# A MULTIPLE GENERATION LIFE HISTORY STUDY ON REARING A GENETICALLY ALTERED (EGFP) STRAIN OF PINK BOLLWORM (LEPIDOPTERA: GELECHIIDAE)

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## Abstract

The genetic transformation of a strain of PBW (APHIS strain) did not cause major changes in time sequenced life history parameters of the transgenic strain (EGFP strain of PBW) when compared to its non-transformed ancestors, the APHIS strain of PBW. However, there was a loss of fitness evidenced in the female's ability to produce eggs. Egg survivability was also reduced in the EGFP strain. This loss of fitness under simulated mass rearing conditions is most likely due to the debilitating effects of extensive handling of the larvae and the disruption of the larval feeding cycle rather than effects associated with the transgeneic change. The maintenance protocol for selection of a homozygous strain of the EFGP colony required larval extraction from the diet in the 3rd and /or 4th instar, examination of the larvae under a microscope for gene expression then larval return to the diet. This involved considerable handling and was a major interruption of the normal larval feeding cycle.

## Introduction

The San Joaquin Valley of California remains the last cotton growing area in the Southwest that is not generally infested with pink bollworm, *Pectinophora gossiepiella* (Saunders). Prevention of its establishment in this valley is attributed primarily to the ongoing sterile-insect program (SIT) established in 1968 jointly by the USDA, California Department of Food and Agriculture, and the California cotton growers (Miller et al.1984), (Staten et al 1992). All sterile pink bollworm (PBW) used in this project are mass-reared in a facility located in Phoenix, AZ.

Miller et al. (1994) reported the advantages a genetically marked mass reared strain of PBW would provide to SIT program managers in their daily insect deployment strategies. Though, the PBW-SIT program has implemented the use of genetic markers through mutants produced by classical genetic techniques, homozygosity was not maintained in these genetically marked strains of PBW when exposed to the highly selective processes of mass rearing.

The development and implementation of a mass reared strain of PBW with a genetically engineered marker demonstrates the ability to rear transgenic insects and sets the stage for future development and deployment of a genetically transformed mass-reared strain of PBW containing a dominant conditional lethal trait. This type of technology would significantly alter the strategies used in traditional SIT programs. Klassen (et al. 1970) and more recently, Fryxell and Miller (1995) report on the merits of implementing this type of strategy to control pest populations.

We report here on rearing a strain of PBW that was genetically altered to expresses Enhanced Green Fluorescent Protein (EGFP), (Peloquin et al. 2000). The EGFP gene was constructed using a model Green Fluorescent Protein originally derived from the jellyfish *Aequora victoria.* The transgenic PBW with the EGFP gene fluoresces with an emission peak of 510 nm under excitation illumination of 460 to 500 nm, (Peloquin et al. 2000). The objective of our study was to determine the fitness of a transgenic strain of PBW when reared under laboratory conditions similar to those employed in a mass rearing facility.

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## Materials and Methods

### Colony Maintenance

The following protocols were used to rear 20 generations of the EGFP transgenic strain of PBW. The methodologies used here are similar to those recently developed and employed in the Pink Bollworm Mass Rearing Facility (PBWMRF).

The transgenic PBW used in this study were derived from original stock taken from the (PBWMRF) and genetically altered at the University of California at Riverside by John Peloquin. Fourth generation PBW pupae (267 individuals) from transgenic line 35 were received at our Animal Plant Health Inspection Service (APHIS) quarantine rearing facility in March of 1999. Family 35 was started from a single EGFP male that was outcrossed to a mass reared APJHIS females for 4 generations before introgression through mass mating of all EGFP progeny at our location was begun. Each pupa was examined with a stereo microscope to determine its sex. The pupae were then placed in a standard 5.7- liter PBWMRF oviposition cage. The cages were placed in an oviposition room / chamber maintained at 25.6°C, 80 ± 5% RH. Pupal eclosion generally occurred 3-4 days after setup. Oviposition substrate was placed on the screened lid of the caged adults and eggs harvested daily for 10 days from each cage. The donut shaped oviposition substrate consisted of (in order of placement on the cage): an 18 X 16 mesh fiberglass screen, an embossed paper towel (American Tissue Chicago, IL), and a silicon weight to insure complete contact with the screen top of the cage. Placed in the center of the donut shaped oviposition substrate was a 5 x 6.4 cm sanitary napkin saturated with a 6% sucrose solution containing 0.1% methyl paraben to prevent microbial growth. The sucrose served as the adult stage food source and the pads were changed daily. Harvested eggs were incubated for 2 d in a 17.8 cubic foot growth chamber (Precision Instruments Growth Chamber model 818, Winchester, VA) held at 26.7°C, 45 % RH, 24-h dark cycle.

After incubation, egg pads were placed, egg side facing up, in the bottom of a 13.4 x 13.4-cm thermoformed plastic tray made of 25-mil PVC plastic. PBW diet (≈ 240 g) was placed over the eggs. Immediately after diet placement the top of the tray was covered and sealed with a paper material (part #35563 Oliver Products, Grand Rapids, MI). The paper lid was sealed to the tray using an iron, which activated a heat sensitive adhesive imbedded on the paper material. After sealing, the lids on each tray were perforated using a metal roller fitted with 24-0.6cm long x 0.31-cm diameter pins. Trays with the inoculated diet were placed in a growth chamber (Precision Scientific model 818, Winchester VA) maintained at 29°C, 50 ±10% RH, 24-h dark cycle for 10 days. On day 11, trays were transferred to a second Precision Growth Chamber maintained at 29°C, 50 ±10% RH, 24-h light cycle at 4 −6 lux. After 2 days the containers were removed from the chamber, the paper lid removed and all larvae separated from the diet. Individual larva were then examined under a stereo microscope (Ziess model SV6) equipped with a light supply (Opti-Quip model 1600) fitted with a 100 watt mercury bulb with illumination passed through a 480 nm wave length filter. GFP gene expression causes transgenic PBW larvae to fluorescence strongly green in the second through fourth instar larval stage of development when viewed under 460-500 nm illumination. Therefore, each larva was screened, and those with positive expression of the gene were placed in a new tray with fresh diet and sealed in the same manner as described above. Larvae not expressing the gene were disposed of by freezing at -20°°C for 24-h.The larval screening was timed so that 90 % of the larval population was in the third instar when examined. EGFP gene expression is most prominent in this stage of growth. The purpose of screening individual larvae was to establish a homozygous EGFP strain.

Trays containing the EGFP positive larvae were positioned vertically in a 3.02 liter Rubber maid container and covered with a plastic top and returned to the growth chamber for an additional 6 days. The bottom of the Rubber maid container contained a piece of honeycomb 8.9 X 15.3 cm in size placed over a piece of cardboard of the same dimension. The honeycomb/cardboard substrate served as a pupation site for the larvae as they "cutout" from the tray. In the field, PBW feeds to larval maturity, then chews its way out of a cotton boll and drops to the ground where it pupates in soil cracks or plant litter at the base of the plants. Insects reared on artificial diet placed in a rearing container exhibit the same behavior.

Pupae were harvested by separating the honeycomb sheets from the cardboard backing. This allowed the pupae to fall from the honeycomb into our collecting tray. The pupae were then counted, their sex determined then placed in a standard 5.7- liter PBWMRF oviposition cage. Oviposition cages (2-4) were set up each generation. The number of pupae per cage per generation ranged from 12 –973 over the course of the study.

Data collected from each generation was compared to a historical database maintained in the PBWMRF and included: 1) Female fecundity 2) Mean percentage implanted eggs producing late instar larvae 3) Mean egg to adult stage development time and 4) Mean pupae weight. The purpose of the comparisons was to determine if genetic alteration caused any biotic dissimilarity between the EGFP strain and its ancestors who had not been genetically transformed.

**Life History Comparisons of EGFP Strain and APHIS Strain PBW**

Comparing our EGFP colony to a historical database maintained by the PBWMRF provided us with a gross indicator of how the EGFP strain performed when compared to its mass reared non -transformed ancestors. However, to obtain a more precise comparison we conducted a study comparing several life history parameters of the two strains using identical insect densities in rearing containers, identical rearing equipment and diets. Due to requirements issued in the APHIS permit to rear the EGFP strain we were unable to rear the control insects in the exact same facilities. However, with the use of electronic environmental controls fitted to environmental chambers we were able to maintain near identical environmental conditions in the rearing areas for the two PBW strains.

For each replicate 50 female pupae and 30 male pupae of the EGFP and APHIS strains were placed individually in plastic vials (10 by 75-mm). All adults that eclosed were sexed again, and placed in 473-ml paper cages fitted with screen tops in-groups of 5-females and 3- males. Five cages were set up for each treatment. Moths in each cage were fed a 6 % sucrose solution in 0.5-dram (0.89 g) vials inserted in the screen tops. Eggs were harvested daily from each cage for 10 consecutive days using the same oviposition substrate used on the standard 5.7- liter PBWMRF oviposition cage but cut into 5.1 by 3.8 cm wedges to fit the top of the smaller cages. All collected eggs were counted and recorded. Eggs collected on days 4 and 5 were implanted on diet and those collected on days 1 through 3 and 7 through 10 were disposed of by freezing. Eggs collected on day 6 were used to determine egg hatch. They were counted and held for 3 days at 29°C, 50 RH and 24 dark cycle. On day 3 they were examined and a hatch rate determined. Methods used for larval rearing and pupae harvest were the same as those described in colony maintenance. Harvested pupae from each strain were placed individually in plastic vials (10 by 75-mm).

The pupae in vials were examined daily and eclosed adults from each strain placed separately in groups of 10 in 40-dram plastic cages with screen lids and one screened side. Each cage was fitted with a feeder vial (0.5-dram (0.89 g)) filled with a 6% sucrose solution inserted in the screen top of the cage. The cages were checked daily for 20 days to determine adult survival rates.

## Results and Discussion

**Colony Maintenance**

As indicated in table 1 the EGFP strain showed reduced fitness when to its non- transformed APHIS strain ancestors maintained in the PBWMRF. In the critical arenas of insect mass rearing, female fecundity and egg to late instar larval survival, the mass reared APHIS strain produced 19.8 % more eggs per female and had 26% higher egg to larval survivability than did the EGFP strain. EGFP pupa weight was similar to the average weight maintained in the mass reared APHIS colony. In fact it was slightly larger (7.3%). It's larger size is most likely attributed to lower egg and larvae densities in the 240 grams of diet maintained in each rearing container. Life cycle development time (egg to adult) was also very similar between the two strains with the EGFP strain averaging 26.6 days (mean for 20 generations) and the APHIS mass reared colony averaging 26 days.

As noted in table1 it required 14 generations of selection in our facility to develop a homozygous EGFP strain (99-100 % EGFP positives). However, as early as the 5th generation in our facility we had reached 96% EGFP positives, but regressed to 72% EGFP positives over the next 5 generations. This initial loss of homozygosity can most likely be explained, upon intensive Quality Assurance investigations, by the inadvertent selection of false positive larvae. The visual selection process was hindered by auto-fluorescence of ingested diet in the larval gut tract and further complicated by the gradual degradation of the mercury light source. An average of 3866 larvae were individually screened each generation, therefore a margin of error as small as 0.5 of 1% could conceivably allow ca. 19 false positive insects (homozygous mass reared type insects) to be integrated into the EGFP breeding stock cages.

**Life History Comparisons of EGFP Strain and APHIS Strain PBW**

EGFP strain PBW females had significantly lower fecundity rates than female PBW from the APHIS strain. Egg hatch was also significantly lower in the EGFP when compared to the APHIS strain (Table 2). However, mean pupal weights and eclosion rates were not significantly different between the two strains.

Life cycle development (egg to adult) time between the two strains did not differ significantly with the EGFP strain developing in 25.7 days compared to the 25.6 days for the APHIS strain (table 3). Results of a adult longevity study also indicated, no significant difference between the two strains with 50.8% of caged adults alive 20 days after eclosion in the EGFP strain compared to 56.9% of the adults alive in the APHIS strain (table 3).

Under the conditions of our studies the genetic transformation of the APHIS strain did not cause major deviations between time sequenced life history parameters of the transgenic EGFP strain of PBW and its non-transformed ancestors, the APHIS strain of PBW. However, there was a loss of fitness evidenced in the female's ability to produce eggs. Egg survivability was also reduced in the EGFP strain. This loss of fitness in the EGFP strain under simulated mass rearing conditions is most likely due to the debilitating effects of extensive handling of the larvae and the disruption of the larval feeding cycle rather than effects caused by the gene transformation.. The maintenance protocol for selection of a homozygous strain of the EFGP colony required larval extraction from the diet, examination of the larvae under a microscope for gene expression, then larval return to the diet which interrupted the normal larval feeding cycle. It is interesting to note that this procedure did not appear to impact larval weight. Further testing is currently in progress to determine the definitive impact this procedure has on the insect.

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Table 1. Comparison of life history parameters of a transgenic (EGFP) strain of PBW and the current mass reared stain of PBW maintained in the APHIS, Pink Bollworm Mass Rearing Facility.

| Gen. # | Mean eggs per female | Total larvae exam. | % eggs on diet produc. larvae | % total larvae with EGFP gene | Mean pupal weight | Mean days egg to adult |
|---|---|---|---|---|---|---|
| 1 | 160.0 | 28 | 0.78 | 53.6 | - | - |
| 2 | 76.0 | 446 | 2.56 | 79.8 | 17.35 | 29.0 |
| 3 | 148.0 | 4157 | 24.21 | 73.4 | 20.90 | 26.5 |
| 4 | 137.0 | 5034 | 17.80 | 78.9 | 17.40 | 27.5 |
| 5 | 70.0 | 4570 | 13.22 | 96.8 | 17.88 | 28.5 |
| 6 | 84.0 | 2230 | 8.81 | 96.2 | 19.03 | 26.3 |
| 7 | 150.0 | 7095 | 32.67 | 96.0 | 15.60 | 27.0 |
| 8 | 140.0 | 4000 | 11.97 | 91.7 | 14.27 | 26.0 |
| 9 | 168.0 | 6691 | 17.79 | 91.8 | 15.60 | 27.3 |
| 10 | 103.0 | 6388 | 31.62 | 72.0 | 14.91 | 25.3 |
| 11 | 151.1 | 5950 | 17.17 | 86.6 | 15.31 | 26.7 |
| 12 | 159.6 | 3798 | 41.39 | 88.4 | 15.36 | 26.0 |
| 13 | 204.9 | 3154 | 17.57 | 98.3 | 15.78 | 26.5 |
| 14 | 174.0 | 2272 | 18.11 | 99.7 | 14.16 | 27.0 |
| 15 | 166.2 | 2563 | 30.36 | 99.6 | 16.20 | 27.0 |
| 16 | 182.2 | 4525 | 27.14 | 100.0 | 15.14 | 26.0 |
| 17 | 227.0 | 3174 | 21.91 | 99.8 | 15.33 | 26.0 |
| 18 | 152.4 | 3326 | 27.39 | 100.0 | 16.99 | 26.0 |
| 19 | 150.8 | 5044 | 26.20 | 100.0 | 16.41 | 25.5 |
| 20 | 161.7 | 2865 | 31.50 | 100.0 | 16.32 | 25.5 |
| EGFP* | 148.30 | 3866 | 21.01 | | 16.32 | 26.61 |
| APHIS** | 177.61 | | 47.10 | | 15.13 | 26.0 |

* Mean of 20 generations of EGFP production

**Data reported here obtained from the PBWMRF, 1999 produc1tion season. Data represents a season long daily average for a single oviposition cage.

Table 2. Comparison of fecundity, egg hatch, pupae weight and pupae eclosion in EGFP and APHIS strains of pink bollworm.

| Strain | Mean # eggs/female | Mean % egg hatch | Mean pupae weight | Mean % pupae eclosion |
|---|---|---|---|---|
| EGFP | 109.0± 14.32 | 60.9± 3.10 | 20.6± 1.56 | 96.6± 3.52 |
| APHIS | 193.1± 44.03** | 93.7± 4.82** | 20.8± 1.46 | 95.7 ± 2.48 |

Means in columns followed by ** are highly significantly different (t-test = P<0.01).

Table 3. Comparison of length of life cycle (egg to adult) and adult longevity (% alive after 20 days) in EGFP and APHIS strains of pink bollworm.

| Strain | Mean days to develop From egg to adult. | % adults alive 20 days after eclosion |
|---|---|---|
| EGFP | 25.7 ± 0.89 | 50.8 ± 19.24 |
| APHIS | 25.6 ± 2.83 | 56.9 ± 8.49 |

Means in columns followed by ** are highly significantly different (t-test = P<0.01).