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Embryonic development of the olive fruit fly, *Bactrocera oleae* Rossi (Diptera: Tephritidae), in vivo

Hanife GENÇ*
Department of Agricultural Biotechnology, Faculty of Agriculture, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

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Abstract: The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is a pest that infests olive fruits. The female oviposits in large green olives and larvae hatch inside the fruit, where they feed upon the fruit tissues. Larval development is completed inside the fruit. These flies cause great damage to olive production worldwide. Traditionally, insecticides have been directed against the adult stage, but the results are not efficient. This present work is a study of embryogenesis in the olive fruit fly. The external morphology of the *Bactrocera oleae* Rossi (Diptera: Tephritidae) egg is described from light microscopy without dechorionation. The observations were made in vivo and were photographed. The eggshell of *B. oleae* contains a smooth chorion with a cup-shaped anterior pole. The average length of eggs is 0.738 ± 0.01 mm and the average diameter is 0.21 ± 0.06 mm. The embryonic developmental progress is described as formation of the zygote, blastoderm and gastrulation, and organogenesis. The embryogenesis is completed within 65–70 h at 25 ± 1 °C under laboratory conditions. External egg morphology can be useful in estimating the age of *B. oleae* eggs for purposes such as introducing genes into embryos by germline transformation in future studies.

Key words: *Bactrocera oleae*, embryogenesis, fruit flies, chorion, egg

1. Introduction

Tephritid fruit flies are economically important and serious pests wherever olives are grown. The genus *Bactrocera* is one of the largest within the family Tephritidae, with more than 500 described species capable of attacking a wide variety of commercially produced fruits (White and Elson-Harris, 1992). Although there have been extensive studies on the biology of these flies, detailed descriptions of egg stages are rare. Embryonic development of *Bactrocera tryoni* has been previously described (Anderson, 1962). Relatively little is known regarding external egg morphology within the genus *Bactrocera*, and most studies have been based on dissection of eggs to document some aspects of the developing embryos (Fytizas and Mourikis, 1973; Margaritis, 1985; Mouzaki and Margaritis, 1991).

Embryological development has been investigated in *Anastrepha fraterculus* (Wiedemann), *A. sororcola* Zucchi, *A. serpentina* (Wiedemann), *A. obliqua* (Macquart), *A. nigrifascia*, and *A. pittieri*, and egg descriptions are available for *A. ludens* (Loew), *A. obliqua*, *A. striate* Schiner, and *A. serpentina* (Emmart, 1933). Ferrar (1987) reviewed eggs from frugivorous and nonfrugivorous tephritids and reported general egg morphologies in some species. External differences in the anterior and posterior poles of eggs in some dipterans in Cyclorrhapha have been described, with some having a sculptured chorion, some having a smooth chorion, and some having reticulation that is barely visible. A respiratory horn or appendages are present in the eggs of some dipteran species, such as *A. obliqua* (Norrbom, 1985).

Because of the economic importance of the olive fruit fly, a number of control methods have been developed to reduce the pest population. The traditional approach is chemical control, but insecticide resistance has been reported because of the intensive use of chemicals (Vontas et al., 2002; Skavdis et al., 2008). The most promising control approach is the sterile insect technique through construction of germline-transformed stable strains and efficient transformation systems, as in *Ceratitis capitata* (Louis et al., 1987; Loukeris et al., 1995). Germline transformation requires careful knowledge of the age of eggs for the molecular studies needed in gene transformation; hence, there is a need for the study of development of the egg in the olive fruit fly.

The purpose of this study is to examine and describe the external morphology of the egg of *Bactrocera oleae* in vivo, based on observations with light microscopy. A brief outline of the development of the *B. oleae* live embryo is

* Correspondence: hgenc@comu.edu.tr

presented with photographic illustration. The results will be useful in identifying developmental stages in the olive fruit fly and may possibly serve as a guide for other *Bactrocera* species.

2. Materials and methods

The laboratory colony of *Bactrocera oleae* was started from infested olive fruits in Çanakkale Province, Turkey. The colony was maintained on an artificial diet (Tsitsipis and Kontos, 1983; Tzanakakis, 1989; Genç and Nation, 2008). Adult flies were fed with water and a 3:1 mixture of sugar and yeast hydrolysate, and were kept at 25 ± 1 °C with an 18:6 (L:D) photoperiod and 65% relative humidity (Tzanakakis, 1989). Paraffin domes were used as artificial oviposition substrates to obtain eggs (Tzanakakis, 1989). For embryo analysis, eggs were collected from 10-day-old gravid females maintained in the laboratory, which were fed the above adult diet to ensure high oviposition rates and embryo viability. Adults were exposed to oviposition domes for 5 minutes. The eggs were obtained from paraffin domes with a 0.3% propionic acid solution. After the eggs were collected, timing of development was started, and development of the embryo was observed continuously during 3 days in vivo, as well as overnight, and was photographed every hour with a phase contrast Olympus SZX16 light microscope attached to an Olympus C7070 digital camera. Randomly collected eggs (25) were transferred diagonally onto each glass coverslip and covered with halocarbon oil (Sigma) to prevent desiccation of the eggs. The length and diameter of eggs (n = 100) were measured with a scale embedded in the microscope ocular. The coverslips were previously prepared by sticking them to 2 strips of double-sided tape (Scotch). A rectangular marking was made on each coverslip with a China marker (Phano China Marker 77, black) to prevent leaking of halocarbon oil. Timing was used as a method of staging, but modifications in the embryos' external morphology were also used to determine the developmental progress, as previously used in *D. melanogaster* (Campos-Ortega and Hartenstein, 1985).

3. Results

A creamy white chorion is secreted onto each egg before it is fertilized in the oviduct of the female. Eggs (n = 100) are slightly curved. The average length of eggs is 0.738 ± 0.01 mm, with a mean diameter of 0.21 ± 0.06 mm. The egg is broader from the middle toward the anterior pole and slightly tapers toward the posterior pole.

The embryogenesis of *B. oleae* is basically divided into 3 stages. The first stage involves egg maturation and zygote formation. At this stage, the egg is uniformly dark in the center and light at the periphery (Figure 1A). Cleavage soon begins. Yolk is evenly distributed within the egg. The cup-shaped anterior end of the egg (Figure 1A) contains an opening, possibly the external opening of the micropyle, the microchannel that provides an entrance for sperm. It is difficult to view zygote formation with a light microscope because it does not allow sufficient depth of resolution, but in this first stage, a space appears between the chorion and vitelline membrane at the posterior end (Figure 1B). The space disappears before blastoderm formation. This stage lasts about 15–20 min after oviposition. The chorion presents well-developed asymmetric grid ridges in polygonal organization, like reticulations covering the entire egg (Figure 1B). Variations in the chorion were not observed. The micropyle is not quite visible, but is probably located in the anterior pole of the embryo where the head eventually develops.

The second stage is the formation of the blastoderm and gastrulation. Cellularization of the blastoderm began

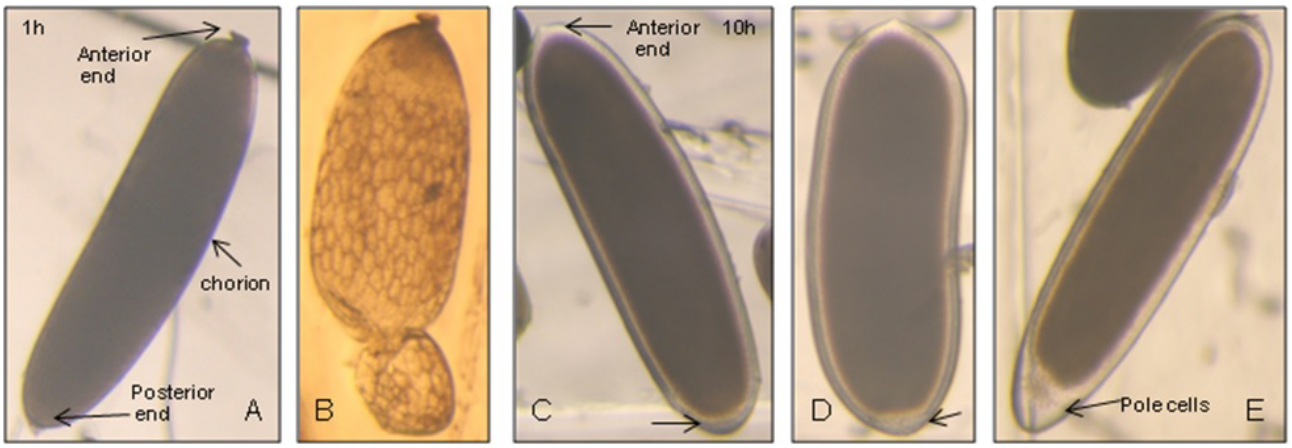

Figure 1. In vivo photographic illustration of *Bactrocera oleae* eggs. A) Anterior and posterior ends of the egg (1 h old); B) the chorion; C), D), and E) sequence of pole cell formation in a living embryo. The arrow indicates the posterior tip of the egg.

by 6 h (Figure 1C). Pole cells could be seen at the posterior end of the egg, and the blastoderm stage was basically completed (Figure 1D). Pole cells are the first cells to become committed and differentiate into germ cells to form the future gonads of the organism. They form in the space between the posterior of the egg and the vitelline membrane, which is not visible (Figure 1E). The series of photographic illustrations in Figures 1C, 1D, and 1E shows the formation of the pole cells close to blastoderm cells. About 10 h after oviposition, a layer of cells around the outer perimeter of the yolk becomes visible as the blastoderm formation. This stage can easily be followed in the living embryo.

The third stage is the formation of organogenesis (Figure 2). The formation of the ventral furrow was started by 22 h; it was not visible at 22 h (Figure 2A), although a space was apparent between the vitelline envelope and the embryo. The cephalic furrow was seen across the embryo by 28 h (Figure 2B). The head and abdominal lobe masses were visible by 46 h. The cephalic furrow disappeared as head involution began and tracheal pits became visible (Figure 2C). Gut formation and mouth hook formation were evident and light brown in color by 52 h (Figure 2D). The mandibles, maxillae, and labium became visible immediately. The gut formed a closed tube; development and differentiation of gut, nervous system, and tracheal systems were evident by 60 h (Figure 2E). The tracheal tree and longitudinal trunks were clearly visible. Tracheal branches and tracheal tubes appeared to darken when the tracheae filled with air (Figure 2E). Development of the nervous system, gut, Malpighian tubules, and tracheal system were completed in the organogenesis stage.

The embryo is visible at the anterior end just before hatching; the embryo does not turn around inside the egg. The larva ecloses through a longitudinal slit beginning close to the anterior end of the egg (Figure 3). The series of photographic illustrations in Figures 3A–3D shows hatching. The movements of larval mouth hooks were visible just before hatching. The larva appeared to move its head for biting or chewing. The larvae may ingest the remaining anterior mass of the yolk before eclosion. The median embryo viability was 68.3% (Figure 3).

Many fundamental processes occur in embryogenesis that specify the future of the organism. Not all eggs are in the same developmental stage when laid because sometimes females retain eggs; thus, timing embryos from egg-laying is not the best method to measure developmental progress. Eclosion can also occur at variable times after the laying of eggs. Thus, observations of morphological changes are more important than timing to estimate the age of the egg. However, evaluating developmental stages required continuous (day and night) examinations of embryos under the microscope. Embryonic development of *B. oleae* lasted about 66–70 h in this study.

4. Discussion

Eggs of frugivorous (Margaritis, 1985) and nonfrugivorous Tephritidae (Haseler, 1965) have some common features (Hinton, 1981; Ferrar, 1987), including respiratory appendages and the decoration of the chorion. The eggshell of *B. oleae* has a smooth chorion and is probably important in providing air to the developing embryos inside the fruit, as is known to be the case in other insect eggs (Hinton, 1981). The anterior and posterior poles that were photographed in living embryos in this study were described previously by Margaritis (1985) and Mouzaki and Margaritis (1991). The results of the present study were in agreement with their results by showing the anterior pole as inverted and cup-shaped. Mouzaki and Margaritis (1987) reported that respiratory structures in drosophilids

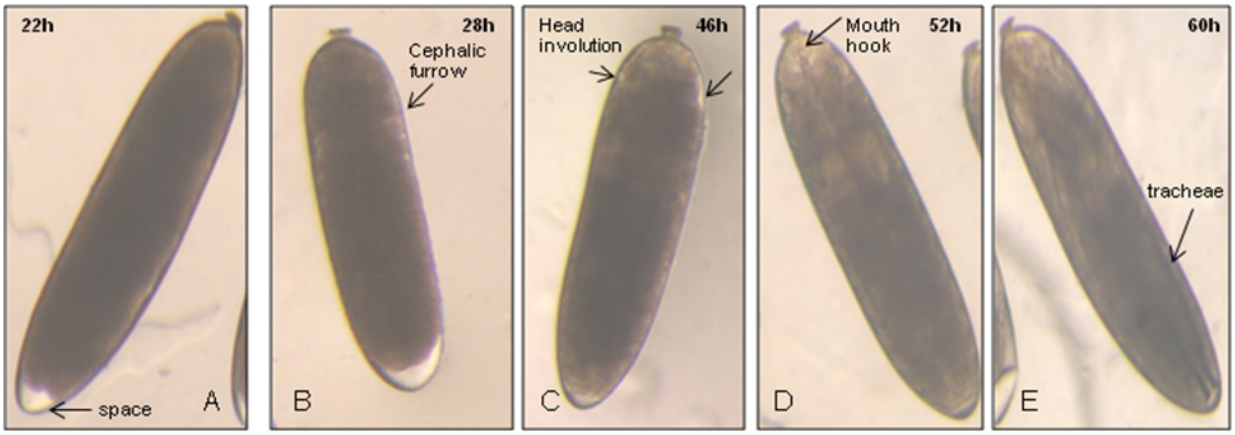

Figure 2. In vivo photographic illustration of *Bactrocera oleae* eggs (22–60 h), gastrulation and differentiation of the blastoderm. A) A space in the posterior pole egg at 22 h; B) cephalic furrow at 28 h; C) head involution at 46 h; D) mouth hook and gut formation; E) digestive, nervous, and tracheal systems.

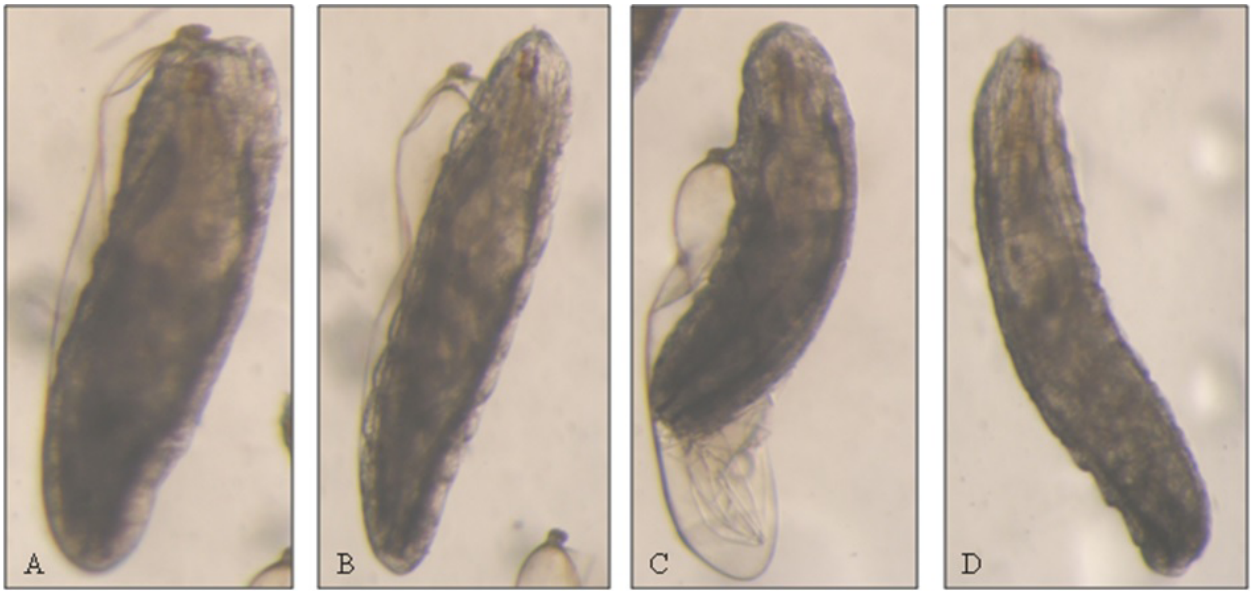

Figure 3. Hatching of *Bactrocera oleae* egg by 66 h (A, B, C, and D).

have been replaced throughout the entire egg's surface or the anterior part of the egg in Tephritidae. However, the *B. oleae* egg has no respiratory horns or appendage on the anterior end like those found in other fruit flies such as *D. melanogaster*, *Anastrepha obliqua*, *A. nigrifascia*, *A. pittieri*, and *Paracantha gentilis* (Emmart, 1933; Norrbom, 1985; Headrick and Goeden, 1990; Murillo and Jirón, 1994). In addition to these findings, the present study showed that there were clear differences in the external morphology of *B. oleae* eggs during embryogenesis, as demonstrated by in vivo photographic illustrations of eggs 1–60 h after oviposition.

Larvae of *B. oleae* hatch through a longitudinal slit at the anterior end of the egg, as in many other Diptera (Margaritis, 1985; Ferrar, 1987). Some tephritid species such as *Anastrepha fraterculus* (Nascimento and Oliveira, 1996), *A. sororcola*, *A. serpentina*, *A. obliqua*, and *A. ludens* eclose through a slit located in the posterior end (Carroll and Wharton, 1989). After a *B. oleae* larva hatches, it moves into internal fruit tissues, so eclosion at the anterior end may be advantageous to reach deeper fruit tissue around the seed of the olive fruit, where first instars were usually found in previous studies (Genç and Nation, 2008).

It is important to understand the stage of the egg for many laboratory studies such as genetic transformation by microinjection applications. The gene should be injected before cellularization of the blastoderm so that the gene becomes incorporated into the pole cells (the future gonads), so that the gene can be passed on to progeny at reproduction.

Data for the present study were averaged from different embryos observed on an hourly basis. It is very difficult to determine precisely the beginning and ending of the observed stages under light microscope, because it is a dynamic process.

External surface features of *B. oleae* observed with light microscopy were seen in great detail; however, further studies need to be done on the detailed embryonic development of *B. oleae*.

Acknowledgments

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