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Completion of metamorphosis after adult emergence in *Ceratitis capitata* (Diptera: Tephritidae)

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ABSTRACT

The ecdysis of the imago is a crucial step in the development of holometabolous insects. However, no information on several aspects of *Ceratitis capitata* imago emergence and subsequent body maturation is available. We analysed behavioural events and evaluated the oxygen consumption and the dynamics of carbohydrate and lipid reserves during puparium extrication and in newly emerged imago until full wing expansion. A system for recording images with the corresponding software for image analysis was built for this purpose. After extrication, *C. capitata* showed two early postemergence phases: walking (6.56 ± 4.01 min, 6.2% of the wing spreading period, WSP) and the phase without locomotor motion (98.75 ± 26.04 min; 93.8% WSP). Three main events were recognized during the last phase. The first involved an initial expansion of the wings (11.12 ± 4.32 min). The second event was the progressive tanning of the body cuticle in general and the wing veins in particular, and the last entailed the definitive expansion of the wings to attain the characteristic arrow-shaped wing aspect. Our studies here complement previous descriptions of the tanning process of newly emerged medfly adults (Pérez et al., 2018). As a consequence of the results presented here, we consider that the initial events of the newly emerged adult could be interpreted as the last steps of metamorphosis.

1. Introduction

The metamorphosis of Diptera Cyclorrhapha produces radical morphological, physiological and behavioural changes. Three ontogenetic stages could be recognized in postembryonic flies (larva, pupa, and adult). Within the puparium, the pupa is an inactive instar, interposed between the larva and the pharate adult. Conventionally, instars are well defined as organisms between two successive moults (Carlson, 1983; Chapman, 1982; Borror el at., 1989, Sojack, 1995; de la Torre-Bueno, 1989). According to this definition, the pharate adult and the imago should not be considered different instars.

At the end of the pharate adult stage, changes in ecdysone levels initiate a hormonal cascade that ultimately leads to adult emergence. The first hormone to be released in response to decreasing ecdysone levels is ecdysis-triggering hormone (ETH), which in turn triggers an increase in eclosion hormone (EH) levels. Together, ETH and EH are responsible for preparatory behaviour called pre-ecdysis (Scott et al., 2020; Davis et al., 2007). EH causes the release of the so-called crustacean cardioactive peptide that is thought to control emergence behaviour. Acting downstream of the above sequence of regulatory peptides, the hormone bursicon is involved in apoptotic removal of wing epidermal cells after adult emergence and is a key factor in the expansion of the wings and tanning the cuticle (Cottrell, 1962; Fraenkel and Hsiao, 1965; Peabody and White, 2013; Flaven-Pouchon et al., 2020).

The emergence of the imago from the puparium is a crucial step in the development of dipterans, universally accepted as the end of metamorphosis. During this stage, insects are extremely vulnerable to predators and ambient aggressions, eventually provoking the failure of emergence (Mitchell and Petersen, 1982; Yocum et al., 1994; Barry et al., 2002, 2003; Fairbrother et al., 1981; Berni et al., 2003; Pujol-Lereis et al., 2010). To ensure survival, the newly emerged imago needs to sclerotize its cuticle soon to be able to acquire locomotion and to avoid desiccation or another adverse climate condition.

The pattern of emergence from the puparium was described in detail in flesh flies (Reid et al., 1987; Zdarek et al., 1986), in blood-sucking flies (Zdarek and Denlinger, 1987, 1992) and vinegar flies (Johnson and Milner, 1987). At the end of metamorphosis, pharate adults use ptilinum to open the puparium operculum, thus initiating the process of extrication, i.e., the transition from pharate to newly emerged adults (Miyan, 1989, Denlinger and Zdarek, 1994). Some authors qualify this moment of

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the holometabolous lifecycle as ecdysis although emergence from the puparial case does not imply moult or cuticle shedding of the emerging insect. Other authors indistinctly use eclosion or ecdysis for emergence (Peabody and White, 2013). The newly emerged flies undergo a stereotyped pattern of behaviours (walking, grooming, air swallowing, abdominal contraction, etc.) that leads adults to reach the final body shape and expansion of wings (Zdarek and Denlinger, 1987, 1992; Peabody and White, 2013). Studies in several flies have shown that wing expansion requires an increase in haemolymph pressure (Johnson and Milner, 1987; Zdarek and Denlinger, 1992). We previously studied *Ceratitis capitata* wing expansion followed by final steps of cuticle tanning and colouration (Perez et al., 2018). After emergence, the pale and folded wings are expanded and sclerotized to acquire the definitive form and to stabilize the cuticle. The wings of this fly show a specific pattern of brownish and black spots. We demonstrated that N-beta-alanydopamine (NBAD) is responsible for the colouration of brownish spots. We determined that the timing of NBAD synthesis and deposition occurred in the absence of wing epidermal cells. Haemolymph circulation through the veins is required to tan the wings.

The Mediterranean fruit fly is one of the most important orchard pests worldwide. Control strategies to prevent establishment include eradication of medflies by continuous releases of males that were sterilized by irradiation (Franz et al., 1996). The main drawback of this technique is that it usually presents a lower percentage of adult emergences due to the effects of irradiation (Mastrangelo et al., 2010, Sobrinho et al., 2006; Barry et al., 2002, 2003). Furthermore, to the best of our knowledge, no publications have reported a detailed analysis of the emergence and postemergence behaviour of medflies, despite a great deal of effort invested in the eradication of this pest. Thus, we focused our studies on the pre- and postemergence behaviour together with some concomitant metabolism markers (oxygen consumption, glycogen, trehalose and lipids) of the medfly. To monitor macroscopic events during and after the emergence of *C. capitata*, we built a novel imaging recording system and developed special software.

2. Materials and Methods

2.1. Insects

Colonies of 100 wild-type *C. capitata* (strain "Mendoza") adults were maintained in 3.75 L flasks with free access to sucrose-dry yeast (3:1) and 1% agar as food and water sources, respectively. Eggs laid in plastic fruits during a 4 h period were collected to start new fly cultures. Larvae were reared in a pumpkin-based medium (Pujol-Lereis et al., 2006). Flies were kept in a chamber (Conviron CMP 3244; Winnipeg, Manitoba, Canada) at 23 ± 1°C and 50–60% relative humidity with a photoperiod of L16:D8 h. Lights were turned on at 8:00 h (ZT-0) and turned off at 24:00 h (ZT-16). In our culture conditions, stages within the puparium lasted 13 days.

2.2. Video recording of adult emergence and postemergence behaviour

To record *C. capitata* extrication and postemergence behaviour of isolated individuals, we used plastic Petri dishes of 35 mm diameter and 4.5 mm height as experimental arenas. Each fly was prevented from flying as the Petri dish was an enclosed space, where the fly was able to make free movements except flying. To initiate each experiment, a single puparium was glued to the centre of the Petri dish with a drop of 2% agar with sugar and yeast (3:1) (Supplementary Figure S1C). The experimental setup at 23°C and 50-60% relative humidity consisted of an oval platform (80 cm long axis; 40 cm short axis) surrounded by light filter paper (40-45 g/m^2) illuminated with RGB LED stripes (smd 5060, 30 LEDs per metre). The flies were recorded using two Logitech-C625 cameras (Lausanne, Switzerland); one camera was set to film during the day and the other at night. Each camera was able to simultaneously record twelve arenas of 35-mm diameter for the simultaneous analysis of 12 individuals at once (Supplementary Figure S1A and B). Images were recorded at 30 fps (640 × 480) using our Fly recorder camera interface programme.

To determine the time of subjective day on which the largest number of adult emergences occurred, medflies were recorded for 24 h (Supplementary Figure S1B). White light was used during the subjective day, and red light was used during the subjective night (Donelson et al., 2012). To record *C. capitata* extrication and postemergence behaviour, the individuals were filmed from ZT-2 to ZT-8 (10:00 to 16:00 h), and white light was used.

The timing and events of extrication behaviour were analysed through observation of the recorded videos (Supplementary Figure S3). After extrication, the timing and length of the pathways were continuously analysed, including the stops, by our Fly tracker programme. The initial position was considered when the insect stood on all its legs (t0), and the final position (tf) was when the flies completed final wing expansion (Fig. 1A and 1C). In our current analysis, *in situ* movements such as ptilinum pumping, proboscis bobbing, grooming with the legs, abdominal pulses, expansion of the wings, etc., which do not involve locomotion, were interpreted as equivalent to stationary behaviour. The representation of the time of resting was proportional to the radii of circumferences in Fig. 1B (Bubble plot, Microsoft Excel 2013). The Fly tracker analysis software allowed us to establish the real position of the fly in a Cartesian coordinate system, as well as the time that remained at any point. Empirically, movement (as a parameter) was defined as a 152 motion speed of 1 cm/min or faster; lower values were considered pause or static. The tracker was written in C++ using OpenCV http://opencv.willowgarage.com, OpenCL (Khronos Group, http://www.khronos.org/opencl), and Visual Basic (Microsoft) libraries. Detection methods considered the same steps described by Branson et al. (2009), alternating two steps: fly detection and identity assignment. At each new frame, flies were first detected, and their positions were computed. Next, each detected fly in frame tx was associated with a fly tracked in the previous frame tx-1. The background and light were constant, and the only objects moving in the video were flies that appeared white in the black background. We considered "eat" as the first event of feeding postemergence, and "eat" was defined as the repeated extension of the proboscis or when the fly stayed 2 sec or more on the meal.

2.3. Data analysis

Insect movement within the experimental arena was analysed using original experimental software developed in our laboratory. The use of this programme allowed us to establish the real position of the fly in a two-dimensional space and interpret it in a Cartesian axis system per unit time (X (t) and Y (t)). The displacement of each insect was estimated using the vector Eq. (1). We define the average velocity of the insect during the interval time (Δt) as the ratio between the displacement and the time interval (Eq. 2). The position data (X (t) and Y (t)) of each fly were collected in a comma-separated value file (.CSV) and analysed using the Excel programme (Microsoft).

$$\Delta r_n = \Delta X_{n(t)} i - \Delta Y_{n(t)} j$$
$$\Delta X_n = X_n(t_n) - X_{n-1}(t_{n-1}) \quad (1)$$
$$\Delta Y_n = Y_n(t_n) - Y_{n-1}(t_{n-1})$$

$$v = \Delta r / \Delta t \quad (2)$$

2.4. Oxygen consumption

To determine oxygen consumption, we generated an ad hoc respirometer that consisted of an airtight container with a screw cap and a 1-ml micropipette inserted across the cap (Bochicchio, 2012). The

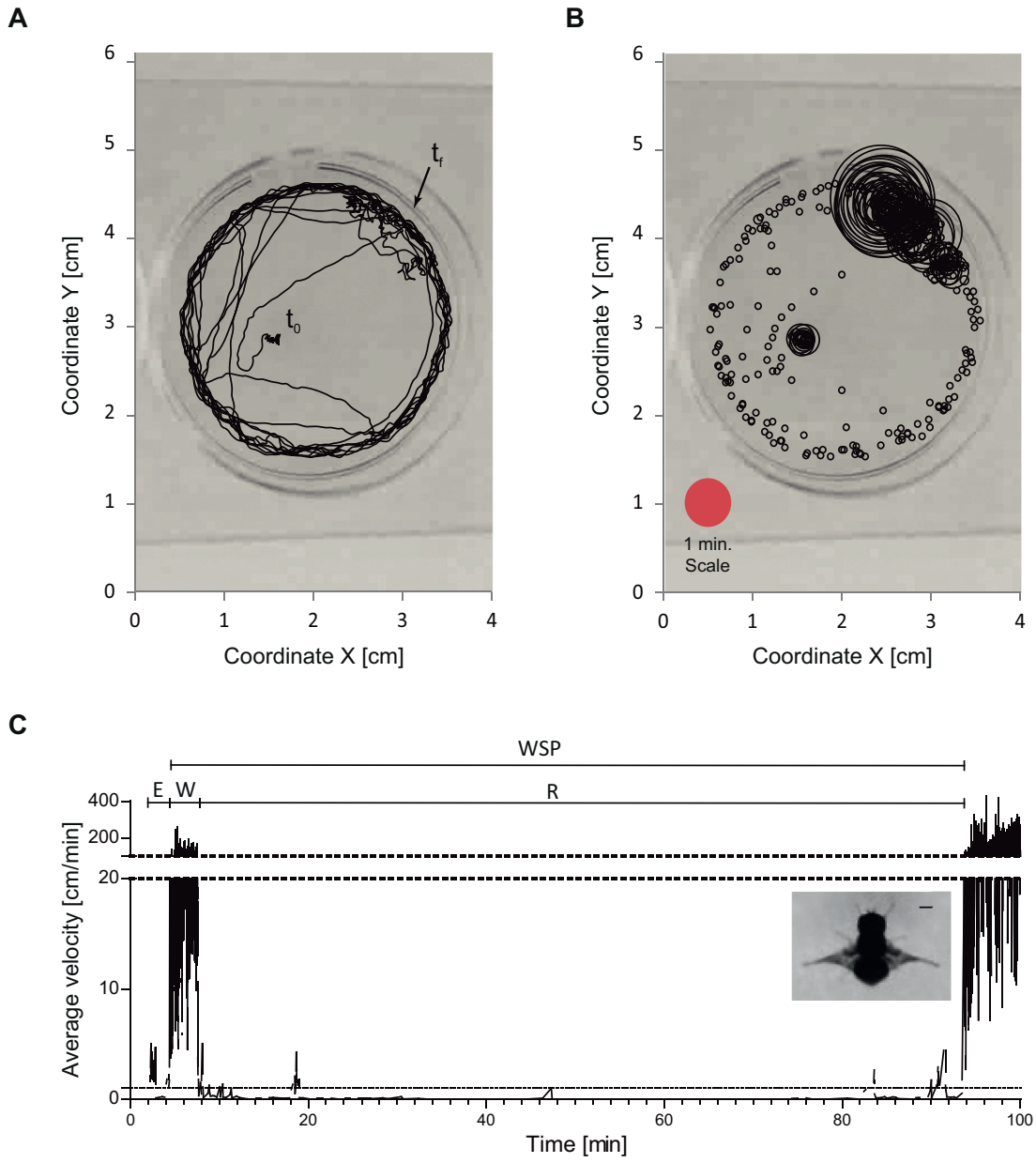

Fig. 1. Example of *C. capitata* imago emergence behavioural patterns registered in 35-mm circular arenas. (A) Records of fly pathways, starting at the extrication point (t0) and ending when the adult attained full wing expansion (tf). *C. capitata* pathway length: 120.8 cm. (B) Diagram of the same pathway as in A, indicating the position and duration of the motionless periods. The diameters of the circumferences in the diagram are proportional to the duration of the resting period. The time of immobility was proportional to the radii of circumferences. (C) Diagram of *C. capitata* adult locomotor activity during extrication (E) and the postemergence period (WSP). In panel C, the walking phase of *C. capitata* (W) and phase without locomotor motion (R) are indicated. Data provided in Supplementary material.

tubes containing 20 male flies were immersed in water (23°C) within an adiabatic chamber. Five replicas were performed for each analysed age. The oxygen consumed was determined by recording the volume change after absorbing carbon dioxide using a piece of cotton moistened with KOH (15% w/v). As oxygen is consumed by flies, the pressure decreases within the respirometer, allowing the water to penetrate into the pipette, which is directly proportional to the volume of oxygen consumed after a certain time interval. We recorded the volume of water entering each respirometer every 5 minutes during 40 minutes of testing. The results were expressed as the oxygen consumption rate per fly. Curve fittings (linear regressions) were performed using Origin v.8.5 (OriginLab, Northampton, MA, USA). Three replicates were performed for each treatment and age.

2.5. Lipid, glycogen and trehalose extraction and quantification

Insects were sampled at different ages using five specimens per replicate for each developmental time. Lipids were extracted from flies as described by Nestel et al. (2003). Flies were homogenized in 0.2 ml of 2% Na_2SO_4. Then, 1.3 ml of chloroform: methanol (1:2) was added to the homogenate, mixed, and centrifuged (200 × g) for 10 min. After centrifugation, aliquots of 0.5 ml of the supernatant were used for lipid estimation using sulfo-phospho-vanillin-reagent (SPV). Briefly, the samples were evaporated to dryness, and the material was resuspended in 0.3 ml of H_2SO_4 and hydrolysed at 100°C for 10 min. An aliquot of 30 μl was reacted with vanillin reagent (270 μl) for 30 min. The amount of total lipids was measured as the absorbance at 490 nm in an

Table 1
Time duration displayed by *C. capitata* during the ecdysis and the postemergence period.

Analysed events	n	Event duration (min)	WSP (%)	Accumulated time (min)
Opening of operculum		-	0	0
Extrication behavior	35	**2.07 ± 0.96**	-	2.07 ± 0.96
Post-emergence behaviour				
1- walking phase	35	**6.56 ± 4.01**	6.2	8.63 ± 4.12
2- motionless phase	35	**98.75 ± 26.04**	93.8	107.38 ± 26.11
(wing expansion)	35	(11.12 ± 4.32)	-	
(arrow shape position of wings + motion activity restored)	35	(95.10 ± 31.18)	-	
Total time (extrication + post-emerg.)		107.38 ± 26.11	100	
First feeding	14	**112.22 ± 18.55**	-	
Definitive colour of wings spots	35	**1440**	-	1443 ± 165

Time: min ± SEM, WSP%: percentage of wing spreading period. Data provided in Supplementary material.

ELISA-reader spectrophotometer (Beckman Coulter, multimode detector DTX 880, California, USA). Regardless of the mechanism, the SPV reaction requires either a C=C double bond or a hydroxyl group present in the lipid molecule to be SPV-positive. Most of the reactive lipids were compounds with one double bond. Only a single carbonium ion is formed per molecule, whereas multiple ions are not stable (Knight et al., 1972). Triglyceride triolein was used as a lipid standard that includes three units of unsaturated fatty. We assume that triglyceride can be hydrolysed to produce glycerol and fatty acids in the presence of sulfuric acid and heat (hydrolyse the lipid esters). Based on the above, we expect 3 SPV-positive signals by one molecule of triolein. Glycogen was extracted from flies as described by Tolmasky et al. (2001). The homogenate was digested at 100°C for 15 min in the presence of 0.9 ml of 33% KOH. After centrifugation to discard nondigested material, the supernatants were separated, and three volumes of 96% ethanol were added to precipitate glycogen at 4°C. Glycogen was obtained by centrifugation at 5000 g, and the pellet was then resuspended in 0.1 ml water. The amount of glycogen was determined by reacting 50 μl of the suspension with 250 μl of I_2/KI/$CaCl_2$ reagent (Krisman, 1962) and measuring the absorbance at 450 nm in an ELISA-reader spectrophotometer (Beckman Coulter, multimode detector DTX 880) (standard: glycogen from rabbit liver; Sigma-Aldrich). For trehalose determination, the commercial kit K-TREH (Megazyme, Ireland) was used. We adapted the volumes proposed by the manufacturer to the volumes necessary to measure the absorbance at 340 nm in an ELISA-reader spectrophotometer (Beckman Coulter, multimode detector DTX 880).

2.6. Statistical analysis

All statistical analyses were performed using R (v 3.2.3). To determine differences in lipids, glycogen, trehalose content, and oxygen consumption rate between the pharate adults and newly emerged flies, we used the generalized least squares (GLS) method with a post hoc Tukey HSD test. In case of lack of homoscedasticity, we employed the varIdent function (package 'nlme'). To fit the frequency of emergence, we performed a Gaussian distribution model with the least squares fitting method, which was included in GraphPad Software. Graphics were made using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

3. Results

Using our novel video recording system (Suppl. Figure S1A), we documented and quantified the timing of emergence from the puparium followed by the postemergence behaviour of *C. capitata* adult males. To establish the duration of the filming, we first determined the time of the day when the highest number of adults emerged under our experimental conditions. Medfly adult emergence occurred mainly during the illumination period, with a mean time of approximately 12.36 ± 1.97 h (Suppl. Figure S2). Medfly pharate adults who completed their development in the afternoon must wait until the next day to eclose.

In this work, we considered that extrication was initiated when the pharate adult pushed with the inflated ptilinum and opened the puparial cap, ending when the insect stood on its legs after emergence. The extrication of *C. capitata* lasted for 2.07 ± 0.96 minutes (Table 1 and Suppl. Figure S1D and S3).

To study the initial period of the adult, we defined the wing spreading period (WSP) as the time between the end of the extrication and the time when the characteristic arrow-shaped wings and body final shape were attained. Thus, the WSP period represents the transition from the newly emerged imago to the adult that is able to move freely (Suppl. Figure S3). The span of this period in *C. capitata* was 107.38 ± 26.11 min and showed two distinct phases: a walking and a motionless phase. Immediately after emergence, medflies displayed continuous walking behaviour that lasted 6.56 ± 4.01 min (i.e., 6.2% of WSP) (Table 1). Eighty percent of the analysed flies showed no interruptions during the entire phase, 16% stopped only once for a few seconds, and 4% stopped two or more times. Only at the end of the walking phase did medflies stop and groom the head with their forelegs. Fig. 1A-B-C shows a representative example of the pattern of activities displayed by *C. capitata* in the restricted experimental circular arena. During the walking phase of *C. capitata,* the average length of the pathway was 150.27 ± 13.47 cm (Fig. 1A). The path represents approximately 300 times the length of its own body. Fig. 1B indicates the duration of the inactive times in a given position, and the diameters of the circumferences in the diagram are proportional to the inactive times. Progressively wider circles observed in Fig. 1B indicate fly changes from the mobile phase to the phase without locomotor motion. In *C. capitata*, the latter lasted 98.75 ± 26.40 min (Table 1), representing 93.8% of the WSP. Three main events were recognized during the phase without locomotor motion. The first lasted on average 11.12 ± 4.32 minutes (Table 1) and involved the initial expansion of the wings in 98% of the flies. The abdomen became inflated, and the wings gradually expanded. This event ended when the wings were spread over the abdomen and the fly moved its hind legs to stroke the wings. In some cases (Fig. 1C), a few walking movements occurred. The second event was the progressive tanning of the body cuticle in general and the wing veins in particular. The third event entailed the definitive expansion of the wings to attain the characteristic arrow-shaped wings aspect. The area of the definitive body shape was approximately 25.6 ± 0.5 mm^2 (Suppl. Fig S4). The second and third events lasted 95.10 ± 31.18 minutes. At the end of this period, the fly walked again, and a few minutes later, it was capable of flying if the fly was induced (Fig. 1C and Table 1). Although full mobility is reached, it may be arguable whether the final body structure of the fly is attained since distinctive wing colours achieved through final tanning are not completed until 24 h (see Table 3 and Discussion). Fig. 1C shows the medfly activity from the beginning of the extrication behaviour until the end of the WSP. Interestingly, the newly emerged imagos never approached the food until the end of the WSP. *C. capitata* started to feed

Table 2
Oxygen consumption during the last days of the pharate stage, the newly emerged adult and mature adult of *C. capitata* male flies. APF: Hours after the puparium formation.

Develpmental stage	Age	N	Insects per replica	Oxygen consumption per fly (µl / min ± SD)
Pharate adult	240 h APF	3	40	0.10 ± 0.01 a
Pharate adult	288 h APF	3	40	0.16 ± 0.01 a
6 h adult	318 h APF	3	20	0.38 ± 0.05 b
Mature adult	5 days [#]	3	20	0.39 ± 0.05 b

[#] Days after adult emergence. ANOVA: Oxygen consumption $F_{3,11}$=48.9 p<0.001. Different letters indicate significant differences between the means (Tukey HSD post hoc, p <0.05). Data provided in Supplementary material.

at 112.22 ± 18.55 min, and 10 min after the first meal ingestion, meconium deposition occurred (122.45 min).

We determined that oxygen consumption in the pharate adults and the first 5 days of the adult stage was significantly different (p <0.001). An increase of 237% in oxygen consumption was registered 6 h after emergence (in which the fly began to move freely) compared to the pharate adult (288 h after puparium formation, APF) (p <0.05) (Table 2).

To determine changes in the levels of the main energy molecules during the last hours of the pharate adult (240 and 288 h APF) and the first 5 days of the adult (10 min, 6 h [318 h APF] and 5 days after emergence), the total content of glycogen, lipids (mainly triglycerides) and trehalose was quantified. The amount of circulating trehalose in haemolymph changed significantly for the ages analysed (p<0.05) (Fig. 2A). During the pharate adult stage, the levels did not change significantly (p> 0.05). However, in the newly emerged adult, the trehalose content was 44% higher than that in the pharate adult (288 h APF) (p<0.01), and 6 h after emergence, the trehalose content was 80% higher (p<0.05). In five-day-old adults, the trehalose levels were similar to those recorded at 6 h. Fig. 2B shows that glycogen levels varied significantly for the analysed ages (p <0.001). Glycogen levels did not change significantly during the analysed pharate adult stage (p> 0.05). However, in the newly emerged adult, we registered a 60% reduction in the glycogen content (p <0.001). Subsequently, the glycogen content increased rapidly in 6-h-old flies (150% higher) compared to the day of emergence (p<0.01) and increased 500% at day 5 (p <0.001) (Fig. 2B). The estimation of total lipid levels significantly changed during the pharate to adult transition (p <0.001) (Fig. 2C). During the pharate adult stage, the levels did not change significantly (p>0.05), and a significant decrease was observed at the time of emergence (p <0.001). The lipid levels increased rapidly at 5 days postemergence (p <0.001).

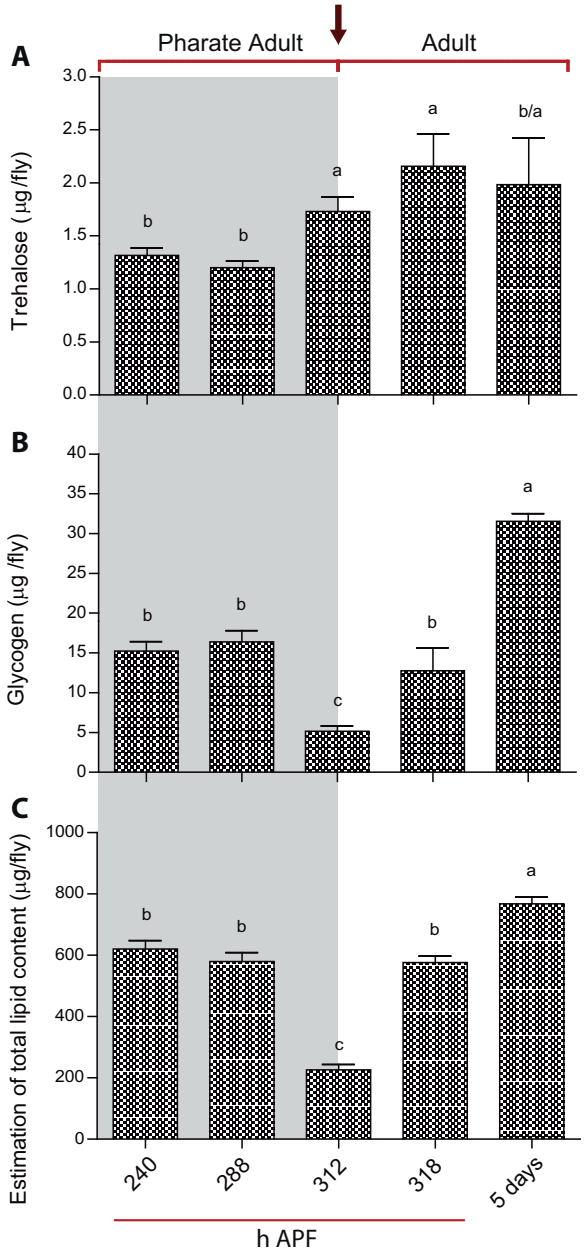

Fig. 2. Mean (± SD) of trehalose (A) and glycogen (B) and the estimation of total lipid content (C) in whole flies during the transition from pharate to mature adults. APF: Hours after the puparium formation. ANOVA: trehalose $F_{4,10}$=5.6 p<0.05; glycogen $F_{4,10}$=35.5 p<0.001; lipids $F_{4,10}$=25.8 p<0.001. Different letters indicate significant differences between the means (Tukey HSD post hoc, p <0.05). Arrow indicates adult emergence. Data provided in Supplementary material.

4. Discussion

The goal of this study was to establish the pattern of events that occur during and after *C. capitata* adult emergence from puparium to fully complete the metamorphosis process. We developed a novel video recording system that records up to 12 flies simultaneously, allowing behaviour, trajectory and resting periods to be analysed in detail. In addition, we proposed a novel scheme of analysis with the incorporation of the Open CL framework intending to increase the performance of analysis, allowing us to evaluate longer videos in less time.

Our results in *C. capitata* are in accordance with the sequence and timing of events during other cyclorrhaphan metamorphoses that appear well conserved among different species (Bainbridge and Bownes, 1981, Rabossi et al., 1991, 1992, Denlinger and Zdarek, 1994). The pattern and timing of adult emergence in the medfly agree with previous studies in *Sarcophaga* performed by Ždárek et al. (1986) and Ždárek and Denlinger (1987) regarding the two phases and their relative duration (approximately 4.3 and 95.7% for the walking phase and the phase without locomotor motion for *Sarcophaga*, respectively, and 6.2 and 93.8% for *Ceratitis*). Unlike the data reported in *S. crassipalpis* (Zdarek and Denlinger, 1987), most of the newly emerged medflies did not interrupt the walking phase and never groomed their head with the forelegs until the end of this phase, when medfly adults stopped.

Here, we also studied the timing of the full cycle of the different stages of *C. capitata*, particularly the span of instars. Curiously, there are many misinterpretations in the literature of the events occurring within the puparium that we established and reported in Table 3. These misinterpretations probably arise from the old habit of calling pupa (a very specific term for an instar) the organism encased within the puparium. The origin of each instar can be defined with high accuracy by the synthesis and deposition of new cuticle proteins. In *Drosophila,* while the

Table 3
Stages of metamorphosis in *C. capitata* since definitive immobilization of the larva (23°C).

Stages of metamorphosis in *Ceratitis capitata* (at 23°C)			
Time (hours)[a]	% [b] (this paper)	Time	Developmental event
0	0	-	Onset of pupariation. White puparium.
16	4.7	-	Puparium fully coloured.
20.5	6.1	-	Larval-pupal apolysis. The insect positively buoyant.
40	11.9	-	Crytocephalic pupa. Onset of pupal stage.
48	14.3	-	Imaginal head sac, wings and legs are everted.
72	21.4	-	Bright yellow-eyes.
160	47.6	-	Pupal-adult apolysis. Onset of pharate adult stage.
192	57.1	-	The red eye pigment (Drosopterin-like) appears.
216	63.3	-	Bristle pigmentation begins dorsally on head and thorax.
240	71.4	-	The tips of the folded wings become grey. Bristle pigmentation begins in abdominal segments. Pigmented bristles are visible on legs.
264	78.6	-	Wings are fully grey. Tarsal bristles darken and claws become dark.
288	85.7	-	Wings become black.
312	92.8	2.07 min	**Puparium extrication**. Imago eclosion.
		6.56 min	**Newly emerged adult walking phase.**
313.6	93.3	1.39 h	**Newly emerged adult motionless phase** (Wings expansion. Wings epidermal cells disappeared. Brownish and black spots of the wings became visible. Definitive body shape is attained).
336	100	24 h	Definitive colour of brown and black spots of the wings. **End of metamorphosis.**

[a] hours since the beginning of pupariation,
[b] accumulated % of time since puparium formation.

electrophoretic patterns of cuticle proteins from first- and second-instar larvae were essentially identical, the patterns for third-instar larvae, pupae, and juvenile adults were each shown to be unique (Chihara et al., 1982). In the medfly, Boccaccio and Quesada Allué (1989) demonstrated that the patterns of SDS-extractable cuticle proteins of third instar larvae, pupae and pharate adults were completely different. As expected, the pharate adult pattern of cuticle proteins did not change significantly after imago emergence (Rabossi, 1991).

The metabolic rates in cyclorrhaphous flies have been demonstrated to decrease after pupariation, remain low during the pupal and beginning of the pharate adult stages and, at the end of this stage, increase again (Merkel et al, 2011). The respiration rate during *C. capitata* metamorphosis has been described as a U- shaped curve by Nestel et al. (2007), and our results showing an important increase in O_2 consumption 6 h after the emergence of the adult seem to coincide with the increase registered by these authors at the end of the pharate stage.

Here, we correlated the first behavioural events with few molecular landmarks. In particular, we determined the period when the flies begin to recover glycogen and trehalose reserves, the main energy reserves of adult males. *C. capitata* undergoes larval development in the pulp of the fruit, and in contrast to insects that develop on protein-rich substrates, adults emerge with relatively low nutrient reserves (Kaspi et al., 2000). The accumulation and variations of energy reserves in different situations in young adult medflies have been published (Nestel et al. 1985, 2003, 2004; Warburg and Yuval, 1996 and 1997, Kaspi et al., 2000). In a previous study, we had analysed the variations in energy molecules during the stages within the puparium of *C. capitata* (Nestel et al., 2003), and in the present work, we extended that study to the period of time limited to the transition from pharate to newly emerged adults. The level of trehalose and the O_2 consumption rate differ significantly between pharate and emerged adult flies. In insects, trehalose is the main circulating energy source; therefore, as expected, the energy demands in the adult transition seem to be covered by this disaccharide. This was the case since the trehalose level was already significantly elevated at the moment of emergence from the puparium. The O_2 consumption rate was increased 237% in 6-h-old adults and surely was also increased in newly emerged adults. However, we were unable to measure O_2 consumption during this developmental stage since the times required in the technique (40 min) exceeded the emergence time (2 min). The rapid decrease in glycogen recorded during adult emergence would cover the demand for the synthesis of circulating trehalose. Thus, between 288 h APF and emergence, glycogen content decreased 68.7%, much faster than lipids (34%). Therefore, the similarity in consumption profiles between lipids and glycogen indicates that, as expected, both reserves are being metabolized to cover the energy demand required for extrication, the initial walking phase, and wing expansion. The important increase in lipids and glycogen content recorded from 6 h emerged adults coincided with the food intake of the males. Several studies have suggested that lipids are synthesized only during larval stages (Garcia et al., 1980, Municio et al., 1973) and that adults have no lipogenic capabilities (Pagani et al., 1980; Nestel et al., 1985). These authors concluded that young adults appear to be incapable of lipid synthesis. However, Warburg and Yuval (1996) and Jacome et al. (1995) registered lipogenic activity in adults of medflies and *Anastrepha serpentina*, respectively. Our results agree with these last authors since after the reduction in the lipid content during emergence, on the fifth day of age, lipid reserves not only recovered but also increased.

Our studies complement previously published studies (Perez et al., 2018) describing the tanning process in the wings of newly emerged adults of *C. capitata*. First, the increased activity of NBAD synthase in the body temporarily coincided with the definitive tanning of the head, thorax, and abdominal cuticles and coincided with the acquisition of a definitive body shape (2 h after adult emergence). Second, the wings of newly emerged flies are highly folded, with a pale and pliable cuticle. At the end of the walking period, expansion of the wings occurs. One hour after ecdysis in adults, the characteristic brown and black spots of *Ceratitis* began to be recognized. However, the definitive pattern of colouration is attained 24 h after emergence (Perez et al., 2018). Our results indicate that while the shape of the body and the final position of the wings would be reached within the first two hours after emergence, the final colouration of the wings was completed after 24 hours. Taking into account these and current results reported here, we propose these events as the real completion/end of *Ceratitis capitata* metamorphosis. We present in Table 3 the timing of the main events of medfly metamorphosis from the formation of the puparium with the addition of new data from the extrication to the final colouration of the wing spots, which we considered the end of the process.

Credit Author Statement

P. A. Bochicchio: Investigation. Experimental set up and video recording analysis. Formal analysis.

M.M. Pérez: Investigation. Review & Editing Writing.

L.A. Quesada-Allué: Resources. Review & Editing Writing.

A. Rabossi: Investigation. Conceptualization. Writing - Original Draft. Review & Editing Writing.

Data availability

Raw Data to reproduce figures are provided in an excel file as supplementary material.

Declaration of Competing Interest

The authors declare no conflict of interest, including financial interests and relationships and affiliations.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2021.100017.

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