# 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 distinguish the differences between two types of eggs.

**Results.** We found that *P. solani* laid two types of eggs in one batch, 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 are very significant. The results suggest that the embryo of the egg undergoes development inside the mother and only a short molting outside the mother.

**Discussion.** Our study reveals that the reproductive pattern of *P. solani* cannot strictly be considered ovoviviparity; we propose the term semi-ovoviviparity to describe the reproductive pattern of this species.

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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 as one of 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 hatch 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*). The viviparous reproductive mode has been reported in earwigs, *Arixeniaesau* (*Benoit et al., 2015*). In some insect populations, a less advanced or "transitional" reproductive pattern called ovoviviparity has been described that is similar to viviparity, but the embryos are nourished by the egg yolk rather than the mother's body, i.e., young are produced by means of eggs, not the body of the mother (*Blackburn, 1999*; *Gaino & Rebora, 2005*; *Lodé, 2012*). Based on 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*), have been 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 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 studies suggest that the phenomenon of ovoviviparity is widely distributed among scale insects (*Tremblay, 1997*; *Gavrilov & Kuznetsova, 2007*). Gavrilov-Zimin (*2018*) overviewed 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 ovoviviparous / viviparous ones with the appearance of new and interesting adaptations for egg protection. In other words, there may be a richness and variety in the reproductive modes of scale insects; 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 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 the population (*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 of 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 the process is relatively concealed (beneath the abdomen), so it has been suggested that this may be 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, 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 and transmission electron microscopy to distinguish the difference between two types of eggs (reproductive products putatively considered to be eggs) laid in one batch. Finally, we further investigated the miRNA and mRNA expression from 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) From the above findings, determined the reproductive mode of *P. solani*.

## Materials & Methods

### Oviposition characteristics

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

### Egg morphology

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

experiment was carried out in an artificial climate chamber with a temperature of 27 ± 1℃, a humidity of 70% ± 5% and a photoperiod of 16 L: 8D.

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

Two treatments were set: 1) eggs with-ES were incubated under the mother's body (Fig. 5A); and 2) eggs with-ES were artificially removed from the mother and kept on the concave slide (Fig. 5C). Then the female adults were continuously observed through a NikonSMZ1500 zooming stereomicroscope every five minutes. When female adults continued to stretch their abdomens, this indicated that they were about to lay eggs. With treatment 2, the upper concave slide was opened immediately, and the mother's body was carefully removed with an insect pin. The hatching time was recorded as the time from when female adult laid eggs to the time when the yolk membrane completely detached. Each egg was bioassayed once; experiment 1 had 30 repeats, and experiment 2 had 47 repeats.

**The difference between two kinds of eggs from a microscopic perspective**

**1 Egg surface and internal structure**

The surface and internal structure of the two kinds of eggs were observed by scanning electron microscopy (SU8010, Hitachi, Japan) and transmission electron microscopy (H7650, Hitachi, Japan). The collected eggs with and without ES were pretreated with liquid nitrogen immediately. The follow-up procedures followed the instrument operation methods for the scanning electron microscope. The transmission electron microscopy was performed as follows: 1) Fixation: eggs were immersed in 2.5% glutaraldehyde fixative solution and then rinsed with buffer solution; 2) Gradient dehydration: the fixed samples were dehydrated radiantly with ethanol; 3) Gradient osmosis: the samples were permeated with a mixture of acetone and Spurr resin penetrant (1:1); 4) Embedding and polymerization: 100% Spurr embedding agent was added, and polymerization occurred for 24 hours; 5) Ultrathin sectioning: the samples were cut to approximately 90 nm; 6) Observation and photography.

**2 RNA sequencing and data analysis**

**RNA extraction, library construction and RNA sequencing**

The newly laid egg samples (with-ES or without-ES) were collected and immediately placed in a 0.5 MIEP tube, and then frozen in liquid nitrogen for RNA extraction. Total

RNA from 6 samples was extracted and constructed library as previously described (*Yin et al., 2018*). The Illumina HiSeq X Ten was used to sequence the libraries and generated 150bp paired-end reads. Raw data were deposited in the National Center for Biotechnology Information (NCBI) and can be accessed in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA554708.

**Unigene denovo assembly, functional annotation and data analysis**

Transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). Raw data (raw reads) were processed using Trimmomatic (*Bolger et al., 2014*) to obtain clean data (clean reads). Then, the clean reads were assembled into expressed sequence tag clusters (contigs) and de novo assembled into the transcript using Trinity (*Grabherr et al., 2011*) (vesion: 2.4) with the paired-end method. The longest transcript was chosen as a unigene based on the similarity and length of the sequence for subsequent analysis. The function of the unigenes was annotated by the alignment of the unigenes with the NCBI nonredundant (NR), SwissProt, Clusters of orthologous groups for eukaryotic complete genomes (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 fold change < 0.5 was set as the threshold for significantly differential expression. Hierarchical cluster analysis of DEGs was performed to explore transcript expression patterns.

## Results & Discussion

### Oviposition characteristics

Female adults of *P. solani* produced eggs in one generation through thelytokous parthenogenesis, and the eggs were long, oval and lacking oocysts, similar to those described by Zhi *et al*. (*2018*), but the female adults produced wax. After egg laying, the yolk membrane was detached from the egg (eggshell), followed by the appearance of 1st instar nymphs quickly crawling away from the lower part of the mother. It took a short time (< 30 minutes) to complete this process, so the researchers thought the first nymph was produced by scale insects such as *P. solani* and another invasive mealybug *Phenacoccus solenopsis*. Because both eggs and first 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*). Moreover, in a batch of eggs laid by female adults of *P. solani*, two kinds of eggs with distinct morphological differences appeared (Fig. 2A/B), i.e., two reddish-brown eyespots were seen on one type of egg (eggs with-ES; Fig. 2D), while the other type did not display these eyespots (eggs without-ES; Fig. 2C). In *Matsucoccus matsumurae*, there are also two types of eggs, those with and without eyespots, but the eggs with eyespots were similar to the type of eggs without eyespots, and the egg types showed different developmental periods but all hatched normally (*Xie et al., 2014*). The hatching process of eggs with-ES was described as follows: 1) in the first 5 minutes, eggs began to show considerable peristalsis beneath the mother, and at 10-15 minutes, the yolk membrane was gradually detached and wax powder appeared on the surface of body; 2) at approximately 20 minutes, antennae and feet were starting to become visible, and the hatching process was basically completed within 25 minutes, while the detached yolk membrane could be seen at the abdominal end of the 1st instar nymph (Fig. 3).

### Egg morphology

In the present study, we observed that female adults could lay two types of eggs, those with-ES and without-ES, under either treatment. The ratio of eggs with-ES was significantly higher than that of eggs without-ES (on concave slide, 91.56 ± 2.14 vs.

8.44 ± 2.14，$\chi 2$ = 86.01, n = 15, $p$< 0.001; on leaf, 87.31 ± 2.90 vs. 12.69 ± 2.90, $\chi 2$ = 13.07, n = 15, $p$< 0.001) (Fig. 4A). 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. 4C), and with widths of 0.146 ± 0.002 mm (with-ES) and 0.147 ± 0.003 mm (without-ES) (t = 0.24, n = 25; Fig. 4D). Furthermore, the hatching rate of eggs with-ES was 100%, and no eggs without-ES hatched (Fig. 4B). Generally, eggs are under stress from external factors, which may cause them to not hatch 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 found 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*). Another type of insect egg that does not hatch is the nutritive egg that is common in social insects, such as the ant group. The nutritive eggs of ants are unfertilized eggs that cannot hatch and are eaten in colonies containing a queen (*Heinze et al., 1996, 1999*). However, for this parthenogenetic and thelyotokous species of mealybug, the factors causing *P. solani* to lay eggs that cannot hatch require further study.

**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 and did not hatch, just as a chick cannot hatch without a hen. Therefore, we further tested whether eggs with-ES hatched after they were also removed from the area beneath the mother's body. In the present study, we found that eggs with-ES could hatch normally under any treatment (Fig. 5 A/B/C), but the hatching times were different. Inside the mother's body, the hatching time was 24.30 ± 0.60 minutes (Fig. 5D), but when the mother's body was removed, the hatching time was reduced by nearly 5 minutes to 19.47 ± 0.45 minutes (Fig. 5E). 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, which secrete a small amount of wax. Thus, it is not surprising that the eggs of *P. solani* hatched so quickly, especially when the eggs were isolated.

**The difference between the two types of eggs from a microscopic perspective**

**1 Egg surface and internal structure**

The surface and internal structure of eggs with and without-ES were observed using SEM and TEM. It was found that the contour of appendages could be clearly seen across the yolk membrane, 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. 6); moreover, there were complete blood cells, cytoplasm, mitochondrion and myocutaneous filaments inside. Eggs without-ES had a smooth surface and only lipid droplets, endoplasmic reticulum and free ribosomes inside (Fig. 6). At this point, we suggest that eggs with-ES are alive, with features such as conical receptors and blood cells, and that they no longer resemble an egg on a microscopic level.

**2 RNA sequencing and data analysis**

Illumina sequencing generated approximately 45 M reads per sample after removal of the low-quality reads. These reads were assembled randomly and produced 55,558 unigenes within an N50 of 1026 nt. After annotating unigenes with several databases and calculating the expression of unigenes through FPKM, correlation coefficients between samples were calculated and used to estimate biological repeatability and differences between groups. The correlation coefficient of 3 biological repetitions in the group with-ES and 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 threshold of a *P* value < 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). These results revealed the differences between eggs with and without ES in terms of unigene expression.

## Conclusions

Although the reproductive mode of *P. solani* has been described previously, the actual process may not be simple. We found that there is no difference between the two types of eggs by visual observation, but the physiological and molecular differences are very 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 is not complete or has not started at all, and these types of eggs are not vital. Before determining the reproductive mode of *P. solani*, one thing needs to be made clear. The egg begins to hatch and shed its yolk membrane (immediately) after leaving the mother's body, i.e., this species lays eggs and does not experience live birth. However, ovoviviparity is defined as the mother giving live birth, basically to larvae. Therefore, we conclude that the reproductive "product" of *P. solani* is an egg, but the embryo of the egg has already been developed inside the mother, and only a short molting occurs outside the mother. Strictly speaking, the reproductive pattern of *P. solani* cannot be described as ovoviviparity, and we suggest the term semi-ovoviviparity to describe the reproductive pattern of this species.

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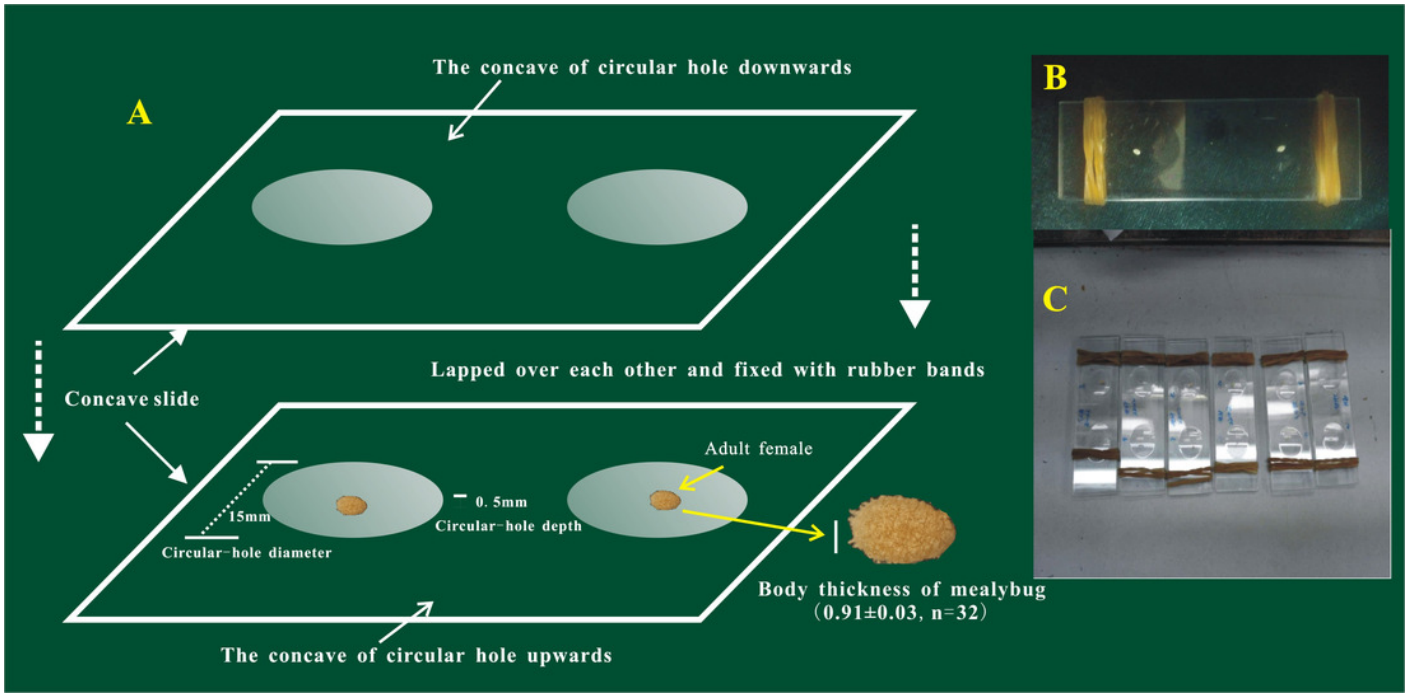
# Figure 1

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

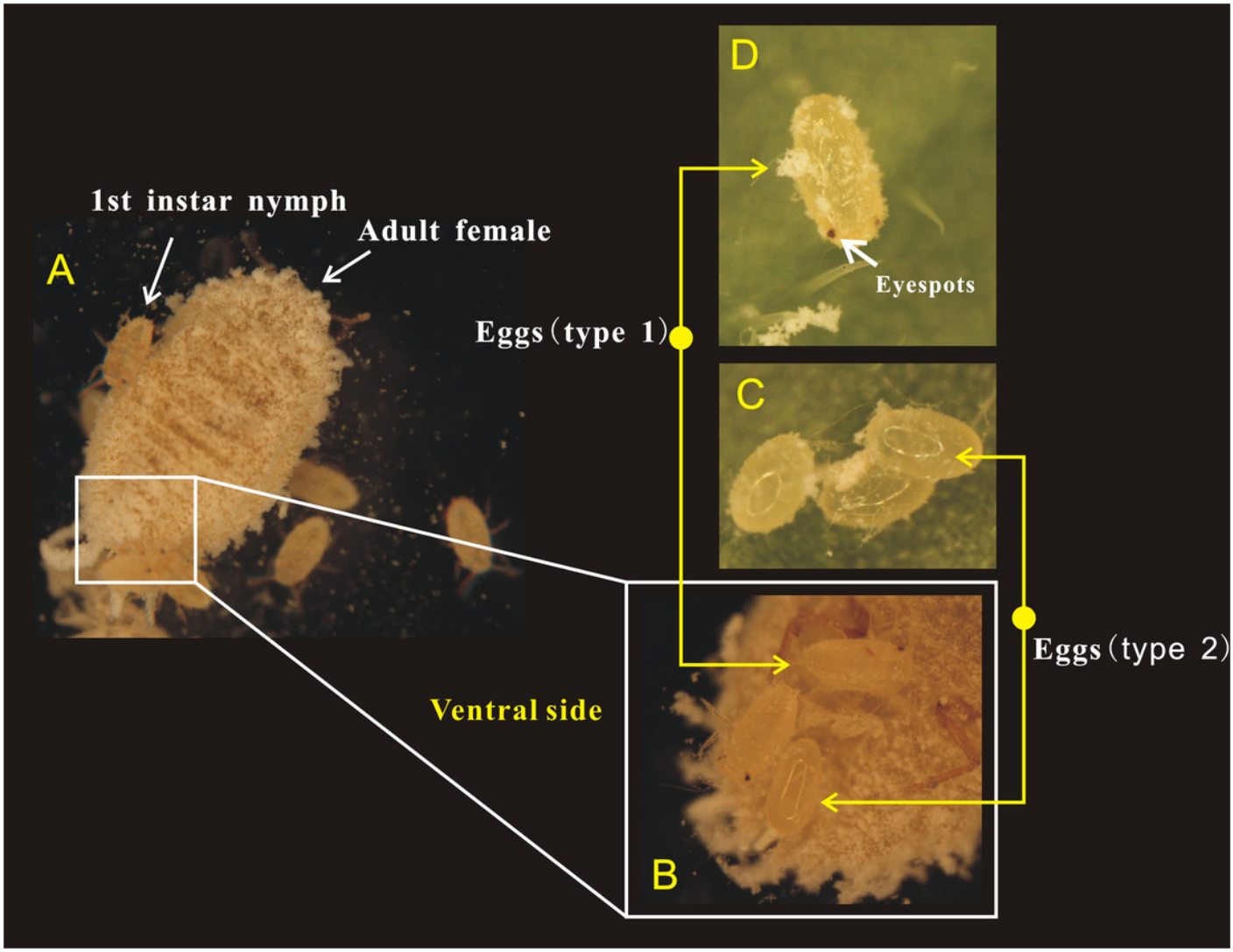
(A) Position and operation diagram. Two concave slides overlapped seamlessly, and adult female was placed into the middle of the hole of the concave slide; concave slides were fixed with rubber bands at both ends. (B) Finished observation device, and contained two separate adult females. (C) Multiple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



# Figure 2

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

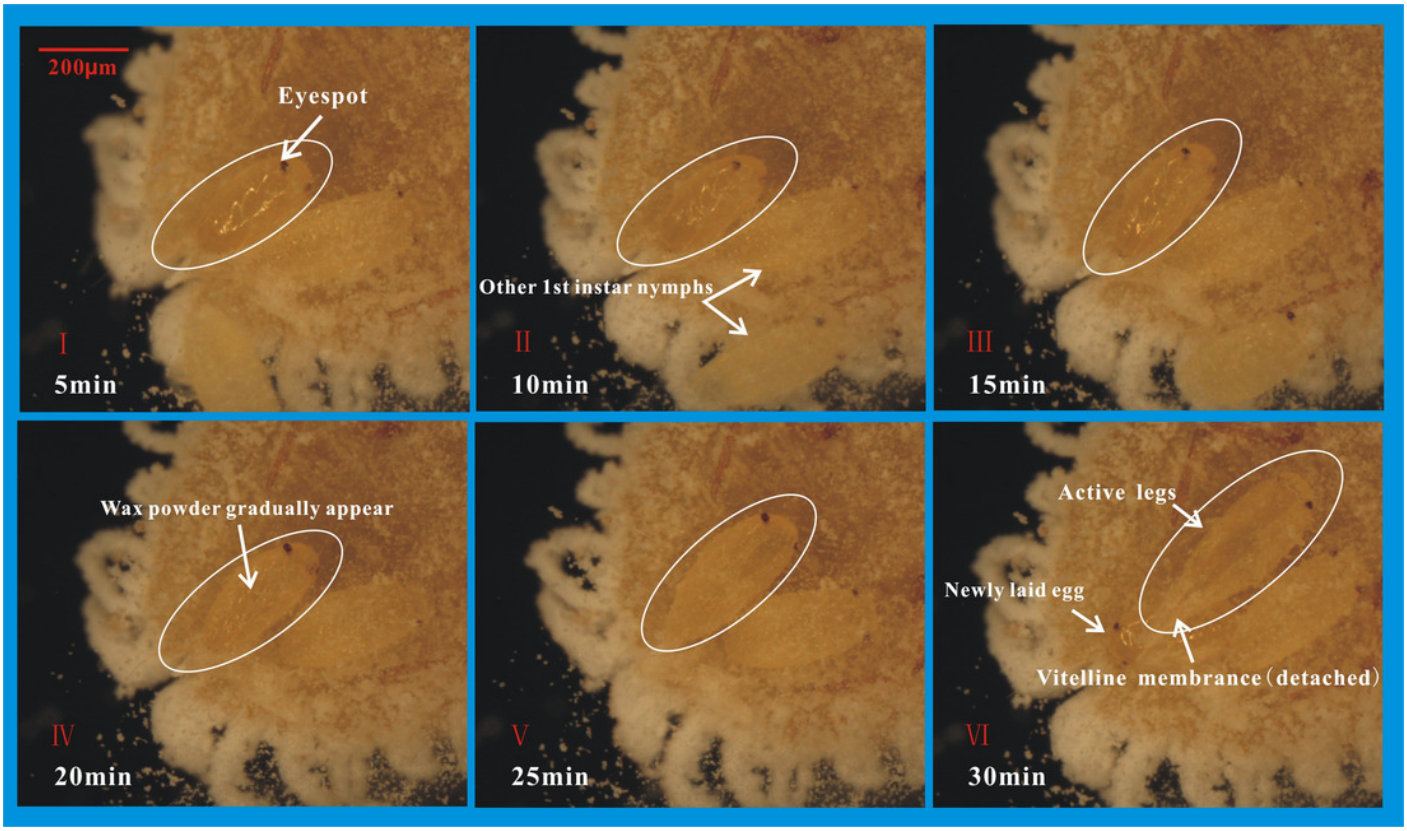
(A) Adult female laying eggs, and some eggs have rapidly hatched into 1st instar nymphs; (B) Newly laid two types of eggs below the abdomen of adult female; (C) The eyespots of eggs (type 2) were not visible; (D) Eggs (type 1) before hatching, the eyespots were clearly visible.



# Figure 3

The hatching process of eggs.

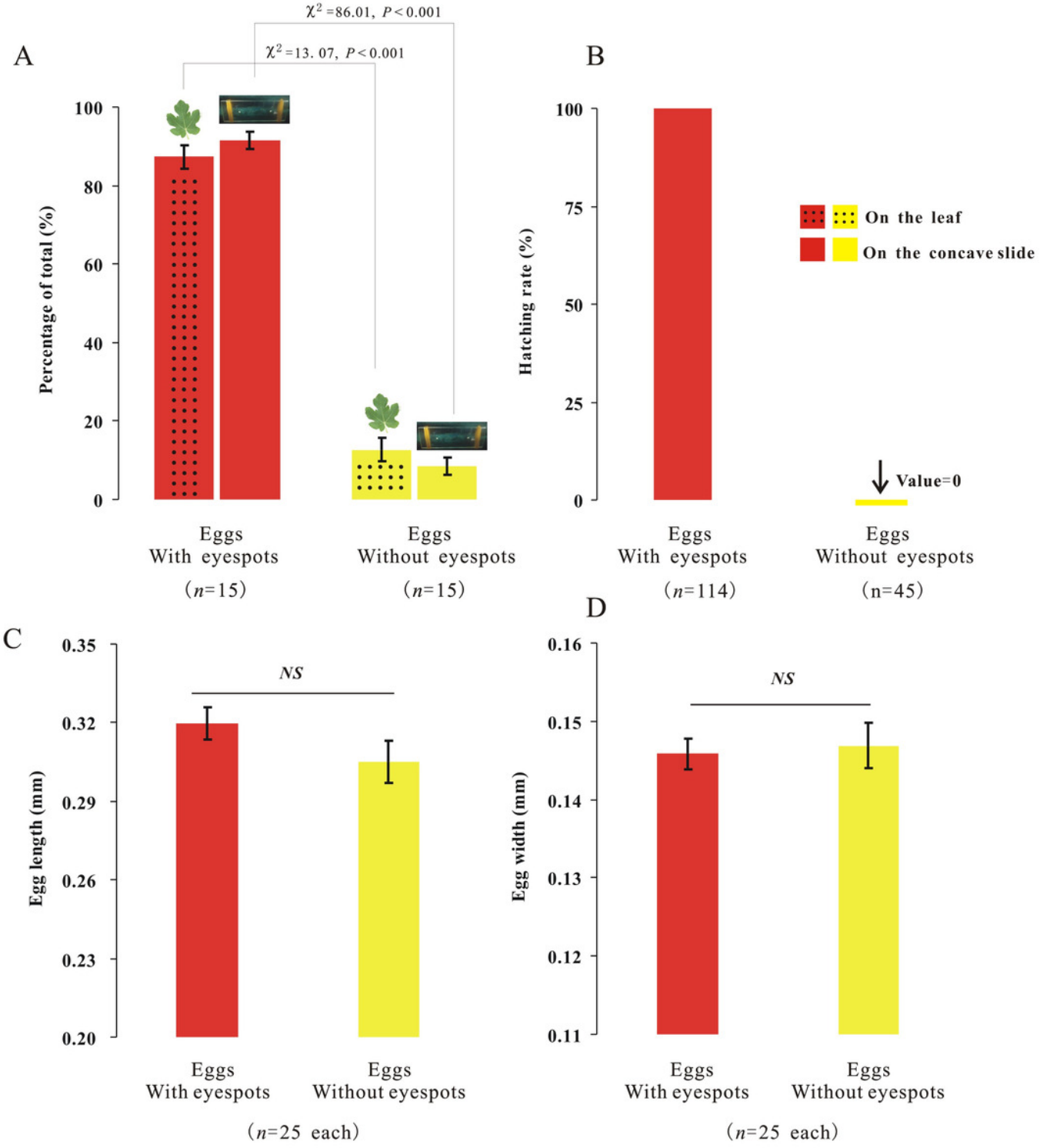
I) The first 5 minutes, eggs began to show considerable peristalsis beneath the mother; Ⅱ/Ⅲ) At 10-15 minutes, the yolk membrane was gradually detached and wax powder appeared on the surface of body; Ⅳ) At 20 minutes, antennae and feet were starting to become visible; V/VI) The hatching process was basically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



# Figure 4

Percentage of the total, hatching rate, and egg appearance for the two types of eggs with different treatments.

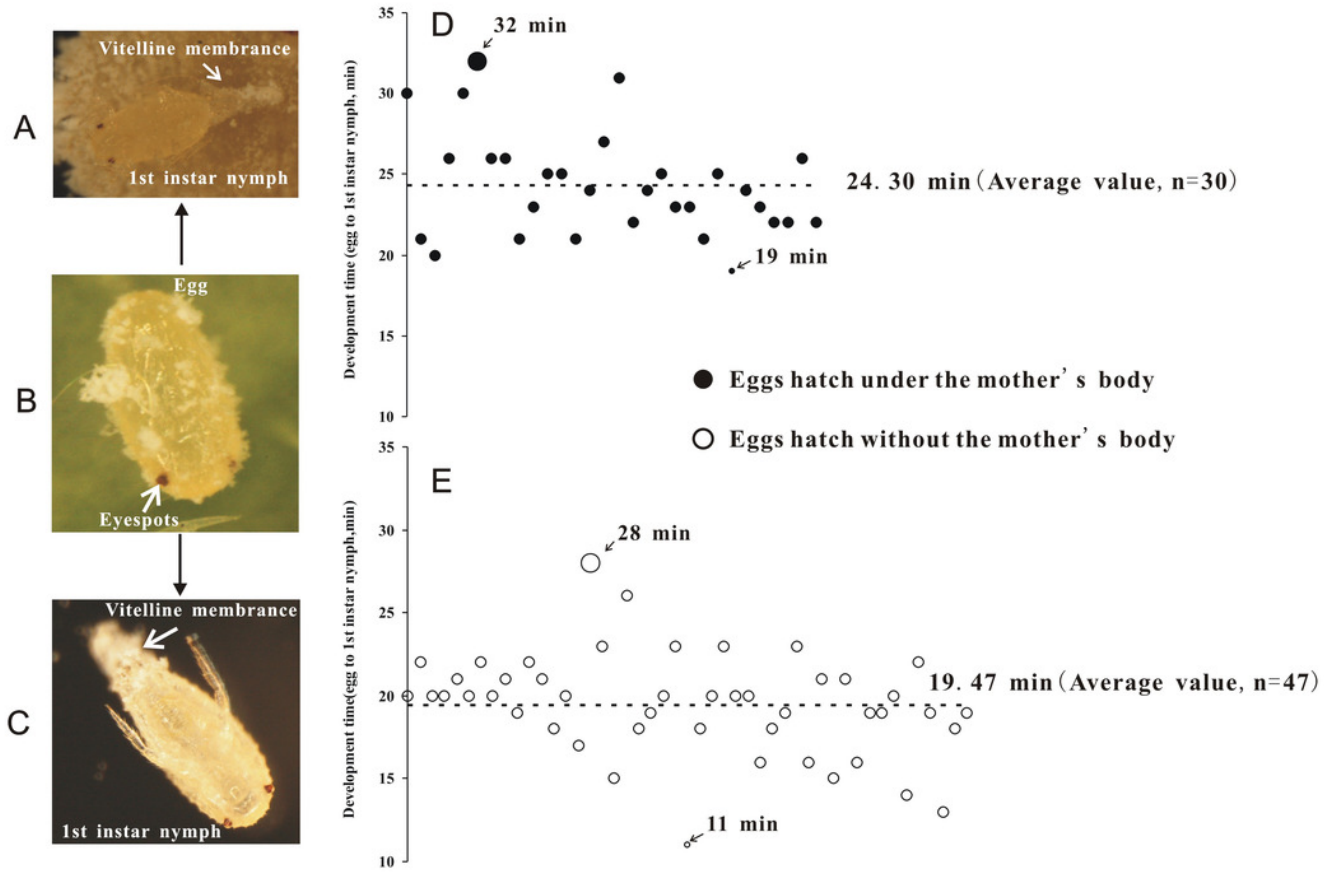
Percentage of the total for the two types of eggs with the treatment of placing female adults on leaves or concave slide (A), and hatching rate of eggs (B); The difference between the two types of eggs with same treatment was analyze d using Chi-SquareTest. The length (C) and width (D) of two types of 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
$\chi^2$ =86.01, $P$ < 0.001
$\chi^2$ =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)
0.35
0.32
0.29
0.26
0.23
0.20
NS
Eggs With eyespots
Eggs Without eyespots
($n$=25 each)
D
Egg width (mm)
0.16
0.15
0.14
0.13
0.12
0.11
NS
Eggs With eyespots
Eggs Without eyespots
($n$=25 each)

# Figure 5

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

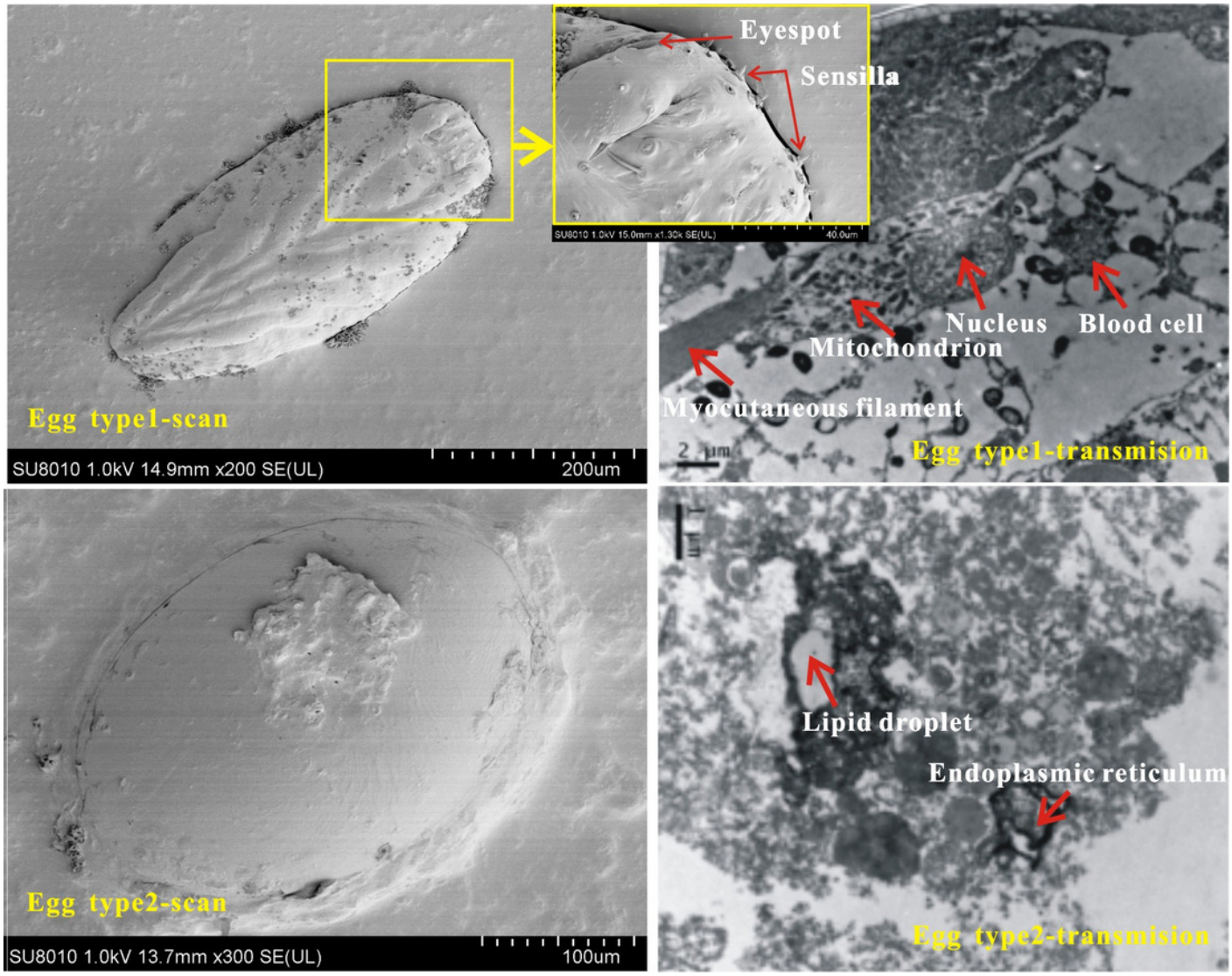
(A) Egg hatching beneath the abdomen of adult female, and the vitelline membrance had been detached. (B) Eggs with eyespots; (C) Egg hatching in isolation (without mother's body); (D) Hatching time of eggs beneath the abdomen of adult female; (F) Hatching time of eggs without the mother's body or in isolation.



# Figure 6

Egg surface and internal structure between two kinds of eggs

The surface (left) and internal structure (right) of eggs with eyespots (type 1) and without eyespots (type 2)



# 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).