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RESEARCH

Description of *Rhagoletis cerasi* (Diptera: Tephritidae) Pupal Developmental Stages: Indications of Prolonged Diapause

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ABSTRACT. The European cherry fruit fly, *Rhagoletis cerasi* (L.) (Diptera: Tephritidae), is the key pest of sweet and sour cherries in many European countries and west Asia. It is a univoltine species of the west Palaearctic zone that undergoes obligatory pupal diapause. In this study, the development of *R. cerasi* pupae that were brought to an optimum temperature for postdiapause development following a long chilling period is described. The six most representative developmental stages within the puparium are illustrated, and the developmental progression among the stages after the end of the chilling period is quantified. Within 20 d postchilling, there was a gradual progress from stage I to pharate adult. However, ~30% of the pupae remained at the transitional stage II, after 20 d at 25°C (optimum temperature for development). This suggests that a proportion of pupae remain at an intermediate developmental stage for an extended period of time that goes beyond 20 d postchilling. The pupal stage II might be related to diapause termination and responsiveness to environmental cues. It may also define the time before developmental progress to pharate adult. This finding agrees with previous studies proposing that a number of *R. cerasi* pupae undergo prolonged diapause, though the morphological characteristics of these pupae have never been described before.

Key Words: European cherry fruit fly, morphology, pharate adult

Obligatory diapause is a programmed developmental inhibition that is expressed regardless of environmental stimuli (Kostal 2006). The main characteristics of obligatory diapause in insects are 1) arrest of morphogenesis and growth, 2) hormonally mediated metabolic suppression, and 3) a duration that requires a period of time and accumulated environmental cues to be terminated, so that diapausing individuals emerge at the right time of year (Tauber et al. 1986). Although diapause is often thought of as a strategy for avoiding adverse conditions, it also plays a critical role in synchronizing life cycles with favorable times of the year (Dambroski and Feder 2007).

The European cherry fruit fly, *Rhagoletis cerasi* (L.) (Diptera: Tephritidae), is a major pest for sweet and sour cherries in many European countries including Greece (Fimiani 1989). It is univoltine and oligophagous (mainly *Prunus* and *Lonicera* hosts) (Boller and Prokopy 1976). After completing feeding, larvae burrow into the soil where they enter obligatory pupal diapause. The duration of the obligatory pupal diapause synchronizes the adult emergence of this univoltine species with host fruit phenology (Papanastasiou et al. 2011). Although most pupae terminate diapause and emerge in the next fruiting season, a portion of *R. cerasi* pupae remain in diapause for longer than 1 yr (Wiesmann 1950, Moraiti et al. 2012).

Previous studies in *Rhagoletis* species have described several physiological mechanisms associated with diapause (Teixeira and Polavarapu 2005, Lopez-Martinez and Denlinger 2008, Papanastasiou et al. 2011, Ragland et al. 2011) and the environmental cues affecting postdiapause development (Baker and Miller 1978, Ragland et al. 2009). However, most of these studies focus either on the allocation of energetic reserves during diapause or on the factors affecting diapause termination and postdiapause development. The progress of morphological changes of diapausing pupae and postdiapause pharate adult development has only been illustrated for *Rhagoletis pomonella* species (Ragland et al. 2009).

In this study, we provide information on the developmental stages of *R. cerasi* pupae and detailed pictures of each stage. The progress of pupae development has been followed closely for a period ranging from the end of cold storage up to adult emergence (in laboratory overwintering conditions), and the timing of morphological changes during postdiapause development has been recorded. Therefore, six representative developmental stages within the puparium are illustrated. Data and empirical observation concerning the morphology of cherry fruit flies immediately after pupation and throughout the chilling period were used to complete our study. We also estimated the proportion of pupae progressing to each developmental stage during their maintenance at 25°C after the end of a chilling period that is known to terminate diapause. A gradual progress from stage I to pharate adult was observed within 20 d postchilling, but ~30% of the pupae remained at the transitional stage II, after 20 d at 25°C. Apparently, a proportion of pupae remain at an intermediate developmental stage for an extended period of time suggesting prolonged diapause.

Materials and Methods

Flies used in this study were collected from field-infested cherries during June 2006 in Kala Nera Magnisias, a coastal area of central Greece. Infested cherries were transferred to the laboratory of Entomology and Agricultural Zoology, University of Thessaly, placed in plastic boxes on a layer of dry sand, and covered with organdie cloth. Fully grown, mature larvae left the cherries and pupated within the sand. Pupation was recorded daily. In total, 300 pupae were collected by sieving the sand and were maintained under laboratory conditions (25 ± 1°C, 65 ± 5% RH and a photoperiod of 14:10 (L:D) h with the photophase starting at 0700 hours) for 3 mo. After the 3-mo prewinter warm period, pupae were stored in an incubator at 3 ± 1°C for almost 7 mo (chilling period). Finally, pupae were transferred back to 25°C, a postwinter warm period, where a sample of 25 individuals was dissected every second day, and morphological changes were recorded. The mortality of pupae during the chilling period varied from 4 to 29% depending on the sample.

Each pupa was individually transferred under a Nikon SMZ – U stereoscope (Nikon, Japan), and the puparium was gently dissected and removed. Dorsal and ventral pictures of bare pupae were taken with a

Nikon cool Pix 4500 camera adjusted to the stereoscope, and all pupae were classified according to their developmental stage. We used a laboratory guide of *Ceratitis capitata* (Quesada-Allue 1993) as a classification key, and we determined six different developmental stages for *R. cerasi*. In addition, 20 pupae were dissected 2–3 h after pupation to find out that these individuals were in prepupal stage. Furthermore, data were collected by dissecting a sample of 20 pupae after concluding the exposure at the prewinter warm period, 1 d before being exposed to chilling conditions. Twenty overlaying pupae (second year in diapause) were also dissected at the end of the prewinter warming period, and 20 more individuals were dissected during the chilling period of the second year. No pupae were found to terminate diapause prematurely neither during the prewinter warm period nor during the chilling period (S.A.P. and N.T.P., unpublished data).

Statistical analyses were performed with IBM SPSS 20.0 software package for Windows (SPSS Inc., Chicago, IL). A cubic-curved estimation model was applied to predict the probability of pupae pertaining to the transitional developmental stage II in relation to the days exposed to 25°C after the end of the chilling period.

Results and Discussion

Diptera pass through three universal developmental stages during metamorphosis. For *R. cerasi*, metamorphosis is tightly associated with obligatory diapause. Thus, after larva immobilization, the puparium is formed, and at the end of the prepupal stage, it begins to separate from the underlying epidermis (larval–pupal apolysis). Somewhere during these developmental events, diapause starts. Then, metamorphosis occurs (phanerocephalic pupal stage), wings and legs extend, and the pupal cuticle separates from the pharate adult epidermis (pupal–adult apolysis that indicates diapause termination). Finally, eye and body pigmentation begins, and setae appear (pharate adult development–postdiapause progress) that is completed with the opening of the operculum and the eclosion of the adult (Robertson 1936, Fraenkel and Hsiao 1968, Fraenkel

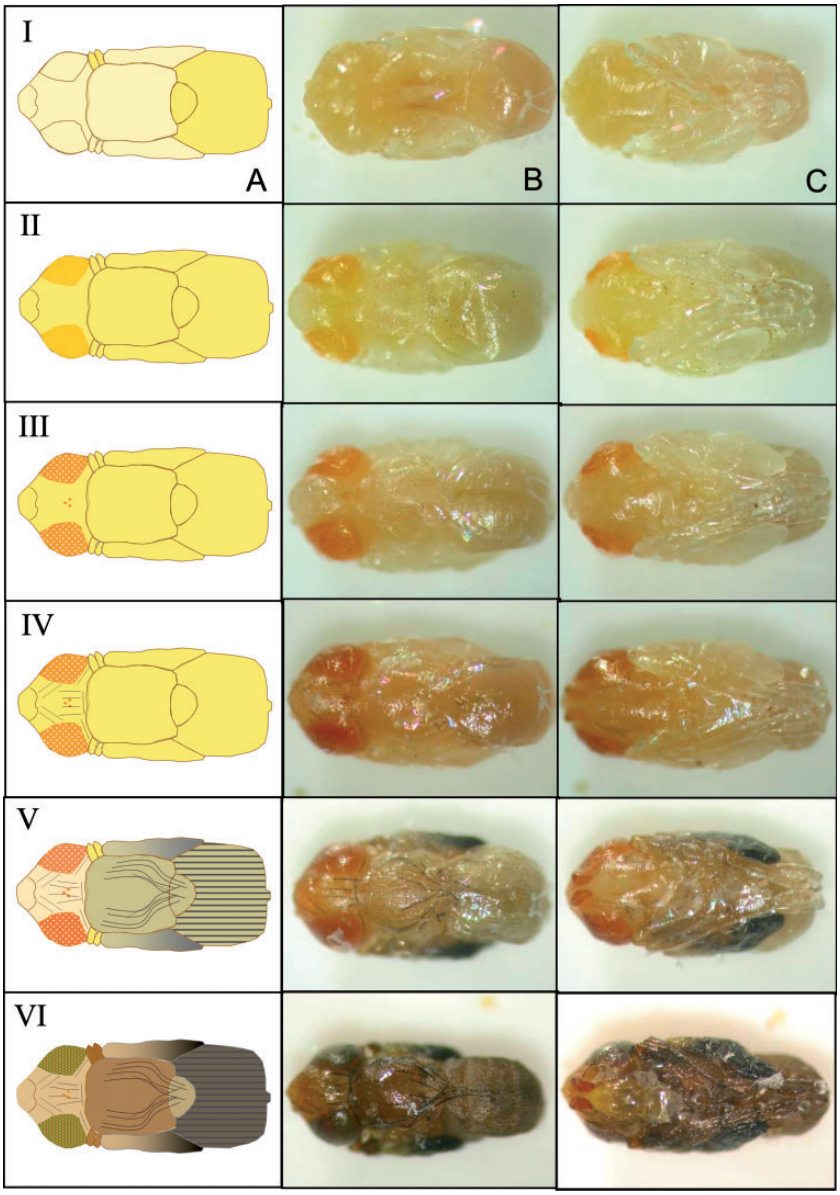

Fig. 1. Progress of *R. cerasi* pupae morphology. (A) Graphic dorsal representation. (B and C) Dorsal and ventral picture of the pupa. Stage I: yellowish pupa during diapause. Stage II: first indications of morphological alterations. Change of composite eyes color from pale yellowish to orange. Stage III: pharate adult. Appearance of ocelli. Stage IV: outgrowth of setae and darkening of composite eyes. Stage V: change of wing color from yellowish to gray and appearance of more setae. Stage VI: change of color of thorax, abdomen, legs from yellowish to pale brown and eyes from orange to deep silver green.

and Bhaskaran 1973, Rabossi et al. 1992, Denlinger and Zdarek 1994).

In this study, we took pictures of the pupae, after exposing them to a 7-mo chilling period, in both dorsal and ventral positions, and we distinguished six different pupal and pharate adult developmental stages (Fig. 1). Pupae at stage I have clearly separated body parts (head, thorax, and abdomen) and apparent body accessories (wings and legs). Stage I pupae are characterized by a light yellowish color, and the majority of them are most probably still in diapause. This is supported by the results we obtained after dissecting pupae before exposing them to a chilling period (90% pertained at stage I, and 10% were dead) and pupae undergoing prolonged diapause (75% pertained at stage I, and 25% were dead). However, as the transition from diapause maintenance to termination is not explored in this study, it is likely that an unknown fragment of stage I pupae had already terminated diapause without any obvious morphological advances and would soon proceed to the next developmental stage (Ragland et al. 2009).

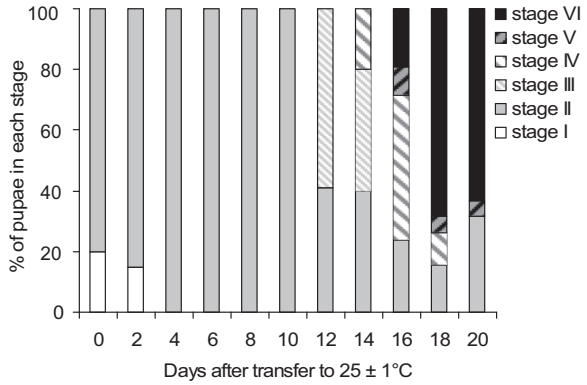

Fig. 2. Progress of development and respective changes in the composition of the six pupal developmental stages after the end of the chilling period (3°C) and transfer of pupae to optimum for development conditions (25°C). Twenty-five individuals were sampled every second day after the end of the chilling period.

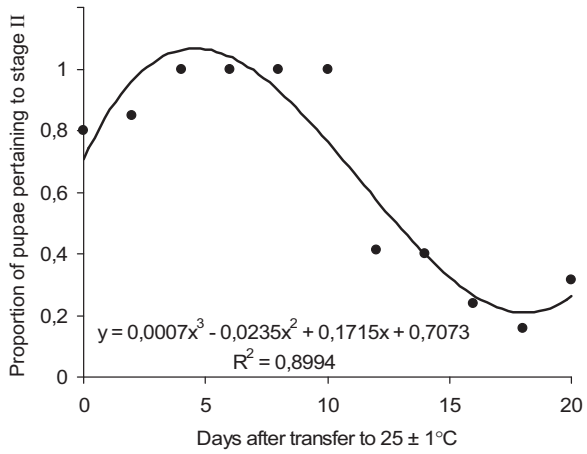

Fig. 3. Proportion of pupae at the transitional stage II in relation to the duration of their maintenance at room temperature after the end of the chilling period.

We also designated stage II as a transitional state from pupa to pharate adult, seeing that the first morphological differentiations begin to be apparent. In transitional stage II, the pupa exhibits a light orange pigmentation of the composite eyes. Whether stage II pupae have undergone pupal–adult apolysis indicating pharate adult development could not be derived from this study. However, our data clearly suggest that a proportion of pupae pertain to dormancy (or developmental arrestment) at least for 20 d postchilling (Figs. 2 and 3). Surprisingly, none of the dissected individuals going through the chilling period for the first or for the second time belonged to stage II. This indicates that the initiation of eye pigmentation may be reversible and may define the period just before the pupae–adult apolysis.

Moreover, we described the morphological changes that pharate adults underlie during postdiapause development in stages III–VI. The pharate adult of stage III is characterized by an orange pigmentation of the ocelli that renders them visible and by the color intensity of composite eyes. In stage IV, the first thoracic setae appear while ventrally the antennae are colored light orange. The dark pigmentation of the wing tips is visible both dorsally and ventrally in stage V when also the density of setae increases. Finally, in stage VI, a dark coloration of the full body and of the accessories appears, and composite eyes acquire the final silver-green coloration (Fig. 1).

At the end of the chilling period (day 0), the majority of *R. cerasi* pupae (80%) advanced morphologically from stage I (yellowish body, lack of color discrimination among body parts) to transitional stage II (start of orange coloration of composite eyes; Fig. 2). This morphological change is probably related to a period of responsiveness to environmental cues. Previous studies have shown that the *R. cerasi* population used in our study terminates diapause after 5 mo in local field conditions (Papanastasiou et al. 2011) and after the combination of 2 mo at 25 ± 1°C and 5 mo at 5 ± 1°C in a laboratory incubator (Papanastasiou 2007). Therefore, most of the pupae dissected on day 0 had most probably fulfilled requirements in low temperatures for diapause

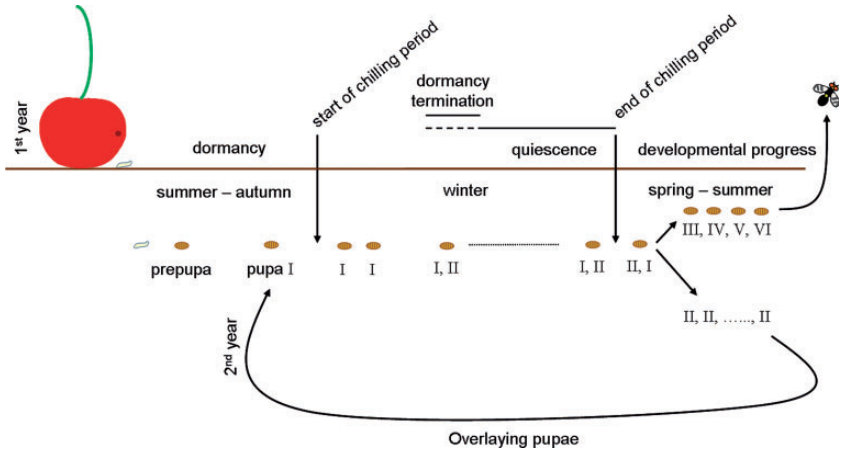

Fig. 4. Possible scenarios of *R. cerasi* pupae developmental progress.

termination and had proceeded to the transitional stage II, which, however, rather defines the end of stage I than the beginning of pharate adult (stage III). After 4 d at 25°C, no more pupae were at stage I.

Within 4–10 d at 25 ± 1°C, all sampled pupae have advanced to the transitional stage II exhibiting light orange pigmentation of composite eyes. After 12 d in 25°C, 60% of the sampled population has developed to stage III (appearance of ocelli). Pupae sampled after 20 d at room temperature were found to pertain to stages II, V, and VI (Fig. 2). It is worth mentioning that at this point, most of the pupae were at the final stage of pharate adult with a significant percentage (almost 30%) still remaining at the transitional morphological stage II, while no pupae were classified at stages III and IV (Fig. 3). Lately, it has been confirmed that a rather high percentage (15–90% depending on biotype and treatment) of *R. cerasi* pupae remains in diapause for >1 yr (prolonged diapause; Moraiti et al. 2012). Our results are in agreement with this finding and provide insights on morphological alterations of overlaying dormant pupae. Pupae at developmental stage II enter a sensitive phase where high temperature can stimulate morphogenesis and development. Prolonged exposure to chilling may initiate a second cycle of dormancy in a proportion of individuals resulting in overlaying pupae (Moraiti et al. 2014). Therefore, it seems that a proportion of pupae at stage II can still follow an alternative path and "return" in dormancy exhibiting plastic response to environmental cues. Initiation of eye pigmentation, which is the sole morphological alteration observed at stage II, may precede the pupa–adult apolysis. In addition, contrary to our expectations, all pupae ($n = 20$) undergoing diapause for a second period have been classified to developmental stage I when dissected during the chilling period of the second "winter period."

It seems that the transitional stage II is a reversible dynamic condition that enables pupae to follow two radically opposite developmental directions (Fig. 4). Whether the initiation of the composite eye pigmentation is the morphology key indicator of diapause termination per se needs to be defined in future studies. Earlier studies suggest that several metabolic paths of energetic metabolites (glycogen–polyols) are reversible and are affected by external stimuli during the dynamic state of diapause (Hahn and Denlinger 2007, Papanastasiou et al. 2011). Pupae morphology could also respond to environmental or to metabolic stimuli with a narrow range of oscillation between two morphological stages being possible. Dormant pupae may become receptive to diapause-terminating environmental cues for a particular time window with the ability to become refractory again if the cues are not received. However, despite the evidences given above, this is a first approach of describing the pupal developmental stages of *R. cerasi* and especially the overlaying ones. Additional data and in-depth studies should be initiated to define the relationship of the light eye pigmentation with pupae–pharate adult apolysis, diapause termination, and prolonged dormancy. Future studies should enrich the present data with the morphology of pupae throughout the diapausing chilling period, as well as with information regarding the combination of cold or warm period that leads to a prolonged dormancy. Possible genetic differentiations of individuals that diapause for one or two seasons could also enlighten the present findings.

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