The effect of methoprene treatment on Vth instar silkworm

larvae reared on artificial diet

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Silkworm as a bioreactor

"The expression of pharmaceutically relevant proteins, using silkworm larvae or cocoons has become very attractive. Silkworm biotechnology is an innovative and easy approach to achieve high protein expression levels and is a very promising platform technology in the field of life science" (Kato et al., 2010).

Table 1
Expression of recombinant proteins in silkworm larvae and pupae (Kato et al., 2010)

Proteins	Used viruses or bacmids	References
Firefly luciferase	BmNPV	Palhan et al. (1995)
Human interferon-α	BmNPV	Maeda et al. (1985)
Human macrophage colony-stimulating factor	BmNPV	Qiu et al. (1994)
Human growth factor	BmNPV	Kadono-Okuda et al. (1995)
Rat interleukin-5	Cysteine protease depleted BmNPV	Ishihara et al. (1999)
Human butyrylcholinesterase	BmNPV	Wei et al. (2000)
Bovine interleukin-21	HyNPV	Muneta et al. (2004)
Bovine interferon-t	Cysteine protease depleted BmNPV	Nagaya et al. (2004)
Porcine lactoferrin	HyNPV	Wang et al. (2005)
Human granulocyte macrophage colony-stimulating factor	BmNPV	Chen et al. (2006)
GFP_{uv}-β3GnT2 fusion protein	BmNPV bacmid	Park et al. (2007)
EGFP-spider dragline silk fusion protein	BmNPV bacmid	Zhang et al. (2008)
Cholera toxin B	BmNPV	Gong et al. (2005)
Human stem cell factor	BmNPV	Han et al. (2004)
anti-BSA scFV	Cysteine protease and chitinase depleted BmNPV	Ishikiriyama et al. (2009)
Human anti-BSA IgG1	Cysteine protease and chitinase depleted BmNPV	Park et al. (2009)
Human α2,6-sialyltransferase	Cysteine protease and chitinase depleted BmNPV	Ogata et al. (2009b)
Human (pro)renin receptor	Cysteine protease depleted BmNPV	Du et al. (2008)
Human prorenin-(pro)renin receptor complex	Cysteine protease depleted BmNPV	Du et al. (2009b)

Other transgenesis methods

Involving the use of an attenuated recombinant baculovirus or a *piggyBac* transposon-derived vector (Tamura et al. 2000; Yamao et al. 1999) or with a method combining the two systems (Yamamoto et al. 2004):

1) Human type III procollagen and feline interferon were produced in cocoons using transgenic silkworms (Kurihara et al. 2007; Tomita et al. 2003).
2) Human μ-opioid receptor was expressed in the silk glands and fat bodies of transgenic silkworms, which were screened by the *GAL4/UAS* system (Tateno et al. 2009).

Why using artificial diet?

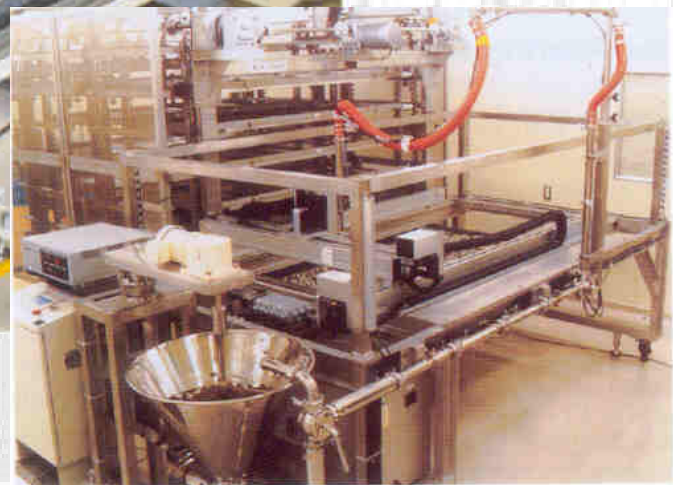
“Rapid advances in biotechnology and realization of transgenic silkworms to create new materials for pharmaceutical or bio-medical applications have highlighted the importance of artificial diets for silkworm larvae. Traditional rearing on mulberry leaves is largely dependent on natural environmental conditions and demanding extensive workers’ labor, it does not warrant efficient and predictable production(Cappellozza et al., 2011).
In mass-rearing of silkworms, diseases from bacterial and viral infections are frequent causes of total loss of cocoon harvest.

NPV in Vth instar larvae

Bacterial flacherie

Why using artificial diet?

- Mechanization of rearing is easier, standardization of production is higher, continuous cycles are better in order to continuously employ rearing rooms, machineries and manpower

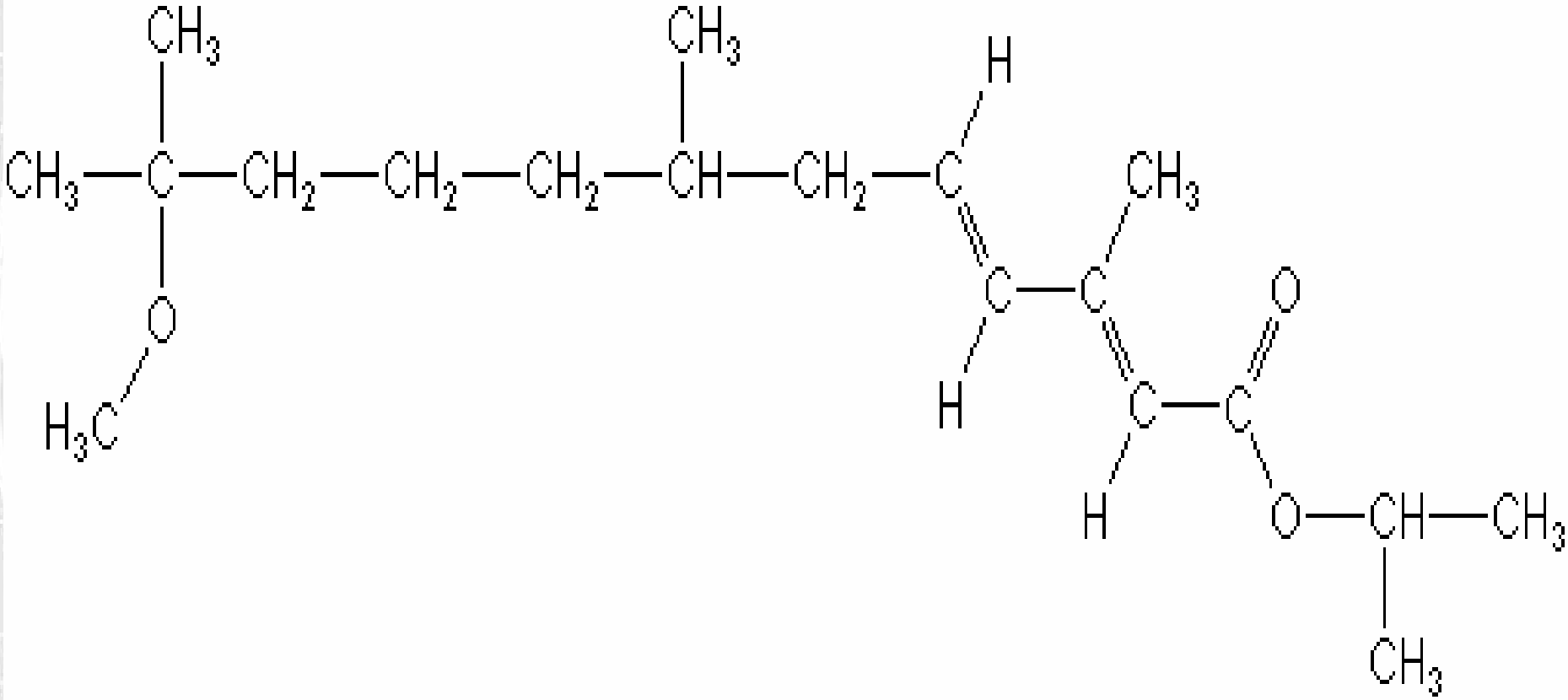


- Nevertheless silk production is reduced in diet-reared larvae in comparison to leaf-reared larvae

Possible solutions

- To improve the nutritional quality of the diet
- To use Juvenile Hormone Analogues

Methoprene (J.H.A. = Juvenile Hormon Analogue)



Structural formula

Characteristics

- Although methoprene differs greatly in its chemical structure from natural insect juvenile hormones, it is extremely similar in its biological activity.
- Its use is suggested for topical application during a period of 48-60 hrs following the first feeding of the fifth instar silkworms.
- At the suggested doses (2.5 ppm) it should prolong of one day the eating period and delay of one day the spinning.
- At this doses it should cause a 10% increase in the cocoon weight

Materials and methods

- Polyhybrid larvae were reared under 12:12 D/L photoperiod, decreasing temperature from 29°C to 25°C, according to the progression of the larval instars from the first to the fifth and decreasing relative humidity, until reaching 70±5% in the last instar.
- Silkworms were reared with artificial diet (CRA-API Patent, Cappellozza et al., 2005), containing decreasing quantity of mulberry leaf (from 25% in the first instar to 5% in the last).
- Larvae were topically treated with a solution of Methoprene diluted in ethanol. Pestanal (methoprene, analitycal standard) was purchased by Sigma-Aldrich.
- Accordingly to the concentration (2.5 ppm) suggested by the Manta production company (Zoecon Corporation, Palo Alto, California), different concentrations were tested. Furthermore, even though treatment from the 48^{th} to the 60^{th} hr of the last instar was suggested by Zoecon, in our experiments it ranged in a period from the 0 (immediately after moulting) to the 72^{nd} hr.
- However, some preliminary tests were performed in order to evaluate which were the doses causing no increase in mortality even though administered in a later stage.
- Groups of larvae were made by at least 10 larvae per each sex.
- ANOVA analysis was carried out and the significance of the interactions: Time of Treatment x Doses x Sex and Time of Treatment x Doses were evaluated. Tukey's test was performed in order to distinguish significant differences among means.

Results

- Production data of the first experimental set are presented in Table 1. For the two tested doses, the improvement of silk production over the control increased with time elapsing, reaching its maximum around the 48th hours, with some differences according to the sex, and with a marked effect related to the day more than to the doubling of the treatment doses.
- The limited increase in silk production for the treatment at hour 0 as well as the scarce delay in spinning time is probably related to quick metabolism of the chemical.
- No increase in mortality was recorded in treated lots, even though some non-transformed larvae (larval-pupa intermediates) were found into the cocoons of 24 and 48 hr treated groups.
- In general the increase of the cocoon weight was higher than the increase in the silk shell, but even this physiological behaviour was related to the time of treatment and the hormone doses.

TAB.1

Treatment	Sex	Cocoon weight (g)	Shell weight (g)	Silk percentage (%)	Delay in spinning(hr)
Control (Et-OH)	M	1.227 ± 0.081	0.239 ± 0.019	19.5 ± 1.8	-
	F	1.541 + 0.095	0.267 ± 0.016	17.3 ± 4.0	-
Hour 0 – 156 ng/larva	M	1.416 ± 0.108 15%	0.259 ± 0.027 8%	18.3 ± 1.3	12
	F	1.787 ± 0.228 16%	0.298 ± 0.040 12%	16.7 ± 1.1	12
Hour 0 – 313 ng/larva	M	1.419 ± 0.096 16%	0.279 ± 0.016 8%	19.7 ± 4.0	12
	F	1.867 ± 0.180 21%	0.306 ± 0.027 15%	16.5 ± 1.5	12
Hour 24 – 156 ng/larva	M	1.597 ± 0.171 30%	0.290 ± 0.039 21%	18.2 ± 19.0	48
	F	2.135 ± 0.235 39%	0.330 ± 0.031 24%	15.6 ± 2.3	48
Hour 24 – 313 ng/larva	M	1.646 ± 0.163 34%	0.279 ± 0.049 17%	17.1 ± 2.8	48
	F	1.866 ± 0.220 21%	0.283 ± 0.033 6%	15.2 ± 2.3	48
Hour 48 – 156 ng/larva	M	1.713 ± 0.110 40%	0.323 ± 0.038 35%	18.8 ± 1.8	48
	F	2.119 ± 0.216 37.5%	0.341 ± 0.056 28%	16.1 ± 2.1	48
Hour 48 – 313 ng/larva	M	1.682 ± 0.164 37%	0.314 ± 0.065 31%	18.5 ± 2.4	48
	F	2.029 ± 0.284 31%	0.332 ± 0.048 24%	16.3 ± 4.0	48

- In the second experimental set were tested decreasing treatment doses (469 – 313 -156 – 78 ng) with time elapsing in the fifth instar (0 – 24 –48 – 72 hr)
- The interaction Time of treatment x Doses x Sex was not significant, for the cocoon weight or for the cocoon shell, while it was for the Time of treatment x Doses.

TAB. 2

Treatment	Sex	Cocoon weight (g)	Shell weight (g)	Silk percentage (%)	Delay in spinning in comparison to control (hrs)
Control (Et-OH)	M	1.284 ± 0.214	0.221 ± 0.036	17.3 ± 1.2	-
	F	1.632 ± 0.091	0.281 ± 0.020	17.2 ± 0.1	-
Hour 0 – 469 ng/larva	M	1.850 ± 0.129 44%	0.302 ± 0.034 37%	16.6 ± 2.0	48
	F	2.144 ± 0.418 31%	0.315 ± 0.046 12%	14.9 ± 1.8	48
Hour 24 – 313 ng/larva	M	1.769 ± 0.290 38%	0.296 ± 0.042 33%	16.9 ± 1.8	48
	F	1.973 ± 0.302 21%	0.306 ± 0.041 9%	15.4 ± 1.2	48
Hour 48 – 156 ng/larva	M	1.792 ± 0.227 40%	0.299± 0.038 35%	16.8 ± 2.3	48
	F	2.208 ± 0.327 35%	0.347 ± 0.054 23%	15.7 ± 1.3	48
Hour 72 – 78 ng/larva	M	1.320 ± 0.229 3%	0.253 ± 0.059 14%	18.4 ± 4.2	6
	F	1.992 ± 0.237 22%	0.305 ± 0.054 9%	15.4 ± 2.9	48

•Table 3 shows the value for the second experiment, where we cumulated data obtained for male and female cocoons together

•In the second experimental set were tested decreasing treatment doses (469 – 313 - 156 – 78 ng) with time elapsing in the fifth instar (0 – 24 –48 –72 hr)

• Male and female data were analysed together.

TAB. 3

Treatment	Cocoon weight (g)	Shell weight (g)	Silk percentage (%)
Control (Et-OH)	1.522 e	0.265 dc	17.4 a
Hour 0 – 469 ng/larva	1.976 a	0.307 ab	15.5 a
Hour 24 – 313 ng/larva	1.834 ab	0.300 ab	16.4 a
Hour 48 – 156 ng/larva	1.972 a	0.281abcd	14.2 a
Hour 72 – 78 ng/larva	1.645 edcb	0.278 dc	16.9 a

Value followed by different letters significantly differ at $P<0.01$

CONCLUSIONS

• It is possible to apply methoprene treatment even in the case of diet rearing.

• The increase of the silk production is generally remarkable.

• However the doses should be better analysed in order to find a good balance between the increase in the shell weight and in the pupal weight.

• Our results are quite different from those of Miranda et al. (2002), where the quantity of applied methoprene was rather low in comparison with ours. This could also depend on the purity of the product we used. On the other part, ours agree with Mamatha et al. (2006), which used 1μg/larva.

• Fast degradation of the chemical due to the insect metabolism was proven by the fact, that very high doses at the beginning of the last instar were not harmful to the larvae and they did not cause very big delay in spinning.

• Treating larvae at the beginning of the last instar is very convenient from a practical point of view, as they have to be transferred to the new diet after the last moult, so that it is necessary to handle them, and that is the perfect time to topically treat them, in order to not repeat the operation more than once.

• In case of rearing under germfree conditions (Sumida et al., 2007), larvae should be allowed to eat in the dark until the end of the fifth instar, so that treating them at the beginning of the instar appears to be very opportune.

• In addition, we noticed that there was a direct relationship between the doses, the moment of treatment and the incapability of some larvae to transform into adults, even though they spun their cocoons. Therefore, anticipating the treatment is safer, as larvae have longer time in order to metabolize the chemical into their bodies.

To sum up, we think that Methoprene treatment of diet-reared larvae could be convenient especially in case of production of particular importance (recombinant proteins) where the high added value of the final harvest justifies the cost of the chemical and of the treatment.

In this specific case ethanol treatment was implemented, but even water spraying on just-moulted larvae could be studied in order to avoid individual treatments

Thank you for your kind attention