

Biology of *Diadiplosis multifila* (Diptera: Cecidomyiidae) in *Planococcus citri* under constant temperatures

Alexandre Martins dos Santos^{1*}, José Eudes de Morais Oliveira², Andréa Nunes Moreira de Carvalho³, Martin Duarte de Oliveira², Carla Patrícia Oliveira de Assis², Fabiana Soares Cariri Lopes²

¹Federal University of Piauí, Bom Jesus, Brazil

²Embrapa Semi-Árido, Petrolina, Brazil

³Instituto Federal de Educação, Ciência e Tecnologia do Sertão Pernambucano, Petrolina, Brazil

*Corresponding author, email: alexandre.agro2012@hotmail.com

Abstract

Diadiplosis multifila was recently discovered feeding on *Planococcus citri* eggs in vineyards in the semi-arid northeast region of Brazil. The objective of the present paper was to study the biology of *D. multifila* in *P. citri* under constant temperatures of 22, 25, 28, and 31 °C. We evaluated its embryonic stage, egg viability, development period, survival of larva and pupa, longevity, average number of eggs, and sex ratio. *D. multifila* completed its life cycle in all temperatures except for 31 °C. The length of the embryonic period ranged from 4 to 7 days. The larval stage was longer at a temperature of 22 °C (8.6 days) and shorter at 28 °C (6.4). The pupal stage exhibited durations of 12.9, 10.4, and 8.2 days for temperatures of 22, 25, and 28 °C, respectively. The average viability in the larval stage was 97% and 83% in the pupal stage. The total life cycle took 16.7 (28 °C), 20 (25 °C), and 27 (22 °C) days to complete. The adults lived for approximately 2 days and the females produced on average 34, 25, and 19 eggs at temperatures of 22, 25, and 28 °C, respectively. The sex ratio varied from 0.46 to 0.54.

Keywords: biological control, citrus mealybug, predator, Lower-Middle São Francisco Valley

Introduction

The Lower-Middle São Francisco Valley, home to the largest center of irrigated fruit growing in Brazil (Leão & Moutinho, 2014), is renowned for its grape and wine production, being responsible for approximately 95% of Brazilian table grape production and 15% of its fine wine production (Zanini & Rocha, 2010). However, in this region the grape crops have suffered production losses owing to attacks from the citrus mealybug, *Planococcus citri* (Risso, 1813) (Hemiptera: Pseudococcidae) (Soares & Leão, 2009).

To control this pest, some producers apply organophosphate insecticides, which present elevated toxicity and are not selective

of the pest's natural enemies (Botton et al., 2007; Arshad et al., 2015). Currently, there is a push to produce foods using fewer chemicals, with the aim to reduce impacts both human health and the environment (Zagati & Pagliuca, 2014). For this reason, employing natural enemies for pest control is expanding in Brazil. Various natural enemies of the citrus mealybug have been recorded, including 25 parasitoid species and 33 predators such as insects and Acari (CPC, 2015).

The Cecidomyiidae family has 6,100 species (Tokuda, 2012), with only six referred to as predators (CPC, 2015). The species *Diadiplosis multifila* (Felt, 1907) (Diptera: Cecidomyiidae) was found in South America, the Caribbean

and Fiji feeding on eggs of *P. citri* (Risso, 1813) (Hemiptera: Pseudococcidae) and *Icerya montserratensis* (Riley and Howard, 1890) (Hemiptera: Monophlebidae) (GAGNÉ, 1994; HARRIS, 1997).

In Brazil, *D. multifila* was found in the State of Espírito Santo, in the city of Vitória, feeding on different species of cochonilhas (CULIK et al., 2009). Recently, in vineyards of the Lower-Middle São Francisco Valley, the species *Diadiplosis multifila* (Diptera: Cecidomyiidae) was recorded in *P. citri* colonies present in grape bunches (Oliveira et al., 2013), however, little is known regarding the biology of this predator.

The present study, which investigated the biology of *D. multifila* feeding on *P. citri* eggs, contributed to the understanding of this small group of Cecidomyiidae. The literature presents information on biological parameters of the predatory species *F. acarisuga* (Gillespie et al., 2000; Mo & Liu, 2006) and *A. aphidimyza* (Havelka, 1980; Kim & Kim, 2004) which are commercialized in Europe and North America for Acari (Acari: Tetranychidae) and aphid (Hemiptera: Aphididae) control, respectively, in protected environments (Jandricic et al., 2013).

Thus, in the present study the biological data investigated for *D. multifila* was compared to these other two species of Cecidomyiidae. Thus, it is of great importance to study its bioecology, as well as the potential of *D. multifila* as an agent for *P. citri* control, with the aim to implement an applied biological control program. The present study has the objective to investigate the biology of *D. multifila* in *P. citri* under constant temperature regimes.

Material and Methods

The biological study of *D. multifila* in *P. citri* was conducted at the Entomology Laboratory of the Center of Agricultural Research for Semi-Arid Tropics – EMBRAPA SEMIÁRIDO, in the Petrolina municipality, Pernambuco state, Brazil. Initially, colonies of the two species involved in the present study were established, with the rearing protocols explained below.

Rearing of P. citri (Risso, 1813) (Hemiptera: Pseudococcidae)

The rearing of *P. citri* began with the collection of females in the reproductive phase from commercial vineyards in Petrolina municipality, Pernambuco state. The mealybugs were taken to the laboratory and transferred to squash (*Curcubita maxima*) of a variety known as "Jacarezinho." Prior to infestation, the squash were cleaned using a damp sponge and neutral detergent, rinsed in running water, and dried at ambient temperature. The infestation occurred via the transfer of approximately 500 females per squash with the aid of a flexible fine point brush.

The infested squash were maintained in plastic containers (10.5 cm diameter and 7 cm height) with perforated sides, which were placed in racks with shelves lined with paper. Approximately 30 to 50 days after infestation, the squash were replete with mealybugs at all phases of development. To increase the abundance of *P. citri*, squash without mealybugs were placed in contact with those already infested for five days. This allowed the nymphs of the first instar to move from one squash to another. The infested squash were air-conditioned at a temperature of 25 ± 1 °C, $70 \pm 10\%$ relative humidity, and a photoperiod of 12 h light and 12 h dark (12L:12D).

Rearing of D. multifila (Felt, 1907) (Diptera: Cecidomyiidae)

D. multifila were obtained from collecting bunches of grapes infested with citrus mealybugs in vineyards located in Petrolina municipality, Pernambuco state. The infested plant sections were placed inside entomological cages for observation of the emergence of flies. After identifying them as *D. multifila*, the colony was established on *P. citri* mealybugs in Jacarezinho variety squash in wooden cages (53.5 × 43 × 47.5 cm), with a glass top, sides of fine nylon screen, and a front covered with "voile" cloth, under controlled conditions of temperature (25 ± 1 °C), relative humidity ($70 \pm 10\%$) and photoperiod (12L:12D). Approximately four squash infested with reproducing *P. citri* females were placed into each cage. Reproducing females are easily identified by the presence of an ovisac, which is formed by wax filaments containing eggs and is located on the lower abdomen of the mealybug.

Approximately 50 male and 50 female

D. multifula were released into each cage to potentially couple and take their appropriate positions. The sex classification was determined by observing the ovipositor apparatus. After hatching, *D. multifula* larvae fed on *P. citri* eggs until the pupal stage. After the emergence of *D. multifula* adults, new squash infested with *P. citri* females in the reproduction phase were placed in the cage to ensure an adequate food supply for the new generation.

Biology of *D. multifula* at different temperatures

The biological aspects of *D. multifula* were studied under constant temperatures of 22, 25, 28, and 31 °C, relative humidity of 60 ± 10%, and photoperiod of 12L: 12D. Initially, ten mealybugs with ovisacs from the reared stock were placed inside plastic containers (8.5 cm diameter and 5.5 cm height) with the bottom lined with filter paper. This density of mealybugs in the container was determined using results from previous experiments evaluating the oviposition of *D. multifula* in different densities of *D. multifula*. Following this, five females and five males adults of *D. multifula* were transferred from the reared stock to the plastic container using an entomological vacuum apparatus. This procedure was performed to obtain mating and oviposition. After this transfer, the container was sealed with plastic film and the adults were confined for 24 h.

After the *D. multifula* larvae hatched, which lasted in half five days, two each individual (up to 3 h old) was transferred with the aid of a fine pointed craft knife to a Petri dish (5 cm diameter), which contained ten ovisacs full of eggs, randomly selected from the rearing of *P. citri*, that were the food source. Soon after, the dishes were sealed with plastic film and placed in air-conditioned chambers. Every day, *D. multifula* larvae were offered mealybug eggs (average of 50 eggs) collected from the rearing stock to ensure food *ad libitum*. After emergence, a *D. multifula* female was transferred and released again into the plastic container holding ten mealybugs with ovisacs, droplets of *P. citri* honeydew was offered as food, and five *D. multifula* males from the rearing stock were made available for coupling and later oviposition.

Biological aspects were evaluated for each temperature in at least 50 individuals, with each one of these corresponding to one replication. The following factors were then evaluated: the embryonic period, egg viability, development period and survival of larva and pupa, as well as longevity, average number of eggs, and sex ratio.

Statistical Analyses

The egg-adult period of *D. multifula* observed at the different temperature ranges, was employed to determine the lower threshold temperature (LTT) or base temperature (T-base) and the thermal constant (K) in degree-days (DD). The hyperbole method (Haddad et al., 1999) was used through the SAS version 2.8 program (SAS Institute, 2001). The results were then subjected to analysis of variance (ANOVA) following a randomized experimental design with four treatments (temperatures) and 50 replications. When significant differences from the ANOVA occurred, the averages of the treatments were subjected to Tukey's HSD test to 5% probability for averages comparison.

Results and Discussion

The development periods (days) embryonic period, the larval and pupal stages, and the total life cycle (egg to adult period) of *D. multifula*, fed from *P. citri*, decreased as temperature increased (Table 1). When subjected to a temperature of 31 °C, all *D. multifula* individuals died at three days of age, high temperatures have been a limiting factor for the predators of the Cecidomyiidae family (Kim & Kim, 2004).

At 22°C, the development periods were 7.0, 8.6, 12.9 and 26.9 days for the embryonic period, larval and pupal stages, and total life cycle (egg to adult) periods, respectively. Here were significant differences ($P < 0.05$) in the temperature ranges, from 28°C to 22°C for all periods of development (Table 1). Mo & Liu (2006) observed that at a temperature of 26.7 °C and relative humidity of 75%, the embryonic phase of *F. acarisuga* lasted for 2.6 days, on average. Gillespie et al. (2000) found that at temperatures of 20, 27, and 30 °C, the embryonic period of *F.*

acarisuga was on average 1.7, 1.5, and 2.2 days, respectively.

The length of the larval stage of *D. multifila* showed significant differences between the temperatures (Table 1). At 28 °C, the larval stage was shorter (6.4 days), while at 22 °C the larval stage was the longest (8.6 days). The larval stage lasted for 7.6 days on average with a range of 6 to 8 days. Gillespie et al. (2000) established that

F. acarisuga feeding on *Tetranychus urticae* eggs at 20 and 30 °C showed a larval stage of 12.9 and 7.9 days, respectively. Mo & Liu (2006) observed that, when *F. acarisuga* fed on *T. cinnabarinus* eggs, the larval stage lasted approximately seven days at a temperature of 26 °C. The larval stage of these species of Cecidomyiidae predators varies from 5 to 12 days, at temperatures between 20 and 30 °C (Kim & Kim, 2004; Havelka, 1980).

Table 1. Length (days) of the embryonic, larval and pupal stages, and total life cycle (egg to adult) of *Diadiplosis multifila* reared in *Planococcus citri* at temperatures of 22, 25, and 28 °C at the Embrapa Semiarid Entomological Laboratory, Petrolina, Pernambuco state

Temp. (°C)	Length (average ± SE) of the stages			
	EEmbryo	Larva	Pupa	Egg–adult
22	7.0 ± 0.02 a	8.6 ± 0.11 a	12.9 ± 0.15 a	26.9 ± 0.15 a
25	5.0 ± 0.02 b	8.0 ± 0.16 b	10.4 ± 0.50 b	20.0 ± 0.24 b
28	4.0 ± 0.02 c	6.4 ± 0.09 c	8.2 ± 0.24 c	16.7 ± 0.20 c

Averages (± SE) followed by the same letter in the column do not differ between themselves using the Tukey's HSD test at 5% probability.

The pupal stage of *D. multifila* was also influenced by the temperatures (Table 1), presenting significantly different durations of 12.9, 10.4, and 8.2 days for temperatures of 22, 25, and 28 °C, respectively (Table 1). On average, the pupal stage was 10.5 days, with a range of 8 to 13 days. Gillespie et al. (2000) observed that the pupal stage of *F. acarisuga* at temperatures of 20, 27, and 30 °C was 7.5, 4.9, and 4.9 days, respectively. Mo & Liu (2006) showed that the pupal stage of *F. acarisuga* lasted, on average, 6.7 days at a temperature of 26 °C.

Havelka (1980) observed that at temperatures of 15, 20, and 25 °C and relative humidity of 80–100% the embryonic period of *A. aphidimyza* lasted on average 1.8, 2.6, and 5 days, respectively and pupal stage of *A. aphidimyza* at 20 and 25 °C was 10.9 and 7.9 days, respectively. Thus, the embryonic period of *D. multifila* corresponds, on average, to double the number of days of *F. acarisuga* and *A. aphidimyza* at approximately 25 °C.

The length of the total life cycle (egg to adult) of *D. multifila* diminished significantly with an increase in temperature, presenting the highest development period at 22 °C (27 days) and the lowest at 28 °C (17 days) (Table 1). These results are in agreement with those obtained by Gillespie et al. (2000) and Havelka (1980), who observed that the total life cycle length of *F. acarisuga* and *A. aphidimyza*, respectively, was inversely

proportional to the increase in temperature. *F. acarisuga* completed its development between 20 and 16 days at temperatures of 20 to 30 °C (Gillespie et al., 2000), whereas the total life cycle length of *A. aphidimyza* was 28 and 16 days at 20 and 30 °C, respectively. Thus, the total life cycle length of *D. multifila* is similar to that of the species *A. aphidimyza*.

The viability of *D. multifila* eggs at the three temperatures (22, 25, and 28 °C) was high, corresponding to 99%. The pupal stage viability of *D. multifila* was 83% on average, being less than the larval viability (Table 2). *Diadiplosis multifila* have three larval instars, observed by the skin casting process, which is typical of members of the family Cecidomyiidae. However, the duration of each instar was not measured because after hatching the larvae moved into the ovisac, where generally they were found covered by the mealybug eggs.

To observe the change at each instar, it was necessary to take out the larvae from the inside of the ovisac. In performing this daily procedure, there was a high degree of mortality of the individuals. Therefore, it was only possible to determine the larval period, as during this phase manipulation was less frequent. Madahi et al. (2013), studying the predation efficiency of *A. aphidimyza*, were also not able to differentiate the instars and conducted their experiment using the specific age of the larvae instead.

Table 2. Viability (%) of egg, larva* and pupal stages of *Diadiplosis multifila* reared in *Planococcus citri* at temperatures of 22, 25, and 28°C at the Embrapa Semiarid Entomological Laboratory, Petrolina, Pernambuco state

Viability Parameter	Temperature (°C)					
	22		25		28	
	%	N	%	N	%	N
Egg	99	600	99	600	99	600
Larva*						
1 day	100	69	100	150	100	171
2 days	91.3	63	100	150	91.2	156
3 days	92.0	58	97.3	146	98.4	154
4 days	100	58	100	146	91.2	143
5 days	100	58	99.3	145	97.5	140
6 days	91.3	53	100	114	100	113
7 days	100	40	100	82	-	-
8 days	100	39	100	59	-	-
Larval stage	97.5	69	96.9	150	96.3	171
Pupal stage	88.0	42	76.7	112	84.9	133

* Viability of the larva, data corresponding to the days after hatching.

From the first day of life to the last before becoming a pupa, larval survival was high at all temperatures, varying from 91 to 100% (Table 2). Average larval viability of *D. multifila* was 97%. At the completion of the juvenile phase, the larvae suspended movement and feeding. Instead, they started secreting a silky substance around their bodies, which formed a cocoon and is where they remained until emergence.

Kim & Kim (2004) observed that the pupal stage of *A. aphidimyza* at temperatures of 20, 25, and 30 °C lasted on average 17.5, 8.4, and 9.7 days, respectively. Thus, the pupal stage of *D. multifila*, *F. acarisuga*, and *A. aphidimyza* varies from 5 to 17 days between 20 and 30 °C. The lowest pupal viability was observed at a

temperature of 25 °C (76%) and the highest was at 22 °C (88%).

The longevity of adult male and female *D. multifila* varied with temperature. At temperatures of 22 and 25 °C, the longevity of the male was similar (approximately 2.5 days) and significantly greater than at 28 °C where longevity was one day shorter (1.5 days) (Table 3). The adult male of *F. acarisuga* lives, on average, for 12 days at a temperature of 26.7 °C (Mo & Liu, 2006), a longevity much greater than that seen in *D. multifila*.

The female longevity of *D. multifila* showed values significantly greater at 22 °C than at 28 °C, having averages of 2.2 and 1.5 days, respectively (Table 3).

Table 3. Longevity (days; average ± SE), fertility, and sex ratio of *Diadiplosis multifila* reared in *Planococcus citri* at temperatures of 22, 25, and 28 °C at the Embrapa Semiarid Entomological Laboratory, Petrolina, Pernambuco state

Parameter	22		25		28		Fp
	Average	N	Average	N	Average	N	
Long. ♂	2.52±0.17a	17	2.41± 0.19a	17	1.47±0.15b	17	11.18 ^{0.001}
Long. ♀	2.20±0.20a	20	2.15±0.18ab	20	1.55±0.15b	20	4.06 ^{0.024}
Eggs/♀	34.23±3.62a	17	25.40±3.12ab	25	19.58±2.47b	29	5.44 ^{0.0064}
Sex ratio	0.54	37	0.50	86	0.46	113	-

Averages (± SE) followed by the same letter in the column do not differ between themselves using the Tukey's HSD test at 5% probability

Gillespie et al. (2000) showed that the longevity of the female *F. acarisuga* varied from 4.6 to 5.6 days between temperatures of 20 and 25 °C. Kim & Kim (2004) discovered that *A. aphidimyza* females survived for approximately 5.0 to 6.8 days at a temperature of 25 °C. Therefore, *D. multifila* adults are ephemeral

and present lesser longevity than other Cecidomyiidae predator species. In general, as *D. multifila* adults survive for only two days, the coupling must occur soon after emergence and as soon as the females perform oviposition. The *D. multifila* females confined with males in containers with mealybugs in reproduction phase,

deposited their eggs on or inside the ovisacs and on the white filter paper, which was used to line the bottom of the container.

In evaluating the fertility of *D. multifila*, it was shown that the females produced significantly more eggs when they developed at 22 and 25 °C. Thus, the fertility of *D. multifila* varied from 34 to 19 eggs, respectively, and displayed a reduction in egg production with increase in temperature (Table 3). Gillespie et al. (2000) indicated that *F. acarisuga* produced, on average, 42 eggs at 20 and 25 °C, and that the temperature did not affect fertility. Mo & Liu (2006) showed that *F. acarisuga* deposited on average 32.4 eggs during its cycle at 26.7 °C. These results show that the species *D. multifila* and *F. acarisuga* have similar fertility, with each female able to deposit between 20 and 45 eggs.

The individuals that hatched on or inside the ovisac quickly found the eggs and fed on them until the pupal stage. In contrast, the larvae that hatched on the filter paper moved around the container, were unable to locate the ovisac, and died after one day of life owing to lack of food. Thus, the selection of the place of oviposition is revealed as a determining factor in the survival of *D. multifila* larvae.

The sex ratio of *D. multifila* varied from 0.46 to 0.54 (Table 3). It occurred a reduction in the number of *D. multifila* females with an increase in temperature. In terms of the sex ratio of other Cecidomyiidae, *F. acarisuga* showed a female rate of 60% at 26.7 °C (Mo & Liu, 2006), while *A. aphidimyza* revealed a rate of 45% at 22 °C (Van Lenteren, 2003). Therefore, the *D. multifila* sex ratio is slightly lower than that of *F. acarisuga* and comparable to that of *A. aphidimyza* under similar temperature conditions.

D. multifila develop in temperatures between 22 and 28 °C. This Cecidomyiidae species was discovered in grape bunches infested with *P. citri* collected in vineyards in the Petrolina municipality, Pernambuco state (Oliveira et al., 2013), which is located in a semiarid region where the average annual temperature has variations from 20.7 to 26.8 °C (Ramos et al., 2011). Thus, *D. multifila* are adapted to develop in these temperature conditions of the semiarid northeast of Brazil. One of the characteristics of this region is the presence of two climatic seasons: wet

and dry. In the latter, from August to October, the temperature can reach peaks equal to or greater than 31 °C (Santos et al., 2013).

D. multifila do not complete their development cycle at 31 °C. Therefore, how can we explain the survival of *D. multifila* in the semiarid region during periods of high temperatures? In nature, climatic conditions are quite different, as during the day there are variations in temperature. Therefore, *D. multifila* may present behavioral adaptations, which allow its survival when there is a wide temperature range. For example, *D. multifila* adults may seek refuge in a section of the grape vine and/or oviposition sites that have a microclimate with a more amenable temperature, such as shaded branches or grape clusters.

D. multifila present several biological similarities with the Cecidomyiidae species *F. acarisuga* and *A. aphidimyza*, which are reared en masse and commercialized. However, the species *D. multifila* warrants attention, requiring further studies to evaluate its potential as a predator, with a view to the implementation of a program of applied biological control.

Conclusions

Temperatures of 22, 25 and 28 °C favored the development of all phases of *D. multifila*. At 31 °C, there was a high mortality of insects, especially in the larva stage.

It was evidenced that the constant temperatures of 25°C and 28°C, provide a reduction of the biological cycle of *D. multifila*, but presented a lower fecundity, in relation to 22°C.

D. multifila presented biological similarities in relation to the species of Cecidomyiidae, *F. acarisuga* and *A. aphidimyza*. In this way *D. multifila* deserves attention, and further studies are needed to evaluate its predation potential aiming at the implementation of an applied biological control program.

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