An Acad Bras Cienc (2023) 95(1): e20210122 DOI 10.1590/0001-3765202320210122
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ANIMAL SCIENCE

Effect of environmental conditions on the changes of voltinism in three lines of *Bombyx mori*

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Abstract: This work aims to estimate the voltinism differences in the studied lines and the influence of biological and environmental variables on this trait. Three *Bombyx mori*. lines were used. One polyvoltine (Indian, C. Nichi) and two bivoltine (Chinese6-C6 and Japanese7-J7). Silkworms were reared under controlled conditions of temperature (24±1, 26±1, 28±1° C) and photoperiod (8-16, 12-12, 16-8 light/darkness) during the life cycle. The measured biological characters were duration of larval period (DLP), larval weight on fifth day of V instar (LW(V-5)), rate of weight (RWV), duration of life cycle (DLC), fecunditiy (NE), fertility (F) and voltinism (V). Voltinism was estimated using a linear regression model; the variables that influence voltinism were found using a multiple regression analysis; and the effect of environmental conditions on change of voltinism employing an ANOVA. The results show that the variables that affect voltinism are different for each line, which demonstrates the effect of the line factor. Nichi voltinism was affected by the six measured variables, while in C6 and J7 where voltinisms depends on the duration of diapause, some variables were excluded from the model. In Indian tropical polyvoltine, the temperature was the influencing factor, while in Chinese and Japanese bivoltines lines was the photoperiod.

Key words: *Bombyx mori*, environmental factors, photoperiod, temperature, voltinism.

INTRODUCTION

The silkworm, *Bombyx mori*, according to geographical origin, is classified as seasonal and tropical, consistent with the type of voltinism that occurs (univoltine, bivoltine and polyvoltine) (Pescio et al. 2008). In this context, the silkworm found in tropical regions is polyvoltine and its embryos develop continuously, while that of temperate zones, called univoltine, exhibits a type of facultative diapause and the bivoltine type presents an obligatory diapause type modified by environmental factors. Voltinism is therefore an important factor in discussing silkworm evolution and adaptation (Muniraju & Mundkur 2018).

Diapause is considered a development strategy for temperate zone insects (Denlinger 1986); and it is also shown that it is not a physiological response caused by adverse conditions, but by signals that warn of a change in external conditions (e.g. temperature, photoperiod) unfavorable for survival in an environment of normal activity (Cifuentes & Sonh 1998). Selective pressure for the presence of diapause can vary considerably between different areas of the tropics. Many species that show no diapause in one geographic location show it in another, and diapause increases with latitude (Denlinger 1986). As such, selection experiments with tropical species indicate that diapause can be modified; by selection for non-diapause, incidence decreased from 80% to 20% in five generations (Denlinger 1979), while by selection for diapause, it increased from 43% to 94% in four generations (Kikukawa & Chippendale 1983). According to Denlinger (1986) the introduction of species to the tropics can generate the loss or retention of a residue thereof, while properly tropical species may show no diapause or manifest it in a low, variable way.

The embryonic diapause of the silkworm is regulated by the diapause hormone secreted by the suboesophageal ganglion (Grenier et al. 2004, Kubota et al. 1979) and plays an important role in the accumulation of 3-hydroxycinurenine (Kai & Kawai 1981), which acts on the mature oocytes in the pupal stage and generates interruption in embryonic development, once approximately 12,000 cells are reached at 20-22 hours after oviposition (Muniraju & Mundkur 2018). It is induced by the temperature and photoperiod in the egg state of the previous generation (Tsuchida & Yoshitake 1983a). As a result, eggs subjected to high temperatures and long days produce adults that lay diapaused eggs (and some mixed layings) (Kobayashi et al. 1986), while adult females coming from eggs exposed to low temperatures and short days lay non-diapaused eggs (Takamiya 1974, Tsurumaki et al. 1999).

Voltinism is a genetic characteristic that defines the number of generations in a year under natural conditions (Porto et al. 2004, Cifuentes & Sonh 1998) and is considered maternally inherited, influenced by light and temperature in the different stages of the worm. (Hemmatabadi et al. 2016, Hussain et al. 2011, Seidavi & Bizhannia 2008). The presence of diapause limits to one or a maximum of two offspring per year (uni or bivoltines) and has longer life cycles, while its absence determines a greater number of offspring per year (polyvoltins) and shorter cycles (Gaviria et al. 2006). Change in voltinism depends on these environmental factors because insects require adequate conditions to maintain their physiology under normal conditions (Kumar et al. 2003). In light of this, the fluctuation of temperature and humidity is favorable for growth and development in larval instars, but high conditions of these two factors negatively affect pupation and productivity (Kumar et al. 2003). Environmental variations emphasize the need for temperature management for sustainable cocoon production (Rahmathulla 2012).

Voltinism studies focus on modeling the effects of climate change in some species of insects (Chen et al. 2011, Long et al. 2017, Stoeckli et al. 2012), because these species are an appropriate model due to their high temperature dependence (Lowry & Lowry 1989, Wagner et al. 1984). However, other factors, both biotic and abiotic, can influence the seasonality of insects, such as the photoperiod (Tauber et al. 1986, Denlinger 2002). However, the non-linear interaction of temperature and photoperiod makes it difficult to research and understand changes in insect voltinism in response to climate change (Tobin et al. 2008).

Voltinism in silkworm emerges as an opportunity to understand the biology of the species after centuries of domestication for the production of thread and adaptation to new regions where sericulture is proposed as an economic alternative. The present work aims to determine the biological variables that influence voltinism for lines of Chinese, Japanese and Indian origin, and determine the effect of environmental conditions on change of voltinism.

MATERIALS AND METHODS

The experimental research was carried out at the Center for Plant Studies in La Rejoya, attached to the Faculty of Agrarian Sciences of the University of Cauca. It is located northeast of Popayán in the hamlet of La Rejoya (Cauca), at 1800 meters above sea level, an average temperature of 18 °C and an average annual rainfall of 1750 mm (Vivas & Morales 2005).

Biological material

Three lines were used. Two of these were bivoltine (Chinese 6 (C6), Japanese 7 (J7)) and one polyvoltine (Indian, C. Nichi). The Chinese and Japanese lines were selected for their high yield in characters of productive interest and the C. Nichi line for being polyvoltine. All three lines were imported (National Environmental Licensing Authority (ANLA) Resolution 00431 of March 2018) from the Experimental Station of Sericulture and Agriculture, SAES in Vratsa, Bulgaria, specifically from the department of research in genetics, in the area of improvement for *B. mori*.

Assembly and breeding

The lines were kept in optimal feeding and sanitary conditions based on the protocols of Cifuentes & Sonh (1998) and Pescio et al. (2008), reared under controlled environmental conditions, through the use of incubators, from egg to complete pupation (according to modified protocols of Kumar et al. 2002, Lakshminarayana et al. 2002) and during the number of generations that would allow the estimation of the voltinism, two for J7 and three for C6 and C. Nichi. The incubators were designed specifically for silkworm rearing in this research, equipped with instruments to control and monitor temperature, humidity, light, air speed and CO_2 concentration. The technical characteristics are described in Duque et al. (2018). The experimental phase was conducted from July 2018 to April 2020.

Temperatures in the incubator were 24±1°C, 26±1°C and 28±1°C and the photoperiod, with illuminance of 20 lux (lumen/m^2), 8-16, 12-12 and 16-8 of photophase/scotophase respectively. The temperature and the photoperiod were chosen taking into account the effect of these variables in the different stages of development and their expression in the voltinism of *B. mori* (Kai & Hasegawa 1971, Kamili & Masoodi 2004, Kobayashi et al. 1986, Takamiya 1974). The levels of each environmental variable were chosen according to the maximum and minimum limits taking into account the range identified for silkworms (Benchamin et al. 1983, Rahmathulla 2012, Vermana et al. 2003). Relative humidity (75±5%), feeding (fresh mulberry), CO_2 concentration (<2%) and air speed (0.5–1m/s) were constant, at a level considered optimal for the rearing of *B. mori* (Rahmathulla 2012).

Variables

The biological characters of the life cycle were chosen because they show variation according to the type of voltinism of the worms (Denlinger 1986, Lakshminarayana et al. 2002, Rahmathulla 2012, Singh et al. 2008) and are described in Table I. Larval weight was recorded using a precision scale. Fecundity and fertility were measured using ImageJ software (version 1.52).

Data analysis

The samples of the *B. mori* lines were defined independently, randomly, with 3 repetitions (70-100 individuals per repetition) and 9 treatments (temperature*photoperiod). Registering the information of the biological variables was carried out independently by repetition and the means of the variables by treatment of each line were estimated. In each incubator, the brood of the three lines was maintained under a single

Table I. Biological variables for the study of voltinism in *B. mori*.

Variable	Description	Measure per repetition
DLP: Duration of larval period - (hours)	Time of the larval period (includes residence time of the larva in each of the instars and molts).	15 larvae
LW(V-5): Larval weight - (grams)	Weight of the larva. Taken on the fifth day of the fifth instar	15 larvae per instar
RWV: Rate of weight gain V instar	Weight on fifth day of V larval instar divided by the weight on the first day of V larval instar.	15 larvae
DLC: Duration of life cycle - (hours)	Time elapsed from the hatching of the eggs to the emergence of the adult (> 80%).	Population of 70-100 individuals
NE: Fecundity - (number)	Number of eggs per laying.	5 layings
F: Fertility - (percentage)	Percentage of fertile eggs per laying. Identified by gray color days after the oviposition.	5 layings
PD: Percentage of diapause	Percentage of diapause eggs per laying following the treatment. Ranges <20, 20-50, 50-80 and 80-100.	5 layings
Diapause - (hours)	Embryonic diapause. Used to estimate the DLC	Population of 70-100 individuals
V: Voltinism	Number of generations per year.	2-3 generations

treatment. The order in the trays within the incubator was defined with random numbers.

The following statistical procedures were performed to estimate the voltinism differences in the studied lines and the influence of biological and environmental variables on this trait. In first place, lines voltinism were estimated using linear regression models, considering life cycle duration (hours - includes diapause duration) per generation as the response variables, and generations number as the regressor. With the obtained data, an analysis of variance was performed to determine if there were differences in the silkworm lines voltinism. Tukey test was used to compare the means ($p<0.05$).

A second analysis was performed to evaluate the effect of biological variables on voltinism through multiple linear regressions. In first place, to evaluate the association between biological variables, a Pearson correlation analysis was estimated within each line (J7, C6 and C. Nichi). Subsequently, multiple linear regression models were evaluated, in order to identify the biological variables that most influenced voltinism per line, where the biological variables, (DLP, DLC, LW(V-5), RWV, NE and F) were taken as the regressors and voltinism as the response. Previous correlation analysis showed associations between regressor, so multicollinearity tests through the variance inflation factor (VIF<10), tolerance (≥ 0.1) and collinearity diagnosis (eigenvalue: close to 0; condition index >0) were evalauted using the PROC REG of SAS 9.4. Results indicated the presence of multicollinearity within some lines. In order to avoid eliminating related variables indiscriminately, the regressions were adjusted with the Lasso regularization method (Least Absolute Shrinkage and Selection Operator) (Tibshirani 1996), and the best analysis model was selected based on the Akaike Information Criterion (AIC). The analysis was carried out using the PROC GLMSELECT of SAS 9.4 statistical software.

The third statistical procedure was made to evaluate the effect of temperature and photoperiod on voltinism. In this case, it was necessary to use a bifactorial design without repetitions because obtaining voltinism records in the evaluated lines, did not allow to obtain repetitions for the combination of temperature and photoperiod factors, which limits the estimation of the interaction between them. Thus, in order to carry out the analysis, it was assumed that the two factors influence the voltinism independently. In this sense, voltinism data was analyzed under a complete random design without repetitions through a conventional ANOVA with fixed temperature and photoperiod effects. Least square difference was used for mean differentiation (p<0.05).

Finally, a complement analysis in C6 line was performed due to the high voltinism variation observed as a consequence of diapause presence in the treatments. In this case, the non-parametric Chi-square test of independence was carried out, with a contingency table and four ranges (<20, 20-50, 50-80 and 80-100) of the diapause percentage of the layings obtained during three generations after the treatments (reduction of the presence of diapause). The independence hypothesis was considered significant at 5%. The procedure was estimated using the statistical software InfoStat (version 2017).

RESULTS AND DISCUSSION

The means of the biological characters that show variation according to the type of voltinism of the worms (DLP, DLC, LW(V-5), RWV, NE and F) for the three lines for each treatment are presented in Table II. The best values for the duration of the larval period and the life cycle were those of the shortest time in rearing, because it implies a decrease in food consumption and less time in the activities of the rearing management. The highest values were for the variables (LW(V-5), RWV, NE, F), because the higher the weight of larvae, fecundity and fertility, the higher the production of worms. The polyvoltine line C. Nichi presented the highest average of percentage fertility, as well as the lowest average for the variables larval period and life cycle duration, while of the bivoltine lines, J7 presenting the greatest larval weight and average rate of weight gain in the fifth instar and the highest average duration of life cycle. Line C6 presented the highest average number of eggs per laying.

Voltinism estimation

The construction of a regression model from the DLC (including duration of diapause) for each treatment during two or three generations depending on the line (Table III), allowed estimating the number of generations in a year for each genetic material (Table II). The polyvoltine line presented the highest number of generations in one year (6,944 to 8,991), followed by the bivoltine lines C6 (2.56 to 5.13) and J7 (1.48 to 4.58), results that depend on the presence or absence of diapause. The average duration of diapause for C6 was shorter compared to J7 (160.64 and 175.36 days, respectively). Line J7 presented the longest diapause time (149 to 285 days). The highest average of generations in a year was with a temperature of 28 °C (3.45) and photoperiod 8-16 (4.18), while the lowest average of generations per year was at 24 °C (2.60) and photoperiod 16-8 (1.57). The latter photoperiod is recommended to reduce the incubation time (Datta et al. 1996, Dingle et al. 2005).

According with the ANOVA results, a significant differences between lines voltinism was found (F=69.53; p=0.0001). Tukey test defines two groups, the first one brings together the two bivoltine lines (C6 and J7) and the second the polyvoltine line. The difference lies in

Table II. **Mean (± standard error) of the biological characters associated with voltinism and estimated value of voltinism for the nine treatments (temperature*photoperiod) of lines C6, J7 and C. Nichi. DLP: duration of the larval period, DLC: duration of the life cycle, LW(V-5): larval weight of the fifth day of the fifth instar, RWV: rate of weight gain of the fifth instar, NE: number of eggs, F: fertility, V: voltinism.**

Treatments	DLP (h)	LW(V-5) (g)	RWV	DLC (h)	NE (number)	F (%)	Diapause* (hours)	V
C6								
24°/8-16	724.98±6.37	2.88±0.1	2.79±0.09	1116.83±11.5	423.64±14.2	97.78±1.21	3984.00±0	3.44
24°/12-12	684.40±6.37	3.05±0.1	2.64±0.09	1037.87±11.5	500.71±14.2	99.28±0.83	3984.00±0	3.84
24°/16-8	689.94±6.37	2.87±0.1	2.85±0.09	1058.11±11.5	482.40±14.2	96.87±3.46	3984.00±0	3.74
26°/8-16	529.02±6.37	2.98±0.1	2.58±0.09	855.72±11.5	444.11±17.6	98.83±1.04	3764.37±371.75	3.60
26°/12-12	556.81±6.37	2.93±0.1	2.26±0.09	874.98±11.5	489.07±17.6	99.01±1.2	3878.64±405.44	2.91
26°/16-8	567.43±6.37	2.64±0.1	2.18±0.09	877.28±11.5	474.78±17.6	97.66±3.49	3581.19±26.57	4.73
28°/8-16	531.72±6.37	3.17±0.1	2.36±0.09	837.65±11.5	425.09±16.3	98.48±1.59	4003.56±478.14	2.60
28°/12-12	538.57±6.37	3.11±0.1	2.28±0.09	828.58±11.5	399.31±16.3	98.32±2.46	3567.40±10.48	5.13
28°/16-8	539.56±6.37	2.77±0.1	2.18±0.09	814.63±11.5	438.69±16.3	99.18±0.69	3818.26±0.01	4.92
J7								
24°/8-16	693.91±2.8	2.94±0.1	2.74±0.08	1125.91±7.24	452.10±11.1	95.35±4.11	3964.66±0	3.51
24°/12-12	662.37±12.7	2.63±0.1	2.61±0.08	1038.26±24.4	455.43±13.1	97.71±2.31	4044.34±159.32	2.63
24°/16-8	581.58±11.7	3.11±0.1	2.79±0.09	971.55±5.86	475.37±14.6	97.75±2.0	4354.46±428.5**	1.66
26°/8-16	582.99±2.8	2.91±0.1	2.19±0.08	946.64±7.24	348.37±11.1	93.98±5.67	3578.32±2.04	4.45
26°/12-12	577.69±12.6	3.17±0.1	2.68±0.08	934.50±24.4	387.80±13.1	92.12±8.9	3792.98±3.41	4.30
26°/16-8	548.29±11.7	2.83±0.1	2.77±0.09	867.43±5.86	467.30±14.6	95.71±3.65	4912.57±1458.1**	1.59
28°/8-16	573.74±2.8	3.04±0.1	2.64±0.08	922.70±7.24	355.37±11.1	93.39±7.59	3578.32±2.04	4.58
28°/12-12	534.13±12.6	2.81±0.1	2.45±0.08	862.40±24.4	370.87±13.1	93.25±4.95	3798.28±330.86	2.17
28°/16-8	578.46±11.7	3.07±0.1	2.51±0.08	938.25±5.86	427.17±14.6	96.65±3.37	4882.82±1463.5	1.48
C. Nichi								
24°/8-16	618.78±5.08	2.37±0.07	2.69±0.08	962.72±3.43	416.07±8.6	99.68±0.86	-	6.94
24°/12-12	560.15±5.08	2.27±0.07	2.38±0.08	856.85±3.43	491.36±8.6	99.76±0.39	-	7.53
24°/16-8	596.33±5.08	2.14±0.07	2.37±0.08	914.39±3.43	432.11±8.6	99.99±0.02	-	7.35
26°/8-16	532.06±5.08	2.24±0.07	2.52±0.08	772.60±4.83	421.62±13.6	95.64±4.17	-	8.90
26°/12-12	563.23±5.08	2.49±0.07	2.53±0.08	819.83±4.83	487.00±13.6	99.50±0.46	-	8.39
26°/16-8	573.93±5.08	2.92±0.07	2.88±0.08	857.56±4.83	405.96±13.6	95.69±4.08	-	8.02
28°/8-16	541.14±5.08	2.68±0.07	2.89±0.08	806.50±11.1	464.38±9.0	98.77±1.14	-	8.49
28°/12-12	500.30±5.08	2.15±0.07	2.56±0.08	755.00±11.1	388.60±9.0	97.93±2.88	-	8.96
28°/16-8	513.86±5.08	2.25±0.07	2.15±0.08	765.69±11.1	415.38±9.0	99.42±0.63	-	8.99

*** Mean (± standard error) of the diapause was estimated only in treatments that presented it.**
**** All repetitions of treatment with diapause.**

the absence of diapause in C. Nichi and in its presence in the bivoltine lines.

Correlations among biological variables

Correlation value was interpreted according to Hinkle et al. (2003), correlation coefficients whose magnitude are between 0.9 and 1.0 indicate variables which can be considered very highly correlated, magnitude between 0.7 and 0.9 can be considered highly correlated, magnitude between 0.5 and 0.7 indicate variables moderately correlated and magnitude less than 0.5 have little if any correlation. Among the three lines, very high and positive correlations between DLC-DLP were found (r = 0.990 to 0.972). Within the C. Nichi line inverse associations between V-DLP (r = -0.930) and V-DLC (r = -0.977) were estimated. High correlation in C6 line were observed between RWV-DLP (r = 0.816) and RWV-DLC (r = 0.865). In J7 line, variables like RWV-NE (r = 0.707) and NE-F (r = 0.811) presented significant associations, while in C. Nichi line, RWV-LW(V-5)

Table III. **Regression equations for estimating the number of generations per year of the three lines and the coefficient of determination obtained in each case (R^2). Variable predicted = total hours per year, x = number of generations. AIC: Akaike information criterion.**

Treatments		Intercept (*P*-value)	Coefficient (*P*-value)	AIC	R^2
24°/8-16	C6	3816.2 (<0.0001)	1436 (<0.0001)	120.82	0.985
	J7	3600 (<0.0001)	1472 (<0.0001)	52.14	0.999
	C. Nichi	- 240 (<0.0001)	1296 (<0.0001)	89.69	0.999
24°/12-12	C6	3652.3 (<0.0001)	1328.8 (<0.0001)	105.29	0.998
	J7	2742.6 (0.327)	2289.4 (0.215)	111.21	0.352
	C. Nichi	- 144.39 (<0.0001)	1143.9 (<0.0001)	79.48	0.999
24°/16-8	C6	3746.1 (<0.0001)	1340.1 (<0.0001)	98.04	0.998
	J7	- 620.02 (<0.0001)	5636 (<0.0001)	67.11	0.999
	C. Nichi	- 176 (<0.0001)	1216 (<0.0001)	79.81	0.999
26°/8-16	C6	2490.2 (0.061)	1739.5 (0.012)	157.86	0.618
	J7	3272.6 (<0.0001)	1232 (<0.0001)	61.04	0.998
	C. Nichi	198.59 (<0.0001)	962.1 (<0.0001)	86.14	0.999
26°/12-12	C6	1475.2 (0.289)	2502.8 (0.004)	160.40	0.716
	J7	3464 (<0.0001)	1232 (<0.0001)	47.97	0.999
	C. Nichi	170.66 (<0.0001)	1024 (<0.0001)	84.40	0.999
26°/16-8	C6	3322.5 (<0.0001)	1149.1 (<0.0001)	95.00	0.998
	J7	- 2120 (0.147)	6840 (<0.0001)	102.43	0.954
	C. Nichi	177.22 (<0.0001)	1070.2 (<0.0001)	92.48	0.998
28°/8-16	C6	544.85 (0.602)	3161.8 (<0.0001)	155.86	0.870
	J7	3296.6 (<0.0001)	1192 (<0.0001)	62.11	0.998
	C. Nichi	124.88 (0.016)	1016.7 (<0.0001)	97.66	0.998
28°/12-12	C6	3338.6 (<0.0001)	1056.6 (<0.0001)	81.80	0.999
	J7	808.21 (0.715)	3663.7 (0.048)	109.10	0.664
	C. Nichi	162.67 (0.133)	959.25 (<0.0001)	113.63	0.985
28°/16-8	C6	3590 (<0.0001)	1051 (<0.0001)	104.71	0.995
	J7	- 739.96 (0.833)	5427.9 (0.059)	114.69	0.630
	C. Nichi	56.001 (0.006)	968 (<0.0001)	79.81	0.999

(r = 0.732) showed a direct relationship. The other correlations were low or negligible (Table IV).

Influence of biological variables on voltinism

Lasso regression coefficients to estimate voltinism within each line are present in Table V. According to the Lasso regression method, biological variables influencing voltinism varied depending of the genetic line. The regression model showed that C6 voltinism was influenced by DLP, DLC, LW(V-5) and NE, and it explains 19.4% of the variance proportion, the lower adjustment is due to the high variability of the diapause duration data. Furthermore, it reveals a negative effect of DLC, LW(V-5) and NE, while DLP had a positive effect.

According to the regression model, voltinism in J7 line was influenced by DLP, LW(V-5), NE and F variables, and it explains 65.7% of the variance proportion. Furthermore, it reveals a negative effect of NE and F, while DLP and LW(V-5) had a positive effect on voltinism (Table V).

The regression model showed that C. Nichi voltinism was influenced by six variables DLP, DLC, LW(V-5), RWV, NE and F, and it explains 98.8% of the variance proportion. Furthermore, it reveals a negative effect of DLC and NE, while DLP, LW(V-5), RWV and F had a positive effect (Table V).

Table IV. Matrix of correlation between biological variables (estimated values and their significance are below and above the diagonal, respectively). Very high correlations are shown in bold. DLP: duration of the larval period, DLC: duration of the life cycle, LW(V-5): larval weight of the fifth day of the fifth instar, RWV: rate of weight gain of the fifth instar, NE: number of eggs, F: fertility, V: voltinism.

Variables		DLP (h)	DLC (h)	LW(V-5) (g)	RWV	NE (num.)	F (%)	V
DLP (h)	C6	1.000	<0.0001	0,69	0,007	0,38	0,24	0,68
	J7		<0.0001	0,52	0,56	0,29	0,31	0,60
	C. Nichi		<0.0001	0,57	0,51	0,63	0,44	0.0002
DLC (h)	C6	**0.990**	1.000	0,78	0,002	0,38	0,22	0,52
	J7	**0.978**		0,76	0,54	0,29	0,30	0,61
	C. Nichi	**0.972**		0,76	0,61	0,78	0,31	<0.0001
LW(V-5) (g)	C6	-0.157	-0.107	1.000	0,67	0,27	0,39	0,25
	J7	-0.257	-0.123		0,76	0,54	0,34	0,43
	C. Nichi	0.216	0.115		0,02	0,82	0,29	0,88
RWV	C6	0.816	0.865	0.144	1.000	0,67	0,25	0,33
	J7	0.218	0.229	0.112		0,03	0,42	0,44
	C. Nichi	0.252	0.196	0.732		0,86	0,23	0,71
NE (num.)	C6	0.334	0.331	-0.406	0.166	1.000	0,93	0,58
	J7	0.392	0.393	-0.245	0.707		0,01	0,04
	C. Nichi	0.187	0.107	0.093	-0.065		0,21	0,58
F (%)	C6	-0.431	-0.449	0.319	-0.426	0.036	1.000	0,90
	J7	0.385	0.388	-0.362	0.307	0.811		0,04
	C. Nichi	0.294	0.383	-0.390	-0.441	0.465		0,24
V	C6	-0.157	-0.249	-0.429	-0.362	-0.216	-0.048	1.000
	J7	0.203	0.199	0.301	-0.301	-0.688	0.676	
	C. Nichi	**-0.930**	**-0.977**	-0.061	-0.138	-0.216	-0.435	

The models obtained show that the biological variables that affect voltinism are different according to the line studied. Only C. Nichi shows the effect of the six variables measured, while in the lines where voltinism depends on the duration of diapause (J7 and C6), some variables were excluded from the model. For example, for J7, DLC was excluded (not including the egg stage), a line that presented a longer diapause time and lower percentage of laying without diapause and mixed (6.2%) compared to C6, which agrees with the effect of line found by Kinoshita et al. (1982).

The DLP variable positively affected the voltinism of the three lines, which indicates that the greater the voltinism (shorter duration of the generations), the longer the larval period, contrary to what happens with the DLC (except for J7), which negatively affected the voltinism. This indicates that the higher the voltinism (the shorter the duration of generations), the shorter the life cycle duration. The NE variable also affected the three lines but negatively, this shows that the lower the voltinism (longer generations), the higher the fecundity. The variables LW(V-5), RWV and F also showed a positive effect on the C. Nichi line, which suggests that the higher the voltinism (shorter duration of the generations), the higher the larval weight, the rate of weight gain in the fifth instar and fertility. The variable LW(V-5) for C6 had a negative effect on voltinism, which shows that the higher the voltinism (shorter duration of the generations), the lower the larva weight,

Table V. Intercept and coefficients of the Lasso regression model by line. *Variable not selected. AIC: Akaike information criterion, DLP: duration of the larval period, DLC: duration of the life cycle, LW(V-5): larval weight of the fifth day of the fifth instar, RWV: rate of weight gain of the fifth instar, NE: number of eggs, F: fertility.

Line	Intercept	R^2	AIC	DLP	LW(V-5)	RWV	DLC	NE	F
C6	15.92	0.194	9.52	0.027	-2.313	*	-0.020	-0.009	*
J7	19.59	0.657	8.78	0.013	0.948	*	*	-0.013	-0.228
C. Nichi	8.28	0.988	-33.38	0.019	0.034	0.214	-0.021	-0.006	0.089

contrary to the effect on J7, which also showed a negative effect on fertility.

Effect of temperature and photoperiod on voltinism

Analysis by line showed the differential effect of temperature and photoperiod according to the type of voltinism presented in the evaluated lines (bivoltine or polyvoltine), data that agree with Kinoshita et al. (1982) who argue that the effect of temperature and photoperiod varies according to race.

The polyvoltine line C. Nichi showed that the temperature presents significant differences (p=0.022) and two homogeneous subsets of 24 °C and 26-28 °C (Figure 1). The offspring obtained at 24 °C presented the lowest average value of voltinism (7.28 generations per year) compared with 26 °C and 28 °C (8.44 and 8.82 generations per year respectively) (Table VI). The C. Nichi offspring were obtained from layings without diapause, therefore, the number of generations per year depended exclusively on the life cycle length (from the moment of oviposition of the eggs to the emergence of the adult, including incubation time) and the effect of temperature on it. DLC (not including incubation time) and DLP had a longer duration in the treatments at 24 °C and less than 28 °C. At higher temperatures up to 28 °C, they accelerated growth, shortened the larval period and the life cycle and in turn increased larval weight in fifth instar and the number of eggs per laying was reduced.

Temperature has a direct correlation with the silkworms growth. An increase in temperature increases physiological functions, accelerates growth and shortens the larval period, while a decrease in temperature generates a reduction in physiological activities (Rahmathulla 2012), slowing growth and prolonging the larval period. Considering that polyvoltine breeds reared in tropical countries develop tolerance to slightly higher temperatures and adapt to climatic conditions (Hsieh et al. 1995), Nagaraju (2002) notes that polyvoltine breeds from tropical India (Pure Mysore, C. Nichi and Nistari) are more tolerant to high temperatures than bivoltine breeds of seasonal origin, although unlike polyvoltines, bivoltine breeds have better yield potential and produce superior silk quality (Gaviria et al. 2006). Studies in tropical Indian lines show that when subjected to high temperatures, cells respond to heat shock with the synthesis of a new set of proteins (heat shock protein-HSP) (Craig 1985), making C. Nichi a thermo tolerant line, adapted to changes in temperature.

The breeding of C. Nichi was carried out from non-diapause layings, however in 15% of the layings diapause was occurred. Although the percentage of diapause did not show homogeneous groups by temperature or photoperiod, the 24°C temperature presented fewer diapause layings and the 12-12 photoperiod more diapause layings; in this regard, Tsurumaki et al. (1999) recorded that the lower the incubation temperature, the higher the number of non-diapause eggs and Kobayashi (1990) recorded

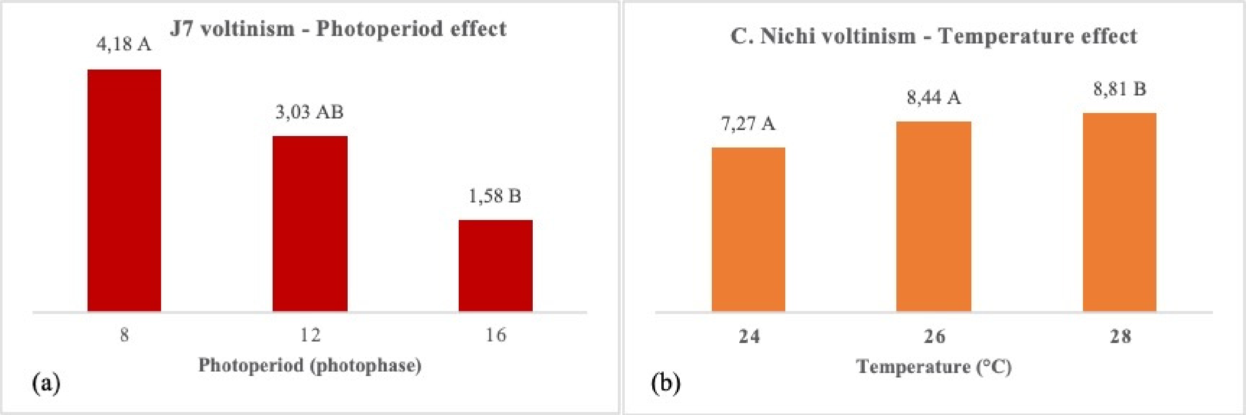

Figure 1. Voltinism means for lines with significant effect of (a) photoperiod (J7) and (b) temperature (C. Nichi). Means with different letter differ statistically at p<0.05.

that the 12-12 photoperiod presents the highest number of diapause eggs.

Several tropical species, when diapause occurs, are not affected by the length of day (e.g. Yponomeutidae: *Plutella xylostella*) (Campos 2008) and depend on temperature signals to regulate it (e.g. the Figitidae parasitoid wasp: *Leptopiliana boulardi* and Sarcophagidae flies) (Carton & Claret 1982, Denlinger 1979). The introduction of seasonal species into the tropics has caused (1) the loss of diapause (e.g. Pieridae: *Tatochila xanthodice*) (Shapiro 1978), (2) intraspecific geographic variation of diapause expression (e.g. Gelechiidae: *Pectinophora gossypilla*, whose populations near the Equator do not respond to photoperiod signals) (Pradhan et al. 2020, Ando 1983) or (3) show a low and variable incidence of diapause (e.g. Lygaeidae, Oncopeltus) (Dingle et al. 1980). Variability in diapause response decreases toward the temperate zone, possibly as selection pressure for diapause increases; the high variability of the diapause response observed near the equator provides flexibility in the life cycle, allowing species to respond to a less predictable seasonal environment (Denlinger 1986).

Polyvoltine breeds are recommended for breeding in tropical countries because they tolerate slightly higher temperatures and adapt to adverse climatic conditions (Sarkhel et al. 2017), and due to the effect of climate change in the coming decades the practices and economics of sericulture in the temperate region may drastically change and could have a beneficial effect in the tropical region (Ram et al. 2016).

Contrary to what was observed in the polyvoltine line, the J7 bivotine line showed that photoperiod presents significant differences (p=0.026) on voltinism; it shows overlap between subsets (8-16, 12-12 and 12-12, 16-8) but the decrease in voltinism from 8-16 to 16-8 was evident (Figure 1). The lowest average value of voltinism was shown by the 16-8 photoperiod with 1.57 generations per year and the highest was in 8-16 with 4.18 generations (Table VI). The number of generations per year depended on the life cycle duration, from the moment of eggs oviposition to the emergence of the individual adult, including the diapause time. The photoperiod that increased DLP and DLC was 8-16. Although in the 16-8 photoperiod these two variables were lower, this photoperiod had a higher number of eggs per laying and fewer changes in the occurrence of diapause, thus maintaining the lowest number of generations per year.

The results of the C6 line showed that temperature and photoperiod did not influence the voltinism (Table VI). This effect is possibly

Table VI. **Effect of temperature and photoperiod on voltinism. Means with different letter differ statistically at p<0.05. NS: Non-significant effect.**

Line	Temperature (°C)	Mean	Photoperiod (photophase/scotophase)	Mean
C6	24	3,67	8-16	3,21
	26	3,75	12-12	3,96
	28	4,22	16-8	4,46
	P-value	NS	P-value	NS
J7	24	2,6	8-16	4,18 A
	26	3,45	12-12	3,03 AB
	28	2,74	16-8	1,58 B
	P-value	NS	P-value	0,0263
C. Nichi	24	7,27 A	8-16	8,11
	26	8,44 A	12-12	8,29
	28	8,81 B	16-8	8,12
	P-value	0,0216	P-value	NS

attributed to the observed voltinism variability when diapause occurs. Unlike J7, C6 presented a higher percentage of non-diapause (42%) and mixed layings (4.5%), while J7 did not exceed 4% and 2.2% respectively. Considering the photoperiod effect, voltinism varied between 1.58 - 4.18, while in the temperature effect the voltinism ranged between 2,60 – 3,45 generations per year (Table III).

In order to understand the voltinism variability presented in C6 line, an independence chi-squared test was performed. In C6 line, the generation number per year depended on the life cycle duration since eggs oviposition moment to the adult emergence, including the diapause time. In light of the above, variable percentages of diapause were used (the ranges <20, 20-50, 50-80 and 80-100) to observe the influence of unobservable environmental factors with the voltinism variable. The result of the chi square test with four ranges of diapause percentage (diapause reduction) for temperature and photoperiod (Figure 2) showed an association between temperature and/or photoperiod in reducing the presence of diapause (Chi^2=39.73; gl=24; p=0.023). The photoperiod is the factor that presents the association (Chi^2=16.35; df=6; p=0.012). The layings of the hatchlings maintained in the 12-12 photoperiod had the highest percentage of diapause eggs, unlike those maintained at 8-16 and 16-8; this agrees with Kobayashi (1990) who records that females reared in the 12-12 photoperiod generate more diapause eggs.

In temperate zones, the dominant pattern is that insects use the day length to predict the arrival of winter (Denlinger 1986). Day length is an important seasonal indicator, since the species can distinguish a difference of even 1/2 hour in day or night length to induce diapause (Saunders 1982). Additionally, photoperiod can exert a dual function by inducing diapause and influencing its duration (Wolda & Denlinger 1984). Closer to the equator in the tropics, the ability to signal the seasonal change in day length seems to be lost (Carton & Claret 1982).

In *B. mori*, the diapause hormone is responsible for the induction of embryonic diapause (Grenier et al. 2004, Yamashita 1996). The mechanism through which the brain controls diapause hormone secretion can be modified by photoperiod conditions during the larval stages (Tsuchida & Yoshitake 1983b). According to Shimizu & Hasegawa (1988), the photoreceptor is located extraocularly in the head. The light is

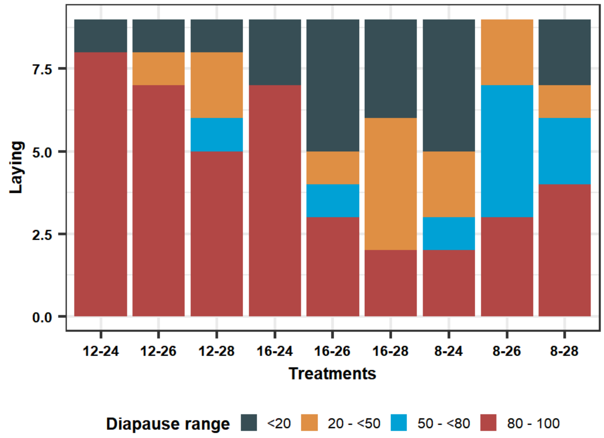

Figure 2. Distribution of the percentage of diapause for line C6 in four ranges (<20, 20-50, 50-80 and 80-100) by combination of photoperiod and temperature. The x-axis labels represent the treatments, the first number the photoperiod and the second the temperature °C.

admitted through the translucent clypeus of the head in the first instance and in the later stages the light enters the entire larval body, including the head and reaches the brain lobe.

Using molecular markers (microsatellites), Trochez et al. (2019) found significant differences in the genetic relationships between lines C6 and J7. The C6 line was found in a group of Chinese lines and hybrids with tropical parents, which would reflect one of the dispersal events of sericulture in southern China and India (Xiang et al. 2018) or one of the evolution hypotheses from bivoltines to polyvoltines (Gamo & Ohtsuka 1980), while J7 was within a group containing exclusively lines of Japanese origin and widely separated from the others, suggesting the high number and frequency of exclusive alleles present in these materials that may be the result of reproductive isolation and significant genetic differentiation in relation to the Chinese, European and tropical race (Yukuhiro et al. 2011).

The Chinese line (C6) showed greater susceptibility to environmental changes (Kinoshita et al. 1982), particularly to photoperiod, an interpretation given by the changes in the percentage of diapause, since 46.5% of the layings were non-diapause or mixed. This shows that altering the incidence of diapause may be easier in some lines than in others and selecting the non-diapause for several generations can decrease its incidence (Denlinger 1979). It would also allow increasing the number of offspring per year or having material available at any time for use in programs of breeding (germplasm banks) or improvement.

CONCLUSIONS

Biological variables (DLP, DLC, LW(V-5), RWV, NE, F) influence voltinism differently in the C. Nichi, C6, and J7 silkworm lines. In the case that the percentage of diapause is high (J7), the changes in duration of life cycle (not including the duration of the diapause) due to the effect of environmental conditions were not significant for voltinism. Otherwise, when the percentage of diapause is reduced, the duration of the life cycle has a significant negative effect.

The duration of the life cycle from the moment of laying to the emergence of the new adults and the number of times it can be repeated in a year, are affected by environmental conditions (temperature and photoperiod) and their incidence depends on the origin and voltinism of silkworm lines. The results showed that for the Indian tropical polyvoltine line, temperature was the influencing factor in voltinism, while for the Chinese and Japanese bivoltine lines, it was photoperiod.

The Chinese line (C6) presented greater variability in the diapause response influenced by the photoperiod imposed on the breeding for three generations, which makes C6 a useful line for decisions of management and selection. The Japanese line (J7) meanwhile shows little variability possibly resulting from isolation and differentiation with other races.

Acknowledgments

The authors wish to thank the University of Cauca and the following research groups: the Geology, Ecology and Conservation research group (GECO), and the Integrated Production Systems Research Group (SISINPRO). Also, to the projects InnovAccion-Cauca and Technical Development for Organic and Innovative Silk Products Project, from the General Royalties System, and the Government of Cauca.

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How to cite

ZAMBRANO-GONZÁLEZ G, ALMANZA M, VÉLEZ M & RUIZ-ERAZO X. 2023. Effect of environmental conditions on the changes of voltinism in three lines of *Bombyx mori*. An Acad Bras Cienc 95: e2021-0122. DOI: 10.1590/0001-3765202320210122.

Manuscript received on January 26, 2021;
accepted for publication on August 18, 2022

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