Reproductive pattern in the solanum mealybug, *Phenacoccus solani*: A new perspective

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Background. The reproductive pattern of most scale insects is ovoviviparity. The solanum mealybug, *Phenacoccus solani* (Hemiptera: Pseudococcidae), is known as a thelytokous parthenogenetic species, but there is still debate about the reproductive strategies of this species.

Methods. Here, we investigated the oviposition characteristics of *P. solani* and used scanning/transmission electron microscopy and RNA-seq to identify the differences between two types of eggs.

Results. We found that *P. solani* laid two types of eggs in one batch, with no significant difference in apparent size: one with eyespots that hatched and another without eyespots that failed to hatch. Furthermore, the physiological and molecular differences between the two types of eggs were highly significant. KEGG enrichment analysis revealed significant enrichment for the JAK-STAT, Notch, Hippo, and Wnt signaling pathways and dorsoventral axis formation, wax biosynthesis, cell cycle, insulin secretion, and nitrogen metabolism pathways. The results suggest that the embryo of the egg undergoes development inside the mother and only a short molting period outside the mother.

Discussion. There is a continuum between full viviparity and full oviparity, and ovoviviparity has been used to variably describe any number of states along the continuum between these two extremes. Therefore, we suggest that the reproductive pattern of *P. solani* can be described as ovoviviparity.

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Abstract

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Subjects Agricultural Science, Entomology, Insect Bioecology

Keywords mealybug, reproductive pattern, oviposition, egg, ovoviviparity, *Phenacoccus solani*

Introduction

The reproductive strategies of most insects have been described with three main patterns: oviparity, viviparity and ovoviviparity (*Meier et al., 1999*; *Wheeler, 2003*; *Gullan & Granston, 2014*). In oviparous species, egg development occurs in the external environment after oviposition, and hatching occurs outside the mother's body, whereas in viviparous species (which are relatively rare among insects), egg development occurs inside the mother's body, which provides gas exchange and, more importantly, nourishment for the embryos which are born alive (*Andrews & Rose, 1994*; *Tworzydlo et al., 2013*). Based on nutritional relationships between maternal and embryonic organisms, two modes of viviparity are recognized: matrotrophic and lecidotrophic (*Ostrovsky et al., 2016*). Ovoviviparity is in fact a specific type of viviparity where developing eggs are retained within the body of the mother, and the offspring are nourished by the reserve materials accumulated in the eggs during oogenesis (*Blackburn, 1999*; *Gaino & Rebora, 2005*; *Lodé, 2012*). Therefore, the recent view is that there are truly only two main reproductive strategies: viviparity and oviparity (*Ostrovsky, 2013; Ostrovsky et al., 2016*). In previous studies, some insects, such as cockroaches (*Warnecke & Hintze-Podufal, 1996*), aphids (*Ortiz-Rivas et al., 2004*), tsetse flies (*Meier et al., 1999*), thrips (*Kranz et al., 2002*), and scale insects (*Gavrilov & Kuznetsova 2007*; *Ngernsiri et al., 2015*), were described as ovoviviparous species. Scale insects (Hemiptera: Sternorrhyncha: Coccoidea), like many Hemiptera, feed on sap drawn directly from the plant vascular system and secrete a waxy coating for defense; in addition, many scale insect species are major quarantine pests of agricultural or ornamental plants in tropical/subtropical climates as well as in greenhouses in temperate zones worldwide (*Gullan & Kosztarab 1997*; *Gavrilov-Zimin, 2018*). All previous relevant studies suggest that the phenomenon of ovoviviparity is widely distributed among scale insects (*Tremblay, 1997*; *Gavrilov & Kuznetsova, 2007*). Gavrilov-Zimin (*2018*) provided an overview of the distribution of different variants of ovoviviparity/viviparity among scale insect families and demonstrated that the evolution of scale insects shows multiple cyclic conversions of the oviparous reproduction pattern to ovoviviparity/viviparity, with the appearance of new and interesting adaptations for egg protection. In other words, the reproductive modes of scale insects may be rich and

variable; however, the understanding of the course of evolution of reproductive patterns in these insects is not complete (*Gavrilov & Kuznetsova, 2007*). Therefore, the identification of reproductive patterns has great heuristic value in terms of both reproductive and evolutionary biology.

The solanum mealybug, *Phenacoccus solani* (Hemiptera: Pseudococcidae), is native to North America (*Chatzidimitriou et al., 2016*) and is a newly recorded species in China. Furthermore, this mealybug species is quite polyphagous and considered to be a major threat to agricultural production and the ecological environment, causing significant problems (*Ben-Dov, 2005a*; *Zhi et al., 2018*). *P. solani* is a thelytokous parthenogenetic species, and no male individuals are found in its populations (*Lloyd, 1952*; *Ben-Dov, 2005b*; *Zhi et al., 2018*). Regarding the birth strategies of *P. solani*, McKenzie reported that this species was viviparous (*McKenzie, 1967*); however, Kosztarab (*1996*) and Chatzidimitriou *et al*. (*2016*) considered this species to be ovoviviparous. Vennila *et al*. (*2010*) found that in another species of mealybug in the same genus, parthenogenesis via ovoviviparity (96.5%) was dominant over oviparity (3.5%). Many scholars have found that the hatching period of eggs laid by mealybugs is very short and that the hatching process is relatively concealed (beneath the abdomen), as a result, short and concealed hatching has been suggested as the main reason for the divergence of reproductive modes in mealybugs (*Tremblay, 1997*; *Lagowska & Golan, 2009*; *Vennila et al., 2010*; *Zhi et al., 2018*). According to our previous observations, we believe that the reproductive mode in mealybugs, at least in *P. solani,* is complex and not simple to define.

Here, we investigated the oviposition characteristics of *P. solani* and used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to distinguish between two types of eggs (i.e., reproductive products putatively considered to be eggs) laid in one batch. Finally, we further investigated miRNA and mRNA expression in the two types of eggs. We aimed to answer the following questions: 1) What life stage is *P. solani* laying – an egg, a nymph, or something in between? 2) If different reproductive "products" coexist, do they differ in appearance, physiology and molecular biology? 3) According to the above findings, what is the reproductive mode of *P. solani*?

126 ## Materials & Methods

127 ### Oviposition characteristics

128 The oviposition process of female adults of *P. solani* was observed on two transparent,
129 single-well, concave slides (Fig. 1) (length * width = 76.2 mm * 25.4 mm; the thickness
130 of each concave slide was 1.3 mm; the diameter and depth of the circular hole were 15
131 mm and 0.5 mm, respectively). The steps of the procedure were as follows: 1) a
132 concave slide was placed face up on a flat table and a female adult was gently placed
133 into the middle of the hole of the concave slide with a brush; 2) quickly another concave
134 slide was placed face down, on top of the bottom concave slide; and 3) finally, rubber
135 bands were used to bind the ends of the two concave slides to secure them. Preliminary
136 experiments showed that the thickness of female adults was 0.91 ± 0.03 mm, so the
137 above observation method did not harm the bodies of female adults. The egg hatching
138 process was further tracked and observed under a NikonSMZ1500 zoom
139 stereomicroscope (Nikon, Japan), and photographs were taken every 5 minutes from
140 the start of the egg laying process.

141 ### Egg morphology

142 The number, length, width and hatching rate of eggs with eyespots (hereafter eggs with-
143 ES) or eggs without eyespots (hereafter eggs without-ES) were further observed in the
144 same batch of eggs under a NikonSMZ1500 zoom stereomicroscope. There were two
145 kinds of treatments: one employed the concave slide method (placing a female adult
146 between two concave slides as mentioned above), and the other employed the blade
147 method, i.e., placing a female adult on detached potato leaves, with the petiole
148 wrapped with defatted cotton to maintain leaf freshness and the placing the whole
149 treated leaves in Petri dishes (diameter = 9 cm, thickness = 1.4 cm). The oviposition of
150 female adults was observed every 30 minutes from 9.00 a.m. to 4.00 p.m, and the
151 numbers of the two kinds of eggs were counted. Each female adult was biometrically
152 tested once, and each treatment was repeated 15 times. A total of 25 eggs were
153 randomly selected from the two kinds of eggs, and their lengths and widths were
154 measured. After 72 hours, the hatching of the two kinds of eggs was observed. Eggs
155 with-ES hatched 114 individuals, while eggs without-ES hatched 45 individuals. The

156 experiment was carried out in an artificial climate chamber with a temperature of 27 ±
157 1°C, a humidity of 70% ± 5% and a photoperiod of 16 L: 8D.
158 **Does the mother's body affect the hatching of eggs?**
159 Two treatments were established: 1) eggs with-ES were incubated under the mother's
160 body (Fig. 2A), and 2) eggs with-ES were artificially removed from the mother and kept
161 on the concave slide (Fig. 2C). Then, the female adults were continuously observed
162 through a NikonSMZ1500 zoom stereomicroscope every five minutes. Continuous
163 stretching of the abdomen by female adults indicated that they were about to lay eggs.
164 For treatment 2, the upper concave slide was removed immediately, and the mother
165 was carefully removed with an insect pin. Hatching time was recorded as the time from
166 when the female adult laid eggs to the time when the eggshell was completely
167 detached. Each egg was bioassayed once; experiment 1 included 30 replicates, and
168 experiment 2 included 47 replicates.
169 **Microscopic differences between the two kinds of eggs**
170 **1 Egg surface and internal structure**
171 The surface and internal structure of the two kinds of eggs were observed by SEM
172 (SU8010, Hitachi, Japan) and TEM (H7650, Hitachi, Japan). The collected eggs with
173 and without ES were pretreated with liquid nitrogen immediately. The follow-up
174 procedures followed the instrument operation methods for the scanning electron
175 microscope. TEM was performed as follows: 1) fixation, eggs were immersed in 2.5%
176 glutaraldehyde fixative solution and then rinsed with buffer solution; 2) gradient
177 dehydration, the fixed samples were dehydrated with an ethanol; 3) gradient osmosis,
178 the samples were permeated with a mixture of acetone and Spurr resin penetrant (1:1);
179 4) embedding and polymerization, 100% Spurr embedding agent was added, followed
180 by polymerization for 24 hours; 5) ultrathin sectioning, the samples were cut to
181 approximately 90 nm; and 6) observation and photography.
182 **2 RNA sequencing and data analysis**
183 **RNA extraction, library construction and RNA sequencing**
184 Samples of newly laid eggs (with-ES or without-ES) were collected and immediately
185 placed in a 0.5 MIEP tube and then frozen in liquid nitrogen for RNA extraction. Total
186 RNA was extracted from 6 samples, and a library was constructed as previously

described (*Yin et al., 2018*). The libraries were sequenced on the Illumina HiSeq X Ten platform, and 150 bp paired-end reads were generated. Raw data (raw reads) in fastq format were first processed using Trimmomatic (*Bolger et al., 2014*). Reads containing poly-N stretches and reads oflow quality were removed to obtain clean reads. After removing adaptor and low-quality sequences, the clean reads were assembled into expressed sequence tag clusters (contigs) and de novo assembled into transcripts by Trinity (*Grabherr et al., 2011*) (version: 2.4) with the paired-end method. The longest transcript was chosen as a unigene based on similarity and length for subsequent analyses. Raw data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA554708.

Unigene de novo assembly, functional annotation and data analysis

Transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). The functions of the unigenes were annotated by alignment of the unigenes with the NCBI nonredundant (NR), SwissProt, EuKaryotic Orthologous Groups (KOG), Gene Ontology (GO) and Kyoto-Encyclopedia of Genes and Genomes (KEGG) databases. Differentially expressed unigenes (DEGs) were identified using the DESeq (*Anders & Huber, 2013*) functions “estimate size factors” and “nbinom test”. A p value < 0.05 and fold change > 2 or < 0.5 were set as the thresholds for significant differential expression. Hierarchical cluster analysis of DEGs was performed to explore transcript expression patterns, and KEGG pathway enrichment analysis of DEGs was performed using R based on a hypergeometric distribution.

Results

Oviposition characteristics

Female adults of *P. solani* produced eggs in one generation through thelytokous parthenogenesis, and the eggs were long and oval, similar to those described by Zhi *et al*. (*2018*). Female adults secreted silken wax, but it never formed an ovisac. After egg laying, the eggshell was detached from the nymph, followed by the appearance of 1st-instar nymphs that quickly crawled away from the lower part of the mother. Moreover, in a batch of eggs laid by female adults of *P. solani*, two kinds of eggs with distinct

morphological differences appeared (Fig. 3A/B): two reddish-brown eyespots were seen on one type of egg (eggs with-ES; Fig. 3D), while the other type did not display these eyespots (eggs without-ES; Fig. 3C). The hatching process of eggs with-ES was as follows: 1) in the first 5 minutes, the eggs began to show considerable peristalsis beneath the mother, and at 10-15 minutes, the eggshell was gradually detached, and wax powder appeared on the surface of the body; and 2) at approximately 20 minutes, antennae and feet began to appear, and the hatching process was generally completed within 25 minutes, while the detached eggshell could be seen at the abdominal end of the 1st instar nymph (Fig. 4).

Egg morphology

In the present study, we observed that female adults could lay two types of eggs, those with-ES and those without-ES, in either treatment. The ratio of eggs with-ES was significantly larger than that of eggs without-ES (on concave slides, 91.56 ± 2.14 vs. 8.44 ± 2.14, respectively, $\chi 2 = 86.01$, n = 15, $p < 0.001$; on leaves, 87.31 ± 2.90 vs. 12.69 ± 2.90, respectively, $\chi 2 = 13.07$, n = 15, $p < 0.001$) (Fig. 5A). The eggs were long and elliptical, with lengths of 0.320 ± 0.006 mm (with-ES) and 0.305 ± 0.008 mm (without-ES) (t = 1.42, n = 25; Fig. 5C) and widths of 0.146 ± 0.002 mm (with-ES) and 0.147 ± 0.003 mm (without-ES) (t = 0.24, n = 25; Fig. 5D). Furthermore, the hatching rate of eggs with-ES was 100%, and no eggs without-ES hatched (Fig. 5B).

Does the mother's body affect the hatching of eggs?

To investigate the extent to which the mother's body affected the hatching of eggs, eggs without-ES were removed from the area beneath the mother's body after they were laid. These eggs did not hatch, just as a chick cannot hatch without a hen. Therefore, we further tested whether eggs with-ES hatched after they were removed from the area beneath the mother's body. We found that eggs with-ES could hatch normally under any treatment (Fig. 2 A/B/C), but the hatching times were different. Inside the mother's body, the hatching time was 24.30 ± 0.60 minutes (Fig. 2D), but when the mother's body was removed, the hatching time was reduced by nearly 5 minutes to 19.47 ± 0.45 minutes (Fig. 2E). Therefore, we suggest that the mother's body has no effect on the success of egg hatching, or it could be inferred that the failure of eggs without-ES to hatch was largely due to internal factors. Many species of scale insects secrete abundant wax and

form an ovisac that covers eggs and prevents their adhesion, but in some species, ovisacs are not built, and the time of egg development outside of the maternal body is decreased (*Gavrilov-Zimin, 2018*). *P. solani* belongs to the latter group, secreting a small amount of wax and never forming an ovisac. Thus, it is not surprising that the eggs of *P. solani* hatched so quickly, especially when the eggs were isolated.

Microscopic differences between the two types of eggs

1 Egg surface and internal structure

The surface and internal structure of eggs with and without-ES were observed using SEM and TEM. The contour of appendages could be clearly seen across the eggshell, and the bristles, tubular glands and six conical receptors (which were symmetric, with three on each side) could be seen on the surfaces of eggs with-ES (Fig. 6A); moreover, complete blood cells, cytoplasm, mitochondria and myocutaneous filaments were observed inside (Fig. 6B). Eggs without-ES had a smooth surface (Fig. 6C) and contained only lipid droplets, endoplasmic reticulum and free ribosomes (Fig. 6D).

2 RNA sequencing and data analysis

Illumina sequencing generated approximately 45 M reads per sample after the removal of low-quality reads. These reads were assembled randomly and produced 55,558 unigenes with an N50 of 1,026 nt. After annotating unigenes with several databases and calculating the expression of unigenes as fragments per kilobase of exon model per million reads mapped (FPKM), correlation coefficients between samples were calculated and used to estimate biological repeatability and differences between groups. The correlation coefficient of 3 biological replicates in the group with-ES and the group without-ES was > 0.8, and the sample correlation coefficient between these two groups was only 0.4, showing an obvious difference between these two groups (Fig. 7A). DEGs were identified and screened with the thresholds of $p < 0.05$ and fold change > 2 (or fold change < 0.5) (Fig. 7B/C). There were 13,164 DEGs between the with-ES and without-ES groups, including 9,243 up regulated DEGs and 3,921 down regulated DEGs. The DEGs are shown with a volcano plot, and a heat map was generated from hierarchical cluster analysis to show the expression patterns of the DEGs (Fig. 7B). KEGG enrichment analysis of the DEGs was performed to determine the main pathways associated with these DEGs. We found that the JAK-STAT, Notch, Hippo, and Wnt

signaling pathways and dorso-ventral axis formation, wax biosynthesis, progesterone-mediated oocyte maturation, cell cycle, eukaryote ribosome biogenesis, insulin secretion, and nitrogen metabolism pathways were significantly enriched (Fig. 8).

Discussion

Oviposition and hatching characteristics

In the present study, we found eggs laid by parthenogenetic *P. solani*, but soon emerged 1st-instar nymphs following hatch. Because this hatching process is fast (< 30 minutes), researchers might think that 1st-instar nymphs are produced by *P. solani* female adults. A similar phenomenon was reported in another invasive mealybug, *Phenacoccus solenopsis*, and because both eggs and 1st-instar nymphs were found in oocysts, Vennila *et al*. (*2010*) considered the scale insect to be able to reproduce in both ways, i.e., via ovoviviparity and oviparity. In *Coccus hesperidum*, naked nymphs appeared from the vulvar orifice, but thin eggshells were shown to remain in the female reproductive tract (*Hagan, 1951*), and in *P. solani*, the eggshells were kept beneath the mother.

In *Matsucoccus matsumurae*, there are two types of eggs, those with and without eyespots, but the eggs with eyespots are similar to those without eyespots, and the egg types show different developmental periods with normal hatching (*Xie et al., 2014*). However, *P. solani* laid two types of eggs in one batch, with no significant difference in apparent size: one with eyespots that hatched and another without eyespots that failed to hatch. Generally, eggs are under stress from external factors, which may prevent them from hatching properly. For example, some of the eggs laid by heat-treated females of *Nilaparvata lugens*were unable to hatch due to failure during blastokinesis (*Lee & Roger, 1987*). Further research revealed that yeast-like symbiotes in *N. lugens* play an important role in the embryonic and postembryonic development of eggs, especially the ventral differentiation of the embryo (*Wilkinson & Ishikawa, 2001*; *Nan et al., 2016*). The symbiotic bacteria in the bodies of most mealybug subfamily insects are *Tremblaya princeps* (*Gruwell et al., 2010*), play a substantive role in the host plant specificity of their hosts (*Baumann, 2005; Hansen & Moran, 2014*), and are correlated with host development (*Huang et al., 2015*); therefore, we suggest that the absence of

311 symbiotes might explain the presence of nondeveloping eggs. Another type of insect
312 egg that does not hatch is the nutritive egg common in social insects, such as ants. The
313 nutritive eggs of ants are unfertilized eggs that cannot hatch and are eaten in colonies
314 containing a queen (*Heinze et al., 1996, 1999*). However, for this parthenogenetic and
315 thelyotokous species of mealybug, the factors causing *P. solani* to lay eggs that cannot
316 hatch require further study.
317 **Physiological and molecular differences between the two types of eggs**
318 According to these observations of egg surface and internal structure, we suggest that
319 eggs with-ES are alive, with features such as conical receptors, blood cells and
320 myocutaneous filaments, and that they still have an eggshell and are close to hatching,
321 even though they no longer resemble an egg on a microscopic level. Although some
322 important organelles such as endoplasmic reticulum and free ribosomes were present in
323 the eggs without-ES, mitochondria had never been found. Mitochondria are a special
324 organelle that contain their own genomes and plays an important role in oocyte
325 maturation and embryo development (*Ferenz, 1993; Lieber et al., 2019*). Moreover,
326 mitochondria are inherited only in the maternal line, i.e., mitrochondrial DNA is passed
327 only through the mother's egg cell (*Ma et al., 2014; Lieber et al., 2019*). Lack of
328 mitochondrial redistribution in cytoplasm was a sign of immature oocyte and was closely
329 related to low developmental of eggs (*Bavister & Squirrell, 2000*). If mitochondria were
330 missing, it could be fatal to the development of egg cell and later embryonic
331 development. Therefore, we hypothesized that the development of egg cell might be
332 arrested for the eggs without-ES in the absence of mitochondria.
333 We further determined the differences between eggs with and without ES at the
334 molecular level. The result revealed that the differences in terms of unigene expression
335 between two types of eggs were highly significant, and we also found that the JAK-
336 STAT, Notch, Hippo, and Wnt signaling pathways and some important pathways related
337 to metabolism and nutrition were significantly enriched. Recently, the JAK-STAT, Notch,
338 Hippo and Wnt signaling pathways were found to independently or interactively
339 participate in the regulation of egg production (*Hombria & Brown, 2002*; *McGregor et al.,*
340 *2002*). For example, mutual antagonism between the Notch and JAK/STAT signaling
341 pathways provides a crucial facet of follicle cell patterning and ultimately helps establish

the polarity of the egg chamber (*Assa-Kunik et al., 2007*), and the Hippo pathway controls polar cell specification by repressing Notch activity (*Chen et al., 2011*). Moreover, some of these important signaling pathways are involved in aspects of cell development and metabolic function, such as dorsal-ventral axis formation, wax biosynthesis, insulin secretion and nitrogen metabolism.

Conclusions

Although the reproductive mode of *P. solani* has been described previously, there is still no clear agreement on its definition. We found no differences between the two types of eggs by visual observation, but the physiological and molecular differences were highly significant. The results suggest that the embryonic development of eggs with-ES is complete when the eggs are laid beneath the abdomen, i.e., the embryo of the egg develops inside the mother. However, the embryonic development of eggs without-ES seems to be incomplete. Although there are no direct data on the entire process of embryonic development, we can at least be sure that the cell development and physiological metabolism of eggs without-ES are hindered or arrested.. Moreover, we found that eggs with-ES begin to hatch and shed their eggshell (immediately) after leaving the mother's body: i.e., this species lays eggs and does not experience live birth. Ovoviviparous species oviposit eggs at an advanced stage of embryological development, and the larva exits the eggshell during or immediately following oviposition (*Meier et al., 1999*). We determined that there is a continuum between full viviparity (live birth) and full oviparity (eggs laid immediately following fertilization), and ovoviviparity can be used to variably describe any number of states along the continuum between these two extremes. Therefore, we suggest that the reproductive pattern of *P. solani* can be described as ovoviviparity.

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Figure 1

The device used for the observation of egg-laying and egg hatching of adult females of *Phenacoccus solani* (Double-concavity slide method).

(A) Position and operation diagram. Two concave slides overlapped seamlessly, and adult female was placed into the middle of the hole of the concave slide; concave slides were fixed with rubber bands at both ends. (B) The final observation device contained two separate adult females. (C) Multiple devices together.

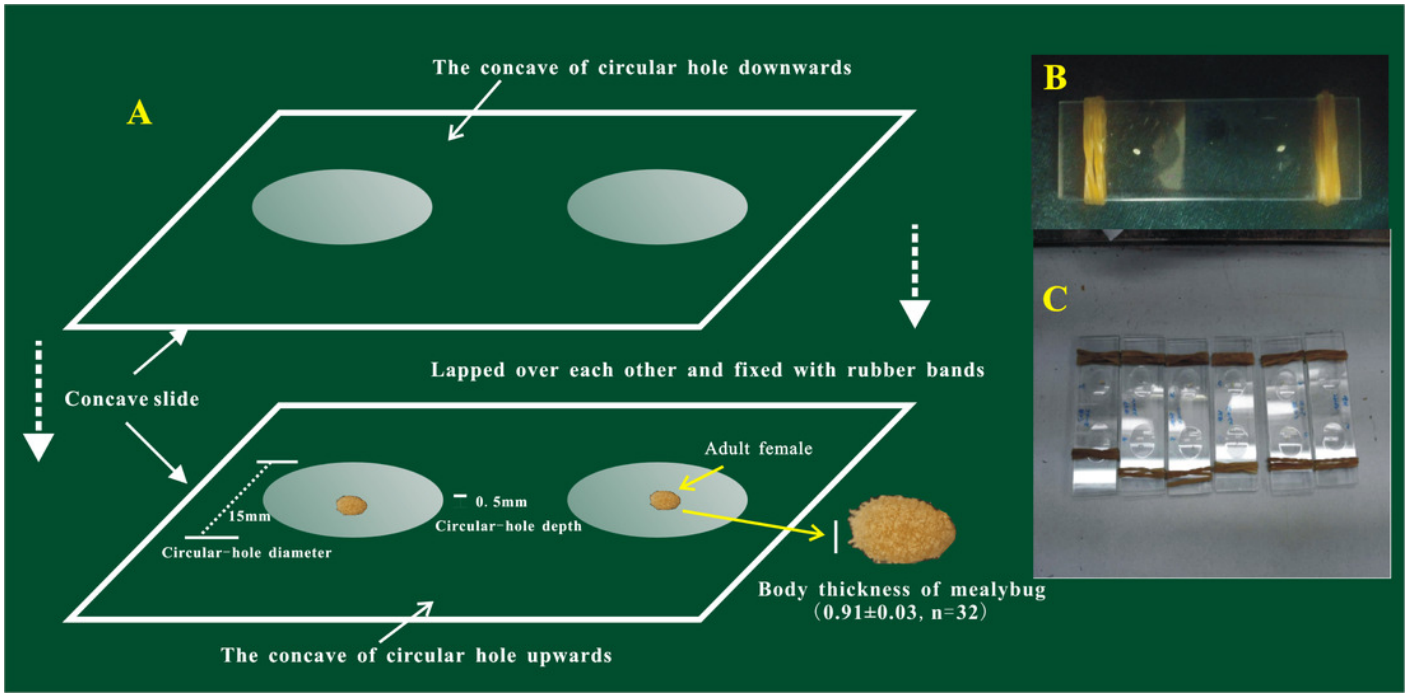

Figure 2

The hatching time of eggs beneath the abdomen of adult female or in isolation

(A) Egg hatching beneath the abdomen of adult female, at the point at which the eggshell detached; (B) Eggs with eyespots; (C) Egg hatching in isolation (without mother's body); (D) Hatching time of eggs beneath the abdomen of adult female; (E) Hatching time of eggs without the mother's body or in isolation.

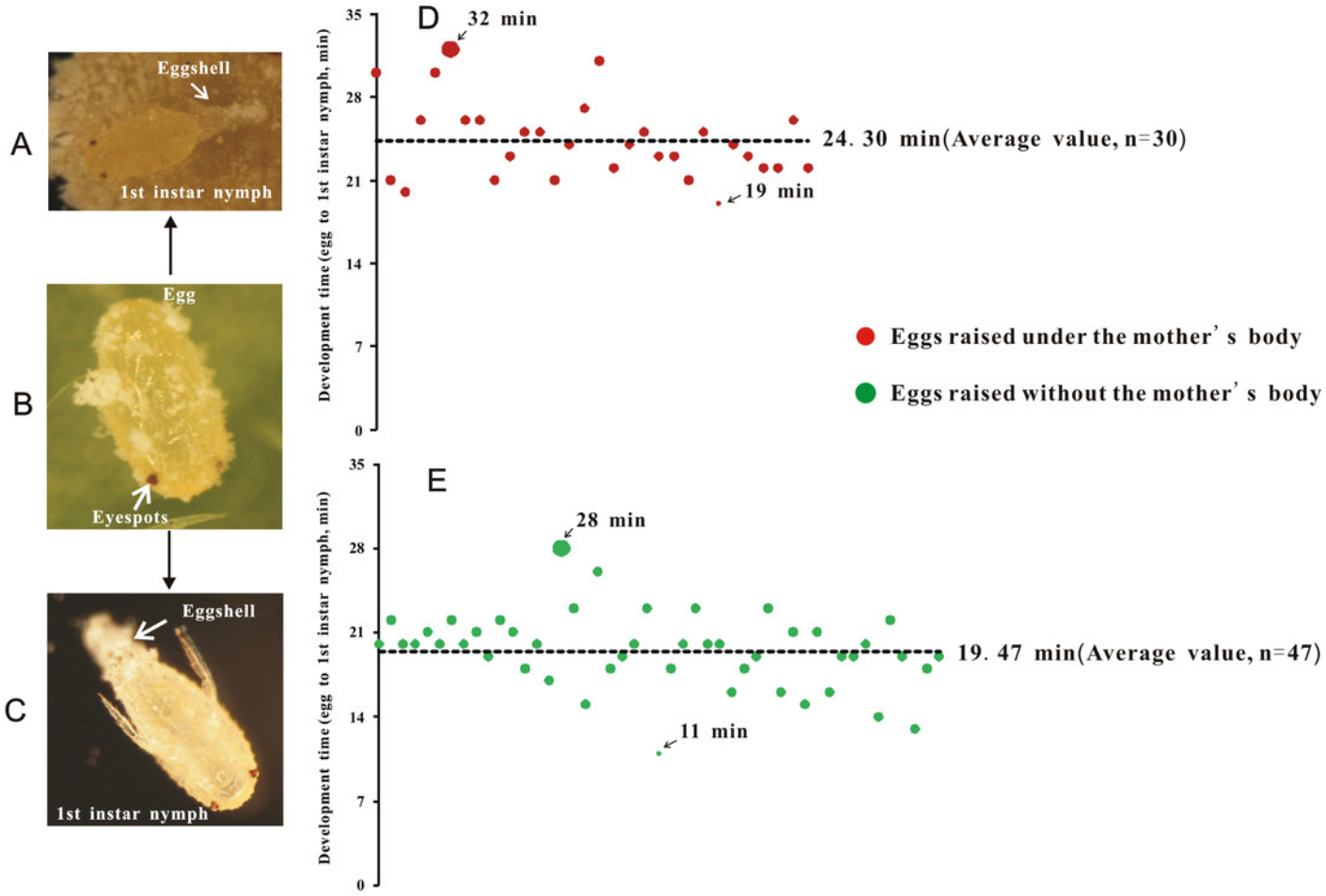

Figure 3

Adult female of *Phenacoccus solani* laid two types of eggs in one batch, one with eyespots (type 1), and another without eyespots (type 2)

(A) Adult female laying eggs, and some eggs have rapidly hatched into 1st instar nymphs; (B) Newly laid two types of eggs below the abdomen of adult female; (C) The eyespots of eggs (type 2) were not visible; (D) Eggs (type 1) before hatching, the eyespots were clearly visible.

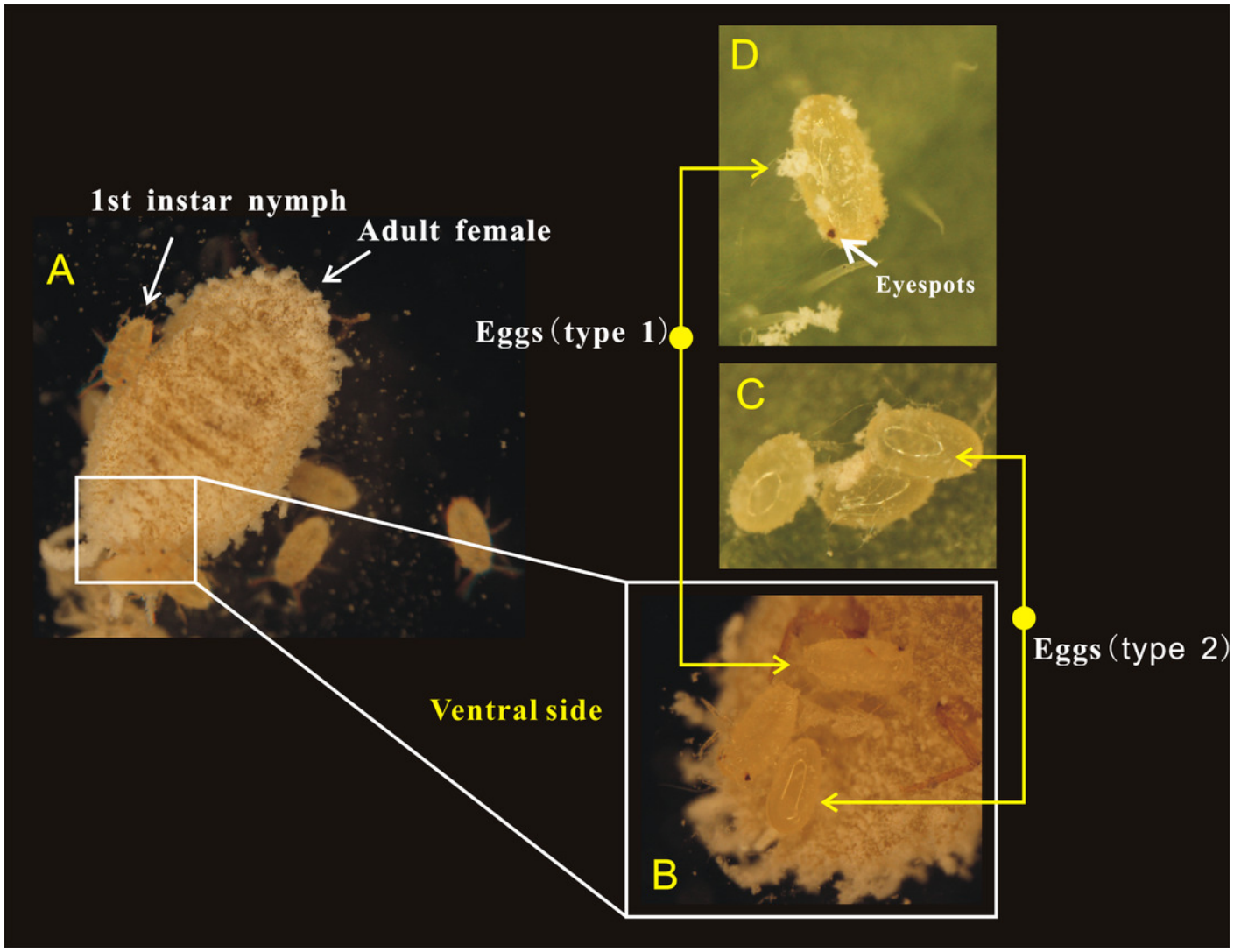

Figure 4

The hatching process of eggs

I) After the first 5 minutes, eggs began to show considerable peristalsis beneath the mother; Ⅱ/Ⅲ) At 10-15 minutes, the eggshell was gradually detached and wax powder appeared on the surface of body; Ⅳ) At 20 minutes, antennae and feet were starting to become visible; Ⅴ/Ⅵ) The hatching was basically completed, while the detached eggshell could be seen at the abdominal end of the 1st instar nymph.

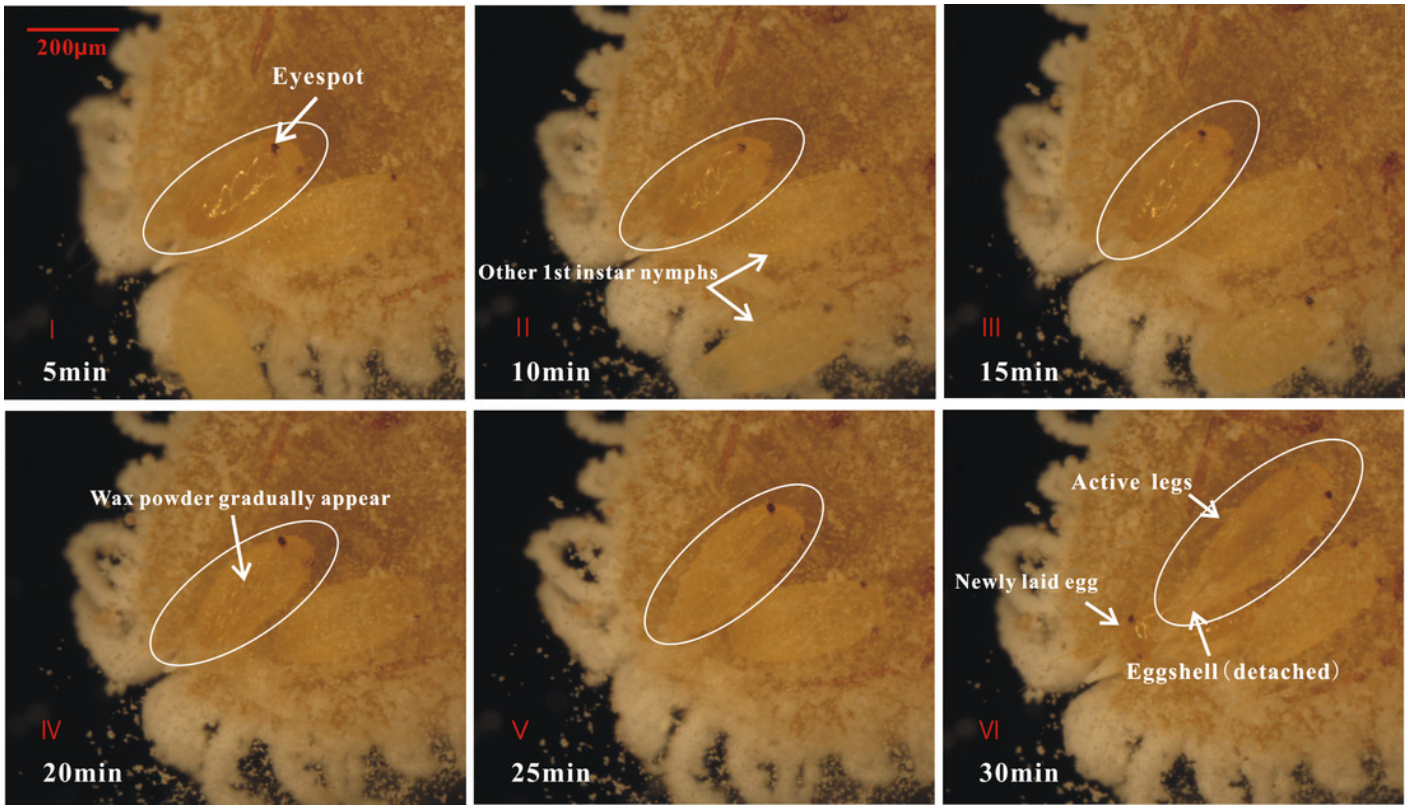

Figure 5

The percentage, morphology and hatching of the two types of eggs with different treatments

Percentage of the total for the two types of eggs with the treatment of placing female adults on leaves or concave slide (A), and hatching rate of eggs (B); the difference between the two types of eggs with same treatment was analyze d using Chi-SquareTest. The length (C) and width (D) of two types of eggs; the difference between the two types of eggs was analyze d using T-Test, and "*NS*" on the two bars indicate not significantly different from each other ($p > 0.05$).

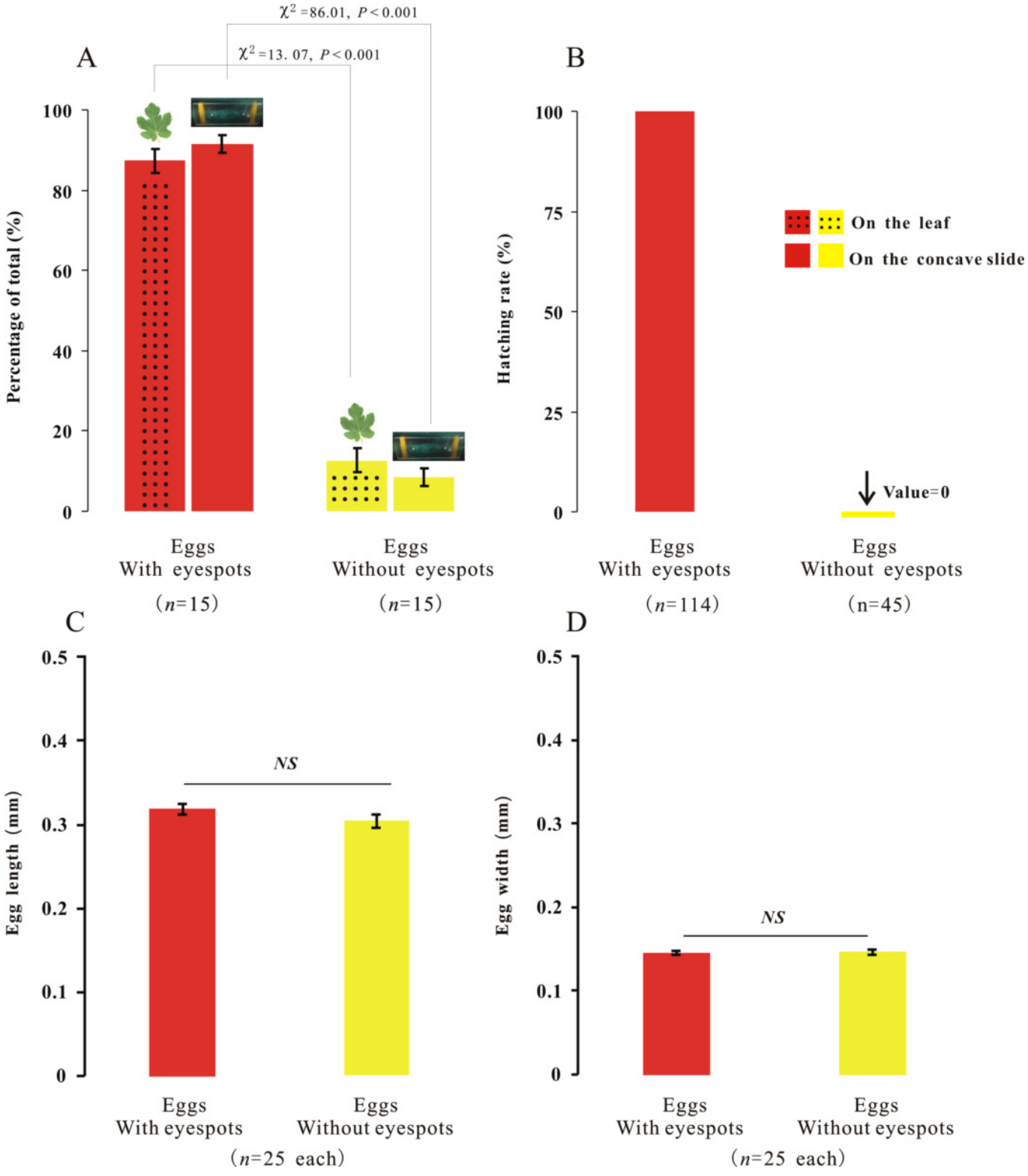
A
χ^2 =86.01, P < 0.001
χ^2 =13. 07, P < 0.001
Percentage of total (%)
100
80
60
40
20
0
Eggs
With eyespots
(n=15)
Eggs
Without eyespots
(n=15)
B
Hatching rate (%)
100
75
50
25
0
On the leaf
On the concave slide
Value=0
Eggs
With eyespots
(n=114)
Eggs
Without eyespots
(n=45)
C
Egg length (mm)
0.5
0.4
0.3
0.2
0.1
0
NS
Eggs
With eyespots
Eggs
Without eyespots
(n=25 each)
D
Egg width (mm)
0.5
0.4
0.3
0.2
0.1
0
NS
Eggs
With eyespots
Eggs
Without eyespots
(n=25 each)

Figure 6

The surface and internal structure of eggs with eyespots and without eyespots

The surface of eggs with eyespots (A) and without eyespots (C) was observed by scanning electron microscope, and the internal structure of eggs with eyespots (B) and without eyespots (D) was observed by transmission electron microscope.

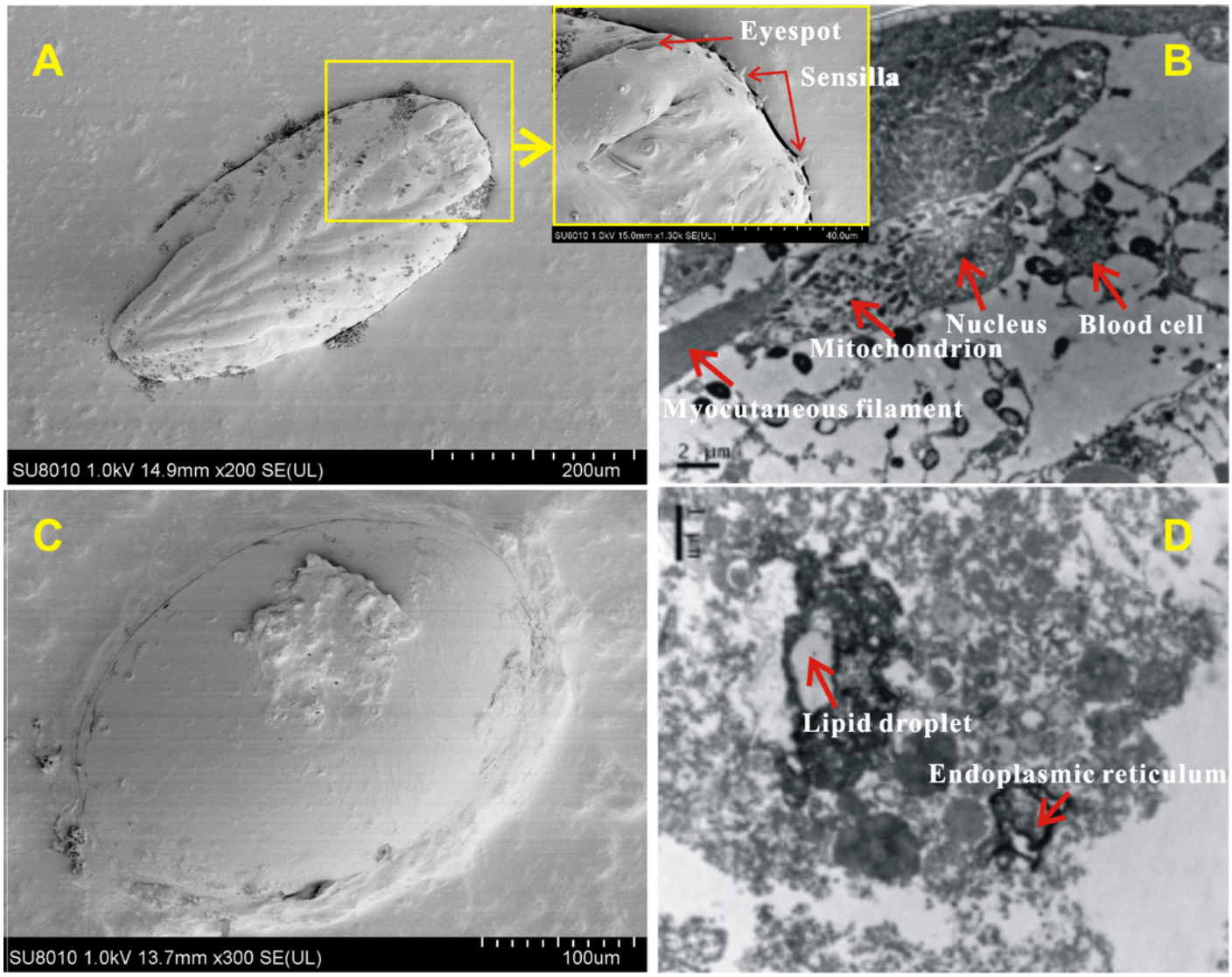

Figure 7

RNA-seq to distinguish the differences between two types of eggs.

Heat-map coefficient matrix (A); the abscissa indicates the sample name, the ordinate indicates the corresponding sample name, and the color indicates the correlation coefficient. The closer the correlation coefficient is to 1, the higher the similarity in expression patterns between samples. Volcano plots of different differentially expressed unigenes (DEGs) between two groups. Green dots indicate down-regulated unigenes, red dots indicate upregulated unigenes, and grey dots indicate no differential unigenes (B). Cluster analysis of DEG levels. Expression differences are shown in different colors. Red and blue indicate up-regulation and down-regulation, respectively (C).

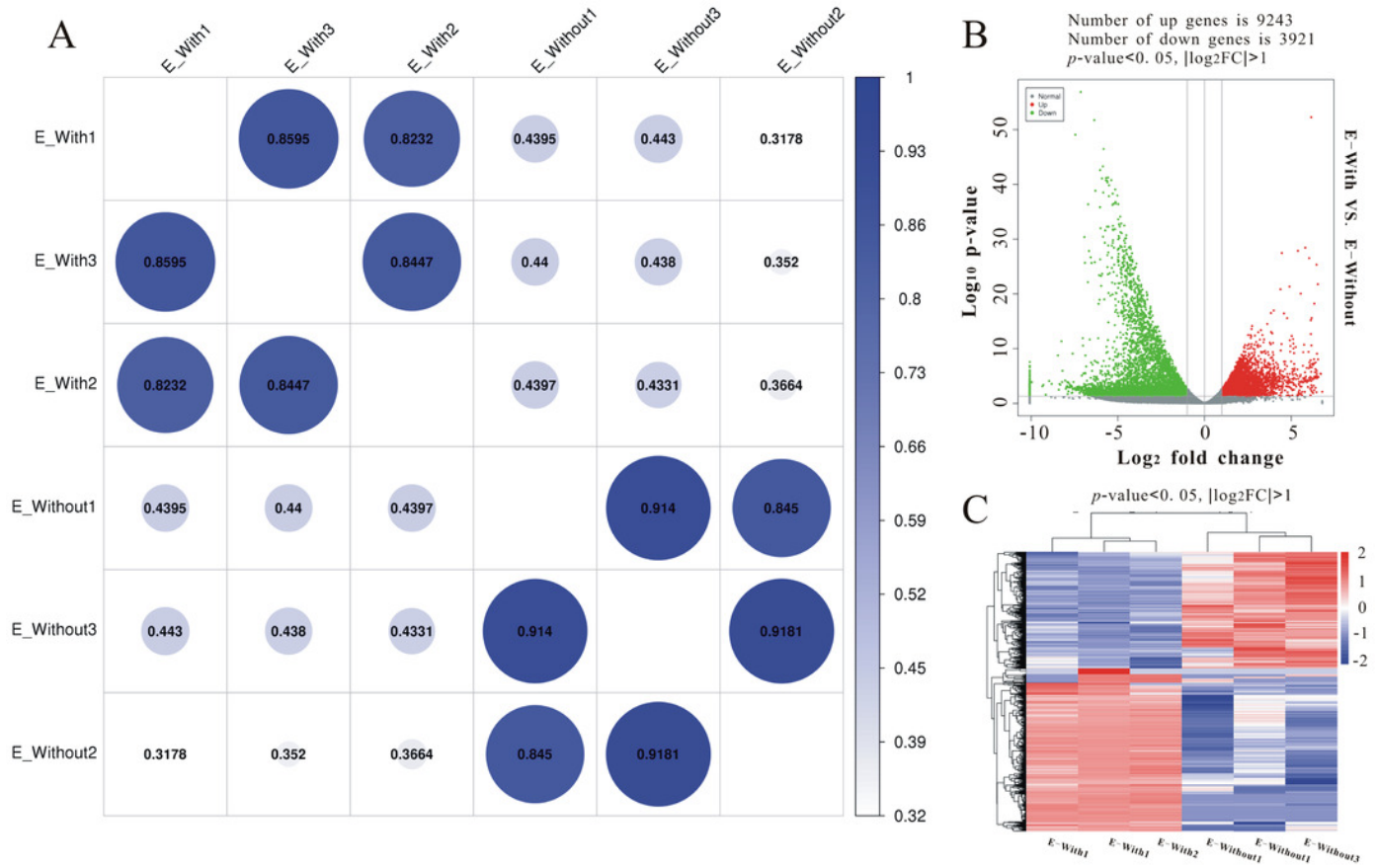

Figure 8

KEGG pathway enrichment analysis of differentially expressed genes (DEGs)

Only the top 20 pathways in KEGG enrichment function were listed in the figure. The ordinate is the name of the KEGG metabolic pathway, and the abscissa is the Enrichment Score to the pathway. The larger the bubble is, the more different the number of Unigene is contained, and the bubble color changes from purple-blue-green-red, with smaller Enrichment *p* value and greater significance.