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Development of *Fannia pusio* (Diptera: Fanniidae) Under Controlled Temperature Conditions and its Enforcement in the Estimate of the Post-mortem Interval (PMI)

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Original article

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Fannia pusio (Wiedemann, 1830) is a species belonging to the family Fanniidae, which is of great forensic, sanitary, and veterinary interest. The behavioral peculiarities of this species, depending on the temperature at which it is found, may provide additional information for future research. The application of entomology in the forensic field has focused especially on the early colonizing taxa of corpses that are in the initial stage of decomposition. However, species occurring at more advanced stages can contribute to further knowledge, as is the case with *F. pusio*. In addition, the species has the ability to colonize buried corpses that are inaccessible to larger dipterans. On the other hand, the sanitary and veterinary interest of this species is due to the performance of females as phoretic hosts of *Dermatobia hominis* eggs that cause myiasis in both animals and humans. In the current study, the behavior of *F. pusio* was observed at a temperature range of 5ºC to 40ºC. We found that its viability range is limited between 15ºC and 35ºC; above and below these temperatures, adults survive but oviposition does not take place. Data collected by statistical analysis were subsequently applied to calculate the post-mortem interval (PMI) using isomorphen and isomegalen diagrams. The results show a directly proportional relationship between growth rate and temperature increase. However, a slowdown in the growth of individuals was observed at extreme temperatures (5ºC and 35ºC). The results shown in this manuscript, together with the existing bibliography of other species, help to broaden the knowledge of *F. pusio*, which has not been studied in such depth until now.

Key words: forensic importance, growth rate, isomegalen-diagrams, isomorphen-diagrams, sanitary importance, veterinary importance.

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Forensic entomology studies the association of arthropods in evidence with judicial examinations (GRZYWACZ *et al.* 2017). Arthropods colonize a corpse in a specific order according to the stage of decomposition (faunal succession) and climatic conditions (BARRIOS & WOLFF 2011). The data obtained following this behavior are essential for the determination of the post-mortem interval (PMI) (AMENDT *et al.* 2011; CHARABIDZE 2012; CAVALLARI *et al*. 2015; FARIS *et al*. 2020; WANG *et al*. 2020).

There are various methodologies for the estimation of PMI, however, the isomorphism and isomegalen diagram are the simplest and most intuitive techniques. Diptera are the order of insects with the greatest application in forensics as they are the first colonizers (AMENDT *et al*. 2011). That is why there are many studies carried out on different families: Calliphoridae (BENECKE & LESSIG 2001; OLIVEIRA-COSTA & MELLO-PATIU 2004; MICHAUD & MOREAU 2009), Muscidae (BENECKE & LESSIG 2001; BENECKE *et al.* 2004; GARCÍA-ROJO *et al*. 2009), Sarcophagidae (NASSU *et al.* 2014), and Fanniidae (BARROS *et al*. 2008; AMAT 2010; MATUSZEWSKI *et al.* 2010; QUIROGA & DOMÍNGUEZ 2010; ABALLAY *et al*. 2012; AKBARZADEH *et al*. 2012; GRZYWACZ & PRADO E CASTRO 2012; BAZ *et al.* 2015; CAVALLARI *et al*. 2015). The most commonly used species for PMI estimation are those belonging to the first colonizing families (BENECKE & LESSIG 2001; GARCÍA-ROJO *et al*. 2009; NASSU *et al*. 2014; DEFILIPPO *et al*. 2019). However, there is also a need for research on insect species that occur in the most advanced stages of decomposition due to their increasingly frequent occurrence (VASCONCELOS *et al*.

2017). Fanniidae is one of the examples that highlight this need, since despite preferring very advanced stages of decomposition, there is also evidence of their appearance in early stages (AMAT *et al*. 2013).

In this work we focused on *Fannia pusio* (Wiedemann, 1830), a species belonging to the family Fanniidae. This species was established under controlled environmental conditions in the laboratory after being found in a cat carcass in an advanced state of decomposition in an urban area of the Region of Murcia (Spain). It is also known as the "chicken dung fly" due to its presence on laying hen farms. They are native to the Nearctic and Neotropical regions (SMITH 1986; BÉLO *et al*. 1998; COURI & SOUSA 2019), but are widespread in the tropical and warm temperate areas of the Old World as well. They have also adapted to survive low temperatures and wet periods (LINHARES 1978; LEANDRO & D'ALMEIDA 2005). Therefore, *F. pusio* is considered a synanthropic species (MONTEIRO & DO PRADO 2006) and is found in all seasons (SOUZA & LINHARES 1997; BARBOSA *et al.* 2009; KRÜGER *et al*. 2010; ABALLAY *et al*. 2012; FARIA *et al*. 2013).

The forensic interest of this species is its ability to colonize buried bodies that have been partially protected from colonization by larger diptera, which provides a notable added point of forensic interest (INTRONA *et al.* 2011; GRZYWACZ *et al*. 2017). The sanitary and economic interest of this species is also relevant as it acts as an egg carrier of *Dermatobia hominis* Linnaeus, which causes myiasis in both domestic animals and humans (GOMES *et al*. 2002; DE CARVALHO *et al*. 2003; ESPINDOLA & COURI 2004; AMAT 2010; QUIROGA & DOMÍNGUEZ 2010; AMAT *et al*. 2013; VELÁSQUEZ *et al*. 2013). Unfortunately, before now there were no studies on the effect of temperature on the development of *F. pusio* (FARIS *et al*. 2020). Therefore, the aim of this work was to evaluate the effect of temperature on its development, for the first time, providing data to specialists for determining its suitability for a more accurate PMI determination. The importance of this information for medical, veterinary, and forensic purposes (MARCHIORI 2014) is also discussed.

Material and Methods

A colony of *F. pusio* was established under controlled environmental conditions (25±2ºC, 60±5% relative humidity, and a 12-hour light/dark cycle) in the Laboratory of Necrophagous Diptera at the University of Murcia (Spain). Water and sugar *ad libitum*, supplemented with canned cat food, were provided to adults (COURI 1991) for three hours to induce oviposition and obtain eggs (MARCHIORI & DO PRADO 1996). Canned cat food was chosen after unsuccessfully testing various substrates such as dog and human faeces and chicken and pork liver (D'ALMEIDA 1994). Its composition is based on meat and meat by-products, fish and fish by-products, and minerals, similar to that used by MARCHIORI & DO PRADO (1996).

Eggs obtained after the three-hour oviposition period were then moved to cages (25 cm x 25 cm x 10 cm) with this substrate and sand to allow larvae growth and pupariation, after which the pupae were returned to the adult cage (40 cm x 30 cm x 30 cm). Throughout this process, a sample of n=20 individuals were taken every 12 hours, until the end of the experiment. All samples are independent, therefore, the difference in individual length is due to the variation of temperature during the growth period.

The eggs cages were kept at constant temperatures (eight different temperatures were tested: 5±2ºC, 10±2ºC, 15±2ºC, 20±2ºC, 25±2ºC, 30±2ºC, 35±2ºC, and 40±2ºC) and 60±5% of relative humidity, until hatching after which pupae were transferred to plastic jars to rear into adults. Three replicates were carried out for each experiment and twenty individuals were sampled twice a day, until more than 50% of the larvae had reached the pupae stage, this fact allows to ensure each immature stage has reached the next developmental stage in a significant level. Immediately upon collection, samples (larvae, prepupae, and pupae) were immersed in boiling water (100ºC) for a few seconds, and then preserved in 70% ethanol. Each individual was measured with a stereomicroscope connected to a video camera, according to the protocol described by BYRD & ALLEN (2001) and CHARABIDZE *et al*. (2008).

Data analysis was performed with SPSS v20 statistical software (IBM, Armonk, New York, USA) (IBM CORP, 2011). Statistical analyses were made taking larval stage and pitting length (mm) as dependent variables and temperature (ºC) as an independent one. First, the Kolmogorov-Smirnov test was performed in order to check normality samples. Second, descriptive statistic and Box and Whisker plots were calculated to get a visual displaying of the data distribution through their quartiles. Third, the Kruskall Wallis test was used to analyze differences within means, and post hoc tests (Mann-Whitney U) were carried out for pointing out differences among the groups.

In addition, the relationship between temperature and growth rate were analyzed using regression analyses. Although several models were tested, only a quadratic model gave high values for the R^2 assessing goodness of fit in the regression model. Is this paper, a constant term (y-intercept) was not present. A biological meaning can hardly be assigned for the y-intercept; besides, it allowed for the increase of the R^2 values.

Quadratic regression is used to model variance as a function of the mean for sample counts of spatially aggregated insect populations (CANDY 2000). Therefore, they are used to determine the growth rate of different factors such as different populations, studies of predator-prey, or food competition as is the case (CHONG *et al.* 2006), or even the growth of different variables (LAGOS-BURBANO & BENAVIDES-ARTEAGA 2012).

Longitudinal measurements of the specimens were used to calculate PMI using the isomorphen and

isomegalen diagrams. These methodologies are graphical representations that cover information from oviposition until the pre-imaginal stages are reached (RICHARDS *et al.* 2008; BAMBARADENIYA *et al*. 2018). The isomorphen diagram shows the duration of development of the various stages at different temperatures where each line shows the transition time of each stage according to temperature; the spaces between these lines are the periods corresponding to each stage. The isomegalen diagram shows the development time needed to reach certain lengths versus temperature (REITER 1984; GENNARD 2007). Each line represents groups of larvae of the same size within each temperature range studied.

Results

This study is based on the longitudinal data of 7389 individuals (including 57 eggs, 606 L1, 590 L2, 2916 L3, 2402 prepupae, and 818 pupae) of *F. pusio* established under laboratory conditions at different temperatures (Table 1) and their application to the calculation of the PMI by isomegalen and isomorphen diagrams.

Table 1

Descriptive statistics for length of *Fannia pusio* from egg to pupae and time since hatching at constant temperatures. T – temperature (°C); n – sample size; X±SD – mean±standard deviation (mm); V – variance; Min-Max – minimum and maximum values (mm); TSH (h/d) – time since hatching (hours/days); NT – total sample size; L1 – larval stage 1; L2 – larval stage 2; L3 – larval stage 3

T (°C)	n	Length			TSH (h/d)
		X ±SD	V	Min-Max	
Eggs					
25	57	0.74±0.61	0.04	0.59-0.91	–
NT	57				
L1					
15	163	1.06±0.35	0.126	0.44-1.91	9-165 h/0.4-6.9 d
20	163	1.07±0.35	0.126	0.44-1.90	9-69 h/0.4-2.9 d
25	124	1.25±0.41	0.168	0.58-2.39	9-68 h/0.5-2.8 d
30	81	0.82±0.31	0.10	0.36-1.63	9-33 h/0.4-1.4 d
35	75	0.83±0.26	0.07	0.40-1.64	9-33 h/0.4-1.4 d
NT	606				
L2					
15	156	1.94±0.45	0.20	1.24-3.18	9-237 h/0.4-9.9 d
20	156	1.94±0.45	0.20	1.24-3.18	9-69 h/0.4-2.9 d
25	90	2.75±0.47	0.23	1.52-3.67	9-84 h/1.5-3.5 d
30	70	1.68±0.74	0.55	0.74-3.67	12-81 h/0.9-3.4 d
35	118	1.31±025	0.64	0.68-1.95	9-45 h/0.4-1.9 d
NT	590				
L3					
15	816	5.41±1.03	1.07	2.14-7.13	81-549 h/3.4-22.9 d
20	825	5.39±1.04	1.09	2.14-7.13	33-285 h/1.4-11.9 d
25	369	5.11±1.14	1.30	2.15-7.15	36-189 h/1.5-7.9 d
30	371	5.10±1.32	1.74	1.13-7.01	21-165 h/1.9-6.9 d
35	535	4.73±1.49	2.23	1.00-7.24	9-537 h/0.4-22.4 d
NT	2916				
Prepupae					
15	690	4.87±0.63	0.40	3.24-6.22	137-549 h/5.7-22.9 d
20	690	4.87±0.63	0.40	3.24-6.22	105-285 h/4.4-11.9 d
25	346	5.39±0.65	0.48	3.53-6.95	96-224 h/4-9.3 d
30	214	4.77±0.69	0.47	3.15-6.89	57-165 h/2.4-6.9 d
35	462	4.96±0.03	0.36	3.20-6.88	57-537 h/2.4-22.4 d
NT	2402				
Pupae					
15	167	4.07±0.55	0.31	2.27-5.10	321-537 h/13.4-22.4d
20	199	4.08±0.56	0.31	2.27-5.44	129-285 h/5.4-11.9 d
25	139	4.57±0.39	0.15	3.05-6.06	92-248 h/3.8-10.3 d
30	94	4.07±0.74	0.55	2.53-5.54	81-165 h/3.4-6.9 d
35	219	4.25±0.78	0.62	2.13-5.94	81-720 h/3.4-30 d
NT	818				

Statistical analyses

No normality was found by the Kolmogorov Smirnov test. Descriptive statistics are summarized in Table 1, with the measurements of each stage and the time each one took depending on the temperature studied. However, at 5ºC, 10ºC, and 40ºC it was observed that adult *F. pusio* can survive in poor conditions, but that oviposition does not occur. Thus, the "viable temperature range" for the species is from 15ºC to 35ºC and this is where further analysis will be focused. Box and whisker plots (Fig. 1) show data distribution through their quartiles.

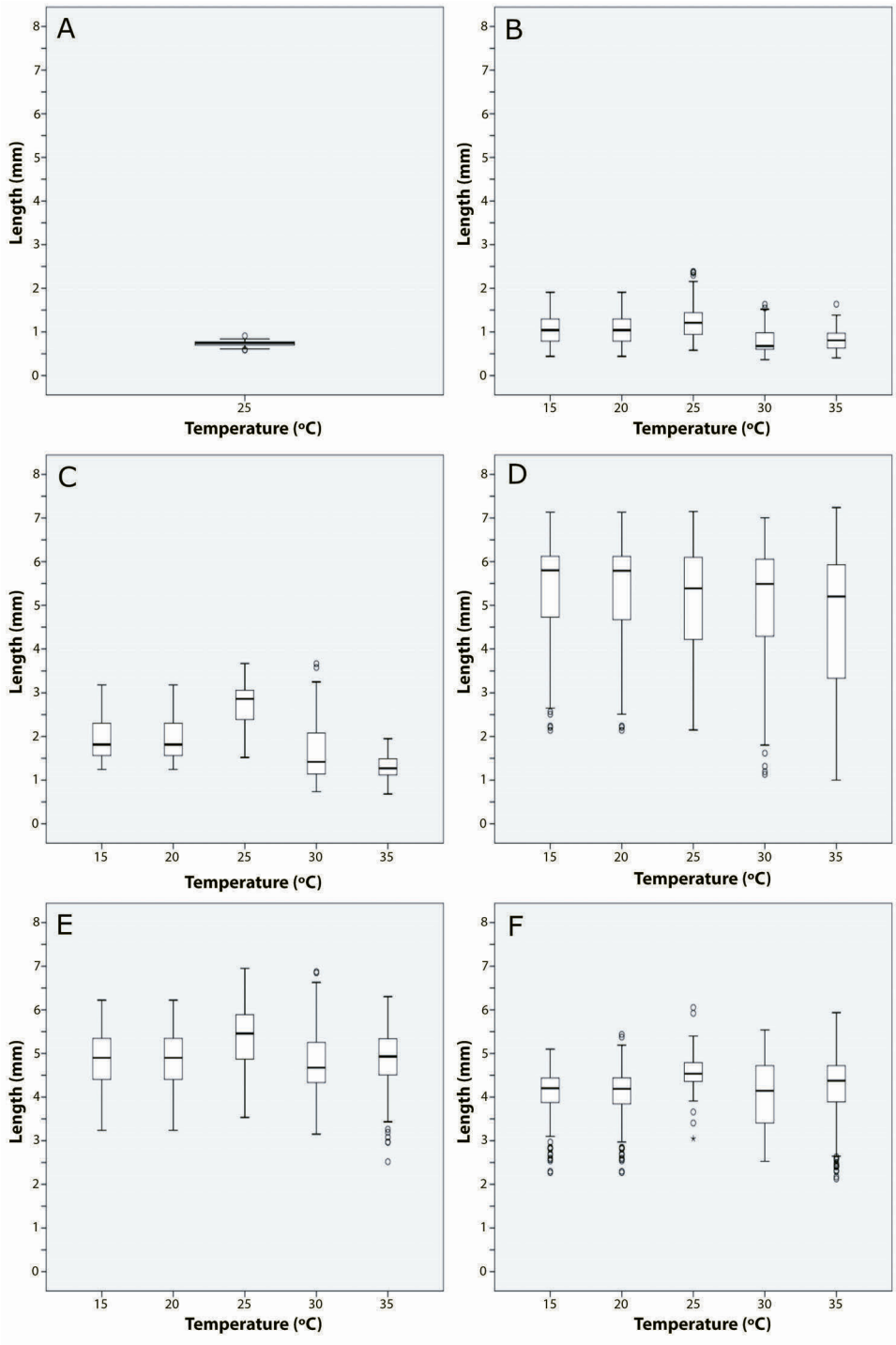

Fig. 1. Box and Whisker Plots for the immature stages of *Fannia pusio.* a) Eggs; b) L1, c) L2, d) L3, e) Prepupae, and f) Pupae at different temperatures (ºC) on the x-axis and length (mm) on the y-axis. The lines that run parallel to the boxes are the "whiskers," which indicate variability outside the upper and lower quartiles, which are the upper and lower edges of each box. The median is the horizontal line that appears inside the box. Outliers are represented as individual points aligned with the whiskers. Note: In the Eggs plot, the x-axis scale is different to the rest due to their smaller size.

The length of the first instar larvae (L1) varies at the temperatures tested (Kruskal Wallis H=95.17, df=4; p<0.05). Mann-Whitney U tests were carried out to find the differences among the L1 larvae. There were significant differences among larvae reared at different temperatures except those reared at 15ºC and 20ºC (bilateral asymptotic significance p>0.05) and L1 at 30ºC and 35ºC that were equal means (p>0.05). The largest L1 was obtained at 25ºC while it wasn't possible to determine when the smaller larvae became established since at 30ºC and 35ºC rather similar data were observed (Fig. 1, Table 1). The same applies to the second instar larvae (L2) (Kruscal Wallis, H=277.37, df=4, p<0.05). All L2 reared at the studied temperatures were different in size (U-Mann Whitney, p<0.05) although at 15ºC and 20ºC the means were equals (U Mann Whitney, p>0.05). At 25ºC the largest larvae were obtained and at 35ºC, clearly, the smallest (Fig. 1, Table 1).

The third instar larvae (L3) represent a very extended period of the larval growth process. There are significant differences in L3 length (Kruskal Wallis, H=93.86, df=4, p<0.05) and in addition, in a comparison between two groups of L3 reared at the studied temperatures (U Mann Whitney, p<0.05), all groups had statistical differences, except the L3 reared at 15ºC and 20ºC, (U Mann Whitney p>0.05), and the L3 bred at 25ºC and 30ºC, (U Mann Whitney p>0.05). The largest larvae were found at 15ºC/20ºC while, the smallest ones were reared at 35ºC (Fig. 1, Table 1). These results are in line with those obtained in the prepupae groups since these two states (L3 and prepupae are developed in a synchronized way (Fig. 1, Table 1). Significant differences were found in prepupae length (Kruskal Wallis, H=59.43, df=4, p<0.05). In the same way, Mann-Whitney U tests were developed to find differences within the groups reared at the studied temperatures (p<0.05). The pupae were all different in length except for those reared at 15ºC and 20ºC (p<0.05), similar to the previous results, and for prepupae obtained at 20ºC and 35ºC (p<0.05).

Finally, significant differences were observed in the length of the pupae reared at the temperatures tested (Kruskal Wallis, H=83.62, df=4, p<0.05; U Mann Whitney, p<0.05). In this state, the largest larvae were also observed at 15ºC, while the smallest were observed between 30ºC and 35ºC (Fig. 2). When measuring pupae, the curvature that modifies the length of individuals must be taken into account. The highest number of curved pupae (70.2%) was obtained at 30ºC; while the lowest number (6.9%) was obtained at 25°C.

Body length of the preimaginal forms of *Fannia pusio* and the time from egg hatching to pupation (age of specimens) varies with the temperature (Fig. 2). Linear regression model did not yield a significant coefficient of determination in the process of testing the equation that fits best the data set. However, quadratic models (Table 2, Fig. 3) are a suitable alternative

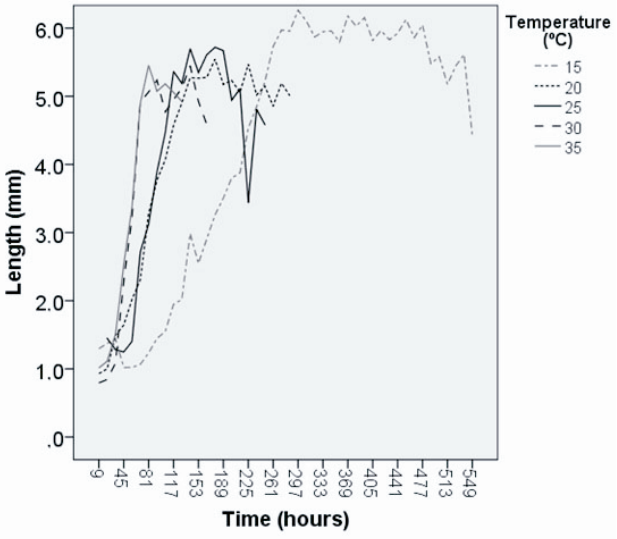

Fig. 2. Effect of temperature on the growth rate in *Fannia pusio*. The different lines describe mean length of immature stages through the time in each temperature.

Table 2

Quadratic regression models obtained for the relationship between the growth rate of *Fannia pusio* (dependent variable) and temperature (independent variable). T – temperature (ºC); R^2 – explained variance of data; F – F value; df 1 and df2 – degree of freedom; * – statistically significant value p<0.001

Model summary					
T	R^2	F	df1	df2	p
15º	0.971	33984.525	2	1996	0.000*
20º	0.970	33046.193	2	2037	0.000*
25º	0.967	15965.203	2	1099	0.000*
30º	0.944	7406.656	2	884	0.000*
35º	0.910	7096.945	2	1412	0.000*

when the studied phenomenon can be approximated with a parabolic shape, as in this case. The y-intercept was omitted, as a result R^2 values were higher than 0.90, which means that the variance in the data set was explained well by our quadratic model.

Application in the calculation of the PMI

First, Fig. 4 displays the time (x-axis) required for *F. pusio* to complete its cycle at each temperature (y-axis) within its viable range. It can be seen how at high temperatures this time is less, while at low temperatures the time is greater. However, at 35ºC a slowing down of the pupal stage can be seen, prolonging

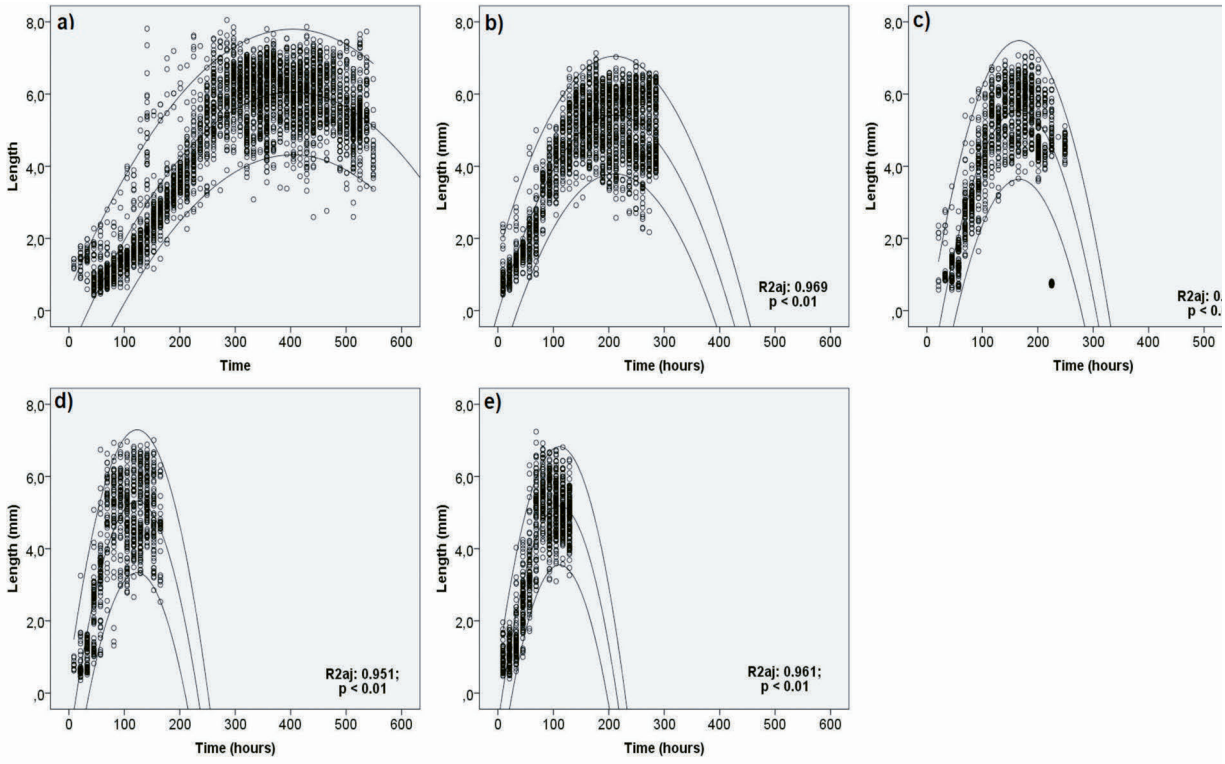

Fig. 3. Regression models between length (y-axis) and time in hours (the x-axis) at studied the temperatures. a) 15ºC, b) 20ºC, c) 25ºC, d) 30ºC, and e) 35ºC.

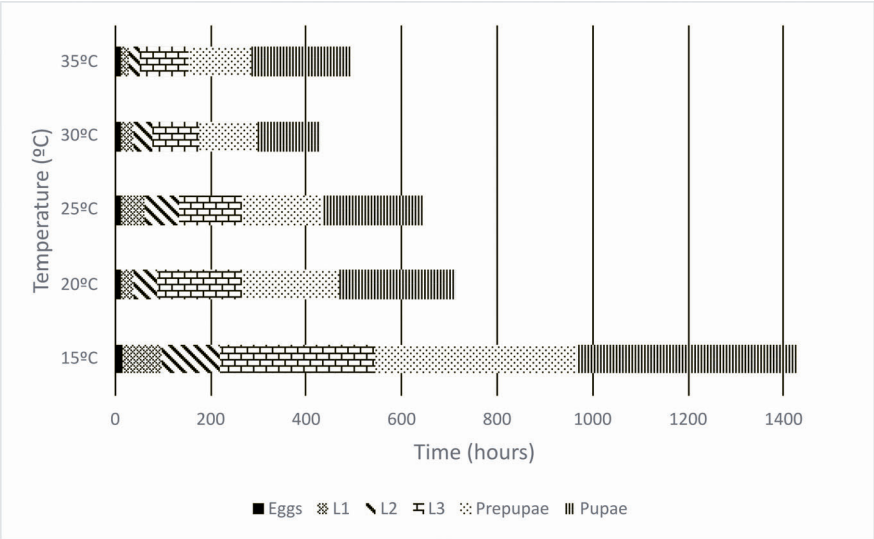

Fig. 4. Mean of hours needed (x-axis) to get to the next developmental stage of *Fannia pusio* (eggs, L1, L2, L3, prepupa, and pupa) at the viable temperatures studied (15ºC, 20ºC, 25ºC, 30ºC, 35ºC) represented on the y-axis.

the complete time of development more than at 30ºC, although the pupae from that group were not able to open or give viable adults.

The inverse variation between temperature and time can also be seen in the isomorphen (Fig. 5a) and the isomegalen (Fig. 5b) diagrams. That is, at a lower temperature, the development of *F. pusio* takes longer, while at higher temperatures, it takes less time. Egg development was not altered by the temperatures analyzed; however, we documented that 10ºC and 40ºC are the limits at which clutch was obtained and larvae were able to hatch and develop (Fig. 5).

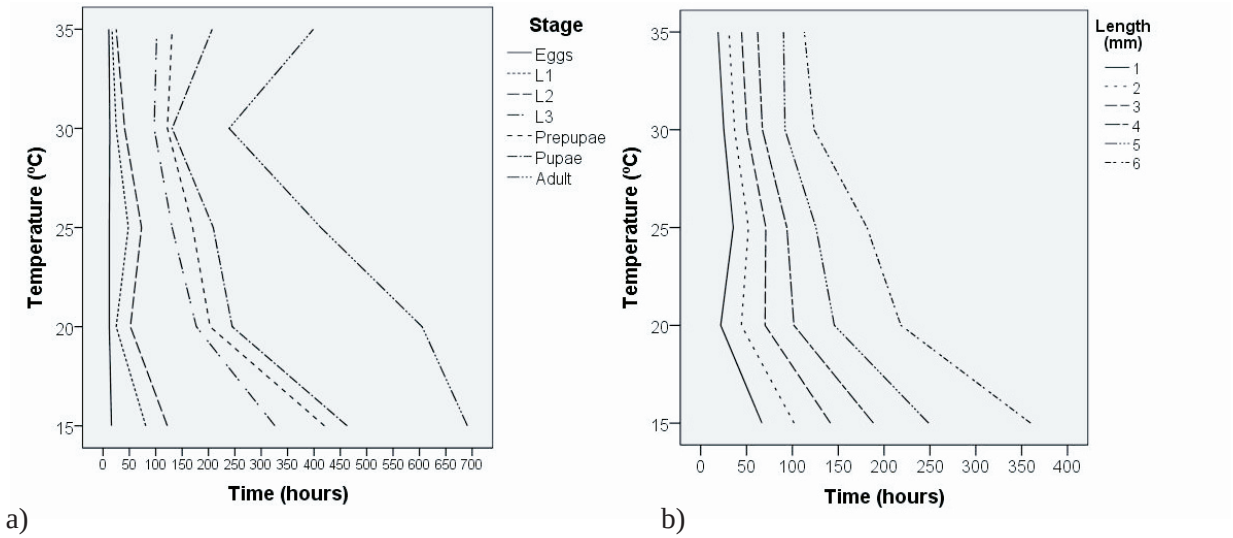

Fig. 5. a) Isomorphen diagram of *Fannia pusio*: representation of the time expressed in hours (x-axis) required for the development of each stage of the biological cycle within each test temperature range (y-axis); b) Isomegalen diagram of *Fannia pusio*: representation of the time expressed in hours (x-axis) needed to reach a certain length expressed in millimetres within each range of temperatures studied (y-axis).

The isomorphen diagram shows a similar behavior in L1 and L2, undergoing the same drop at 20ºC. L3 and prepupae also show the same behavior pattern. However, the results of the pupal stage do show differences, with a decrease in the development time at 25ºC which rises again at 30ºC. This means that the adult's emergence is also prolonged in time (Fig. 5a). In addition, the isomegalen diagram shows other peculiarities; at 20ºC a very noticeable acceleration of development was observed for the smallest lengths (1-4 mm), but for individuals of 5 mm a deceleration was registered (Fig. 5b).

Discussion

There are few studies on the oogenesis, oviposition, and hatching of *F. pusio* eggs (LINHARES 1978; D'ALMEIDA 1994; MARCHIORI & DO PRADO 1996, 1999) and on their use to calculate the post-mortem interval (ABALLAY *et al.* 2012). In this study, the behavior of *F. pusio* was observed at different temperatures for its subsequent application in the calculation of PMI. We recorded that the viable temperature range of the species is between 15ºC and 35ºC; below and above these thresholds adults can just barely survive but oviposition does not occur. It has also been found that the minimum time of embryonic development is 19 hours and that eggs begin to hatch between 9-12 hours, results which differ from those presented by MARCHIORI & DO PRADO (1996). These differences could be explained by the sample size (n) of the analyses since MARCHIORI & DO PRADO (1996) used only 10 individuals, whereas we used 20 individuals per cage with 3 replicates. Regarding the immature stages, unexpected results were also obtained. The non-existent differences between eggs and L1 larvae suggest that the youngest larvae seek a good place to feed, which they remain in until reaching the minimum size required to undergo the moult to L2 (Fig. 1, Table 1). Furthermore, the largest individuals were the L3 and the time that this species spends to enter the pupal stage is generally longer than in other dipteran families such as Calliphoridae, Sarcophagidae, or Muscidae, regardless of temperature (REITER 1984; GRASSBERGER & REITER 2001; GRASSBERGER *et al*. 2003; DEFILIPPO *et al*. 2019).

In this study, remarkable data previously unknown were obtained. In the pupal stage, a natural dehydration occurs, which causes tissue contraction. The higher the temperature, the lower the relative humidity is, the more pronounced this curvature is, affecting pupal viability. In addition, the larval masses of larger larvae increase the temperature of their microenvironment due to their own metabolism (HIGHLEY & HASKELL 2001; CHARABIDZE *et al*. 2011; DÍAZ-MARTÍN *et al.* 2014). This is why, at 30ºC and 35ºC, the highest percentage of curvature and non-viability was obtained at 35ºC. These results improve at 25ºC where larger and more viable larvae are observed (Table 1, Fig. 2), coinciding with the predominant temperature in their areas of origin (PONT 1977; DE CARVALHO *et al*. 2003). New results on the effect of temperature have been found with quadratic regression models, this regards the growth of the larvae and the decline in the length of prepupae and pupae, and this behavior is observed at all the temperatures tested.

Insects are ectothermic animals and their metabolic activity depends on environmental conditions (GRASSBERGER & REITER 2001; NASSU *et al*. 2014). Therefore, the forensic activity of different dipteran species depends on these conditions. Often, they must save energy to keep their metabolic processes active in stressful situations such as low temperatures, which leads to a slowdown in development (Fig. 2 and 4). However, at extremely high temperatures they behave in the same way (VELÁSQUEZ *et al*. 2013; DÍAZ-MARTÍN *et al*. 2013) as can be observed in *F. pusio* at 35°C. These same behaviors can be observed in the PMI calculation diagrams (Fig. 5) which coincide with the behavior of other species (REITER 1984; GRASSBERGER & REITER 2001; GRASSBERGER *et al*. 2003; DÍAZ-MARTÍN *et al*. 2014; YANMANEE *et al*. 2016; WANG *et al*. 2017; YANG *et al*. 2017; DEFILIPPO *et al.* 2019). It is worth noting however that *F. pusio* presents certain peculiarities that unfortunately cannot be contrasted due to the lack of studies in this species.

Both the isomorphen (Fig. 5a) and isomegalen diagrams (Fig. 5b) show the same patterns. The only variation shown in the egg phase by temperature is hatching time, as the morphology does not vary. The results obtained for L1 and L2 show a comparable pattern of behavior with an increase in developmental speed and length at 20ºC. L3 and prepupae also grow in the same way with a decreasing rate as temperature increases. In addition, pupae and adults accelerate their development at 30°C, which is not the case for the other temperatures tested. All these behaviors are consistent with other species of forensic interest (BAMBARADENIYA *et al*. 2018). In other words, the two methodologies used to calculate the PMI are complementary to each other and show the same patterns of behavior.

In summary, this work expands the knowledge of the behavior of *F. pusio* at different temperatures, offering data relevant to its forensic application that have been little studied so far. The peculiarities shown by this species, such as the curvature shown in the pupae and the behavior at different temperatures, may offer additional information in the resolution of forensic cases in court.

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Author Contributions

Research concept and design: Y.B.-P., E.R.; Collection and/or assembly of data: Y.B.-P., E.R.; Data analysis and interpretation: Y.B.-P., E.R.; Writing the article: Y.B.-P., J.G.; Critical revision of the article: J.G., E.R.; Final approval of article: J.G., E.R.

Conflict of Interest

The authors declare no conflict of interest.

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