Comparative Study of Biological and Technological Characters in Three Generations of Silkworm *Bombyx mori* L. Ameiotic, Parthenogenetically Cloned Lines

H. Greiss,^{1,2} J. Vassilieva,¹ N. Petkov,¹ and Z. Petkov¹

Submitted March 30, 2004; accepted July 28, 2004

Purpose: Detect any deviation in biologic and technologic characters of eight ameiotic-parthenogenetically cloned lines of *Bombyx mori* L. from different origins from a normal sexually reproduced control line in three generations.

Methods: Comparative study of the three generations was conducted in SES, Vratza, unit of the National Center for Agrarian Sciences of Bulgaria after fixing all environmental rearing conditions.

Results: The ameiotic-parthen-clones displayed good parthenogenetic development, although total hatchability was significantly less than the sexually reproducing control populations. Survival rates between clones and control were not significantly different. All clones displayed significantly longer larval periods. Slight decline in second generation, and a steeper one in the third generation were observed for all eight cloned lines in cocoon weight, shell weight, and shell ratio and these differences were statistically significant. Cocoon yield was significantly lower than the control throughout the three generations.

Conclusions: Our parthen-cloning method has a high rate of success in comparison to other cloning methods, the cloned progeny populations although were weaker technologically (cocoon weight, shell weight, and shell ratio), the biological characters (parthenogenetic development and survival rate) were not compromised. Further study is needed to determine the thermal needs of the cloned embryos and metabolic rate of all stages.

KEY WORDS: Ameiotic; *Bombyx mori* L.; cloning; parthenogenesis.

INTRODUCTION

One of the goals of silkworm reproduction is to produce exact replicas of female genotypes by means of ameiotic parthenogenesis (1). Astaurov (2) noticed for the first time spontaneous parthenogenesis in silkworm *Bombyx mori* L. in the mid-1940s at a rate less than 0.1% and he started testing different factors that controlled and influenced this phenomenon. According to the same author (3) high aptitude for

parthenogenetic development is determined multigenically and in most cases acts in a semidominant or dominant manner.

In an important advance for the field, Strunnikov (4) developed the technique for inducing spontaneous parthenogenesis by thermal disruption of the meiotic spindles at the first reduction division. All his experiments were conducted on pure races, but Vasileva and Tzenov (5,6) received improved results when applying this technique to hybrid silkworm moths, originating from crossing Japanese and Chinese type races. Heat induced meiotic parthenogenesis duplicates the genotype of the mother, the hatched offsprings are only female and with identical genotypes (7–13). Different clones, produced by thermal

¹ Sericulture Experiment Station, 24 Mito Orozov St., 3000 Vratza, Bulgaria.

² To whom correspondence should be addressed at 4 Norman Ct, Burr Ridge, Illinois 60527; e-mail: agromier@yahoo.com.

disruption of meiosis, have been selected with high hatchability and pupation ratio (3,5,6,11,14–16).

Ameiotic parthenogenetic clones produce identical female twins (ZW genotype). A clone line lacks genetic variation and accordingly phenotypic variations are exclusively due to environmental factors. During the initial construction of the clonal lines, genetic advances concerning the biological and quantitative characters were achieved by back crossing ameiotic parthenogenetic clones with full-sib males of the same batch (1,17).

The aim of the present study was to determine the manifestation of the biological and quantitative characters of eight-silkworm parthenogenetic cloned populations at their third generation.

MATERIALS AND METHODS

The study was carried out during the period of 2001–2003 at SES, Vratza, a unit of the National Center for Agricultural Sciences of Bulgaria. Eight ameiotic-parthenogenetically cloned lines from different origins, viz. four Egyptian (Parthen₁, Parthen₂, E₁₁, and E₁₂), two Bulgarian (Joanna and H₂xS₁-P) and two Ukrainian (P_{11/22} and P_{15/18}) were used in the study. The control strain was a normal sexually reproducing line, 157-K.

Parthenogenetic clones were created through thermal disruption of meiosis as follows: the eggs were extracted from ovarioles of unfertilized virgin moths and we preserved them at 20–25°C for 12 h before thermal treatment to induce parthenogenetic development. The extracted eggs were placed in a water bath at 46°C temperature for 18 min; they were then immersed in water at 18°C for 10 min. Then, the eggs were dried and preserved for 3 days at 15–17°C and 80–90% RH as in Strunikov (11). Subsequently the eggs were stored for 12 months according to the regime for silkworm egg preservation as shown in Table I. After this period the eggs were incubated according to the standard regime (Table II) for hibernated eggs.

Percentage of parthenogenetic development was determined by Astaurov's method (2). Parthenogenetic lines were tested in two replications, beginning with 200 individuals counted after second molting. During the first three instars the larvae were reared under a polyethylene sheet and fed three times per day. During the 4th and 5th instars the larvae were fed two times per day with fresh mulberry leaves. Survival rates were calculated by the number of viable healthy

Table I. Regime for Silkworm Egg Preservation

Period	Ecological parameters for eggs preservation	
	Temperature (°C)	Relative humidity (%)
Till end of August	23–24	75–80
September	20	75–80
October	12–10	75–80
November	10–8	75–80
01–10 December	8	75–80
01 December–10 January	5	75–80
10 January–20 March	2.5	75–80
20–25 March	15	75–80
26 March–20 April	2.5	75–80

cocoons obtained from 200 larvae counted just after the second molt.

All parthenogenetic cloned lines and control strain have plain larva and white cocoons, the only exception is the Bulgarian parthen-clone, H₂xS₁-P, which has larval markings and white cocoon. ANOVA two-factor dispersion analysis was performed to determine effects and significances.

RESULTS AND DISCUSSION

Successful induction of parthenogenetic clones by thermal method varied among eight different lines of silkworms ranging from 77 to 89%, averaging about 85%, as demonstrated by change of eggs color similar to fertilized eggs. An average of 45% of the eggs completed development and healthy viable embryos were detected after hibernation and preincubation (Table III). The Bulgarian hybrid parthenogenetic clone H₂xS₁-P line manifested significantly

Table II. Regime for Egg Incubation

Day	Temperature (°C)	Humidity (% RH)	Lighting regime
1	15	75	Natural lighting
2	18	75	Natural lighting
3	24	75	Natural lighting
4	24	75	Natural lighting
5	24	75	16 h luminous day
6	24	75	16 h luminous day
7	25	80	18 h luminous day
8	25	80	18 h luminous day
9	25	80	18 h luminous day
10	25	80	Dim out
11	25	85	Full darkness
12	25	85	Full darkness
13	25	85	Morning illumination

Table III. Silkworm Parthenogenesis Development (%)

Lines	Succesful parthenogenesis	Parthenogenetic eggs includes			Failed Parthengensis	
		Complete healthy	Death at late embryonic stage	Death at early embryonic stage		
P _{11/22}	88.37	47.02	13.29	39.69	11.63	
P _{15/18}	88.87	45.22	15.29	39.49	11.13	
Joana	77.00	50.30	18.64	31.06	23.00	
H ₂ x S ₁ -P	89.33	24.77	26.03	49.20	10.67	
Parthen ₁	85.32	49.12	14.67	35.17	14.68	
Parthen ₂	81.17	48.76	18.80	32.44	18.83	
E ₁₁	83.86	48.28	14.62	37.05	16.14	
E ₁₂	87.56	49.63	11.87	38.77	12.44	
Average	85.19	45.39	16.65	37.86	14.82	
Source of variation	SS	df	MS	<i>F</i>	<i>P</i> -value	<i>F</i> crit
Lines	0.358	7	0.051	0.001	1.000	2.764
Parthen-stages	3552.566	2	1776.283	28.824	0.000	3.739
Error	862.754	14	61.625			
Total	4415.678	23				

Note. SS: Sum square, df: Degree of freedom, MS: Mean square, F: Fisher value, P-value: Probability value, F crit: Critical Fisher value.

lower healthy eggs at 24.77% despite having the highest initial success rate at 89.33%; this line also exhibited the highest early embryonic death rate at 49%.

High significance was exhibited between different stages (death in early and late embryonic stages and complete embryo genesis), however no significance was shown among the eight tested lines concerning these phases.

Egg hatchability ranged from 26.54% for line E₁₁ in 2001 to 94.49% for line E₁₂ during 2003 (Table IV). In contrast, sexually fertilized eggs had more than 99% hatching, despite using the same standard regimes for both types of eggs. Differences in hatchability between successive years did not prove to be statistically significant, but the individual parthenogenetically cloned lines were statistically different from each other, with a *P*-value of 0.006.

Several authors (1,13) have demonstrated that hatchability is influenced by the regimes used for egg preservation and subsequent incubation. It is possible that parthenogenetic embryos have different requirements than that determined for sexually produced embryos. In addition, it should be noted that parthenogenetically developing eggs are all female whereas sexually produced eggs are a mixture of male and female offspring. These considerations suggest that the thermal regime used for normal sexually fertilized may have to be adapted to fit the requirements of the parthenogenetically produced embryos. Further studies will have to be performed to determine the proper preservation conditions.

We analyzed a number of additional features relative to the control line of silkworms for further characterizing these parthenogenetically derived cloned lines. Survival rate is determined by resistance to the adverse conditions as described by Petkov and Penkov (18) and Lim *et al.* (19) and figures were comparable to those obtained for the control line (Table V), indicating that female parthenogenetic lines did not suffer any deterioration in resistance during the 3 years of the study. Four lines, Parthen₁, Parthen₂, E₁₁, and E₁₂, actually manifested higher

Table IV. Silkworm EGGS Hatchability (%)

LINES	2001	2002	2003	Average		
P _{11/22}	70.51	75.05	74.87	73.48		
P _{15/18}	61.52	67.19	71.11	66.61		
Joana	49.25	52.51	50.53	50.76		
H ₂ x S ₁ -P	61.73	64.11	66.13	63.99		
Parthen ₁	82.04	86.93	87.06	85.34		
Parthen ₂	79.98	81.89	84.01	81.96		
E ₁₁	26.54	93.99	90.85	70.46		
E ₁₂	71.05	93.83	94.49	86.46		
Average	62.83	76.94	77.38	72.38		
Control	99.81	99.8	99.76	99.79		
Source of variation	SS	df	MS	F	P-value	F crit
Lines	5104.687	8	638.086	4.290	0.006	2.591
Generations	973.915	2	486.957	3.274	0.064	3.634
Error	2380.083	16	148.755			
Total	8458.685	26				

Note. SS: Sum square, df: Degree of freedom, MS: Mean square, F: Fisher value, P-value: Probability value, F crit: Critical Fisher value.

Table V. Silkworm Survival (%)

LINES	2001	2002	2003	Average
P _{11/22}	76.23	93.11	89.21	86.18
P _{15/18}	97.01	87.04	81.16	88.40
Joana	83.54	89.03	93.89	88.82
H ₂ x S ₁ -P	80.72	86.40	75.36	80.83
Parthen ₁	94.34	89.45	94.11	92.63
Parthen ₂	96.80	89.67	92.17	92.88
E ₁₁	90.91	91.75	95.50	92.72
E ₁₂	97.20	86.57	92.02	91.93
Average	89.59	89.13	89.18	89.30
Control	89.52	76.02	97.43	87.66

Source of variation	SS	df	MS	F	P-value	F crit
Lines	382.456	8	47.807	1.071	0.429	2.591
Generations	29.390	2	14.695	0.329	0.724	3.634
Error	714.063	16	44.629			
Total	1125.909	26				

Note. SS: Sum square, df: Degree of freedom, MS: Mean square, F: Fisher value, P-value: Probability value, F crit: Critical Fisher value.

survival ratios in comparison to the sexually reproducing control. For all tested lines this character varied from 80.83% in H₂xS₁-P to 92.88% in Parthen₂. The survival ratio of E₁₁ and Joanna lines increased during the period of investigation whereas the other lines showed a slight decrease in the value of this biological character.

The silkworm *Bombyx mori* L. is a poikilotherm animal and its metabolic rate depends on the ambient temperature. Various temperature-rearing regimes result in different larval durations, even when all other conditions are standardized (20). In our experiment we used the most commonly accepted rearing protocol as the standard regime (21). On average, the developmental time needed for the sexually reproduced 157-K control line for the 3 years of experiments was within the international average duration of 665 h, the ameiotic-parthenogenetic clones had longer larval duration by 29–68 h (Table VI). Clone H₂xS₁-P had the shortest larval duration (704 h) among the cloned lines, which is probably due to the hybrid origin of this line, because hybrids usually manifest shorter life cycles than pure inbred races. High statistically significant difference was found between generations and between lines concerning larval duration. We noted that all of the parthenogenically-derived clones had longer instars, molting, and spinning durations.

Individual cocoon weight varied between cloned lines from 1.742 to 2.216 g (Table VII), which suggests that weight depends upon genetic background differences among the cloned lines. A statistically

Table VI. Silkworm Larval duration (h)

LINES	2001	2002	2003	Average
P _{11/22}	724	695	749	723
P _{15/18}	735	725	749	736
Joana V	739	718	762	740
H ₂ x S ₁ -P	704	694	714	704
Parthen ₁	744	724	743	737
Parthen ₂	744	720	749	738
E ₁₁	726	725	749	733
E ₁₂	726	726	746	733
Average	730	716	745	730
Control	670	661	664	665

Source of variation	SS	df	MS	F	P-value	F crit
Lines	14375.407	8	1796.926	23.776	0.000	2.591
Generations	3122.741	2	1561.370	20.659	0.000	3.634
Error	1209.259	16	75.579			
Total	18707.407	26				

Note. SS: Sum square, df: Degree of freedom, MS: Mean square, F: Fisher value, P-value: Probability value, F crit: Critical Fisher value.

significant decline in this important quantitative character in the third generation was exhibited by all lines except E₁₁ and H₂xS₁-P. The control pure line maintained its values during the three generations. The lower standard deviation for the clone individuals corresponds to their absolute genetic homogeneity, as compared to the control animals.

Similarly, shell weights exhibited the same pattern of statistically significant decline in value during successive generations for all parthenogenetic clones

Table VII. Silkworm Cocoon Weight (gm)

LINES	2001	2002	2003	Average
P _{11/22}	2.048	2.042	1.821	1.970
P _{15/18}	1.923	1.917	1.792	1.877
Joana	1.942	1.936	1.702	1.860
H ₂ x S ₁ -P	2.293	2.304	2.051	2.216
Parthen ₁	1.951	1.897	1.632	1.827
Parthen ₂	2.011	1.967	1.703	1.894
E ₁₁	1.850	1.864	1.858	1.857
E ₁₂	1.905	1.987	1.333	1.742
Average	1.990	1.989	1.737	1.905
Control	2.153	1.929	2.212	2.098

Source of variation	SS	df	MS	F	P-value	F crit
Lines	0.516	8	0.064	3.724	0.012	2.591
Generations	0.258	2	0.129	7.450	0.005	3.634
Error	0.277	16	0.017			
Total	1.051	26				

Note. SS: Sum square, df: Degree of freedom, MS: Mean square, F: Fisher value, P-value: Probability value, F crit: Critical Fisher value.

Table VIII. Shell Weight (gm)

LINES	2001	2002	2003	Average		
P _{11/22}	0.371	0.380	0.322	0.358		
P _{15/18}	0.371	0.376	0.341	0.363		
Joana	0.367	0.402	0.292	0.354		
H ₂ x S ₁ -P	0.410	0.448	0.333	0.397		
Parthen ₁	0.449	0.366	0.352	0.389		
Parthen ₂	0.397	0.371	0.351	0.373		
E ₁₁	0.402	0.350	0.259	0.337		
E ₁₂	0.391	0.374	0.167	0.311		
Average	0.395	0.383	0.302	0.360		
Control	0.451	0.330	0.399	0.393		
Source of variation	SS	df	MS	<i>F</i>	<i>P</i> -value	<i>F</i> crit
Lines	0.019	8	0.002	1.128	0.397	2.591
Generations	0.037	2	0.019	8.852	0.003	3.634
Error	0.034	16	0.002			
Total	0.090	26				

Note. SS: Sum square, df: Degree of freedom, MS: Mean square, F: Fisher value, P-value: Probability value, F crit: Critical Fisher value.

with a *P*-value of 0.003 (Table VIII). The decline pattern was $0.395 > 0.383 > 0.302$ g in average for all parthenogenetic lines in the three successive generations. There was also a similar tendency for the shell ratio, which is the ratio of the silk shell in relation to the whole cocoon weight, to decline in successive generations from 19.90 to 19.27 to 17.34 at *P* value of 0.03 (Table IX). It should be noted that the silk shell determines the amount of energy the silkworm spares for the pupal protection

Table IX. Shell Ratio (%)

LINES	2001	2002	2003	Average		
P _{11/22}	18.13	18.61	17.68	18.14		
P _{15/18}	19.30	19.61	19.03	19.31		
Joana	18.91	20.76	17.16	18.94		
H ₂ x S ₁ -P	17.87	19.44	16.24	17.85		
Parthen ₁	23.01	19.29	21.57	21.29		
Parthen ₂	19.74	18.86	20.61	19.74		
E ₁₁	21.73	18.78	13.94	18.15		
E ₁₂	20.52	18.82	12.53	17.29		
Average	19.90	19.27	17.34	18.84		
Control	20.95	17.11	18.04	18.70		
Source of variation	SS	df	MS	<i>F</i>	<i>P</i> -value	<i>F</i> crit
Lines	0.0034	8	0.0004	1.0649	0.4327	2.5911
Generations	0.0031	2	0.0015	3.8495	0.0432	3.6337
Error	0.0064	16	0.0004			
Total	0.0130	26				

Note. SS: Sum square, df: Degree of freedom, MS: Mean square, F: Fisher value, P-value: Probability value, F crit: Critical Fisher value.

Table X. Cocoon Yield (Kg)

LINES	2001	2002	2003	Average		
P _{11/22}	22.02	28.54	24.33	24.96		
P _{15/18}	22.95	22.42	20.68	22.02		
Joana	15.98	18.10	16.15	16.74		
H ₂ x S ₁ -P	22.85	25.52	20.44	22.94		
Parthen ₁	30.20	29.50	26.74	28.81		
Parthen ₂	31.14	28.89	26.37	28.80		
E ₁₁	8.93	32.15	32.24	24.44		
E ₁₂	26.31	32.28	23.18	27.26		
Average	22.55	27.18	23.77	24.50		
Control	38.47	29.27	43.00	36.91		
Source of variation	SS	df	MS	<i>F</i>	<i>P</i> -value	<i>F</i> crit
Lines	752.218	8	94.027	2.910	0.033	2.591
Generations	43.017	2	21.509	0.666	0.528	3.634
Error	517.007	16	32.313			
Total	1312.243	26				

Note. SS: Sum square, df: Degree of freedom, MS: Mean square, F: Fisher value, P-value: Probability value, F crit: Critical Fisher value.

Finally, the cocoon yield per box (containing $20,000 \pm 200$ eggs) was determined for each clone line and control. This value, which combines both silkworm biological and cocoon technological characters, showed statistically significant differences between lines with a *P*-value of 0.033 (Table X). The different genetic background used for constructing different lines is assumed to be responsible for this phenomenon. The productivity of the eight parthenogenetic cloned lines was considerably lower than the sexually fertilized control.

CONCLUSION

Our parthen-cloning method has a high rate of success in comparison to other cloning methods. Ameiotic parthenogenetical clones were tested for main silkworm biological and cocoon technological traits. Although there was good total parthenogenetic development, total hatchability was significantly less than the sexually fertilized control. Survival rate of clones and sexually reproduced control manifested no significant difference, and four out of the eight cloned lines had higher survival ratio than control. All clones manifested significantly longer larval duration.

Decline of individual cocoon weight, shell weight, and shell ratio in almost all eight lines was manifested in the direction generation $1 > 2 > 3$ and differences were statistically proven. Difference between the parthenogenetically produced lines and the sexually

reproduced control increased in the same direction and was steeper in the third generation. The cocoon yield was significantly lower than the sexually reproduced control during the three generations.

Further study is needed to determine the thermal needs of the cloned embryos and metabolic rate of all stages.

REFERENCES

1. Strunnikov VA, Lejenko SS, Stepanova NL: Silkworm *Bombyx mori* L. cloning. *Genetics* 1983;19(1):82–94
2. Astaurov BL: Artificial Silkworm Parthenogenesis. Moscow, AS USSR, 1940, pp 238–302
3. Astaurov BL: Selection choice for thermal artificial parthenogenesis development ability in silkworm. *Genetics* 1973;9(9): 93–105
4. Strunnikov, V: Male generation receiving in silkworm *Bombyx mori* L., Reports of Academy of Sciences, Moscow, 1990, vol. 18, pp. 5–32.
5. Vasileva J, Tzenov P: Heterosis, depression and degrees of dominance at silkworm *Bombyx mori* L. characters pigmented and non-pigmented parthenogenetic eggs. *Anim Sci* 2003;1/2:103–105
6. Vasileva J, Tzenov P: Study on parthenogenetic development of some silkworm *Bombyx mori* L. F₁ hybrids. *Anim Sci* 2003;3/4: 128–131
7. Astaurov, B: Artificial partheogenesis in silkworm *Bombyx mori* L., Academy of Science Publishing, Moscow, pp 240.
8. Astaurov BL: Experimental alterations of the developmental cytogenetic mechanisms in the mulberry silkworm: Artificial parthenogenesis, polyploidy, gynogenesis and androgenesis. *Adv Morphol* 1967;6:199–257
9. Vereiskaya V: Meioses and changes in thermo activated silkworm *Bombyx mori* L ovarioles. *Bull Moscow Soc Nat Defender* 1975;4:31–50
10. Klymenko VV: Mechanism silkworm artificial parthenogenesis. I. Dependence between thermoactivation effect and speed of after activation cooling. *Genetics* XVI, 1980;6:1002–1010
11. Strunnikov V: Genetic methods of sex selection in silkworm. Moscow, 1987, pp 327.
12. Vasileva J, Tzenov P: Study of influence of before and after activating the ovariole silkworm eggs preservation at thermal ameiotic development ability. In Scientific Conference “Sciences for Life at New Millennium,” Agricultural University, Plovdiv, Bulgaria, 2000, pp 76–82.
13. Murakami A: Genetic studies on parthenogenesis in *B. mori* L. thermal activation and its significance. *Annu Rep Natl Inst Genet Jpn* 1985;36:48–49
14. Vasileva J, Mladenov G: Investigation on possibilities for creation of ameiotic parthenogenetic silkworm individuals from Super1xHessa2. *Anim Sci* 1998;Suppl.:79–81
15. Klymenko VV: Parthenogenesis and cloning in the silkworm, *B.mori* L. Problems and prospects. *J. Insect Biotech Seric* 2001;70:155–165
16. Ravindra S, Raghavendra RD, Kariappa, BK, Prabhati S, Premalatha V: Studies on parthenogenetic development, hybrid vigor and cocoon shape variability in bivoltine F₁ hybrids of the silkworm, *B.mori* L. *Int J Ind Entomol* 2002;5(1): 59–65
17. Strunnikov VA, Gubanov EA, Zvyaginceva TV, Strunnikova LV: Silkworm eggs activation at different combination of thermal effects. *Acad Sci Rep* 1996;348(6):849–850
18. Petkov M, Penkov I: Sericulture Reference Book. Sofia, Bulgaria, Zemizdat, 1980, pp 200
19. Lim S, Kim Y, Lee S, Rhee I, Lim J, Lim B: Sericulture Training Manual. Rome, FAO, 1990, pp 200
20. Greiss H, Sohn HR, Lim JS: Study on the effect of different temperatures on the main productive characters of the silkworm larvae *Bombyx mori* L. under the Egyptian conditions. *Agric Res Bull Kyungpook Natl Univ* 2000;18(12):43–51
21. Kipriotis E, Petkov N, Tzenov P, Grekov D: Practical Sericulture. National Agricultural Research Foundation. Komotini, Greece, 1999, pp 130.