

Development, Life History

Effect of Temperature on Biological Parameters of the West Nile Virus Vector *Culex pipiens* form 'molestus' (Diptera: Culicidae) in Greece: Constant vs Fluctuating Temperatures

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Abstract

Studying the biology of *Culex* species is crucial to understanding their role in arbovirus transmission and for the development of efficient control strategies. Assessments of survival, development, adult longevity, fecundity and egg hatching of *Culex pipiens* form 'molestus' (Forsskål), were conducted, under nine constant and fluctuating temperatures ranging from 15 to 35 ± 0.5°C. Higher survival rates were observed at constant temperature of 25°C as well as fluctuating with the same mean. Complete mortality occurred at 35°C in both constant and fluctuating temperature regimes. Development rate from egg to adult increased between 15 and 32.5°C, in a linear fashion. Adult longevity ranged from 1.4 d at 32.5°C to 73.5 d at 15°C. Females lived significantly longer compared to males at all temperature regimes with the exception of constant 32.5°C where adult longevity was similar between males and females. Fecundity was higher at moderate constant and fluctuating temperatures compared to high temperatures, where females laid a significantly smaller number of eggs. Likewise, egg hatching was significantly lower at the highest tested temperature regimes compared to low and moderate ones. The lowest developmental thresholds of the species in different developmental stages ranged between 11.17 and 11.95°C at constant temperatures and between 11.09 and 12.74°C at fluctuating ones. Differences between constant and fluctuating temperatures were observed concerning developmental time, fecundity, and male adult longevity at the two lowest tested temperatures, highlighting the importance of testing also fluctuating temperatures that simulate field conditions.

Key words: *Culex pipiens*, mosquito, temperature, biology

Culex pipiens (L.) exists in two forms, 'pipiens' and 'molestus', that exhibit substantial differences in behavioral and physiological characteristics, but are morphologically indistinguishable (Vinogradova 2000). While form 'pipiens' is described as anautogenous, eurygamous, heterodynamous (diapausing) and predominantly ornithophilic, form 'molestus' is stenogamous, autogenous, anthropophilic, prefers underground water bodies with high organic contents and does not diapause (Vinogradova 2000, Di Luca et al. 2016). *Culex pipiens* form 'molestus' (Forsskål) often develops in subterranean habitats, such as sewers or underground railway tunnels, where temperature in the near-surface (e.g., above 10 m depth) is clearly affected by the seasonal change of above ground temperature (Ohtani et al. 2015). However, the water temperature in sewer systems and underground tunnels fluctuate in the order of 2–3°C,

and temperatures remain above 10°C even during winter (Byrne and Nichols 1999, Cipolla and Maglionico 2014).

The medical importance of *Cx. pipiens* is due to the fact that it has a worldwide distribution and is considered one of the primary bridge vectors of the West Nile Virus (WNV), and other arboviral pathogens which cause serious disease in humans and animals (Hamer et al. 2008, Hubálek 2008, Brugman et al. 2018). WNV causes sporadic outbreaks of neuroinvasive disease, with Europe and North America being most affected in recent decades (Chancey et al. 2015, Brugman et al. 2018). In 2010, 262 cases of West Nile neuroinvasive disease were reported in Greece, of which 35 were fatal (HCDCP 2010). In 2018, after a short period of relative silence of the disease, an ongoing outbreak has been reported; 312 patients tested positive in serological essays, and

44 died of acute aseptic meningoencephalitis (HCDCP 2018). In Greece WNV transmission to humans occurs between May and June, while the peak of disease notifications is from July to September (HCDCP 2018). The minimum and maximum air temperatures during these months are reported to be approximately 15 and 35°C, respectively, while the daily temperature fluctuations are relatively low (5–10°C) (HNMS 2018). With disease outbreaks becoming more frequent in Europe, early detection is essential to controlling the virus and limiting its' spread in a vector population (Chancey et al. 2015). Although some European countries have established enhanced surveillance programs for the disease, present surveillance tools do not provide accurate data of the distribution of vectors of WNV (Engler et al. 2013).

Temperature has a major influence on the ecology and biology of mosquitoes, as it directly affects their life history traits, such as developmental time, body size, fecundity, hatching success, adult longevity, as well as mortality (Shelton 1973, Ward and Stanford 1982, Rueda et al. 1990, Bayoh and Lindsay 2003, Delatte et al. 2009, Andreadis et al. 2014, Ciota et al. 2014). In fact, any significant temperature variations from the optimal range for mosquito development may cause stress, resulting in potential developmental inconsistencies (Mpho et al. 2002). In addition, temperature affects mosquitoes' ability to transmit pathogens since unfavorable developmental conditions can cause significant effects on mosquito biological parameters and fitness that can lead to variations in vector competence (Shelton 1973, Kilpatrick et al. 2008, Delatte et al. 2009, Mohammed and Chadee 2011).

The majority of studies dealing with the temperature-dependent development of insects has been conducted at constant temperatures, rather than fluctuating ones (Spanoudis et al. 2015). However, natural conditions are characterized by daily thermal cycles which influence the development of insects in a different manner, compared to exposure to constant temperatures in the laboratory (Beck 1983, Mironidis and Savopoulou-Soultani 2008). Thus, results of studies conducted with constant temperatures can hardly be extrapolated to natural conditions (Beck 1983), which can be simulated more efficiently by using fluctuating temperatures (Fischer et al. 2011, Spanoudis et al. 2015). On the other hand, it is reported that even constant temperatures can adequately predict development rate functions of insects in natural conditions (Howe 1967, Bursell 1974). Differences in biological parameters of mosquito species after developing at constant and fluctuating temperatures have been reported through the years (Huffaker 1944, Bradshaw 1980, Joshi 1996, Ragland and Kingsolver 2008, Lyons et al. 2013).

The aim of the present study was to evaluate the impact of constant and fluctuating temperature regimes on biological parameters (e.g., development, survival, adult longevity, fecundity) of one of the primary vectors of WNV, *Cx. pipiens* form 'molestus', under controlled conditions. It is the first time that a detailed study on the effects of constant and fluctuating temperatures on *Cx. pipiens* form 'molestus' has been conducted. The temperature regimes examined in the current study simulate the daily temperature fluctuations that occur in Greece and other Mediterranean countries during the WNV transmission season. This information can provide important insights into the dynamics, regulation and prediction of the species' populations in many natural systems. As a result, they contribute to the development of efficient control strategies against *Culex* species, in relation to the pathogens they transmit, especially WNV, which causes sporadic outbreaks of disease in Greece and other European countries.

Materials and Methods

Mosquito Culture

Culex pipiens form 'molestus' mosquitoes were obtained from a colony maintained in the laboratory of Benaki Phytopathological Institute, Kifissia, Greece at 24–26°C, with high relative humidity (RH) (>80%), and a photoperiod of 14:10 (L:D) h for more than 25 yr (Michaelakis et al. 2011). The culture was then established in the laboratory of Applied Zoology and Parasitology at the Aristotle University of Thessaloniki and maintained in a controlled environment at 25–26°C, 65–70% RH and at a 16:8 (L:D) h period for more than two generations prior to experimentation. The levels of RH were calibrated with a room humidifier (HUMI-ED, Solerpal, Spain). Adults were kept in plastic cages with mesh sides (20 × 20 × 20 cm) and fed on 10% sucrose solution, through a cotton wick. Females laid eggs, without being provided a bloodmeal, in round, plastic 30 ml containers (5 cm diameter × 3 cm height) filled with distilled water. Egg rafts were removed daily with a fine brush (Artist's Loft™, MSPCI, TX, United States) and placed in plastic trays (20 × 30 × 10 cm) filled with approximately 2 liters of distilled water, where larvae were reared until pupation. Larvae were fed with fish food in flakes (SuperVit-8Mix-Tropical, VPG Sweden AB), daily with a quantity of approximately 0.5 g food per tray (Hill et al. 2009). Pupae were collected daily in 30 ml containers (5 cm diameter × 3 cm height) and transferred to the cages that adults were maintained in.

Effect of Temperature on *Cx. pipiens* form 'molestus'

Newly laid eggs (<24 h old) were transferred to controlled environment chambers (GRW 1000SB CMP, E. Crisagis, Athens, Greece) at nine constant and nine fluctuating (with the same mean) temperatures: 15, 17.5, 20, 22.5, 25, 27.5, 30, 32.5, and 35 ± 0.5°C. The detailed fluctuations for each temperature regime are shown in Fig. 1. The photoperiod was constantly 16:8 (L:D) h in all treatments, while relative humidity was held above 65% with the use of saturated solution of sodium chloride (Sigma-Aldrich, St. Louis, MO) enclosed in a glass chamber. We define the terms low, moderate and high temperature as temperature regimes ranging between 15–17.5, 20–25, and 27.5–35°C, respectively, throughout the text.

Survival and Developmental Time

Newly collected eggs (<24 h old) were kept in plastic containers (5 cm diameter × 3 cm height) in controlled environment chambers, as described above. The number of egg batches used to start each of the temperature treatment varied from one to five, depending on the tested temperature, as a higher number of eggs were required for temperatures above 30°C, due to high egg mortality. Larvae hatched from each tested temperature regime, were used for the developmental studies. Each first instar larva was placed individually as a single replicate in a plastic 30-ml container (5 cm diameter × 3 cm height), covered with fine mesh, that contained distilled water up to 20–25 ml and was reared to one of the tested temperature regimes until adult emergence. When necessary, additional water was added to keep the water volume at the desired levels. Larvae were fed daily with fish flakes as described above. Each individual was monitored daily, except for those reared at temperatures above 27.5°C, which were examined every 12 h, to record survival, larval development, molting, pupation and adult emergence. The survival rate at each developmental stage (S_{j-x}) was calculated as:

$$S_{j-x} = \frac{n_x}{n_j}$$

Mean temperature (°C)	Temperature fluctuations			
	Photophase			Scotophase
	08:01-14:00	14:01-18:00	18:01-24:00	00:01-08:00
15	→ 15°C →	18°C →	15°C →	→ 13°C →
17.5	→ 17.5°C →	21°C →	17.5°C →	→ 15°C →
20	→ 20°C →	25°C →	20.5°C →	→ 16°C →
22.5	→ 22.5°C →	26°C →	25°C →	→ 17°C →
25	→ 25°C →	27.5°C →	26.5°C →	→ 22.5°C →
27.5	→ 27.5°C →	31.5°C →	28°C →	→ 25°C →
30	→ 29.5°C →	33°C →	31°C →	→ 27.5°C →
32.5	→ 32.5°C →	35°C →	32.5°C →	→ 31°C →
35	→ 35°C →	38°C →	35°C →	→ 33°C →

Fig. 1. Schematic diagram of fluctuating temperatures.

where n_x is the number of insects that survived and developed to stage x and n_j is the number of insects that survived to previous developmental stage j , as described by Ning et al. (2017).

Adult Longevity and Reproduction

After adult eclosion, mosquitoes were transferred in pairs (one female and one male) in plastic cages ($6 \times 11 \times 6$ cm) provided with 10% sucrose solution and a plastic container (2 cm diameter \times 3 cm height) for the female to oviposit and kept in the growth chambers under the same conditions of temperature, humidity and photoperiod. Adults were monitored daily, to record adult longevity, fecundity, and egg hatching.

Lower Developmental Threshold and Thermal Constant

Lower developmental thresholds (t) and thermal constants (K) for egg, larval, pupal, and total development of the species were estimated, based on the linear regression equation of the form $y = a + bT$, as described by Campbell et al. (1974). Equations and methods used for the calculation of lower thresholds, thermal constants and their standard errors (SE) were similar to previous studies conducted on other insects (Eliopoulos and Stathas 2003, Andreadis et al. 2017).

Upper Developmental Threshold and Optimum Temperature

Estimations of the upper developmental thresholds (T_m) and optimum temperature (T_o) for egg, larval, pupal, and total development of the species were based on the Logan I nonlinear model (Logan et al. 1976) and the equation of Logan et al. (1976), respectively, as described in previous studies (Eliopoulos and Stathas 2003, Andreadis et al. 2017).

Statistical Analysis

The effect of temperature, either constant or fluctuating, on developmental time, fecundity, egg hatching and adult longevity of *Cx. pipiens* form 'molestus' was examined through a one-way analysis of variance (ANOVA), using the Tukey's Honestly Significant Difference (HSD) test to compare means. A log transformation was conducted for egg hatching to obtain normal distributed data: untransformed means are presented in the tables. Mean adult longevity between males and females was compared using the independent Student's t -test. Likewise, statistical differences on developmental time, fecundity and adult longevity between constant and fluctuating temperatures were estimated using the independent Student's t -test. Results are presented as mean \pm SE. All abovementioned statistics were performed using IBM SPSS Statistics 25 Software (IBM Corp. 2017). Estimations for the nonlinear model were performed using the statistical program JMP (SAS Institute 2007). Survival of the species at each temperature regime was tested by applying a generalized linear model (GLM) with a binomial error distribution followed by Tukey's HSD. Results are presented as means \pm confidence intervals (CI). Statistical tests for survival were performed using the R software version 3.5.1 (R Core Team 2016).

Results

Survival

Survival rates for each developmental stage and temperature regime, constant and fluctuating, are presented in Figs. 2 and 3, respectively. *Culex pipiens* form 'molestus' achieved complete development between temperatures of 15 and 32.5°C, at both constant and fluctuating temperature regimes. Survival from egg to adult at both constant and fluctuating temperature of 32.5°C was low and only few individuals pupated and emerged successfully. Complete mortality was observed at 35°C for both constant and fluctuating temperature

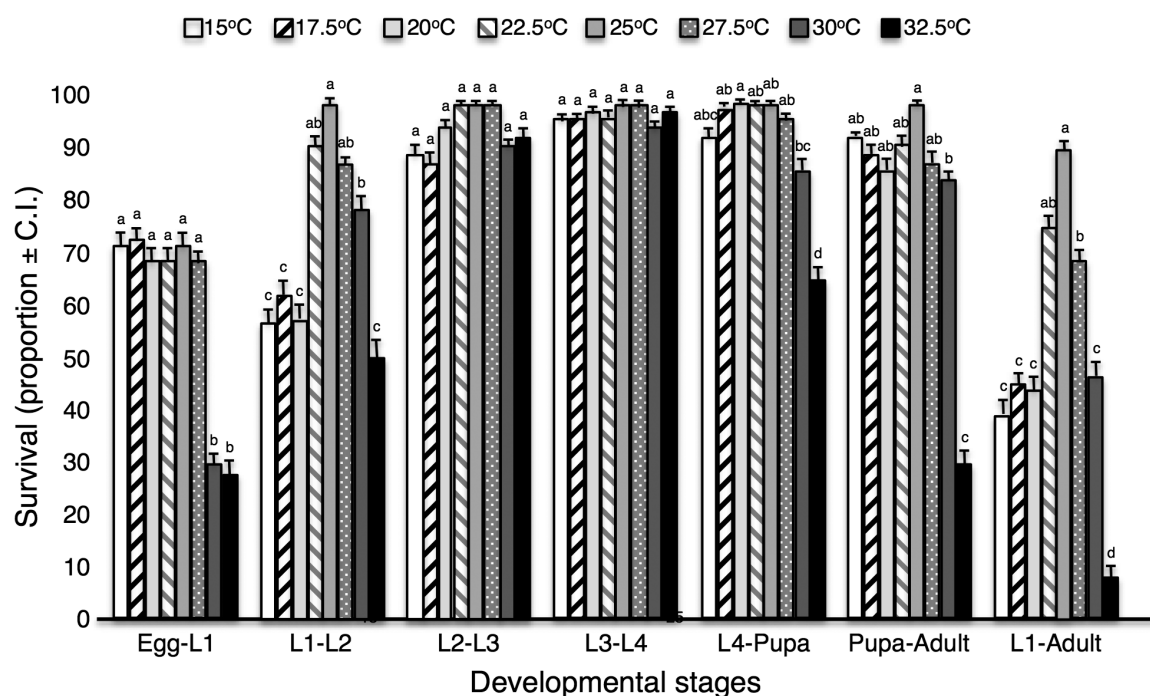


Fig. 2. Proportion ($\pm 95\%$ binomial CI) of *Cx. pipiens* form 'molestus' that survived each developmental stage at the different constant temperatures used. Bars with different lettering denote significant differences among means (GLM with binomial error distribution followed by Tukey's HSD, $P < 0.001$).

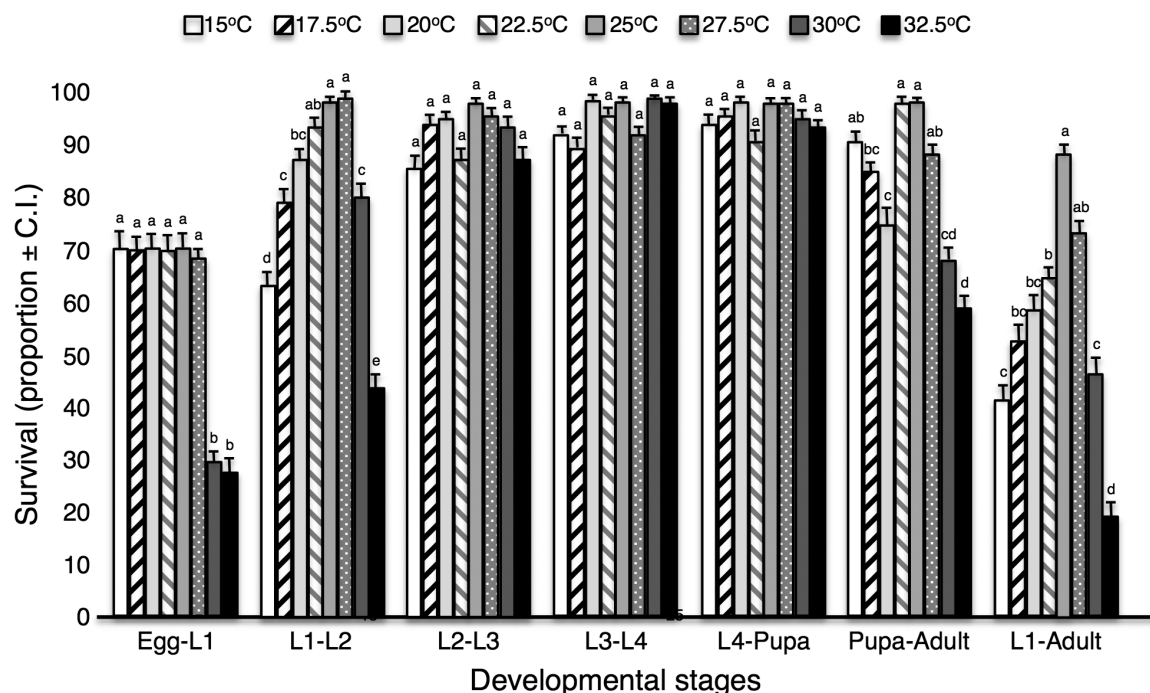


Fig. 3. Proportion ($\pm 95\%$ binomial CI) of *Cx. pipiens* form 'molestus' that survived each developmental stage at the different fluctuating temperatures used. Bars with different lettering denote significant differences among means (GLM with binomial error distribution followed by Tukey's HSD, $P < 0.001$).

regimes. At constant temperatures, the highest rate of survival, from first instar to adult, was observed at 25°C (89.1%) which was statistically similar to the survival at 22.5°C (74%) and statistically different than all other tested temperatures ($X^2_{L1-Adult} = 20.991$; $df = 7$; $P < 0.001$; Fig. 2). Similarly, at fluctuating temperatures, 25°C presented the highest overall survival of the species (87.5%) which was statistically similar to the observed survival at 27.5°C

(73.3%) and statistically different than all other tested temperatures ($X^2_{L1-Adult} = 7.125$; $df = 7$; $P < 0.001$; Fig. 3). High survival rates (86.6–98.2%) were recorded for the second and third instars in all tested temperature regimes, either constant or fluctuating, without any significant differences among them (constant: $X^2_{L2} = 1.508$; $df = 8$; $P = 0.1483$; $X^2_{L3} = 0.695$; $df = 8$; $P = 0.6962$; Fig. 2, fluctuating: $X^2_{L2} = 1.29$; $df = 7$; $P = 0.251$; $X^2_{L3} = 1.524$; $df = 7$; $P = 0.154$;

Fig. 3). Finally, survival was relatively low in egg and pupal stage at temperatures above 30°C (28–58%).

Developmental Time

Developmental time for each developmental stage and temperature regime, constant and fluctuating, are presented in Tables 1 and 2, respectively. Development rate from egg to adult of *Cx. pipiens* form 'molestus' increased between 15 and 32.5°C, in a linear fashion for both constant and fluctuating temperature regimes. At constant temperatures, the developmental times of the species decreased significantly as temperature reached 32.5°C in egg, larval, and pupal stage as well as total development (Table 1). Likewise, at fluctuating temperatures, the developmental time varied inversely with temperature in the egg, larval, and pupal stage (Table 2). The longest total developmental time of the species, was observed at fluctuating temperatures with a mean of 15°C (52.7 d), while the shortest was at constant 32.5°C (10.9 d). At low constant temperatures, larval and total developmental time of immature stages was significantly shorter compared to fluctuating temperatures with similar means (larval: $t_{15} = 6.248$; df = 52; $P < 0.001$; $t_{17.5} = 3.168$; df = 60; $P < 0.01$ / total: $t_{15} = 5.223$; df = 52; $P < 0.001$; $t_{17.5} = 2.983$; df = 60; $P < 0.01$). On the contrary, at 30°C larval and total development was significantly shorter at fluctuating temperatures compared to constant ones (larval: $t_{30} = 3.527$; df = 70; $P < 0.01$; total: $t_{30} = 3.942$; df = 70; $P < 0.01$). Similarly, eggs in fluctuating temperatures with a mean of 27.5 and 30°C, developed significantly faster compared to the corresponding constant temperatures ($t_{27.5} = 5.539$; df = 92; $P < 0.001$; $t_{30} = 4.904$; df = 70; $P < 0.001$). At 32.5°C, developmental times did not differ significantly between constant and fluctuating temperatures at all developmental stages (egg: $t_{32.5} = 0.084$; df = 27; $P = 0.933$, larval: $t_{32.5} = 0.840$; df = 27; $P = 0.408$, pupal: $t_{32.5} = 0.864$; df = 27; $P = 0.406$, total: $t_{32.5} = 0.503$; df = 27; $P = 0.619$).

Adult Longevity

Adult longevity for both sexes at constant and fluctuating temperatures are presented in Figs. 4 and 5, respectively. Longevity for male and female adults decreased with temperature increase in the range 15–32.5°C at all tested constant and fluctuating temperature regimes, with the exception of constant 20°C. At low constant and fluctuating temperatures (15–20°C), adult longevity for both male and female

individuals, was significantly longer compared to higher temperatures (constant: $F_{\text{male}} = 91.87$; df = 7; $P < 0.001$; $F_{\text{female}} = 87.817$; df = 7; $P < 0.001$; Fig. 4, fluctuating: $F_{\text{male}} = 65.93$; df = 7; $P < 0.001$; $F_{\text{female}} = 113.55$; df = 7; $P < 0.001$; Fig. 5). The critical higher temperature for adult longevity was between 30 and 32.5°C. In particular, at constant and fluctuating temperature of 32.5°C, longevity of females and males ranged from 1.5 to 8 d. Although both males and females completed development at 32.5°C, they emerged as weak adults and died within few days. Female adults lived significantly longer compared to males at all tested constant and fluctuating temperature regimes, except for constant 32.5°C (constant: $t_{15} = 4.326$; df = 26; $P < 0.001$; $t_{17.5} = 4.891$; df = 30; $P < 0.001$; $t_{20} = 4.9$; df = 45; $P < 0.001$; $t_{22.5} = 2.953$; df = 35; $P < 0.01$; $t_{25} = 4.626$; df = 40; $P < 0.001$; $t_{27.5} = 4.3$; df = 31; $P < 0.01$; $t_{30} = 4.92$; df = 37; $P < 0.001$; $t_{32.5} = 0.235$; df = 8; $P = 0.846$, fluctuating: $t_{15} = 6.526$; df = 24; $P < 0.001$; $t_{17.5} = 4.331$; df = 28; $P < 0.001$; $t_{20} = 6.369$; df = 31; $P < 0.001$; $t_{22.5} = 3.23$; df = 34; $P < 0.01$; $t_{25} = 5.394$; df = 31; $P < 0.001$; $t_{27.5} = 7.681$; df = 59; $P < 0.001$; $t_{30} = 10.17$; df = 31; $P < 0.001$; $t_{32.5} = 7.247$; df = 17; $P < 0.001$). Adult longevity of males at low temperatures was significantly different between constant and corresponding fluctuating temperatures ($t_{15} = 5.974$; df = 24; $P < 0.001$; $t_{17.5} = 7.288$; df = 31; $P < 0.001$) whereas no statistical differences were observed for most of the other tested temperatures. On the contrary, female adults lived longer in a number of tested fluctuating temperatures compared to constant ones ($t_{20} = 2.483$; df = 37; $P < 0.05$; $t_{22.5} = 5.986$; df = 38; $P < 0.001$; $t_{25} = 8.818$; df = 36; $P < 0.001$; $t_{27.5} = 4.128$; df = 43; $P < 0.001$; $t_{32.5} = 7.337$; df = 5; $P < 0.01$).

Reproduction

Fecundity and egg hatching, at constant and fluctuating temperatures, are presented in Tables 3 and 4, respectively. Female mosquitoes laid a significantly higher number of eggs at moderate constant and fluctuating temperature regimes compared to 27.5 and 30°C (Table 3 and 4). No eggs were laid at 32.5°C in both constant and fluctuating temperatures. A significantly higher number of eggs were laid by females reared at low constant temperatures than fluctuating ones with the same mean temperature ($t_{15} = 7.2$; df = 15; $P < 0.001$; $t_{17.5} = 4.31$; df = 20; $P < 0.01$). At high tested temperatures, no statistical difference in fecundity was observed between constant and

Table 1. Developmental time (days ± SE) of *Cx. pipiens* form 'molestus' at constant temperature regimes

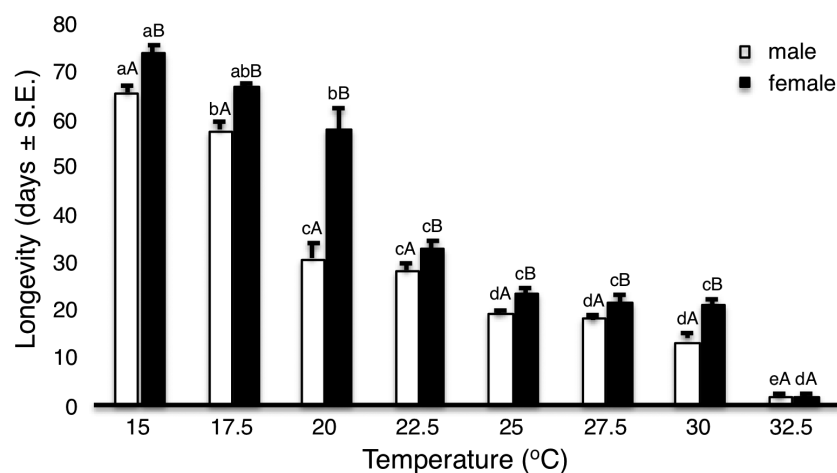
Temperature (°C)	n ^a	Developmental time (days ± SE)							
		Egg stage	Larval instars				Larval stage	Pupal stage	Total
			First	Second	Third	Fourth			
15	28	4.1 ± 0.06a ^b	13.2 ± 0.19a	7.5 ± 0.21a	7.8 ± 0.16a	10.2 ± 0.16a	38.7 ± 0.30a	6.4 ± 0.14a	49.2 ± 0.33a
17.5	32	3.1 ± 0.05b	11.8 ± 0.28b	6.3 ± 0.24b	6.2 ± 0.22b	8.3 ± 0.17b	32.5 ± 0.53b	4.4 ± 0.19b	40.0 ± 0.54b
20	47	2.9 ± 0.05b	11.5 ± 0.32b	5.2 ± 0.16c	4.0 ± 0.2c	6.7 ± 0.13c	27.4 ± 0.30c	3.7 ± 0.10c	34.0 ± 0.35c
22.5	37	2.0 ± 0.03c	6.4 ± 0.25c	4.1 ± 0.16d	4.0 ± 0.16c	3.9 ± 0.21d	18.4 ± 0.43d	2.7 ± 0.08d	23.1 ± 0.44d
25	42	2.0 ± 0.03c	3.7 ± 0.01d	3.0 ± 0.12e	2.6 ± 0.12d	3.6 ± 0.08d	12.9 ± 0.26e	2.1 ± 0.05de	17.1 ± 0.28e
27.5	33	1.5 ± 0.09d	3.6 ± 0.11d	2.7 ± 0.12e	2.0 ± 0.11de	4.0 ± 0.13d	12.2 ± 0.27ef	1.5 ± 0.09ef	15.3 ± 0.25f
30	39	1.3 ± 0.08d	3.5 ± 0.16d	2.7 ± 0.16e	1.7 ± 0.13e	3.4 ± 0.11d	11.4 ± 0.17f	1.6 ± 0.08ef	14.3 ± 0.25f
32.5	10	0.9 ± 0.08e	2.8 ± 0.13d	1.7 ± 0.15f	1.7 ± 0.15e	2.6 ± 0.22e	8.8 ± 0.29g	1.2 ± 0.1f	10.9 ± 0.37g
35	357	-	-	-	-	-	-	-	-
F		243.90	356.29	108.83	153.18	291.86	929.22	215.61	1242.67
df		7	7	7	7	7	7	7	7
P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

^aNumber of individuals.

^bValues in a column followed by the same letter are not significantly different (Tukey's HSD test, $P < 0.05$).

Table 2. Developmental time (days \pm SE) of *Cx. pipiens* form 'molestus' at fluctuating temperature regimes

Temperature fluctuations (°C)	<i>n</i> ^a	Developmental time (days \pm SE)							
		Egg stage	Larval instars				Larval stage	Pupal stage	Total
			First	Second	Third	Fourth			
13–18	26	4.3 \pm 0.09 ^a	10.7 \pm 0.18 ^a	6.4 \pm 0.14 ^a	8.5 \pm 0.26 ^a	17.2 \pm 0.31 ^a	42.8 \pm 0.60 ^a	5.6 \pm 0.11 ^a	52.7 \pm 0.60 ^a
15–21	30	3.0 \pm 0.03 ^b	9.3 \pm 0.28 ^b	4.6 \pm 0.27 ^b	7.5 \pm 0.59 ^a	16.0 \pm 0.68 ^a	37.4 \pm 1.47 ^b	4.3 \pm 0.11 ^b	44.7 \pm 1.51 ^b
16–25	33	2.6 \pm 0.09 ^c	5.5 \pm 0.23 ^c	3.7 \pm 0.26 ^c	4.2 \pm 0.23 ^b	10.8 \pm 0.36 ^b	24.2 \pm 0.45 ^c	2.8 \pm 0.10 ^c	29.6 \pm 0.44 ^c
17–26	36	2.1 \pm 0.06 ^d	4.8 \pm 0.18 ^{cd}	3.7 \pm 0.13 ^c	3.9 \pm 0.16 ^b	7.9 \pm 0.18 ^c	20.4 \pm 0.32 ^d	2.4 \pm 0.08 ^c	24.9 \pm 0.34 ^d
22.5–27.5	33	1.9 \pm 0.05 ^d	4.2 \pm 0.76 ^d	2.2 \pm 0.07 ^d	2.3 \pm 0.08 ^c	4.6 \pm 0.14 ^e	13.4 \pm 0.23 ^e	2.0 \pm 0.03 ^d	17.2 \pm 0.25 ^e
25–31.5	61	1.0 \pm 0.04 ^e	3.1 \pm 0.10 ^e	2.1 \pm 0.08 ^d	2.2 \pm 0.08 ^c	6.5 \pm 0.21 ^d	13.9 \pm 0.32 ^e	1.8 \pm 0.05 ^d	16.8 \pm 0.35 ^e
27.5–33	33	0.9 \pm 0.04 ^e	3.2 \pm 0.11 ^e	1.9 \pm 0.13 ^d	2.0 \pm 0.12 ^c	3.3 \pm 0.14 ^{ef}	10.5 \pm 0.18 ^f	1.6 \pm 0.09 ^d	13.0 \pm 0.20 ^f
31–35	19	0.8 \pm 0.05 ^e	3.0 \pm 0.13 ^e	1.6 \pm 0.12 ^d	1.8 \pm 0.12 ^c	2.8 \pm 0.14 ^f	9.2 \pm 0.27 ^f	1.1 \pm 0.04 ^e	11.1 \pm 0.27 ^f
33–38	402	-	-	-	-	-	-	-	-
<i>F</i>		398.17	273.53	93.50	108.68	267.98	406.24	286.97	559.85
<i>df</i>		7	7	7	7	7	7	7	7
<i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

^aNumber of individuals.^bValues in a column followed by the same letter are not significantly different (Tukey's HSD test, $P < 0.05$).**Fig. 4.** Adult longevity (days \pm SE) of male and female *Cx. pipiens* form 'molestus' reared under constant temperatures. Error bars represent standard errors of the mean. Means between temperatures of the same sex followed by the same lowercase letter are not significantly different (one-way ANOVA followed by Tukey's HSD test, $P < 0.05$). Means between sexes in a particular temperature followed by the same uppercase letter are not significantly different (Student *t*-test, $P < 0.05$).

fluctuating temperatures. Furthermore, egg hatching ranged between 70.9 and 81.9% at low and moderate constant and fluctuating temperatures which was statistically higher, compared to higher temperatures tested, where egg hatching ranged between 18.8 and 54% (Tables 3 and 4).

Lower and Upper Developmental Thresholds, Thermal Constant and Optimum Temperature

Lower developmental thresholds (*t*) and degree-day requirements (*DD*) of each developmental stage are presented in Table 5. The lowest developmental thresholds of the various stages ranged from 11.17 to 11.95°C at constant temperature regimes and from 11.09 to 12.74°C at fluctuating ones. The thermal constant for total development was similar at both constant and fluctuating temperature regimes and was estimated at 243.90 *DD*. The optimum temperature (*T_o*) estimated for all developmental stages of *Cx. pipiens* form 'molestus' ranged between 32.4 and 34.9°C at constant temperatures, whereas the upper developmental thresholds (*T_m*) was calculated to 35°C for all developmental stages. The corresponding

values for the fluctuating temperatures were 31.9–34.9°C and 35°C, respectively (Table 6).

Discussion

Previous studies have clearly demonstrated that temperature affects the biological parameters and the population bionomics of *Culex* species (Shelton 1973; Rueda et al. 1990; Olejníček and Gelbic 2000; Loetti et al. 2007, 2011; Andreadis et al. 2014; Ciota et al. 2014) as well as other mosquito genera (Rueda et al. 1990, Joshi 1996, Alto and Juliano 2001, Impoinvil et al. 2007, Delatte et al. 2009, Mohammed and Chadee 2011, Carrington et al. 2013, Lyons et al. 2013). The current study extended this work by examining the different impact that constant and fluctuating temperatures may have on *Cx. pipiens* form 'molestus', an important but understudied vector of WNV in Greece.

Our results showed that the critical temperature for survival was between 30 and 35°C. At 32.5°C a small number of individuals in both constant and fluctuating temperature regimes, completed

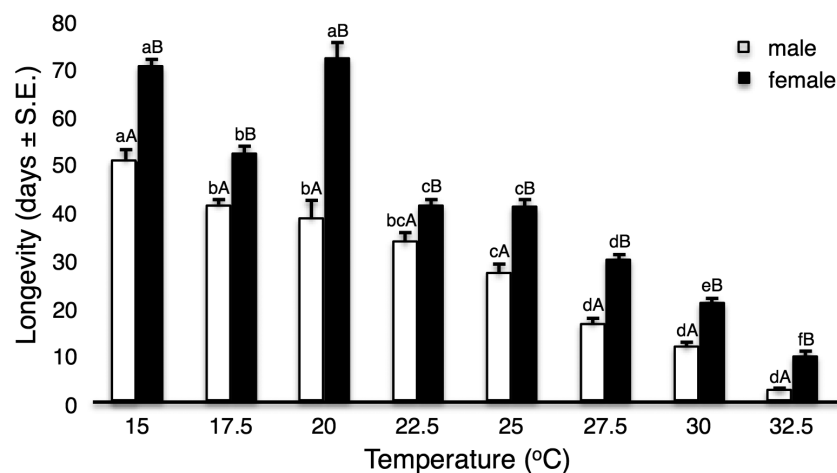


Fig. 5. Adult longevity (days \pm S.E.) of male and female *Cx. pipiens* form 'molestus' reared under fluctuating temperatures. Error bars represent standard errors of the mean. Means between temperatures of the same sex, followed by the same lowercase letter are not significantly different (one-way ANOVA followed by Tukey's HSD test, $P < 0.05$). Means between sexes in a particular temperature followed by the same uppercase letter are not significantly different (Student *t*-test, $P < 0.05$).

Table 3. Fecundity (eggs/female) (mean \pm SE) and percentage (%) of egg hatching (mean \pm SE) of *Cx. pipiens* form 'molestus' at constant temperatures

Temperature (°C)	<i>n</i> ^a	Eggs per female (mean \pm SE)	% egg hatching (mean \pm SE)
15	16	80.2 \pm 1.7cd ^b	78.8 \pm 1.9a
17.5	17	89.7 \pm 3.8bc	81.2 \pm 1.8a
20	24	124.1 \pm 3.3a	78.8 \pm 2.9a
22.5	20	104.9 \pm 3.2b	76.3 \pm 1.4a
25	22	70.8 \pm 3.0cd	70.9 \pm 2.4a
27.5	13	64.6 \pm 2.9d	48.8 \pm 4.2b
30	16	41.2 \pm 14.7e	18.8 \pm 11.2c
<i>F</i>		41.12	30.42
<i>df</i>		6	6
<i>P</i>		<0.001	<0.001

^aNumber of females.

^bValues in a column followed by the same letter are not significantly different (one-way ANOVA followed by Tukey's HSD test, $P < 0.05$); no eggs were laid at 32.5°C; all individuals failed to complete development at 35°C.

development, while at 35°C no development was recorded. These findings agree with another study on *Cx. pipiens* form 'pipiens', where only two individuals reached the adult stage at 33°C (Loetti et al. 2011). On the other hand, a study observed adult emergence at 34°C for *Culex quinquefasciatus* (Say) (Diptera: Culicidae) (Rueda et al. 1990), and even at 37°C for field-collected populations of *Cx. pipiens* form 'pipiens' (Mpho et al. 2002). In our study, survival from first instar larva to adult emergence was higher at moderate constant and fluctuating temperatures compared to low temperatures as well as temperatures above 27.5°C which agrees with other studies conducted on *Cx. pipiens* form 'pipiens' and *Cx. quinquefasciatus* (Suman et al. 2011, Chuang et al. 2012). When *Cx. pipiens* form 'pipiens' was reared under conditions simulating the weather in Egypt, survival rates of the larvae during the winter–spring experiments (mean temperature 20.5°C) were lower as compared to survival during the summer–autumn period (mean temperature 30.2°C) (Abouzied 2017). The variations in survival rates of *Cx. pipiens* among studies can potentially be explained by the different experimental conditions as well as the different geographic strains and forms used in the experiments.

A decrease in total developmental time in a linear fashion as temperature increased was observed. A similar relationship was observed by other authors in various mosquito species including

Cx. pipiens form 'pipiens', *Cx. quinquefasciatus*, and *Culex restuans* (Theobald) (Diptera: Culicidae) (Madder et al. 1983, Rueda et al. 1990, Ribeiro et al. 2004). Loetti et al. (2011) concluded that the immature development of mosquitoes was generally shortened by warm temperatures and lengthened by cooler temperatures. At lower water temperature, larval development is slower, and maturation is delayed (Ray 1960, Atkinson 1994). Water temperature was also the most important parameter affecting the rate of larval development of *Cx. pipiens* form 'pipiens' (Madder et al. 1983) while the mean duration of the larval stage and the mean pupal period of *Cx. quinquefasciatus* decreased significantly at higher temperature regimes compared to lower ones (Grech et al. 2015).

Concerning adult longevity, our results indicated that at temperatures above 27.5°C longevity was drastically reduced compared to lower constant and fluctuating temperature regimes. Similarly, Chuang et al. (2012) reported that higher temperatures had negative effects on the longevity of mosquitoes. In another study, male and female adults of *Cx. pipiens* form 'pipiens' showed prolonged longevity at air temperatures of 20°C as compared to 30°C (Abouzied 2017). Similar results were obtained for male and female *Culex pipiens pallens* (Coquillett) (Diptera: Culicidae) mosquitoes (Oda et al. 2002). However, in adult *Cx. quinquefasciatus*, the mean longevity was higher at 30°C than at 25°C (Oda et al. 2002). In our study, female

Table 4. Fecundity (eggs/female) (mean \pm SE) and percentage (%) of egg hatching (mean \pm SE) of *Cx. pipiens* form 'molestus' at fluctuating temperatures

Temperature fluctuations (°C)	<i>n</i> ^a	Eggs per female (mean \pm SE)	% egg hatching (mean \pm SE)
13–18	12	54.0 \pm 3.2 ^{cb}	76.3 \pm 1.8a
15–21	12	58.2 \pm 6.2c	81.9 \pm 4.6a
16–25	15	109.4 \pm 6.8a	78.2 \pm 1.9a
17–26	20	72.5 \pm 4.4bc	76.6 \pm 2.7a
22.5–27.5	16	92.7 \pm 5.7ab	76.5 \pm 2.2a
25–31.5	32	70.7 \pm 3.6bc	54.0 \pm 3.0b
27.5–33	14	48.9 \pm 2.8c	33.2 \pm 2.2c
<i>F</i>		15.13	29.72
<i>df</i>		6	6
<i>P</i>		<0.001	<0.001

^aNumber of females.^bValues in a column followed by the same letter are not significantly different (one-way ANOVA followed by Tukey's HSD test, $P < 0.05$); no eggs were laid at 32.5°C; all individuals failed to complete development at 35°C.**Table 5.** Linear regression equations for developmental time and temperature, lower developmental thresholds and thermal constants for eggs, larvae, pupae and total development of *Cx. pipiens* form 'molestus'

Developmental stage	Temperature	Regression equation	<i>R</i> ²	<i>t</i> \pm SE	<i>K</i> \pm SE
Egg	Constant	$y = 0.0457x - 0.526$	0.86	11.51 \pm 2.40	21.88 \pm 3.60
	Fluctuating	$y = 0.0588x - 0.7494$	0.92	12.74 \pm 1.65	17.01 \pm 2.07
Larva	Constant	$y = 0.005x - 0.0563$	0.96	11.26 \pm 1.21	200.00 \pm 16.21
	Fluctuating	$y = 0.0051x - 0.0586$	0.97	11.49 \pm 1.09	196.08 \pm 14.54
Pupa	Constant	$y = 0.0382x - 0.4566$	0.97	11.95 \pm 1.06	26.18 \pm 1.94
	Fluctuating	$y = 0.0374x - 0.415$	0.93	11.09 \pm 1.73	26.73 \pm 3.08
Total	Constant	$y = 0.0041x - 0.0458$	0.97	11.17 \pm 1.11	243.90 \pm 18.14
	Fluctuating	$y = 0.0041x - 0.0481$	0.98	11.73 \pm 0.95	243.90 \pm 16.15

*R*²: coefficient of determination (linear); *t*: lower temperature thresholds (°C); *K*: thermal constant (DD); data were recorded at eight constant and fluctuating temperatures (15, 17.5, 20, 22.5, 25, 27.5, 30, and 32.5°C).**Table 6.** Parameter estimates of Logan I nonlinear model for development of *Cx. pipiens* form 'molestus'

Developmental stage	Temperature	ψ	ρ	<i>T</i> _m	ΔT	<i>T</i> _o	<i>R</i> ²
Egg	Constant	0.058 \pm 0.007	0.090 \pm 0.004	35.0 \pm 0.0002	0.0058	34.9	0.9838
	Fluctuating	0.030 \pm 0.007	0.124 \pm 0.009	34.99 \pm 0.054	1.469	31.9	0.9910
Larva	Constant	0.008 \pm 0.0005	0.080 \pm 0.002	35.0 \pm 0.018	0.7709	32.7	0.9840
	Fluctuating	0.008 \pm 0.0006	0.081 \pm 0.003	35.0 \pm 0.0003	0.015	34.9	0.9879
Pupa	Constant	0.004 \pm 0.006	0.097 \pm 0.005	35.0 \pm 0.042	1.035	32.7	0.9882
	Fluctuating	0.066 \pm 0.007	0.080 \pm 0.004	35.0 \pm 0.0001	0.004	34.9	0.9923
Total	Constant	0.007 \pm 0.0003	0.080 \pm 0.002	35.0 \pm 0.012	0.636	32.4	0.9896
	Fluctuating	0.006 \pm 0.0003	0.087 \pm 0.002	35.0 \pm 0.017	0.871	32.6	0.9944

 ψ , ρ , ΔT : Logan equation parameters; *T*_m: maximum temperature threshold (°C); *T*_o: optimum temperature for development (°C); *R*²: nonlinear regression coefficient; data were recorded at eight constant and fluctuating temperatures (15, 17.5, 20, 22.5, 25, 27.5, 30, and 32.5°C).

adults lived significantly longer than males in most constant temperatures tested and all fluctuating ones. This is not surprising, as it is well known that mosquitoes show sexual dimorphism and vary in developmental time, adult longevity and body size, among other characters (Clements 1992). In *Cx. pipiens* form 'pipiens', sex differences in the developmental time were observed between 15 and 30°C; females required more time to reach the adult stage than males because of a longer fourth instar larval stage (Loetti et al. 2011). These differences could be related to different nutritional requirements between life stages since *Cx. pipiens* form 'molestus' is an autogenous species.

As for the effect of temperature on fecundity, our study showed that females laid more eggs at moderate constant and fluctuating temperature regimes compared to 27.5 and 30°C, while no eggs were laid at 32.5°C. Another study indicated that *Cx. pipiens* form

'pipiens' females produced a higher average number of eggs at 20°C than at 30°C (Abouzieed 2017). Similarly, Oda et al. (2002) found that female insemination and egg hatchability in *Cx. pipiens pallens* were significantly higher at 25°C than at 30°C. In contrast, Madder et al. (1983) reported that field overwintering females of both *Cx. pipiens* and *Cx. restuans* laid fewer eggs compared to females of the summer generations. Furthermore, in our study egg hatching was highest at low and moderate constant and fluctuating temperatures, whereas it was significantly reduced in temperatures of 27.5°C and especially 30°C, in both constant and fluctuating temperature conditions. Likewise, egg hatching for *Cx. pipiens pallens* was significantly higher at 25°C than at 30°C (Oda et al. 2002).

The lowest developmental thresholds calculated in this work, ranged from 11.17 to 11.95°C at constant temperature regimes and

from 11.09 to 12.74°C at the fluctuating ones. On the other hand, the lowest threshold temperatures for *Cx. pipiens* form 'pipiens' reported by other authors were calculated around or below 10°C (Madder et al. 1983, Ribeiro et al. 2004, Loetti et al. 2011). To cite just one example, Loetti et al. (2011) reported that the lowest thresholds of *Cx. pipiens* form 'pipiens' were 8.4 and 9.8°C for males and females, respectively. The difference between the results we obtained and the results on previous studies, can be explained by the different experimental conditions applied in each study, such as range of tested temperatures, mosquito populations, generation number, and rearing conditions.

Thermal constant for total development was similar to both constant and fluctuating temperature regimes and was estimated at 243.90 DD which was within that reported by other authors (132–252.5 DD) (Madder et al. 1983, Ribeiro et al. 2004, Loetti et al. 2011). In addition, the estimated optimum temperatures and the upper thermal thresholds were similar between constant and fluctuating temperatures and were calculated to 31.9–34.9°C and 35°C, respectively. In a previous study on *Cx. pipiens* form 'pipiens', the upper thresholds were calculated to 34.4°C in males and 34.2°C in females, values similar to the ones reported in our work (Loetti et al. 2011). In the same study, the estimated optimum rearing temperature for males and females of the species was 28.5°C. However, these values were not estimated by a nonlinear model. It is important to mention that different values on developmental thresholds among studies can be attributed to differences in experimental conditions as well as geographical adaptations of the species.

Insects have been reported to develop faster at fluctuating than at constant temperatures when the fluctuating temperatures are within the optimal range of development for the organism (Hagstrum and Hagstrum 1970, Mironidis and Savopoulou-Soultani 2008). On the contrary, development of certain species at constant temperatures is shorter above 25–30°C and longer below this range, than at fluctuating temperatures with the same means (Hagstrum and Milliken 1991). In our study, at low constant temperatures, larval and total developmental time was significantly shorter compared to fluctuating temperatures. At high temperatures, egg, larval and total development was significantly faster at fluctuating temperature conditions. Differences between constant and fluctuating temperatures in our study were also observed on adult longevity and fecundity. In particular, adult longevity of males at low temperatures was significantly longer at constant temperatures than at corresponding fluctuating temperatures whereas female adults lived longer in fluctuating temperatures compared to constant ones. In addition, a higher number of eggs were laid by females at low constant temperatures than fluctuating ones. Experiments on *Cx. pipiens* complex testing the differences between constant and fluctuating temperatures have not been conducted in the past, however, in a similar study on *Aedes krombeini* (Huang) (Diptera: Culicidae), the developmental time of males, adult longevity for both sexes and fecundity were statistically different between constant and fluctuating temperatures (Joshi 1996).

In conclusion, temperature plays a considerable role in the biology of *Cx. pipiens* form 'molestus'. Data from this study indicate that certain biological parameters of *Cx. pipiens* form 'molestus' differ when measured at constant and fluctuating temperatures. Such findings are important for evaluating geographic distribution, development, reproduction, and longevity of the species in the field, which can benefit the potential development of effective control strategies against the species and associated arboviruses they transmit to human and domestic animals. Mosquito populations reared in the laboratory can be used as reference organisms for comparison with data from similar experiments conducted under semi-field and field

conditions. Furthermore, life tables of *Cx. pipiens* form 'molestus' and other mosquito species, need to be developed in the laboratory, in either constant or fluctuating simulations, and should also be validated under field conditions.

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