

# No evidence for change in oviposition behaviour of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) after widespread adoption of transgenic insecticidal cotton

M.P. Zalucki<sup>1\*</sup>, J.P. Cunningham<sup>1</sup>, S. Downes<sup>2</sup>, P. Ward<sup>1</sup>,  
C. Lange<sup>1</sup>, M. Meissle<sup>3</sup>, N.A. Schellhorn<sup>3</sup> and J.M. Zalucki<sup>4</sup>

<sup>1</sup>School of Biological Sciences, The University of Queensland, St Lucia, Brisbane, 4072, Australia; <sup>2</sup>CSIRO Ecosystem Sciences, Australian Cotton Research Institute, Narrabri, 2390, NSW; <sup>3</sup>CSIRO Ecosystem Sciences, Brisbane, 4001, Australia; <sup>4</sup>School of Environment, Griffith University, Nathan, Brisbane, 4111, Australia

## Abstract

Cotton growing landscapes in Australia have been dominated by dual-toxin transgenic Bt varieties since 2004. The cotton crop has thus effectively become a sink for the main target pest, *Helicoverpa armigera*. Theory predicts that there should be strong selection on female moths to avoid laying on such plants. We assessed oviposition, collected from two cotton-growing regions, by female moths when given a choice of tobacco, cotton and cabbage. Earlier work in the 1980s and 1990s on populations from the same geographic locations indicated these hosts were on average ranked as high, mid and low preference plants, respectively, and that host rankings had a heritable component. In the present study, we found no change in the relative ranking of hosts by females, with most eggs being laid on tobacco, then cotton and least on cabbage. As in earlier work, some females laid most eggs on cotton and aspects of oviposition behaviour had a heritable component. Certainly, cotton is not avoided as a host, and the implications of these findings for managing resistance to Bt cotton are discussed.

**Keywords:** source-sink, host preference, Bt cotton, *Helicoverpa armigera*, natural selection

(Accepted 8 December 2011; First published online 7 February 2012)

## Introduction

Agriculture, in general, and the stability of management of insect pests, in particular, are subject to the vagaries of natural selection and evolution (Hendry *et al.*, 2011; Thrall *et al.*, 2011).

The evolution of pesticide resistance has been a problem for management of insect populations ever since these were first used. It was expected that similar problems would bedevil plants that were engineered to express novel insecticidal toxins. Although recent studies suggest that some populations of at least five crop pests have evolved a degree of resistance to insecticidal toxins engineered into plants (Tabashnik *et al.*, 2005, 2009; Bagla, 2010; Carrière *et al.*, 2010; Tabashnik & Carrière, 2010; Zhang *et al.*, 2011), only three show field failures due to resistance: *Busseola fusca* to Bt corn producing

---

\*Author for correspondence  
Fax: +61 7 33651655  
E-mail: m.zalucki@uq.edu.au

Cry1Ab in South Africa, (Van Rensburg, 2007), *Spodoptera frugiperda* to Bt corn producing Cry1F in Puerto Rico (Matten *et al.*, 2008) and *Pectinophora gossypiella* to Bt cotton producing Cry1Ac in India (Tabashnik & Carriere, 2010). Thus, despite their deployment throughout the world, in some parts since the mid-1990s, resistance to transgenic insecticidal crops has not developed within the 5–7 years predicted by skeptics.

Transgenic cottons, expressing the *Bacillus thuringiensis* (Bt) toxin genes, target the pest noctuid *Helicoverpa armigera* (Hübner) (Noctuidae). Bt cotton has been widely adopted in Australia, where it currently comprises nearly 90% of all cotton crops (Zalucki *et al.*, 2009), as it does in other parts of the world (Wu *et al.*, 2008). This landscape level change began in the late 1990s with the introduction of one-gene cotton transformations (INGARD), followed by two-gene Bollgard II in 2004 (see discussion). At a landscape level, cotton has effectively become a sink crop, as virtually no offspring survive from eggs laid in the crop (Rochester *et al.*, 2002), except in those cases where refuges of non-Bt cultivars or other crop hosts are deliberately planted across the landscape with cotton. From time to time, in some fields, *H. armigera* develop to adults on Bt cotton crops, although this survival does not appear to be due to physiological resistance to Bt *per se*, and most likely reflects poor expression (Lu, 2011). The most commonly used deliberately planted non-Bt refuge in Australia is pigeon pea.

For transgenic crops expressing Bt toxins at high levels for target pests, simple population genetic models (Kennedy *et al.*, 1987; Tabashnik, 1994; Gould, 1998) and more complex models (Peck *et al.*, 1999; Storer *et al.*, 2003; Sisterson *et al.*, 2005; Shelton *et al.*, 2008; Jongsma *et al.*, 2010) predict that refuges would substantially decrease the rate at which populations evolve physiological resistance to crops. The prediction is based on relative differences in population densities from different sources, a sink crop expressing toxin at a high enough level to kill any individuals that are heterozygous for resistance genes, extensive movement of moths in the landscape with random mating resulting in very few crosses between rare homozygous resistant individuals, should these arise from Bt crops. These models, by and large, do not directly incorporate information on oviposition behavior and host preferences of the target insects. Rather, they assume that adults do not shift their oviposition behaviour in response to the introduction of Bt crops into the landscape, and instead oviposit randomly with respect to the target crop, or maintain their previous preferences. When shifting host preferences are incorporated into a model (see Jongsma *et al.*, 2010) the resulting prediction is a preference shift in favour of alternative hosts. If moths were to avoid laying on sink crops, then physiological resistance should be slower to evolve, as a smaller fraction of the population will be subject to selection (Jongsma *et al.*, 2010). Concurrently, although growers of Bt cotton would enjoy a lower pest pressure, there should be a reduced sink effect of Bt cotton and, hence, less impact on population densities experienced throughout the landscape.

Host plant selection in Lepidoptera is generally undertaken by the ovipositing adults (Singer, 1984). Females may display a preference hierarchy among potential plant host species (e.g. Nylin & Janz, 1993; Midega *et al.*, 2011), which is thought to arise from the balance between attractants and deterrents (e.g. Renwick & Chew, 1994) and a number of proximate environmental effects on behaviour (e.g. learning: Cunningham *et al.*, 2001). Offspring can display the same oviposition preferences as their mothers, but this is not always the case

(Thompson, 1988; Nylin & Janz, 1996; Mayhew, 1997; Khan *et al.*, 2006). Host preferences may be influenced by a number of evolutionary factors (West & Cunningham, 2002), and it is generally argued that the evolution of more specialist oviposition behaviour would be more rapid in species that are host generalists, and potentially flip-flop between the two extremes (Janz & Nylin, 2008), if host selection is labile and populations become fragmented. *Helicoverpa armigera* (Hübner) (Noctuidae) is an extreme host generalist (Zalucki *et al.*, 1986, 1994). There is experimental evidence for genetic variation in host preference among major lepidopteran cotton pests, including *H. armigera* (Schneider & Roush, 1986; Jallow & Zalucki, 1996; Jallow *et al.*, 2004; Wang *et al.*, 2004), providing the opportunity or raw material for evolution. Given this variation in oviposition behaviour and the assumption of a strong selection against laying on cotton, might we not expect a change in oviposition behaviour as a result of Bt cotton dominating the landscape?

One of the biggest problems with host-selection theory, explanations of why insects prefer to lay their eggs on particular plant species, is that empirical studies frequently fail to match with up with predictions (Mayhew, 2001); in other words, insects appear to make bad choices. This lack of correlation, between insect choice and host suitability, may arise through constraints to the evolution of host-selection behaviour. Ecological (e.g. Walter & Benfield, 1994) or physiological (see Cunningham, 2012) factors may prevent species, particularly generalists, from adapting to changes in host abundance or suitability within their environment. Given this theory of constraints, and the extensive host range of *H. armigera*, might we expect *no* change in oviposition behaviour as a result of Bt cotton dominating the landscape?

Our aim here is to utilize a 'man-made' change in host suitability for a highly polyphagous insect that occurs on a landscape level, and has existed over many generations, to investigate the extent to which change in host suitability can drive evolutionary change. Specifically, we are asking the question: Has there been a change in *H. armigera* egg distribution across different host plants, in populations from environments with widespread Bt cotton? To address this query, we used standard laboratory bioassays to test the behavior of first generation females (G1) from two major cotton-growing locations in Australia and, for a subset of G1 females, two subsequent further generations (using a slightly different but standard cage test methodology) to ascertain if females were consistent in their oviposition choices.

In order to evaluate whether there has been a change in *H. armigera* egg distribution, current evidence must be compared with data taken prior to the introduction of Bt cotton. Firempong & Zalucki (1990b) tested a population from Narrabri for host oviposition preference among flowering plants of sunflower (Hysun 32), tobacco (Q46), maize (sweet corn), soybean (Davis), lucerne (Hunter River), cotton (Deltapine 61), linseed (unknown), pigweed and cabbage (Earliball). Insects were collected on two occasions as third–fifth instars from (non-Bt) cotton in February to March in 1985 and reared to adults. The results were consistent in both separate trials conducted with each cohort. Tests were conducted in a large hexagonal cage (1.6 m at the sides, 2.2 m in height and 4.3 m in diameter maintained within a glasshouse) with *ca.* 15 females in which glasshouse-grown potted plants were randomly repositioned throughout the period. Female moths showed a strong preference for ovipositing on tobacco, which received nearly 60% of eggs over five days consistently

in two trials, followed by sunflower, lucerne, maize, cotton, soybean, linseed, pigweed and cabbage.

In an experiment designed to examine variation in host plant acceptance, Jallow & Zalucki (1996) tested the post-alighting oviposition preference of individual moths from Narrabri collected from cotton, sorghum and sunflower crops. Plant material was presented to females as leaf discs cut from tobacco, lucerne, cowpea, maize, sorghum, and cotton (DP90 & HG660). Moths were tested using a tethered (stick) method (Jallow & Zalucki, 1995). Insects were collected as larvae and reared for 1–2 generations on a standard artificial diet in 1992 before being tested. Of the 20 tested moths from Narrabri, most females ranked maize, sorghum and tobacco as the most preferred. Some 45% of females ranked cotton (DP90 and HG660) as either first ( $n=4$ ), second ( $n=3$ ) or third ( $n=2$ ) most preferred. Jallow & Zalucki (1995) found that the stick test was a good predictor of oviposition choice by free-lying caged moths. This earlier work established a preference hierarchy at the population level (Firempong & Zalucki, 1990b) and demonstrated heritable differences among females in how hosts are ranked (Jallow & Zalucki, 1995, 1996). In our current experiments, we chose plants from the previously highly preferred group (tobacco), mid-ranked (obviously cotton) and low ranked hosts (cabbage). This in part gets around the problem of differences between cultivars across experiments. The changes are either going to be dramatic or not measurable.

## Materials & methods

### Collection and rearing of field insect material

*Helicoverpa armigera* eggs and larvae were collected from cotton fields and refuge crops (mainly pigeon pea) on three neighbouring properties (Genelg, Rosewood, Kilmarnock) in the Upper Namoi (Boggabri some 50 km from Narrabri, 30°19'S, 149°46'E) (mid-December 2009) and Bt cotton fields and refuge crops (pigeon pea and sorghum) on the Darling Downs (early February 2010). In each case, animals were sourced from multiple (6–18) widely separated fields, and immature stages were reared individually on a chickpea-based artificial diet (based on Teakle, 1991). Collection from widely separated fields means we are less likely to have collected closely related individuals. Male and female pupae were separated and each sex was housed in bulk in vermiculite. Temperature conditions were manipulated to ensure synchronous adult emergence.

### Host plants

We used tobacco (*Nicotiana tabacum*, SR1), cotton (*Gossypium hirsutum*, Sicot 71) and head cabbage (*Brassica oleracea*, var Warrior) to test oviposition preference. From previous studies, these plant species represent high, mid and low preference plants (Firempong & Zalucki, 1990b). Plants were grown in 15 cm diameter pots in standard potting mix, watered and fertilized as required.

### Oviposition choice experiments

Upon emergence, adults were allowed to mate with conspecifics from the same location and host source and the females were subsequently used in oviposition choice trials. Two types of choice tests, hereafter referred to as small-cage tests and large-cage tests, were conducted. In all experiments,

adults were given access to cotton wicks soaked in 10% sugar solution.

### G1 tests

Mated *H. armigera* females, from field collected (G1) larvae were used in small-cage and, if they did not lay, in large-cage tests. In small-cage tests, females were released into 45 cm<sup>3</sup> mesh cages, together with whole leaves of tobacco, cotton and cabbage, of approximately the same size, and with stems embedded in floral foam (*Oasis*). In large-cage tests, whole potted plants were used in choice tests for groups of moths tested in mesh cages that were 1 m<sup>3</sup>. For large-cage tests, cotton and tobacco of similar size (50–75 cm) were used. These plants had buds and flowers, and cabbage (non-flowering) was raised to the same height as the other two hosts to prevent height effects (Firempong & Zalucki, 1990a).

G1 moths from the Narrabri population were tested in small cages over two nights. The host plant source from which larvae were collected for this population (cotton or pigeon pea) was noted in individual choice trials, and moths were mated with individuals from the same source crop. If individual moths laid fewer than ten eggs, they were grouped by host source and used in a large-cage test, conducted over two nights.

For further experiments, we selected a subset of G1 females from Narrabri that laid more than ten eggs in small-cage trials that could be categorized as either 'specialist' females ( $n=2$ , one from each source), which placed a majority of eggs on a single host, or 'generalist' females ( $n=2$ , again one from each source), which distributed eggs more evenly amongst hosts (see results). The offspring of each of these females were reared on artificial diet and mated with sibs. These F1 females were then tested for oviposition preference using large-cage tests in sub-groups over eight nights. As no plants with open flowers were available for this test, large pre-flowering plants were used (50–60 cm). Both tobacco and cotton had either buds or squares, and such plants were used in this and the subsequent experiment (below). Cabbage plants were non-flowering but were raised to the same height as the other plants. The distribution of eggs among host plants was counted daily, and plants were randomised amongst cages daily, and we varied the locations within the cage each day. There were between 15 and 28 females in each cage for F1 and subsequent F2 tests.

On the final day of counting, a sample of eggs from each of the F1 groups displaying contrasting oviposition hierarchies was collected. The eggs were reared on artificial diet and subsequently re-tested as F2 adults, following the same (large-cage) test procedures outlined above for F1 adults.

As survival of larvae to the pupal stage was very low for the Darling Downs populations, we ran choice tests on this sample using small-cage tests only and grouped adults from all sources for the initial mating and subsequent testing of individual females.

### Statistical analysis

Factors such as source of moths and host plants available that can affect egg-counts were tested for statistical significance using a regression approach that recognizes the possibility of correlations among egg counts (amongst plant parts or plants within cages, amongst days, amongst moths from the same source, etc.). Such correlations are usually ignored in these types of assays, but this effectively ignores the fact that if

a moth lays on one plant first this may affect subsequent laying and so forth.

*Estimation of regression coefficients*

Predictors for the mean egg count were plant type (tobacco, cotton or cabbage) and the source of the female moth (cotton vs pigeon pea, or Narrabri vs Darling Downs).

A regression model for the  $i^{\text{th}}$  egg-count,  $\mu_i$ , is given by:

$$\mu_i = \exp(\beta_0 + \beta_1 \text{Cotton} + \beta_2 \text{Cabbage} + \beta_3 \text{Source} + \beta_4 \text{Cotton} \times \text{Source} + \beta_5 \text{Cabbage} \times \text{Source})$$

The use of an exponential link ensured that the expected egg counts were non-negative; namely, the exponentiation, on the right hand side of the formula above, ensured that the expected egg counts were always non-negative during the iterative process for estimating the regression coefficients. Each estimated regression coefficient ( $\beta_i$ ) was then tested for significance using a *t*-test, formulated by dividing each coefficient by its respective standard error.

*Allowance for correlations among egg-counts*

Each set of three egg counts taken from the same female(s) within the same cage is considered a block of correlated egg counts. Egg counts are considered independent across blocks but not within blocks.

In order that the regression analysis be carried out in a way that allows for correlation within blocks, the ‘generalized estimating equations’ (GEE) method of data analysis was used (Liang & Zeger, 1986). This method allows simultaneous estimation of regression parameters for the mean and an estimation of regression parameters for the covariance. It also engenders no assumptions about the probability distribution of the data.

Denoting the covariance of egg counts *i* and *j* as  $C_{ij}$ , the model for the variance/covariance matrix within a block is:

$$C_{ij} = \begin{cases} \exp(\alpha_1) & \text{if } i = j \\ \exp(\alpha_1) \tanh(\alpha_2) & \text{if } i \neq j, \end{cases}$$

where  $\alpha_1$  and  $\alpha_2$  are parameters to be estimated from the data. Following Prentice (1988) and Prentice & Zhao (1991), information about  $\alpha_1$  and  $\alpha_2$  is extracted from the cross-products of residuals:  $S_{ij} = (y_i - \mu_i)(y_j - \mu_j)$ .

Within each block of three egg counts, there are six such cross-products of residuals (including the diagonals:  $i=j$ ). A variance/covariance for these six observations is estimated as the observed ‘sum-of-squares and products’ matrix among them, taken across all blocks. For further details, see Prentice (1988) and Prentice & Zhao (1991).

We tested for difference in host plant preference between geographic source: Narrabri vs Darling Downs. Within Narrabri, we tested for differences between collection source: ex-refuge crop (pigeon pea) vs Bt cotton.

*Evidence that preference is heritable*

Evidence was sought for heritability of the distribution of egg counts across the three plant types across three generations. Four females that displayed apparent distinct oviposition patterns in G1 were selected and their F1 progeny tested and inbred to form an F2 generation. Three generations of egg counts across the three plants were thus recorded,

producing four large blocks of (potentially) correlated egg counts, which will reflect heritability of preference due to genetic and maternal effects. Each block consisted of 39 sets of three egg counts.

One method to find evidence for heritability is to take moths in pairs and regress their phenotypic difference (the difference in their egg count distribution) against their kinship; a significantly negative relationship would indicate heritability. In order to get some handle on the concept of ‘preference’, each set of three egg counts was first divided by the total egg count for that set, converting it to three frequencies ( $f_k, k=1, 3$ ). This removed variation in fecundity (total egg count) from this particular analysis, placing the moths onto a common ‘preference’ scale. Information about the total egg count was not completely discarded, however, as the total egg count was used as a weight in the subsequent regression analysis (see below). Each resultant set of three frequencies was then contrasted to every other such set as the euclidean distance ‘ $d(ij)$ ’ between the sets:

$$d(ij) = \text{sum}(k = 1, 3) (f(i, k) - f(j, k))^2.$$

The degree of familial relationship was quantified using the ‘coefficient of kinship’ (Lynch & Walsh, 1998). Within blocks, kinships were calculated using the recursive formulas of Kariql (1981) and are summarized as follows:

Familial relationship	Kinship
F1–G1	0.25
F1–F1	0.25
F2–G1	0.25
F2–F1	0.375
F2–F2	0.375

Kinship was assumed to be zero across blocks. The effects of kinship and day separation upon distance were assessed for statistical significance using the ‘glm’ module of the R statistical package. Each distance, being a sum-of-squares, was considered a gamma variate, i.e. a gamma error structure and an inverse link function were specified. Each distance was weighted in these analyses by the smallest of the two total egg counts relevant to that pair (*ij*). Given the multiple comparison being made, we use the 0.01 level of significance.

**Results**

*Oviposition preference of G1 females*

Of the moths sourced from the Narrabri, 11 females’ ex-pigeon pea and 11 ex-cotton were set up in small cages to assess host preference. When females from both host sources from Narrabri are considered together, 77% of their eggs were laid on tobacco, 15% on cotton and 8% on cabbage ( $n=2068$  eggs laid by all females across two nights). Five of seven (71%) ex-pigeon pea females that laid more than ten eggs showed a preference for tobacco, with one female preferring cotton (68% of 40 eggs) and one female preferring cabbage (54% of 13 eggs). Four of five (80%) ex-cotton females that laid more than ten eggs showed a preference for tobacco, and in one case cotton was clearly preferred (71% of 124 eggs laid). No ex-cotton females preferred to oviposit on cabbage.

For the Narrabri population, six females from cotton and four females from pigeon pea laid very few eggs (1–9) over two nights; but, when grouped in large cages with whole

Table 1. Regression parameters and associated *t*-statistics, for predictors of the mean egg count and for predictors of the covariance of egg counts. Results are presented separately according to whether the 'source' of the female moth refers to its host plant (pigeon pea vs. cotton) or to its geographical location (Darling Downs vs. Narrabri).

Predictor	Source: Host		Source: Location	
	Estimate	<i>t</i>	Estimate	<i>t</i>
<i>Parameters of the mean egg-count</i>				
Intercept	4.746	68.454	4.405	63.597
Cotton	-1.499	-5.280	-1.428	-5.343
Cabbage	-1.688	-4.861	-1.831	-4.489
Source	-0.036	-0.413	0.485	6.249
Cotton × Source	-0.117	-0.311	-0.210	-0.664
Cabbage × Source	-0.808	-1.142	-0.450	-0.860
<i>Parameters of the correlation among egg-counts within a block</i>				
Alpha (1)	6.101	4.553	5.550	2.911
Alpha (2)	0.520	1.098	0.448	0.993

plants, these females ex-cotton and ex-pigeon pea laid 124 and 158 eggs, respectively. The distribution of eggs in these cages for ex-cotton females was 29% on tobacco and the remainder on cotton, while egg distribution by ex-pigeon pea females was 58% on tobacco, 35% on cotton and 7% on cabbage.

Similarly, the eight G1 females ex-Darling Downs tested in small cages laid 989 eggs over four nights with 71% on tobacco, 17% on cotton and 11% on cabbage. One female laid a higher proportion on cotton (51%), and two laid a substantial proportion on cabbage (36% and 37%), with tobacco in these cases having similar egg distributions (44–40%).

The variance of egg counts (as measured by  $\alpha_1$ ) was significantly different from zero, as indicated by *t*-tests ( $t=2.911$ ,  $P<0.005$  for Narrabri vs. Darling Downs populations, and  $t=4.553$ ,  $P<0.001$  for pigeon pea vs. cotton). Alpha two estimates, whether there is any correlation between egg counts amongst the three possible hosts within a cage, and these were not significant (i.e. there was no significant correlation; table 1).

Although there was a significant difference in mean egg counts between the tested Narrabri and Darling Downs G1 females (table 1), this difference did not depend on host (i.e. the source by host interaction was not significant). Narrabri G1 females laid more eggs than Darling Downs G1 females, but moths from both sources ranked the hosts the same way: there were significantly more eggs on tobacco compared to cotton, which received significantly more eggs than cabbage (table 1). For Narrabri females, there was no significant difference between oviposition rankings of females ex-pigeon pea vs. ex-cotton (source and interaction terms were not significant; table 1). Moths from both sources preferred tobacco significantly to cotton, and the latter to cabbage.

#### Oviposition preference of F1 females: specialists vs. generalists

From G1 females ex-Narrabri, we selected a female from each collection host source that either showed a clear preference for tobacco or was more even in her distribution of eggs (table 2).

When F1 females from both host sources and host specialty types are considered together, there was a strong preference in the large-cage trials for oviposition on tobacco (65%), followed

Table 2. Distribution of eggs (proportion) amongst three host plants over two nights by four *H. armigera* females collected as eggs from either cotton (COT) or pigeon pea (PP). These females were classified as either host specialist or generalists and their daughters re-tested as F1 and F2. Each female was given a designated number.

Host	Female	Number of eggs on:			Designation
		Tobacco	Cotton	Cabbage	
COT	8	628 (0.99)	1 (0.01)	0	Specialist
COT	16	81 (0.63)	36 (0.28)	11 (0.09)	Generalist
PP	13	442 (0.98)	3 (0.007)	6 (0.013)	Specialist
PP	15	222 (0.53)	80 (0.19)	111 (0.27)	Generalist

Table 3. Distribution of eggs for females selected in G1 and retested as F1 and F2 in larger cages over eight days. The females are numbered as in table 2.

Host	Source	Female/ designation	Proportion of eggs on:				Total eggs
			Tobacco	Cotton	Cabbage	Total	
COT	8	Specialist	F1	0.75	0.22	0.02	5387
			F2	0.64	0.35	0.01	886
COT	16	Generalist	F1	0.63	0.35	0.02	6720
			F2	0.44	0.55	0.01	683
PP	13	Specialist	F1	0.40	0.52	0.08	18,545
			F2	0.25	0.74	0.01	1329
PP	15	Generalist	F1	0.69	0.20	0.11	9630
			F2	0.51	0.44	0.05	663

by cotton (27%) and then cabbage (8%); that is, the females behaved in much the same way as the G1 females (their mothers) from Narrabri.

The F1 offspring of the ex-cotton female that preferred tobacco (table 2) laid 5387 eggs, of which 75% were on tobacco, 22% were on cotton and 2% were on cabbage. The F1 offspring of the ex-pigeon pea host specialist behaved more like a cotton specialist/ generalist with 40% of eggs deposited on tobacco, 52% on cotton and 8% on cabbage (out of 18,545 eggs laid over eight days). The F1 offspring of the two females that were generalists laid a lower proportion on tobacco: 69% for the female ex-pigeon pea (out of 9630 eggs) and 63% for the ex-cotton female (6720 eggs). For these females, the remaining eggs were distributed as follows: 20% on cotton and 12% on cabbage for the ex-pigeon pea generalist and 35% on cotton and 2% cabbage for the ex-cotton generalist.

The oviposition behaviour of F2 offspring (granddaughters of G1) from generalists vs. specialists was statistically similar to that of F1 offspring from generalists vs. specialists (their mothers; table 3). In the F2 offspring, ex-cotton tobacco specialist laid 64% of eggs on tobacco, whereas for the generalist this was 44%. In the F2 test, offspring from the ex-pigeon pea female originally classified as a tobacco specialist continued to lay more eggs on cotton (74%) as it had done in the F1 test. In the F2 test, offspring from the ex-pigeon pea generalist did not show a strong preference for a particular host (e.g. 51% on tobacco; table 3). There was a significant effect of kinship on egg-laying behaviour, but the day of egg lay did not affect this relationship (table 4 and fig. 1).

Table 4. Evidence that preference is heritable based on a kinship analysis. Parameters of the 'distance' between distributions of egg-counts across three generations (see text for details).

Predictor	Estimate	<i>t</i>	<i>P</i> -value
Intercept	5.56528	20.133	$< 2 \times 10^{-16}$
Kinship	7.68939	4.654	$3.5 \times 10^{-6}$
Day separation	-0.18540	-2.268	0.0234

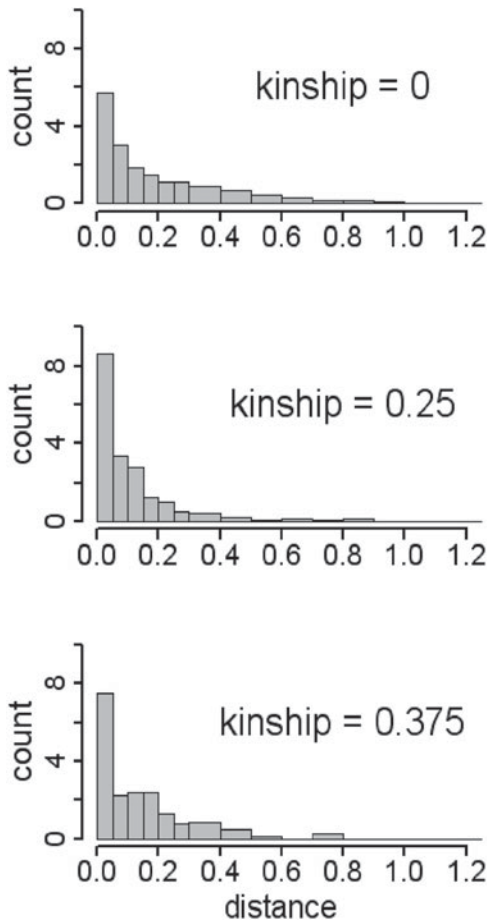


Fig. 1. Histogram of 'distance', separately for each level of kinship for G1, F1 and F2 females. When moths are unrelated, the histogram is flatter, shifting left (i.e. the oviposition preferences are more similar), as moths are more related (see table 4).

**Discussion**

Host preference trials performed in the 1980s and 1990s with *H. armigera* female moths from Narrabri and the Darling Downs demonstrated that tobacco was highly preferred next to cotton and that cabbage received very few eggs (Firempong & Zalucki, 1990b; Jallow & Zalucki, 1996).

Since this work was conducted, nearly the entire irrigated cotton crop in Australia has shifted from being conventional varieties to those with either one or, more recently, two Bt genes expressing Cry toxins in all green leaf tissue (see Zalucki et al., 2009, fig. 2). Consequently, the cotton crop has effectively

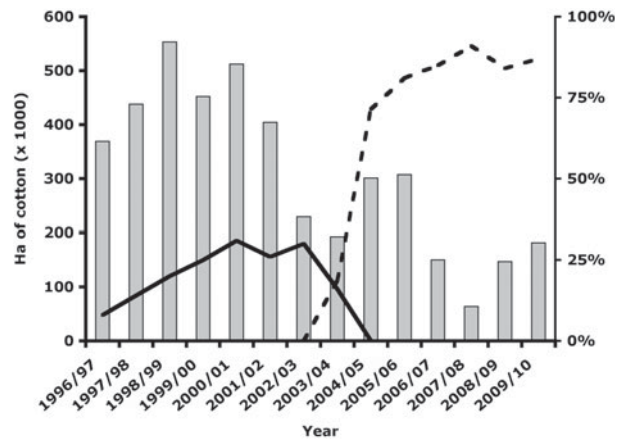


Fig. 2. Area of cotton grown in Australia from 1996/97 to 2009/10 (histogram), as well as the proportion of one (INGARD, solid line) and two-Bt gene (BOLLGARD II, dashed line). Note the collapse of the area grown reflects drought conditions and the lack of water for irrigation. Records sourced from Cotton Yearbook series published by Australian Cotton Grower.

become a sink for the main targets of this technology, *Helicoverpa* spp. Yang et al. (2008) found that 97.5% of first instars died within 72h on two gene Bt cotton, compared to 60% on non-Bt cotton. Has this effective sink or trap crop changed the host plant selection behaviour of the *H. armigera* population in cotton growing areas?

Based on the results of the experiments reported herein, the short answer to the question appears to be 'no'. As in tests performed in the late 1980s and early 1990s on populations of *H. armigera* from both geographic locations tested herein, overall tobacco continues to be the preferred host for oviposition, despite the fact that it is not grown as a commercial crop in Australia. Female *H. armigera* moths continue to lay eggs on cotton, and some females even preferred it among the options that we offered. This was true for both geographic regions. Collection sources within a region also made no difference. This result is regardless of the fact that the current landscape is comprised of a variety of cotton on which virtually all of a moth's offspring will die. We have not tested oviposition by *H. armigera* on Bt cotton vs. non-Bt cotton; we intend to conduct such work shortly. However, work done on *Heliothis virescens* (Fabricius) (Noctuidae) and *Helicoverpa zea* (Boddie) (Noctuidae) in the USA demonstrates no oviposition preference of female moths for non-Bt cotton vs. Bt cotton after almost a decade of widespread planting of Bt cotton (Torres & Ruberson, 2006). Moreover, in both pest species, there were no differences in the spatial distribution of eggs within plants between the two types of cotton, indicating that moths had not shifted their behaviour to deposit on structures of Bt cotton that may be more or less toxic to pests (Torres & Ruberson, 2006). In India in a field study, Kumar & Stanley (2010) found *H. armigera* did not discriminate between Bt and non-Bt cottons in their tests. Anecdotally, in the Australian system, large numbers of *Helicoverpa* spp. eggs continue to be recorded on Bt cotton fields.

Our results demonstrate that, as found by Firempong & Zalucki (1990b) and Jallow & Zalucki (1996), genetic variation for oviposition still exists in *H. armigera* and that this variation is heritable. There are still moths that show more specialized

patterns of oviposition and others that distribute their eggs more evenly. Yet, there appears to have been no change in oviposition behaviour in this generalist pest species. This finding supports the theory of constraints, either ecological or physiological, to the evolution of preference.

Recent molecular studies suggest that populations of *H. armigera* are probably not structured geographically, with high gene flow between populations (Weeks *et al.*, 2010; see also Endersby *et al.*, 2007). Additionally, persistence of *H. armigera* populations throughout a number of cropping seasons might present a barrier to an evolutionary shift in host preference (Jongsma *et al.*, 2010), as subsequent generations of insects are presented with varying abundances of crop and wild host species (Wardhaugh *et al.*, 1980; Walter & Benfield, 1994). If movement is extensive, population mixing and random mating could ensure that no local directional change in behaviour to exploit better hosts or avoid bad ones can be achieved.

Constraints to evolution in host preference may be inherent in the behavioural mechanism of host selection. Recent advances in insect neurophysiology suggest that constraints in olfactory perception might prevent insects from adapting towards (or away from) particular hosts (Cunningham, in press). It is also possible that oviposition preference in generalists is not related to offspring performance, as host quality is not predictable, and variability in neonate performance even within host plants is large (Zalucki *et al.*, 2002).

Quantitative theoretical models (Gould, 1984; Kennedy *et al.*, 1987; Castillochavez *et al.*, 1988) suggest that natural selection for behavioural avoidance as a mechanism of herbivore adaptation to classical host-plant resistance could render a high-dose plus refuge strategy ineffective. Our finding that *Helicoverpa* spp. moths do not avoid cotton as hosts for oviposition verifies the appropriateness of adopting a stringent plan in Australia, which includes mandatory planting of dedicated refuge crops, to retard the evolution of resistance to Bt by this pest. Physiological resistance to Bt cotton has not yet developed in Australia, and between 2002 and 2010 the proportion of the *H. armigera* population that carried genes conferring resistance to either of the two toxins in Bollgard II did not increase (Mahon *et al.*, 2007; S. Downes, unpublished data). Is the resistance management strategy working or has the relatively low area of cotton (fig. 2), and hence the relatively small proportion of the population subject to selection, resulted in relatively low selection for resistance? Since rainfall levels and the price of cotton have improved considerably, the area of cotton grown in Australia is expected to increase. With greater exposure of populations to Bt cotton, the vigilance of resistance monitoring needs to be maintained.

### Acknowledgements

This work was supported in part by ARC grant DP0988150 to MPZ. T. Parker, K. Stanford, W. Finn, N. Winters, D. Jones, L. Howie, A. Hulthen and A. Marcora provided valuable technical assistance. Collection of insect material was supported by CSIRO, Cotton Communities Catchment Cooperative Research Centre through CCC0005 and the Cotton Research and Development Corporation (CRDC) through CSE0002. Dr B. Pyke, CRDC, generously provided many years of records on area of cotton grown.

### References

- Bagla, P. (2010) Hardy cotton-munching pests latest blow to GM crops. *Science* **327**, 1439.
- Carriere, Y., Crowder, D.W. & Tabashnik, B.E. (2010) Evolutionary ecology of insect adaptation to Bt crops. *Evolutionary Applications* **3**, 561–573.
- Castillochavez, C., Levin, S.A. & Gould, F. (1988) Physiological and behavioral adaptation to varying environments: a mathematical model. *Evolution* **42**, 986–994.
- Cunningham, J.P. (2012) Can mechanism help explain insect host choice? *Journal of Evolutionary Biology* **25**, 244–251.
- Cunningham, J.P., West, S.A. & Zalucki, M.P. (2001) Host selection in phytophagous insects: a new explanation for learning in adults. *Oikos* **95**, 537–543.
- Endersby, N.M., Hoffmann, A.A., McKechnie, S.W. & Weeks, A.R. (2007) Is there genetic structure in populations of *Helicoverpa armigera* from Australia? *Entomologia Experimentalis et Applicata* **122**, 253–263.
- Firempong, S. & Zalucki, M.P. (1990a) Host plant selection by *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae): role of certain plant attributes. *Australian Journal of Zoology* **37**, 675–683.
- Firempong, S. & Zalucki, M.P. (1990b) Host plant preferences of populations of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) from different geographic locations. *Australian Journal of Zoology* **37**, 665–673.
- Gould, F. (1984) Role of behavior in the evolution of insect adaptation to insecticides and resistant host plants. *Bulletin of the Entomological Society of America (USA)* **30**, 34–41.
- Gould, F. (1998) Sustainability of transgenic insecticidal cultivars: 420 Integrating pest genetics and ecology. *Annual Review of Entomology* **43**, 701–726.
- Hendry, A.P., Kinnison, M.T., Heino, M., Day, T., Smith, T.B., Fitt, G.P., Bergstrom, C., Oakeshott, J., Jørgensen, P.S., Zalucki, M.P., Gilchrist, G., Southerton, S., Sih, A., Strauss, S., Denison, R.F. & Carroll, S.P. (2011) Evolutionary principles and their practical application. *Evolutionary Applications* **4**, 159–183.
- Jallow, M.F.A. & Zalucki, M.P. (1995) A Technique for Measuring Intraspecific Variation in Oviposition Preference in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Journal of the Australian Entomological Society* **34**, 281–288.
- Jallow, M.F.A. & Zalucki, M.P. (1996) Within- and between-population variation in host-plant preference and specificity in Australian *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Australian Journal of Zoology* **44**, 503–519.
- Jallow, M.F.A., Cunningham, J.P. & Zalucki, M.P. (2004) Intra-specific variation for host plant use in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae): implications for management. *Crop Protection* **23**, 955–964.
- Janz, N. & Nylin, S. (2008) The oscillation hypothesis of host-plant range and speciation. pp. 203–215 in Tilmon, K.J. (Ed.) *Specialization, Speciation, and Radiation: The Evolutionary Biology of Herbivorous Insects*. Berkeley, CA, USA, University of California Press.
- Jongsma, M.A., Gould, F., Legros, M., Yang, L.M., van Loon, J.J.A. & Dicke, M. (2010) Insect oviposition behavior affects the evolution of adaptation to Bt crops: consequences for refuge policies. *Evolutionary Ecology* **24**, 1017–1030.
- Karig, G. (1981) A recursive algorithm for the calculation of identity coefficients. *Annals of Human Genetics* **45**, 299–305.
- Kennedy, G.G., Gould, F., Deponti, O.M.B. & Stinner, R.E. (1987) Ecological, agricultural, genetic, and commercial

- considerations in the deployment of insect-resistant germplasm. *Environmental Entomology* **16**, 327–338.
- Khan, Z.R., Midega, C.A.O., Hutter, N.J., Wilkins, R.M. & Wadhams, L.J.** (2006) Assessment of the potential of Napier grass (*Pennisetum purpureum*) varieties as trap plants for management of *Chilo partellus*. *Entomologia Experimentalis et Applicata* **119**, 15–22.
- Kumar, R.K. & Stanley, S.** (2010) Comparative feeding behavior and ovipositional aspects of cotton bollworms *Helicoverpa armigera* on transgenic and non-transgenic cotton. *Resistant Pest Management Newsletter* **20**, 26–28.
- Liang, K.Y. & Zeger, S.L.** (1986) Longitudinal data analysis using generalized linear models. *Biometrika* **73**, 13–22.
- Lu, B.-Q.** (2011) Thresholds and mechanisms of survival for Bt-susceptible *Helicoverpa* spp. Living on Bollgard II® cotton. PhD thesis, University of New England, Maine, USA.
- Lynch, M. & Walsh, B.** (1998) *Genetics and Analysis of Quantitative Traits*. Sunderland, MA, USA, Sinauer and Associates, Inc.
- Mahon, R.J., Olsen, K.M., Downes, S. & Addison, S.** (2007) Frequency of Alleles Conferring Resistance to the Bt Toxins Cry1Ac and Cry2Ab in Australian Populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **100**, 1844–1853.
- Matten, S.R., Head, G.P. & Quemada, H.D.** (2008) How governmental regulation can help or hinder the integration of Bt crops within IPM programs. pp. 27–39 in Romeis, J., Shelton, A.M. & Kennedy, G.G. (Eds) *Integration of Insect Resistant Genetically Modified Crops within IPM Programs*. New York, USA, Springer.
- Mayhew, P.J.** (1997) Adaptive patterns of host-plant selection by phytophagous insects. *Oikos* **79**, 417–428.
- Mayhew, P.J.** (2001) Herbivore host choice and optimal bad motherhood. *Trends in Ecology & Evolution* **16**, 165–167.
- Midega, C.A.O., Khan, Z.R., Pickett, J.A. & Nylin, S.** (2011) Host plant selection behaviour of *Chilo partellus* and its implication for effectiveness of a trap crop. *Entomologia Experimentalis et Applicata* **138**, 40–47.
- Nylin, S. & Janz, N.** (1993) Oviposition preference and larval performance in *Polygonia c-album* (Lepidoptera: Nymphalidae) – the choice between bad and worse. *Ecological Entomology* **18**, 394–398.
- Nylin, S. & Janz, N.** (1996) Host plant preference in the comma butterfly (*Polygonia c-album*): Do parents and offspring agree? *Ecoscience* **3**, 285–289.
- Peck, S.L., Gould, F. & Ellner, S.P.** (1999) Spread of resistance in spatially extended regions of transgenic cotton: Implications for management of *Heliothis virescens* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **92**, 1–16.
- Prentice, R.L.** (1988) Correlated binary regression with covariates specific to each binary observation. *Biometrics* **44**, 1033–1048.
- Prentice, R.L. & Zhao, L.-P.** (1991) Estimating equations for parameters in means and covariances of multivariate discrete and continuous responses. *Biometrics* **47**, 825–839.
- Renwick, J.A.A. & Chew, F.S.** (1994) Oviposition behaviour in Lepidoptera. *Annual Review of Entomology* **39**, 377–400.
- Rochester, W.A., Zalucki, M.P., Ward, A., Miles, M. & Murray, D.A.H.** (2002) Testing insect movement theory: empirical analysis of pest data routinely collected from agricultural crops. *Computers and Electronics in Agriculture* **35**, 139–149.
- Schneider, J.C. & Roush, R.T.** (1986) Genetic differences in oviposition preference between two populations of *Heliothis virescens*. pp. 163–171 in Huettel, M.D. (Ed.) *Evolutionary Genetics of Invertebrate Behaviour*. New York, USA, Plenum Press.
- Shelton, A.M., Hatch, S.L., Zhao, J.-Z., Chen, M., Earle, E.D. & Cao, J.** (2008) Suppression of diamondback moth using Bt-transgenic plants as a trap crop. *Crop Protection* **27**, 403–409.
- Singer, M.C.** (1984) Butterfly-host plant relationships. pp. 81–88 in Vane-Wright, R.I. & Ackery, P. (Eds) *The Biology of Butterflies*. London, UK, Academic Press.
- Sisterson, M.S., Carriere, Y., Dennehy, T.J. & Tabashnik, B.E.** (2005) Evolution of resistance to transgenic crops: Interactions between insect movement and field distribution. *Journal of Economic Entomology* **98**, 1751–1762.
- Storer, N.P., Peck, S.L., Gould, F., Van Duyn, J.W. & Kennedy, G.G.** (2003) Spatial processes in the evolution of resistance in *Helicoverpa zea* (Lepidoptera: Noctuidae) to Bt transgenic corn and cotton in a mixed agroecosystem: a biology-rich stochastic simulation model. *Journal of Economic Entomology* **96**, 156–172.
- Tabashnik, B.E.** (1994) Delaying insect adaptation to transgenic plants: seed mixtures and refugia reconsidered. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **255**, 7–12.
- Tabashnik, B.E. & Carrière, Y.** (2010) Field-evolved resistance to Bt Cotton: bollworm in the U.S. and pink bollworm in India. *Southwestern Entomologist* **35**, 417–424.
- Tabashnik, B.E., Dennehy, T.J. & Carriere, Y.** (2005) Delayed resistance to transgenic cotton in pink bollworm. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 15389–15393.
- Tabashnik, B.E., Van Rensburg, J.B.J. & Carriere, Y.** (2009) Field-evolved insect resistance to Bt crops: definitions, theory and data. *Journal of Economic Entomology* **102**, 2011–2025.
- Teakle, R.E.** (1991) Laboratory culture of *Heliothis* spp. and identification of diseases. pp. 11–21 in Zalucki, M.P. (Ed.) *Heliothis Research Methods and Prospects*, New York, USA, Springer-Verlag.
- Thompson, J.N.** (1988) Evolutionary ecology of the relationship between oviposition preference and performance of offspring in phytophagous insects. *Entomologia Experimentalis et Applicata* **47**, 3–14.
- Thrall, P.H., Oakshott, J.G., Fitt, G.P., Southerton, S., Burdon, J.J., Sheppard, A., Russell, R.J., Zalucki, M.P., Heino, M. & Denison, R.F.** (2011) Evolution in agriculture: the application of evolutionary approaches to the management of biotic interactions in agro-ecosystems. *Evolutionary Applications* **4**, 200–215.
- Torres, J.B. & Ruberson, J.R.** (2006) Spatial and temporal dynamics of oviposition behavior of bollworm and three of its predators in Bt and non-Bt cotton fields. *Entomologia Experimentalis et Applicata* **120**, 11–22.
- Van Rensburg, J.B.J.** (2007) First report of field resistance by stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. *South African Journal Plant Soil* **24**, 147–151.
- Walter, G.H. & Benfield, M.D.** (1994) Temporal host-plant use in 3 polyphagous *Heliothinae*, with special reference to *Helicoverpa punctigera* (Wallengren) (Noctuidae, Lepidoptera). *Australian Journal of Ecology* **19**, 458–465.
- Wang, C.-Z., Dong, J.-F., Tang, D.-L., Zhang, J.-H., Li, W. & Qin, J.** (2004) Host selection of *Helicoverpa armigera* and *H. assulta* and its inheritance. *Progress in Natural Science* **14**, 880–884.
- Wardhaugh, K.G., Room, P.M. & Greenup, L.R.** (1980) The incidence of *Heliothis armigera* (Hübner) and *Heliothis punctigera* Wallengren (Lepidoptera, Noctuidae) on cotton



- and other host-plants in the Namoi Valley of New South Wales. *Bulletin of Entomological Research* **70**, 113–131.
- Weeks, A.R., Endersby, N.M., Lange, C.L., Lowe, A., Zalucki, M.P. & Hoffmann, A.A.** (2010) Genetic variation among *Helicoverpa armigera* populations as assessed by microsatellites: a cautionary tale about accurate allele scoring. *Bulletin of entomological Research* **100**, 445–450.
- West, S.A. & Cunningham, J.P.** (2002) A General model for host plant selection in phytophagous insects. *Journal of theoretical Biology* **214**, 499–513.
- Wu, K.-M., Lu, Y.-H., Feng, H.-Q., Jiang, Y.-Y. & Zhao, J.-Z.** (2008) Suppression of cotton bollworm in multiple crops in China in areas with Bt toxin-containing cotton. *Science* **321**, 1676–1678.
- Yang, Y.-Z., Johnson, M.-L. & Zalucki, M.P.** (2008) Possible effect of genetically modified cotton on foraging habits of early instar *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) larvae. *Australian Journal of Entomology* **47**, 137–141.
- Zalucki, M.P., Daghli, G., Firempong, S. & Twine, P.** (1986) The biology and ecology of *Heliothis armigera* (Hübner) and *Heliothis punctigera* Wallengren (Lepidoptera: Noctuidae) in Australia: What do we know? *Australian Journal of Zoology* **34**, 779–814.
- Zalucki, M.P., Murray, D.A.H., Gregg, P.C., Fitt, G.P., Twine, P.H. & Jones, C.** (1994) Ecology of *Helicoverpa armigera* (Hübner) and *H. punctigera* Wallengren in the inland areas of eastern Australia: larval sampling and host plant relationships during winter/spring. *Australian Journal of Zoology* **42**, 329–346.
- Zalucki, M.P., Clarke, A.R. & Malcolm, S.B.** (2002) Ecology and behaviour of first instar larval Lepidoptera. *Annual Review of Entomology* **47**, 361–393.
- Zalucki, M.P., Adamson, D. & Furlong, M.J.** (2009) The future of IPM: whither or wither? *Australian Journal of Entomology* **48**, 85–96.
- Zhang, H., Yin, W., Zhao, J., Jin, L., Yang, Y., Wu, S., Tabashnik, B.E. & Wu, Y.** (2011) Early warning of cotton Bollworm resistance associated with intensive planting of Bt cotton in China. *PLoS ONE* **6**(8), e22874 (doi:10.1371/journal.pone.0022874).