity in the honey bee, we found that AmGR10 with 99% identity is involved in nursing or brood care. Expression of AmGR10 was restricted to organs of the hypopharyngeal gland, brain, and ovary in the nurse bee phase. Members of an extended nursing caste under natural conditions continued to express this gene. RNAi knockdown of AmGR10 accelerated the transition to foraging. Our findings demonstrate that this one gene has profound effects on the division of labor associated with the development and physiology of honeybee society.

Introduction
Genetic and epigenetic studies can be fruitful in revealing the social structures of animal species. Diverse social organisms such as insects, fishes, voles, and humans have been used to explore the relations between genes, brains, and social behavior [1–3]. Conserved genes are crucial to understanding a wide range of characteristics from social cognition to clinical disorders; for example, FoxP1 and FoxP2 expression patterns in human fetal brain are similar to those in the songbird [4, 5]. Although some genes influence complex behaviors via pleiotropic effects, manipulation of a single gene or its orthologs can have remarkable effects on behavior [1]; for example, disruption of a single copy of FoxP2 in mice causes modest development delay and a significant alteration in ultrasonic vocalization [6]. Behavioral transitions in *Drosophila melanogaster* food searching behavior from rover to sitter [7] and in adult worker honey bees (*Apis mellifera*) from hive tasks to foraging [8] are caused by expression of the same foraging gene, for.
The honey bee is an important model organism in studies of nursing or brooding behavior. During the first 2–3 weeks of their adult lives, worker bees perform different tasks in the hive, including nursing or caring for larvae and the queen. After that, for remaining 5–7 weeks of their lives, they become foragers, collecting pollen and nectar [9–11]. This transition in behavior involves changes in the physiological processes of hormones and neurochemicals and in the expression of thousands of genes [12–14].

Although exposure to brood pheromone can delay onset of foraging and regulate the expression of genes in the brain [15], and vitellogenin influences social foraging specialization [16], a single nursing or brood care–related gene which causes an adverse effect to the foraging gene, may also be involved in the division of labor. Therefore, we looked for nursing-related genes that influence nursing behavior, in contrast with foraging behavior.

Materials and Methods

Bees

European honeybees were maintained in the hives 1 and 2 at Iwate University and in the hive 3 at the Tropical Biosphere Research Center, University of the Ryukyus. Newly emerged workers were marked with a small spot of enamel paint on the thorax for later identification [9]. Marked nurse bees (seen with their heads in larval cells) aged 0 to 14 days after adult emergence were collected in the hive (hive 1). Marked forager bees (carrying pollen or nectar) aged 19 to 29 days were collected on their return to the hive (hive 2) [9, 16]. An extended-duration nursing caste in 29-day-old adults within the hive (hive 2) was created under natural conditions during October to early November of 2007 [11]. We marked them and also used as the extended-duration nursing caste.

Total RNA extraction and mRNA purification

To investigate the influence of gene action on behavior, we chose the hypopharyngeal gland (HPG), which initially synthesizes and secretes royal jelly, and later shrinks in foragers and synthesizes digestion enzymes, indicating a transition in an organ-level trait that reflects the division of labor [17]. To examine organ-specific expression, we also analyzed ovaries, brains, midguts, mandibular glands, and salivary glands.

Bees were anesthetized in insect saline (0.75% NaCl) on ice, and the organs were dissected out a binocular microscope. Total RNAs from bulked organs were prepared with Isogen (Nippon Gene) and frozen in liquid nitrogen and stored at −80°C until use. Polyadenylated RNA was separated with the aid of oligotex-dT30-coated magnetic beads (TaKaRa Bio), according to the manufacturer’s instructions.

Differential display and subcloning, sequencing, construction of cDNA library, and PCR

These materials in experiments 1 and 2 were performed as described in supporting information.

RT-PCR and cloning of AmGR10 in nurse bees

For RT-PCR, the full-length cDNAs of 7-day-old nurse bees revers-transcribed using Ex–Taq polymerase (TaKaRa Bio) from AmGR10 mRNA were amplified using from primers (forward, 5′–ATGATAGAATCTTCATAAGGC–3′; reverse, 5′–CGTACTTGTTGTCCCTTACT–3′) obtained from the NCBI Honey Bee Genome Resources. PCR conditions were 9 min denaturation at 95°C; 35 cycles of 1 min at 94°C, 1 min annealing at step-down temperatures (72°C×3,
68°C×3, 64°C×3, 60°C×3, 56°C×3, 52°C×20), and 1 min extension at 72°C; and a final 7 min at 72°C. PCR products were separated in agarose gels, excised, purified with a DNA fragment purification kit (Toyobo), subcloned into the pCR 2.1 vector (Invitrogen), and cloned into INFαF0 cells (Invitrogen). The RACE products were sequenced as described in supporting information.

**Life-stage-specific RT-PCR of AmGR10 in organs**

To examine stage-specific expression during from egg to adult forager and the organ-specific distribution of AmGR10 transcripts, we collected randomly 300 eggs within 24 h after oviposition, and 50 larvae and 10 pupae within the hive (hive 2) between June and October of 2007 and 2008. Total RNAs from these samples were frozen in liquid nitrogen and stored at −80°C until use. We subjected all RNA samples to first-strand reverse transcription. The reverse-transcribed cDNA samples were amplified with AmGR10 primers. Expression was normalized to that of the constitutively expressed β–actin gene of A. mellifera, and was confirmed by RT-PCR [18].

**Preparation of double-stranded RNA**

To synthesize double-stranded RNA (dsRNA) for RNA interference, we used a PCR-template method. Forward and reverse primer sequences to amplify a 513- base-pair region were selected from the AmGR10 nucleotide sequence and from A. mellifera putative GR 10 from the NCBI Honey Bee Genome Resources. A T7-promoter sequence (TAATACGACTCACTCAC TATAGGGGAGA) was attached to the 5′ end of each primer to facilitate in vitro transcription of sense and antisense RNAs simultaneously. The following primers were used: forward, primer–5′–TAATACGACTCACTATAGGGAGACCACATAGAACTCTCTAAGGC–3′; reverse, primer–5′–TAATACGACTCACTATAGGGAGACCACAGTAAGGATCACCAAG–3′. PCR was performed with 100 ng plasmid DNA template, 25 pmol of each T7-linked primer, 8 mM MgCl2, 10×PCR buffer, all four deoxynucleotides at 5 mM, and 2.5 U of Taq DNA polymerase in a 50 μL PCR reaction. PCR was performed as for cloning. Amplified products were purified by gel extraction with a Mag Extractor–PCR & Gel Clean up kit (Toyobo) and used as templates for in vitro transcription for dsRNA synthesis in a Megascript RNAi kit (Ambion). Transcription products were treated as instructed by the user manual and re-suspended in nuclease-free water.

**RNA interference (RNAi)**

Seven-day-old nurse bees were divided into three groups. The first group was injected through the neck membrane with 8.2 μg/2.0 μL AmGR10 dsRNA (ds AmGR10 group, n = 100 total in 4 independent experiments), as described previously [19]. As a handling control, the second group (sham group, n = 80 total in 4 independent experiments) received a 2.0 μL of nuclease-free water [19, 20]. The third group were left untreated (n = 80 total in 4 independent experiments). All bees were tagged with paint to identify the treatment. Immediately after the injections, the bees were returned to the same hive (hives 1–2010, hive 2–2011 and 2012, hive 3–2009), which held approximately 5,000–10,000 worker bees of all age classes and an egg-laying queen. Following the transition to foraging (as indicated by the presence of marked bees outside of the hives), they no longer engaged in any within- hive-tasks [9]. The bees were observed daily for a total of 4.5 h (10:00–12:00 and 13:00–15:30) over 3 consecutive days.

**qRT-PCR**

Bees were anesthetized on ice, and their HPGs were removed. RNA was extracted in Isogen buffer according to the manufacturer’s instructions and frozen in liquid nitrogen and stored at...
–80°C until use. After treatment with RAase-free DNase (Promega), 2 μg of RNA was reverse-transcribed with AMV reverse transcriptase XL (RNA LA PCR kit [AMV] v. 1.1, Takara). The first-strand cDNAs were then amplified by quantitative real-time PCR using primers specific for honeybee AmGR10 (forward, 5′–TGTCGGCGAGCTTATTTTCT–3′; reverse, 5′–TCGAA AGCCAGGAGGAA–3′). Values were normalized to those of a β–actin gene of A. mellifera (AB023025; forward, 5′–TGCCAACACTGTCCTTTCTG–3′; reverse, 5′–AGAATTGAC CCACCAATCCA–3′) [18]. Sequences were amplified in a 7500 Real-Time PCR System (Applied Biosystems) using a One Step SYBR PrimeScript RT-PCR kit (Takara) under conditions of 94°C for 10 s, followed by 55 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 30 s. Each sample was analyzed in 4 independent experiments.

Data analysis
We compared performance within an experimental group by Wilcoxon’s signed–rank test, and between groups by Mann–Whitney U–test.

Results and Discussion
First we compared the expression of HPG mRNAs in nurse and forager bees. One candidate band sequence that was strongly expressed in nurse bees was used in a BLAST search of GenBank and the NCBI Honey Bee Genome (S1 Fig). 5′ and 3′ RACE–PCR amplified a gene fragment of 543 bases encoding 181 amino acid residues (S2 Fig) that had 99% sequence identity to gustatory receptor (GR) 10 of A. mellifera and 50% to that of a parasitoid jewel wasp, Nasonia vitripennis (S3 Fig) [21, 22]. The insect GR family was identified in the Drosophila melanogaster genome and named for its expression in gustatory organs such as the mouthparts [23]. So far, candidates for 68 GRs encoded by 60 genes have been identified in D. melanogaster [24] and candidates for 76 GRs encoded by 52 genes have been found in Anopheles gambiae [25]. AmGR1 in the honeybee antennae was recently shown to function as a sweet receptor that responds to some sugars, and AmGR2 in the same organ may act as a co-receptor [26], but Robertson and Wanner [21] revealed a total of 10 genes encoding proteins with 15% to 99% amino acid identity to each other and proposed that these 10 GRs are very limited in terms of gustatory mechanism and capacity. Here, we propose that the GR10 protein is primarily involved in nursing or brood-caring behavior in honey bees.

We monitored the expression of AmGR10 in the HPGs of 7-day-old nurse bees and 29-day-old foraging bees by using RT–PCR (Fig 1A). AmGR10 was highly expressed in the HPGs of the nurse bees, but not of the foraging bees (Fig 1B). We then compared the expression of AmGR10 in total RNAs prepared from newly laid eggs and in larvae and pupae collected randomly. AmGR10 was not expressed in the eggs or larvae, but it was expressed at low levels in the pupae (Fig 1B). It was expressed in the HPGs of nurse bees from age 1 to 14 days, but not in foraging bees from age 19 to 29 days (Fig 1C). These expression profiles reflect the division of labor and physiological processes between nurse and foraging workers [1, 9, 10, 12].

Next, we created an artificially extended nurse state, in which the HPGs are hypertrophied and remain active [11]. AmGR10 remained expressed even in 29-day-old nurse bees, unlike in foragers (Fig 2A). In nurse bees aged 7 days, we found AmGR10 expression not only in the HPGs, but also in the brain and ovary (Fig 2B). Our results provide new evidence for a nursespecific gene linked to the HPG, brain, and reproductive organs.

If AmGR10 is fundamentally involved in worker behavior, its function should be detectable in the transition to foraging. To investigate this possibility, we generated AmGR10–knockdown bees by injecting ds RNA into 7-day-old nurse bees. These workers had significantly lower
levels of AmGR10 mRNA in the HPG than controls (Fig 2C). Monitoring of bee movements showed that this knockdown of AmGR10 activity caused earlier nurse-to-forager transition (Fig 2D, S1 Video). Although the collective activities of foraging workers remain to be demonstrated [12], our data strongly support the notion that the AmGR10 influences nursing behavior.

Complex relations between crucial genes and reversible DNA methylation have challenged our molecular understanding of the division of labor in honey bees [1, 3, 27], but little is known about how such genes or epigenetic changes can explain the complex pathways that determine the division of labor. Examination of the nine genes encoding GRs in A. mellifera by qRT-PCR has revealed that the expression of seven of them is enriched in gustatory organs such as the labial palps and the glossa, and AmGr7 is expressed at high levels in the heads (although AmGR10 was not investigated) [21]. Our finding of AmGR10 as crucial to nursing or brood-caring behavior in the hive will help to explain how the GR protein family in social insects mediates the synthesis of royal jelly, as well as how it contributes to behavior in the hive. Although the ligand–receptor binding of the AmGR10 product remains to be analyzed, our results suggest that AmGR10 is important in the organization of honeybee societies.

In the invertebrate model system D. melanogaster, recent research has uncovered the fact that GR43a is expressed in a group of neurons in the posterior superior lateral protocerebrum; it is both necessary and sufficient to sense hemolymph fructose, and promote feeding in hungry flies but suppress feeding in satiated flies [28, 29]. Trehalose and glucose are the main hemolymph sugars in many insects, but fructose also is a main sugar in honey bee [30, 31]. This unique role of Drosophila GR43a in the sensing of fructose in the diet and the hemolymph may provide new insights into the mechanisms of the division of labor in honey bee and the nutrient receptor function of AmGR10 in the HPG, brain, and ovary.
Supporting Information

S1 Fig. Differential expression patterns of genes by gel electrophoresis. Total RNA samples from 4- to 7-day-old nurse bees and foragers collected randomly outside of the hive were pre-screened according to the Seegene user manual, and some differentially expressed genes were found. (A) In experiment 1, bands in columns GP 21, 23, 32, and 34 are differentially expressed genes between nurse bees and foragers (arrows). These bands were reamplified and directly sequenced (see Materials and Methods). Genes 1, 2, and 7 showed no significant similarity, but gene 6 showed high similarity to a royal-jelly-related milk protein (pRJP57-1) of A. mellifera [17]. (B) In experiment 2, one gene overexpressed in nurse bees (arrow) was subcloned (see S2 and S3 Figs). ACPs 11–20 are alternative primers.

(EPS)

S2 Fig. Partial cDNA and deduced amino acid sequences of a gustatory gene AMGR10 from Apis mellifera. *Terminal codon. A putative polyadenylation signal is boxed. N-glycosylation sites are underlined.

(EPS)
S3 Fig. Comparison of amino acid sequences of gustatory proteins from honey bee and jewel wasp: gustatory receptor 10 (Nurs) in Apis mellifera identified from our experiments; gustatory receptor 10 (GR10) in A. mellifera; and GR10 in Nasonia vitripennis. Identical amino acids are shown in red. Dashes indicate gaps introduced to maximize sequence similarity.

S1 Materials and Methods. Differential display and subcloning. Amplification and PCR products were performed as described previously (Yang P, et al., 2008).

S2 Materials and Methods. Sequencing.

S3 Materials and Methods. Construction of cDNA library.

S4 Materials and Methods. RACE.

S1 Video. Effects of the injection of RNA interference on foraging onset. Seven-day-old nurse bees were injected through the neck membrane with dsRNA (513-bp; ethanol-precipitated, suspended in nuclease-free water at 8.2 μg/2 μL) or with 2 μL of nuclease-free water (handling control), or were not treated (mock). The bees were observed daily for a total of 4.5 h (10:00–12:00 and 13:00–15:30) for 3 consecutive days, and the emergence of newly transitioned forager bees (tagged in white paint on the thorax), was recorded by video.

Acknowledgments

We thank Profs. Kiyoshi Hiruma (Hirosaki University), Ken Sahara (Iwate University) and Jun Nakamura (Tamagawa University) for helpful discussion. We thank Fujiwara and Kakudate Bee Farms in Iwate Prefecture and Arakaki Bee Farm in Okinawa Prefecture.

Author Contributions

Conceived and designed the experiments: YP SI KS. Performed the experiments: YP RUM PY. Analyzed the data: YP SI TY KI HM HT KS. Contributed reagents/materials/analysis tools: TY KI HM. Wrote the paper: YP SI KS.

References

1. Robinson GE, Fernald RD, Clayton DF. Genes and social behavior. Science 2008; 322: 896–900. doi: 10.1126/science.1159277 PMID: 18988841
2. Donaldson ZR, Young LJ. Oxytocin, vasopressin, and the neurogenetics of society. Science 2008; 322: 900–904. doi: 10.1126/science.1158668 PMID: 18988842
3. Herb BR, Wolschin F, Hansen KD, Aryee MJ, Langmead B, Irizarry R, et al. Reversible switching between epigenetic states in honeybee behavioral subcastes. Nature Neurosci. 2012; 15: 1371–1373. doi: 10.1038/nn.3218 PMID: 22983211
4. Enard W, Przeworski M, Fisher SE, Lai CSL, Wiebe V, Kitano T, et al. Molecular evolution of FOXP2, a gene involved in speech and language. Nature 2002; 418: 869–872. doi: 10.1038/nature00125 PMID: 12192408
5. Teramitsu I, Kudo LC, London SE, Gerchwind DH, White SA. Parallel FoxP1 and FoxP2 expression in songbird and human brain predicts functional interaction. J Neurosci. 2004; 24: 3152–3163. doi: 10.1523/JNEUROSCI.5589-03.2004 PMID: 15056695
6. Shu W, Cho JY, Jiang Y, Zhang M, Weisz D, Elder GA, et al. Altered ultrasonic vocalization in mice with a disruption in the Foxp2 gene. Proc. Natl. Acad. Sci. U.S.A. 2005; 102: 9643–9648. doi: 10.1073/pnas.0503739102 PMID: 15983371

7. Osborne KA, Robichon A, Burgess E, Butland S, Shaw RA, Couthard A, et al. Natural behavior polymorphism due to a cGMP-dependent protein kinase of Drosophila. Science 1997; 277: 834–836. doi: 10.1126/science.277.5327.834 PMID: 9242616

8. Ben-Shalar Y, Robichon A, Sokolowski MB, Robinson GE. Influence of gene action across different time scales on behavior. Science 2002; 296: 741–744. doi: 10.1126/science.1069911 PMID: 11976457

9. Toth AL, Robinson GE. Worker nutrition and division of labour in honeybees. Anim. Behav. 2004; 69: 427–435. doi: 10.1016/j.anbehav.2004.03.017

10. Denisorn R, Raymond-Delpech V. Insights into the molecular basis of social behaviour from studies on the honeybee, Apis mellifera. Invert. Neurosci. 2008; 8: 1–9. doi: 10.1016/s10158-008-0066-6 PMID: 18274798

11. Johnson BR. Division of labor in honeybees: form, function, and proximate mechanisms. Behav. Ecol. Sociobiol. 2010; 64: 305–316. doi: 10.1007/s00265-009-0874-7 PMID: 20119486

12. Amdam GV, Page RE. The developmental genetics and physiology of honeybee societies. Anim. Behav. 2010; 79: 973–980. doi: 10.1016/j.anbehav.2010.02.007 PMID: 20541437

13. Whitfield CW, Cziko A-M, Robinson GE. Gene expression profiles in the brain predict behavior in individual honey bees. Science 2003; 302: 296–299. doi: 10.1126/science.1086807 PMID: 14551438

14. Whitfield CW, Ben-Shahar Y, Brillet C, Leoncini I, Crauser D, LeConte Y, et al. Genomic dissection of behavioral maturation in the honey bee. Proc. Natl. Acad. Sci. 2006; 103: 16068–1607. doi: 10.1073/pnas.0606909103 PMID: 17065327

15. Alaux C, Conte L, Adams A, Rodriguez-Zas S, Grozinger CM, Sinha S, et al. Regulation of brain gene expression in honey bees by brood pheromone. Genes Brain Behav. 2009; 8: 309–319. doi: 10.1111/j.1601-183X.2009.00480.x PMID: 19220482

16. Nelson CM, Ihle KE, Fondrk MK, Page RE, Amdam GV. The gene vitellogenin has multiple coordinating effects on social organization. PLOS Biol. 2007; 5: e62. doi: 10.1371/journal.pbio.0050062 PMID: 17341131

17. Ohashi K, Natori S, Kubo T. Changes in the mode of gene expression of the hypopharyngeal gland cells with an age-dependent role change of the worker honeybee Apis mellifera L. Eur. J. Biochem. 1997; 249: 797–802. doi: 10.1111/j.1432-1033.1997.t01-1-00797.x PMID: 9395329

18. Scharlaken B, de Graaf DC, Goossens K, Brunain M, Peelman LJ, Jacobs FJ. Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, Apis mellifera, after a bacterial challenge. J. Insect Sci. 2008; 8 (Article 33): 1 doi: 10.1673/031.008.3301

19. Tanaka H, Suzuki K. Expression profiling of a diapause-specific peptide (DSP) of the leaf beetle Gastrophyta atrocyanea and silencing of DSP by double-strand RNA. J. Insect Physiol. 2005; 51: 701–707. doi: 10.1016/j.jinsphys.2005.03.018 PMID: 15936770

20. Amdam GV, Sim es ZLP, Guidugli KR, Norberg K, Omholt SW. Disruption of vitellogenin gene function in adult honeybees by abdominal injection of double-stranded RNA. BMC Biotechnol. 2003; 3: 1–8. doi: 10.1186/1472-6750-3-1 PMID: 12546706

21. Robertson HM, Wanner KW. The chemoreceptor superfamily in the honey bee, Apis mellifera: Expression of the odorant, but not gustatory family. Genome Res. 2006; 16: 1395–1403. doi: 10.1101/gr.5057506 PMID: 17065611

22. Robertson HM, Gadau J, Wanner KW. The insect chemoreceptor superfamily of the parasitoid jewel wasp Nasonia vitripennis. Insect Mol. Biol. 2010; 19: 121–136. doi: 10.1111/j.1365-2973.2009.00979.x

23. Clyne PJ, Warr CG, Carlson JR. Candidate taste receptors in Drosophila. Science 2000; 287: 1830–1834. doi: 10.1126/science.287.5459.1830 PMID: 10703012

24. Robertson HM, Warr CG, Carlson JR. Molecular evolution of the insect chemoreceptor gene superfamily in Drosophila melanogaster. Proc. Natl. Acad. Sci. 2003; 100: 14537–14542. doi: 10.1073.pnas.2335847100 PMID: 14608037

25. Hill CA, Fox AN, Pitts RJ, Kent LB, Tan PL, Chrysal MA, et al. G protein-coupled receptors in Anopheles gambiae. Science 2002; 298: 176–178. doi: 10.1126/science.1076196 PMID: 12364795

26. Jung JW, Park KW, Ahn Y-J, Kwon AW. Functional characterization of sugar receptors in the western honeybee, Apis mellifera. J. Asia-Pacific Entomol. 2015; 18: 19–26. doi: 10.1016/j.aspen.2014.10.011

27. Ament SA, Wang Y, Chen CC, Blatti CA, Hong F, Liang ZS, et al. The transcription factor Ultraspiracle influences honey bee social behavior and behavior-related gene expression. PLOS Genet. 2012; 8: e1002596. doi: 10.1371/journal.pgen.1002596 PMID: 22479195
28. Miyamoto T, Slone J, Song X, Amrein H. A Fructose receptor functions as a nutrient sensor in the *Drosophila* brain. Cell 2012; 151: 1113–1125. doi:10.1016/j.cell.2012.10.024 PMID: 23178127

29. Miyamoto T, Wright G, Amrein H. Nutrient sensors. Curr. Biol. 2014: 23; R369–37. http://dx.doi.org/10.1016/j.cub.2013.04.002

30. Fell RD. The qualitative and quantitative analysis of insect haemolymph sugars by high performance thin-layer chromatography. Comp. Biochem. Physiol. 1990; 95A: 539–544. doi:10.1016/0300-9629(90)90735-B

31. Leta MA, Gilbert C, Morse RA. Levels of hemolymph sugars and body glycogen of honeybees (*Apis mellifera* L.) from colonies preparing to swarm. J. Insect Physiol. 1996: 42; 239–245. doi:10.1016/0022-1910(95)00106-9