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Female qualities in males: Vitellogenin synthesis induced by ovary transplants into the male silkworm, *Bombyx mori*

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ABSTRACT

Female qualities in males are common in vertebrates but have not been extensively reported in insects. Vitellogenin (Vg) is highly expressed in the female fat body and is generally required for the formation of yolk proteins in the insect egg. Vg upregulation is generally regarded as a female quality in female oviparous animals. In this study, we found that *Bombyx mori* Vg (BmVg) is especially highly expressed in the female pupa. Downregulation of the BmVg gene in the female pupa by RNA interference (RNAi) interfered with egg formation and embryonic development, showing the importance of BmVg in these processes. So, we used BmVg as a biomarker for female qualities in the silkworm. Hematoxylin-eosin staining and immunofluorescence histochemistry showed that ovary transplants induced BmVg synthesis in the male pupa fat body. Ovaries transplanted into male silkworms produced only a few eggs with deformed yolk granules. These results suggested that the amount of BmVg in the male silkworm was insufficient for eggs to undergo complete embryonic development. After 17-beta-estradiol was used to treat male pupae and male pupal fat bodies, *BmVg* was upregulated *in vivo* and *in vitro*. These findings indicated that the male silkworm has innate female qualities that were induced by a transplanted ovary and 17β-estradiol. However, in silkworms, female qualities in males are not as complete as in females.

1. Introduction

Vitellogenin (Vg), the major precursor of the egg-yolk protein vitellin (Vn), is expressed in female oviparous organisms as a nutritional reserve for embryonic development. Vg is induced by 17β-estradiol in males and immature females in the flounder *Platichthys flesus* [1] and Vg expression in aquatic invertebrates is generally used as a biomarker of environmental estrogens [2].

In insects, yolk proteins are the major source of nutrition for embryonic development and account for >90% of total egg proteins. Vn, which participates in yolk formation, is the most abundant protein in insect eggs [3] and is considered essential for ovary development [4]. Vg is the major precursor of Vn and was first identified as a female-specific protein in the hemolymph of the silk moth *Hyalophora cecropia* [5]. In insects, synthesis of Vg is specific to tissue, sex, and developmental stage. Vg is synthesized in the female insect fat body, secreted into the hemolymph, and sequestered by competent oocytes via receptor-mediated endocytosis [6]. The *Bombyx mori* Vg (BmVg) gene was identified in 1994 [7]. BmVg protein, which is a tetramer with a molecular mass of 440 kDa, is composed of two heavy chains and two light chains [8]. BmVg synthesis begins in the female fat body at larval-pupal ecdysis. However, in silkworms, the existence of 30 K proteins (30KPs), a group of species-specific proteins in the Lepidoptera, reduces the proportion of Vn in yolk proteins compared to other insect species. Vg has been suggested as a biomarker for female qualities in silkworm, but has not been confirmed experimentally [9].

In this study, we confirmed BmVg as a biomarker of female qualities in the silkworm by analysis of expression patterns and RNA interference (RNAi). Using BmVg as a biomarker, we detected BmVg synthesis in ovaries transplanted into male silkworms, and observed eggshell surface of formed eggs and the development of embryos from the eggs by artificial parthenogenesis. We also detected BmVg expression and synthesis in male silkworm pupae and male fat bodies after estrogen treatment. The results showed that female qualities in the male silkworm were induced by a transplanted ovary and 17β-estradiol. However, the female qualities of the male silkworm are not same as that of the female.

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2. Materials and methods

2.1. Animal strains and sample preparation

B. mori wild type *dazao* strain was obtained from the silkworm gene bank at Southwest University, Chongqing, China. Larvae were reared on fresh mulberry leaves (*Morus* sp.) under 25–27 °C natural conditions and began wandering 7–8 days after entering the fifth instar stage and pupated 3 days after the wandering stage. Time 0 of wandering was defined as the time at which the larvae stopped feeding. Day 0 for pupae was defined as the time at which larvae molted completely into pupae. Samples of embryos during development and from whole bodies from the first instar larva to the adult moth stage were prepared for *BmVg* transcript detection. Hemolymph samples were collected from female and male fifth instar larvae to day 5 pupae for BmVg protein synthesis profiles. Brain, mid-gut, ovary/testis and fat body of first-day female and male pupae were collected for total RNA and protein extraction.

2.2. RNA interference

RNA interference was done as described [10]. *BmVg* (amino acid positions 1027–1193) and *EGFP* (negative control) gene fragments were amplified. The primers were given in Table S1.

Based on the *BmVg* transcription profile, 50 *dazao* female silkworms at day 1 of wandering were injected with 40 μg dsBmVg by capillary needle into the intersegmental membrane between the eighth and ninth abdominal segments. For a stronger effect, as described by Lin [11], pupae were injected again on day 2 with 60 μg dsBmVg and day 5 with 20 μg. As controls, dsEGFP and double-distilled water were injected into *dazao* female pupae with the same volume at the same time. Injected pupae were maintained at room temperature. Pharate adults were dissected, and then fat bodies were collected for detection of *BmVg* transcripts at 2 days after injection and egg formation was observed at day 7 after pupation. After eclosion, dsRNA-injected moths were mated with untreated males and allowed to lay eggs. Laid eggs were counted and embryonic development was observed.

2.3. Ovary transplantation

Silkworm pharate adults at day 0 were used for transplant experiments. Bodies were sterilized with 70% (v/v) ethanol. Ovaries without fat bodies were removed from females and put into males and the transplant site sealed with nail varnish. As controls, cuts were made and sealed with nail varnish in male and female pharate adults. After surgery, experimental and control pharate adults were maintained at room temperature. At 2 days after surgery, fat bodies were prepared for RNA and protein isolation. After eclosion, moths were dissected to examine ovary development and egg formation. Unfertilized eggs were collected for detection of BmVn protein by immunofluorescence and histochemistry and for artificial parthenogenesis.

2.4. Estrogen treatment

Six day 0 male pupae per experiment were microinjected at 1 μg/g body weight with the active estrogen 17β-estradiol (Sigma, USA) dissolved in 0.1% (v/v) ethanol. Experiments were done in triplicate. Hormone dose was determined according to Keshan [12]. The same volume of corresponding solvent was injected into control pupae. At 24 h after treatment, fat body and hemolymph were collected, frozen immediately in liquid nitrogen and stored at −80 °C.

Fat body was removed from male silkworm pupae at 0 days and incubated in Grace insect medium (Gibco, Invitrogen) supplemented with 2 μM 17β-estradiol with penethamate and streptomycin as described [13]. As a control, fat body was incubated in 0.1% (v/v) ethanol. After treatment, the fat body was kept at 25 °C and 75% relative humidity for 24 h, then frozen in liquid nitrogen and stored at −80 °C. Experiments were performed three times independently.

2.5. RT-PCR

The RT-PCR and quantity RT-PCR (qPCR) were done as described [10]. Primers for RT-PCR and qPCR were given in Table S1.

2.6. SDS–PAGE and Western blotting

The methods of SDS–PAGE Western blotting were as described [11]. Anti-BmVg (maintained in our laboratory) was diluted 1:10,000 in blocking buffer TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20) with 1% (w/v) bovine serum albumin and second antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG diluted 1:20,000 (Beyotime, China) in the same blocking buffer. Bound HRP-conjugated antibodies were detected by an enhanced chemiluminescence system (ECL; Thermo, USA) and photographed using a Clinx ChemiScope 3400 Mini (China Scientific, China).

2.7. Immunofluorescence histochemistry of eggs

Egg immunohistochemistry was as described by Zhang [14]. Eggs from acceptor male moths using normal female moths as control and from dsBmVg-treated female moths with dsEGFP-treated female moths as control were fixed overnight at 4 °C in 4% (v/v) formaldehyde. Tissues were embedded in paraffin and sliced into 5 μm sections. Portions were stained with hematoxylin-eosin (HE) to determine the shape of the yolk granules; other portions were treated with anti-BmVg as primary antibody and Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Invitrogen, Carlsbad, CA) as secondary antibody. Antibody incubations were for 1 h. Primary and secondary antibodies were diluted 1:200 in PBST (PBS with 0.2% (v/v) Tween-20). Sections were examined under a fluorescence microscope (DMI4000B, Leica).

3. Results

3.1. BmVg is highly expressed in the female silkworm pupa

Microarray data [15] show that *BmVg* is expressed in female silkworm from 24 h after wandering stage to moth stage, with a peak at 60 h after wandering. However, *BmVg* transcription signals were weak in males (Fig. 1A). RT-PCR showed that the level of *BmVg* mRNA was higher in female pupae than at other times in females or at all times in males (Fig. 1B1). Transcription of *BmVg* was high in fat bodies of day 1 female pupae. Transcription was very low in embryos, with almost no detection in male larvae or pupae. In addition, a weak transcription signal was detected in other tissues including the epidermis and midgut in females. Transcription was detected in some tissues in males but with a weak signal (Fig. 1C1).

Large amounts of BmVg protein were detected in female fat bodies from day 3 of wandering. However, the protein was barely detectable in female larvae or in males at any stage (Fig. 1B2). Very little signal was detected in the epidermis, midgut or ovaries of female pupae (Fig. 1C2).

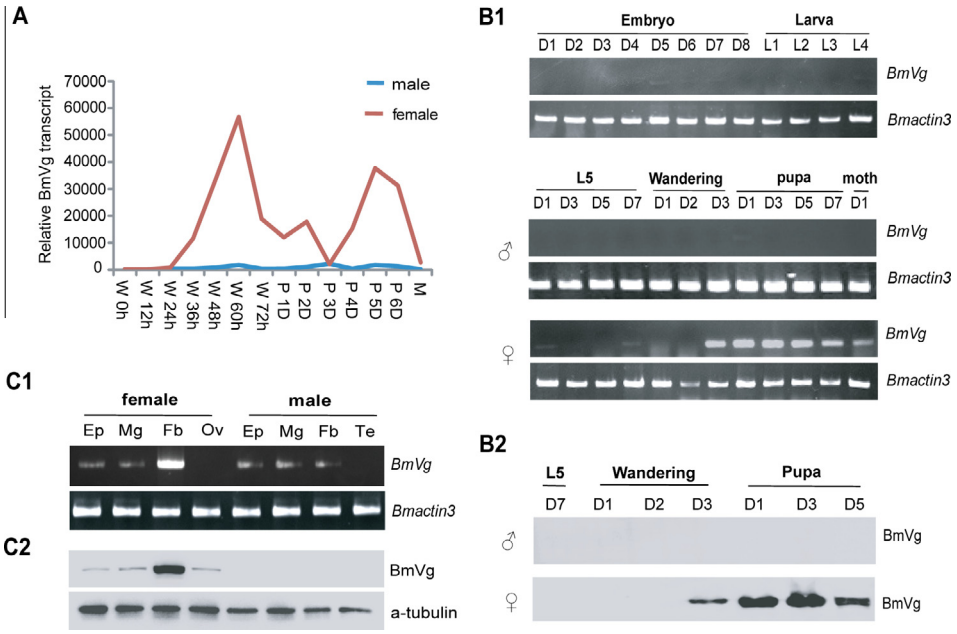

Fig. 1. Expression patterns of BmVg in silkworm stages and tissues. (A) *BmVg* expressed specifically in silkworm female pupae determined by microarray. (B1) *BmVg* transcriptional profiles for the entire silkworm life cycle by RT-PCR (E1–E8, embryo; L1–L5, larva; P1–P8, pupa; M, moth). (B2) BmVg protein in hemolymph was analyzed by Western blotting from day 5 larvae to day 3 pupae; 20 μg total hemolymph proteins were loaded in every lane. (C1) *BmVg* transcriptional profiles of tissue in day 1 pupae by RT-PCR (Ep, epidermis; Mg, mid-gut; Fb, fat body; Ov, ovary; Te, testis). (C2) BmVg expressed in different tissues in day 1 pupae by Western blotting.

3.2. Downregulation of BmVg affected egg formation and embryonic development in female silkworm

To examine the function of BmVg in egg formation and embryonic development, dsRNAs of *BmVg* and *EGFP* were synthesized and injected into female pupae; 10% of dsBmVg-treated pupae died (Table S2). In survivors, oviducts of dsBmVg-treated moths were shorter than oviducts of wild type female moths that were treated with ddH_2O or dsEGFP (Fig. 2A). Eggs from dsBmVg-treated moths were smaller and whiter than the pale yellow eggs of controls (Fig. 2B). The dsBmVg-treated moths laid fewer eggs than dsEGFP-treated moths (Fig. 2C and D). Of 36 surviving female moths, 16 moths accounted for nearly 50% of eggs, but with an average <200 eggs; 8 moths laid an average of 200–300 eggs; and 12 moths laid an average >300 eggs, which was similar to the number of eggs from control moths (Table S2). Eggs of 9 female moths from each treatment group were hatched artificially and embryos developed. Embryonic development was also affected by downregulation of BmVg. Some embryos from eggs laid by moths with low BmVn died in the shell and a few larvae developed to mature larval stage but were unable to climb out of the shell (Fig. 2E). The hatching rate of eggs from dsBmVg-treated moths yielded fewer embryos than eggs from ddH_2O and dsEGFP-treated moths (Fig. 2F). The hatching rate of dsBmVg-treated embryos was 29.70%. The rate was significantly lower than the 63.75% for ddH_2O-treated and 61.42% for dsEGFP-treated embryos (Table S2).

RT-PCR and qPCR showed that *BmVg* transcripts were reduced in dsBmVg-treated silkworms almost to 35% (Fig. 2G) and Western blotting showed protein quantities were decreased significantly to 40% of the level seen in dsEGFP-treated silkworms (Fig. 2H). These results showed that reduction of BmVg synthesis affected egg formation and embryonic development.

3.3. BmVg synthesis in male silkworm induced by ovary

Based on the above results, we chose the *BmVg* gene as a biomarker for studying female qualities in males. We used transplantation to determine whether ovaries developed in male. Ovaries from females were removed and transplanted into male pupa (Fig. 3A). Ovaries developed in male silkworms and formed four complete oviducts with eggs (Fig. 3B1). However, eggs from acceptor male moths were slightly whiter than the pale yellow eggs produced by normal female moths (Fig. 3B2). RT-PCR and qPCR showed *BmVg* transcription was induced in acceptor male fat bodies (Fig. 3C). Total proteins including BmVg/BmVn were detected by SDS–PAGE and Western blotting in tissues during transplantation surgery. Before surgery, BmVg synthesis was just beginning in the female fat body, with little BmVn in ovaries. BmVg was low in the male fat body. After surgery, BmVg synthesis was induced in fat bodies of males with transplanted ovaries. Abundant BmVn was present in acceptor eggs (Fig. 3D1 and D2). Nonetheless, the amount of BmVn in acceptor eggs was lower than the amount in normal silkworm eggs (Fig. 3D2).

To detect the development of the embryo, 6675 unfertilized eggs from acceptor male silkworm was treated by artificial parthenogenesis. However, none of the embryos developed into normal larvae. Embryonic development stopped at an early stage (Fig. S1A). The number, shape and size of yolk granules in eggs produced by ovaries transplanted into acceptor male silkworms were different from eggs produced by normal female silkworms. And the content of BmVn protein in yolk granules of acceptor male silkworm eggs was lower than in normal female silkworm eggs (Fig. S1B). These results suggested that the quantity of BmVg was important for normal development of embryos, and that female qualities were incomplete in the male silkworm.

3.4. Expression of BmVg induced by 17beta-estradiol

Estrogen secreted from ovaries can induce *Vg* transcription in vertebrates [1,16] and some evidences suggest that silkworm should have estradiol [17–19]. Therefore, we injected 17β-estradiol into male silkworms at 0 day pupae. Results from RT-PCR and qPCR showed 17β-estradiol induced *BmVg* transcription in the male fat body (Fig. 4A). The result is the same as cultured fat bodies

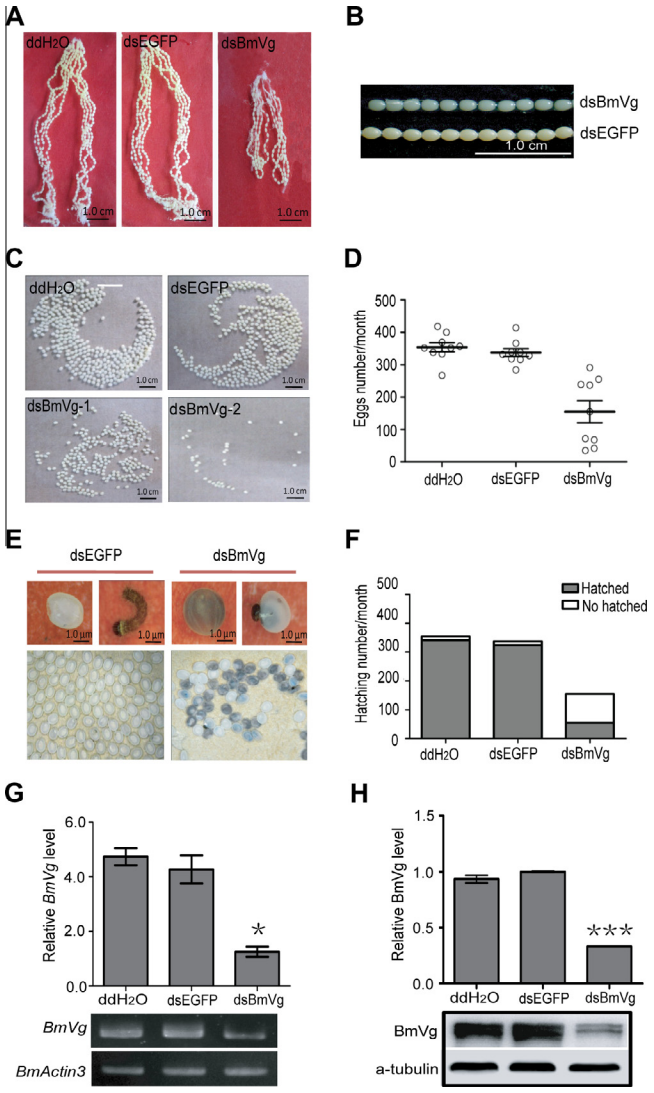

Fig. 2. Effect of RNAi silencing BmVg in silkworm pupae. (A) Differentiation of wild type and dsRNA-treated silkworm ovaries. (B) Eggs from dsBmVg-treated moths were smaller and whiter than from dsEGFP-treated moths. (C) Eggs from wild type and dsBmVg-treated female moths. (D) Number of eggs from dsBmVg-treated moths after fertilization. Each group had 9 samples. (E) Embryonic development following dsBmVg treatment; some embryos died in the shell and others were unable to leave the shell. (F) Hatching statistics of embryos from dsBmVg-treated moths after fertilization. Each group had 9 samples. (G) qPCR of *BmVg* transcripts in the fat body 2 days after dsBmVg-treatment. (H) Western blotting of BmVg in eggs from dsBmVg-treated female moths. Relative amount of BmVg was analyzed by Quantityone software 4.62. $^{*}P < 0.05$, $^{***}P < 0.001$ (dsBmVg treatment versus dsEGFP control; *t*-test).

in vitro (Fig. 4B). These results suggested that BmVg was induced in the silkworm male fat body by 17β-estradiol *in vivo* and *in vitro*.

4. Discussion

Vg, egg specific protein (ESP), and 30 KPs are essential for egg formation and embryonic development in the silkworm and large amounts of these proteins are present in eggs [4]. The existence of 30 KPs in silkworm eggs reduces the proportion of Vn in yolk proteins compared to other insect species [20]. In this study, down-regulation of BmVg in female pupae by RNAi resulted in abnormal egg formation and embryonic development. This phenomenon was likely due to the absence of BmVg leading to insufficient and malformed yolk granules, so eggs could not provide enough nutrition for embryonic development. We also found that oviducts of dsBmVg-treated silkworms were shorter than oviducts in wild type silkworms. When the size or number of eggs was reduced, treated silkworm oviducts seemed shorter than in wild type silkworms. However, this phenomenon should be investigated further. Furthermore, the defective Vg receptor affects egg formation and embryonic development by preventing Vg transport into eggs. Thus, the silkworm oogenesis recessive mutation *scanty vitellin* (*vit*) strain causes and embryonic lethality and smaller and whiter eggs compared to wild type [21]. The recessive female-sterile mutation *yolkless* (*yl*) in *Drosophila melanogaster* [22] and downregulation of VgR in the American dog tick *Dermacentor variabilis*, or in *Blattella germanica* or *B. mori* result in similar phenotypes, with Vn reduction resulting in abnormal egg formation and embryonic development [11,23,24]. These results indicate that Vg is essential for egg formation and embryonic development. The absence of Vg results in embryonic lethality. Although *B. mori* has less BmVn in eggs than other insects, BmVg/BmVn is essential for nutrition for silkworm embryonic development.

We chose BmVg/BmVn as a biomarker for silkworm female qualities based on five factors. First, BmVn is the most abundant protein in the silkworm egg, although the amount of BmVn is low compared to the amount in other insect species. Yolk proteins of mature silkworm eggs have 40% Vn, 25% ESP and 35% 30 KPs [4]. Second, synthesis of BmVg in fat body is specific to sex and developmental stage [6,25]. Third, we found that BmVg was essential for silkworm egg formation and embryonic development. Fourth, although 30 KPs and storage proteins are expressed in the fat body, they are not specific to females or pupal stage. In addition, ESP is expressed specifically in females, but only in the ovary [26]. Last, Vg in aquatic invertebrates is a biomarker for detecting environmental estrogenic compounds [2]. Therefore, for this study, BmVg was selected as a biomarker for female qualities in silkworm.

Transplanted ovaries develop well in acceptor male silkworms, with each ovary differentiating into four complete oviducts with eggs. In our study, however, no normal silkworms were obtained by artificial parthenogenesis. We suggest that artificial parthenogenesis likely has a strain bias. Vg expression is induced in transplanted oocytes in male cockroaches (*Diploptera punctata*) and butterflies (*Pieris brassicae*) [27,28]. A study of *Aedes aegypti* showed that synthesis of Vg is controlled by ovaries [29]. In our study, immunohistochemistry showed fewer and malformed yolk granules in eggs from acceptor male silkworms. It indicated that the male silkworms have innate female qualities and ovaries could induce them. However, in the male silkworm, these qualities are not as complete as in the female silkworm.

Normally female-specific functions are induced in the male silkworm by 17β-estradiol treatment. In *A. aegypti*, ovaries secrete ecdysone and induce Vg expression [29]. Ecdysone regulate cascade and in lepidopteran insects ovarian development [30]. Juvenile hormones are required for the synthesis of Vg by fat bodies and for initiation of blood protein uptake by *Eublaberus posticus* oocytes [31]. When eggs form, ecdysone and juvenile hormone titers are similar in the female and male silkworm during wandering and early pupal stages [32]. We propose that ecdysone is not likely the key factor for inducing BmVg expression in male silkworms because the key factor must be sex-specific during ovary development. In vertebrates, the ovary is the source of estrogen, which induces Vg synthesis ahead of schedule and is highly expressed in fish and the wood frog *Rana sylvatica* [1,16]. Some evidence suggests that estradiol should exist in the silkworm [17–19]. We treated day 0 male pupae *in vivo* and fat bodies *in vitro* with 17β-estradiol. The results showed that *BmVg* transcription was induced by 17β-estradiol. However, determining whether silkworm ovaries produce estrogen requires further experiments.

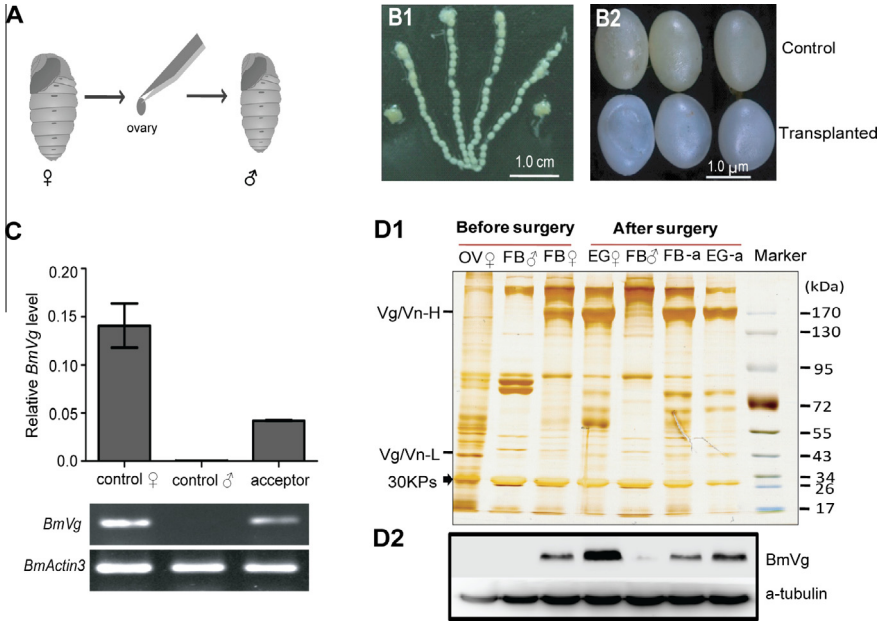

Fig. 3. Expression of BmVg in male pupae induced by ovary transplanted for egg formation. (A) Day 0 male silkworm pupae with transplanted ovaries. (B) Ovary phenotypes in male pupae. B1, ovary transplanted into a male that developed four complete oviducts with eggs. B2, eggs were slightly whiter than control eggs from female moths. (C) RT-PCR and qPCR for *BmVg* transcription in fat body 2 days after transplant. Acceptor, male pupae transplanted with an ovary; Control♀♂, normal female and male pupae cut and sealed with nail varnish. (D) Analysis of total proteins in silkworm tissues before and after transplant. SDS–PAGE and Western blotting for BmVg (before surgery, day 0 pupa; after surgery, fat body from day 2 pupae and eggs from moths; OV, ovary; FB, fat body; EG, egg; EG-a, FB-a, egg and fat body from the acceptor silkworm; Marker, standard protein marker).

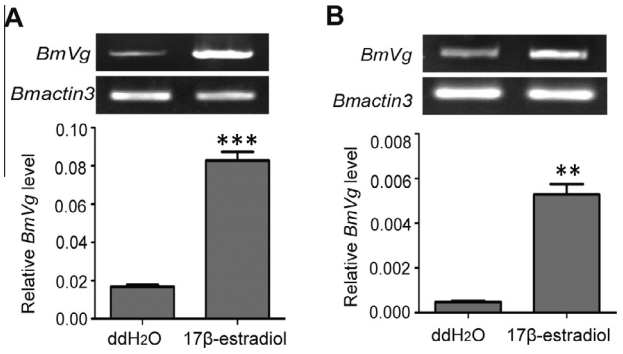

Fig. 4. Expression of BmVg induced by estrogen in male fat body *in vivo* and *in vitro*. (A) Transcription of *BmVg* in fat body of day 0 male pupae. (B) *BmVg* transcription in male fat body. $^{**}P < 0.01$ and $^{***}P < 0.001$ for 17β-estradiol treatment versus ddH_2O control; *t*-test.

The most important finding from this study was that female characteristics could be induced by estrogen in the male silkworm.

5. Conclusions

In this study, we demonstrated that BmVg can be used as a biomarker for female qualities in silkworm. Synthesis of BmVg was induced by transplanting ovaries into male silkworms and 17β-estradiol resulted in egg formation. These results indicated that the male silkworm has female qualities that can be induced under certain conditions.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.044.

References

[1] A.P. Scott, I. Katsiadaki, M.F. Kirby, J. Thain, Relationship between sex steroid and vitellogenin concentrations in flounder (*Platichthys flesus*) sampled from an estuary contaminated with estrogenic endocrine-disrupting compounds, Environ. Health Perspect. 114 (Suppl. 1) (2006) 27–31.
[2] V. Matozzo, F. Gagne, M.G. Marin, F. Ricciardi, C. Blaise, Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review, Environ. Int. 34 (2008) 531–545.
[3] M.L. Pan, W.J. Bell, W.H. Telfer, Vitellogenic blood protein synthesis by insect fat body, Science 165 (1969) 393–394.
[4] J. Zhu, L.S. Indrasith, O. Yamashita, Characterization of vitellin, egg-specific protein and 30 kDa protein from Bombyx eggs, and their fates during oogenesis and embryogenesis, Biochim. Biophys. Acta 882 (1986) 427–436.
[5] W.H. Telfer, Immunological studies of insect metamorphosis. II. The role of a sex-limited blood protein in egg formation by the Cecropia silkworm, J. Gen. Physiol. 37 (1954) 539–558.
[6] A.S. Raikhel, T.S. Dhadialla, Accumulation of yolk proteins in insect oocytes, Annu. Rev. Entomol. 37 (1992) 217–251.
[7] K. Yano, M.T. Sakurai, S. Izumi, S. Tomino, Vitellogenin gene of the silkworm, *Bombyx-Mori* – structure and sex-dependent expression, FEBS Lett. 356 (1994) 207–211.
[8] S. Izumi, S. Tomino, H. Chino, Purification and molecular-properties of vitellin from the silkworm, *Bombyx-Mori*, Insect Biochem. 10 (1980) 199–208.
[9] J. Zhu, Q.Z. Meng, Biology of hemolymph and yolk proteins, in: Z.H. Xiang (Ed.), Biology of Sericulture, first ed., China Forestry Publishing House, 2005.
[10] C. Yang, Y. Lin, H. Liu, G. Shen, J. Luo, H. Zhang, Z. Peng, E. Chen, R. Xing, C. Han, Q. Xia, The broad complex isoform 2 (BrC-Z2) transcriptional factor plays a critical role in vitellogenin transcription in the silkworm *Bombyx mori*, Biochim. Biophys. Acta 2014 (1840) 2674–2684.
[11] Y. Lin, Y. Meng, Y.X. Wang, J. Luo, S. Katsuma, C.W. Yang, Y. Banno, T. Kusakabe, T. Shimada, Q.Y. Xia, Vitellogenin receptor mutation leads to the oogenesis mutant phenotype 'scanty vitellin' of the silkworm, *Bombyx mori*, J. Biol. Chem. 288 (2013) 13345–13355.
[12] B. Keshan, A.K. Ray, Estradiol-17beta in *Bombyx mori*: possible significance and its effect on silk production, J. Insect Physiol. 46 (2000) 1061–1068.

[13] A.S. Raikhel, K.W. Deitsch, T.W. Sappington, Culture and analysis of the insect fat body, in: J.M. Crampton, C.B. Beard, C. Louis (Eds.), The Molecular Biology of Insect Disease Vectors: A Methods Manual, Chapman and Hall, 1997, pp. 507–523.
[14] Y. Zhang, P. Zhao, H. Liu, Z. Dong, Q. Yang, D. Wang, Q. Xia, The synthesis, transportation and degradation of BmLP3 and BmLP7, two highly homologous *Bombyx mori* 30K proteins, Insect Biochem. Mol. Biol. 42 (2012) 827–834.
[15] Q.Y. Xia, D.J. Cheng, J. Duan, G.H. Wang, T.C. Cheng, X.F. Zha, C. Liu, P. Zhao, F.Y. Dai, Z. Zhang, N.J. He, L. Zhang, Z.H. Xiang, Microarray-based gene expression profiles in multiple tissues of the domesticated silkworm, *Bombyx mori*, Genome Biol. 8 (2007) R162.
[16] A.R. Tompsett, S. Wiseman, E. Higley, J.P. Giesy, M. Hecker, Effects of exposure to 17alpha-ethynylestradiol during larval development on growth, sexual differentiation, and abundances of transcripts in the liver of the wood frog (*Lithobates sylvaticus*), Aquat. Toxicol. 126 (2013) 42–51.
[17] E. Ohnishi, M. Ogiso, K. Wakabayashi, Y. Fujimoto, N. Ikekawa, Identification of estradiol in the ovaries of the silkworm, *Bombyx mori*, Gen. Comp. Endocrinol. 60 (1985) 35–38.
[18] Y. Fujimoto, N. Ikekawa, M. Ogiso, E. Ohnishi, Characterization of 17β-estradiol 3-(β-D-glucopyranoside) and 17-(α-D-glucopyranoside) as the metabolites of 17β-estradiol in the cultured ovaries of the silkworm, *Bombyx mori*, Experientia 42 (1986) 567–568.
[19] S. Roy, J. De, S. Kundu, A. Biswas, M. Pramanik, A.K. Ray, Estradiol-17beta: tracing its metabolic significance in female fat body of fifth instar larvae of silkworm, *Bombyx mori* L. (race. Nistari), Life Sci. 80 (2007) 446–453.
[20] O. Yamashita, K. Irie, Larval hatching from vitellogenin-deficient eggs developed in male hosts of the silkworm, Nature 283 (1980) 385–386.
[21] K. Fujikawa, Y. Kawaguchi, Y. Banno, K. Koga, H. Doira, Yolk of a "scanty vitellin" mutant, *vit*, of *Bombyx mori* is lacking in vitellin and 30 kDa proteins, Comp. Biochem. Physiol. 112 (1995) 585–589.
[22] P.J. DiMario, A.P. Mahowald, Female sterile (1) yolkless: a recessive female sterile mutation in *Drosophila melanogaster* with depressed numbers of coated pits and coated vesicles within the developing oocytes, J. Cell Biol. 105 (1987) 199–206.
[23] R.D. Mitchell 3rd, E. Ross, C. Osgood, D.E. Sonenshine, K.V. Donohue, S.M. Khalil, D.M. Thompson, R. Michael Roe, Molecular characterization, tissue-specific expression and RNAi knockdown of the first vitellogenin receptor from a tick, Insect Biochem. Mol. Biol. 37 (2007) 375–388.
[24] L. Ciudad, M.D. Piulachs, X. Belles, Systemic RNAi of the cockroach vitellogenin receptor results in a phenotype similar to that of the *Drosophila* yolkless mutant, FEBS J. 273 (2006) 325–335.
[25] T.W. Sappington, A.S. Raikhel, Molecular characteristics of insect vitellogenins and vitellogenin receptors, Insect Biochem. Mol. Biol. 28 (1998) 277–300.
[26] E. Mine, S. Izumi, M. Katsuki, S. Tomino, Developmental and sex-dependent regulation of storage protein-synthesis in the silkworm, *Bombyx-Mori*, Dev. Biol. 97 (1983) 329–337.
[27] A. Karlinsky, M. Lamy, Juvenile hormone and vitellogenesis in male of *Pieris brassicae* L. (Lepidoptera) (author's transl) (proceedings), Ann. Endocrinol. (Paris) 37 (1976) 529–530.
[28] E.C. Mundall, S.S. Tobe, B. Stay, Induction of vitellogenin and growth of implanted oocytes in male cockroaches, Nature 282 (1979) 97–98.
[29] H.H. Hagedorn, A.M. Fallon, Ovarian control of vitellogenin synthesis by the fat body in *Aedes aegypti*, Nature 244 (1973) 103–105.
[30] L. Swevers, K. Iatrou, The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkmoth paradigm, Insect Biochem. Mol. Biol. 33 (2003) 1285–1297.
[31] W.L. Bell, R.H. Barth Jr., Initiation of yolk deposition by juvenile hormone, Nat. New Biol. 230 (1971) 220–222.
[32] B. Calvez, M. Hirn, M. De Reggi, Ecdysone changes in the haemolymph to two silkworms (*Bombyx mori* and *Philosamia cynthia*) during larval and pupal development, FEBS Lett. 72 (1976) 57–61.