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Authors: Nestel, David, Nemny-Lavy, Esther, and Alchanatis, Victor

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GAS-EXCHANGE PATTERNS OF MEDITERRANEAN FRUIT FLY PUPAE (DIPTERA: TEPHRITIDAE): A TOOL TO FORECAST DEVELOPMENTAL STAGE

DAVID NESTEL[1], ESTHER NEMNY-LAVY[1] AND VICTOR ALCHANATIS[2]
[1]Department of Entomology, Institute of Plant Protection, Agricultural Research Organization Volcani Center, P.O. Box 6, 50250 Beit-Dagan, Israel

[2]Department of Sensing, Information and Mechanization, Institute of Agricultural Engineering Agricultural Research Organization, Volcani Center, P.O. Box 6, 50250 Beit-Dagan, Israel

ABSTRACT

The pattern of gas-exchange (CO_2 emission) was investigated for developing Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedemann) pupae incubated at different temperatures. This study was undertaken to explore the usefulness of gas-exchange systems in the determination of physiological age in developing pupae that are mass produced for sterile insect technique projects. The rate of CO_2 emission was measured in a closed flow-through system connected to commercial infrared gas analysis equipment. Metabolic activity (rate of CO_2 emission) was related to pupal eye-color, which is the current technique used to determine physiological age. Eye-color was characterized digitally with 3 variables (Hue, Saturation and Intensity), and color separated by discriminant analysis. The rate of CO_2 emission throughout pupal development followed a U-shape, with high levels of emission during pupariation, pupal transformation and final pharate adult stages. Temperature affected the development time of pupae, but not the basic CO_2 emission patterns during development. In all temperatures, rates of CO_2 emission 1 and 2 d before adult emergence were very similar. After mid larval-adult transition (e.g., phanerocephalic pupa), digital eye-color was significantly correlated with CO_2 emission. Results support the suggestion that gas-exchange should be explored further as a system to determine pupal physiological age in mass production of fruit flies.

Key Words: carbon dioxide emission, sterile insect technique, metabolic rate, pupal respiration, physiological age, irradiation time, digital eye-color

RESUMEN

En el presente estudio se investigaron los patrones de intercambio gaseoso (emisión de CO_2) en pupas de la mosca de las frutas del Mediterráneo (*Ceratitis capitata* Wiedemann) incubadas a diferentes temperaturas. El estudio fue realizado con la finalidad de explorar la utilización de sistemas de intercambio gaseoso en la determinación de la edad fisiológica de pupas durante su producción masiva en proyectos de mosca estéril. La proporción de emisión de CO_2 fue medido en un sistema cerrado de "flujo a través del sistema" conectado a un detector infrarrojo de gases. La actividad metabólica de la pupa (emisión de CO_2) fue contrastado al color del ojo de la pupa en desarrollo, que constituye la actual técnica de determinación de la edad fisiológica. El color de ojos en pupa fue determinado digitalmente, usando tres variables (Tendencia, Saturación e Intensidad). Los colores fueron separados utilizando el análisis discriminatorio. Los patrones de emisión de CO_2 durante el desarrollo de la pupa sugieren una tendencia de U: una alta actividad metabólica durante la fase inicial de pupación y transformación y durante la fase final del adulto. La temperatura de incubación afecto el tiempo de desarrollo pero no el patrón básico de actividad metabólica. La proporción de emisión de CO_2 uno y dos días antes de la emergencia del adulto fue muy similar para pupas mantenidas en las diversas temperaturas. El color digital del ojo de la pupa se correlaciono significativamente con los patrones de emisión de CO_2 detectados a partir de la fase media de la transformación de larva a adulto. Los resultados soportan la utilización de sistemas de intercambio gaseoso como un sistema auxiliar para la determinación de la edad fisiológica en cría masiva de moscas de la fruta.

Translation provided by the authors.

The sterile insect production capacity has greatly expanded during the last decades and evolved into an industrial process. For fruit flies, many mass-produces sterile insects for field release to control the damage exerted by these pest insects; released sterile males copulate with wild fertile females curtailing their ability to produce a new generation of wild flies. The success and increased interest in the SIT, is based on scientific achievements and technological innovations de-

veloped during the last decades, and on the ability to produce and deliver sterile fruit flies of good quality and sexual competitiveness (Fisher 1997; Hendrichs et al. 2002; Tween 2002).

This technological advance has opened the doors for the participation of the private sector in this enterprise, which was solely financed in the past by the public sector. New facilities, some of them based on private capital (such as "Bio-Fly" in Israel and "Insecta" in Europe) are being opened, where labor is expensive and production is capital intensive. These new tendencies in the industrial production process of sterile insects are driving research and development in the direction of process automation, labor saving activities, and the reduction in production uncertainties. Our aim in this study was to support this tendency by exploring the ability of available gas-exchange measuring technologies to forecast important biological events of the developing flies. Specifically, we characterized the gas-exchange patterns of developing pupae and explored analytical methods to forecast and monitor key events in the production process.

Key biological events during mass production of Mediterranean fruit flies (medflies) *Ceratitis capitata* (Wiedemann) for SIT purposes include, among others, egg hatching, larval jumping, pupation (Quesada-Allue et al. 1996), stage of pupal development, and adult emergence. Knowledge on the precise pupal physiological age is of importance for the management of mass-production and for sterilization purposes (Ruhm & Calkins 1981). Medflies are sterilized by exposing pupae to gamma-radiation. The outcome of such exposure on induced sterility and fly quality depends on the physiological age and dose at which pupae are irradiated (Ruhm & Calkins 1981). Pupae irradiated earlier than the optimal time could be damaged, affecting their adult performance and ability to effectively mate (Ohinata et al. 1971). On the other hand, late irradiation could compromise sterility (FAO/IAEA/USDA 2003), leading to the release of fertile flies. These fertile flies may copulate with wild ones, affecting the sterile/wild relationship and effectiveness of the program. In addition, the ability to monitor and forecast the physiological stage of the developing pupae could be of use during the daily activity and working program of mass-rearing facilities. As an example, mass-rearing facilities manipulate the environmental conditions (such as temperature) of developing pupae to synchronize, and adjust, production timing to field logistics and release schedules (Ruhm & Calkins 1981). In this sense, a system able to automatically determine the physiological stage of the developing pupae could serve to manage and set incubation temperatures (i.e., automatically) as required by field timetables.

The current system to determine pupal physiological age consists of removing the pupal case and visually inspecting the color of the eye (FAO/IAEA/USDA 2003). This subjective system is based on the fact that eye-color is known to change with pupal physiological age (Ruhm & Calkins 1981; Resilva et al. 2007). Eye color in developing pupae becomes apparent during mid-pupation with the eversion of the head and the initiation of the "phanerocephalic" pupal stage (Quesada-Allue et al. 1996). At this stage, pupal eyes are whitish. Eye coloration starts to change at the end of the pupal stage and beginning of the pharate-adult stage, with the increase in metabolic activity: at this stage, eyes become yellowish (Quesada-Allue 1994). Subsequently, eye color rapidly changes from yellow to orange, to red, to brown orange and, before adult emergence, to iridescent (Ruhm & Calkins 1981; Quesada-Allue 1994; Quesada-Allue et al. 1996). At 23°C, the whole larval-adult transition may be accomplished in 13 d (Quesada-Allue et al. 1996). At this temperature, the onset of the phanerocephalic stage, where eyes have a whitish coloration, occurs 48 h after larval immobilization, while the finalization of the pupal stage and initiation of the pharate adult stage, with the subsequent acceleration of metabolism and change of eye color to yellow, is observed 120 h (5 d) after larval immobilization (Quesada-Allue et al. 1996).

Recently, Donoso et al. (2003) suggested monitoring temperature in pupal incubation rooms to determine physiological age and time of irradiation. These authors proposed that since insect development is dependent upon temperature (Ratte 1984), degree-day models could be used to determine emergence time and the precise time for irradiation. While this method could provide us with a good approximation of physiological age, it is an indirect estimation of age which is based on parameters estimated under constant conditions. As a result, it could be insensitive to temperature oscillations and other environmental factors inside incubation rooms that could affect development.

Direct measurements of metabolic activity, on the other hand, could provide a more reliable system for the estimation of physiological age. Oxygen consumption and carbon dioxide (CO_2) production in living organisms, as an example, are the direct outcome of metabolic activity. The rates at which these gases are consumed or produced are directly related to the rate at which metabolism proceeds in the organism (Keister & Buck 1973; Lighton & Wehner 1993). Thus, the measurement of gas exchange is expected to be a good indication of metabolic activity. The rate of gas exchange during metamorphosis of some Diptera species (e.g., *Calliphora erythrocephala* Macquart) has already been described and was shown to follow a U-shaped curve (Agrell & Lundquist 1973), with a high metabolic activity during early and late developmental stages and a slow-down during mid-pupal stage. This same U-shaped

curve was described for the oxygen consumption of developing medfly pupae (Langley 1970).

The present study is based on this previously generated knowledge. We suggest that the measurement of metabolic activity in medfly pupae through gas exchange systems could provide us with a reliable tool to forecast physiological age and adult emergence time in mass-rearing facilities. In order to investigate this idea, we first digitally characterized the eye-color of developing medfly pupae as a reference, and to have an objective method of comparison. We then characterized the daily patterns of CO_2 emission on pupae developing at different constant and variable incubation temperatures (ranging from 15 to 30°C). Finally, the ability of the gas-exchange system to determine pupal physiological age was inferred from correlating and contrasting digital eye color with pupal respiration patterns.

Materials and Methods

Study Insects

Larvae were obtained from the colony of the medfly strain 'Sade' (more than 20 years old) of the Board of Fruit and Vegetable Growers, Israel. This is a bisexual strain, reared on artificial diet, which is regularly refreshed with material from the wild (2-3 times a year). Larvae were collected as they were leaving the diet ("crawling phase"). Collections were conducted during a short period of time to synchronize immobilization and pupation.

Digital Determination of Medfly Pupal Eye-color

Approximately 330 pupae from different ages (and developing under different temperatures) were sampled, dissected, and their eyes exposed. Dissected pupae were positioned, always following the same orientation on a mini-stage (5 cm in diameter), where the background was always the same, illumination was provided from the same sources and from the same directions, and shade was reduced by a series of mini-reflectors surrounding the stage. Pupae were photographed at a magnification of 20× with a stereoscopic microscope equipped with a 3-CCD color digital camera (Sony DXC 990P). A small area of the pupal eye was focused and a picture taken. The digital image consisted of 3 components: Red, Green, and Blue (RGB space). The digital image was analyzed with Image-Pro PLUS version 4.5 software (Media Cybernetics, Inc.). The 3 basic color components, RGB, were transformed to an alternative color space, defined by Hue, Saturation, and Intensity (HSI). In the HSI color space, the information on the object's color is expressed mainly through Hue, because this variable is not affected by the illumination intensity. The Saturation expresses the vividness of the color, while the Intensity is affected mainly by the illumination intensity. Eye colors included: white, yellow, orange, red-orange, brown, dark-brown, and iridescent. For each color category we analyzed at least 20 specimens.

Eye color data derived from all the samples were analyzed by "Discriminant Analysis" (Statgraphics 5 Plus 2000, Manugistics, Inc.). This analysis produced 3 canonical variables (F1, F2, and F3) that are derived from the original variables (Hue, Intensity and Saturation). These canonical variables are used to classify the data into groups ("standardized digital eye-color"). Resulting groups were correlated with their respiration patterns.

Respiratory Patterns of Medfly Pupae as Affected by Incubation Temperature

CO_2 emission was measured with a closed flow-through system connected to commercial infrared gas analysis equipment (Model No. S-151, Qubit Systems, Inc., Kingston, Ontario, Canada). Pupae were placed inside a glass Erlenmeyer flask (250 mL), which was hermetically sealed with a rubber stopper with lure connectors. Air was pumped (0.4 L/min) through the flask to collect the pupal-emitted CO_2, which was directed to the infrared carbon dioxide analyzer (Qubit Systems, Model No. S-151, with a resolution of ±1 ppm CO_2). Air emerging from the CO_2 analyzer was pumped back into the Erlenmeyer flask. The whole system was kept closed with vinyl tubing. Closed circulation of air provided the cumulative amount of CO_2 emitted by the pupae in a period of time. In order to measure the rate of CO_2 emission, we recorded its accumulation in a period of 10 min and obtained the rate from the slope. The data-logger connected to the CO_2 analyzer (Vernier Software and Technology, Beaverton, Oregon, USA) generated 1 measurement per min.

We measured the rate of CO_2 production in pupae incubated at several constant temperatures (15, 20, 25, and 30°C), and at variable room temperature (R.T.), with temperatures oscillating between 18-25°C. This design was expected to create different pupal physiological ages independent of chronological age. Each experiment (e.g., replicate) consisted of simultaneously incubating 5 g of recently immobilized larvae at the different temperatures. Incubation in the different environments was kept constant from the moment of larval immobilization until adult emergence.

Gas-exchange measurements took place around mid-day. During the last days of pupal development, there are differences in metabolic rate throughout the day (e.g., circadian rhythms), which tend to increase after mid-day (Nestel, unpublished data). Due to this we kept constant the time of the day at which gas-exchange was measured. Batches of pupae were taken from the incubator, weighed (pupae lose water throughout development, reduc-

ing their initial weight at larval immobilization by approximately 20%, Nestel et al. 2003), placed inside the Erlenmeyer flask, and connected to the flow-through Erlenmeyer flask system to measure CO_2 production during a period of 10 min. At the end of the measurement, pupae were returned to their respective incubator until the following measurement next day. This experiment was repeated 2 to 3 times on different dates. For room temperature (R.T.), we only conducted 1 replicate.

CO_2 emission patterns during pupal development under the different temperatures were obtained by fitting the data to quadratic functions (Statgraphics 5 Plus 2000, Manugistics, Inc.). Average rate of CO_2 production 1 and 2 d before adult emergence were extrapolated from the calculated functions. During all the experiments, we sampled more than 10 pupae per temperature and day. These were dissected and their eye-color determined with the digital ImagePro System. Canonical variables were derived from the original variables (Hue, Intensity and Saturation) as explained earlier, and the derived standardized digital eye-color was later correlated with rate of CO_2 emission during the specific day and temperature regime (see the following section).

Relationship between Standardized Digital Eye-color and Respiration Patterns

Pupae collected in the above experiment were immediately dissected and their eye-color digitally characterized by the 3 variables with ImagePro. Based on the derived canonical variables, a "standardized digital eye-color" was determined for each pupa. The average "standardized digital eye-color" for a specific temperature treatment during a specific date was related to the rate of CO_2 production measured during that day based upon linear regression (Statgraphics 5 Plus 2000, Manugistics, Inc.). Due to the typical U-shape pattern of gas exchange, we decided to investigate this relationship with data on CO_2 emission obtained in all temperatures and replicates from mid-pupal development period until adult emergence. Thus, digital eye-color was correlated with CO_2 emission from d 10 in pupae developing at 15°C, from d 6 in pupae developing at 20°C, from d 4 in pupae developing at 25°C, from day 2 in pupae developing at 30°C, and from d 6 in pupae developing at room temperature (R.T). These dates corresponded in all of the treatments with pupae having white-eyes.

Results

Digital Characterization of Pupal Eye-color and Standardization

After pupariation and the eversion of the head during mid-pupal stage, pupal eyes have a whitish coloration. As pupal development proceeds, eye-color changes from white to yellow, then orange, red-orange, brown, dark-brown, and finally iridescent (slightly before adult emergence). Fig. 1a shows the scatter graph derived from the digital characterization of pupal eye-color using the Hue, Saturation and Intensity system. As can been seen, the colors are not well separated by these 3 variables. As a result we applied a multivariate analysis to separate between groups. The canonical discriminant analysis resulted in 3 significant "canonical variables" able to discriminate between the 7 levels of pupal eye-color (Variable F1, $\chi^2 = 1377$, $df = 18$, $P < 0.01$; Variable F2, $\chi^2 = 651$, $df = 10$, $P < 0.01$; Variable F3, $\chi^2 = 101$, $df = 4$, $P < 0.01$). Fig. 1b shows the clusters of pupal eye colors formed by using the 2 first canonical variables F1 and F2. The ability of canonical variable F1 to predict pupal eye-color from digital data of Hue, Saturation and Intensity used for the calibration is provided by Table 1. The overall classification accuracy was 74.01%. The classification accuracy decreases (to 53% of cases accurately predicted) when the pupal eye-color obtains an iridescent coloration.

Effect of Incubation Temperature upon Pupal Development Time and CO_2 Emission

Fig. 2 shows the daily patterns of CO_2 emission at mid-day as affected by incubation temperature and chronological pupal age. The figure shows actual measurements and the fitted quadratic functions. In all the cases, the fitted quadratic function resulted in a high coefficient of determination (above 0.85). Rate of gas exchange patterns followed a typical U-shape, with a high metabolic activity during the first hours and days of metamorphosis, and a lower level by mid-pupal period. After this drop in metabolic activity, CO_2 emission steadily increases up to adult emergence. Using the resultant quadratic functions obtained for each incubation temperature, we derived the level of CO_2 emission 1 and 2 d before adult emergence by extrapolation (Table 2). Rate of CO_2 emission 2 d before adult emergence ranged from 22.5 to 30.8 nmol CO_2/g of pupae/minute while 1 d before adult emergence it ranged from 30.3 to 39.4 nmol. In elevated incubation temperatures, the rate of CO_2 emission was higher than at lower temperatures. In contrast, at lower temperatures, rate of emission was more reduced (Table 2). At 25 and 30°C, rate of CO_2 emission 1 and 2 d before adult emergence was comparable. At 20°C rate of CO_2 emission was slightly lower than at these 2 temperatures. Incubation at room temperature produced the lowest rates of CO_2 emission for 1 and 2 d before adult emergence.

Relationship between Standardized Pupal Eye-color and Patterns of CO_2 Emission

Fig. 3 shows the relationship between standardize pupal eye-color and rate of CO_2 emission.

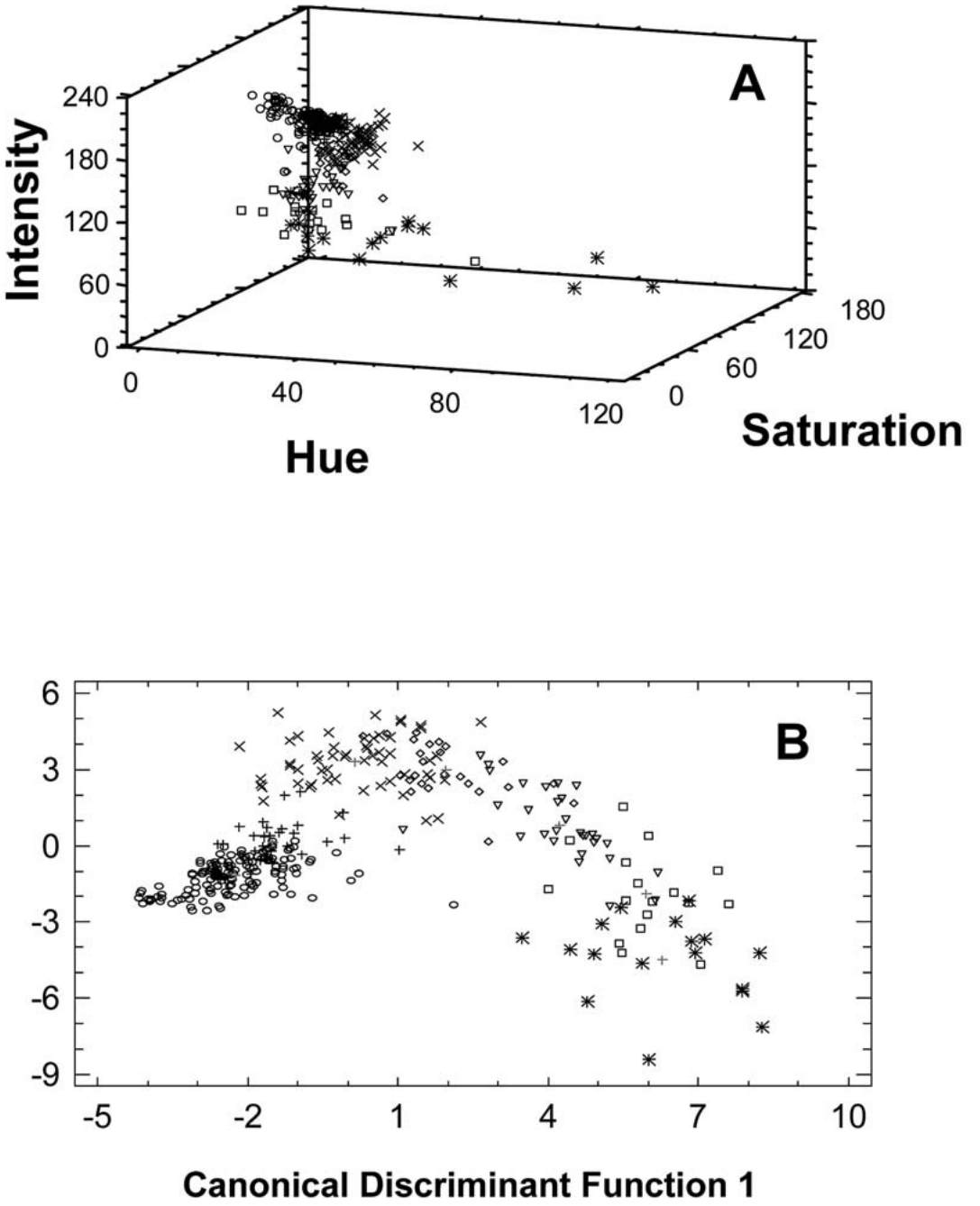

Fig. 1. (A) Scatter plot of medfly pupal eye-color (visually determined) separated by 3 digital parameters (Hue, Saturation and Intensity) derived from the digital-photographic characterization. (B) Classification of pupal eye-color (visually determined) by canonical (unstandardized) variables 1 (F1) and 2 (F2): [F1 = (-0.0272642 × hue) + (-0.0286977 × saturation) + (-0.078355 × intensity) + 15.7097] and [F2 = (-0.010854 × hue) + (0.0815401 × saturation) + (0.04218 × intensity) – 13.5662]. ○ white pupal eye-color, + yellow, x orange, ◊ red-orange, Δ brown, □ dark-brown, * iridescent.

Eye color was standardized based upon canonical variable F1 (see previous section). As explained in the methodology section ("Relationship between Standardized Digital Eye-color and Respiration Patterns"), the relationship between the 2 variables was performed from mid-pupal stage until

TABLE 1. CLASSIFICATION ACCURACY OF EYE COLOR (VISUALLY DETERMINED) BY DISCRIMINANT ANALYSIS BASED ON CANONICAL VARIABLE F1 [F1 = (-0.220486 × HUE) + (0.470136 × SATURATION) + (-1.2807 × INTENSITY)]. THE DIAGONAL ELEMENTS IN THE TABLE REPRESENT THE PERCENTAGE OF CASES ACCURATELY CLASSIFIED.

Actual eye color visually determined	Predicted pupal eye-color from digital parameters						
	White	Yellow	Orange	Red-orange	Brown	Dark-brown	Iridescent
White (n = 150)	73.3	24.0	0.0	0.0	0.0	0.0	0.0
Yellow (n = 33)	15.2	78.8	6.1	0.0	0.0	0.0	0.0
Orange (n = 55)	0.0	3.6	76.4	20.0	0.0	0.0	0.0
Red-Orange (n = 25)	0.0	0.0	1 2.0	76.0	12.0	0.0	0.0
Brown (n = 30)	0.0	0.0	0.0	16.7	70.0	13.3	0.0
Dark-Brown (n = 17)	0.0	0.0	0.0	0.0	17.7	70.6	11.7
Iridescent (n = 17)	0.0	0.0	0.0	0.0	0.0	41.7	52.9

adult emergence (eyes in the pupae are only distinguished after the "phanerocephalic" stage). As can be seen from the figure, there is a good linear relationship between these 2 variables. The coefficient of determination (R^2) was 0.78, suggesting that at least 78% of the variability can be explained by the linear relationship between these 2 variables.

DISCUSSION

The typical U-shape metabolic (and CO_2 emission) pattern during metamorphosis is basically the manifestation of the early histolysis and later histogenesis and differentiation processes of the larval-adult transition (Agrell & Lunquist 1973). During the pre-pupal and early pupal stage, all the machinery involved in tissue histolysis (e.g., proteolytic enzymes) is highly active (Agrell & Lunquist 1973; Rabossi et al. 2000; Tolmasky et al. 2001). This was manifested in our study by the high levels of CO_2 production during the first hours and days of metamorphosis. Following the evagination of the head and thoracic appendages, and formation of definite body proportions (i.e., end of the pupation process), metabolic activity and CO_2 emission drops (Agrell & Lunquist 1973; Rabossi et al. 2000; Tolmasky et al. 2001). At this stage, pupal eyes are whitish. Metabolic activity rises again during the pharate adult stage, when tissues are increasing and further differentiating (Agrell & Lundquist 1973). During this period eyes start to change in coloration (Quesada-Allue 1994). As expected and as previously reported for the medfly (Langley et al. 1972), reduced incubation temperatures delayed the metamorphosis process and the time needed for the completion of development. However, the basic U-shape metabolic patterns did not differ for any of the tested temperatures, and metabolic activity as measured from CO_2 production followed the expected trend.

The ability of the digital system and discriminant function to predict eye-color was within acceptable ranges (overall, more than 70% of the cases were correctly classified). The success of this system to correctly classify pupal eye-color decreased when the eye color became fully iridescent. The main source of misclassifications was with iridescent eyes, when 43% of the cases where classified as dark-brown when our subjective classification was iridescent. The lack of accuracy in this case could be related to our inability of subjectively classifying eye color correctly, or to the inability of the discriminant function to completely separate between these 2 similar classes of eye-color. In any case, this misclassification at a sensitive moment of production highlights the problem of the system to rely on a subjective measurement to make decisions, which is mainly based on the perspective of the human eye.

The significant linear relationships after mid-pupal stage between standardized pupal eye-color and average pupal emission of CO_2 strengthened the working hypothesis of this study that the rate of gas exchange, and therefore of metabolic activity, is an indication of mid-pupal and pharate adult physiological age. The variability of the estimated regression (mainly at advanced pupal ages and larger rates of CO_2 emission) can probably be attributed to 2 aspects: (1) the reduced synchronicity of pupal age resulting from incubations at low temperatures (e.g., 15 and 20°C), and (2) the mentioned misclassification of eye-colors close to adult emergence.

At low temperatures, adult emergence usually extends over a longer period of time (Nestel, unpublished data). In contrast, pupae incubated simultaneously at higher temperatures result in adult emergence occurring more synchronously within a shorter period of time (Nestel, unpublished data). This lack of synchronicity at lower incubation temperatures, and the resulting mixture of physiological ages within the samples may explain the slightly lower rate of CO_2 emission observed 1 and 2 d before adult emergence in pupae incubated at 15°C, 20°C, and room temperature.

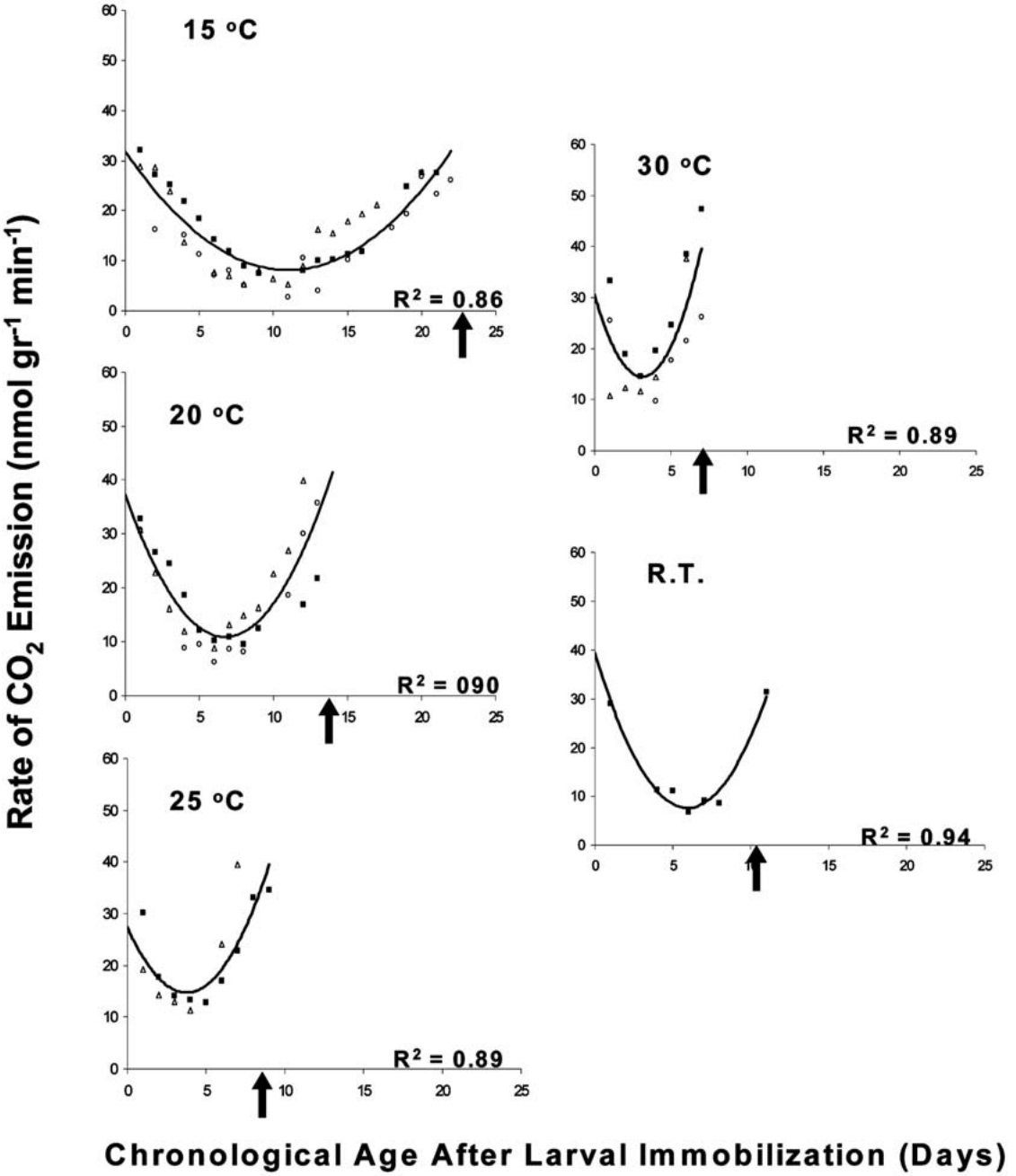

Fig. 2. Patterns of CO_2 emission (rate of emission) by medfly pupae incubated at different constant temperatures throughout development, and at room temperature (variable temperature): 15°C, 20°C, 25°C, 30°C, and R.T. (room temperature). Marks (rectangles, triangles, and circles) show the result of actual CO_2 measurements in each of the replicated experiments. Solid line is the average gas-exchange trend per incubation temperature, and was obtained from quadratic functions (R^2, coefficient of determination for the fitted function). The arrow marks the day before adult emergence.

This aspect requires further studies, corrections and fine-tunings before the gas-exchange system can be suggested for mass-rearing facilities.

The present study was conducted under laboratory conditions and a colony of a bisexual strain was used. Production levels in this colony are

TABLE 2. AVERAGE CO_2 EMISSION 1 AND 2 D BEFORE ADULT EMERGENCE FROM PUPAE INCUBATED AT SEVERAL CONSTANT TEMPERATURES, AND AT VARIABLE ROOM TEMPERATURE. THE LEVEL OF CO_2 WAS DERIVED BY EXTRAPOLATION FROM THE QUADRATIC FUNCTIONS FITTED TO THE DATA (SEE FIG. 2).

Conditions of pupal incubation	nmol CO_2/g of pupae/min	
	2 d before adult emergence	1 d before adult emergence*
15°C (constant temperature)	27.9	32.0
20°C (constant temperature)	27.1	33.6
25°C (constant temperature)	30.8	39.4
30°C (constant temperature)	27.1	38.1
Room Temperature (variable)	22.6	30.3

small, and sample size was tailored to the availability of pupae for experimentation. If the gas exchange system is going to be adopted by the SIT industry however, modifications and adaptation would be required. One possibility includes the sampling of developing pupae at critical stages and measurement of CO_2 emission to determine physiological age in a similar way to the one presented in this study. A completely different approach may include the establishment of incubation rooms in the mass-rearing facilities for advanced pupal ages, and the automatic monitoring of CO_2 accumulation in the room air. These options, and other possibilities, however, would need to be further explored.

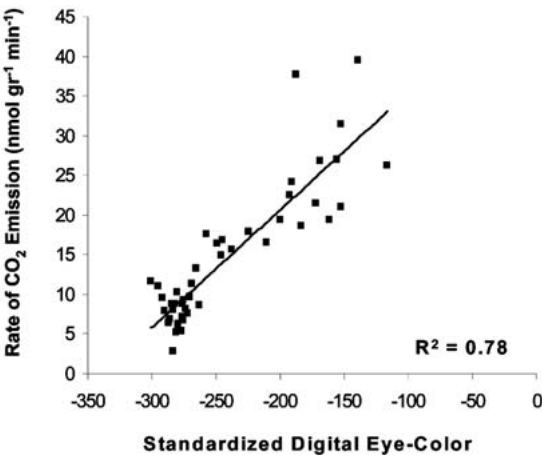

Fig. 3. Relationship between the average "standardized digital pupal eye-color" (derived from canonical variable 1 based on the measured Hue, Saturation, and Intensity) and the rate of emitted CO_2 by pupae. The linear relation was calculated for pupae that have already completed the first half of their development, and that are starting to increase their metabolic rate (i.e., the ascending portion of the polynomial functions in Fig. 2). Data represent measurements of CO_2 and eye-color from pupae developing at several constant temperatures, and at room temperature. R^2 stands for the coefficient of determination for the linear regression.

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