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Postmortem Interval Estimation and Validation Through a Comparative Study of South American Flies Reared in the Field Versus Laboratory Conditions

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Abstract

Studies under constant temperatures are the most common to estimate the Postmortem Interval (PMI). It is imperative that forensic sciences have data from studies carried out in the field. Therefore, this work aims to: (1) evaluate the parameters (weight, length, development time) associated with the life cycles of *Lucilia ochricornis* (Wiedemann) (Diptera: Calliphoridae) and *Lucilia purpurascens* (Walker) under experimental conditions in the field considering fluctuating temperatures, and (2) compare these results with those known and published by the same authors for cultures realized in the laboratory under constant temperatures; which will permit us to contrast the most widely used existing methodologies for forensic application in estimating the minimum postmortem interval (PMI_{min}). For each season of the year, cultures of both species were made in the field, collecting information on temperature, humidity, and photoperiod to perform laboratory cultures, later comparing: development time, length, weight, and Accumulated Degree-Hours (ADH) in both types of cultures. Methods for estimating the PMI were obtained and validated with the information of the cultures grown in the field. The two types of cultures showed differences between each other for both species. The forensic use methods to estimate PMI were enhanced and their precision increased when maximum larval length data were used, and it was also concluded that feeding larval stages are the most accurate to be used in making estimates because the larva is growing. The estimation of the PMI through the use of necrophagous flies development remains reliable for obtaining the PMI_{min}.

Resumen

Estudios bajo temperaturas constantes son los más comunes para estimar el Intervalo Postmortem (IPM). Es imperativo que las ciencias forenses cuenten con datos de estudios llevados a cabo en el campo. Por ello, los objetivos de este trabajo son: (1) evaluar los parámetros (longitud, peso, tiempo de desarrollo) asociados a los ciclos vitales de las moscas *Lucilia ochricornis* (Wiedemann) (Diptera: Calliphoridae) y *Lucilia purpurascens* (Walker) bajo condiciones experimentales en el campo considerando temperaturas fluctuantes, y (2) comparar estos resultados con aquellos ya conocidos y publicados por los mismos autores para cultivos realizados en laboratorio bajo temperaturas constantes; lo cual nos permitirá contrastar las metodologías existentes más utilizadas de aplicación forense en la estimación del intervalo postmortem mínimo (IPM_{min}). Para cada estación del año, se realizaron cultivos de ambas especies en el campo colectando información de temperatura, humedad y fotoperíodo para realizar cultivos en laboratorio, comparándose posteriormente: tiempo de desarrollo, longitud, peso y los Grado-Hora Acumulados (GHA) en ambos tipos de cultivo. Se obtuvieron métodos de estimación del IPM y se los validó con la información de los cultivos realizados en campo. Los dos tipos de

cultivos mostraron diferencias entre sí para ambas especies. Los métodos de uso forense para estimar el IPM aumentaron su precisión cuando se usaron datos de longitud larval máximos, asimismo se concluyó que los estadios larvales alimentarios son los más precisos para ser usados en la realización de estimaciones debido a que la larva está creciendo. La estimación del IPM a través del uso del desarrollo de moscas necrófagas sigue siendo fiable para la obtención del IPM_{min}.

Key words: PMI, fluctuating temperature, constant temperature, Calliphoridae, *Lucilia*

Palabras clave: IPM, temperatura fluctuantes, temperatura constantes, Calliphoridae, *Lucilia*

Forensic science increasingly demands the development of reliable approaches regarding useful tools for the discipline. One of the major concerns is the estimation of time to death (Faris et al. 2020), which is generally expressed through the Minimum Postmortem Interval (PMI_{min}). PMI_{min} refers to the minimum time between colonization of a carcass by insects and its discovery (Merritt 2020). Most of the studies carried out to obtain data to estimate the PMI_{min} are fulfilled by culturing preimaginal stages of flies in the laboratory, at constant temperatures (Chen et al. 2019). Therefore, one way to validate the methods and results obtained in the laboratory is to compare them with those obtained in the field, under natural conditions, where the temperature fluctuates daily (Anderson 2000, Tarone and Foran 2008, Lecheta et al. 2015, Yang et al. 2016, Faris et al. 2020).

To predict the activity and density of insects, it is essential to be able to understand the variation of the climatic factors that act on them (Speight et al. 2008). Thus, biotic and abiotic factors such as temperature, precipitation, humidity, and photoperiod influence the rate of development of organisms (Speight et al. 2008, Wells and LaMotte 2010, Bauer et al. 2020). This rate of development provides essential information for understanding the ecological dynamics of insects. Of those variables, the temperature is the most relevant and has been intensively studied (Ikemoto and Takai 2000, Wu et al. 2015).

On the other hand, the photoperiod effect on the development and ecology of insects that live in seasonal climates, for example, the length of the day provides information on the progression of the seasons and conditions for growth and development rates of the insects, allowing them to synchronize efficiently with the most favorable conditions (Speight et al. 2008, Bauer et al. 2020). However, to date, very little progress has been made in studying its effect on species of forensic importance (Bauer et al. 2020).

In forensic entomology, it is often assumed that a microclimate exists around the different stages of development of an insect. In this way, it is possible to correlate this microclimate with the ambient temperature, assuming a constant average temperature, and thus estimate the age of the insect (Grassberger and Reiter 2001). One problem is that ambient temperatures fluctuate daily and seasonally, making it difficult to estimate the PMI_{min} using data obtained at a single temperature (Yang et al. 2016, Chen et al. 2019). For this reason, data obtained at different constant temperatures are often used to make these estimates (Bourel et al. 2003). Thus, different methods involve either: (1) the analysis of the size of the larvae (length and weight), such as isomegalendiagrams (Reiter 1984, Grassberger and Reiter 2001) or growth models (Day and Wallman 2006, Lecheta and Moura 2019); (2) the analysis of the total times for each development event, such as isomorphodiagrams (Grassberger and Reiter 2001), or thermal summation models that involve the calculation of the Accumulated Degree-Hours (ADH) (Ikemoto and Takai 2000, Kipyatkov and Lopatina 2010).

Alternatively, there are more sophisticated methods (mathematical models, molecular genetic techniques, among others) for estimating the time of death using insects of forensic interest, these methods are just being developed and few are feasible to replicate widely and systematically in Latin America (Acosta et al. 2021).

If we define precision as the ability of the prediction interval to cover the real age (Faris et al. 2020), the analysis of the entomological evidence to estimate the PMI_{min} is one of the most precise available and can be applied long after the first days after the death of an individual, contrary to the methods available to pathologists (Bauer et al. 2020).

Blowflies (Diptera: Calliphoridae) are necrophagous flies that arrive on the corpse, often within minutes after death. This family of flies is one of the most important at the forensic level for the estimation of PMI, and those of the genus *Lucilia* (Robineau–Desvoidy) stand out (Byrd and Tomberlin 2020). In Argentina, two native species of *Lucilia* have been described (*L. ochricornis* and *L. purpurascens*), and have been actively studied in recent years (Acosta et al. 2020a, b, 2021) to uncover aspects of forensic importance in entomological investigations.

Thus, this work aims to: (1) evaluate the parameters (weight, length, development time) associated with the life cycles of *L. ochricornis* and *L. purpurascens* under experimental conditions in the field considering fluctuating temperatures, and (2) compare these results with those known and published by the same authors for cultures realized in the laboratory under constant temperatures; which will permit us to contrast the most widely used existing methodologies for forensic application in estimating the minimum postmortem interval (PMI_{min}).

Materials and Methods

Obtaining Specimens and Field Experimental Design

L. ochricornis and *L. purpurascens* were captured in the months of January, April, and October 2018 for the autumn, spring, and summer experiments, respectively. The catches were made in the town of La Caldera (24° 35' 57 "S, 65° 22' 22"W) in Salta province, Argentina.

The methods for capturing adult flies, obtaining eggs, and establishing cultures are extensively described in Acosta et al. (2020b, 2021). Briefly, during the spring, summer, and autumn seasons, female flies of both species were captured in their natural habitat and placed in individual polyethylene vessels, with fresh liver inside to achieve oviposition. Immediately after the hatching, 750 larvae from different mothers were divided into three replicates of 250 each. In each replica, the larvae were placed on a 300 g piece of fresh liver inside an aluminum foil package that opens to the outside at its upper end. Each package was placed in a translucent plastic container with a 3 cm high layer of soil sterilized at 180°C and covered with a mesh cloth lid to facilitate breathing.

In each replication, from the emergence of 50% of the larvae, 10 larvae were collected from a different portion of the bait every

12 hr until reaching the feeding peak and they stopped feeding. To facilitate stretching, these larvae were immersed in hot water at 80°C (Tantawi and Greenberg 1993) for 3–5 s depending on the size of the larva, and were subsequently fixed in 70% ethyl alcohol. A change of larval instar (verified through the number of slits on the posterior spiracle) was registered when this phenomenon occurred in more than 50% of the collected individuals. Once the burial phenomenon of the III instar larvae began, the time in which the postfeeding larvae (PF) took to form the first pupa was recorded with daily morning observations, as well as the time it took for the first adult to emerge.

Morphometric data (length and weight) were obtained following Adams and Hall (2003) and Donovan et al. (2006) within the first hour of material collection. Body length was recorded by software using a Celestron MicroCapture PRO v2.3 digital microscope and weight with Acculab ALC precision analytical balance with a resolution of 0.0001 g.

Three cultures per species at fluctuating temperature regimes in the field (natural environmental conditions) were carried out on a property belonging to the Universidad Nacional de Salta, during each season of the year, except winter. The cultures were placed inside a 40 × 30 × 30 cm cage with mosquito net sides (Fig. 1A), thus avoiding the access of other animals, especially flies that could carry out new ovipositions. The cages were placed in a wired and secured enclosure for protection against scavenging vertebrates (Fig. 1B). The latter had a transparent greenhouse plastic roof and a black half-shade that had the function of avoiding insolation and the heavy rainfall that occurs during the summer. The entire enclosure was directly exposed to the outside environment (Fig. 1C). There, temperature, humidity, and light were recorded as climatic variables throughout the experiments. The temperature and humidity of the experimentation site were recorded every half hour using a CEM Brand Data Logger, DT-171, and the light every 60 seconds through a Luxmeter with CEM Brand Data Logger, DT-8809-A, both instruments placed in situ.

The experiments from cultures could not be carried out during the winter because the studied species do not reproduce during that period of the year (Acosta et al. 2020a) and the method of maintaining their populations in the laboratory is still unknown. Despite this, the data of the climatic variables in that season of the year were recorded for 62 d (1 July 2017 to 31 Aug 2017) to know the extreme values that would limit the activity of these flies.

Fig. 1. Establishment of cultures in the field (at fluctuating temperatures): (A) entomological boxes with cultures protected from external ovipositions, (B) enclosure prepared to protect against scavengers, and (C) panoramic view of the enclosure in the natural environment.

Obtaining the Information of Laboratory Cultures (Constant Temperatures)

The results obtained here were compared with those already known and published by the authors at constant temperatures (in laboratory) (see Acosta et al. 2020b, 2021). These allowed us to make estimates of the PMI_{min} from different forensic application methods. Thus, the information of length and body weight, isomegalen diagram, and Logistic growth model from the work of Acosta et al. (2020b) was used; and the development time, Isomorphodiagram, Thermal summation model 1 (Kipyatkov and Lopatina 2010), and Thermal summation model 2 (Ikemoto and Takai 2000) of Acosta et al. (2021).

Data Analysis from Field Cultures (Fluctuating Temperatures)

Several Multivariate Analysis of Variance (MANOVA) were performed using the SPSS program version 25 (IBM 2017) at a significance level of $P < 0.05$. When a MANOVA indicated a significant combination effect, Tukey's honestly significant difference (HSD) post hoc test was used to test pairwise differences.

Thus, a MANOVA was used to analyze the differences for the climatic variables considered in each season of the year studied. In this way, comparisons were made between the means of the daily averages, amplitudes, minimum and maximum for the variables of temperature and humidity. In the same way, the analysis was carried out for the variable light, the start and end times of the day, the amplitude of hours of light (photoperiod), and the average daily lux.

MANOVA were also carried out to detect if there were differences between species and between phenological seasons analyzed concerning the data of development time, length and body weight, and ADH for all instar/stages and total development time for each species.

The growth curves for each species and per season were constructed using the means and their associated standard deviations of the length and weight data measured every 12 hr, from the hatching of the larvae to the feeding peak.

ADHs were obtained for cultures at fluctuating temperatures, adding the temperature (°C) at each hour during the period of each instar/stage and the total development time, because field temperatures undergo variations with the change of the hours of the day.

Data Analysis to Compare Between Field (Fluctuating Temperatures) and Laboratory (Constant Temperatures) Cultures

Several MANOVA analyses were performed to detect if there were differences between the different types of cultivation (field-laboratory) for each species, concerning the data of development time, body length, body weight, and ADH for all instar/stages and total development time.

A Principal Component Factor Analysis using the SPSS program (IBM 2017) was used to comparatively validate the results obtained in the field and the laboratory, taking into account the development time. This analysis gathers the common variability explained by correlated variables by reducing the number of variables in factors or components (Arriaza-Balmón 2006). For this, the development times of each instar/stage obtained were used, on the one hand, for each of the seasons (autumn, spring, and summer) of the field cultures, and on the other hand, for the cultivation temperatures in the laboratory (13.4, 23.6 and 22.3°C, respectively). Subsequently, a Simple Linear Regression was performed, using the Past 3.23 program (Hammer et al. 2001), using as an independent variable the component extracted from the fluctuating temperature data and as a dependent variable the component obtained by the data at constant temperatures. These analyses were carried out for each species separately.

Comparison of Methods to Estimate PMI

For each season and species, from the cultures at fluctuating temperatures, mean values of each instar/stage were extracted for: (1) length and weight, with their associated exact development times; (2) the total time spent by each stage of development and its associated ADHs. These were contrasted with the time estimate provided by the different PMI estimation methods already obtained for the species under study: Isomegalendiagram, Logistic growth model, Isomorphodiagram, Thermal summation model 1, Thermal summation model 2.

Then, the same procedure was performed, but using only the individual maximum values of larval length (among the three replicates) for each species. Body weight data were not used because for this variable only the mean values were obtained per sample and not individual values for each larva.

To evaluate which method was more appropriate for each case (data on length, weight, ADH, or total times of each stage), the following criteria were established:

- For the larval feeding stages (Instar I, Intar II, and Instar III) a tolerance deviation of ±3 hr was established for the value obtained by the methods.
- For the postfeeding stages (PF Larva, Pupa) and the Total time, a tolerance deviation of ±72 hr was established concerning the value obtained by the methods, since these stages usually carry a larger error in the estimation of the PMI.
- If the method analyzed for a given instar/stage and station fell within the established ranges, an asterisk was added.
- In turn, if the method, in addition to being within the allowed ranges, was the one that most accurately approached the data obtained at fluctuating temperatures, a second asterisk was added.

Subsequently, the number of asterisks accumulated by each method was divided by the total number of asterisks for each case. In this way, the method that obtained the highest value was selected as the most accurate.

The value of 3 hr and 72 hr were selected based on consultations with forensic experts with extensive experience in Argentina (Centeno Néstor and Ayón Rosana, pers. Com.) and because in that period the chance of a change from one instar/stage to the next is minimal, based on previous experiments (Acosta, pers. obs.).

Results

The experiment during the autumn lasted 73 d (from 25 April 2017 to 4 July 2017), in spring 49 d (from 30 October 2017 to 18 December 2017), and in summer 53 d (from 21 January 2018 to 15 March 2018).

Climatic Variables

For each season of the year studied, the mean values of the daily average, the amplitude, the minimum, and the maximum for temperature and humidity were collected (Table 1). The MANOVA analysis ($F = 30.024$, $P = 0$) showed differences of statistical significance between the seasons of the year with respect to the amplitude ($F = 14.808$, $P = 0$) and the daily average of the temperature ($F = 117.948$, $P = 0$), as well as for the amplitude ($F = 10.597$, $P = 0$) and the daily average of the humidity ($F = 27.147$, $P = 0$) (Table 1). During spring and summer, there were no differences in the daily average temperature. In winter the highest temperature and humidity amplitude was registered, also presenting the lowest daily average humidity.

Table 1. Temperature and humidity in the four seasons of the year. Different letters in the same column indicate differences of statistical significance (Tukey's test, $P < 0.05$)

Season	Temperature (°C)								Humidity (%)							
	Daily average		Amplitude		Minimum		Maximum		Daily average		Amplitude		Minimum		Maximum	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Autumn	13.4**a**	2.8	14.6**a**	6.7	7.9	3.7	22.5	5.4	74.9**a**	7.3	40.2**bc**	15.9	50.0	15.6	90.1	2.6
Winter	15.1**b**	3.4	19.9**b**	8.4	7.8	3.9	27.7	7.1	63.4**b**	10.2	48.8**a**	14.2	34.7	15.7	83.5	6.0
Spring	23.6**c**	2.6	14.9**a**	5.0	17.6	1.7	32.5	5.4	69.9**c**	8.7	42.3**b**	12.0	45.4	1.9	87.7	4.1
Summer	22.3**c**	2.2	12.5**a**	3.5	17.3	1.5	29.7	3.5	76.0**a**	6.2	35.1**c**	8.7	54.6	9.1	89.7	2.8

The MANOVA for the variable light ($F = 29.767$, $P = 0$) showed differences of statistical significance both for the amplitude ($F = 446.047$, $P = 0$), as well as for the beginning ($F = 253.963$, $P = 0$) and the end of the day in each season ($F = 297.883$, $P = 0$). The daily light averages did not show differences between stations ($F = 1.117$, $P = 0.345$) (Table 2). It should be noted that spring was the season with the greatest number of hours of light; whereas the autumn had the fewest.

Table 2. Light in the four seasons of the year. Different letters in the same column indicate differences of statistical significance (Tukey's test, $P < 0.05$)

Season	Light			
	Daily Average (lux)	Amplitude (h)	Start (h)	End (h)
Autumn	1916**a**	10:35**a**	8:02**a**	18:37**a**
Winter	2292**a**	10:52**b**	8:02**a**	18:55**b**
Spring	2276**a**	13:15**c**	6:39**b**	19:57**c**
Summer	1985**a**	12:25**d**	7:13**c**	19:47**d**

Field Cultures (Fluctuating Temperatures)

Development Time

There are differences between both species in the development time of each instar/stage and in the Total time ($F = 7.987$, $P = 0.007$). So too, differences in this variable between stations were evidenced for *L. ochricornis* ($F = 19.993$, $P = 0.005$) and *L. purpurascens* ($F = 12.865$, $P = 0.012$) in: the I Instar ($F_{Lo} = 22.400$, $P = 0.002$; $F_{Lp} = 99.200$, $P = 0$), the II Instar ($F_{Lo} = 19.111$, $P = 0.002$; $F_{Lp} = 9.538$, $P = 0.014$), the III Instar ($F_{Lo} = 96.800$, $P = 0$; $F_{Lp} = 45.778$, $P = 0$), the PF Larva ($F_{Lo} = 7.339$, $P = 0.024$; $F_{Lp} = 74.400$, $P = 0$), the Pupa ($F_{Lo} = 486.093$, $P = 0$; $F_{Lp} = 195.928$, $P = 0$) and the Total time ($F_{Lo} = 270.343$, $P = 0$; $F_{Lp} = 84.452$, $P = 0$) (Table 3). For *L. ochricornis* no differences were exhibited between spring and summer until Larva III; however the Pupa and Total time differed markedly between all seasons, taking more total development time in the autumn and less in the spring. For *L. purpurascens* there were no differences between spring and summer. In the same way, as in the other species, a longer development time was needed in the autumn, although the time to pupate (represented by the PF Larva) was much shorter in the autumn than in the warm season (spring–summer).

Body Length

The MANOVA showed that there were differences regarding the body length of the larvae between species ($F = 21{,}781$, $P = 0$). However, when analyzing the effect of the seasons for each instar concerning length, no differences of statistical significance were found for *L. ochricornis* ($F = 3.189$, $P = 0.051$), or *L. purpurascens* ($F = 1.489$, $P = 0.275$) (Table 4).

Regarding the relationship between body lengths and development times in each species (Fig. 2), in general, the warm season (spring–summer) resulted in maximum lengths reached more quickly, especially for *L. ochricornis* in the spring. On the contrary, the low temperatures in autumn significantly slowed development times for both species.

Body Weight

The body weights of the larvae between both species showed differences of statistical significance ($F = 56{,}360$, $P = 0$). In each species, there was a difference between seasons. For *L. ochricornis* ($F = 11.065$, $P = 0.001$) this occurred in all larval stages: I Instar ($F = 53.444$, $P = 0$), II Instar ($F = 8.997$, $P = 0.016$) and III Instar ($F = 9.203$, $P = 0.015$). For *L. purpurascens* ($F = 4.015$, $P = 0.026$), this only ocurred in Larva I during the autumn ($F = 15.621$, $P = 0.004$) (Table 5).

When analyzing both species, a common pattern was found in the relationship between development time and body weight (Fig. 3); maximum body weights were quickly reached in the warm season (spring–summer), with the development time for *L. ochricornis* being even shorter in the spring. In the spring season, the maximum body weights were not recorded due to the rapid growth. On the contrary, the low autumn temperatures mean that a greater amount of time is needed to reach the maximum body weights of each species (Fig. 3).

Accumulated Degree-Hours (ADH)

ADHs differed between species ($F = 46{,}200$, $P = 0$). Differences were also found between ADHs for *L. ochricornis* ($F = 336.552$, $P = 0$) and *L. purpurascens* ($F = 1182.449$, $P = 0$) between stations (Table 6). In the different instar/stage of development, the differences were evidenced in the I Instar ($F_{Lo} = 402.732$, $P = 0$; $F_{Lp} = 125.130$, $P = 0$), the II Instar ($F_{Lo} = 58.538$, $P = 0$; $F_{Lp} = 6.130$, $P = 0.028$), the III Instar ($F_{Lo} = 1482.334$, $P = 0$; $F_{Lp} = 144.903$, $P = 0$), the PF Larva ($F_{Lo} = 11.968$, $P = 0.08$; $F_{Lp} = 1313.376$, $P = 0$), the Pupa ($F_{Lo} = 117.128$, $P = 0$; $F_{Lp} = 12.170$, $P = 0.08$) and the Total ADH for *L. ochricornis* ($F = 151.915$, $P = 0$). In the case of *L. purpurascens*, no differences were recorded in Total ADH ($F = 0.126$, $P = 0.884$), which indicates that the total ADH for this species was stable, although it varied between instar/stage.

Comparison Between Types of Cultures (Field Vs. Laboratory)

When the development times were compared at fluctuating (field) and constant temperatures (laboratory), it was observed that there were differences of statistical significance in both species, with the

development time at fluctuating temperatures being longer for both *L. ochricornis* ($_{Lo}$) ($F = 113.820$, $P = 0$) as for *L. purpurascens* ($_{Lp}$) ($F = 20.807$, $P = 0$). In each species these differences were mainly evidenced for the III Instar ($F_{Lo} = 14.400$, $P = 0.003$; $F_{Lp} = 11.139$, $P = 0$), PF Larva ($F_{Lo} = 11.532$, $P = 0.005$; $F_{Lp} = 75.783$, $P = 0$), Pupa ($F_{Lo} = 63.746$, $P = 0$; $F_{Lp} = 71.876$, $P = 0$), and the Total time ($F_{Lo} = 115.872$, $P = 0$; $F_{Lp} = 84.276$, $P = 0$). There were no differences of statistical significance in the I Instar ($F_{Lo} = 2.667$, $P = 0.128$; $F_{Lp} = 0$, $P = 1$) and in the II Instar ($F_{Lo} = 0.212$, $P = 0.654$; $F_{Lp} = 0.286$, $P = 0.603$).

The Factorial Analysis of main components of development time extracted for each species a single component for each type of culture (field/laboratory). Thus, for each species, all the treatments (seasons/constant temperatures) included in each component were equally represented. On the one hand, the component of field cultures explained that 99.5% and 97.2% of the total variance for *L. ochricornis* and *L. purpurascens*, respectively. On the other hand, the component of laboratory cultures represented 99.9% and 99.8% of the total variance for each species, respectively. The results of the Linear Regression Analysis were highly significant ($P = 0$) and showed a positive relationship. These analyses showed a linear relationship between the types of cultures: $R^2 = 0.997$ for *L. ochricornis* and $R^2 = 0.995$ for *L. purpurascens*.

Comparisons of body length measurements showed that there were no differences between the types of cultures for both species ($F_{Lo} = 1.003$, $P = 0.431$; $F_{Lp} = 2.524$, $P = 0.117$). Conversely, body weight does show differences between the types of cultures, for *L. ochricornis* ($F = 16{,}982$, $P = 0$) that only occurred in the I Instar ($F = 56{,}889$, $P = 0$); and for *L. purpurascens* ($F = 11.556$, $P = 0.001$) occurred in the I Instar ($F = 19.565$, $P = 0.001$), II Instar ($F = 11.535$, $P = 0.005$) and III Instar ($F = 6.989$, $P = 0.022$).

Regarding ADH, these also differed between the types of cultures for *L. ochricornis* ($F = 265.717$, $P = 0$) and for *L. purpurascens* ($F = 71.205$, $P = 0$) in: the I Instar ($F_{Lo} = 100.210$, $P = 0$; $F_{Lp} = 9.686$, $P = 0.009$), the II Instar ($F_{Lo} = 25.005$, $P = 0$), the III Instar ($F_{Lo} = 578.265$, $P = 0$; $F_{Lp} = 337.407$, $P = 0$), the PF Larva ($F_{Lo} = 108.293$, $P = 0$; $F_{Lp} = 291.032$, $P = 0$), the Pupa ($F_{Lo} = 151.191$, $P = 0$; $F_{Lp} = 39.773$, $P = 0$) and the Total time ($F_{Lo} = 426.842$, $P = 0$; $F_{Lp} = 220.766$, $P = 0$); not so for Larva II ($F = 3.994$, $P = 0.069$) of *L. ochricornis*.

Comparison of Methods to Estimate PMI

Applying the procedures provided by each method, the PMI_{min} estimates were obtained, which are summarized in Table 7 for *L. ochricornis*, and Table 8 for *L. purpurascens*.

For *L. ochricornis*, the Isomegalen diagram method (7/12 = 0.58) was the one that best approached the values of the data obtained under fluctuating temperatures for body length. For body weight, the Logistic growth model method (7/13 = 0.54) was more appropriate. For the larval feeding stages, the Isomorphodiagram method (8/18 = 0.45) was the best for the total development time data, whereas the Thermal summation model 1 (6/15 = 0.40) was the best for the postfeeding stages.

For *L. purpurascens*, the Isomegalen diagram method was the one that best approximated the data values obtained under fluctuating temperatures for length (12/22 = 0.55) and body weight (6/8 = 0.75), whereas the method of Thermal summation model 1 (8/16 = 0.50) was the best method for the data of total development times for the larval stage until the feeding peak. Finally, the Thermal summation model 2 method (4/10 = 0.40) was adequate when considering the postalimentary stages.

Table 3. Mean development time (hours) of *Lucilia ochricornis* (Lo) and *Lucilia purpurascens* (Lp) between seasons. Different letters in the same column indicate differences of statistical significance (Tukey's test, $P < 0.05$)

Season	Time (h)																			
	I Instar Lo		I Instar Lp		II Instar Lo		II Instar Lp		III Instar Lo		III Instar Lp		Postfeeding larva Lo		Postfeeding larva Lp		Pupa Lo		Pupa Lp		Total Lo		Total Lp	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Autumn	36a	0	36a	0	40a	6.9	40a	6.9	68a	6.9	68a	6.9	240a	0	240a	0	776a	36.7	744a	24.0	1160a	36.7	1128a	24.0
Spring	12b	0	12b	0	12b	0	16b	6.9	24b	0	32b	6.9	132b	24.0	312b	0	232b	13.9	288b	48.0	412b	27.7	660b	48.0
Summer	20b	6.9	16b	6.9	16b	6.9	20b	6.9	24b	0	24b	0	172ab	55.4	300b	83.1	336c	0	336b	0	568c	55.4	696b	83.1

Table 4. Mean values of body length of each instar of *Lucilia ochricornis* (Lo) and *Lucilia purpurascens* (Lp) in different seasons of the year. There were no statistically significant differences between them

	Length (mm)											
Season	I Instar Lo		I Instar Lp		II Instar Lo		II Instar Lp		III Instar Lo		III Instar Lp	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Autumn	2.878	0.497	3.121	0.525	5.944	1.129	6.860	1.224	11.710	1.947	13.959	2.640
Spring	2.361	0.330	3.518	0.910	5.385	0.224	7.117	2.044	12.394	1.276	14.324	2.832
Summer	3.065	0.589	2.840	0.795	5.855	1.481	6.850	1.373	12.345	2.032	14.076	2.585

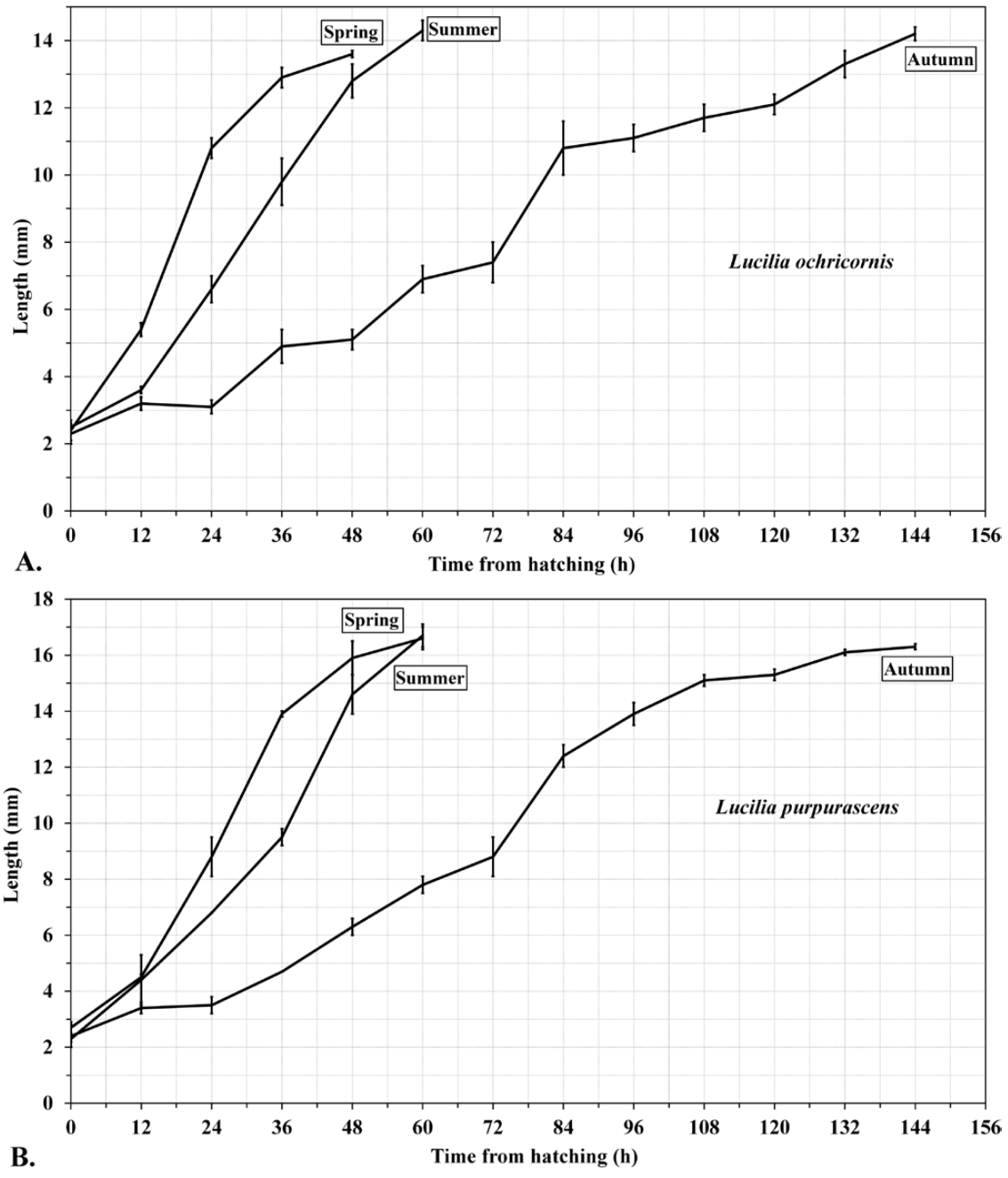

Fig. 2. Growth curves for body length from young in the field in different seasons of the year as a function of developmental time (in hours and its standard deviation), corresponding to (A) *Lucilia ochricornis* and (B) *Lucilia purpurascens*.

Table 5. Mean values of body weight of each instar of *Lucilia ochricornis* (Lo) and *Lucilia purpurascens* (Lp) in different seasons of the year. Different letters in the same column indicate differences of statistical significance (Tukey's test, $P < 0.05$)

	Weight (g)											
Season	I Instar Lo		I Instar Lp		II Instar Lo		II Instar Lp		III Instar Lo		III Instar Lp	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Autumn	0.0012**a**	0.0005	0.0013**a**	0.0006	0.0044**a**	0.0016	0.0052**a**	0.0024	0.0288**a**	0.0103	0.0388**a**	0.0150
Spring	0.0002**b**	0.0001	0.0006**b**	0.0004	0.0020**b**	0.0002	0.0058**a**	0.0040	0.0240**b**	0.0086	0.0404**a**	0.0216
Summer	0.0005**b**	0.0004	0.0005**b**	0.0004	0.0041**a**	0.0020	0.0052**a**	0.0029	0.0292**a**	0.0142	0.0354**a**	0.0176

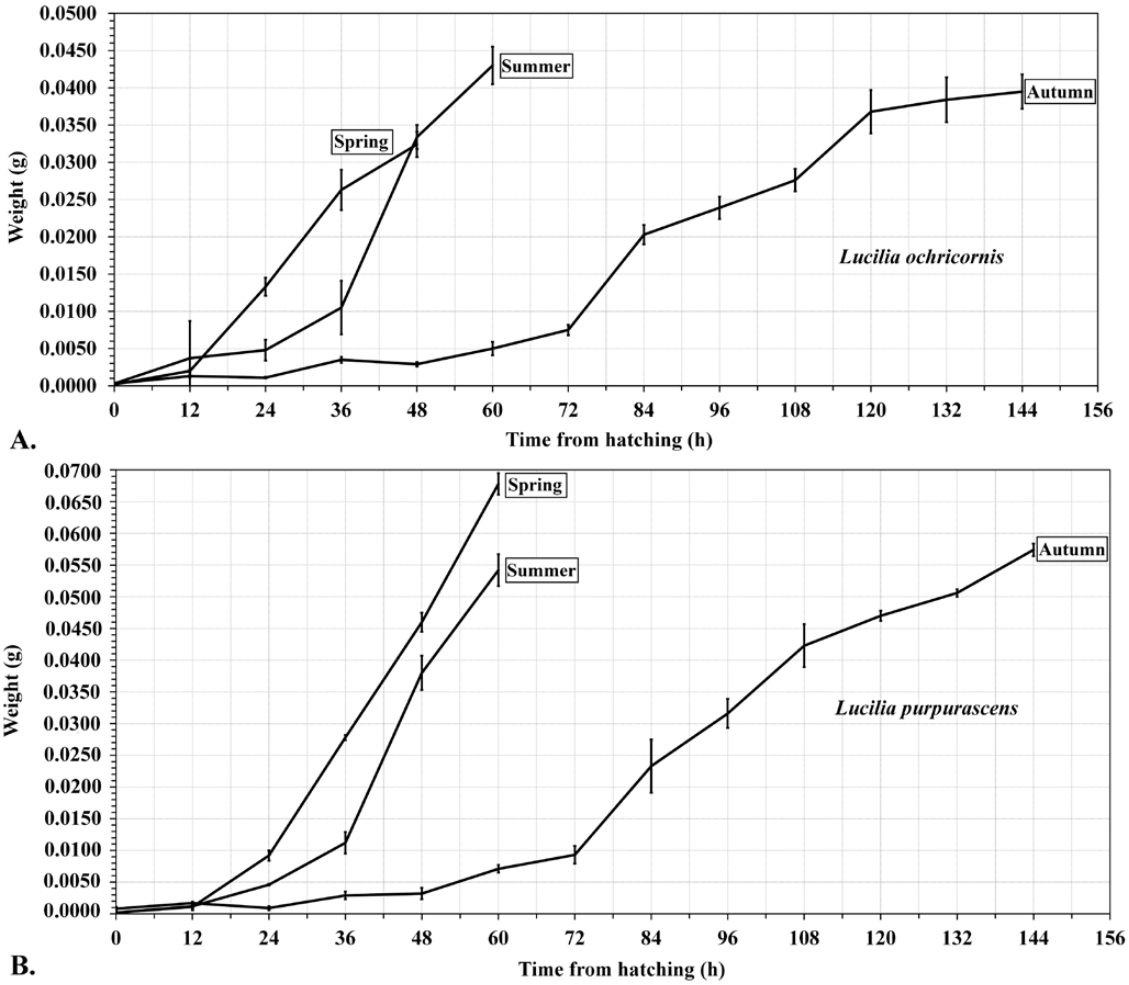

Fig. 3. Growth curves for body weight from young in the field in different seasons of the year as a function of developmental time (in hours and its standard deviation), corresponding to (A) *Lucilia ochricornis* and (B) *Lucilia purpurascens*.

In general terms, field data underestimates body length, whereas it overestimates body weight. When considering the maximum measurements made for body length (Table 9), the method that best approximates the field data was the Isomegalendiagram, both for *L. ochricornis* (11/20 = 0.55) and *L. purpurascens* (14/23 = 0.61). By using these extreme values, more precise data is obtained, increasing the number of positive fits to the models (number of asterisks).

Discussion

The results obtained in this work are among the first to be recorded from breeding experiments of native species of forensic importance, under natural conditions in the field and exposing them to real fluctuating temperatures. In this way, they differ from other published studies where fluctuating ambient temperatures in the brood chamber are simulated in a laboratory by programming increasing and decreasing temperatures for various hourly ranges (Niederegger et al. 2010, Chen et al. 2019, Faris et al. 2020), the latter still being scarce in the literature (Niederegger et al. 2010, Chen et al. 2019).

The importance of carrying out studies outside the controlled conditions of the laboratory is that insects are exposed to the natural climatic conditions of the place where they live. There, they are affected by a set of variables that unpredictably influence their biology, making it difficult to analyze or differentiate the direct effect of an environmental variable on others with which it interacts, generating

Table 6. Total Accumulated Degree-Hours (ADH) of *Lucilia ochricornis* (Lo) and *Lucilia purpurascens* (Lp) in different seasons of the year. Different letters in the same column indicate differences of statistical significance (Tukey's test, $P < 0.05$)

	ADH (°Ch)																					
Season	I Instar Lo		I Instar Lp		II Instar Lo		II Instar Lp		III Instar Lo		III Instar Lp		Postfeeding larva Lo		Postfeeding larva Lp		Pupa Lo		Pupa Lp		Total Lo		Total Lp	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Autumn	513.9a	0	513.9a	0	526.0a	153.7	526.0a	153.7	1165.0a	153.7	1165.0a	153.7	4076.3a	0	4076.3a	0	9813.0a	462.3	9402.2a	303.7	16100.9a	459.9	15690.0a	313.5
Spring	263.6b	0	263.6b	0	363.7b	0	446.2b	142.8	568.2b	0	722.3b	142.8	2776.8b	637.0	7419.5b	0	5899.4b	305.6	6848.8b	1157.8	9871.7b	544.1	15700.4a	1157.8
Summer	488.6a	134.5	411.0c	134.5	419.2c	134.5	496.8ab	134.5	616.2c	0	616.2c	0	4123.5a	1295.0	6756.1c	1632.4	7070.3c	105.6	7154.2b	125.5	12717.8c	1189.4	15434.4a	1757.9

microclimatic conditions that produce changes in the development of insects (Speight et al. 2008). Due many times to the impossibility of replicating these interactions in laboratory conditions, we selected to measure, under field conditions a set of environmental variables that surely have a marked influence on the life cycle of insects, such as temperature, humidity, amount of light, and seasonality.

Comparison of the results obtained here with our results of cultures of the same species in the laboratory (Acosta et al. 2020b, 2021) leads us to propose that, in general, field offspring have a longer development time than those grown in the laboratory, which is consistent with Chen et al. (2019). For this reason, it is important to note that seasonality is a key factor to consider since the cultures made in the autumn need a longer development time, contrary to the spring ones that need less time. Temperature and light are two of the main factors in determining seasonality (Gomes et al. 2006). In this way, the photoperiod causes the duration of the different stages of the life cycle to vary (Saunders 2010). Thus, the development will be faster as daylight hours and temperature increase; instead, it slows down when both decrease (Speight et al. 2008). This explains why the lowest average temperature of the year and the fewest hours of light per day were recorded in autumn. Changes in these variables can be used by insects as a signal to enter the diapause period (Saunders 2010). Diapause prevents potentially adverse environmental conditions from affecting their survival, favoring the synchronization of life cycles, producing the appearance of larval or adult stages with the seasons (Gomes et al. 2006, Speight et al. 2008). This one may also explain why the pupariation time of *L. purpurascens* is shorter in autumn when temperatures are lower; equating to that of *L. ochricornis*, which generally has shorter development times.

We also observed that winter presents a greater amplitude in both temperature and humidity, having very low absolute temperatures (down to −1.8°C) for the area under study. These marked daily climatic fluctuations are possibly the determining factor that leads to the sharp decrease in the abundances of *L. ochricornis* and *L. purpurascens*, until their disappearance, during the winter (Acosta et al. 2020a). These findings would be in agreement with Speight et al. (2008), when as temperatures fall below some critical threshold, the survival of insects tends to decline, with their life cycles being affected by these extreme temperatures. This leads us to think that the marked daily fluctuation of climatic conditions during winter strongly affect the activity of these flies in nature. This assertion leads us to corroborate the idea that experiments carried out exclusively in laboratory conditions do not reflect the dynamics that occur in the natural environment, making it necessary to corroborate and contrast the conclusions obtained with those of cultures carried out in field conditions, under fluctuating temperatures (Lecheta et al. 2015, Faris et al. 2020). The use of mean temperature is habitual to reach valid conclusions in forensic entomology. While it is correct and widely accepted, we should also pay attention to the dynamics of the behavior of the environmental variables as a whole, as it will allow us to achieve a holistic view of the changes in the phenology of flies during their development (Acosta et al. 2020a, Faris et al. 2020).

Currently, existing PMI_{min} estimation methods are not universal and vary. Each method seems to better fit the behavior of a particular species and/or type of data. For *L. ochricornis*, the isomegalendiagram method gives better estimates for body length, the logistic model for body weight, the isomorphodiagram for the total development times of the feeding stages, and the Thermal Summation method 1 for the ADH of the stages postal items. In contrast, for *L. purpurascens*, the isomegalendiagram is more suitable for length and larval body weight, the Thermal Summation method 1 for ADH from larval alimentary stages, and the Thermal Summation

Table 7. PMI calculated from the development data of the different instars/stages of *Lucilia ochricornis* using the different proposed methods: M1: Isomegalen diagram, M2: Logistic growth model, M3: Isomorphodiagram, M4: Thermal summation model 1, M5: Thermal summation model 2. (A) Body length, (B) Body weight, (C) Total developmental times from I Instar to III instar and (D) Total developmental times from Postfeeding (PF) larva to Pupa

A.

Season/Mean Temperature	Body length								
	I Instar – Field	I Instar – M1	I Instar – M2	II Instar – Field	II Instar – M1	II Instar – M2	III Instar – Field	III Instar – M1	III Instar – M2
Autumn/13.4°C	3.1 mm – 24.0 hr	(3 mm) 12.0 hr	19.9 hr	6.9 mm – 60.0 hr	(7 mm) 60.0 hr **	56.1 hr	11.7 mm – 108.0 hr	(12 mm) 92.0 hr	94.2 hr
Spring/23.6°C	3.9 mm – 6.0 hr	(4 mm) 5.0 hr *	6.4 hr **	8.0 mm – 18.0 hr	(8 mm) 17.0 hr **	16.3 hr *	12.9 mm – 36.0 hr	(13 mm) 30.0 hr	28.7 hr
Summer/22.3°C	3.0 mm – 6.0 hr	(3 mm) 2.0 hr	5.2 hr **	6.6 mm – 24.0 hr	(7 mm) 22.0 hr **	19.0 hr	12.8 mm – 48.0 hr	(13 mm) 39.0 hr	40.0 hr

B.

Season/Mean Temperature	Body weight								
	I Instar – Field	I Instar – M1	I Instar – M2	II Instar – Field	II Instar – M1	II Instar – M2	III Instar – Field	III Instar – M1	III Instar – M2
Autumn/13.4°C	0.0010 g – 24.0 hr	(0.0010 g) 31.0 hr	32.1 hr	0.0050 g – 60.0 hr	(0.0050 g) 62.0 hr *	60.8 hr **	0.0276 g – 108.0 hr	(0.0300 g) 106.0 hr **	100.5 hr
Spring/23.6°C	0.0010 g – 6.0 hr	(0.0010 g) 4.0 hr **	8.1 hr *	0.0076 g – 18.0 hr	(0.0080 g) 25.0 hr	17.4 hr **	0.0263 g – 36.0 hr	(0.0250 g) 26.0 hr	26.0 hr
Summer/22.3°C	0.0019 g – 6.0 hr	(0.0020 g) 14.0 hr	17.2 hr	0.0048 g – 24.0 hr	(0.0050 g) 22.0 hr *	22.5 hr **	0.0324 g – 48.0 hr	(0.0300 g) 39.0 hr	38.6 hr

C.

Season/Mean Temperature	ADH – Total developmental times											
	I Instar – Field	I Instar – M3	I Instar – M4	I Instar – M5	II Instar – Field	II Instar – M3	II Instar – M4	II Instar – M5	III Instar – Field	III Instar –M3	III Instar –M4	III Insta – M5
Autumn/13.4°C	513.9 ADH – 36.0 hr	36.0 hr **	32.9 hr *	34.8 hr *	526.0 ADH – 40.0 hr	36.0 hr	42.5 hr **	43.2 hr	1165 ADH – 68.0 hr	60.0 hr	58.2 hr	60.0 hr
Spring/23.6°C	263.6 ADH – 12.0 hr	12.0 hr **	11.6 hr *	11.3 hr *	363.7 ADH – 12.0 hr	12.0 hr **	13.1 hr *	13.2 hr *	528.2 ADH – 24.0 hr	12.0 hr	13.6 hr	14.4 hr
Summer /22.3°C	488.6 ADH – 20.0 hr	12.0 hr	12.6 hr	12.3 hr	419.2 ADH – 16.0 hr	16.0 hr **	14.3 hr *	14.5 hr *	616.2 ADH – 24.0 hr	20.0 hr	15.0 hr	16.0 hr

Table 7. Continued

D.

Season/Mean Temperature`	ADH – Total developmental times											
	PF larva – Field	PF larva – M3	PF larva – M4	PF larva – M5	Pupa – Field	Pupa – M3	Pupa – M4	Pupa – M5	Total – Field	Total – M3	Total – M4	Total – M5
Autumn/13.4°C	4076.3 ADH – 240.0 hr	216.0 hr **	266.7 hr *	277.7 hr *	9813.0 ADH – 776.0 hr	696.0 hr	698.0 hr	700.0 hr	16100.9 ADH – 1160.0 hr	1044.0 hr	1096.0 hr **	1089.3 hr *
Spring/23.6°C	2776.8 ADH – 132.0 hr	72.0 hr *	74.3 hr **	73.3 hr *	5899.4 ADH – 232.0 hr	192.0 hr **	184.0 hr *	183.3 hr *	9871.7 ADH – 412.0 hr	300.0 hr	299.4 hr	300.2 hr
Summer/22.3°C	4123.5 ADH – 172.0 hr	84.0 hr	81.8 hr	80.9 hr	7070.0 ADH – 336.0 hr	192.0 hr	203.0 hr	202.4 hr	12717.8 ADH 568.0 hr	324.0 hr	329.9 hr	330.7 hr

*PMI estimates that fall within the established precision range.

**PMI estimates that fall within the established precision range and are also closer to the real value obtained in the field.

Table 8. PMI calculated from the development data of the different instars/stages of *Lucilia purpurascens* using the different proposed methods: M1: Isomegalen diagram, M2: Logistic growth model, M3: Isomorphodiagram, M4: Thermal summation model 1, M5: Thermal summation model 2. (A) Body length, (B) Body weight, (C) Total developmental times from I Instar to III instar and (D) Total developmental times from Postfeeding (PF) larva to Pupa

A.

Season/Mean Temperature	Body length								
	I Instar – Field	I Instar – M1	I Instar – M2	II Instar – Field	II Instar – M1	II Instar – M2	III Instar – Field	III Instar – M1	III Instar – M2
Autumn/13.4°C	3.5 mm – 24.0 hr	(4 mm) 30.0 hr	23.3 hr **	7.8 mm – 60.0 hr	(8 mm) 60.0 hr **	60.2 hr *	15.1 mm – 108.0 hr	(15 mm) 106.0 hr *	108.7 hr **
Spring/23.6°C	3.6 mm – 6.0 hr	(4 mm) 6.0 hr **	5.6 hr *	6.7 mm – 18.0 hr	(7 mm) 17.0 hr **	16.2 hr *	13.9 mm – 36.0 hr	(14 mm) 34.0 hr *	35.6 hr **
Summer/22.3°C	3.3 mm – 6.0 hr	(3 mm) 4.0 hr **	8.5 hr *	6.8 mm – 24.0 hr	(7 mm) 22.0 hr **	19.6 hr	14.6 mm – 48.0 hr	(15 mm) 41.0 hr	39.7 hr

Table 8. Continued

B.

Season/Mean Temperature	Body weight								
	I Instar – Field	I Instar – M1	I Instar – M2	II Instar – Field	II Instar – M1	II Instar – M2	III Instar – Field	III Instar – M1	III Instar – M2
Autumn/13.4°C	0.0009 g – 24 hr	(0.0010 g) 36.0 hr	37.1 hr	0.0071 g – 60.0 hr	(0.0070 g) 67.0 hr	67.1 hr	0.0423 g – 108.0 hr	(0.0400 g) 104.0 hr	103.5 hr
Spring/23.6°C	0.0008 g – 6.0 hr	(0.0010 g) 8.0 hr **	9.2 hr	0.0050 g – 18.0 hr	(0.0050 g) 19.0 hr **	19.4 hr *	0.0271 g – 36.0 hr	(0.0250 g) 31.0 hr	32.1 hr
Summer/22.3°C	0.0008 g – 6.0 hr	(0.0010 g) 12.0 hr	12.8 hr	0.0046 g – 24.0 hr	(0.0050 g) 22.0 hr **	21.8 hr *	0.0380 g – 48.0 hr	(0.0400 g) 40.0 hr	37.7 hr

C.

Season/Mean Temperature	ADH – Total developmental times											
	I Instar – Field	I Instar – M3	I Instar – M4	I Instar – M5	II Instar – Field	II Instar – M3	II Instar – M4	II Instar – M5	III Instar – Field	III Instar – M3	III Instar – M4	III Insta– M5
Autumn/13.4°C	513.9 ADH – 36.0 hr	40.0 hr	34.6 hr **	38.1 hr *	526.0 ADH – 40.0 hr	36 .0 hr	43.7 hr **	47.5 hr	1165 ADH – 68.0 hr	56 .0 hr	59.3 hr	57.9 hr
Spring/23.6°C	263.6 ADH – 12.0 hr	12.0 hr **	11.8 hr *	11.1 hr *	446.2 ADH – 16.0 hr	12.0 hr	15.1 hr *	15.3 hr **	722.3 ADH – 32.0 hr	24.0 hr	10.8 hr	11.1 hr
Summer/22.3°C	411.0 ADH – 16.0 hr	12.0 hr	12.9 hr **	12.2 hr	496.8 ADH – 20.0 hr	24.0 hr	16.5 hr	16.8 hr **	616.2 ADH – 24.0 hr	12.0 hr	12.1 hr	12.3 hr

D.

Season/Mean Temperature	ADH – Total developmental times											
	PF larva – Field	PF larva – M3	PF larva – M4	PF larva– M5	Pupa – Field	Pupa – M3	Pupa – M4	Pupa – M5	Total – Field	Total – M3	Total – M4	Total – M5
Autumn/13.4°C	4076.3 ADH – 240.0 hr	368.0 hr	370.9 hr	366.8 hr	9402.2 ADH – 744.0 hr	664.0 hr	671.5 hr	675.6 hr **	15690.0 ADH – 1128.0 hr	1164.0 hr *	1162.6 hr **	1170.9 hr *
Spring/23.6°C	7419.5 ADH – 312.0 hr	124.0 hr	125.9 hr	125.9 hr	6848.8 ADH – 228.0 hr	216.0 hr **	207.4 hr *	207.5 hr *	15700.0 ADH – 660.0 hr	388.0 hr	376.7 hr	379.4 hr
Summer/22.3°C	6756.1 ADH – 300.0 hr	140.0 hr	137.4 hr	137.4 hr	7154.2 ADH – 336.0 hr	216.0 hr	227.5 hr	227.5 hr	15434.0 ADH 696.0 hr	404.0 hr	412.3 hr	415.2 hr

*PMI estimates that fall within the established precision range.

**PMI estimates that fall within the established precision range and are also closer to the real value obtained in the field.

Table 9. PMI calculated using the methods M1 (Isomegalen diagram) and M2 (Logistic growth model) for the individual maximum values of body length for (A) *Lucilia ochricornis* and (B) *Lucilia purpurascens*

A.

Season/Mean Temperature	Body length								
	I Instar – Field	I Instar – M1	I Instar – M2	II Instar – Field	II Instar– M1	II Instar – M2	III Instar – Field	III Instar – M1	III Instar – M2
Autumn/13.4°C	3.8 mm – 24.0 hr	(4 mm) 29.0 hr	28.1 hr	7.4 mm – 60.0 hr	(7 mm) 60.0 hr **	60.0 hr **	12.7 mm – 108.0 hr	(13 mm) 103.0 hr	103.7 hr
Spring/23.6°C	3.9 mm – 6.0 hr	(4 mm) 5.0 hr *	6.4 hr **	8.0 mm – 18.0 hr	(8 mm) 17.0 hr **	16.3 hr *	13.5 mm – 36.0 hr	(14 mm) 34.0 hr **	30.9 hr
Summer/22.3°C	3.0 mm – 6.0 hr	(3 mm) 2.0 hr	5.2 hr **	7.8 mm – 24.0 hr	(8 mm) 25.0 hr **	22.7 hr *	14.5 mm – 48.0 hr	(14 mm) 48.0 hr **	49.9 hr *

B.

Season/Mean Temperature	Body length								
	I Instar – Field	I Instar – M1	I Instar – M2	II Instar – Field	II Instar – M1	II Instar – M2	III Instar – Field	III Instar – M1	III Instar – M2
Autumn/13.4°C	4.0 mm – 24.0 hr	(4 mm) 30.0 hr	28.8 hr **	8.3 mm – 60.0 hr	(8 mm) 60.0 hr **	63.6 hr *	15.8 mm – 108.0 hr	(16 mm) 114.0 hr	114.2 hr
Spring/23.6°C	3.6 mm – 6.0 hr	(4 mm) 6.0 hr **	5.6 hr *	6.7 mm – 18.0 hr	(7 mm) 17.0 hr **	16.2 hr *	14.7 mm – 36.0 hr	(15 mm) 38.0 hr **	38.1 hr *
Summer/22.3°C	3.3 mm – 6.0 hr	(3 mm) 4.0 hr **	8.5 hr *	8.3 mm – 24.0 hr	(8 mm) 24.0 hr **	23.3 hr *	16.3 mm – 48.0 hr	(16 mm) 46.4 hr **	46.3 hr *

*PMI estimates that fall within the established precision range.

**PMI estimates that fall within the established precision range and are also closer to the real value obtained in the field.

method 2 for postalimentary stages. This leads us to conclude that the statement (Donovan et al. 2006) that the use of ADH is a reliable estimator to estimate the PMI, even in situations of fluctuating temperatures, is not fulfilled, coinciding with other studies (Wu et al. 2015, Chen et al. 2019, Acosta et al. 2021).

Another important aspect to highlight is that the maximum larval size values obtained in the field are better predictors to obtain more precise estimates of the PMI than the use of the mean values (Donovan et al. 2006, Tarone and Foran 2008, Núñez-Vázquez et al. 2013), thus allowing to enhance the use of the methods obtained here. This could be explained because there is inherent variability in the growth rates of flies, with a proportion of individuals that grow at a slower rate than the rest (Donovan et al. 2006) and that could give a biased estimate, especially in natural environments.

Larval length and body weight are the best predictors of age while the larva is feeding, and mainly during the first two stages, these findings coincide with other studies (Tarone and Foran 2008, Núñez-Vázquez et al. 2013, Acosta et al. 2020b, Faris et al. 2020), thus allowing more precise estimates to be obtained (Acosta et al. 2020b). In contrast, the postalimentary stages, where growth is reduced and body measurements do not change over time, report only a one-time range, with innumerable possible values within it. Therefore, alternative developmental data are necessary to increase the precision of the PMI, such as the morphology of the embryo within the pupa (Tarone and Foran 2008, Faris et al. 2020). This may be because alimentary larvae, I and II mainly, have shorter development times, which improves the precision of age estimates. However, the postalimentary development stages, with longer development times, give less precise estimates, since the error increases (Tarone and Foran 2006, 2008; Núñez-Vázquez et al. 2013; Faris et al. 2020). To make estimates more accurate, it would be preferable to use length over body weight to make estimates, since its variation is low and it is a more reliable estimator, as has been reported here and in other works (Núñez-Vázquez et al. 2013, Lecheta and Moura 2019). Based on our studies, we can conclude that body weight is an unstable variable between types of cultures and between seasons since it does not present behavior of regular increase during its growth (Acosta et al. 2020b), as does body length, which is evident by the low adjustments to the estimation methods, overestimating the PMI times.

The use of constant temperatures is an unrealistic approach to study the thermal responses of insects that inhabit thermally variable environments. This makes it necessary to incorporate fluctuating temperatures into predictive growth models that estimate the PMI (Chen et al. 2019). Since forensic application methods respond to the temperatures of the place where they were built, if the mean temperatures obtained in the field do not represent the development at constant temperatures, it is predicted that no method fits only the data of cultures in the laboratory will give accurate results. This assertion is consistent with the conclusions made by other authors (Catts 1992, Wu et al. 2015), who emphasizes that the average temperature is not enough to predict the development of organisms. However, the use of the average temperature has allowed the construction of models that, with greater or lesser precision, are of great forensic utility, so these models are not disposable but adjustable, which is supported by the very good fit obtained by the linear regression between both types of cultures.

As a possible solution, Chua (2013) proposes sinusoidal and exponential equations to obtain a suitable temperature that is used in other methods (such as ADH calculation), providing a more realistic option than the mean temperature. However, the information necessary to solve the equations is difficult to obtain (for example temperatures and times of the beginning and end of the day, times from noon to the thermal maximum, among others), which limits the use of this tool from multiple points of view, at least for most cases. Doing cultures in the field in a systematic way is a difficult task in terms of infrastructure, handling, transfer, equipment, and safety; which is not a recommended option to perform regularly or for each particular forensic case. As other authors state (Tarone and Foran 2008, Hu et al. 2019), it is imperative to have standardized procedures and methods in forensic entomology. For the aforementioned, we propose as a possible alternative, to carry out field studies that consider the most influential climatic variables of a certain place and summarize them or integrate them into some parameter or factor within more realistic and easy to use mathematical models. This will allow correcting the data obtained at constant temperatures since temperature by itself does not explain all the development of flies and the prediction value increases significantly when incorporating more variables (Tarone and Foran 2008).

The estimation of the PMI through the use of necrophagous flies development remains reliable, even if the precision in its estimation is affected by the number of available studies and the methods proposed by them. The high value of the use of these insects resides in that they act as eyewitnesses once the death has occurred and this allows forensic entomology to obtain with great reliability at least a minimum postmortem interval, especially in the absence of accessory evidence.

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