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Insulin-like peptides regulate vitellogenesis and oviposition in the green lacewing, *Chrysopa septempunctata*

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Abstract

Insulin-like peptides (ILPs) act through a conserved insulin signaling pathway and play crucial roles in insect metabolism, growth, reproduction, and aging. Application of bovine insulin is able to increase vitellogenin (Vg) mRNA and protein levels in female insects. Here, we first show that injection of bovine insulin into previtellogenic *Chrysopa septempunctata* female adults promoted ovarian growth, increased Vg protein abundance, elevated reproductive performance, and enhanced protease activity. These data suggested that ILPs play crucial roles in reproductive regulation of the green lacewing, *C. septempunctata*.

Keywords: Bovine insulin, ILPs, reproduction, *Chrysopa septempunctata*

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Introduction

In insects, endocrine hormones regulate many physiological and developmental processes (Nijhout, 1994; Riddiford, 1994; Bellés & Maestro, 2005; Raikhel *et al.*, 2005; Goodman & Cusson, 2012). Endocrinology of insect reproduction has traditionally focused on the two most studied groups of insect hormones: juvenile hormones (JH) and ecdysteroids (Wang *et al.*, 2004; Bellés, 2005). JH is a sesquiterpenoid with JH III being the primary hormone produced by the corpora allata (CA) of most insects (Goodman & Cusson, 2012; Hartfelder & Emlen, 2012). JH has central roles in the regulation of metamorphosis and reproduction in insects. The presence of JH during larval molting prevents metamorphosis, and then it reappears in the adult to regulate oocyte maturation and vitellogenin synthesis in the fat body and its absorption by the developing oocytes (Wyatt & Davey, 1996; Sheng *et al.*, 2011; Jindra *et al.*, 2013).

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Many physiological processes regulated by JH are also under the control of other hormones such as insulin-like peptides (ILPs). ILPs are encoded by multi-gene families that are expressed in the brain and other tissue (Wu & Brown, 2006). Insect ILPs are analogous to vertebrate insulin. In mammals, the primary action of insulin is to decrease circulating glucose by promoting synthesis of glycogen and triglyceride (Krieger *et al.*, 2004). For insects, insulin like peptide was first isolated from a Lepidoptera species, *Bombyx mori* (Nagasawa *et al.*, 1984). So far, genes encoding ILPs have been cloned in species from different insect orders, including Orthoptera, Diptera, Lepidoptera and Hymenoptera (Wu & Brown, 2006; Garelli *et al.*, 2012; Xu *et al.*, 2015). Over the last decade, genetic studies revealed that in insect, ILPs act through a conserved insulin signaling pathway to sense nutrient status and to regulate various physiological processes such as development, longevity, metabolism, diapause and female reproduction (Masumura *et al.*, 2000; Claeys *et al.*, 2002; Garofalo, 2002; Ikeya *et al.*, 2002; Goberdhan & Wilson, 2003; Sim & Denlinger, 2008; Geminard *et al.*, 2009; Parthasarathy & Palli, 2011; Süren-Castillo *et al.*, 2012). In *Drosophila melanogaster*, changes in expression of ILPs led to phenotypic effects on growth and organs. Overexpression of *dilp* genes produced bigger progenies with increased cell size and cell number in individual organs (Brogiolo *et al.*, 2001). On the contrary,

suppression of ILPs or their receptors reduced metabolism, strengthen stress resistance and extended life-span (Tatar *et al*., 2003). Recently, reports revealed that ILPs were involved in regulation of growth plasticity and in switch of wing morphs (Garelli *et al*., 2012; Xu *et al*., 2015).

Insulin is conserved in sequence and function. Even insulins from invertebrates and mammals have similar amino acid sequence and physiological effects (Nagata *et al*., 1995; Mohan, 2002). For example, injection of bovine insulin into the previtellogenic, starved, or JH deficient female adults of *Tribolium castaneum* increased Vg mRNA and protein level, suggesting a participation of ILPs in regulation of Vg synthesis (Sheng *et al*., 2011).

Taken together, what we know about ILPs and the signaling pathway is merely based on a few insect pests. It is also important to characterize ILPs in natural enemy insects, as theoretical insight into their endocrine hormones may lead to development of artificial methods applicable in reproductive regulation. The green lacewing, *Chrysopa septempunctata* Wesmael, is one of the most effective entomophagous predators and can be found in most agricultural areas of the world (Tauber *et al*., 2000). Here, we first report the functions of ILPs in a natural Neuroptera predator, *C. septempunctata* by using bovine insulin as a substitute. Injection of insulin promoted ovary development, increased Vg synthesis, elevated productive performance and improved protease activity in *C. septempunctata* female adults. Our results suggested that ILPs are crucial players in regulation of *C. septempunctata* reproduction and are potential reproductive stimuli in mass rearing of this natural enemy insect.

Materials and methods

Experimental insects

The *C. septempunctata* culture used in the study was a laboratory strain kindly provided by Dr Zhang Fan, Beijing Academy of Agriculture and Forestry Sciences. The colony was fed with pea aphid (*Acyrthosiphon pisum*) at 25°C, 70% relative humidity under a 16:8 h light:dark photoperiod.

Preparation and microinjection of bovine insulin

Bovine insulin (Nacalai, Kyoto, Japan) was solubilized in 25 mM HEPES (Ph 8.2) at two concentrations, 10 and 5 mg ml^{-1}. Two days after eclosion, each female was injected 1 µl insulin at the conjunctive between the fourth and fifth abdominal segment. InjectMan® NI 2 and FemtoJet express Microinjector system (Eppendorf, Hamburg, Germany) with glass needles were adopted to conduct injection in 0.2 s under a pressure of 400 mP. A second injection at the same concentration was performed 15 days following the first injection (10 mg ml^{-1}). Female adults injected with equal volume of HEPES buffer were served as control. After injection, female was kept with a male adult in a container maintained at 25°C, 70% relative humidity with 16:8 h light: dark cycle and adequate preys were supplied daily. At least 30 individuals were injected for each treatment. Preoviposition period, oviposition period, longevity, fecundity and egg hatching rate were accessed. Individuals died before oviposition were abandoned and not recorded for above parameters.

Microscopic observation

Injected females were dissected 6, 21 days after eclosion (DAE). Ovaries were dissected in saline solution, disrupted with a needle and observed at 20-fold magnifications (SZX16-ILLT, Olympus, Tokyo, Japan).

RNA isolation and quantitative real-time-polymerase chain reaction (qRT-PCR)

Real-time PCR was performed to analyze Vg mRNA level in injected females on day 7, 14, 21, 28 and 35 after emergence. Total RNAs were extracted from larvae using Tranzol reagents (Transgene, Beijing, China). DNA contaminations were removed by treating RNA solution with RNase-free DNase (Takara, Kyoto, Japan). cDNA was synthesized using TransScript First-Strand cDNA Synthesis SuperMix (Transgene, Beijing, China) with anchored Oligo(dT)18 primer and M-MLV reverse transcriptase. qRT-PCR was performed in a 20 µl total reaction volume containing 200 nM each of forward and reverse gene specific primers, 10 µl of 2 × SYBR Green Real-time PCR Master Mix (Toyobo, Shanghai, China), cDNA produced from 2 µg total RNA. The housekeeping gene actin was used as internal control for normalization. Primers specific to *C. septempunctata* Vg (GenBank Accession Number: JX286617) and actin (GenBank Accession Number: KC505163) were shown in table 1. Quantification of the relative changes in gene transcript level was performed according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Means and standard errors for each time point were obtained from the average of four independent sample sets.

Protein preparation and enzyme-linked immunosorbent assay (ELISA)

Polyclonal antibodies against *C. septempunctata* Vg protein were produced in Japanese white rabbits. Crude Vg protein was extracted from *C. septempunctata* eggs. The protein suspension was separated on a 12% SDS-PAGE gel. After a vacuum freeze-drying, the Vg protein band was ground and mixed with FCA (Freund's incomplete adjuvant). Four injections were made at an interval of 1 week (100 µg in each injection). The rabbit was bled at 7 days after the last injection. Crude protein was extracted from whole female body on day 7, 14, 21, 28 and 35 after emergence. For this, insects were weighted, ground completely in extraction buffer (20 mM Tris/HCl, 0.2 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF, pH 7.0)). The obtained liquid was centrifuged at 4°C, 12,000 rpm for 30 min. The supernatant was diluted in coating buffer (0.05 M Carbonate buffer: 1.59 g $NaCO_3$ and 2.93 g $NaHCO_3$ in 1000 ml water, pH, 9.6) and added to each well of the microtitre immunoassay plates (100 µl per well) and incubated at 4°C overnight. To establish a standard curve, reference samples of Vg protein (2 mg ml^{-1}) suspended in coating buffer at 20, 8, 4, 2 and 1 mg ml^{-1} were added to wells and incubated as above procedure. The wells were briefly rinsed with washing buffer (0.15 M PBS suspended with 0.05% Tween-20, pH7.4) for 3 times. Blocking buffer (300 µl washing buffer suspended with 2% BSA) was added to each well and incubated at 37°C for 2 h. After a washing step as described above, a 100 µl polyclonal primary antibody diluted in PBS buffer (0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4{\cdot}12H_2O$, 8 g NaCl and 0.2 g KCl in 1000 ml H_2O, pH, 7.4) was placed to each well

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Table 1. Primers used in the quantitative PCR.

Gene	Forward	Reverse	Fragment size (bp)
C. septempunctata Vg	TAATGGCATAGGAAGTGGAAAC	GGTCGAACTGCAACTAAAGAAC	168
C. septempunctata actin	TCCAGAAGAACACCCAATCC	ATACACCATCACCAGAGTCAAGT	188

and incubated at 37°C for 2 h. The plate was washed again as above and enzyme-conjugated secondary antibodies (100 µl) diluted with PBS by 10,000 times was transferred to each well. After maintaining at 37°C for 1 h, the plates were rinsed with washing buffer for 5 times. Color was developed by adding 100 µl of freshly prepared TMB substrate buffer (0.05 ml TMB buffer (2 mg ml^{-1} TMB in ethanol), 0.95 ml substrate buffer (1.84 g $Na_2HPO_4{\cdot}12H_2O$, 0.51 g $C_6H_8O_7{\cdot}H_20$ in 100 ml H_2O, pH, 5.0), 1 µl 30% H_2O_2) to each well and keeping in the dark for 5–20 min. To terminate the reaction, 50 µl of 2 M H_2SO_4 was added to each well. Immunoassay plates were analyzed for absorbance with FlexStation 3 (Molecular Devices, California, USA) at 450 nm.

Measurement of general protease activity

General protease activity was monitored according to the methods described by Leplé *et al*. (1995). Seven, 21 and 35 days post eclosion, female adults were ground individually in cold phosphate buffered saline (PBS) at a 1:8 (w:v) ratio and centrifuged at 12,000 rpm (4°C, 30 min). The supernatants were measured for protein concentration using a BCA™ Protein Assay Kit (Pierce, Chicago, USA) and then maintained at 4°C for use in 2 days. For general protease activity detection, casein was dissolved in PBS at 20 mg ml^{-1} and used as a substrate. 20 µl of extract was mixed with 150 µl casein substrate and kept at 37°C for 1 h. A heat denatured sample was served as a negative control. The reaction was terminated by add of 150 µl trichloroacetic acid (10% w/v). After a centrifugation at 14,000 rpm for 15 min, supernatant was measured for absorbance at 450 nm in Flexstation 3. The protease activity as expressed as absorbance unit per mg of protein and the measurement was repeated three times.

Statistical analysis

Preoviposition period, oviposition period, longevity, egg weight, egg hatching rate and total fecundity (number of eggs per female) were analyzed by one-way analysis of variance (ANOVA). Differences in weekly fecundity (number of eggs per week), Vg expression level and enzyme activity was analyzed by factorial ANOVA. Means were separated using least significant difference (LSD) test.

Results

Promotion of fecundity after bovine insulin application

Female adults injected with 10 mg ml^{-1} insulin oviposited 0.41 and 0.38 day earlier than those treated with 5 mg ml^{-1} insulin and buffer, respectively. However, bovine insulin caused no significant effects on *C. septempunctata* preoviposition period ($F = 0.76$; $df = 2, 94$; $P = 0.47$). Compared with injection of buffer, application of 10 mg ml^{-1} insulin resulted in significant difference in oviposition period ($F = 4.43$; $df = 2, 86$; $P = 0.016$) and adult longevity ($F = 2.99$; $df = 2, 85$; $P = 0.056$).

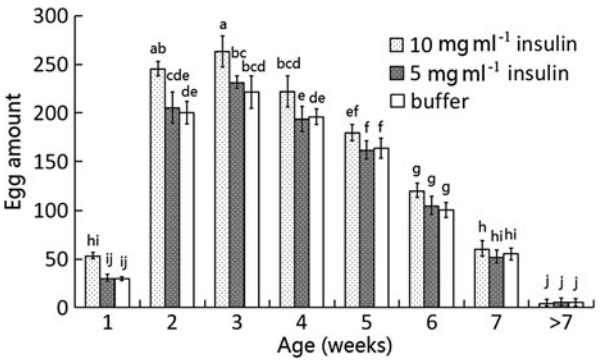

Fig. 1. *C. septempunctata* weekly fecundity (number of eggs per week) after application of bovine insulin. Bars (mean ± SE) with the same letter are not significantly different (Least Significant Difference test, $P < 0.05$).

Weekly fecundity was significantly affected by both insulin treatment ($F = 13.30$; $df = 2, 680$; $P < 0.01$) and female age ($F = 258.11$; $df = 2, 680$; $P < 0.01$) (fig. 1). The total fecundity (number of eggs per female) was significantly different between 10 mg ml^{-1} insulin treated group and 5 mg ml^{-1} insulin or buffer treated group ($F = 5.25$; $df = 2, 85$; $P = 0.007$). Within the whole oviposition period, 10 mg ml^{-1} insulin, 5 mg ml^{-1} insulin and buffer treated females laid total 1150.4, 987.2 and 971.4 eggs, respectively (table 2). Egg hatching rate of 10 mg ml^{-1} insulin treated females was significantly higher than that of 5 mg ml^{-1} insulin and buffer treated individuals ($F = 6.9$; $df = 2, 21$; $P = 0.005$).

Not significant difference in egg weight was observed between insects treated with 10 mg ml^{-1} insulin versus 5 mg ml^{-1} insulin or buffer ($F = 0.04$; $df = 2, 18$; $P = 0.97$). The second injection performed 15 days following the first injection failed to increase fecundity significantly during the remaining oviposition period. When dissection was conducted on day 6 after eclosion, female adults treated with insulin (10 mg ml^{-1}) showed more mature ovaries and egg chambers and larger ovary scale compared with that treated with buffer. Twenty-one days post eclosion, follicles in insulin treated adults showed an even gradient of maturation. However, the primary and secondary follicles in buffer treated adults showed a sharp difference in size, suggesting a lag in maturation of the secondary and later follicles (fig. 2).

Increase of Vg mRNA and protein in insulin treated insects

The *C. septempunctata* Vg transcripts were affected significantly by both insulin treatment ($F = 26.39$; $df = 2, 45$; $P < 0.01$) and adult age ($F = 972.21$; $df = 4, 45$; $P < 0.01$). Seven days post emergence, 10 mg ml^{-1} bovine insulin resulted in significantly higher Vg transcripts level compared with 5 mg ml^{-1} bovine insulin and buffer. The maximum Vg mRNA abundance of all treatments occurred 14 days after eclosion. On day 14

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Table 2. Effects of bovine insulin on *C. septempunctata* reproductive parameters.

Parameters	Bovine insulin (10 mg ml^{-1})	Bovine insulin (5 mg ml^{-1})	Buffer
Preoviposition period (days)	5.68 ± 0.26a	6.09 ± 0.28a	6.06 ± 0.29a
Oviposition period (days)	36.73 ± 0.90a	33.70 ± 0.90b	33.28 ± 0.91b
Longevity (days)	42.40 ± 1.06a	39.38 ± 1.12b	39.03 ± 1.05b
Total fecundity (number of eggs per female)	1150.4 ± 41.10a	987.2 ± 37.69b	971.4 ± 50.80b
Egg weight (mg)	0.0997 ± 0.0056a	0.0976 ± 0.0058a	0.0979 ± 0.0063a
Hatching rate (%)	87.3 ± 1.74a	79.08 ± 1.83b	79.5 ± 1.73b

Note: Statistical significance was analyzed by one-way ANOVA (Least Significant Difference test). Mean ± SE followed by the same letter are not statistically different ($P < 0.05$).

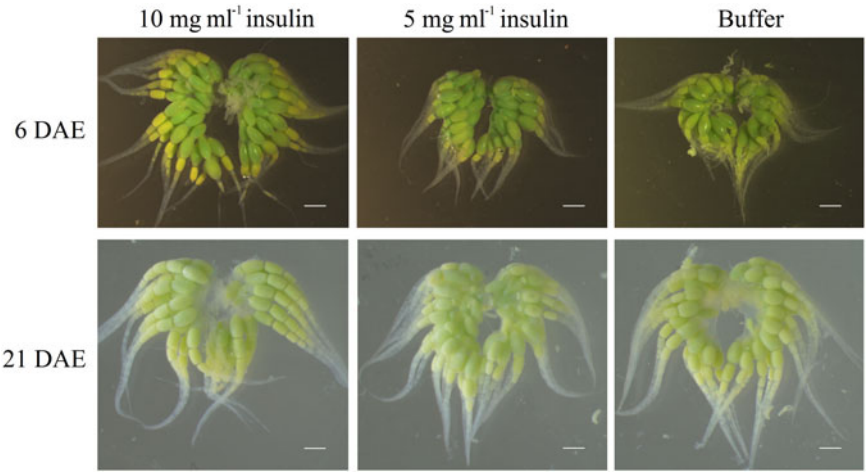

Fig. 2. *C. septempunctata* ovarioles and follicles after injection of bovine insulin. Six days after eclosion (DAE), bovine insulin (10 mg ml^{-1}) treated adults showed bigger ovary scale and more matured follicles compared with buffer treated ones. Twenty-one DAE, bovine insulin (10 mg ml^{-1}) treated females exhibited younger ovarioles characterized by an even gradient of maturation. There are still matured follicles in ovarioles of buffer treated control, but development of the secondary and later follicles were obviously arrested. Application of 5 mg ml^{-1} bovine insulin resulted in similar ovaries compared with buffer. (scale bar, 1 mm)

and 21, individuals applied with 10 mg ml^{-1} insulin accumulated significantly more Vg transcripts than those treated with 5 mg ml^{-1} insulin and buffer (fig. 3a).

Vg protein expression showed a pattern consistent with that of Vg mRNA transcription with significant difference caused by both insulin treatment ($F = 11.00$; $df = 2$, 60; $P < 0.01$) and adult age ($F = 542.58$; $df = 4$, 60; $P < 0.01$). On day 14 after eclosion, all tested insects showed maximum Vg protein expression. On day 7, 14 and 21, individuals treated with 10 mg ml^{-1} insulin showed significantly higher Vg protein level than those treated with 5 mg ml^{-1} insulin and buffer (fig. 3b).

General protease activity after injection

General enzyme activity varied significantly between different insulin treatments ($F = 3.56$; $df = 2$, 45; $P = 0.037$) and adult ages ($F = 585.83$; $df = 2$, 45; $P < 0.01$). Seven days post emergence, general protease activity in females injected with 10 mg ml^{-1} bovine insulin was significantly elevated compared with those injected with buffer. Twenty-one days post emergence, females applied with 10 mg ml^{-1} insulin still showed higher protease activity than those treated with 5 mg ml^{-1} insulin and buffer, but the difference was not significant (fig. 4).

Discussion

Here, we first report the function of ILPs in a Neuroptera species, *C. septempunctata*. Injection of bovine insulin accelerated ovary maturation, promoted Vg expression and elevated reproductive performance in previtellogenic females, suggesting the crucial roles of ILPs in reproductive regulation of the green lacewing, *C. septempunctata*.

Bovine insulin exhibited a continuous effect on egg production. Though significant difference in fecundity was only observed from the 2nd to 4th week post eclosion, the stimulating role did not disappear until the end of the oviposition period (fig. 1). Ovary developmental status (fig. 2) and Vg expression curve (fig. 3) after hormone application led us to conclude that bovine insulin increased lacewing fecundity by promoting vitellogenesis and oogenesis. Aside from egg production, the egg hatching rate in insulin-treated group was also promoted. The possible reason is that hormone stimulated deposit of Vg in oocytes. The evidence is the slight increase in weight of eggs oviposited by insulin-injected insects (table 2).

Our study showed that application of bovine insulin improved protein digestive capacity in *C. septempunctata*. (fig. 4). In fact, insulin signaling pathway has been implicated in glycogen metabolism (Wu & Brown, 2006). In *B. mori* larvae, injection of ILP raised trehalase and glycogen phosphorylase

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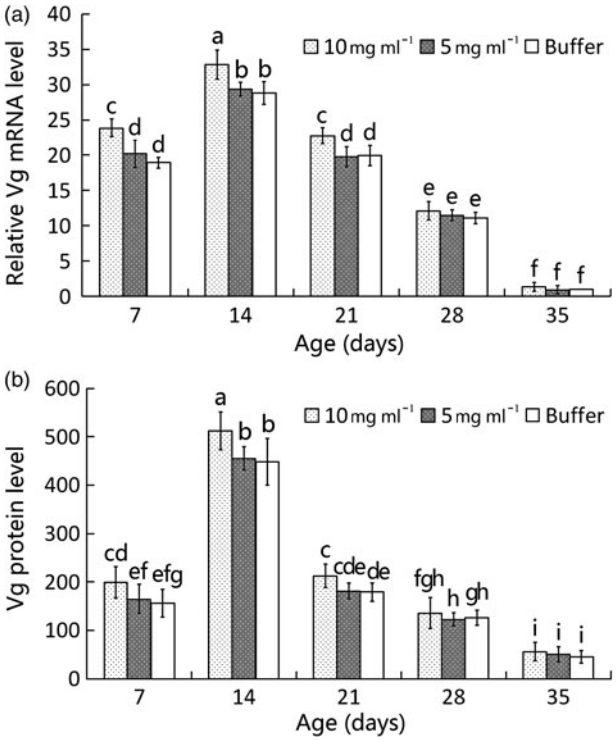

Fig. 3. *C. septempunctata* Vg expression after application of bovine insulin. *C. septempunctata* Vg mRNA level was monitored at different adult stages by qRT-PCR. On day 7, 14 and 21 after emergence, insulin (10 mg ml^{-1}) injected insects accumulated significantly more Vg transcripts than buffer treated control. The day 35 Vg mRNA level in buffer treated female are expressed as 100% (a). *C. septempunctata* Vg protein exhibited a similar curve with Vg mRNA as detected by ELISA at different adult stages. On day 7 and 14 after emergence, insulin (10 mg ml^{-1}) injected insects synthesized more Vg protein than buffer treated ones (b). Mean ± SE are presented. Different letters above the bars indicate a significant difference (Least Significant Difference test, $P < 0.05$).

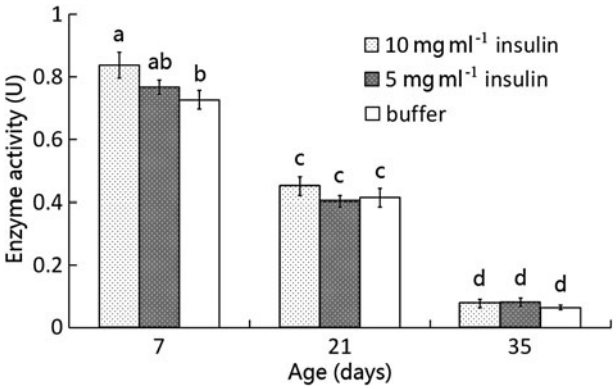

Fig. 4. General protease activity after application of bovine insulin. General protease activity was monitored by using casein as substrate. Mean ± SE of each bar followed by the same letter are not statistically different (Least Significant Difference test, $P < 0.05$).

activity (Satake *et al.*, 1997, 1999). As Vg is a major protein in insect eggs (Tufail & Takeda, 2008), and what is more, promotion of Vg synthesis means increase of protein requirement, we inferred that application of insulin indirectly enhanced protease activity in *C. septempunctata*. Toward this end, the total protease activity after hormone application was monitored and the hypothesis was verified. In gut of neuropteran insects, serine protease is dominant among digestion enzymes (Mochizuki, 1998; Mulligan *et al.*, 2010). In addition, trypsin and chymotrypsin-like proteases are present in adult *C. septempunctata* (Liu *et al.*, 2013). These reports led us to presume that prevailing of these proteases in gut resulted in elevation of the general protease activity in *C. septempunctata*.

To date, vertebrate insulin, like bovine insulin, was widely used to investigate the physiological functions of ILPs and the related signaling pathway in insects (Tatar *et al.*, 2001; Sheng *et al.*, 2011). This is not only because insulin structure and function are conserved in different species, but also what we know about insect ILPs is scarce. However, conservation is a relative concept and prominent differences do exist in primary structure between insect ILPs and mammalian insulins (Brogiolo *et al.*, 2001; Riehle *et al.*, 2006; Wu & Brown, 2006). More importantly, insect ILPs participate in more physiological processes and act through different ways compared with mammalian insulins. Now, ILPs have been only characterized in minority species from several insect orders (Wu & Brown, 2006; Xu *et al.*, 2015). The investigation should be spread to representatives of other insect orders, such as Neuroptera, which contains important predatory species. In present study, application of bovine insulin significantly promoted oviposition of the green lacewing, *C. septempunctata*, implicating a new function of ILPs in reproductive physiology. As *C. septempunctata* is a famous predator with great potential of commercial use, it is very urgent to identify its ILPs and to demonstrate the respective functions. Understanding *C. septempunctata* ILPs may contribute to development of artificial approaches for reproductive regulation and to improvement in rearing techniques of this natural predator.

The significant rise of fecundity after hormone application indicated that bovine insulin could be used a reproduction stimulant. As 5 mg ml^{-1} insulin did not induce significant difference in all of the reproductive parameters compared to HEPES buffer, 10 mg ml^{-1} should be a threshold concentration to improve lacewing fecundity. Because green lacewing usually shrinks egg production on week 3 post eclosion, a second injection was performed 15 days post the first injection. Disappointedly, the second injection did not increase egg amount in the rest of oviposition period. We conclude that excessive dose of hormone could not promote vitellogenesis further. A more promising promotion in fecundity will be expectable if *C. septempunctata* ILPs, rather than bovine insulin is applied. This is because ILPs usually have much higher efficiency than bovine insulin. For instance, ILP3 in *Aedes aegypti* stimulated yolk uptake by oocytes at much lower concentrations than bovine insulin (Brown *et al.*, 2008).

Presently, there are mainly three methods to deliver hormone into insects. Injection has been proved feasible in several insects, including *T. castaneum* and *A. aegypti* (Brown *et al.*, 2008; Sheng *et al.*, 2011). Fumigation is also a good method and very fit for JH and its volatile analog methoprene (Tatar *et al.*, 2001). Certainly, feeding was also much used and has been confirmed feasible at least for hydroprene (Sheng *et al.*, 2011). Aside from injection, we have tried another method in present study, spraying insulin liquid onto lacewing surface.

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Regretfully, no significant increase in fecundity was observed compared with spraying of buffer. Maybe this is because the insulin protein molecule cannot penetrate insect cuticle to play its roles. Frankly, separate injection is not applicable in mass rearing of natural enemies. As insulin is a kind of stable molecule, feeding might be a solution if artificial diet is available for the green lacewing.

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